The Prokaryotes

Second Edition

A Handbook on the Biology of Bacteria:
Ecophysiology, Isolation,
Identification, Applications

Edited by
ALBERT BALOWS
HANS G. TRÜPER
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KARL-HEINZ SCHLEIFER

Volume IV

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Volume IV

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Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These four volumes on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

This edition of *The Prokaryotes* recognizes the almost unbelievable impact that the work of Carl Woese has had in defining a phylogenetic basis for the microbial world. The concept that the ribosome is a highly conserved structure in all cells and that its nucleic acid components may serve as a convenient reference point for relating all living things is now generally accepted. At last, the phylogeny of prokaryotes has a scientific basis, and this is the first serious attempt to present a comprehensive treatise on prokaryotes along recently defined phylogenetic lines. Although evidence is incomplete for many microbial groups, these volumes make a statement that clearly illuminates the path to follow.

There are basically two ways of doing research with microbes. A classical approach is first to define the phenomenon to be studied and then to select the organism accordingly. Another way is to choose a specific organism and go where it leads. The pursuit of an unusual microbe brings out the latent hunter in all of us. The intellectual challenges of the chase frequently test our ingenuity to the limit. Sometimes the quarry repeatedly escapes, but the final capture is indeed a wonderful experience. For many of us, these simple rewards are sufficiently gratifying so that we have chosen to spend our scientific lives studying these unusual creatures. In these endeavors many of the strategies and tools as well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

In this school, the principles of the selective, enrichment culture technique have been developed and diversified; they have been a major force in designing and applying new principles for the capture and isolation of microbes from nature. For me, the "organism approach" has provided rewarding adventures. The organism continually challenges and literally drags the investigator into new areas where unfamiliar tools may be needed. I believe that organism-oriented research is an important alternative to problem-oriented research, for new concepts of the future very likely lie in a study of the breadth of microbial life. The physiology, biochemistry, and ecology of the microbe remain the most powerful attractions. Studies based on classical methods as well as modern genetic techniques will result in new insights and concepts.

To some readers, this edition of the *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have

evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the "nitrosome," a unique nitrogen-fixing organelle. Study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become "dog-eared" and worn as students seek basic information for the hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, i.e., from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator these volumes should generate excitement.

Happy hunting!

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Preface

In 1962 R. Y. Stanier and C. B. van Niel formulated the view that bacteria represented a definable, biologically coherent group of organisms called the prokaryotes. They pointed out that, up to that time, "the abiding intellectual scandal of bacteriology has been the absence of a clear concept of a bacterium" (Archiv für Mikrobiologie 42:17–35, 1962). In addition to formalizing the distinction between the eukaryotes and the prokaryotes, Stanier and van Niel also emphasized the diversity of the prokaryotes.

The early view of Ferdinand Cohn that bacteria were simply a group of unicellular microorganisms that divide by binary fission had gradually been supplanted, so that by 1962, Stanier and van Niel could point out that bacteria were "photosynthetic or non-photosynthetic; motile by any one of three different mechanisms or permanently immotile; unicellular, multicellular or coenocytic; multiplying by binary transverse fission, or by formation of gonidia or conidia." Although Stanier and van Niel never actually defined the bacteria as a group, their seminal article explicitly emphasized the wide variety of metabolic, physiological, and morphological types among the bacteria, a variety which was reflected in cellular organization, modes of cell division, mechanisms of locomotion, and patterns of energy-yielding metabolism. Nevertheless, the succeeding decades saw a narrowing of the scope of research on the bacteria, since the incredible power and successes of molecular biology required intense study of only a few suitable model organisms. The hypothesis that there was a small group of typical bacteria whose mechanisms and processes were accurately representative of the bacteria as a whole became tacitly accepted. Most areas of human activity have a dialectic quality, and the evolution of scientific ideas is no exception. Thus, the narrow focus on a small group of bacteria is now being broadened to recognize new mechanisms, new strategies for coping with the environment, newly expanded limits to the abilities of the microbe, and new experimental systems. The publication in 1981 of the first edition of *The Prokaryotes* played an important role in this broadening of the perspective of the microbial world. Ten years later, the second edition continues to emphasize the diversity of the prokaryotes while adding the entirely new perspective of prokaryotic phylogeny. As far as possible, in this edition the chapters dealing with individual groups and genera of bacteria are arranged in strictly phylogenetic order.

The pioneering work of Carl Woese in cataloging and sequencing the ribosomal ribonucleic acid of prokaryotes has, for the first time in the history of biology, provided a means of establishing a truly phylogenetic system for living organisms—a goal previously thought to be impossible. The use of the oligonucleotide sequences of 16S rRNA as a molecular/evolutionary chronometer has revealed unsuspected phylogenetic affiliations. Furthermore, it has shown that the uniform concept of the prokaryotes must give way to a dichotomy dividing the prokaryotes into two groups (the archaebacteria, or Archaea, and the eubacteria, or Bacteria) no more closely related to each other than either of them is to the eukaryotes. In essence, the molecular approach has added an entirely new dimension to prokaryotic diversity, providing a fascinating opportunity for gaining insight into the origin of life.

The work of Woese and his coworkers has uncovered so many inconsistencies in classical prokaryotic systematics that an entirely new system for the taxonomy of the prokaryotes will undoubtedly emerge. These phylogenetic conclusions, drawn mainly from 16S rRNA analyses, have been strongly supported by nucleic acid hybridization studies and comparative se-

quence analysis of 23S rRNA, of the beta subunit of adenosine triphosphatase, and of the elongation factor Tu. The phylogenetic relationships of bacteria are also reflected by studies of chemotaxonomic markers such as peptidoglycan and lipid.

A reader familiar with the first edition of *The Prokaryotes* will note that, in the new edition, the subtitle has been changed from "A Handbook on Habitats, Isolation, and Identification of Bacteria" to "A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications." This change reflects developments in bacteriology over the past 10 years as well as the growing awareness of the important role that prokaryotes play in determining and stabilizing the global environment. The term "Habitats" has been changed to "Ecophysiology" to emphasize the interactions of microorganisms both with their habitats and with other organisms that occupy those habitats. In this context, the number of chapters dealing with symbiotic and syntrophic associations of prokaryotes with other prokaryotes and with eukaryotes has been substantially increased in the new edition.

The terms "Isolation" and "Identification" have been maintained because they reflect essential aspects of the second edition of *The Prokaryotes*. However, the individual chapters have undergone considerable change so as to include new techniques for the handling of fastidious anaerobes, for chemotaxonomic analysis, for molecular genetic methods of identification, etc. In addition, the results of another 10 years of experience in preserving and maintaining cultures have been included in many of the chapters. An important addition has been the new key term "Applications." This reflects the conscious move by microbiology into biotechnology. The use of microbes to produce new and useful products and to recycle undesirable products is now of worldwide economic and ecological importance.

But the organization of the second edition of *The Prokaryotes* differs even more fundamentally than the subject matter of the first edition. The decision to organize the Handbook on the basis of phylogenetic relationships generated two problems. First, bacteria have traditionally been divided into groups that share one or more phenotypic properties. Thus, microbiologists are used to dealing with collective entities such as the "gliding bacteria," "the autotrophs," and "the photosynthetic bacteria." These are groups that are similar in their overall physiology, behavior, or metabolism, or that inhabit similar ecological niches. However, the new phylogenetic analyses have made it clear that these groups are not necessarily related to each other in any evolutionary sense. Thus, an organization that reflected the true evolutionary relations among the prokaryotes would necessarily have sacrificed the familiar and intellectually useful associations of phenotypic properties, but retention of the traditional arrangements would have ignored the phylogenetic revolution that has taken place. A second problem is that there are still a number of genera or groups whose position in the phylogenetic scheme have not been determined.

The solution to this dilemma was to divide the present edition of *The Prokaryotes* into six parts. Part I contains introductory essays dealing with the broader aspects of microbiology that underlie the rest of the Handbook. Part II contains general chapters that cover life cycles, prokaryotic behavior, anaerobic growth, syntrophism, and a series of synoptic chapters that describe the familiar phenotypically organized groups. This has allowed us to retain discussions of those useful physiological, metabolic, and ecological generalizations that characterize the bacteria. Parts III and IV of the Handbook consist of phylogenetically arranged chapters devoted to genera, families, and sometimes higher taxa of related prokaryotes. For example, the archaebacteria (Archaea) have been strictly separated from the eubacteria, and within these major groups the individual genera are arranged phylogenetically.

These phylogenetic groupings have generated some phenotypically strange bedfellows. Nevertheless, the editors hope that the juxtaposition of organisms that are phylogenetically related, but appear phenotypically dissimilar, may stimulate microbiologists to seek taxonomically relevant similarities that otherwise would not have been evident. Following the major groups of generic chapters, Part V covers those microorganisms that have established firm symbiotic relationships. Finally, those genera that have not been phylogenetically allocated as yet are dealt with in Part VI. In order to help the reader find a specific genus, an alphabetical listing of genera and other higher taxa follows the Contents.

Microbiology is in the midst of the most significant conceptual revolution it has experi-

enced in the past few decades. We have the privilege of a new way of thinking about the evolution of bacteria that may even help us to understand the origin of life. We also have a set of molecular tools that allow us to probe the innermost details of the workings of the cell and that may also allow us to ask ecological questions, the answers to which have thus far eluded us. Nevertheless, in the final analysis, it is only the ability to isolate and cultivate microbes individually and as consortia that will lead to a full understanding of the strategies, mechanisms, and processes of biodiversity. We dedicate this Handbook to the efforts of those who pursue these goals.

A scientific work of this magnitude requires support, advice, and assistance from many people with a wide range of skills and knowledge. We would be remiss if we failed to express our thanks and appreciation for the suggestions and hard work provided by many secretaries, typists, graphic artists, and photographers who worked with all of the authors of *The Prokaryotes,* second edition. We are indebted to the staff of Springer-Verlag who encouraged and indulged us and demonstrated remarkable patience with us. We are equally grateful to Thomas D. Brock and the staff of Science Tech Publishers, especially Carol Bracewell and Ruth Siegel, whose copyediting expertise and problem-solving capabilities lightened our load considerably. Finally, we are beholden to the more than 300 people throughout the world who gave of their time, knowledge, and experience to serve as authors in this international effort.

Albert Balows
Hans G. Trüper
Martin Dworkin
Wim Harder
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CHAPTER 163

The Genus *Xylophilus*

ANNE WILLEMS, MONIQUE GILLIS, and JOZEF DE LEY

The genus *Xylophilus* comprises strains pathogenic for the grapevine, *Vitis vinifera. Xylophilus ampelinus,* the only species, is the causal agent of bacterial necrosis and canker of grapevine and is therefore responsible for important economic losses to viticulture in both Europe and South Africa. Although the disease was first described in France in 1895 as "Maladie d'Oléron" (Ravaz, 1895), the causal pathogen was not isolated until 1969.

In 1939 Sarejanni had reported on "Tsilik marasi", a disease of Cretan grapevines which he attributed to a fungus of the family Pythiaceae, and which he considered to be different from the "Maladie d'Oléron", the "Gommose bacillaire" in France and from the "Mal nero" in Italy. The former three diseases of grapevines and the similar South African "Vlamsiekte" were at that time all attributed to *Erwinia vitivora* (Sarejanni, 1939; Du Plessis, 1940). This bacterium (syn. *Erwinia lathyri* and *Erwinia herbicola*) was subsequently shown to be an ordinary saprophyte, commonly isolated from infected plant material (Lelliott, 1974). In 1969, Panagopoulos reported the isolation of a very slow growing yellow-pigmented bacterium from grapevines with "Tsilik marasi" symptoms. This organism proved to be pathogenic upon reinoculation of healthy plants. It was named *Xanthomonas ampelina* (Panagopoulos, 1969) and was shown to be identical to the causal agent of "Maladie d'Oléron" (Prunier et al., 1970), "Vlamsiekte" (Erasmus et al., 1974) and "Mal nero" (Grasso et al., 1979).

The pathogen was originally classified in the genus *Xanthomonas* because it is a Gram-negative, aerobic, nonsporeforming, monotrichously flagellated rod-shaped, plant pathogenic bacterium that produces a yellow water-insoluble pigment and metabolizes sugars oxidatively (Panagopoulos, 1969). Gradually however, it became evident that *Xanthomonas ampelina* was not a genuine *Xanthomonas* (Bradbury, 1984) because of 1) the absence of xanthomonadins in *Xanthomonas ampelina* (Starr et al., 1977); 2) the differences in the regulation pattern of the aromatic amino acid biosynthesis (Byng et al., 1980; Whitaker et al., 1981); and 3) the dissimilarity between the rRNA cistrons of *Xanthomonas ampelina* and the other *Xanthomonas* species (De Vos and De Ley, 1983). By means of hybridizations between a labelled rRNA from the type strain of the vine pathogen and DNA from several reference taxa, it was definitely shown that *Xanthomonas ampelina* is not related to members of the genus *Xanthomonas,* but forms a separate genus in the acidovorans rRNA complex. *Xanthomonas ampelina* was therefore transferred to a new genus as *Xylophilus ampelinus* (Willems et al., 1987).

Habitats

Bacterial Necrosis and Canker of Grapevines

Xylophilus ampelinus strains have been isolated exclusively from different cultivars of *Vitis vinifera,* displaying symptoms of bacterial necrosis and canker. The disease can be detected beginning in early spring, when buds on affected shoots fail to open. The most typical symptoms on affected shoots are longitudinal cracks and cankers that develop from hyperplasiae in the cambial tissue. The underlying vascular tissue shows a brown discoloration and eventually dies. Other parts of the shoot, less severely affected, can survive. Similar cracks can develop on the petioles, flower stalks, and fruit stalks, causing death of leaves, flowers, or fruits. Leaves infected through hydathodes or stomata show reddish-brown lesions. Roots can also be affected, resulting in retarded growth of the shoots. Severity of the symptoms may vary considerably with different varieties (Panagopoulos, 1969; Ridé, 1984; European and Mediterranean Plant Protection Organization data sheets on quarantine organisms, list A2, No. 133, 1984; López et al., 1987b; Grasso et al., 1979).

Agricultural practices and local climatological conditions strongly affect the occurrence of the disease. Since *Xylophilus ampelinus* survives in the vascular tissue, transmission occurs mainly through pruning lesions by the use of contaminated tools and infected grafting material. Wind and rain (and overhead spraying) not only favor the spreading of bacterial ooze from infected leaves, but often cause wounds and thus provide additional access routes for the pathogen. Exceptionally dry seasons may result in partial recovery of infected vineyards (Ridé, 1984; Panagopoulos, 1987; López et al., 1987b).

Geographical Distribution

Xylophilus ampelinus has been isolated from diseased vines in Greece, France, Sardinia, Sicily, Spain and South Africa (Panagopoulos, 1969; Prunier et al., 1970; Garau et al., 1987; Grasso et al., 1979; López et al., 1978; Erasmus et al., 1974). Similar disease symptoms, very likely also caused by *Xylophilus ampelinus,* were reported from Argentina, Austria, Bulgaria, Canary Islands, Portugal, Switzerland, Tunisia, Turkey, Yugoslavia, and the USSR (Bradbury, 1984; Panagopoulos, 1987). According to Panagopoulos, the actual distribution of bacterial necrosis of grape vine is probably much larger, since its symptoms may be confused with those of other diseases (Panagopoulos, 1987). By means of several techniques it was shown that strains from Crete, France, Greece, Spain and South Africa are very similar (Willems et al., 1987).

Isolation

A major problem in the isolation of *Xylophilus ampelinus* strains is the extremely slow and poor growth of these strains on virtually all isolation media (Panagopoulos, 1969). Very often, fast-growing saprophytes, accompanying the pathogen, rapidly overgrow the whole culture. This may partly account for the long time period before *Xylophilus ampelinus* was isolated and characterized.

The most common isolation sources are small pieces of infected wood tissue, taken aseptically from diseased grapevines and soaked for 20 minutes in sterile water. The resulting bacterial suspension is plated on nutrient agar (Panagopoulos, 1969; Erasmus et al., 1974). After five to six days of incubation at 26°C, small pale-yellow colonies appear (0.1 to 0.3 mm diameter). After eight to ten days they can reach a diameter of 0.4 to 0.6 mm. The colony diameter never exceeds 1 mm on nutrient agar (Panagopoulos, 1969). On this medium, colonies are round with an entire margin, semitranslucent, slightly raised, and glistening. According to Panagopoulos, in Greece the pathogen can be isolated from plants the whole year around.

For better growth, the addition of 5% sucrose to nutrient agar is advised. Best growth is reported on yeast extract-galactose-calcium carbonate (YGC) medium containing 1% yeast extract, 2% galactose, 2% $CaCO_3$, and 2% agar (Panagopoulos, 1969; Bradbury, 1973). We have also obtained good growth on glucose-yeast extract-calcium carbonate-agar (GYCA) medium containing 0.5% yeast extract, 1% glucose, 3% $CaCO_3$, and 2% agar (Willems et al., 1987). The presence of calcium carbonate, which prevents the transmission of light, makes the latter two media less suitable for the study of colony morphology. More convenient and also providing good growth, is Yeast Extract/Galactose (YEGAL) medium (Starr et al., 1977).

Composition of YEGAL Medium

Yeast extract	5 g
Galactose	10 g
K_2HPO_4	3.01 g
NaH_2PO_4	4.55 g
NH_4Cl	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Ferric ammonium citrate	0.05g
$CaCl_2$	0.005 g
Distilled water	1 liter

Yeast extract and galactose, each dissolved in 100 ml, should be autoclaved separately as concentrated solutions.

On a purely synthetic medium, the addition of 0.1% glutamic acid is required as a growth factor (Bradbury, 1973). In general, the use of a large inoculum is required for good growth. *Xylophilus ampelinus* strains fail to grow on media that have been liquified and sterilized twice (Panagopoulos, 1969).

Xylophilus strains may produce two different stable colony types: one type (t1) producing relatively large yellow colonies (colony diameter 0.8 to 2.0 mm after 15 days on GYCA), the other type (t2) producing smaller, paler colonies that grow more slowly (colony diameter 0.4 to 1.0 mm after 15 days on GYCA). Microscopically, cells of both types look identical. Based on the results of comparative whole-cell protein gel electrophoresis and DNA:DNA hybridizations, it was established that these types are merely morphological colony variants which are phenotypically and genotypically highly similar (Willems et al., 1987).

Cultures can be maintained on screw-capped slants at 4°C; they should be transferred every

two months. For long-term preservation, strains can be lyophilized.

Identification

A few relatively simple bacteriological tests (Table 1) will establish whether strains, isolated from grapevines displaying the typical disease symptoms, belong to the genus *Xylophilus.* Most typical is the extremely slow and poor growth on most media at the optimal growth temperature of 24°C. The tiny yellow colonies, occurring after six to 15 days, produce a brown diffusible pigment on YGC medium. Cells are Gram-negative, straight to slightly curved rods, 0.4 to 0.8 μm by 0.6 to 3.3 μm. They occur singly, in pairs, or short chains, and are motile by means of one polar flagellum. Filamentous cells may occur in older cultures.

Additional features for differentiating *Xylophilus* from most *Xanthomonas* species are: growth on K, Na tartrate, Ca lactate, D-glucose, use of L-glutamine as sole carbon and nitrogen source, and no production of H_2S from L-cysteine (M. Van den Mooter, unpublished observations).

Because of their slow and poor growth, *Xylophilus* strains require relatively long incubation periods in conventional bacteriological tests. We tried to overcome this disadvantage by applying miniaturized API test systems (API Systems S.A., France). The enzymatic tests provided in the API ZYM gallery and in the experimental galleries Osidases, Esterases, and Aminopeptidases AP1 to AP6 (103 tests in total) could be used to distinguish *Xylophilus* from related taxa and from *Xanthomonas* (Willems et al., 1987). The 32 *Xylophilus* strains we tested formed a separate cluster using numerical analysis of the data. For identification purposes, however, these systems are not very suitable because of the large number of negative reactions and the lack of reference data. The auxanographic tests, available from API Systems, could not be used since *Xylophilus* does not grow on the standard medium these tests require.

Table 1. Diagnostic features for *Xylophilus ampelinus.*

Growth at 33°C	−
Mucoid growth	−
Acid from glucose	−
Alkali from Na propionate	−
Alkali from tartrate	+
Urease production	+
Esculin hydrolysis	−
Brown, diffusible pigment on YGC	+

From Panagopoulos, 1987.

Regardless of their geographic origin, *Xylophilus* strains produce a unique pattern in sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell proteins and can therefore be identified by means of this technique (Willems et al., 1987).

In specialized phytopathological laboratories, the identity of suspected *Xylophilus ampelinus* strains is verified by techniques such as indirect immunofluorescence and indirect Enzyme-linked immunosorbent assay (ELISA) (López et al., 1987a). Erasmus et al., (1974) used Ouchterlony gel diffusion, Wassermann agglutination, and hemagglutination plate tests to identify the causal agent of the South African "Vlamsiekte" as *Xylophilus ampelinus.* Application of such fast techniques is becoming more and more widespread.

A fast, very reliable, and decisive identification method is based on hybridization of a radioactively labelled rRNA probe of a representative *Xylophilus ampelinus* strain with DNA of the strain to be identified. When rRNA from the type strain is used, *Xylophilus ampelinus* strains have Tm(e) values from 78 to 81°C (Willems et al., 1987). Tm(e) is the temperature at which half of the DNA:rRNA duplex is thermally denatured.

Physiology

Xylophilus strains have a strictly aerobic chemoorganotrophic metabolism. They use only a limited number of carbohydrates, organic acids, and amino acids for growth: on a total of 60 substrates tested, growth could be recorded only on D-glucose, D-galactose, L-glutamic acid, Na succinate, Na fumarate, K, Na tartrate, Na L-malate, Na_3 citrate, and Ca gluconate (M. Van den Mooter and J. Swings, manuscript submitted to Int. J. Syst. Bacteriol.). L-glutamate is required as a growth factor.

Taxonomic Position

Based on rRNA cistron similarities, as determined by DNA-rRNA hybridizations, the genus *Xylophilus* belongs to the acidovorans rRNA complex within rRNA superfamily III (see Chapter 100). Its closest genotypic neighbors are the genera *Comamonas* and *Hydrogenophaga,* several generically misnamed *Pseudomonas, Alcaligenes,* and *Aquaspirillum* species, and several unnamed clinical isolates. The genus *Xylophilus* constitutes a separate rRNA subbranch within this group. It can be

differentiated readily from all these taxa by its slow and poor growth.

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CHAPTER 164

The Genus *Acinetobacter*

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Current Taxonomic Status

Members of the genus *Acinetobacter* have previously been classified by various authors under a variety of different names (reviewed by Henriksen, 1973), and, consequently, much of the early literature concerning this group of organisms is difficult to interpret owing to confusion over nomenclature and the lack of a widely accepted classification scheme. The genus *Acinetobacter* originally proposed by Brisou and Prévot (1954) included a heterogenous collection of nonmotile Gram-negative saprophytes that could be distinguished from other similar bacteria by their lack of pigmentation (Ingram and Shewan, 1960). The Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria subsequently proposed (Lessel, 1971) that the genus *Acinetobacter* should include only the oxidase-negative strains. This division has been supported by the use of transformation tests (Juni, 1972), which now forms the basis for inclusion in the genus. *Acinetobacter* is classified in the family Neisseriaceae, and the current generic description (Juni, 1984) allows unambiguous identification of strains to the genus level.

In contrast, delineation of species within the genus *Acinetobacter* is not satisfactory and is still the subject of much research. Only one species *(A. calcoaceticus)* is included in the current generic description, although it is known that the genus is biochemically and genetically heterogenous (Baumann et al., 1968; Johnson et al., 1970). DNA hybridization studies have identified 12 phenotypically distinct hybridization groups (genospecies) (Bouvet and Grimont, 1986). Species names have been proposed for six of these groups (Table 1), but phenotypic identification can be complex and time-consuming (Bouvet and Grimont, 1986). Five further genospecies have been similarly identified by Bouvet and Jeanjean (1989), but these cannot, as yet, be differentiated solely on the basis of phenotypic properties. There is an urgent need for a simple identification and differentiation scheme suitable for routine taxonomic and epidemiological use.

Table 1. Proposed subdivision of the genus *Acinetobacter* into genospecies.

Genospecies	Proposed species name	Number of strains[a]
1	*Acinetobacter calcoaceticus*	8
2	*Acinetobacter baumannii*	121
3		15
4	*Acinetobacter haemolyticus*	23
5	*Acinetobacter junii*	17
6		3
7	*Acinetobacter johnsonii*	23
8	*Acinetobacter lwoffii*	34[b] (8 and 9)
9		
10		4
11		4
12		3
13–17		20[c]

[a]The number of strains shown is the total of those identified phenotypically in the cited studies and provides an indication of the relative frequencies of the different genospecies.

[b]Genospecies 8 and 9 cannot be differentiated solely on the basis of phenotypic properties (Bouvet and Grimont, 1986).

[c]Genospecies 13–17 are phenotypically distinguishable from genospecies 1–12, but cannot themselves be differentiated phenotypically (Bouvet and Jeanjean, 1989).

Adapted from Bouvet and Grimont (1986) and Bouvet and Jeanjean (1989).

Habitats and Pathogenicity

Acinetobacters are ubiquitous organisms that are present in soil, water, and sewage (Blaise and Armstrong, 1973; Baumann, 1968; Warskow and Juni, 1972). It has been estimated that *Acinetobacter* may constitute as much as 0.001% of the total heterotrophic aerobic population of soil and water (Baumann, 1968). They have been found at densities exceeding 10^4 organisms per 100 ml in freshwater ecosystems and 10^6 organisms per 100 ml in raw sewage (LaCroix and Cabelli, 1982). They can be isolated from

heavily polluted water but are found more frequently near the surface of fresh water and where fresh water flows into the sea (Droop and Jannasch, 1977).

Members of the genus are also found in a variety of foodstuffs, including milk products (Koburger, 1964) and fresh meat (Eribo and Jay, 1985). Acinetobacters have been frequently isolated from eviscerated chicken carcasses and other poultry meats, even following gamma irradiation (Barnes and Thornley, 1966). Lahellec et al. (1975) reported that *Acinetobacter* constituted 22.7% of the total microflora of chicken carcasses. Members of the genus are responsible for spoilage of a number of foods, including chickens, eggs, bacon, and fish, even when the food is stored under refrigerated conditions (Gardner, 1971; Jay, 1982; Shewan et al., 1960; Thornley et al., 1960).

Acinetobacter is a normal inhabitant of human skin and has consequently been implicated as a presumed causal or contributory agent in numerous infectious disease processes, but sometimes is found as a result of sample contamination. They are particularly found in moist skin areas such as toe webs, the groin, and the axilla (Al-Khoja and Darrell, 1979; Noble and Pitcher, 1978; Taplin et al., 1963). Their pathogenicity is generally low but they may cause occasional serious opportunistic infections, including meningitis, septicemia, and pneumonia, particularly in patients with reduced natural resistance (reviewed by Glew et al., 1977). Increasing numbers of nosocomial infections due to *Acinetobacter* are now being reported (Bergogne-Bérézin et al., 1987). Hospital reservoirs of the organism may include waterbaths, disinfectants, room humidifiers, peritoneal dialysis fluid, wet mattresses, respirometers, and the hands of hospital staff (Buxton et al., 1978; Cunha et al., 1980; French et al., 1980; Sherertz and Sullivan, 1985; Smith and Massanari, 1977).

Isolation

Isolation of members of the genus *Acinetobacter* can be accomplished using standard laboratory media such as trypticase soy agar or brain heart infusion agar. A differential medium such as MacConkey agar may be helpful in recognizing colonies of *Acinetobacter* on primary isolation. The optimum growth temperature for most strains is 33–35°C. Although most strains will grow reasonably well at 37°C, some strains have considerably lower optimum growth temperatures and may be unable to grow at 37°C (Breuil and Kushner, 1975).

Selective Enrichment

Most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source such as acetate, lactate, or pyruvate. Baumann (1968) described the use of an enrichment culture procedure for isolating members of the genus from soil and water. Liquid enrichment cultures containing 20 ml of medium (see below) are inoculated with a 5 ml sample of water or of a filtered 10% soil suspension and vigorously aerated at either 30°C or room temperature. Cultures are examined microscopically after 24 or 48 h and streaked onto suitable isolation media. Strains of *Acinetobacter* have a slightly acid pH optimum for growth, and vigorous aeration at a pH of 5.5 to 6.0 favors their enrichment.

Baumann's Enrichment Medium (per liter)

Sodium acetate (trihydrate)	2 g
KNO_3	2 g
$MgSO_4 \cdot 7H_2O$	0.2 g

dissolved in 0.04M KH_2PO_4-Na_2HPO_4 buffer (pH 6.0) containing 20 ml per liter of Hutner's mineral base (Cohen-Bazire et al., 1957).

Differential Selection on Solid Media

Selective liquid enrichment is rarely used in isolation from clinical specimens. For clinical isolation, general-purpose rich media such as blood agar or MacConkey agar are usually preferred because of their broad bacterial coverage. However, Holton (1983) described a selective differential medium (modified from an earlier medium described by Mandel et al., 1964) which may be suitable for the specific isolation and growth of *Acinetobacter* strains from clinical sources.

Holton's Selective Medium (per liter)

Agar	10 g
Casein pancreatic digest	15 g
Peptone	5 g
NaCl	5 g
Desiccated ox-bile	1.5 g
Fructose	5 g
Sucrose	5 g
Mannitol	5 g
Phenylalanine	10 g
Phenol red	0.02 g

Adjust to pH 7.0.

After autoclaving, the medium is cooled to 50°C and the following filter-sterilized ingredients added (final concentration in g per liter):

Vancomycin	0.01 g
Ampicillin	0.016 g
Cefsulodin	0.03 g

Following overnight incubation at 37°C, red colonies on the medium are tested for oxidase reaction and phenylalanine deamination (10% ferric chloride method). Colonies giving a negative reaction with both of these tests are presumptive *Acinetobacter* isolates.

Identification

Morphology

Short, plump, Gram-negative rods, typically 1.0–1.5 by 1.5–2.5 μm in the logarithmic phase of growth, but often becoming more coccoid in the stationary phase. Occasionally difficult to destain. Cells commonly occur in pairs, but also in chains of variable length. No spores formed and flagella absent. Although generally considered to be nonmotile, "twitching" and "gliding" motility has been reported to occur, particularly on semisolid media (Barker and Maxsted, 1975; Henrichsen and Blom, 1975; Mukherji and Bhopale, 1983). Many strains are encapsulated, and the capsule may be readily seen in India ink wet mounts. Colonies are usually nonpigmented, but some strains form white- to cream-colored colonies, which vary in consistency from butyrous to smooth and mucoid and from 1–2 mm in diameter.

Biochemical Identification

All members of the genus *Acinetobacter* are strict aerobes and can grow at a wide range of temperatures. Most strains will grow at 33°C, but some environmental isolates prefer incubation temperatures from 20 to 30°C. Clinical isolates of *Acinetobacter* will normally grow at 37°C and some strains can also grow at 42°C. All strains of *Acinetobacter* are oxidase-negative and catalase-positive. The negative oxidase reaction serves to distinguish the genus from other genera grouped in the family Neisseriaceae. Most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source. A wide variety of organic compounds can be used as carbon sources by particular strains, although relatively few strains can use glucose (Baumann et al., 1968). There is no single biochemical test that enables ready differentiation of this genus from similar bacteria, but the non-fastidious nature and wide biochemical activities of the members of the genus make them readily distinguishable from other bacteria, using the combination of nutritional tests applied for nonfastidious, nonfermentative organisms in general, including commercially available diagnostic devices (Dowda, 1977; Oberhofer et al., 1977; Oberhofer, 1979; Towner and Chopade, 1987). Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay. A few clinical strains may show hemolysis on blood agar plates due to the production of a phospholipase C (Lehmann, 1971). Most strains are resistant to penicillin due to the production of β-lactamase (Baumann et al., 1968; Gilardi, 1973).

Genetic Identification

Members of the genus are themselves only rarely transformable, but can be easily identified by testing the ability of their isolated DNA to transform a nutritional or antibiotic resistance marker to the naturally competent strain BD413 isolated by Juni (1972). Transformation of this highly competent strain occurs readily either on semi-solid media or in liquid culture and has been used as the basis of a test for the identification of *Acinetobacter* in clinical specimens (Brooks and Sodeman, 1974). DNA samples from unrelated bacteria have consistently failed to transform *Acinetobacter* auxotrophs to prototrophy (Juni, 1972), and this test is consequently believed to allow unambiguous attribution of unknown strains to the *Acinetobacter* genus.

Biochemistry and Physiology

The main identifying biochemical and physiological characteristics of the genus have been outlined earlier in this chapter and only the most significant aspects are therefore reiterated here.

Although rare strains of *Acinetobacter* showing growth factor requirements have been isolated (Baumann et al., 1968; Warskow and Juni, 1972), the vast majority of isolates resemble saprophytic pseudomonads in being able to use any one of a large range of organic compounds as a carbon and energy source in an otherwise mineral medium. Although the utilization of carbohydrates is relatively uncommon, the major biochemical feature of the genus is that many strains are able to metabolize a range of compounds including aliphatic alcohols, some amino acids, decarboxylic and fatty acids, unbranched hydrocarbons, sugars, and many relatively recalcitrant aromatic compounds such as benzoate, mandelate, n-hexadecane, cyclohexanol, and 2,3-butanediol (Juni, 1978). Members of the genus are therefore particularly suitable organisms for studying a variety of unusual biochemical pathways, and they may also have a role to play in degrading a variety of pollutants and industrial products (see "Applications").

As indicated above, most strains are unable to utilize glucose as a carbon source, but occasional rare strains are able to do so via the Entner-Doudoroff pathway (Taylor and Juni, 1961). Many acinetobacters are, however, able to acidify media containing sugars, including glucose, via an aldose dehydrogenase (Hauge, 1960). This property has been previously considered to have major taxonomic significance in the subdivision of the genus, but DNA hybridization studies (Bouvet and Grimont, 1986) now suggest that this is not the case unless considered in combination with other unrelated biochemical properties.

Although most strains of *Acinetobacter* are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay, both nitrate and nitrite can be used as nitrogen sources by means of an assimilatory nitrate reductase (Jyssum and Joner, 1965). All acinetobacters are oxidase-negative, since they lack cytochrome *c*, but they do contain cytochromes *a* and *b* (Whittaker, 1971). All the enzymes of the glyoxylate cycle and the tricarboxylic acid cycle are normally present (Juni, 1978).

One important difference from many other organisms is that *Acinetobacter* cannot incorporate extracellular thymine or thymidine into DNA. Enzyme analysis has revealed that *Acinetobacter* lacks the enzymes thymidine phosphorylase, nucleoside deoxyribosyltransferase, and thymidine kinase, but does contain enzymes for conversion of thymidine-5′-monophosphate to thymidine-5′-triphosphate (Ovrebo and Kleppe, 1973).

Further details of the biochemical pathways mentioned in this section can be found in the detailed review by Juni (1978).

Genetics

All three of the major modes of gene transfer are known to occur in *Acinetobacter* and are discussed briefly below.

Transformation

The transfer of genetic material by transformation in a strain of *Acinetobacter* was first demonstrated by Juni and Janik (1969) and forms the basis of the genetic test for the identification of members of the genus (Juni, 1972). Genetic competence for transformation seems to be a rare trait in *Acinetobacter* (Juni, 1978), but has been used for preliminary genetic mapping of a few genes. Sawula and Crawford (1972) mapped the genes for tryptophan biosynthesis in strain BD413 and showed that they were arranged in a unique pattern consisting of three distinct clusters, as opposed to the single operon found in enteric bacteria. Similarly, Ginther (1977) analyzed proline auxotrophs in strain BD42 and showed that all the mutants belonged to three genetically distinct groups. Cruze et al. (1979) studied the conditions for quantitative transformation in strain BD413 and showed that competence occurred throughout the life cycle, but with a peak early in the exponential growth phase. The highest transformation frequencies (0.5 to 0.7%) were obtained in an aspartate-containing medium which allowed the most rapid growth of strain BD413. Recipient cell concentrations of 1×10^6 to 6×10^6 cells per ml were found to give the highest transformation frequencies, regardless of the DNA concentration. Similar findings were reported by Ahlquist et al. (1980) who demonstrated two peaks of competence for strain NCIB 8250, one during the early stages of batch culture and a second minor peak at the beginning of the stationary phase, and also reported that the presence of cyclic AMP increased the transformation frequencies obtained.

Transduction

A large variety of bacteriophages for various strains of *Acinetobacter* have been isolated from sewage (Blouse and Twarog, 1966; Herman and Juni, 1974; Twarog and Blouse, 1968). Most *Acinetobacter* phages are lytic, but one temperate phage (P78), which lysogenizes its host and is capable of mediating generalized transduction, has been isolated (Herman and Juni, 1974). Phage P78 is specific for its host strain and failed to lysogenize 389 other independently isolated strains of *Acinetobacter,* including the transformation-competent strain BD413. This narrow host specificity may be accounted for by the large number of different surface antigens found in this genus (Marcus et al., 1969). At present, neither P78 nor any other bacteriophage has been used for extensive genetic studies in *Acinetobacter.*

Conjugation

Conjugation in this genus was first reported by Towner and Vivian (1976a) using strain EBF 65/65 and the broad-host-range plasmid RP4 as a mobilizing vector. RP4 is capable of mobilizing the *Acinetobacter* chromosome and transferring chromosomal genes between different mutant derivatives of EBF 65/65. Transfer of the chromosome occurs at detectable frequencies only on solid surfaces (not in liquid matings) and mobilization occurs from at least two different chromosomal locations (Towner and

Vivian, 1976b). This system has been used to map 23 different mutations on a circular linkage group (Towner, 1978), and it has been suggested that the chromosomal organization of *Acinetobacter* differs from enteric bacteria, but is similar to *Pseudomonas,* in that it shows an absence of clustering of functionally related genes. Vakeria et al. (1984) subsequently added several genes concerned with mandelate metabolism to this map using conjugation mediated by RP4 and an indigenous self-transmissible plasmid designated pAV1 (Hinchliffe and Vivian, 1980). A variety of plasmids belonging to different incompatibility groups have now been shown to be capable of transfer by conjugation to *Acinetobacter* from enteric bacteria, although not all are stably maintained (Chopade et al., 1985; Towner and Vivian, 1977). Cloning vectors that are capable of transfer to and maintenance in *Acinetobacter* have also been described (Ditta et al., 1985; Singer et al., 1986).

Applications

Biodegradation of Industrially Relevant Aromatic Compounds

The biodegradation of aromatic compounds produced as industrial pollutants has been reviewed by Fewson (1981). The wide metabolic versatility of *Acinetobacter* means that particular strains have the potential to play an important role in the biodegradation of a wide range of aromatic compounds such as phenols, cresols, toluene, polyethylene glycol, polychlorinated biphenyls, and cyclohexane. Many such degradative pathways are still in the process of being elucidated, but it can already be envisaged that it may also be possible to isolate metabolic intermediates of commercial interest following the construction of mutant strains blocked at appropriate stages in a particular pathway. An important additional advantage is that members of the *Acinetobacter* genus are easy to isolate, cultivate, and manipulate genetically in the laboratory.

Production and Uses of Emulsan

The *Acinetobacter* strain RAG-1 is capable of utilizing a wide variety of hydrophobic growth substrates including crude oil, gas oil, several triglycerides, and middle-chain-length alkanes (Rosenberg et al., 1979a; Shabtai et al., 1985). Emulsan is the extracellular form of a polyanionic, cell-associated heteropolysaccharide produced by strain RAG-1 (Rosenberg et al., 1979b; Zuckerberg et al., 1979). The biopolymer has the property of stabilizing emulsions of hydrocarbons in water and seems to be required in a cell-bound form for growth on crude oil (Pines and Gutnick, 1986). Purified emulsan has a number of potential applications in the petroleum industry, including viscosity reduction during pipeline transport following formation of heavy oil/water emulsions, and production of fuel oil/water emulsions for direct combustion with dewatering (Gutnick and Minas, 1987). However, the affinity of purified emulsan for the oil/water interface also has implications for the stability of oil emulsions during transport and storage, and also for their biodegrability following accidental spillage. The effect of emulsan on the biodegradation of crude oil by pure and mixed bacterial cultures has been investigated in detail by Foght et al. (1989).

Production and Uses of Biodispersan

Most microbial surfactants that have been isolated and characterized act by either lowering the interfacial tension between oil and water or by stabilizing (or destabilizing) hydrocarbon/water emulsions (as described above). Rosenberg et al. (1988a) investigated the possibility that bacteria might also produce surfactants which adhere to the surfaces of inorganic materials. Two strains of *Acinetobacter* were isolated which produced extracellular polymers (termed biodispersans) capable of dispersing limestone in water. The active component of biodispersan has been purified and shown to be an anionic polysaccharide with an average molecular weight of 51,400 (Rosenberg et al., 1988b). Limestone is used in a wide variety of commercial industries, and purified biodispersan may have potential applications in several manufacturing processes producing common products such as paints, ceramics, and paper. It should be noted that whereas the production of hydrocarbon-in-water emulsifiers appears to be widespread among acinetobacters, emulsifying and dispersing activities appear to be due to different materials, with production of dispersants apparently restricted to a relatively small number of *Acinetobacter* strains (Rosenberg et al., 1988a).

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CHAPTER 165

The Family Azotobacteraceae

JAN HENDRIK BECKING

The family Azotobacteraceae is represented by two genera, *Azotobacter* (Beijerinck, 1901a, 1901b) and *Azomonas* (Winogradsky, 1938). The removal of the genera *Beijerinckia* and *Derxia* from the Azotobacteraceae was based on rRNA cistron analysis (De Smedt et al., 1980) and rRNA cistron similarities as observed in DNA-rRNA hybridization experiments (De Vos et al., 1985). Such experiments showed that *Beijerinckia* and *Derxia* are not closely related to the genera *Azotobacter* and *Azomonas,* but that they belong to other subdivisions or groups, i.e., the alpha and the beta subclasses, respectively of the Proteobacteria (see Chapter 100). On the other hand, investigations on rRNA similarities of various Gram-negative bacteria based on $T_{m(e)}$ values of DNA-rRNA hybrids showed that *Azotobacter* and *Azomonas* are closely related to one another and to the *Pseudomonas fluorescens* rRNA branch. The latter branch or group belongs to the Superfamily I in the nomenclature of De Ley and coworkers (see Chapter 100; De Smedt et al., 1980; and De Vos et al., 1985) or the gamma group/subdivision or subclass of the purple bacteria as defined by Woese et al. (1985a, 1985b) or the Proteobacteria, as defined by Stackebrandt et al. (1988).

Members of the Azotobacteraceae are primarily characterized as nonsymbiotic (i.e., free-living), aerobic, heterotrophic bacteria whose main property is the ability to fix molecular (atmospheric) nitrogen in a nitrogen-free or nitrogen-poor medium with an organic carbon compound (preferentially sugars, alcohols, or organic acids) as energy source. However, dinitrogen fixation is not unique to this family, as this property can be observed in quite a number of other unrelated bacteria (see Chapters 107 and 109). Moreover, some representatives of this family can produce associative growth (which is different from symbiotic growth) with higher plants. For these reasons, representatives of this family are usually called nonsymbiotic nitrogen-fixers.

General Habitats

Representatives of the Azotobacteraceae are regular inhabitants of soil, including aerially transported dust, of water habitats, and of plant surfaces such as the external environment of roots (rhizosphere) and leaves (phyllosphere). *Azotobacter chroococcum* and *A. vinelandii* also occur in marine habitats. Some species occur in much larger numbers in the rhizosphere of higher plants than in the soil itself, and it has been shown in some cases that this associative growth is beneficial for the plant because fixed nitrogen becomes available to the plant (Döereiner, 1966, 1968). Some investigators (see "Applications") report the same for *Azotobacter chroococcum* and a number of agricultural crops in India.

For *Azotobacter paspali* and the grass *Paspalum notatum,* the association seems to be species-specific (Döbereiner, 1966, 1970). Also, on leaf surfaces, members of this group (especially, *Azotobacter chroococcum*) often occur as nonpathogenic epiphytic flora, particularly on older or aged leaves. The bacteria probably proliferate at the expense of the sugar-rich and nitrogen-poor exudates of the plant; the exudates act as a kind of enrichment medium (see below). For the phyllosphere, it has been suggested that the fixed nitrogen becomes available to the plant (Ruinen, 1961), but an unambiguous proof using $^{15}N_2$ has not yet been presented.

Most species of this family occur in soil as well in water, but two *Azomonas* species, *A. agilis* and *A. insignis,* have so far only been isolated from water habitats. The pH value of the soil or water environment often governs the occurrence of a certain species. Therefore, the pH of the medium, in combination with selective carbohydrate utilization, are important factors for selective isolation of the various species in enrichment media.

General Identification

Morphological and physiological properties are both very important for the identification of Azotobacteraceae. All representatives have the ability to fix nitrogen in simple media. Cells are usually large, blunt to oval in shape, 2 μm or more in diameter. The morphology may change with various growth conditions. The cells are normally in pairs, but single cells or short chains may also occur. Motile by peritrichous or polar flagella or nonmotile. Intracellular poly-β-hydroxybutyrate is produced (Fig. 1). Cells are Gram-negative to Gram-variable (rarely). Endospores are not formed. Microcysts are formed in one genus *(Azotobacter).* They are chemoheterotrophic, preferentially utilizing sugars, alcohols, and salts of organic acids. Cultures are obligately aerobic and catalase-positive. They fix molecular nitrogen in a nitrogen-free or nitrogen-poor medium in air, but also under reduced oxygen pressure. Organic growth factors are not required. Trace elements (e.g., molybdenum or vanadium) are required because they are involved in nitrogenase activity. Normally, cells fix 10 mg of nitrogen per g of a suitable carbohydrate (usually glucose) in synthetic media containing 1–2% carbohydrate. However, the efficiency of dinitrogen fixation can be markedly increased (sometimes doubled up to 20–25 mg N/g carbohydrate) by lowering the carbohydrate levels (Becking, 1971), to levels such as usually occur in soils, or when under low oxygen tension (Meyerhof and Burk, 1928; J. H. Becking, unpublished observations; see also "Physiological and Biochemical Aspects").

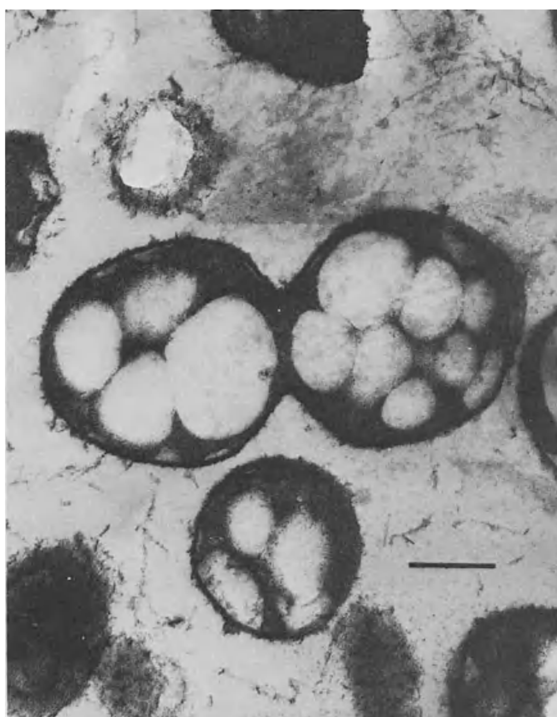


Fig. 1. *Azotobacter chroococcum.* Thin section through cells demonstrating the presence of large, poly-β-hydroxybutyrate globules as reserve material. Many cells occur in pairs in the so-called diplococcus stage. Transmission electron micrograph. Bar = 0.5 μm.

They can utilize various sources of combined nitrogen, but some species utilize nitrate poorly or not at all. Water-insoluble and water-soluble pigments or fluorescent pigments are produced by some species.

In the genus *Azotobacter,* microcysts are formed in older cultures grown with sugar as carbon source. In some species, a medium containing butan-1-ol as organic substrate (0.1–0.2 ml is added prior to pouring of the agar plates) enhances cyst formation (Tchan and New, 1984). A microcyst can be distinguished from an endospore by its characteristic structure: a central body surrounded by a cyst coat, consisting of an exocystorium and an exine. In contrast to a spore, the cell inside the cyst is similar to the vegetative form and there are no cytological changes in the cell prior to its germination (Socolofsky and Wyss, 1961; Tchan et al., 1962). The primary habitat is soil, water, and the plant rhizosphere and phyllosphere. In this family, the GC content of the DNA ranges from 52–67.5 mol%. The type genus is *Azotobacter* Beijerinck 1901b, 567.

Differentiation from Other Nitrogen-Fixing Bacteria

Although some rhizobia may fix nitrogen nonsymbiotically, unlike *Azotobacter,* they can only do so under reduced oxygen tension. Furthermore, their cells are generally smaller than *Azotobacter* cells (*A. paspali* excepted). Moreover rhizobia need a more complex medium (supplemented with growth substances, etc.) for growth. Other nonsymbiotic nitrogen-fixing organisms have a different cell morphology and widely different physiological and nutritional requirements depending on the taxonomic group of the Prokaryote class to which they belong.

Genus Differentiation of Azotobacteraceae

Azotobacter: Microcysts formed; GC content is 63–67.5 mol% (T_m).
Azomonas: Microcysts not formed; GC content is 52–59 mol% (T_m).

Isolation

Species of the Azotobacteraceae are typical aerobic, chemoheterotrophic, dinitrogen-fixing bacteria. Therefore, any medium of suitable pH value that contains an organic carbon source, minerals (especially phosphate), some trace elements (in particular, molybdenum and/or vanadium), and no combined nitrogen is suitable for enrichment, since only organisms that can grow at the expense of atmospheric nitrogen are able to develop.

Members of this group fix dinitrogen better at low oxygen tension, probably because fixation is a reductive process (i.e., nitrogenase is inhibited or inactivated by oxygen). However, in enrichment cultures, care should be taken to provide sufficient aeration of the medium, in order to suppress the development of anaerobic or facultatively anaerobic bacteria (evident by a characteristic butyric acid smell of the enrichment culture), which may develop when the oxygen pressure is low or when the oxygen is totally exhausted. Therefore, thin-liquid layers in Erlenmeyer flasks or petri dishes are recommended to allow sufficient oxygen access. This precaution is not absolutely obligatory for enrichment of most *Azotobacter* species, since in static culture, they usually form a pellicle on the liquid surface. It is advisable to leave this pellicle undisturbed, because careless handling or shaking of the enrichment culture may rupture the pellicle and cause the cells to precipitate as a sediment, which stimulates the development of the above-mentioned anaerobic or facultative anaerobic contaminating bacteria always present in enrichment cultures.

General Enrichment Procedures

Members of the family Azotobacteraceae can be enriched from soil or water by adding phosphate (0.1%) and an organic carbon source, usually 1–2% sugar or a calcium (or sodium) salt of an organic acid, to one of the above substrates as will be described below. Usually, an organic carbon source and phosphate are the minimum nutrients that must be added to obtain the development of Azotobacteraceae under natural conditions in soil or water. Using this principle, a number of simple enrichment procedures can be designed for the successful isolation of members of this group.

ISOLATION OF AZOTOBACTERACEAE BY THE SOIL-PASTE PLATE METHOD. A small amount of soil (about 30–50 g) is mixed in a porcelain mortar with about 0.5–1.0 g of organic carbon (e.g., sucrose, glucose, or mannitol), about 0.5 g of chalk ($CaCO_3$) to assure an alkaline reaction, 4 drops (0.12 ml) of a 10% aqueous K_2HPO_4 solution, and 4 drops of a 10% aqueous $MgSO_4$ solution. When the water content of the soil is low, some extra distilled water is also added in order to obtain a soil paste. If the soil is too sandy, or is difficult to make into paste for other reasons, sterile clay mineral (kaolinite) is added. The soil paste is transferred with a sterile knifepoint or spatula to a small, low-rimmed container, watch glass, or hollow gypsum block, and the soil is firmly pressed into the hollow surface. With the aid of a knife or spatula, preferentially sterile, the surface of the soil is neatly smoothed. The small container with the soil is subsequently placed in a petri dish containing a wet piece of filter paper and incubated at 27–30°C. Care should be taken that the top of the soil does not touch the upper lid of the petri dish, thus causing anaerobic conditions. After 3–7 days incubation, glistening, slimy *Azotobacter* colonies develop on the smooth soil surface. Colonies of *A. chroococcum* turn brown in a few days.

ISOLATION OF AZOTOBACTERACEAE BY THE SIEVED-SOIL PLATE METHOD. In this method (Winogradsky, 1932), silica-gel plates are prepared by treating a sodium-silicate solution with acid to have a complete inorganic substrate. The plates are impregnated with nitrogen-free, carbohydrate-containing nutrient solution suitable for the cultivation of Azotobacteraceae. Before impregnation, the excess salt (NaCl, when HCl is used for gel preparation) is washed out in running tap water, and then the plates are rinsed with sterile, distilled water. The solid medium is allowed to dry in an incubator. Subsequently, the plates are impregnated with medium, the superfluous medium is poured off, and the remaining water is evaporated in an incubator. The dry plates are seeded with soil crumbs; sieved soil is used preferentially (either sieved directly over the plate or distributed on the plate with a spatula) in order to obtain small soil particles and an even distribution. After incubation at 27–30°C, colonies of members of soil-inhabiting Azotobacteraceae develop on the gel around the soil particles.

The preparation of silica gel is rather laborious, but good quality agar can also be used, i.e., agar low in combined nitrogen. Other agars can also made sufficiently low in combined nitrogen by some preparations, i.e., by fermentation of the agar in distilled water and by repeated removal and renewal of the rinsing solution. The treated agar (10–20 g/l) can be made up with Azotobacteraceae nitrogen-free medium (see below), and the nutrient agar plates inoculated with sieved soil particles in

the manner mentioned above for the silica plates. As Azotobacteraceae medium, Winogradsky's nitrogen-free medium can be used. It has the following composition (g/l): KH_2PO_4 50.0; $MgSO_4 \cdot 7\ H_2O$, 25.0; NaCl, 25.0; $FeSO_4 \cdot 7H_2O$, 1.0; $Na_2MoO_4 \cdot 2\ H_2O$, 1.0; and $MnSO_4 \cdot 4H_2O$, 1.0; the pH is adjusted to 7.2 with NaOH. This is a stock solution, which can be stored indefinitely at room temperature; the medium is prepared by using 5.0 ml of stock solution and 0.1 g $CaCO_3$ per liter of distilled water (see Tchan and New, 1984). The medium is sterilized at 120°C for 20 min. The organic substrate (10–20 g/l) is added to this organic medium. Some sugars, including glucose, must be sterilized separately before addition to the sterilized mineral medium. The medium is usually solidified with 15.0 g agar/per liter.

ISOLATION OF AZOTOBACTERACEAE BY THE NUTRIENT SOLUTION METHOD. A complete nutrient medium is used (see e.g., Winogradsky's nitrogen-free mineral medium supplemented with a carbohydrate source as mentioned above). This medium is preferably sterilized in order to eliminate contamination from other sources. The liquid medium is usually placed in shallow layers in Erlenmeyer flasks or in petri dishes, and about 0.3–0.5 g of wet soil of the sample to be tested is used to inoculate 100–150 ml of enrichment medium.

For the investigation of distribution of members of Azotobacteraceae in water samples, usually an organic carbon source (5–20 g/l) is added to the water sample itself. Sometimes the sample is supplemented with some inorganic nutrients (particularly phosphate and molybdenum). The water sample can also be mixed with an equal volume of sterile nutrient solution of double strength. These two methods, in general, gave better results than the inoculation of a complete medium with a small amount of the water sample (e.g., 1–5 ml water sample added to 100–150 ml medium), probably because of the lower density of Azotobacteraceae in normal natural water sources compared to soil.

As already mentioned with use of nutrient solutions, the depth of the liquid layer employed should not be too high; if the liquid layers are too thick, anaerobic conditions may occur at the bottom of the flasks, resulting in growth of contaminants which inhibit the growth of Azotobacteraceae.

Representatives of the Genus *Azotobacter*

These are soil, water, plant rhizosphere, and phyllosphere organisms; therefore, all these substrates can be used as inoculum. Many members of this genus produce copious amounts of capsular slime (polysaccharide). No endospores are formed, but some form thick-walled microcysts, which, unlike spores, are encysted vegetative cells without cytological changes prior to their germination. The cells show motility by peritrichous flagella (Fig. 2) or they are nonmotile. The cells are Gram-negative. Although some species appear to be Gram-variable (e.g., Jensen and Peterson, 1954; Kirakosyan and Melkonyan, 1964; Norris and Kingham, 1968; Johnstone, 1974), but Thompson and Skerman (1979) attribute these results to incomplete decolorization of thick smears.

Identification of *Azotobacter* Species

Cells are motile in *Azotobacter chroococcum, A. vinelandii, A. armeniacus,* and *A. paspali,* but motility is absent in *A. beijerickii* and *A. nigricans.*

Nonmotile variants have been described in *A. vinelandii.*

Excretion of water-soluble, yellow-green, fluorescent pigment occurs in *A. vinelandii* and *A. paspali* and that of red-violet or brownish-black pigment in *A. nigricans, A. armeniacus,* and sometimes *A. paspali* under certain conditions.

Rhamnose as the sole source of carbon can only be utilized by *A. vinelandii* and not by any other species. For a list of other specific carbon compounds and inhibitory substances, see the specific enrichment media given below for the various species.

Azotobacter chroococcum

Cells of *A. chroococcum* are pleiomorphic, bluntly rod-, oval-ovoid-, or coccus-shaped. Mean dimensions are 3.0–7.0 μm long × 1.5–2.3 μm wide. The cell shape changes dramatically in time or with changes in growth (medium) conditions. Cells are often in pairs. Young cells are motile by peritrichous flagella. Microcysts and capsular slime are formed. Colonies are moderately slimy, turning black or black-brown on aging. The pigment produced is not water-diffusible.

SOIL-PASTE METHOD FOR ISOLATION OF *AZOTOBACTER CHROOCOCCUM.* The soil-paste method described under the general techniques is a quick and easy method to obtain this species from soil. Also the sieved-soil plate method and the nutrient solution method are adequate for obtaining *A. chroococcum* from soil. Appar-

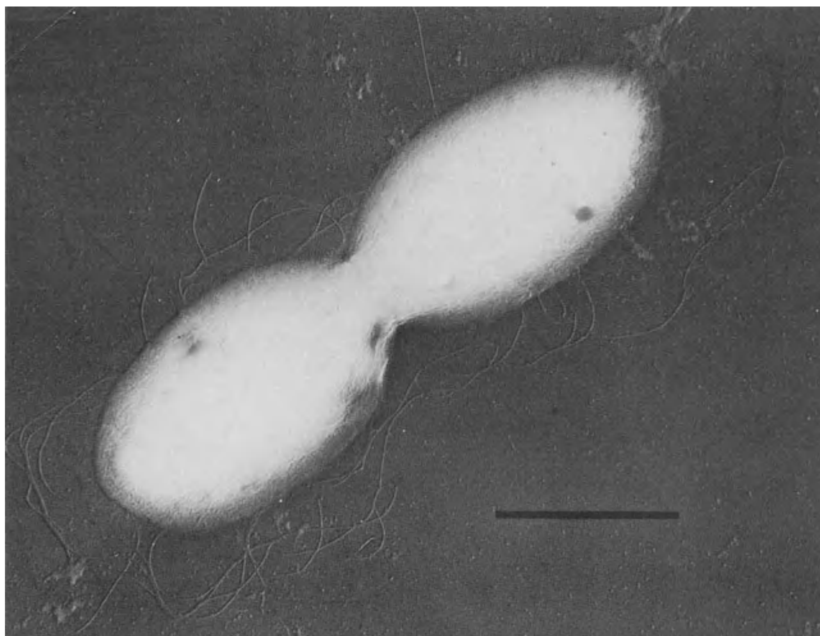


Fig. 2. *Azotobacter chroococcum.* Two cells in a pair ("diplococcus" stage) showing peritrichous flagella. Preparation shadowed with an alloy of gold and manganese. Transmission electron micrograph. Bar = 2.0 μm.

ently, because *A. chroococcum* is the most common and predominant *Azotobacter* in soil, no special enrichment techniques are necessary to make it selectively dominant.

NUTRIENT SOLUTION FOR ISOLATION OF *AZOTOBACTER CHROOCOCCUM.* The nutrient solution method, originally described by Beijerinck (1901a, 1901b) for the isolation of *A. chroococcum,* is also a good and well-established method for enrichment and isolation of *A. chroococcum* from soil and water samples.

Nutrient Solution Method

To a 500-ml Erlenmeyer flask, about 100 ml of nitrogen-free nutrient solution of the following composition is added:

Distilled water	1 liter
Glucose	20.0 g
$CaCO_3$	20.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g

Adjust to pH 7.2-7.6.

In some cases it is advisable to also add trace elements, especially molybdenum ($NaMoO_4 \cdot 2\ H_2O$, 0.005 g/l), to the medium. The medium is inoculated with about 0.3–0.5 g of soil; for water samples, the distilled water of the prescription is replaced by the surface water tested. After 1–3 days, an *Azotobacter* pellicle forms on the liquid surface.

Due to anaerobic conditions below the pellicle, nitrogen-fixing butyric acid bacteria (e.g., *Clostridium pasteurianum*) may develop later at the bottom of the flask, and eventually throughout the medium. It is therefore advisable to subculture the *Azotobacter* pellicle according to the normal procedures (by applying appropriate dilutions in sterile tap water) as soon as possible to nitrogen-free agar plates in order to reduce contamination. Such subculturing is also advisable because combined nitrogen produced by *Azotobacter* development may make it possible for non-dinitrogen-fixing contaminants to develop.

The following agar medium can be used for the isolation, purification, and further subcultivation of *Azotobacter chroococcum* found in the enrichment cultures:

Agar Medium for *Azotobacter chroococcum*

Distilled water	1 liter
Glucose	20.0 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeCl_3 \cdot 6H_2O$	0.10 g (or 0.05 g)
$CaCl_2 \cdot 2H_2O$	0.05 g
or $CaCO_3$	20.0 g
$NaMoO_4 \cdot 2H_2O$	0.05 g
Agar	20.0 g

Adjust to pH 7.4–7.6.

With $CaCl_2$, the agar medium is translucent; with $CaCO_3$ it is opaque white. The latter substance is, however, in some cases an advantage for detecting *Azotobacter* colonies, because they do not produce acid on it. Acid-producing colonies, as evident by the dissolution of the calcium carbonate around the colony, are certainly not *Azotobacter* species. A further advantage for

adding $CaCO_3$ to the medium is that it has a good buffering capacity at the alkaline side; an alkaline reaction is necessary for the development of *Azotobacter chroococcum.*

The presence of *A. chroococcum* in soil or water is strongly governed by the pH value of these substrates. In an environment below pH 6.0, *Azotobacter* is generally rare or totally absent (see Becking, 1961). H. L. Jensen (1965) tested 264 Danish soils and found that practically all of the soils above pH 7.5 contained *Azotobacter* (predominantly *A. chroococcum*) varying in numbers between 10^2 and 10^4 per gram of soil. Of the 148 tropical soils tested by Becking (1961), all soils above pH 7.5 (pH range 7.5–9.0) contained *Azotobacter* (also mainly *A. chroococcum*) and, in the pH ranges of 7.0–7.4, 6.5–6.9, and 6.0–6.4, the percentage of *Azotobacter*-positive soils was 89, 57, and 32%, respectively. In nitrogen-free nutrient media, the lower pH limit for growth of *A. chroococcum* strains in pure culture is between pH 5.5 and 6.0 (Jensen and Petersen, 1955).

As already mentioned earlier (Becking, 1962, 1981), according to the author's experience, not all *Azotobacter* strains that produce a brown or brown-black pigment on aging (Fig. 3) belong to one species, because *Azotobacter chroococcum* strains are very pleomorphic (see Fig. 4). It was therefore likely that this species comprises a more complex group consisting of several species, whose delimitations have not yet been sorted out. The latter is confirmed by Thompson and Skerman (1979) who examined 151 strains of Azotobacteraceae of different provenance in 230 variant tests and analyzed the data according to numerical methods in a hierarchical classification of groups. They obtained evidence that at least two distinct black-brown-pigmented *Azotobacter* species occur next to *A. chroococcum,* i.e., *A. nigricans* and *A. armeniacus* (see below).

Azotobacter nigricans

This species, originally isolated by Krasil 'nikow (1949), possesses, in contrast to *A. chroococcum* (but like strains of *A. beijerinckii*), nonmotile cells. Cells are bluntly rounded rods in shape, occurring singly or in pairs. Mean cell dimensions are 4.1–4.9 μm long $\times$ 1.5–2.7 μm wide. Moreover, they differ from *A. chroococcum* in a number of nutritional properties, such as the inability to utilize ethanol, pentan-1-ol, propionate, caproate, and benzoate and to produce brown-black or red-violet diffusible pigments. They differ from *Azotobacter armeniacus* by the inability to utilize caprylate, which compound can be used as carbon source by the

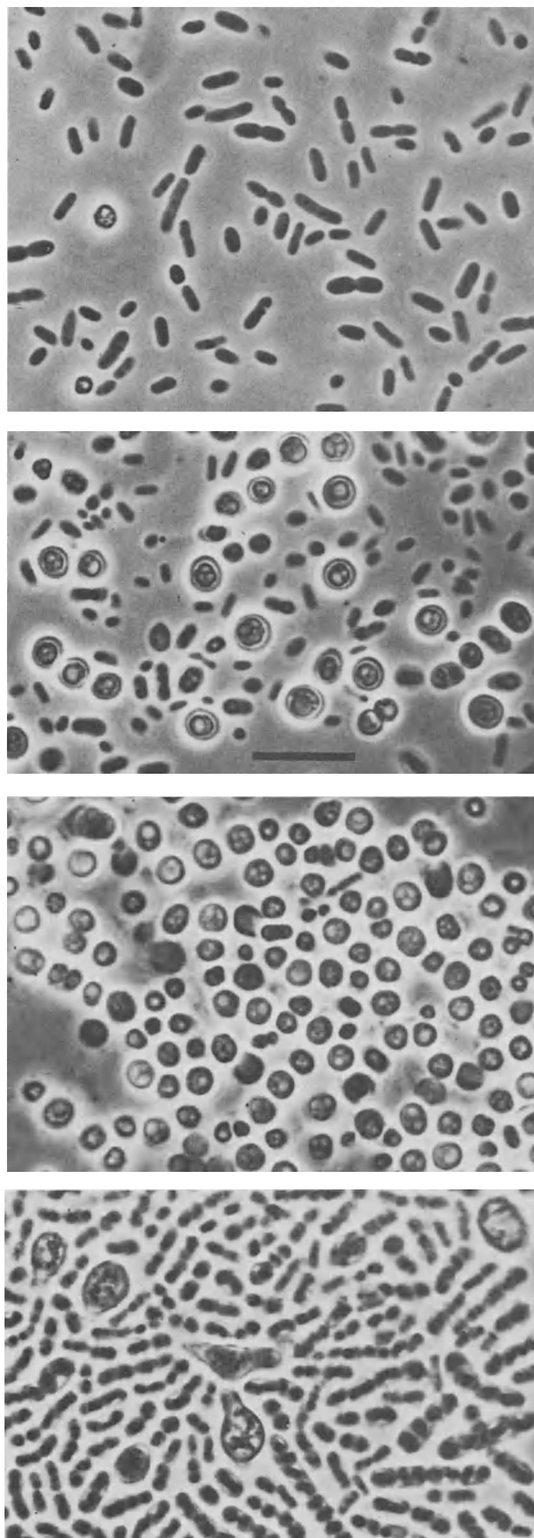


Fig. 3. *Azotobacter chroococcum* cells. Four different strains of the same age growing on an identical medium (nitrogen-free, mineral glucose agar with 2% calcium carbonate). Note the presence of lipoid-filled cells, cysts, and germinating cysts in some of the strains. Living preparations; phase contrast micrographs. Bar = 10 μm.

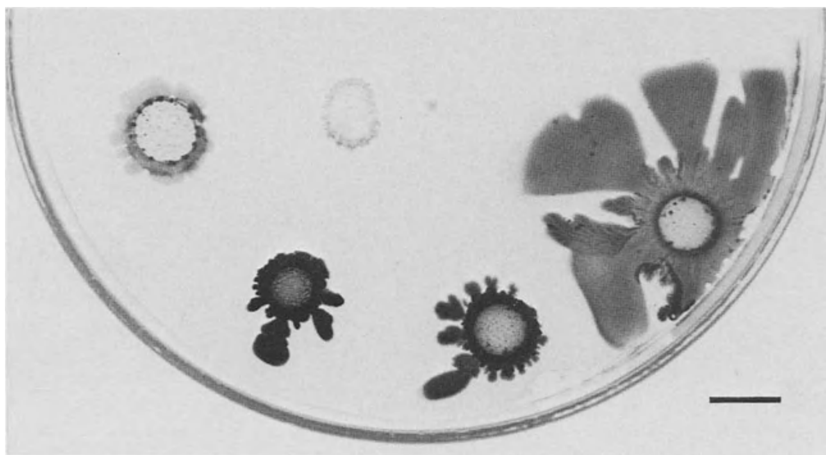


Fig. 4. *Azotobacter chroococcum* colonies. Strains growing on the same medium (nitrogen-free, mineral glucose agar with calcium carbonate) and of the same age, showing the variation of the chromogenesis of the colonies. Bar = 1 cm.

latter species. In *A. nigricans,* the production of agar-diffusible homopolysaccharides from sucrose or raffinose is strain variable, but no strain produces any colony-retained homopolysaccharide. On aging, colonies of *A. nigricans* turn black-brown due to the same diffusible pigment, but a variant formerly called *A. beierinckii* subsp. *achromogenes* Jensen and Petersen 1954, but now proposed to be *A. nigricans* subsp. *achromogenes* (Thompson and Skerman, 1979) produces only a yellow nondiffusible pigment within the colony and no diffusible pigment.

Strains of this species has been isolated from European soils, particularly from East European soils.

There is no species-specific selective enrichment method for isolating *A. nigricans.*

Azotobacter armeniacus

This is a poorly understood species that has been described by Thompson and Skerman (1981). Cells are bluntly rounded rods, occurring singly or in pairs. Mean cell dimensions are 5.0–5.7 μm × 1.7–2.0 μm. Strains of this species differ from *A. nigricans* by having motile cells, but they have in common with the latter species the production of a diffusible brown-black or red-violet pigment into the medium. In contrast to *A. nigricans,* they are able to use citrate or D-galacturonate as sole carbon source and most strains also can use *n*-valerate and caprylate, but like *A. nigricans,* capronate is not utilized. All *Azotobacter* species, including *A. nigricans,* can utilize ammonium and nitrate as sole source of nitrogen, and these N-sources are assimilated in preference to molecular nitrogen, *A. armeniacus* is unable to use ammonium, nitrate, or glutamate as sole source of nitrogen for growth (Thompson and Skerman, 1979).

There is no special enrichment medium for *A. armeniacus,* but probably it can be enriched with caprylate as sole source of carbon, but *A. vinelandii* also can be grown on this medium. Further isolation, however, will differentiate between these two species.

Azotobacter beijerinckii

Formerly, this organism was not regarded as a distinct species, but merely as a nonpigmented variant of *A. chroococcum.* In the eighth edition of *Bergey's Manual* (Buchanan and Gibbons, 1974), it has, however, been restored to the species level. Cells are bluntly ended rods or ellipsoidal, occurring singly or in pairs, sometimes in short chains. Mean cell dimensions are 3.2–5.3 μm long × 1.7–2.7 μm wide.

The main differences between *Azotobacter beijerinckii* and *A. chroococcum* are that colonies of *A. beijerinckii* produce, on aging, a yellowish or cinnamon pigment (in *A. chroococcum,* it is brown or blackish-brown) and its cells are nonmotile (motile in *A. chroococcum*). Moreover, it invariably lacks the ability (in contrast to *A. chroococcum*) to utilize starch as the sole source of carbon. Sometimes the inability to utilize mannitol is also mentioned as a determinative character (see Johnstone, 1974), but 9 out of 10 strains tested by Jensen and Petersen (1955) showed good growth on this carbon source. Thompson and Skerman (1979) mentioned in addition, that caproate is utilized by *A. chroococcum,* but not by its satellite species, *A. nigricans* and *A. armeniacus.* The differences between *A. nigricans,* which is also a species with nonmotile cells, and *A. beijerinckii* are not so clear with respect to carbon source utilization, except that malonate is used by all strains of *A. beijerinckii,* but only by a minority of strains of *A. nigricans,* and that D-glucuronate, D-galacturonate, and benzoate are utilized by *A. beijerinckii* but not by *A. nigricans*

(Thompson and Skerman, 1979). The main difference remaining is the pigment, which in *A. nigricans* is black-brown to red-violet, whereas the excretion of any water-soluble pigment into the substrate is completely absent in *A. beijerinckii.* The physiological differences between *A. chroococcum* and *A. nigricans* or *A. armeniacus* are already outlined under the headings of the latter two species.

Tchan (1953) described a variant of *A. beijerinckii,* named *A. beijerinckii* subsp. *acido-tolerans,* which could grow and fix dinitrogen at a pH of 4.75. Later, Tchan and New (1984) delineated this characteristic more in detail and distinguished two subgroups. An acid-tolerant subgroup and another subgroup distinguished by its sensitivity to 0.05% phenol or 40 μg/ml diamond fuchsin, its inability to utilize sorbitol or aconitate, and its failure to produce diffusible homopolysaccharides. V. Jensen and Petersen (1954) have also described another form of *A. beijerinckii, A. beijerinckii* subsp. *achromogenes,* in which under the cultural conditions applied, no pigment ever was produced. The latter strains were isolated from Danish calcareous forest soils in a survey including all types of Danish soils (see later).

Jensen and Petersen (1954) also showed that acid tolerance is a rather common characteristic of *A. beijerinckii,* since all strains isolated could grow and fix dinitrogen to a pH of 5.1. The nitrogen-fixation data published by Jensen and Petersen (1955) showed, however, that considerably more atmospheric nitrogen was fixed under alkaline than under acidic conditions.

For selective enrichment of *A. beijerinckii* and elimination of *A. chroococcum,* a selective nitrogen-free medium of the same composition as used for *A. chroococcum,* but with $CaCl_2$ (instead of $CaCO_3$) and with a slightly acid pH (pH 4.9–5.5), can be employed. So far, no experience has been gained with such a medium since all *A. beijerinckii* strains so far known are casual isolates coming from *A. chroococcum* plates. It might be surmised that *A. beijerinckii* would favor somewhat acidic soils. In this respect it is remarkable that, in the above-mentioned survey of Danish forest soils by V. Jensen and Petersen (1954), *A. beijerinckii* was found predominantly in soil samples of two localities with beech *(Fagus sylvatica)* forest on calcareous soil with pH values of 7.0–8.0 and 7.8–8.0, respectively. In these and nearly all other forest soils tested, *A. chroococcum* was absent or nearly absent. These observations indicate that some calcareous forest soils are a favorable and probably selective habitat for *A. beijerinckii* and that the occurrence of *A. chroococcum* is confined to the more alkaline agricultural soils.

Azotobacter vinelandii

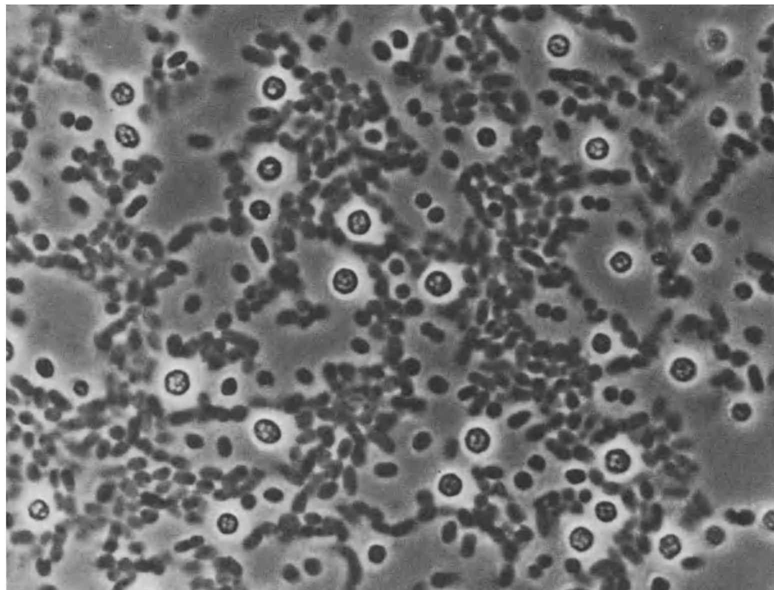
Cells are rounded-ended rods, occurring singly or in pairs. Mean cell dimensions are 3.0–4.5 μm long × 1.5–2.4 μm wide (Fig. 5). Cells are motile with numerous peritrichous flagella; very rarely, some are nonmotile. Colonies are nonpigmented. They excrete a yellow-green, fluorescent, water-soluble pigment into medium.

Fewer strains of *Azotobacter vinelandii* have been isolated in comparison with strains of *A. chroococcum* and *A. beijerinckii.* Most of the strains were casual isolates obtained from soil, such as the original strain of Lipman (1903a) from New Jersey (USA) soil (pH unknown). Wilson's strain O(P), used for many physiological and biochemical studies (e.g., Wilson and Knight, 1947; Shutter and Wilson, 1955), is probably a subculture of this strain. Bortels (1930) isolated another strain from German soil (originally described as *Azotobacter agile*), and Winogradsky (1932) obtained one strain from soil in France. Later, Winogradsky (1938) also underlined the relative rarity of this species.

The present author has obtained a number of strains—often in association with *A. chroococcum*—from Dutch soil, but also from freshwater samples using the nutrient solution method for *A. chroococcum* (preferentially with ethanol as carbon source) for enrichment (J. H. Becking, unpublished observations). *A. vinelandii* was obtained mostly from rather alkaline Dutch and European soils, such as calcareous soils and soil derived from marine sediments, sea sludge, or sea muds that had been pumped up for leveling land. The latter soils, usually also rather rich in sodium chloride, showed pH values of 8.0–9.5.

The water samples from which this organism was obtained were pond, lake, or marsh water of alkaline reaction (pH 7.5–8.0). This species was also numerous in some tropical soils, such as alkaline sea muds and mainly calcareous soils of Indonesia and certain very alkaline soils of Bolivia and other localities in South America (Becking, 1961; J. H. Becking, unpublished observations). Clearly, alkaline conditions are favorable for the occurrence of *A. vinelandii* and probably selective for its distribution. Further, this species may be halophilic to some degree, or at least resistant to salinity, in view of isolation from alkaline sea muds. The presumed halophilic properties need, however, experimental confirmation.

Derx (1951b) designed a method for selective isolation of this species from soil and water sources. The underlying principles of this method were the addition to the enrichment medium of sodium benzoate (1.0%) as an inhibitor in order to suppress the development of



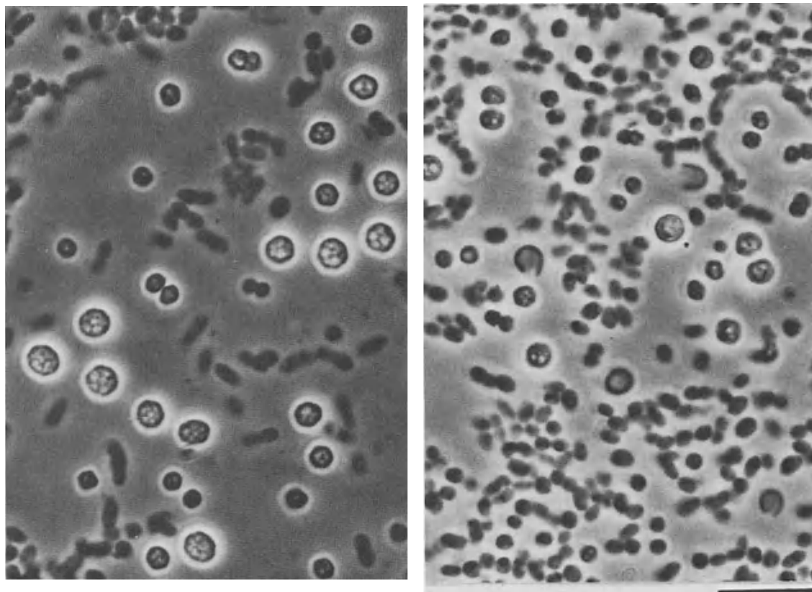


Fig. 5. *Azotobacter vinelandii* cells. Three different strains growing on the same medium (nitrogen-free, mineral glucose agar) showing some variation in the cell size of the strains and in the number of lipoid-filled cells, cysts, and germinating cysts. Living preparation, phase contrast micrographs. Bar = 10 μm.

A. chroococcum, and the use of a special carbon source, such as mannitol or ethanol, which is very readily assimilated by *A. vinelandii.* Derx's method was a further extension of earlier work of Reuszer (1939), who observed that by applying benzoate, benzoic acid, or another phenolic compound to soil, the normal population of *Azotobacter chroococcum* and *A. beijerinckii* was replaced completely by a green-pigment-producing form of *Azotobacter,* which had not been found before in the soil.

Derx's Medium for Selective Enrichment of *Azotobacter vinelandii* (Derx, 1951b)

Distilled water	100 ml
Mannitol	0.5 g
or ethanol	1.0 ml
K_2HPO_4	0.5 g
Sodium benzoate	1.0 g
Adjust to pH 7.5–8.0.	

For the isolation of this species from soil, ethanol is preferentially used as carbon source; for its isolation from water samples, the use of mannitol as carbon source is recommended since *Azomonas agilis* (see "Representatives of the Genus *Azomonas*") is usually unable to assimilate this compound. According to the present author, the rather alkaline reaction induced by the addition of sodium benzoate may also

be responsible for the selective properties of this medium for the enrichment of *A. vinelandii.*

Numerous strains of *A. vinelandii* were obtained by the present author from Dutch water habitats, such as the Rhine River, and pond and lake water (usually of pH 7.0–7.5) by using Derx's medium with mannitol as carbon source and the supplement of sodium benzoate. In all cases, the enrichment medium was adjusted to pH 8.0–9.0. Use was made of 300-ml Erlenmeyer flasks with 50 ml of medium or 100-ml Erlenmeyer flasks with 20 ml of medium. For the water samples, the sample itself, instead of distilled water, was used as liquid to which the carbon source and salts were added.

V. Jensen (1961) mentioned that L-rhamnose (1.0%) could be used as a carbon source for the selective enrichment of *A. vinelandii,* since only a very small fraction of the strains of the other *Azotobacter* and *Azomonas* species tested could utilize this compound. When grown in pure culture on L-rhamnose, *A. vinelandii* develops profusely within 3–5 days at 25°C, while the other *Azotobacter* species that can utilize this compound only produce some development after 1–2 weeks. Therefore, in spite of the presence of other *Azotobacter* species, *A. vinelandii* soon becomes dominant in such an enrichment medium.

Using the L-rhamnose enrichment medium, V. Jensen (1961) observed *A. vinelandii* to be sparsely distributed in normal soil. It was only present in a very few garden soils and seemed to be restricted to the most fertile soils. Although Johnstone (1974) reported that *A. chroococcum* and *A. beijerinckii* do not utilize L-rhamnose as sole source of carbon, according to the present author, this is not always true. Utilization of L-rhamnose is not a common feature of strains of these species, when these are obtained as random isolates with the more common sugars, but some strains utilizing this carbon source can be secured in enrichment media by using L-rhamnose as sole source of carbon. Also, Thompson and Skerman (1979) observed that one of the 19 *A. chroococcum* strains tested could utilize L-rhamnose, while Claus and Hempel (1970) isolated both *A. chroococcum* and *A. beijerinckii* from soil and water samples with L-rhamnose as sole substrate.

Claus and Hempel (1970) observed that resorcin, ethylene glycol, or glutarate, all in 0.1 or 0.2% (wt/vol) concentration, are very selective carbon sources for *A. vinelandii,* and that a number of strains could be isolated from soil by using these compounds as sole source of carbon in enrichment media. The above-mentioned carbon compounds apparently cannot be utilized by the more common *Azotobacter* species.

A. vinelandii strains have numerous peritrichous flagella, in general, but Derx (1951b) isolated nonflagellated strains of *A. vinelandii* for which he proposed the name *Azotobacter non-vinelandii,* which name is, however, considered not to be validly published under Rule 23 of the Bacteriological Code (Lapage et al., 1975). Another characteristic of these strains, which distinguishes them from typically flagellated strains of *A. vinelandii,* is the ability to produce a black pigment in the presence of benzoate (Derx, 1951b).

In multiple tests for numerical analysis, Thompson and Skerman (1979) confirmed that all *A. vinelandii* strains tested could readily utilize rhamnose as sole source of carbon, in contrast to most strains of other species. Moreover, they observed that *A. vinelandii* could utilize caproate, caprylate, and *meso*-inositol, compounds which generally are not utilized by the other *Azotobacter* species. Furthermore, 0.1% phenol can be used in enrichment cultures to inhibit the growth of other *Azotobacter* species, and incubation at 37°C particularly favors the development of this species. According to these authors, the nonmotile *Azotobacter non-vinelandii* strains may be regarded as a special subgroup that differs from typical *A. vinelandii* strains by the inability to utilize raffinose and by the utilization of pimelate, suberate, and sebacate as sole sources of carbon. In addition, in contrast to typical *A. vinelandii,* these strains were resistant to brilliant green at a concentration of 10 μg/ml.

The presence of *A. vinelandii* in enrichment media can be detected by a color change of the medium, which turns yellow, green, or violet. On nitrogen-free, mineral agar plates, a water-soluble yellow, green, or violet fluorescent pigment is excreted into the medium, but they do not produce any nondiffusible pigment. Pigment production into the substrate is stimulated by low-iron concentration or iron deficiency of the medium (Becking, 1962). As shown by Johnstone (1955, 1957b) and Johnstone and Fishbein (1956), the fluorescence of the pigment under ultraviolet light shifts with pH changes, and different fluorescence curves are obtained with the diffusible pigments of *A. vinelandii* and *Azomonas agilis.* Thus, fluorescence measurements also have diagnostic value in distinguishing between both species.

Azotobacter paspali

Cells are long, bluntly ended rods. Mean cell dimensions are 7.0–10.9 μm long × 1.3–1.7 μm

wide; sometimes shorter rods of 3.2–4.2 μm long × 1.6–1.9 μm wide are seen. Cells in young cultures often form long filaments. A yellow-green, fluorescent or red-violet, water-soluble pigment is excreted into the medium.

This species, described by Döbereiner (1966), was originally isolated on Winogradsky's silica-gel plates impregnated with a mineral salt solution (see sieved-soil plate method) with a pH of 6.5 and calcium citrate as sole source of carbon. Later (Döbereiner, 1970), a N-free, mineral sucrose agar (a modification of Lipman's [1903b, 1905] medium) was recommended, because it gave satisfactory results and it was much easier to prepare.

Nitrogen-free, Mineral Sucrose Agar for Isolation of *Azotobacter paspali* (Döbereiner, 1970)

Distilled water	1 liter
K_2HPO_4	0.05 g
KH_2PO_4	0.15 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$CaCl_2$	0.02 g
$CaCO_3$	1.0 g
$Na_2MoO_4 \cdot 2H_2O$	0.002 g
$FeCl_3$ (10%, aqueous solution)	1 drop
Bromthymol blue (0.5%, ethanol solution)	10 ml
Sucrose	20 g
Agar	20 g

Adjust the pH to ca. 7.0.

Plates with this medium were inoculated with root-surface (socalled rhizoplan) soil of a grass species, *Paspalum notatum.* The soil (about 20–50 mg per plate) was usually scattered directly from the roots on the plates. However, the soil also can first be passed through a sieve (0.5-mm mesh), and then 50 mg can be weighed out and scattered over the plates for quantitative tests. The plates were incubated at 35°C.

Differentiation of *A. paspali* from the other *Azotobacter* species on silica-gel plates with calcium citrate as carbon source is relatively easy. Colonies appear 4–5 days after inoculation and these produce an intense yellow pigment, which diffuses into the silica gel below the colony. The colonies are relatively small and raised and readily solubilize the opaque white calcium citrate layer at the top of the silica gel, giving the appearance of many little holes. Later, the colonies spread rapidly and become flat.

On the above-mentioned agar medium, *A. paspali* colonies appear 2–3 days after inoculation and incubation at 37°C. The colonies are dense, raised, and yellow in color due to acid production, because bromthymol blue is added as an indicator to the medium.

The acid-producing ability of this species is not found in any other *Azotobacter* species. Moreover, the ability of *A. paspali* to use organic compounds (only 17 out of 159 tested by Thompson and Skerman, 1979) is much more limited than that of other *Azotobacter* species. *A. paspali* cells are usually 7–12 μm long and 1.3–1.7 μm wide, but occasionally exceptionally long rods (up to 60 μm × about 2.0 μm) are seen. These are typical for this species and are a significant morphological feature, which is not seen in any other *Azotobacter* species. A further characteristic of this species is that cells of the microcolonies are often dimorphic, some colonies possess cells 7–11 μm long, whereas other colonies have cells 3–4 μm in length (Thompson and Skerman, 1979). Cells of older colonies on nitrogen-free agar may reach a length of 60 μm (see above).

From numerical analysis, Thompson and Skerman (1979), concluded that *A. paspali* is not closely related to other *Azotobacter* species. Moreover, they observed an antagonism toward Gram-positive bacteria in this species. In addition, their very restricted habitat—they occur solely in the plant rhizosphere—is unique among all the other *Azotobacter* species. In view of these differences, these authors proposed a new genus for this species, *Azorhizophilus* (Thompson and Skerman 1981). This opinion is, however, opposed by De Smedt et al. (1980), who found that the rRNA cistron of *A. paspali* is almost identical with those of *A. chroococcum, A. beijerinckii, A. vinelandii,* and *A. nigricans.* Moreover, Tchan et al. (1983), using rocket-line immunoelectrophoresis, showed that *A. paspali* is not immunologically separable from other members of this genus. In view of this, it is not desirable to place *A. paspali* in a separate genus.

Strains of *A. paspali* excrete a diffusible, yellow green, in UV-light fluorescent pigment into the substrate, particularly on iron-deficient media.

The association between *Azotobacter paspali* and the grass, *Paspalum notatum,* is highly species-specific; this association is found only in a few other *Paspalum* species (i.e., *P. plicatulum, P. dilatatum,* and *P. virgatum*) of 16 other *Paspalum* species tested for this bacterium (Döbereiner, 1970). It was never found in other Gramineae examined and in some Leguminosae and other dicots tested.

The original isolate came from a broad-leaved and hairy *Paspalum notatum* variety "Batatais" (common Bahia grass) in Brazil, but it was later also found in the same and other *Paspalum* species and varieties in Florida (USA) and Puerto Rico, but not in rhizosphere soil of *Paspalum* from Argentina, Paraguay, and South Africa (Döbereiner, 1970). However, according to the present author, no definite conclusion from its absence in the latter three regions can be made,

because only a very restricted number of rhizosphere soil samples (one to five) of these localities have actually been examined.

Representatives of the Genus *Azomonas*

Members of the genus *Azomonas* are primarily aquatic. *Azomonas agilis* and *A. insignis* are obligately aquatic organisms, since so far they have only been isolated from freshwater habitats. The only exception is *A. macrocytogenes*, which has been isolated from soil. The latter species was first isolated and described by H. L. Jensen (1955). For a long time, it was only known from a single strain and two variants derived from it, but according to Thompson and Skerman (1979) a total of 7 strains is now available. However, probably all, except one strain, are derivatives or subcultures of Jensen's original strain!

Azomonas species differ from those of the genus *Azotobacter* by a number of morphological and physiological characteristics. They all possess relatively large cells, which frequently occur singly and which have a special type of flagellation that gives the cells a high motility. Microcysts are never formed. Colonies on agar are generally opaque, glistening, smooth, and without insoluble pigment. On iron-deficient agar media, a yellow-green diffusible pigment is formed; and in some other media, often more red-violet or purple diffusible pigments are produced. Usually pigment production is very pronounced in liquid media and less pronounced on solid media. In *A. agilis* and *A. macrocytogenes*, the pigments are fluorescent in UV light; *A. insignis* has a nonfluorescent pigment.

Identification of *Azomonas* Species

Apart from cell shape and dimensions, which will be covered later in the descriptions of the separate species, *A. macrocytogenes* can readily be distinguished by the formation of enlarged filamentous cells in media with ethanol, a phenomenon absent in *A. agilis* and *A. insignis*. Moreover, flagellation is peritrichous in *A. agilis*, lophotrichous in *A. insignis*, and usually monotrichous (sometimes, however, two flagella at one pole) in *A. macrocytogenes*. With regard to carbon sources as sole source of carbon. It has been observed that mannitol is utilized by *A. macrocytogenes* and not by *A. agilis* and *A. insignis*, and malonate gives just the reverse outcome, i.e., it is utilized by *A. agilis* and *A. insignis*, but not by *A. macrocytogenes*. Finally, maltose is readily utilized by *A. macrocytogenes*, but not by *A. insignis* and only by a minority of the *A. agilis* strains. All species produce water-diffusible pigments, but in contrast to the pigments of *A. agilis* and *A. macrocytogenes*, the pigment of *A. insignis* does not produce fluorescence in UV light.

Azomonas agilis

This species has large, ovoid, ellipsoidal or coccoid cells, often giving it a protist-like appearance (Fig. 6). Cells are seldom found in pairs. Cells are usually 2.5–6.4 μm long and 2.0–2.8 μm wide; sometimes giant cells up to 10.0–13.5 μm long have been observed (Fig. 6). The cells are motile by means of peritrichous flagella. No microcysts are formed. A nondiffusible pigment is not produced, but a diffusible yellow-green or red-violet pigment, particularly on iron-deficient media. In UV light, the pigment gives a bluish-white fluorescence.

This species was first isolated and described by Beijerinck (1901a, 1901b), who obtained it from Dutch (Delft) canal water. Beijerinck used the following enrichment medium:

Enrichment Medium for *Azomonas agilis* (Beijerinck, 1901a, 1901b)

Canal water	100 ml
Mannitol	2.0 g
K_2HPO_4	0.02 g

The incubation temperature is 25–30°C.

Later, Kluyver and van Reenen (1933) and Kluyver and van den Bout (1936) obtained other strains with the same method. A strain isolated by the latter authors from Delft canal water (ATCC 7494) is now the neotype, because Beijerinck's original strain has been lost. It is remarkable that *A. agilis* in pure culture cannot utilize mannitol, so this carbon source must first be degraded by other microbes before it can become available to *A. agilis*. It is also possible that immediately after isolation, the strains can actually utilize mannitol, but that this ability is later lost during purification and therefore absent in pure cultures. Kluyver and van Reenen (1933) observed that, when cultures were streaked immediately from the enrichment medium on plates containing tap water, 2% mannitol, 0.02% K_2HPO_4, and 1.5% agar, and incubated at 20°C, *A. agilis* develops in 2–3 days to very small colonies about 1 mm in diameter. Growth on agar plates that contain glucose (2%) instead of mannitol as source of carbon was luxurious and with much slime production, but isolation from these plates was not recom-

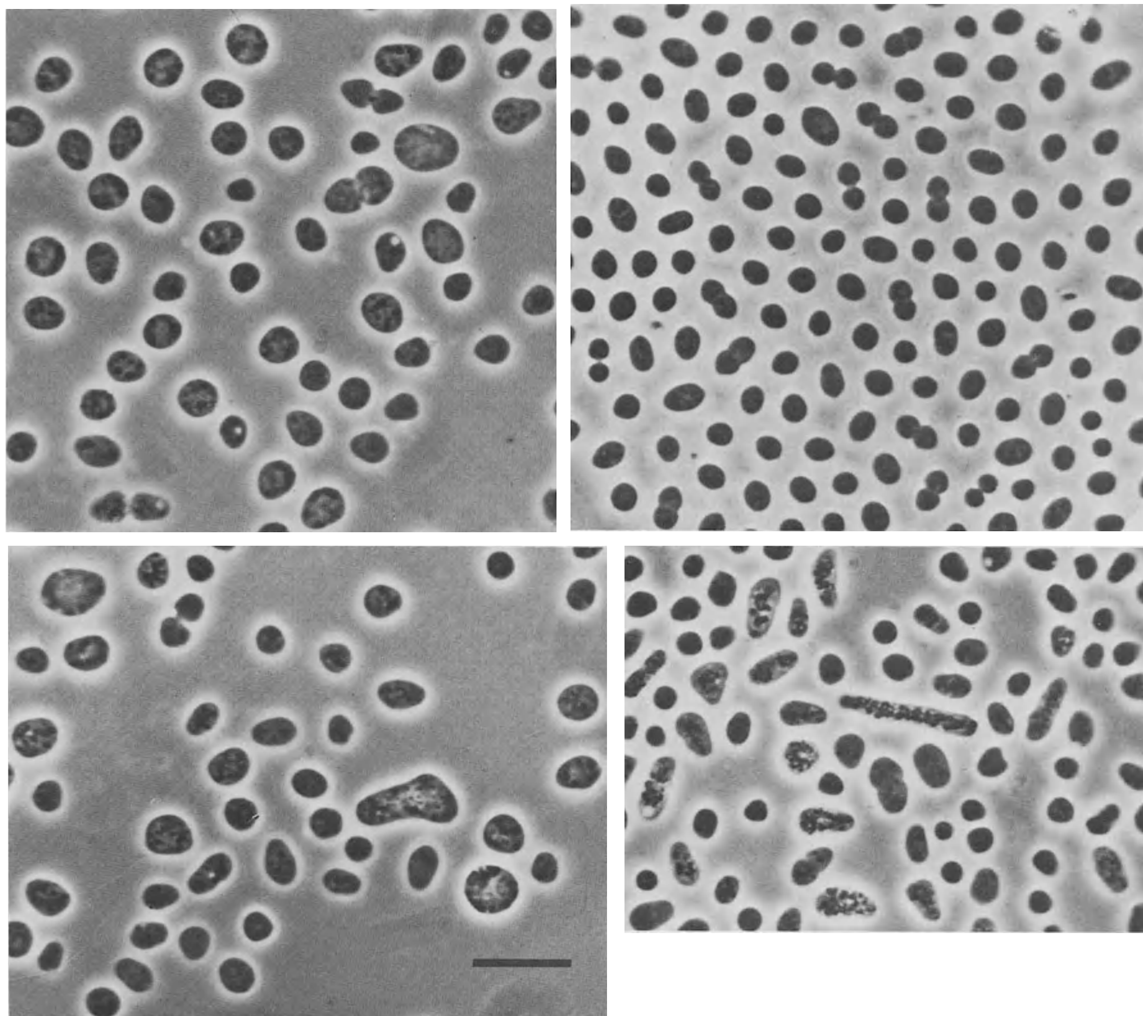


Fig. 6. *Azomonas agilis.* Cells of two strains demonstrating the large, protist-like appearance of the cells and the sporadic occurrence of aberrantly shaped, pleomorphic cells. Living preparations, phase contrast micrographs. Bar = 10 μm.

mended because many accompanying contaminants may develop in the capsular slime produced.

Derx (1951b) designed specific methods for the enrichment of *Azomonas agilis* by eliminating, so far as possible, the development of the more common *Azotobacter* species and of other *Azomonas* species. The underlying principle of Derx's method is that *Azomonas agilis,* in contrast to the more common *Azotobacter* species, is remarkably tolerant to the presence of 1% sodium benzoate in the medium, although it cannot utilize this compound as a carbon source. Derx also added a carbon compound readily utilizable by *A. agilis.* Mannitol cannot be used as the carbon source because it is normally not utilized by *A. agilis,* but it is assimilated by *Azotobacter vinelandii.* The latter organism is frequently also present in water samples and, moreover, is resistant to 1% sodium benzoate. Therefore, Derx recommended the use of ethanol as sole source of carbon for the isolation of *Azomonas agilis.* The following enrichment medium gave good results.

Selective Medium for *Azomonas agilis* (Derx, 1951b)

Water sample (canal, river, or lake water)	100 ml
Ethanol	1 ml
K_2HPO_4	0.05 g
Sodium benzoate	1.0 g

The enrichment medium is placed in relatively thin layers in Erlenmeyer flasks to allow good oxygen access, e.g., 10–15 ml of medium in 100-ml flasks, or 50 ml medium in 250-ml flasks.

Following Derx's method, the author was able to isolate a large number of *Azomonas agilis*

strains from various water sources. The organism proved to be particularly common in strawboard factory wastewater, as already observed by K. T. Wieringa (personal communication; see also Smit, 1954) and by Johnstone (1957a), and in some heavily polluted waters. In clear and clean water of rivers, lakes, and turbulent or rapidly moving, oxygen-rich, mountain brooks and rivulets, it was rarely present and frequently totally absent. In this habitat, it is entirely replaced by *Azomonas insignis.*

The tolerance of *A. agilis* strains to salt concentration up to 1.0% suggest that they are able to live in contaminated waters where concentrations of organic matter and mineral salts can be relatively high. Also its resistance to iodoacetate (1 μM) points in this direction, and this compound could be used for the selective enrichment and isolation of this species (Thompson and Skerman, 1979).

In agreement with this general pattern of being able to survive in polluted environment, nitrate is never or rarely reduced to nitrite by strains of *A. agilis* in contrast to strains of *A. insignis.* Growth experiments using *A. agilis* strains on nitrate-containing media incubated in air revealed that nitrate is an inert, nonutilizable compound for them, and thus they are able to fix molecular nitrogen in the presence of nitrate in the medium (Becking, 1962). In 19 out of 20 *A. agilis* strains tested, vanadium was unable to replace molydenum in nitrogen fixation (Becking, 1962), indicating that they predominantly have only a molybdenum-activated nitrogenase system.

Azomonas insignis

Colonies on nitrogen-free media are usually small, smooth, translucent, and low convex. They show a low proportion of extracellular polysaccharides. Cells are round or ellipsoidal and are usually 2.5–3.9 μm in length and 1.7–2.6 μm in width. On average, they measure 3.1 × 1.9 μm (Derx, 1951a). The cells have lophotrichous flagella. No microcysts are formed. On iron-deficient media, a yellow-green, red-purple, or violet pigment may be produced. The pigments are, however, not UV fluorescent.

As will be shown in the enrichment methods for this species, ethanol and salts of organic acids are preferential carbon sources for the isolation of *A. insignis.* On the whole, the utilization of organic carbon compounds as sole source of carbon for growth and energy is very restricted in this species (see later). The type strain of Derx (1951a), which is no longer extant, could not utilize glucose as sole source of carbon. It was probably lost by subcultivation on glucose-containing agar slants. New isolates (see V. Jensen, 1955) could utilize glucose, but with regard to carbohydrate utilization, in all strains tested, it is limited to only two, D-glucose and D-fructose (Thompson and Skerman, 1979).

This species was first isolated and described by Derx (1951a). It was obtained from water of clear, rapidly flowing mountain streams and small rivulets of the Cibodas Nature Reserve, Western Java, Indonesia (ca. 1,400 m altitude). These rather turbulent streams have limpid, oxygen-rich water, which contains much soluble calcium. *Azomonas agilis* could never be isolated from these sources (see above).

Derx (1951a) recommended the following medium for the specific enrichment of *A. insignis* from these water sources:

Selective Medium for *Azomonas insignis* (Derx, 1951a)

Water sample	100 ml
Calcium formate	0.5 g
Ethanol	1.00 ml

The enrichment medium is placed in rather thin layers in Erlenmeyer flasks, i.e., 20–25 ml in 100-ml flasks or 50 ml in 250-ml flasks. Incubation is at 25–30°C. Calcium formate is added to the medium to inhibit the development of various other microbes, including *Azotobacter* species and other *Azomonas* species. *A. insignis* does not utilize calcium formate but is rather resistant to it. Ethanol is added as sole source of carbon because it is a preferential carbon source for *A. insignis.* According to Derx (1951a), his strains of *A. insignis* could not utilize glucose or mannitol. A nonvolatile carbon source, which also gives good growth and can be used for maintenance in pure culture on agar slants is calcium malate or calcium succinate. Therefore, the use of nitrogen-free, mineral agar plates with calcium malate (0.5%) or calcium succinate (0.5%) as sole carbon source can also be recommended for isolation of *A. insignis* from enrichment cultures.

Pure cultures can also be grown in liquid medium: tap water (for trace element supply), to which is added K_2HPO_4 (0.05%) and ethanol (0.5–1.0%, or about 0.5–1.0 ml per 100 ml medium). Growth is obtained in 24 h at 25–30°C. After several days, the medium turns milky white, later blue-gray, and after about a week sometimes to violet (see above).

V. Jensen (1955) isolated six strains of *A. insignis* from surface water of fast-flowing brooklets and streams with very limpid fresh water in Denmark, but occasionally also from stagnant and somewhat polluted water (e.g., his strains 7 and 9). None of his isolates could utilize mannitol but, in contrast to Derx's strains, all the Danish strains could utilize glucose as sole source of carbon. Two of Jensen's strains were also able to grow in a medium that contained sodium benzoate (1.0%).

Enrichment Medium for *Azomonas insignis* (V. Jensen, 1955)

Water sample	25 ml
K_2HPO_4	0.02%
Ethanol	1.0% (or about 0.25 ml)
or	
Water sample	25 ml
K_2HPO_4	0.02%
Ethanol	1.0% (or about 0.25 ml)
Sodium benzoate	1.0%

The water samples are placed in 100-ml Erlenmeyer flasks.

Thompson (see Thompson and Skerman, 1979) isolated *A. insignis* from a rusty-brown, probably iron-oxide-rich water of a slow-flowing Australian creek with a pH of 6.7, using an enrichment medium recommended by V. Jensen (1955), by adding to the water sample 0.02% K_2HPO_4 and 1% ethanol. The strain isolated (WR-51) was very similar to Derx's type strain as this strain also could not utilize glucose and excretes a violet diffusible pigment into the medium. In this respect it can be regarded as a neotype of Derx's original strain.

The acid tolerance of *Azomonas insignis* was also tested by V. Jensen (1955) in the strains isolated by him and compared to that of the other *Azomonas* and *Azotobacter* species. Most *A. insignis* strains could not grow in a medium with a pH lower than 5.7–5.9, although a few could produce faint growth at pH 5.5. Of the two *Azomonas agilis* strains tested, one could produce faint growth at pH 5.3, and one *Azotobacter beijerinckii* strain could even grow normally at pH 5.2. The one *Azotobacter chroococcum* strain tested in the survey could not grow below pH 6.1.

Other experiments also showed that *Azomonas insignis* strains have, in general, a preference for slightly alkaline conditions for optimal growth and nitrogen fixation. Of nine strains tested, three could grow at pH 6.0, but all strains grew at pH 10.0 (Thompson and Skerman, 1979). With respect to temperature requirements for growth, *A. insignis* strains seems to be able to grow at relatively lower temperature in comparison to other Azotobacteraceae. Nine of the 10 *A. insignis* strains tested by Thompson and Skerman (1979) could grow at 9°C, while the maximum growth temperature was 32°C.

A considerable reduction of the capacity to utilize carbon compounds by *A. insignis* compared to other *Azomonas* and *Azotobacter* species was first noted by V. Jensen (1955). Of the 26 compounds studied, *Azotobacter chroococcum* could utilize 16, *Azotobacter beijerinckii* on the average about 14, *Azomonas agilis* 11, and *Azomonas insignis* only 8. In a general survey, Thompson and Skerman (1979) tested 161 organic carbon compounds as sole source of carbon for *A. insignis.* Of these, 21 compounds could be utilized by nearly all *A. insignis* strains, 9 compounds could be utilized by a few strains, and no strain could utilize any of the 131 other organic carbon compounds tested.

Azomonas macrocytogenes

In contrast to the two previous *Azomonas* species, *A. macrocytogenes* is isolated from soil. The species is primarily known from one isolate, called strain O (original), and two variants derived later, strains M (mutant) and I (intermediate). Although Thompson and Skerman (1979) described seven strains, most of them are subcultures or derivatives of the original strains, and only one, a strain designated as the cotype of *Azotobacter agilis* subsp. *jakutiae* Krasil'nikov (1949), had another origin.

The original strain was isolated by H. L. Jensen (1955) in an attempt to isolate *Beijerinckia* from Danish soil. The isolate appeared on a nitrogen-free, mineral sucrose agar of about pH 5.5, seeded with garden soil (a fertile loam, pH 7.5) of the State Laboratory of Plant Culture, Lyngby, Denmark.

As could be anticipated from its isolation procedure, the type strain (type O and its variants) can produced good growth and nitrogen fixation in a nitrogen-free medium in the pH range of 4.6–6.9; one variant grew also at pH 4.3. This species is therefore even more acid tolerant than Tchan's (1953) and Jensen and Petersen's (1955) strains of *Azotobacter beijerinckii.*

Apart from morphological properties, such as ellipsoidal or rod-shaped cells, usually 2.5–3.5 μm in length and 1.6–2.1 μm wide, occurring singly or in pairs and occasionally forming short chains, and on media containing ethanol, the formation of very large coccoid, spindle-shaped or filamentous (up to 100 μm) cells, also physiological properties are important. The type strain showed no utilization of starch and rhamnose, but mannitol is utilized. Moreover, *A. macrocytogenes* strains can be differentiated from other *Azomonas* species by the utilization of mannitol (negative in *A. agilis* and *A. insignis*) and maltose (not utilized by *A. insignis* and most strains of *A. agilis*), and by its nonutilization of malonate (utilized by both *A. agilis* and *A. insignis*). In addition, *A. macrocytogenes,* being a soil organism, is more resistent to desiccation than *A. agilis* and *A. insignis,* which generally do not survive on silica gel for more than 2 days (Thompson and Skerman,

1979). However, no microcysts are formed, although microcyst-like structures occasionally are produced. These structures are more like capsules lacking the characteristic exine layer of a microcyst. Moreover, the cells sometimes have the tendency to become Gram-variable, i.e., to change from Gram-negative to Gram-positive.

The cells bear monotrichous flagella, but sometimes there are two flagella at one pole. Colonies do not produce a nondiffusible pigment, but on iron-deficient media, yellow-green or red-violet diffusible pigments are produced, which give a blue-white fluorescence in UV light.

In a numerical analysis and in the constructed dendrogram of relationships, Thompson and Skerman (1979) showed that *Azomonas macrocytogenes* fused at a low hierarchical level with *Azotobacter paspali*. At a higher level, the *Azomonas macrocytogenes–Azotobacter paspali* group fused with the two other *Azomonas* species before fusing with *Azotobacter*. This was a motive for these authors to propose a new genus, *Azomonotrichon* (Thompson and Skerman 1979) for *Azotomonas macrocytogenes*. However, De Smedt et al. (1980) showed that the rRNA cistrons of *Azomonas insignis* and *A. agilis* differ as much from each other as they do from *Azomonas macrocytogenes* and from *Azotobacter*. Therefore, there is no reason to create a new genus for *Azomonas macrocytogenes*, although more genome comparisons of the various members of the Azotobacteraceae would probably be useful in clarifying its exact taxonomic position. It is noteworthy that Rubenchik (1959) transferred *Azomonas macrocytogenes* to the genus *Beijerinckia* as *B. macrocytogenes* because it fixed nitrogen at pH values of 4.5–5.0 and produced acid from certain carbohydrates. In support of this, Thompson and Skerman (1979) noted that *Azomonas macrocytogenes* produces colony-retained homopolysaccharides from sucrose and saccharose, and this is only found in this group and in most strains of the group containing the *Beijerinckia* species.

Preservation of Cultures

For routine maintenance, *Azotobacter* and *Azomonas* cultures should be subcultured at monthly or bimonthly intervals on nitrogen-free mineral agar containing glucose or sucrose (1 or 2%). For those strains which cannot assimilate glucose (e.g., *Azomonas insignis*), another appropriate carbon source (e.g., an organic acid such as calcium malate or calcium succinate) can be chosen.

Poor results were obtained by lyophilization in skim milk or dextran-sodium glutaminate solution on filter paper in small glass vials under vacuum with storage of the closed vials at room temperature in the dark. Antheunisse (1972, 1973) compared this method with storage of cultures on the usual agar media in normal culture tubes, plugged with sterile rubber seals, and stored in the dark at room temperature. Tests for viability of the cultures were made after periods of 1 to 10 years. The outcome showed that lyophilization of *Azotobacter* and *Azomonas* gave very poor results. Only 32% of the cultures were viable after 6 years. The rate of survival of *Azotobacter vinelandii* was relatively high, but that of *A. chroococcum* was low and that of *Azomonas agilis* was nil. In contrast, the 65 *Azotobacter* and *Azomonas* strains kept on sealed agar slants for 3–10 years gave an average viability of 60%. In this case, both *Azotobacter vinelandii* and *Azomonas agilis* gave good survival rates (86 and 78%, respectively).

In the author's laboratory, *Azotobacter* and *Azomonas* cultures are usually kept in normal culture tubes (with cotton plugs) under a seal of sterile liquid paraffin or mineral oil at room temperature (sometimes also at 4°C); such cultures generally survive for at least 3–5 years (J. H. Becking, unpublished observations). Care should be taken that the oil completely covers the agar slant, because if the agar medium remains in contact with air, the agar will dry out and the culture may die.

Physiological and Biochemical Aspects

Azotobacteraceae are aerobic, heterotrophic, nitrogen-fixing organisms and therefore possess all oxidative enzymes for the degradation of the numerous organic carbon compounds utilizable by the various species as sole source of carbon and energy. Many of these enzymes are constitutive, but some are induced by the particular carbon substrate involved. Many publications have appeared on these enzymes, including some on the kinetics of their induction, beginning with the Wisconsin school (Stone and Wilson, 1952a, 1952b; Repaske and Wilson, 1953; Repaske, 1954; Williams and Wilson, 1954; Wilson and Wilson, 1955; Shutter and Wilson, 1955; Alexander and Wilson, 1956; Marr and Marcus, 1962), but they will not be discussed here in view of space limitations. Only some characteristic enzymes associated with nitrogen-fixation in Azotobacteraceae and the relation between the oxidative pathway and nitrogen fixation will be discussed.

The oxidative pathway in Azotobacteraceae as source of energy (ATP) follows the TCA cycle. It has been studied in many investigations (see above) using whole-cell suspensions or cell-free extracts to test the various intermediates of the TCA cycle, such as acetate, malate, succinate, α-ketoglutarate, and citrate. Also, the effect of oxygen on nitrogen fixation and the high respiratory activity of Azotobacteraceae have received special attention, since Meyerhof and Burk (1928) and Burk (1930), first oberved it, and it was the subject of many later studies (Tschapek and Giambiagi, 1955; Parker and Scutt, 1958, 1960; Dilworth and Parker, 1961). All the studies found that high oxygen levels, even atmospheric levels of oxygen, inhibited the nitrogen fixation activity in *Azotobacter*. Moreover, the exceptionally high respiratory quotients of *Azotobacter* species obtained by manometric methods were remarkable; Q_{O_2} values of 20,000 μl O_2/h or 4,000–5,000 μl O_2/mg dry wt/h have regularly been measured (Williams and Wilson, 1954; Shutter and Wilson, 1955). Both observations have led to detailed investigations on the role of oxygen in the nitrogenase complex. Experiments revealed that in *Azotobacter* a special mechanism operates which protects the oxygen-sensitive nitrogenase from oxygen damage. The high respiration rate of Azotobacteraceae was explained as a mechanism which scavenges oxygen from the dinitrogen-fixing site of nitrogenase (Dalton and Postgate, 1969a). This process, called the "respiratory protection" of nitrogenase, is coupled with a multitude of cytochromes and redox proteins (Haddock and Jones, 1977), thus maintaining the nitrogenase in an essentially anoxic environment inside cells that nevertheless derive energy from aerobic metabolism.

The significance of respiratory protection has been argued, and alternative mechanisms have been suggested. A prominent argument was that the nitrogenase complex, which consists of two components (see later), was extremely sensitive to oxygen, being rapidly and irreversibly inactivated upon exposure to air, but nitrogenase in cell-free extracts of *Azotobacter* is relatively oxygen stable (Bulen et al., 1964; Kelly, 1969). The Brighton, England nitrogen-fixation school also suggested that if O_2 enters the cell more rapidly than it can be removed by respiration (i.e., by "respiratory protection"), an alternative control mechanism operates for which they proposed the name "conformational state protection," i.e., a protection by some steric arrangement of the components (Dalton and Postgate, 1969a). In the latter mechanism, during O_2 stress, nitrogenase binds to a 2Fe-2S protective protein, also called Fe/S II, to give an O_2-stable complex that is protected from O_2 damage, but that is inert to nitrogenase substrates. When the oxygen stress is low or lowered by excessive respiration, the protected complex dissociates to give active nitrogenase. Such protective proteins have been isolated and characterized for *A. chroococcum* (Robson, 1979) and for *A. vinelandii* (Scherings et al., 1983).

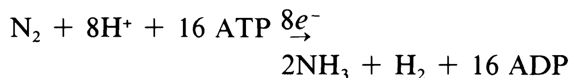
However, some alternative mechanisms have been proposed. Oppenheim et al. (1970a, 1970b) suggested that protection against oxygen was due to a major cytoplasmic membrane component that appears in these organisms when grown in nitrogen-free medium, while Yates (1970) suggested a form of respiratory control by nucleotides (ATP) operating in *Azotobacter*. Also, Kuhla and Oelze (1989) found a dependence of nitrogenase switch-off upon oxygen stress on the nitrogenase activity in *Azotobacter vinelandii*. Their results suggested that the flux of electrons to the nitrogenase complex, rather than cellular oxygen consumption (qO_2), stabilizes nitrogenase activity against O_2 inactivation in aerobically growing *A. vinelandii*.

From the observations mentioned above, it may be concluded that the mechanism of O_2 control and the absence of a serious inhibition of functioning nitrogenase by O_2 is a rather complex process, which may involve several different systems.

Although nitrogenase is the most important enzyme for aerobic nitrogen-fixing bacteria like the Azotobacteraceae, this enzyme is also found in a great number of other microorganisms belonging to quite-different taxonomic groups and affiliations. The main properties of nitrogenase (i.e., of the main species of nitrogenase, see later) can be summarized as follows: it consists of two proteins; it is sensitive to oxygen or it can be destroyed by oxygen; it contains the transition metals Fe and Mo; it needs Mg^{2+} ions to be active; it converts ATP to ADP when functioning; it is inhibited by ADP; it reduces nitrogen and several other small triply bonded molecules (such as C_2H_2 and CN); and it reduces H^+ ions to gaseous H_2 even when N_2 is present. The two proteins forming this nitrogenase are a large one (MW of about 220,000) and a smaller one (MW of about 60,000). Both proteins contain Fe (amounts variable), and this metal is essentially accompanied by S atoms. The large protein contains in addition two atoms of Mo and is therefore usually called the molybdoprotein. The proteins of the nitrogenase complex are often also called component I and component II. Furthermore, it is interesting that the two proteins are very similar in different nitrogen-fixing organisms. Their similarity is so great that it is possible to inter-

change these proteins with the proteins of other nitrogen-fixing microbes, regardless of their origin (e.g., *Klebsiella pneumoniae, Bacillus polymyxa,* and *Clostridium pasteurianum*) yielding fully active preparations (Bulen et al., 1966; Detroy et al., 1968; Dahlen et al., 1969).

Nitrogenase reduces N_2 to NH_3 and, for this function, it needs adenosine triphosphate (ATP). In vitro, 16 ATP molecules are consumed to convert one N_2 to two NH_3 molecules, and eight electrons are also involved:



$$N_2 + 8H^+ + 16\ ATP \xrightarrow{8e^-} 2NH_3 + H_2 + 16\ ADP$$

Actually, the reduction of N_2 to two NH_3 requires six electrons, equivalent to 12 ATP molecules. It has been shown that two electrons or four ATPs are used for the formation of one molecule of H_2. In growth experiments with *Azotobacter chroococcum* in continuous culture, Dalton and Postgate (1969b) demonstrated that nitrogen fixation entrained a maintenance coefficient of 1.06 g substrate/g organism/h compared with about 0.40 for ammonia assimilation. Assuming that most of this maintenance was directed to respiratory protection of nitrogenase, an extrapolated maximum requirement of 4 moles ATP/mole N_2 fixed was observed.

In other growth experiments, Dalton and Postgate (1969a) observed that, at 0.03 atm O_2, nitrogen-limited, continuous cultures of *A. chroococcum* fixed about twice as much N_2/g carbon source utilized than at atmospheric pressure (i.e., 0.20 atm O_2), confirming earlier growth experiments by other authors (Becking, 1971). The specific effects of different O_2 levels on nitrogen-fixation efficiency of *Azotobacter* have already been discussed (see "General Identification").

As noted before, the nitrogenase system in *Azotobacter* consists of two nonheme iron proteins, i.e., a MoFe protein called Component I, which has been crystallized (Burns et al., 1970; Shah and Brill, 1973) and was shown to be a tetramer of 245,000 daltons (Swisher et al., 1977; Shah and Brill, 1977) containing two Mo atoms per molecule, and a protein called Component II, which is a Fe protein, a dimer of two identical subunits of 31,200 daltons, containing 289 amino acids (Hausinger and Howard, 1980). Thus, apart from Fe, the uptake of Mo by the organism is essential and a prerequisite for active functioning of the nitrogenase complex. Particularly, Mo defiency or Mo depletion of the medium of Azotobacteraceae cultures limit their growth under nitrogen-fixing conditions. In growth experiments, it was shown by Becking (1962) that the Mo requirements of various *Azotobacter* and *Azomonas* species differ. Half-maximal nitrogen fixation was obtained in *A. chroococcum* at about 0.05 ppm Mo and in *A. vinelandii* at 0.0004 ppm Mo, whereas the same half-maximal growth value is reached in *Azomonas agilis* at 0.0002 ppm Mo. In these growth experiments, Becking (1962) further observed that in the majority of *Azotobacter chroococcum* and *A. vinelandii* strains, vanadium could replace molybdenum under nitrogen-fixing conditions. Of the 10 *A. chroococcum* strains tested, only three were unable to utilize vanadium as a substitute for molybdenum in nitrogenase and, in 19 out of the 20 *A. vinelandii* strains tested, vanadium was able to replace molybdenum in the nitrogen-fixation process. However, in all except one of 20 *Azomonas agilis* strains simultaneously tested, vanadium was unable to replace molybdenum in nitrogen fixation. Moreover, nitrogen fixation with vanadium was reduced to two-thirds of that produced by molybdenum. Experiments also demonstrated that for nitrate assimilation, molybdenum was also required in those species, which could assimilate nitrate (most *Azomonas agilis* strains are unable to utilize nitrate). But in the nitrate reductase system, molybdenum could not be replaced by vanadium. Finally for ammonium assimilation, neither of the two metals is required (Becking, 1962).

In this context, it is of interest that an alternative nitrogenase system lacking molybdenum has been discovered and its existence proved biochemically and genetically. First evidence of such an alternative nitrogen-fixing system came from the genetic studies of Bishop et al. (1980, 1982), who obtained Nif^+ pseudorevertants of the Nif^- strains UW6 and UW10 (see Shah et al., 1973) of *A. vinelandii.* These pseudorevertants displayed growth on nitrogen-free medium at a lower growth rate than the wild type and fixed nitrogen at a rate of 3–4% of the Nif^+ control. Moreover, phenotypic reversion of Nif^- mutants to Nif^+ occurred when they were grown on media lacking molybdenum but containing tungsten, vanadium, or rhenium salts (Bishop et al., 1980, 1982). Under these conditions, there was nitrogen fixation at a low rate although the cells lacked the typical EPR signal of the MoFe protein. These observations led Bishop et al. (1980, 1982, 1986) to propose an alternative pathway for nitrogen fixation in *A. vinelandii,* whose functioning was independent of molybdenum.

Following this, Eady et al. (1987) and Dilworth et al. (1988) demonstrated the existence of a vanadium nitrogenase in *A. chroococcum.* Miller and Eady (1988) showed that, for both the Mo and V nitrogenases present in *A. chroo-*

coccum, low temperature favors the nitrogen reduction by the V nitrogenase. The vanadium nitrogenase of *A. chroococcum* was purified and the properties of the VFe protein were studied (Eady et al., 1987). These authors demonstrated that the VFe protein of the vanadium nitrogenase contained an iron-vanadium cofactor forming the substrate-reducing site (Smith et al., 1988). Also, the Fe protein of the vanadium nitrogenase was purified and characterized (Eady et al., 1988). Raina et al. (1988) characterized the gene for the iron-protein of the vanadium-dependent alternative nitrogenase of *A. vinelandii* and constructed a Tn*5* mutant. Finally, the structural genes for the vanadium nitrogenase from *A. chroococcum* have been cloned and the nucleotides have been sequenced (Robson et al., 1989). There is also genetic evidence obtained using deletion mutant analysis that shows that at least in *Azotobacter vinelandii,* there is also a third nitrogenase that lacks both Mo and V (Pau et al., 1989). This has been independently verified, and the existence of an FeFe protein has been demonstrated (Smith, 1989; B.E. Smith, personal communication). Thus, in conclusion, three distinct nitrogenases are now known, each with its own, genetically distinct, Fe protein which acts as an electron transfer agent to either a MoFe, VFe, or FeFe protein in an ATP-hydrolyzing reaction. The MoFe and VFe proteins have been shown to contain cofactors (FeMoco and FeVco) that form the substrate-reducing sites.

Besides oxygen, hydrogen has also been intensively studied in relation to nitrogenase (Robson and Postgate, 1980). Hydrogen evolution has received attention in the context of the efficiency of the nitrogen-fixing system (Schubert and Evans, 1976; Schubert et al., 1977). It was suggested that in a fully efficient system, all electrons would be used for ammonia production and no hydrogen would be evolved. All Azotobacteraceae have very powerful hydrogenases and therefore, in these organisms, the role of hydrogen in nitrogen fixation was of particular interest. Hydrogen-dependent mixotrophic growth of *A. vinelandii* has been observed (Wong and Maier, 1985). Moreover, hydrogen-mediated enhancement of hydrogenase expression (Prosser et al., 1988) and hydrogen-mediated mannose uptake (Maier and Prosser, 1988) have both been reported for *A. vinelandii.* Recently, competition studies in continuous culture between a Hup$^-$ (= Hydrogen-uptake$^-$) mutant of *A. chroococcum* and its presumed isogenic Hup$^+$ recombinant showed that Hup activity benefitted the organism under nitrogen-fixing and sucrose- or phosphate-limiting conditions, but it was ineffective or disadvantageous under O_2, sulfate, or iron limitation (Yates and Campbell, 1989). The physiological aspects as well as the genetics of the hydrogen-uptake hydrogenase has been lately reviewed by Yates (1988) and Yates et al. (1988).

With regard to the assimilation of combined nitrogen such as ammonia, the usual enzymes, including glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutamate synthase (GOGAT), have been reported for *A. chroococcum* (Drozd et al., 1972), and some have also been demonstrated in other *Azotobacter* species. GOGAT was found in both soluble and membrane-bound forms in *A. vinelandii.* Assimilatory nitrate and nitrite reductases have been characterized for *A. chroococcum* and *A. vinelandii* (Guerrero et al., 1973; Spencer et al., 1957; Taniguchi and Ohmachi, 1960; Vega et al., 1973).

Genetic Aspects

The first metabolic mutants of Azotobacter were described for *Azotobacter vinelandii* by Karlsson and Barker (1948), and shortly afterwards mutants of the same species which do not fix nitrogen were reported by Wyss and Wyss (1950). The first DNA-mediated transformation in *Azotobacter* was observed by Sen and Sen (1965), who described an interspecific transformation of pigment production between *A. chroococcum* and *A. vinelandii.* Transformation of Nif$^-$ strains of *A. vinelandii* with *Rhizobium* DNA have also been described (Page, 1977), together with some other intergeneric transformations between *Rhizobium* and *Azotobacter* (Sen et al., 1969; Bishop et al., 1977a; Maier et al., 1978). Although phages (azotophages) of *Azotobacter vinelandii* and *A. chroococcum* have been isolated (Monsour et al., 1955; Chuml et al., 1980), these phages had limited host ranges and apparently had no transducing ability (Bishop et al., 1977b). An earlier positive report (Wyss and Nimeck, 1962) mentioning interspecific transduction in *Azotobacter* could not be confirmed.

Most studies on mutants of *Azotobacter* have involved the *nif* genes. The first stable *nif*$^-$ mutants of *Azotobacter* were described for *A. vinelandii* by Fisher and Brill (1969). These mutants were biochemically analyzed by these authors for the activity of the two nitrogenase components (Shah et al., 1973; Bishop and Brill, 1977).

Regulatory mutants of *Azotobacter* that fix nitrogen in the presence of ammonium have also been isolated and described by Sorger (1968), Gordon and Brill (1972), Bishop and

Brill (1977), and Terzaghi (1980b). Such mutants were found particularly among strains resistant to methylalanine. Ammonia-exporting mutants may be important in industry for ammonia production (see "Applications").

Bishop et al. (1980) obtained *nif*⁺ pseudorevertants of some particular *nif*⁻ mutants (UW 6 and UW 10), which could fix nitrogen at a rate of 2–4% of the *nif*⁺ control. From these observations, they inferred the existence of an alternative pathway for nitrogen fixation functioning independentally of Mo (see "Physiological and Biochemical Aspects"). Moreover, drug-resistant mutants (Page and Sadoff, 1976; Bishop and Brill, 1977) and amino acid and vitamin autotrophs (Leach and Battikhi, 1978) of *A. vinelandii* have regularly been obtained by mutagenesis and the application of selective media.

On the whole, *Azotobacter* species and, in particular, *Azomonas* species seem to be difficult to mutate or the selection procedures used so far have been inadequate. Sadoff et al. (1979) argued that *A. vinelandii* contains 40 chromosomes per cell, and therefore failure to isolate mutants could result from difficulties in segregation rather than mutagenesis. In view of these difficulties, most information on the *nif* genes comes from *Klebsiella pneumoniae.* It has a good transduction system and a plasmid (pRD1) that carries the entire *nif* cluster of *K. pneumoniae.* The plasmid has been isolated and this system was most useful in the development of the *Azotobacter nif* genetics, e.g., by the expression of *Klebsiella nif* genes in *Azotobacter* (Cannon and Postgate, 1976).

At the moment, the homology of *nif* genes of *Azotobacter* and other bacteria species is well established, and the genes encoding the two nitrogenase systems in two species *(A. vinelandii* and *A. chroococcum)* have been identified.

A group of genes spanning 25–30 kb of DNA was characterized in *A. chroococcum* after being cloned and their expression studied in *Klebsiella pneumoniae* (Jones et al., 1984). Hybridization to *nif* gene probes from *K. pneumoniae,* coupled with DNA sequencing and complementation analysis, revealed that the *nif* genes *FMVSUNEKDH* are present in this region (Evans et al., 1985). The genes best characterized for structure and function in *A. vinelandii* and *A. chroococcum* are *nifKDH.* A restriction map for a region of the *A. chroococcum* genome carrying *nif* genes (Jones et al., 1984) confirms close linkage and expression of *nifKDH* as a single transcript, and places *nifV* 15 kb away from *nifKDH.*

The promotors of *nif* genes were observed to share common nucleotide sequences in two regions upstream from the site at which transcription begins in a number of different organisms such as *Azotobacter, Klebsiella, Rhizobium, Thiobacillus,* and *Desulfovibrio.* The structural genes, including that of the VFe protein of the vanadium nitrogenase from *A. chroococcum* (Robson et al., 1989), have been cloned, and nucleotide sequences were determined as already reported. Activator proteins are needed for *nif* gene expression, and genes encoding activator genes have diverged to control expression of the three different enzyme systems responsible for nitrogen fixation. Regulation of *nif* genes in *Azotobacter* is therefore rather complicated.

Similarity of *nif* regulation among *K. pneumoniae* and *A. vinelandii* and *A. chroococcum* has been demonstrated (Kennedy and Robson, 1983). Introduction of the *nifA* gene from *K. pneumoniae* into *Azotobacter* mutants deficient in both components I and II restored the Nif⁺ phenotype to the presumed regulatory mutants but not to a nitrogenase-structural-gene mutant. The results are interpreted to suggest that *nifA* activation of *nif* genes might also be conserved among diazotrophs. Two nitrogen fixation regulatory regions, *nifA* and *nfrX,* in *Azotobacter vinelandii* and *A. chroococcum* have also been identified and characterized (Santero et al., 1988). These investigations are too numerous to cite here fully, but most of them are cited in recent reviews on the genetics of *Azotobacter* and related organisms by Elmerich (1984), Kennedy and Toukdarian (1987), and Kennedy (1989).

Applications

Associative growth of one *Azotobacter* species, *A. paspali,* has been reported to produce growth responses, apparently giving an increase in nitrogen, in forage grasses (Döbereiner, 1970; Döbereiner and Day, 1976).

However, *Azotobacter* inoculants (mainly *A. chroococcum*) also may produce crop responses. Especially in the USRR in the years 1958–1960, numerous field experiments were conducted with *Azotobacter* inoculants (named "Azotobacterin" or "Nitragin") with agricultural crops like spring wheat, winter wheat, barley, oats, and maize. Mishustin and Shilnikova (1969, 1971) have summarized these results, showing that in some trials, significant yield increases were obtained with a beneficial effect varying from 7–12%. Later, however, this practice was no longer recommended and it has now been abandoned.

However, some workers still claim that *Azotobacter* has a positive effect on crops, e.g., *Azotobacter* inoculation of seeds or seedlings of wheat, rice, onion, tomato, brinjal *(Solanum aestivum),* and cabbage. Significant responses with increases that average 10–20% are reported for such crops (Sundara Rao et al., 1963; Lehri and Mehrotra, 1968, 1972; Mehrotra and Lehri, 1971; Joi and Shinde, 1976; Shende et al., 1977).

Some other workers (Vančura and Macura, 1959, 1961; Brown et al., 1962, 1964) performed field and pot experiments in which artificial inoculation produces increases in the yield of crops. They attributed the beneficial responses in addition to fixed nitrogen made available to the plants by the production of phytohormones such as gibberellins, auxins, and some phenolic compounds (Jackson et al., 1964; Hennequin and Blachère, 1966), giving the plants a better health condition. Also, the production of antifungal antibiotics by *Azotobacter* may play a rôle in these yield increases (Mishustin and Shilnikova, 1969, 1971; Laksmi Kumari et al., 1975).

For instance, Meshram and Jager (1983) mentioned an antagonism of isolates of *Azotobacter chroococcum* to *Rhizoctonia solani* on agar plates and *Azotobacter* isolates were tested for their ability to control *R. solani* infection of potato sprouts in sterilized and unsterilized soil. The degree of antagonism exhibited varied strongly among the isolates and was found to be temperature-dependent. Following this, Azad and Aslam (1985) observed that *Azotobacter chroococcum* inoculation increased the yield and the protein content of potato *(Solanum tuberosum)* tubers. Analyses showed that the inoculum increased substantially the population of *A. chroococcum* present in the rhizosphere soil in these experiments.

Bagyaraj and Menge (1978) showed that larger populations of bacteria and actinomycetes were recovered from the rhizospheres of tomato *(Lycopersicon esculentum)* plants inoculated with the mycorrhizal fungus *Glomus fasciculatus* and *Azotobacter chroococcum,* either individually or together, than from those of non-inoculated plants. The dry weights of the tomato plants inoculated with both *G. fasciculatus* and *A. chroococcum* were significantly (62%) greater than non-inoculated plants. These results suggest a synergistic or additive interaction between *Glomus fasciculatus* and *Azotobacter chroococcum.*

Although the practice of using *Azotobacter* inoculants was often unsatisfactory and has been abandoned in many countries, in India a constant stream of publications continues to appear on the beneficial effects of *Azotobacter* on various crops such as sorghum, pearl millet, maize, rize, sesame, wheat, and barley (Ghonsikar et al., 1986; Prasad, 1986; Konde and Shinde, 1986; Shende et al., 1986; Subbian and Chamy, 1984; Rai and Gaur, 1988; Kavimandan et al., 1978; Tiwari et al., 1989). In India, it is also suggested that *Azotobacter* inoculation gives positive growth responses in oak seedlings used in forestry (Pandey et al., 1986).

Outside India, there are now only sporadic reports on the beneficial effects of *Azotobacter* inoculations, and these refer mainly to subtropical regions. Some Egyptian authors have obtained positive results on the growth of the castor oil plant, *Ricinus communis* (Monib et al., 1984), or of wheat (Emam et al., 1986) with *Azotobacter* inoculation.

Monib et al. (1979) also studied the effect of bacterization of barley *(Hordium vulgare)* grains with a selected strain of *Azotobacter chroococcum.* In N-deficient sand, seed inoculation increased plant length, dry weight, nitrogen content in addition to a significant increase in soil nitrogen. In the presence of a mixed soil microflora the beneficial effect of bacterization was less than in monobacterial cultures. Azotobacters naturally present in soil also colonized in heavy densities the rhizoplane (rhizosphere) of the barley plants, but their effect on plant growth and soil nitrogen were less as compared with that of bacterization.

In Spain, the beneficial effects of *Azotobacter chroococcum* on root colonization and grain production of maize have been reported (Martinez-Toledo et al., 1988a, 1988b), and in *Sorghum bicolor,* a specific root association with *A. chroococcum* was demonstrated, but there was no interaction influencing the nitrogenase activity in the rhizosphere, probably due to the lack of excretion of a carbon and energy source by the plant (De La Rubia et al., 1989). In addition, in Israel, positive responses of *Azotobacter* inoculation on the growth of *Setaria italica* have been reported (Yahalom et al., 1984). In Pakistan, Hussain et al. (1985) studied in field experiments the seed inoculation of wheat *(Triticum aestivum)* on the yield in relation to the application of farm yard manure and some N-P-K levels, in which N was given in the form of urea. The results indicated that *Azotobacter* inoculation was more effective as regards to grain yield in the trials where no nitrogen was added, next was farm yard manure and the lowest response was with urea application.

From all these investigations it is clear that, with regard to beneficial effects, the supply of fixed N might not be the sole factor involved or it is only a minor factor and that growth-promoting substances may be involved. In par-

ticular, it appears that plant root colonization by bacteria is favorable, probably due to biological control of plant diseases (see e.g., Kloepper et al., 1989).

Some *Azotobacter* mutants may be important for biotechnology and industrial application. Mutants of *A. vinelandii* have been obtained that fix molecular nitrogen in the presence of excess NH_4^+ excretion into the medium (Gordon and Brill, 1972; Shaw et al., 1973). Terzaghi (1980a, 1980b) tried to produce such mutants in a variety of *Azotobacter/Azomonas* species, i.e., mutants which could excrete excess ammonia in addition to being able to reduce N_2 in the presence of excess NH_4^+. She isolated nitrogenase-derepressed (Nif-Drd) mutants of *Azotobacter* in the two *Azotobacter* species *(A. vinelandii* and *A. beijerinckii)* and the two *Azomonas* species *(A. agilis* and *A. macrocytogenes)* tested. Such mutants could only be obtained in *Azotobacter vinelandii* and to a much lesser degree in one strain of *Azomonas agilis.* These mutants, having up to 100% nitrogenase activity and excreting NH_4^+ into the medium without inhibiting the nitrogenase activity, are of industrial significance, for instance by immobilization in Alginate beads, because they offer the opportunity to produce ammonia (which can be used as plant fertilizer) using *Azotobacter* in a biotechnological process.

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CHAPTER 166

The Genus *Beggiatoa*

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The genus *Beggiatoa* is currently represented by a single defined species *Beggiatoa alba* (Strohl, 1989). However, this species designation is commonly used for any colorless, filamentous, gliding bacterium that deposits internal globules of elemental sulfur but does not form bundles of trichomes within a common sheath. Organisms with a variety of filament widths, ranging from 1–120 μm, have been observed in native material and are assumed to belong to this genus (Jørgensen, 1977; Klas, 1937; Nelson et al., 1989b), but a single phylogenetic affinity group is by no means proven. *Beggiatoa* is of historical importance because Winogradsky's (1887) earliest experiments, which ultimately led to development of the concept of bacterial chemoautotrophy, were performed with natural enrichments of this organism (see Brock and Schlegel, 1989).

Habitats

Beggiatoa is worldwide in its distribution. Macroscopically visible assemblages dominated by members of this genus can be observed in a variety of environments, all of which are characterized by the presence of a detectable level of hydrogen sulfide. Freshwater environments such as sulfur springs and a variety of eutrophic lakes and ponds contain benthic mats rich in *Beggiatoa* spp. (Keil, 1912; Lackey et al., 1965; Winogradsky, 1887). However, in the lighted region of these water bodies the dominance of this organism is often overlooked. This is because its pattern of daily migration typically brings it to the sediment-water interface only after dark (Jørgensen, 1982a; Nelson and Castenholz, 1982).

Marine environments are especially conducive to massive enrichment for *Beggiatoa* due to the greater abundance of sulfate, which channels a major portion of the anaerobic mineralization of organic matter into the production of hydrogen sulfide (Jørgensen, 1982b). The surface sediments of shallow seas, brackish fjords, salt marshes and intertidal sand flats may be covered by mats rich in *Beggiatoa* spp. (Ankar and Jansson, 1973; Grant and Bathmann, 1987, Jørgensen, 1977; Lackey, et al., 1965). Highly localized regions of anaerobic decomposition, ranging from a whale carcass on the ocean floor (Smith et al., 1989) to oil-soaked heads of coral (Chet and Mitchell, 1975), can also lead to enrichment of *Beggiatoa.* Some deep-sea hydrothermal vents (2,000–2,500 m depth) rich in geothermally produced hydrogen sulfide are also locally dominated by representatives of this genus (Nelson et al., 1989b).

In the examples above, the *Beggiatoa*-dominated assemblage is proven or presumed to exist at a very narrow interface between oxygen and hydrogen sulfide (Jørgensen and Revsbech, 1983). A different sort of environment, characterized as being without oxygen but oxidized in its redox potential (i.e., no free hydrogen sulfide), can also harbor a more diffuse distribution of *Beggiatoa* (Jørgensen, 1977). This "oxidized-anoxic" region of marine sediments is more globally extensive than the rich environments that produce luxurious sediment surface "blooms" of *Beggiatoa* spp. (Jørgensen, 1989). Therefore, it should be possible to enrich for this bacterium from a very wide variety of marine habitats.

Isolation

Selective Enrichments

The most consistently successful enrichments for *Beggiatoa* spp. have been made as follows: The bottom of a shallow pan or aquarium (approximately 30 × 30 × 12 cm) is covered with a few centimeters of sand. $CaSO_4$ (approximately 20 g) and K_2HPO_4 (a few grams) are then added, along with a source of complex organic polymers such as seaweed or shredded paper. This is covered with several centimeters of sulfide-rich marine mud, and then enough seawater to cover the entire enrichment to a depth

of 1–2 cm is added. Covering the pan with aluminum foil or incubating in a dark place minimizes competition with phototrophic bacteria. The enrichment is certain to contain the proper sulfide-oxygen interface somewhere in the vessel if air is introduced near the sediment surface using an airstone. Water lost by evaporation should be replaced with distilled water. Alternatively, a slow steady flow of freshly aerated seawater, with a drain maintaining a constant level, will provide the necessary O_2. A similar freshwater enrichment inoculated with mud from a sulfur spring and maintained on a light-dark cycle (10 h:14 h) provided viable tufts of *Beggiatoa* spp. for almost a year (Nelson and Castenholz, 1982).

Sewage treatment plants are also an excellent source of enrichment material (Burton and Lee, 1978; Williams and Unz, 1985). A predictably successful enrichment is achieved using activated sludge freshly collected from an aeration basin of a treatment plant (W. Pfeiffer, personal communication). Approximately 500 ml of this "mixed liquor" is incubated in a 1-liter Erlenmeyer flask at room temperature in the dark. After 5 to 15 days the surface of the settled flocculant material is typically covered with tufts or sheets of *Beggiatoa.*

Another type of enrichment has a long historical association with *Beggiatoa* and is based on the use of extensively extracted dried grasses or hay in an otherwise mineral medium (Cataldi, 1940). The complex polymers such as cellulose residues in the material presumably fuel sulfate reduction localized near the surface of the grasses. This, in turn, provides the hydrogen sulfide necessary to enrich for *Beggiatoa.* The grasses also serve as a physical substrate for these gliding bacteria. Inclusion of soil extract and the enzyme catalase appear to enhance the rate and success of this enrichment (Joshi and Hollis, 1976; Strohl and Larkin, 1978a).

Isolation Procedures

The various enrichments described above are considered successful when they yield "tufts" or "puff balls" comprised mainly of intertwined *Beggiatoa* filaments. The nature and size of these aggregates is similar to that obtained in aerobic growth of certain freshwater strains (Fig. 1d). Several tufts are collected from the sediment-water interface with a Pasteur pipette or pointed forceps and are passed through several rinses in sterile water. These rinses may contain sodium azide to help reduce contamination (Burton and Lee, 1978), and inclusion of sodium sulfide (250–500 μM) has also been suggested. Tufts are then inoculated into the center of well-dried plates (0.8–1.2% agar) of an appropriate medium. After a period of time individual filaments will have moved far enough from the inoculation site so that they can be transferred (along with a small block of agar) using finely pointed watchmakers' forceps. The procedure described by Castenholz (1988) has produced excellent results. Depending on the source of inoculum these single filament isolations can be best accomplished several hours to a few days after inoculation. With practice, up to a half of the filaments isolated are without contaminants and success is greatly facilitated by lightly prescoring the surface of the medium to guide the gliding filaments (Burton and Lee, 1978).

Media for Isolation

As will be discussed later, the marine isolates characterized to date have a different physiology from freshwater *Beggiatoa* strains examined. It is suggested, but not proven, that this is because they have been isolated under very different conditions. All recently studied strains of freshwater *Beggiatoa* were isolated in the presence of full air (O_2 partial pressure approximately 20 kPa) typically using media that contained a low concentration (0.02 to 5 mM) of a single organic compound, principally acetate, lactate, or glucose (Strohl and Larkin, 1978a; Williams and Unz, 1985). Such media sometimes, but by no means always, contained Na_2S or $Na_2S_2O_3$. Successful isolations and sustained maintenance have also been accomplished using agar-gelled but otherwise unsupplemented thiosulfate-mineral medium (Nelson and Castenholz, 1981b). In this instance, trace organic impurities in the agar presumably acted as the carbon source for these nonautotrophic strains. All freshwater isolates currently available are capable of growth in the presence of full air.

By contrast, the half-dozen known strains of marine *Beggiatoa* are obligate microaerophiles that were isolated under microoxic conditions (4 kPa O_2) on medium supplemented with sodium sulfide and sodium acetate. Subsequently it was realized that all strains will grow luxuriantly in sulfide-gradient medium (described below) in the absence of added organics.

Isolation of Lithoautotrophic Marine Strains

The following procedure ensures a Marine Basal Medium (J3) free of precipitates.

Solution 1:

Aged natural seawater (salinity 3.2–3.5%); prefiltered (Whatman #1 or Gelman GF/F) and filtered (0.45 μm)	500 ml

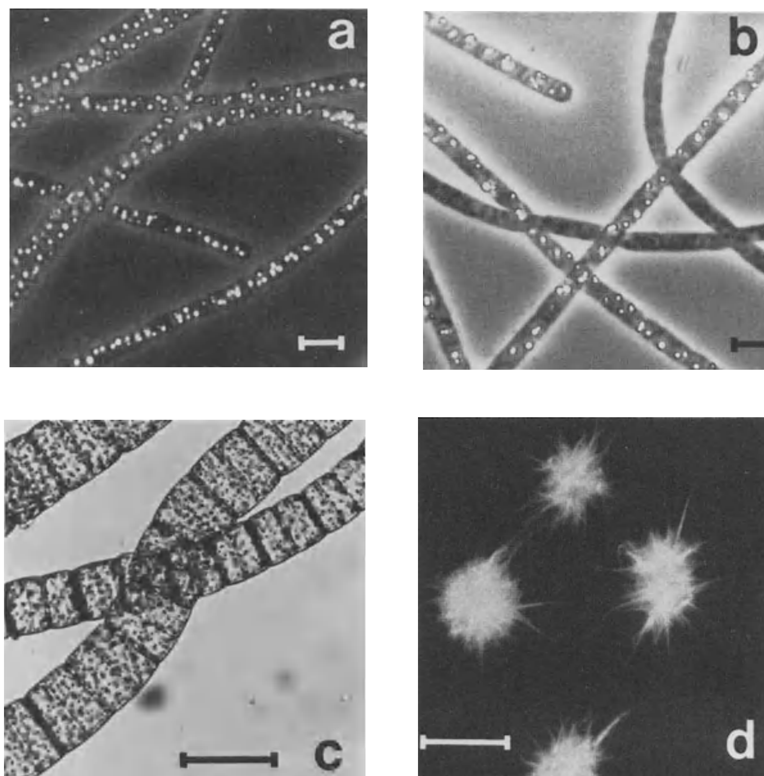

Fig. 1. Light micrographs of pure cultures and natural samples of *Beggiatoa* spp. Part (a) and part (b) are phase contrast; part (c) bright field; part (d) employed reflected illumination and a dissecting microscope. (a) Marine strain MS-81-1c, chemoautotrophic growth, gradient medium. Note refractile S^0 globules. Bar = 5 μm. (b) Freshwater strain OH-75-2a (similar to *B. alba*), stationary phase, liquid DTA medium. Some filaments are devoid of refractile S^0 globules. Nonrefractile inclusions are probably PHB (see Fig. 4). Bar = 5 μm. (c) *Beggiatoa* sp. collected at a deep-sea vent of the Guaymas Basin, Gulf of California. These "hollow," chemoautotrophic filaments (Nelson et al. 1989b) are representative of the wider material frequently found in natural samples. Bar = 50 μm. (Micrograph courtesy of H. W. Jannasch and C. O. Wirsen.) (d) Freshwater strain OH-75-2a aggregated in tufts typical of its aerated growth. Bar = 1 mm.

Solution 2:	
Distilled water	200 ml
Agar	9.0 g
Solution 3:	
NH_4NO_3	0.06 g
Trace elements (SL8, Pfennig and Biebl, 1981)	0.75 ml
Mineral stock	50 ml

The mineral stock contains (per liter): K_2HPO_4, 0.52g; Na_2MoO_4, 0.05 g; $FeCl_3{\cdot}6H_2O$, 0.29 g; $Na_2S_2O_5$ (sodium pyrosulfite), 0.75 g; phenol red, 10 ml of a sterile solution (0.5%, Gibco).

Autoclave solutions 1, 2, and 3 separately in Erlenmeyer flasks. After cooling to 50°C, aseptically combine in the solution 2 vessel (volume > 750 ml). Then supplement with 0.2 ml of Va vitamin solution, which contains (in mg per liter): B_{12}, 1; thiamine, 200; biotin, 1; folic acid, 1; paraaminobenzoic acid, 10; nicotinic acid, 100; inositol, 1; calcium pantothenate, 100.

J3 Basal Medium is amended to produce an Isolation Medium (J-TS) by adding the following sterile stocks (final concentrations in parentheses): 1) 7.5 ml of 200 mM $Na_2S_2O_3$ (2 mM). 2) 3.75 ml of freshly neutralized 200 mM Na_2S (1 mM). This is autoclaved as a basic solution, which is quite stable against autooxidation, and then neutralized with an equimolar quantity of sterile HCl just prior to use. 3) 15 ml of 1 M $NaHCO_3$ (20 mM). To make this stock autoclave 8.4 g of $NaHCO_3$ (dry) and add 100 ml of sterile distilled water when cool.

Immediately after solidification, plates were incubated in a bell jar for 24 h or more under anoxic conditions (99.5% N_2, 0.5% CO_2), with desiccant present to absorb water evaporating from the surface of the medium. The medium is buffered by the bicarbonate in conjunction with the level of atmospheric CO_2. After inoculation with a tuft of *Beggiatoa* spp., plates were placed in a microoxic atmosphere (0.5% CO_2; 0.2% O_2; balance N_2). Exposing the medium and bacteria to full air for approximately 20 min every day or two, as needed for inoculation or single-filament isolations, posed no problem to the success of the technique. Pure cultures resulting from repeated single-filament isolations were maintained in sulfide-oxygen gradient media (see below).

Construction of Gradient Medium

Marine gradient medium (JG8) was constructed as follows: First a 4 ml quantity of J3 Medium (pH 8.4; 1.5% agar; $NaHCO_3$ concentration lowered to 2.0 mM) supplemented with freshly neutralized Na_2S was solidified in the bottom of a 16 × 150 mm screw-capped tube. An initial sulfide concentration of 8 mM in this butt has proven satisfactory for all isolates tested. (The thiosulfate present in the isolation medium need not be included because the tube geometry [Fig. 2] provides a sustained flux of sulfide for several weeks.) This butt was then overlayed with 8.0 ml of semisolid J3 Medium (0.25% agar; $NaHCO_3$ lowered to 2.0 mM; no sulfide or thiosulfate). At this point the gradient of sol-

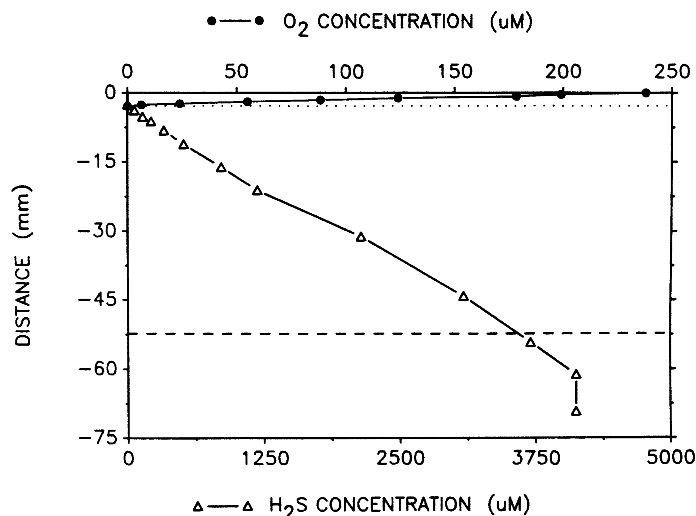

Fig. 2. Microelectrode profiles of O_2 and H_2S in a gradient culture after 47 h of growth at 25°C. The medium was cured 41 h prior to inoculation. Dotted line, location of *Beggiatoa* plate (300 μm vertical thickness). Dashed line, top of initial H_2S butt (8.5 mM). Depth represents distance below air-agar interface. Note different concentration scales. "H_2S" designates sum of soluble sulfide species (e.g., $[H_2S] + [HS^-] + [S^=]$).

uble sulfide (neglecting convective mixing) is theoretically a "step-gradient," i.e., all of the sulfide would be below the dashed line of Fig. 2. The air headspace in the top 8 ml of the tube constitutes an oxygen reservoir. Molecular diffusion and nonbiological reaction between sulfide and oxygen both gradually alter the gradient shapes as described in more detail elsewhere (Nelson et al., 1986a, 1986b).

Aging new gradient media for 2 to 3 days prior to inoculation establishes a sulfide-oxygen interface that is quite stable in both position and rates of nutrient flux. The interface is located approximately as shown in Fig. 2, but the extent of sulfide and oxygen overlap is roughly 6–7 mm in uninoculated medium (Nelson et al., 1986a) as compared with 200μm or less in the cultures (Fig. 3). Whether inoculated at the surface of this medium or stabbed throughout the upper few centimeters, the filaments rapidly proliferate at the sulfide-oxygen interface, forming a marked layer or "plate," which attains a maximum thickness of approximately 1 mm (Fig. 3b). Gliding motility and negative chemotactic responses (discussed later) allow these bacteria to track this interface as it slowly descends due to gradual depletion of the sulfide reservoir.

Isolation of Freshwater Strains

For the freshwater strains currently available, isolations were performed under oxic conditions (air atmosphere) on a variety of media (Nelson and Castenholz, 1981b; Strohl and Larkin, 1978a; Williams and Unz, 1985). A representative medium is shown here:

DTA Medium

ND stock solution (Castenholz, 1988)	50 ml
$(NH_4)_2SO_4$	0.13 g
Sodium acetate	0.68 g
K_2HPO_4	0.027 g
$Na_2S_2O_3 \cdot 5H_2O$	0.50 g
$CaCl_2$	0.10 g
Distilled water	950 ml
Agar	8–12 g

ND stock solution

Distilled Water	1,000 ml
NTA (nitrilotriacetic acid)	2.0 g
Micronutrient solution	10 ml
$FeCl_3$ solution (0.29 g/liter)	20 ml
$CaSO_4 \cdot 2H_2O$	1.2 g
$MgSO_4 \cdot 7H_2O$	2.0 g
NaCl	0.16 g
Na_2HPO_4	1.4 g
KH_2PO_4	0.72 g

Micronutrient solution

Distilled water	1,000 ml
H_2SO_4 (concentrated)	0.5 ml
$MnSO_4 \cdot H_2O$	2.28 g
$ZnSO_4 \cdot 7H_2O$	0.50 g
H_3BO_3	0.50 g
$CuSO_4 \cdot 5H_2O$	0.025 g
$Na_2MoO_4 \cdot 2H_2O$	0.025 g
$CoCl_2 \cdot 6H_2O$	0.045 g

The pH is adusted to 7.0 prior to autoclaving. As in the marine isolations, it is important that the surface of the agar is dry. For purification of single filaments from enrichment-derived tufts, lowering the acetate concentration to 0.5 mM for the initial dispersal of filaments may minimize contamination.

A sulfide-oxygen gradient medium based on DTA Medium has proved effective for maintaining freshwater strains because they require infrequent transfer. The sulfide concentration of the butt should be reduced to 3–4 mM, and thiosulfate can be deleted from the top agar.

Preservation of Cultures

Marine gradient stock cultures require transfer every 3 weeks (25°C) into fresh gradient me-

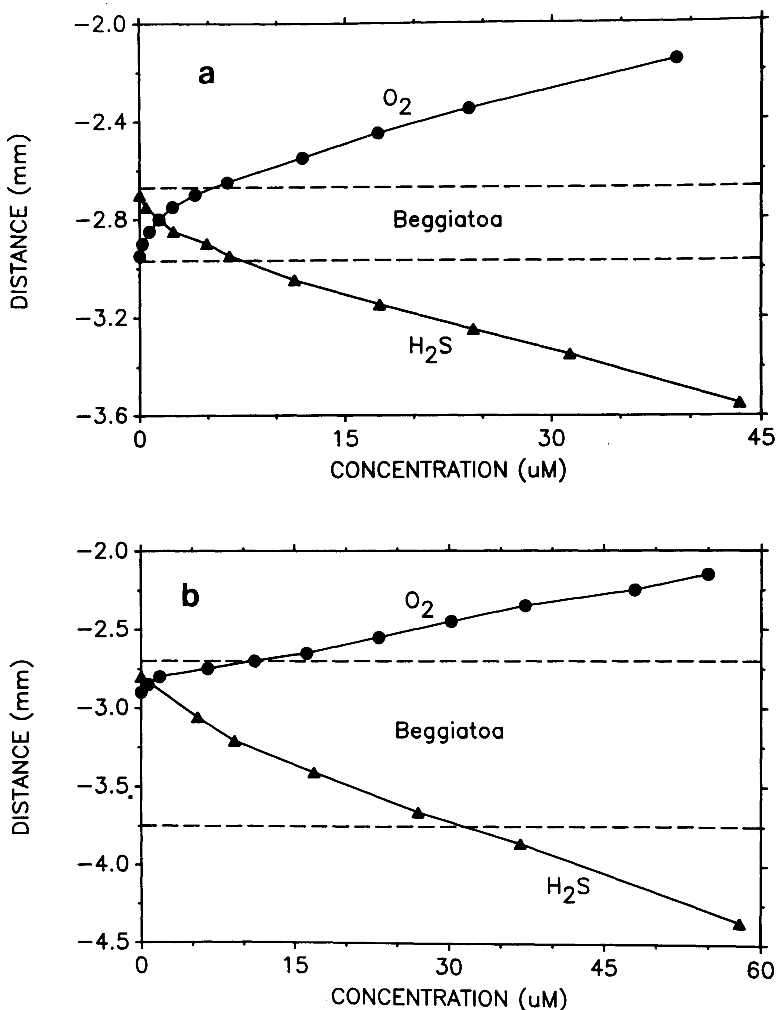

Fig. 3. Fine-scale details of H_2S and O_2 gradients in immediate vicinity of *Beggiatoa* plate. These and other data summarized in Nelson et al. (1986b). (a) Details corresponding to Fig. 2. H_2S flux = 4.8 nmol·mg prot^{-1}·sec^{-1}; O_2 flux = 5.1 nmol·mg prot^{-1}·sec^{-1}. (b) Culture which is a duplicate of part (a) but after 176 h of growth. H_2S flux = 0.46 nmol·mg prot^{-1}·sec^{-1}; O_2 flux = 0.91 nmol·mg prot^{-1}·sec^{-1}.

dium, while freshwater stocks need only be transferred approximately every 6 weeks. Transfers into gradient media aged several weeks will not survive as long as those into freshly prepared media. Stock cultures grown for 1 week at room temperature and then transferred to 10°C maintain viability for 2 to 4 months. Both marine and freshwater strains have been recovered from storage at −80°C for 2 years by inoculating into gradient media. Glycerol (25%) as a cryoprotectant was used along with rapid freezing (solid CO_2-methanol bath).

Identification

Members of the genus *Beggiatoa* are colorless, gliding filaments that contain internal globules of elemental sulfur (S^0). S^0 globules in virtually pure natural assemblages were shown to contain principally S_8 with a small percentage of S_7 (Nelson et al., 1989b). Whenever examined, the S^0 globules were located in the periplasm, surrounded by a nonunit membrane (Strohl et. al., 1982). *Beggiatoa* spp. can be differentiated from other S^0-containing genera as follows: 1) Their lack of photosynthetic pigment and filamentous morphology differentiate them from the purple sulfur bacteria. 2) The other colorless filamentous genera, *Thiothrix* (holdfasts, nonmotile except in the hormogonial stage) and *Thioploca* (common sheath around a bundle of gliding filaments), can also be readily differentiated.

Based on macroscopically visible natural collections, *Beggiatoa* filaments ranging in diameter from 1 μm to approximately 120 μm have been recognized (Strohl, 1989; Nelson et al., 1989b). As summarized elsewhere (Nelson et al., 1989b), it is clear that discrete width classes of this organism exist. Only narrow strains averaging approximately 2 and 4 μm have been isolated in pure culture, and width is aparently an invariant property of these strains.

In the past, different species designations were associated with the various width classes (Leadbetter, 1974); however currently only a

single species, *B. alba,* is recognized (Strohl, 1989). This is a 2–3 μm wide freshwater strain, which, based on 5S rRNA analysis, belongs to the gamma subgroup of the purple bacteria (Stahl et al., 1987). An analysis of the type strain (ATCC #33555) and of a very similar independent isolate (OH-75-2a) indicated a GC content of 37–40 mol% (Nelson, 1989; Mezzino et al., 1984). Although not reported as such, OH-75-2a is the *Beggiatoa* strain tested by Woese et al. (1985), which was found to belong to the gamma group of the purple bacteria based on 16SrRNA oligonucleotide catalogs (J. Gibson, personal communication). Since bulk liquid cultivation of autotrophic marine strains has just become possible (K. Diggs, unpublished observations), the evolutionary relationship of these to the freshwater strains has yet to be established. To date, there have been no studies of DNA homology within this genus.

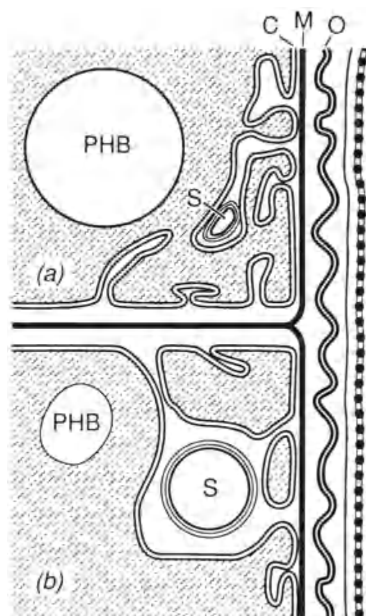

Fig. 4. Schematic of *Beggiatoa alba* strain B15LD (ATCC #33554). Symbols: C, cell membrane; M, presumed murein layer; O, presumed outer membrane layer; S, globule of S^0; PHB, poly-β-hydroxybutyrate granule. (a) Note large PHB inclusion and rudimentary S^0 globule typical of cells grown in acetate-supplemented mineral medium. (b) Note small PHB inclusion and large S^0 globule typical of cell grown in the presence of sulfide or thiosulfate and a low concentrate of acetate. (Figure adapted from Strohl et al. [1982]; by permission of Society for General Microbiology and the author.)

Cell Structure

Narrow *Beggiatoa* filaments examined in pure culture (2–5 μm width) are typically composed of cylindrical cells (length ranging from 1.5–8 × width (Faust and Wolfe, 1961; D. Nelson, unpublished observations; Scotten and Stokes, 1962). However, crosswalls are rarely visible in cultured cells filled with poly-β-hydroxybutyrate (PHB) or S^0 (Figs. 1a and b). Trichomes may exceed 1 cm in length when actively growing in semisolid medium. Terminal cells are rounded in all cultured strains. Exhausting of nutrients results in trichome breakage at necridia or "sacrificial" cells (Pringsheim, 1964; Strohl and Larkin 1978b) to produce trichomes as short as 3 to 10 cells in length. The wider filaments (15–120 μm; Klas, 1937; Jørgensen, 1977; Nelson et al., 1989b) are disk-shaped with cell lengths (5–25 μm) ranging from 0.10–0.90 × cell width (see Fig. 1c). At least some of the wider filaments (e.g., Fig. 1c) are hollow, i.e., composed of a thin cylinder of cytoplasm surrounding a large central vacuole (Nelson et al., 1989b).

The few *Beggiatoa* strains tested are Gram-negative both phylogenetically and by staining; however, they have unusual cell wall structures. Figure 4 shows a schematic diagram that indicates that only the inner layer of the cell wall (presumably murein) plus the cell membrane participate in septation. The outer envelope layers do not participate in cross-wall formation but are apparently continuous over the entire filament length (Strohl et al., 1982).

Three types of inclusions have been reported for various *Beggiatoa* strains: PHB (Pringsheim, 1964; Strohl and Larkin, 1978a; Strohl et al., 1982), polyphosphate (Strohol and Larkin 1878a; Maier and Murray, 1965), and sulfur (Strohl et al., 1981b, 1982; Winogradsky, 1887). The reports of polyphosphate should be regarded as tentative because the staining technique employed is nonspecific (Krieg and Hylemon, 1976). Production of PHB appears, on the other hand, to be a universal feature of the freshwater strains examined. Interestingly, PHB deposition seems to correlate primarily with high aeration (Pringsheim, 1964) and it can account for up to 50% of total dry weight under these conditions in the absence of sulfide (Güde et al., 1981).

The sulfur inclusions of *Beggiatoa* are periplasmic in location, being enclosed in invaginations of the cell membrane (Fig. 4). The S^0 globules in the specific strain diagramed here are enclosed within a multiayered sulfur inclusion envelope of 12–14 nm thickness (Strohl et al., 1982) while in other strains the S^0-globule envelope appears to be composed of a single protein layer 4–5 nm thick (Strohl et al., 1981b). The extraction of S^0 globules with solvents such as pyridine and their refractile appearance when intact cells are viewed under phase contrast mi-

croscopy have proven very useful in confirming their presence (Skerman et al., 1957). As shown schematically (Fig. 4), *Beggiatoa* cells grown in the absence of reduced sulfur compounds apparently contained small, "rudimentary" S^0-inclusion envelopes (Strohl et al., 1982). Since dehydration solvents (e.g., ethanol) necessary for preparation of the electron microscopy specimens dissolve the S^0, it is difficult to determine whether the rudimentary inclusions completely lack S^0.

Physiological Properties

Freshwater Strains

All freshwater strains tested to date could be grown as areobic chemoheterotrophs, and those investigated appear to posses a functional tricarboxylic acid (TCA) cycle with glyoxylate bypass. A very limited number of organic acids, alcohols, and TCA cycle intermediates could serve as the sole source of carbon and energy, with the most universal among these being lactate, acetate, pyruvate, and ethanol. Strain-specific differences are summarized elsewhere (Nelson, 1989; Strohl, 1989).

The earliest studies of freshwater strains reported what we would now interpret as chemoautotrophic growth (Winogradsky, 1887; Keil, 1912). Since that time, repeated attempts to obtain growth of freshwater *Beggiatoa* strains in completely inorganic media have met with failure (Pringsheim, 1967; Strohl, 1989; Nelson, 1989). However, two enzymes diagnostic for the Calvin cycle, ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase, were detected in two of these strains. Demonstration of enzyme activity has also sometimes been confirmed by hybridization with appropriate gene probes (Nelson et al., 1989a). Curiously, the enzyme activities observed were one or more orders of magnitude below those deemed minimally necessary for autotrophic growth. A plasmid location was suggested for the ribulose-1,5-bisphosphate carboxylase/oxygenase gene (Nelson et al. 1989a).

Under the imprecise label of "mixotrophy," lithoheterotrophy has been repeatedly postulated for various freshwater strains (Güde et al., 1981; Strohl and Schmidt, 1984; Pringsheim, 1967). At this time, as discussed in detail elsewhere (Nelson, 1989), there is no clear proof of lithoheterotrophic growth of any freshwater strain.

In gradient media a significant portion of the niche of *Beggiatoa* is anoxic, but microoxic conditions prevail a few hundred micrometers away in another portion of the bacterial plate. A demonstration of this is included here for a marine strain (Fig. 3) and presented elsewhere for freshwater strains (Nelson et al., 1986b). Constant gliding in this medium, which simulates the gradients of this bacterium's natural environment, presumably exposes filaments to alternating anoxic and microoxic conditions. Individual cells will spend on the order of a few seconds to a few minutes in one microniche before passing to the other. Although O_2 is the only electron acceptor proven to sustain growth of *Beggiatoa,* there is evidence that other acceptors are of importance under anoxic conditions, at the very least for sustaining motility. Stored S^0 globules, nitrate, and nitrite are all reduced by some strains under these conditions (Nelson and Castenholz, 1981a; Schmidt et al., 1987; Vargas and Strohl, 1985b). The participation of acetate oxidation in this process has sometimes been demonstrated, but the role of stored PHB has not been carefully addressed. Whether biomass increase occurs under these conditions is not known.

Virtually all freshwater strains tested are capable of fixation of dinitrogen under microoxic conditions (Nelson et al., 1982; Polman and Larkin, 1986). Ammonia, nitrate, nitrite, a few amino acids, and urea are also useful sources of combined nitrogen for some or all strains (Nelson et al., 1982; Vargas and Strohl, 1985a, 1985b). Based on repeated growth in media that contain sulfate as the only sulfur source, there is no evidence of an assimilatory need for reduced-sulfur compounds in freshwater strains.

Marine Strains

Our limited knowledge of the physiology of marine strains is based largely on studies in gradient media. The half-dozen isolates tested to date all grew as chemoautotrophs in a seawater-based medium that contained oppositely sloped gradients of O_2 and H_2S (Fig. 2 and 3) and was devoid of added organic compounds except vitamins (Nelson and Jannasch, 1983; Nelson et al., 1986b). Quantitative insights into this type of growth were obtained using microelectrodes to measure the shapes of O_2 and H_2S gradients and the respective fluxes of these nutrients. Representative data indicate that the "plate" or "lens" formed by an actively growing *Beggiatoa* culture can completely consume H_2S and O_2 fluxes within a vertical distance of 300μm or less (Figs. 3a and b). It has been further demonstrated that bacterially mediated consumption of these two nutrients in their zone of overlap is at least three orders of magnitude more rapid than the purely chemical oxidation of H_2S

in uninoculated control medium (Nelson et al., 1986a). Given also the values for diffusion coefficients, instantaneous rates of H_2S and O_2 consumption can be calculated from the slopes of these gradients immediately below or above the *Beggiatoa* plate, respectively. As cultivation in gradient medium continues, depletion of the H_2S reservoir results in a diminished flux until the rate of transfer is only sufficient to provide maintenance energy of the enlarged *Beggiatoa* population (Fig. 3b). At this point the bacterial plate is considerably thicker, the zone of H_2S and O_2 overlap extremely small, and the cells are, on average, predominantly in an anoxic environment.

Of the two strains studied in detail, one (MS-81-6) is also capable of organoheterotrophic growth with acetate as the sole carbon and energy source in liquid medium under microoxic conditions (Nelson and Jannasch, 1983). This strain also responds to a limited number of organic compounds if they are added singly to sulfide-limited gradient medium. These compounds can augment both O_2 consumption and total biomass production (Nelson, 1989). By contrast, the other strain (MS-81-1c) appears to be an obligate chemoautotroph (Nelson, unpublished observations).

Appropriately high activities of ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoribulokinase are taken as evidence of a functional Calvin cycle in all of these marine strains (Nelson et al., 1989a; Nelson, unpublished observations). Likewise, all strains demonstrated the capacity for nitrogen fixation (Nelson et al., 1982). Determination of the enzymes involved in sulfur-based energy metabolism is currently in progress for these strains. Interestingly, extremely high concentrations of an unusual *c*-type cytochrome have been implicated in H_2S oxidation in very pure natural assemblages of *Beggiatoa* sp. collected from deep-sea hydrothermal vents (Prince et al., 1988).

The sharp upper boundary of a *Beggiatoa* plate (e.g., Fig. 3a and 3b) is almost certainly the result of the negative chemotactic response to O_2 demonstrated by Møller et al. (1985). Likewise, the sharp lower boundary of these plates is taken as presumptive evidence of a negative chemotactic response to excess H_2S. Thus, *Beggiatoa* appears to find its interface niche, somewhat paradoxically, by avoiding high concentrations of its two major nutrients. There is also evidence (Nelson and Castenholz, 1982; Møller et al., 1985) that a negative response to blue light is common. Furthermore, it appears that this response is of ecological importance in helping *Beggiatoa* sp. to locate the H_2S-O_2 interface if it must pass down through a cyanobacterially produced O_2-maximum to do so.

Applications

When growing at or below the sediment-water interface, *Beggiatoa* serves the extremely important function of preventing the aerobic phase of an ecosystem from becoming anaerobic (Kuenen, 1975) as long as it has the capacity to quantitatively harvest the H_2S flux (cf. Figs. 2 and 3). The organisms thus protected can be marine fauna of sediments or the overlying water (Ankar and Jansson, 1973) or rice plants in flooded soils (Joshi and Hollis, 1977). *Beggiatoa* also may channel some of the potential energy of the H_2S flux into dinitrogen fixation. The agricultural importance of this process may have been underestimated due to the difficulty of culturing this organism.

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CHAPTER 167

The Family Halomonadaceae

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The Halomonadaceae (Franzmann et al., 1988) presently contains representatives of two genera, the type genus *Halomonas* and at least one species of the genus *Deleya (D. aesta)* (see Chapter 168). The establishment of the family was suggested because S_{ab} values obtained from 16S rRNA catalogs show that *Halomonas* and *Deleya* are phylogenetically isolated from all other major groups of the gamma subdivision of the Proteobacteria (Stackebrandt et al., 1988 Woese et al., 1985), forming an internally coherent cluster at an S_{ab} of 0.60. Internally, the family contains two subgroups composed of the type species of *Halomonas, H. elongata* and *Halomonas halmophilum* (formerly *Flavobacterium halmophilum*). This cluster forms at an S_{ab} of 0.66. The companion cluster contains *H. subglaciescola* and *D. aesta* and forms at an S_{ab} of 0.67. The position of the other halomonads (Table 1) within the phylogeny is not presently known.

The Genus *Halomonas*

The genus *Halomonas* presently contains four recognized species and two closely related but as yet unnamed strains (Table 1). The type species of the genus is *Halomonas elongata* (ATCC 33173), which has one biotype (ATCC 33174) (Vreeland et al., 1980; Vreeland, 1984; Hebert and Vreeland, 1987). All of the members of this genus are Gram-negative rods that exhibit extreme tolerance to NaCl. These bacteria are rather nonfastidious, being able to grow on a wide variety of sole carbon compounds.

Taxonomy

Vreeland et al. (1980) originally placed these organisms into the family Vibrionaceae based largely upon the fact that the type species produced acid from glucose under anaerobic conditions, indicating some fermentative ability. In addition, the organisms produced numerous curved, highly motile cells. However, since this fermentative ability appeared to be restricted to a single unique situation, the genus was given separate status among the Gram-negative aerobic rods and cocci in the most recent edition of *Bergey's Manual* (Vreeland, 1984). Woese et al. (1985) studied the 165 rRNA and suggested that these bacteria belong to group 3 of the gamma subdivision of the Proteobacteria (Stackebrandt et al., 1988). The genus *Halomonas* possesses seven unique RNA signature sequences, CCUAACUUCG, UUAAUACCCG, AUAAC-

Table 1. Currently recognized species of the genus *Halomonas* and closely related strains.[a]

Species name	Type strain	Biotype	Reference
Halomonas species			
H. elongata[T]	ATCC 33173	ATCC 33174	Vreeland et al., 1980
H. halodurans	ATCC 29686	DSM 30433	Hebert and Vreeland, 1987
H. subglasciescola	UQM 2926	UQM 2927	Franzmann et al., 1987
H. halmophilum	NCMB 1971, ATCC 19717	–	Franzmann et al., 1988
Closely related strains			
Ba1	ATCC 43985	–	Rafaeli-Eshkol, 1968; Huval et al., unpublished observations
Strain NRCC 41227	ATCC 43984		Matheson et al., 1976; Huval et al., unpublished observations

[a]Abbreviations: T, type species of the genus; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung für Microorganismen; UQM, University of Queensland Microbial Culture Collection; NRCC, National Research Council of Canada Culture Collection.

UUG, CCCUCG, UCUCAG, and UUAACG. (Woese et al. 1985) Phylogenetically, the *Halomonas* species and *Deleya aesta* are isolated from the other sublines of the gamma subclass, including such well-defined families as Enterobacteriaceae, Aeromonadaceae, Vibrionaceae, Ectothiorhodopiraceae, and Chromatiaceae (Franzmann et al., 1988). How many other members of the genus *Deleya* also belong to the Halomonadaceae must still be determined. However, in view of the close rRNA similarities known to exist among many of the organisms now contained within the genus *Deleya,* it would seem logical to expect that a significant number will ultimately be shown to belong to the Halomonadaceae.

Habitats

The *Halomonas* were first isolated from a solar saltern on the island of Bonaire in the Netherlands Antilles (Vreeland et al., 1980). The organisms were originally isolated from the saturation ponds and crystallizers in the facility, and were in close association with extremely halophilic archaebacteria. Their extreme salt tolerance and the fact that they are able to survive and prosper with very little NaCl suggests that they are present in virtually any saline environment (Vreeland, 1984).

Halomonas-like strains have been isolated in Canada (J. H. Huval, D. J. Kushner, and R. H. Vreeland, unpublished observations; Matheson et al., 1976), from an American estuary (Hebert and Vreeland, 1987; Rosenberg, 1983), the Dead Sea (J. H. Huval, D. J. Kushner, R. H. Vreeland, unpublished observations; Rafaeli-Eshkol, 1968), manganese nodules in the Pacific Ocean (Hebert and Vreeland, 1987), and from the Antarctic (Franzmann et al., 1988). In addition, several *Halomonas*-like bacteria have been isolated from underground salt formations in the United States (Vreeland and Huval, 1990). As of this writing, no *Halomonas* have been isolated from saline soils, soda lakes, or naturally occurring saline lakes, although several of the moderately halophilic soil isolates described by Quesada et al. (1983) and Ventosa et al. (1982) have properties similar to the *Halomonas.*

Isolation

Due to their wide-ranging biochemical ability, the *Halomonas* are relatively easy to isolate. They can be grown on complex or defined media having a variety of formulations. (See Table 2 and "Laboratory Cultivation"). Because of their salt tolerance, *Halomonas* will grow on these media regardless of the NaCl content used. These organisms have been successfully cultured in NaCl concentrations ranging from 0.016 to 5.5 M in complex media and 0.05 to 3.4 M in defined media (Vreeland et al., 1980; Vreeland and Martin, 1980). The *Halomonas* species actually require the Na^+ cation but not the Cl^- anion, consequently sodium can be supplied as NaBr, $NaNO_3$, Na_2SO_4, Na acetate, or Na glutamate (Vreeland and Martin, 1980; Vreeland et al., 1984). In addition to laboratory media, *Halomonas* strains have been successfully isolated using media prepared by supplementing water taken from the environment. This technique was successfully employed by Franzmann et al. (1987) to isolate *H. subglasciescola.*

While there is no particular technique that can be used to selectively enrich for *Halomonas,* some degree of selection can be obtained by taking advantage of the salt tolerance of the organism. This can be done with relative ease by inoculating samples containing small amounts of NaCl onto media with a high NaCl concentration, or vice versa. The *Halomonas* strains are able to survive sudden NaCl fluctuations of as much as 200-fold (0.2 to 20% w/v NaCl) without experiencing large-scale mortality (Martin et al., 1983), while shocks of this magnitude will quickly kill nonhalotolerant or halophilic bacteria.

Isolation Procedures

In general the *Halomonas* can be isolated using standard bacteriological techniques such as surface inoculation of samples, followed by single colony purification using quadrant streak plates. Some care should be exercised when attempting to isolate these bacteria using plates containing high salt concentrations. First, such plates tend to dry out rapidly, resulting in crystallization of the NaCl. Therefore, high-salt plates should be incubated in high humidity or sealed in plastic bags. Second, plates containing salt concentrations in excess of 25% NaCl have too little free water to support adequate colony growth. Under such conditions, *Halomonas* colonies will be very small and perhaps even transparent, necessitating the use of dissecting microscopes for isolation.

Laboratory Cultivation

Halomonas can be cultivated in any type of glassware, using a rotary or reciprocating shaker to provide oxygenation. Most of the available *Halomonas* strains will grow well on the chem-

Table 2. Recipes for complex media used to grow species of the genus *Halomonas*.[a]

Ingredient	Rosenberg (1984)	Franzmann et al. (1987)	Vreeland et al. (1980)
Casamino acids			7.5
Proteose peptone #3			5.0
Peptone	1.0		
Yeast extract	1.0		1.0
NaCl	0–178.2		80
$MgSO_4 \cdot 7H_2O$			20
K_2HPO_4			0.5
Na citrate			3.0
ASW[b]	26.0		
Agar	15	15	20
pH	7.0	7.0	7.2
Organic lake water (Antarctic)		1 liter	
Distilled water	1 liter		1 liter

[a]All values in grams per liter of final medium.
[b]ASW, artificial sea water.

ically defined medium described by Martin et al. (1983) or Vreeland et al. (1984). Although this medium can be used with virtually any NaCl concentration or carbon source, when low NaCl concentrations are being used growth is more reproducible if the medium is supplemented with 1.0 mM $CaCl_2$ (Vreeland et al., 1984). The basal defined medium has the following composition:

Basal Defined Medium

$MgCl_2 \cdot 6H_2O$	0.026 M
KCl	0.01 M
$(NH_4)_2SO_4$	0.031 M

This basal salts solution is made as a 10× stock for use in both medium and wash solutions. The completed medium also contains:

$CaCl_2$	0.001 M
K_2HPO_4	0.0029 M
Carbon source	0.01 M
NaCl	Variable

The carbon source and NaCl can be added directly to the diluted basal salts solution, the pH adjusted to 7.0 and the solution sterilized by autoclaving. The calcium and phosphate salts must be made as separate solutions (100× is convenient) and sterilized separately from the other components. These latter solutions must not be added until the medium has cooled to room temperature. If they are added to hot medium they will precipitate making the medium unless.

The above medium works well for strains of *H. elongata, H. halodurans,* and Bal, but it must be supplemented with 0.01% yeast extract for NRCC 41227; *H. subglasciecola* and *H. halomophilum* have not yet been grown on this medium. *H. subglasciescola* has been grown on a defined medium described by Franzmann et al. (1987). This defined medium contains:

Medium of Franzmann et al. (1987)

NaCl	80 g
$MgSO_4 \cdot 7H_2O$	9.5 g
KCl	5.0 g
$CaCl_2$	0.2 g
$(NH_4)_2SO_4$	0.1 g
KNO_3	0.1 g
Yeast extract	1.0 g
Distilled water	960 ml

The pH of this solution is adjusted to 7.0 and the solution is sterilized by autoclaving. When the medium is cool, it is supplemented with 20 ml of phosphate solution and 1.0 ml of vitamin mix, which have the following composition:

Phosphate solution

K_2HPO_4	50 mg
KH_2PO_4	50 mg
Distilled water	20 ml

Vitamin mix (filter-sterilized):

Cyanocobalamin	10 mg
Biotin	2.0 mg
Thiamine	10 mg
Ca pantothenate	5.0 mg
Folic acid	2.0 mg
Nicotinamide	5.0 mg
Pyridoxine HCl	10 mg
Distilled water	100 ml

In addition, Franzmann et al. (1987) added 20 ml of Hutner salts solution to the final medium (Staley, 1981). This solution contains:

Distilled water	1 liter
Nitriloacetic acid	10.0 g
$MgSO_4 \cdot 7H_2O$	29.7 g
$CaCl_2 \cdot 2H_2O$	3.3 g
$NaMoO_4 \cdot 2H_2O$	12.7 mg
$FeSO_4 \cdot 2H_2O$	99.0 mg
EDTA*	130 mg
$ZnSO_4 \cdot H_2O$*	550 mg
$FeSO_4 \cdot 7H_2O$*	250 mg
$MnSO_4 \cdot H_2O$*	80 mg

$CuSO_4 \cdot 5H_2O$*	20 mg
$CoCl_2 \cdot 6H_2O$*	10 mg
$Na_2B_4O_7 \cdot 10H_2O$*	9 mg

The nitriloacetic acid must be neturalized with KOH prior to adding the remaining ingredients. In addition, the items marked * can be combined in a separate 1-liter solution (multiply all above weights by 20); then, 50 ml of this solution is added to the Hutner salts.

CULTURE PRESERVATION. *Halomonas* cultures have been stored at 4°C in sealed screw-capped tubes on slants of complex media for up to 3 years without noticeable loss of viability. Cultures can also be stored under liquid nitrogen or frozen using the Protect® system (Pro-Labs, Houston) in which the organisms are adsorbed to ceramic beads.

Halomonas cultures have been lyophilized, although this method is less effective than those listed above. If the cultures are lyophilized, the lyophilization vials should be flame-sealed.

Identification

In order to differentiate between the *Halomonas* and other genera, it is important to consider a wide range of morphological and physiological characteristics. There is no single test that will unequivocally differentiate the *Halomonas* from other eubacteria. Rather, they are differentiated from other genera on the basis of several phenotypic characteristics or by the presence of their rRNA signature sequences. The most useful phenetic characters are extreme salt tolerance, presence of both catalase and oxidase, presence of both lateral and polar flagella on the same cell (giving rise to a helical type of movement), and formation of rather elongated, flexible cells during some portion of their growth cycle (Vreeland et al., 1980; Franzmann et al., 1987; Vreeland and Huval 1990).

The *Halomonas* are Gram-negative and rod-shaped when grown at all salt concentrations. All known species grow in NaCl concentrations from 0.2 to 25% NaCl, depending on the type of medium used. Complex media tend to support growth over a wider range of NaCl concentrations than do defined media. The colonies formed by these organisms are white to yellow in color, although the yellow tends to become more pronounced as the cultures age. *Halomonas* reduces nitrate to nitrite, and most of the species can grow under anaerobic conditions in the presence of nitrate.

H. elongata, H. halodurans, NRCC 41227, and Bal are rather nonfastidious organisms, able to utilize a wide variety of carbon compounds as the sole source of carbon. *H. subglasciescola* tends to have a somewhat more restricted metabolic ability and grows on fewer carbon compounds than do the other *Halomonas* species. The metabolic abilities of *H. halmophilum* have not been tested. The GC content of the *Halomonas* DNA is between 59 to 63 mol% when measured by both buoyant density and thermal denaturation methods.

The fatty acid patterns of several *Halomonas* organisms (*H. elongata, H. halodurans,* NRCC 41227, and Bal) have been studied (J. H. Huval, D. J. Kushner, and R. H. Vreeland, unpublished observations). These four organisms possess dodecanoate ($C_{12:0}$), 3-hydroxydodecanoate (3-OH $C_{12:0}$), *cis*-9-hexadecanoate ($C_{16:1^9}$), hexadecanoate ($C_{16:0}$), heptadecanoate ($C_{17:0}$), 13-methyltetradecanoate (Me + $C_{14:0}$), and *trans*-9-octadecanoate ($C_{18:1^{9t}}$) as their major fatty acids.

Finally, recently completed studies (Hebert and Vreeland, 1987; Huval and Vreeland, unpublished observations) of the effect of NaCl on the phenotype of *Halomonas* species, and salt-tolerant bacteria in general, point to the need to establish optimum growth conditions prior to performing a taxonomic study of these bacteria. During this study, a group of salt-tolerant strains, including *Halomonas,* were subjected to the same testing regime at three different NaCl concentrations. The results showed that, if these bacteria are tested under nonoptimal conditions, identical strains can be mistakenly identified as members of different genera! If, however, the phenotype is established under optimal growth conditions, the similarities between the species are still the same after over 12 years in culture.

Physiology

The physiological properties of salt-tolerant bacteria, including the genus *Halomonas,* has recently been reviewed in some detail (Vreeland, 1987). There are, however, certain unresolved issues that relate to the uniqueness of *Halomonas.* The first issue deals with the mechanisms for osmoregulation in these organisms. The internal ion and amino acid concentration of *H. elongata* following growth in different NaCl concentrations are significantly lower than in its external environment (Vreeland et al., 1983). In short, while the internal Na^+ and glutamate concentrations of the cells do rise in high salts, the increase *is not sufficient* to balance the external osmotic pressures. A similar situation appears to be true for the halotolerant strain NRCC 41227, as well as other salt-tol-

erant organisms (Matheson et al., 1976; Vreeland et al., 1983; Vreeland, 1987).

A significant portion of the physiology of *H. elongata* can be correlated with these cytoplasmic conditions. For instance, in order to utilize electron microscopy to examine the cell walls of cells grown in different NaCl concentrations, it is necessary to use a fixation buffer composed of the ions and amino acid concentrations found by Vreeland et al (1983). Attempts to use buffers not containing NaCl or even to use the growth medium itself cause extensive plasmolysis or shrinkage of the cells. Second, recent work on several enzymes isolated from *Halomonas elongata* has shown that the conditions needed for optimal enzyme activity always correspond to the cytoplasmic conditions predicted by Vreeland et al. (1983) (M. Bylund, J. K. Dyer and E. L. Martin, personal communication). Further, these enzymes are actually inhibited by the KCl or polyol concentrations needed to provide osmotic balance to the cells during growth.

Nuclear magnetic resonance studies with ^{13}C by Wohlfarth et al. (1990) have shown that several *Halomonas* possess high internal concentrations of the novel cyclic amino acid ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carbonic acid; Galinski et al., 1985). This unique compound is present in molar quantities in the cell, it is a zwitterion, so it is charge-neutral for the cells, and it cannot be detected by any of the amino, acid detection techniques used by previous workers (Vreeland et al., 1983, 1984). These properties fit the criteria predicted by Vreeland (1987) during his discussion of osmoregulation in halotolerant bacteria and the possible existence of a previously undetected osmoregulatory compound. Thus it would appear that ectoine provides *Halomonas* with effective osmoregulation (Wohlfarth et al., 1990), obviating the need to postulate the existence of a hypoosmotic lifestyle for salt-tolerant bacteria as discussed by Vreeland (1987). The biosynthesis of ectoine in *Halomonas elongata* has been elucidated (Peters et al., 1990).

Overall, knowledge of the physiological properties of *Halomonas* species is rather sketchy at present. This is probably due to the fact that the studies conducted to date have been directed at trying to develop an explanation for the salt tolerance of these bacteria, rather than strict physiological examination. Two members of the *Halomonas—H. elongata* and strain Ba1—have actually been examined rather extensively. However, their close taxonomic relationship was not recognized until very recently, so no comparative studies have been performed as yet. Most of the work performed on *H. elongata* has been directed toward an examination of how various anatomical features are affected by salt. Work on strain Ba1 on the other hand has been aimed at studies of the salt resistance of the cells' respiratory system and bioenergetics.

From an anatomical perspective, *H. elongata* makes a variety of NaCl-related adaptations (Vreeland et al., 1990). Vreeland et al. (1984) have shown that cells growing in low salt (0.05 M) continuously produce a large number of blebs from their cell wall, while in higher salts (1.37 to 3.4 M), these blebs disappear. Further, freeze-fracture patterns show that the walls of cells grown in low salt contain fracture faces which are not present in high-salt-grown cells (Vreeland et al., 1984). These data indicate that the cell walls tighten up and are more stable in high salt than in low. The tightening of the cell wall is also indicated by recent data on the sensitivity of *Halomonas* species to antibiotics. The experiments, conducted in both my and Dr. E. L. Martin's laboratories, show that low-salt-grown *Halomonas* are susceptible to a variety of cytoplasmically active antibiotics (i.e., chloramphenicol, streptomycin, and tetracyclines). Yet, when the same strains are grown in higher salt concentrations (1.37 M and up), they are resistant to these antibacterials. Subsequent analyses using labeled antibiotics indicated that the inhibitory compounds were simply unable to enter the cells.

The peptidoglycan of *H. elongata,* while not changing in response to NaCl, is unique for

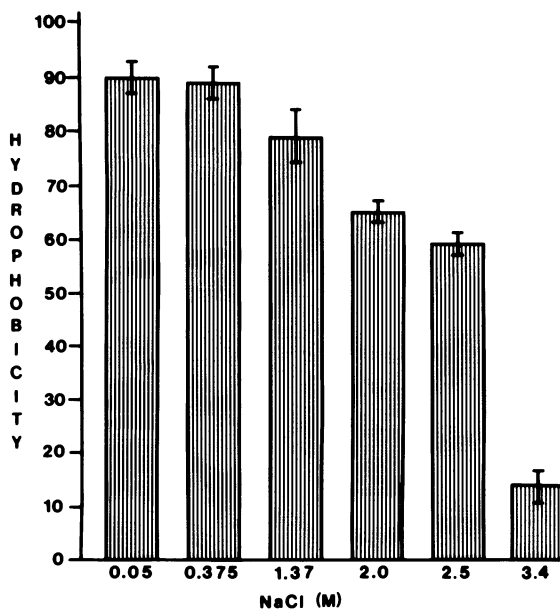

Fig. 1. Differences in the surface hydrophobicity of lag-phase cells of *H. elongata* following growth in different NaCl concentrations. (From Hart and Vreeland, 1988; with permission.)

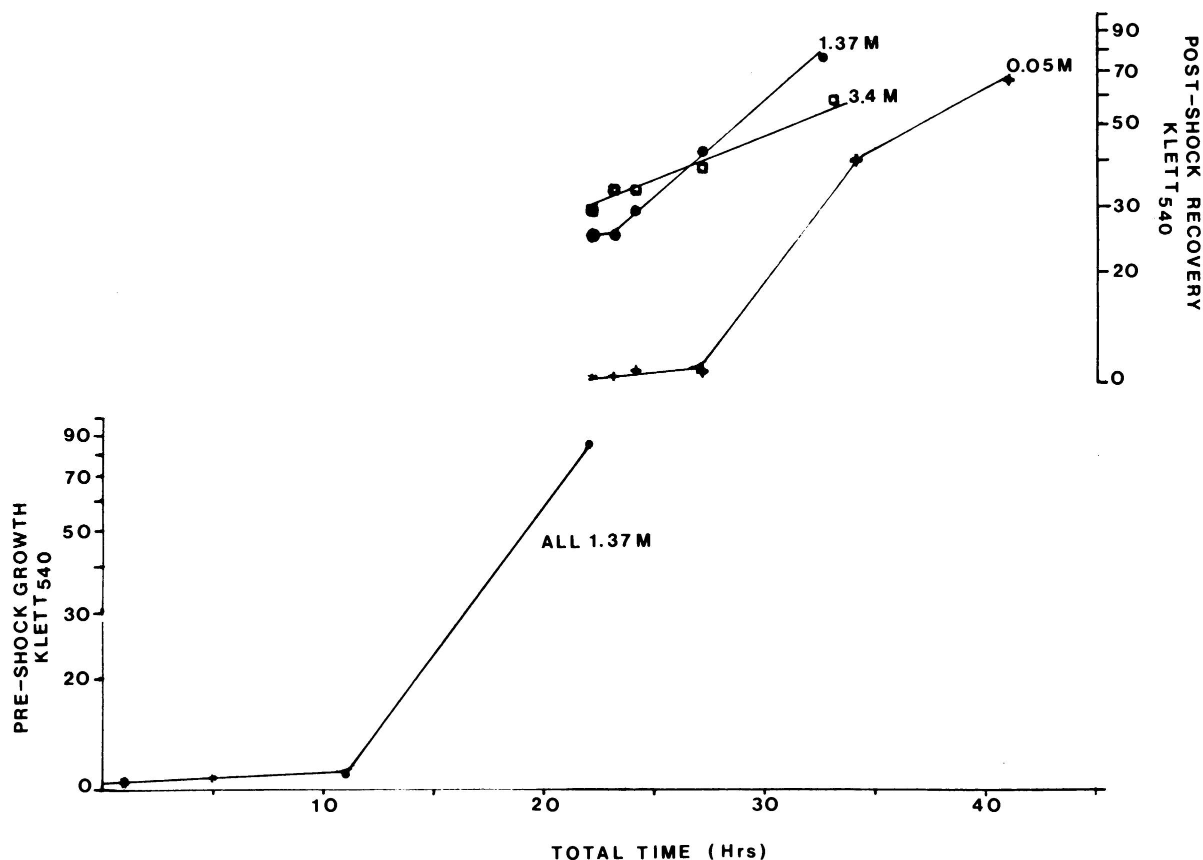

Fig. 2. Growth of *H. elongata* before (pre-shock) and after sudden resuspension (post-shock) in different concentrations of NaCl.

Gram-negative bacteria because it contains a significant amount of leucine. This hydrophobic moiety may be important in the cell wall tightening mentioned above since the strength of hydrophobic bonds increases in high salts. Finally, Vreeland et al. (1984) demonstrated that the phospholipid components were composed of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (C). In low-salt-grown cells, PE makes up 50% of the total phospholipid while PG and C represent 42% and 3% respectively. In high-salt-grown cells, the same ratios are PE, 36%; PG, 44%; and C, 13%. This suggests that as the cells adapt to the high salt, the increased amounts of phosphatidyglycerol and cardiolipin would tend to make the cell surface more negatively charged and hydrophilic. This idea has been confirmed by Hart and Vreeland (1988) (Fig. 1), who demonstrated that high-salt-grown cells were less adherent to hexadecane and therefore more hydrophilic than were low-salt-grown cells (Hart and Vreeland, 1988). The pattern of increased hydrophilicity in high salts does not however, appear to be a wide spread phenomena amongst halotolerant bacteria (D. J. Hart and R. H. Vreeland, unpublished observations).

The salt-related adaptations of *H. elgonata* are physiological in nature (Vreeland et al 1990) and require an adaptation period. When *H. elongata* is grown in 1.37 M NaCl, harvested, and then rapidly resuspended in identical media with low 0.05 M or high 3.4 M NaCl, the cells reenter a lag period (Fig. 2). This lag period may last for up to 12 hours, depending upon the size of the NaCl shock. This sudden shock does not cause a severe noticeable loss of viability (Martin et al., 1983), nor does it cause immediately-detectable changes in the cells' surface hydrophobicity (Hart and Vreeland, 1988). This does not mean however, that the cells are not affected. Electron microscopic studies of cells during a salt-induced lag period (Fig. 3) show that the organisms are definitely subjected to severe stresses, during which water loss causes extensive plasmolysis (Fig. 3, top). After approximately 3 hours, the cells are still plasmolyzed but have a more organized appearance (Fig. 3, middle). Toward the end of the lag period (Fig. 3, bottom), the cells once again ex-

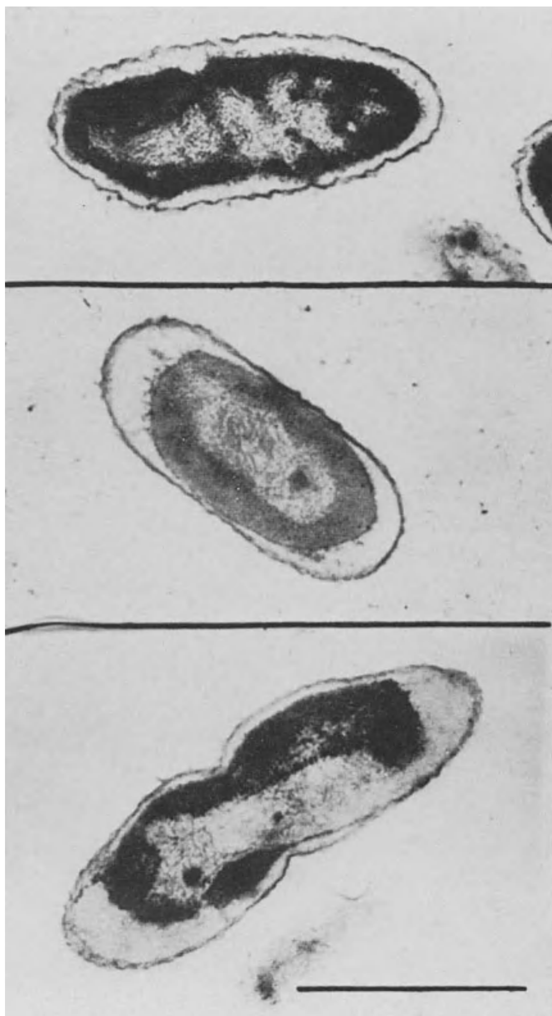

Fig. 3. Plasmolysis stages of cells of *H. elongata* grown in 0.05 M NaCl that have been suddenly exposed to 3.4 M NaCl. Top, immediately after NaCl shock; middle, middle of NaCl-induced lag; bottom, end of NaCl-induced lag just prior to resumption of growth. Bar = 5 μm.

pand to reach the wall, although they still show some level of disruption. During this lag period, cellular protein profiles are also altered and take on the profile of the salt concentration in which the cells are suspended (R. H. Vreeland and R. G. E. Murray, unpublished observations).

The only organism other than *H. elongata* that has been studied at all is strain Ba1. As stated earlier, much of the work on this organism has centered upon the salt resistance of the respiratory chain and on membrane transport systems. As one might expect, the rate of oxygen uptake in Ba1 is significantly affected by the amount of NaCl present in the suspending solution (Rafaeli-Eshkol, 1968). Rafaeli-Eshkol and Avi-Dor (1968) have demonstrated that both choline and its breakdown product, betaine, increased the salt resistance of the respiratory chain. Shkedy-Vinkler and Avi-Dor (1975) have subsequently shown that this stimulatory effect is exerted from outside the cell membrane.

The uptake and extrusion of ions and proline by Ba1 has in fact received some specific attention. Peleg et al. (1980) found that NaCl stimulated the uptake of proline two- to three fold. The K_m and V_{max} data obtained during this study indicated that the same permeases were present in both high- and low-salt-grown cells. Thus the stimulation by Na^+ was not due to an alteration of the cellular transport machinery but to an intrinsic property of the cell permeases.

Recently, Ken-Dror et al. (1986b) demonstrated that the primary site for sodium-mediated respiratory stimulation was the NADH quinone oxidoreductase. In this regard, Bal appears to be similar to *V. alginolyticus.* Further, the sodium transport systems of these bacteria are not linked to the proton motive force. Instead, the electrogenic extrusion of sodium is driven by redox reactions occurring in the respiratory chain (Ken-Dror et al., 1984).

The involvement of Na^+-stimulated respiration in this organism may in fact help Ba1 regulate its internal salt content. Avi-Dor and Schnaiderman (1981) demonstrated that Na^+ stimulation of respiration caused a concomitant increase in the rates of extrusion of Na^+, K^+, and Rb by the cells. Subsequently, Ken-Dror et al. (1986a) showed that the Na^+ pump in these bacteria is not sensitive to uncouplers such as carbonylcyanide-*p*-trifluro-methoxyphenyl-hydrozone, or *N'N'*-dicyclohexyl-carbodimide. As a corollary, Ken-Dror et al. (1986a) noted a distinct volume change in cells adapting to new NaCl concentrations. This suggests that in Bal, and perhaps in *H. elongata* where similar changes have been noted (Vreeland and Martin, 1980), the Na^+ pumps may play a dual role by regulating cell volume (and water?) as well as the cytoplasmic ion content.

Application

At present, *Halomonas species,* like other halophiles, have not been extensively used in biotechnology. Their wide-ranging biochemical ability suggests that they might be useful in the biodegradation of organics in saline areas such as salt marshes, estuaries, and oceans. Some preliminary experiments in the author's laboratory indicate that they may be able to concentrate toxic heavy metals from the environment, particularly in saline regions. Accord-

ingly, *Halomonas* may prove useful for cleaning waste streams contaminated with organic compounds and heavy metals.

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CHAPTER 168

The Genus *Deleya*

KAREL KERSTERS

The genus *Deleya,* along with the genus *Halomonas,* comprises the family Halomonadaceae (Franzmann et al., 1988). This family encompasses various halotolerant and moderately halophilic rod-like Gram-negative nonfermentative, chemoorganotrophs that require 75 mM to 200 mM NaCl for growth. However, some *Deleya* strains grow optimally only in media containing at least 7.5% salts (1.3 M NaCl). "Salt-loving" is a universal feature of all strains belonging to the genera *Deleya* and *Halomonas,* and *Deleya* strains have been isolated from marine environments, solar salterns, saline soils, and salted food.

Within the Proteobacteria, the family Halomonadaceae belongs to the rRNA superfamily II sensu De Ley (1978) (see Chapter 100), i.e., part of the gamma subclass sensu Stackebrandt et al. (1988). The genus *Deleya* consists of six validly published species: *D. aesta, D. cupida, D. halophila, D. marina, D. pacifica,* and *D. venusta* (Baumann et al., 1983; Quesada et al., 1984). The genus is both genotypically and phenotypically heterogeneous; the GC content of the DNA ranges from 52 to 68 mol%. Good evidence exists (see below) that these six *Deleya* species, together with the *Halomonas* species, are part of a single evolutionary lineage. DNA-rRNA hybridizations (De Vos et al., 1989, and this chapter) and chemotaxonomic analyses (Akagawa and Yamasato, 1989; Franzmann and Tindall, 1990) revealed that various organisms misnamed as members of the genera *Pseudomonas, Alcaligenes,* and *Achromobacter* are authentic members of the genus *Deleya.* In the text below, misnamed taxa are enclosed by brackets, [ ], and invalid taxon names are in quotation marks," ".

Intra- and Suprageneric Relationships of *Deleya* Species and Allied Bacteria

In a taxonomic study of more than 200 Gram-negative, nonfermentative, marine eubacterial strains, Baumann et al. (1972) assigned peritrichously flagellated strains of four different phena to four new species of the genus *Alcaligenes ([A.] aestus, [A.] cupidus, [A.] pacificus,* and *[A.] venustus,* respectively). Hendrie et al. (1974) considered *[Achromobacter] aquamarinus* (ZoBell and Upham, 1944) as an acceptable species within the genus *Alcaligenes.* However, it is now well established that all these marine *[Alcaligenes]* strains are genotypically and phenotypically very different from the type species *Alcaligenes faecalis.* The latter species belongs, together with some other *Alcaligenes* taxa and all *Bordetella* species, in the family Alcaligenaceae (De Ley et al., 1986) within the rRNA superfamily III (beta subclass) of the Proteobacteria (see also Chapter 100). On the other hand, all marine *[Alcaligenes]* species are members of the rRNA superfamily II (part of the gamma subclass) (De Ley, 1978; Kersters and De Ley, 1980) and are phylogenetically more related to the authentic *Pseudomonas* species (i.e., *Pseudomonas* section I of Palleroni, 1984). In *Bergey's Manual of Systematic Bacteriology,* the marine *[Alcaligenes]* species were therefore considered as species incertae sedis (Kersters and De Ley, 1984). Evolutionary studies on pathways of carbohydrate metabolism (Sawyer et al., 1977) and on patterns of regulation of aspartokinase activities (Baumann and Baumann, 1974), immunological studies on superoxide dismutases and glutamine synthetases (Baumann and Baumann, 1978; DeLong et al., 1984) as well as chemotaxonomic data (Akagawa and Yamasato, 1989; Franzmann and Tindall, 1990), also yielded strong evidence that the peritrichously flagellated marine *[Alcaligenes]* species constitute a single evolutionary lineage, together with the polarly flagellated species *[Pseudomonas] marina.* This group of bacteria is unrelated to the authentic *Alcaligenes* species and is also distinct from the fluorescent pseudomonads, which are their close relatives. On the basis of all these arguments Baumann et al. (1983) proposed a new genus called *Deleya* to accommodate the marine *[Alcaligenes]* species together with *[P.] marina. D. halophila* was de-

scribed by Quesada et al. (1984) for strains isolated from hypersaline soils in Spain. On the basis of cellular fatty acid profiles, biochemical and physiological features and DNA-DNA hybridizations, Akagawa and Yamasato (1989) proposed that *[Alcaligenes] aquamarinus* was synonymous with *Deleya aesta* and *Deleya aquamarina* be recognized as the type species of the genus *Deleya*. DNA-rRNA hybridizations were performed in order to unravel the inter- and intrageneric relationships of the species of the genus *Deleya*. Fig. 1 summarizes our present knowledge of the position of various taxa and strains on the *Deleya* rRNA branch. All hybridizations were carried out versus labeled rRNA of *Deleya aquamarina* NCMB 557^T (the former *[Alcaligenes] aquamarinus*). The branching point of the genus *Halomonas* at a Tm(e) value of 76°C agrees very well with data from 16S rRNA cataloging (Franzmann et al., 1988). With the latter method, four halophilic species, *Halomonas elongata, H. subglaciescola, H. halmophila* (ex *[Flavobacterium] halmophilum;* Franzmann et al., 1988) and *D. aesta,* form a fairly coherent cluster phylogenetically, with a lowest branching at S_{AB} 0.60 (Franzmann et al., 1988). In the Tm(e) dendrograms, the family Halomonadaceae at 70°C is closest to the authentic *Pseudomonas* group and to *Marinomonas* (Fig. 1).

On the basis of Tm(e) values of DNA-rRNA hybrids versus *D. aquamarina* NCMB 557^T, two groups of strains can be recognized on the *Deleya* rRNA branch (Fig. 1):

1. Strains located in the top 3°C ΔTm(e), including *D. aquamarina* (the type species), *D. aesta* (synonym of *D. aquamarina*), *D. venusta, [A. faecalis]* subsp. *homari* isolated from lobsters, *"[P.] bathycetes," "Agarbacterium*

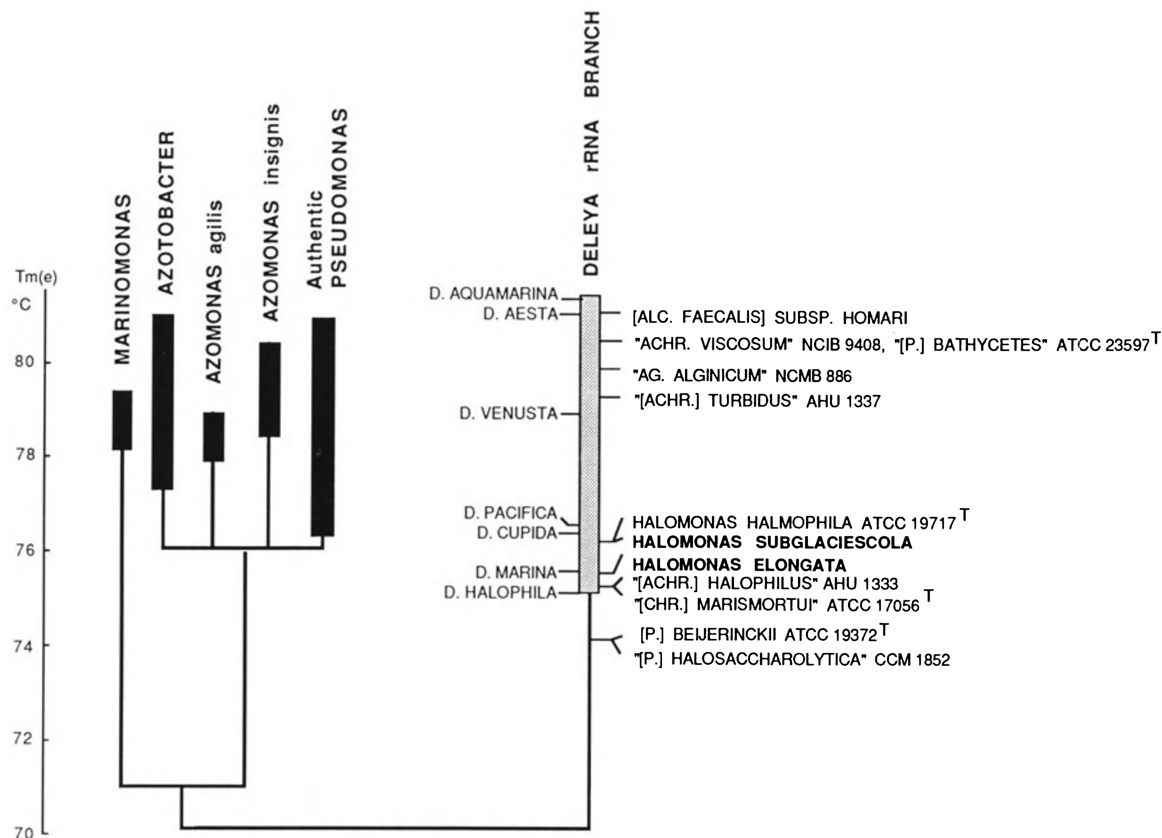

Fig. 1. Simplified rRNA cistron-similarity dendrogram of *Deleya* and related taxa. Tm(e) is the temperature at which 50% of the DNA-rRNA hybrid is eluted. The black bars at the top of the branches represent the ranges of the Tm(e) values of the reference taxa. The position of all validly published *Deleya* species is indicated at the left side of the *Deleya* rRNA branch, whereas three species of *Halomonas* and various generically misnamed taxa and strains are on the right side of this rRNA branch. Abbreviations: ACHR., *Achromobacter,* AG., *"Agarbacterium";* ALC., *Alcaligenes;* CHR., *Chromobacterium;* D., *Deleya;* F., *Flavobacterium;* P., *Pseudomonas.* The position of this group of bacteria within rRNA superfamily II (part of the gamma subclass) of the Proteobacteria can be found in Fig. 5 of Chapter 100. Tm(e) values were taken from De Vos and De Ley (1983), De Vos et al. (1989), Van Landschoot and De Ley (1983), and P. Segers (unpublished observations).

alginicum," and some misnamed *Achromobacter* strains; all these strains possess DNA with a GC content of 52–61 mol%. Akagawa and Yamasato (1989) found that *[A. faecalis]* subsp. *homari* is highly similar to *D. aquamarina.* When more data on their phenotype and genotype will be available, the other misnamed strains will very likely be allocated to the genus *Deleya* either as synonyms of existing species or as separate species.

2. Strains located at the bottom 5–7°C ΔTm(e), including *D. cupida, D. halophila, D. marina, D. pacifica, H. elongata* (type species of the genus *Halomonas*), *H. subglaciescola,* and various misnamed strains (see Fig. 1); the GC content of their DNA ranges from 59 to 68 mol%. More detailed rRNA comparisons are required to elucidate the exact relationships between the different members of this group. In particular, the relationships among this group of strains and all members of the genus *Halomonas* need to be investigated.

Generically Misnamed Taxa and Strains of the *Deleya* rRNA Branch

Genomic and phenotypic studies have indicated that a great number of generically misnamed taxa and strains are authentic members of the *Deleya* rRNA branch (P. Segers and K. Kersters, unpublished observations). The position of these taxa and individual strains is indicated in Fig. 1; additional information on the habitats of these organisms can be found in Table 1.

"[Pseudomonas] bathycetes" originates from deep-sea sediments (Quigley and Colwell, 1968); *"Agarbacterium alginicum"* was isolated from the surface of brown algae and *[Alcaligenes faecalis]* subsp. *homari* is pathogenic for lobsters (Austin et al., 1981). The immunological data of DeLong et al. (1984) on glutamine synthetases indicate that *[Alcaligenes faecalis]* subsp. *homari* is not related to *Alcaligenes,* but it is highly similar to *Deleya.* DNA-rRNA hybridizations (Fig. 1) show that these taxa, together with strains *"[Achr.] viscosum"* NCIB 9408 and *"[Achr.] turbidus"* AHU 1337, belong to the genus *Deleya.* Some misnamed *[Pseudomonas]* and *[Achromobacter]* strains were isolated from salted food, whereas *"[Chromobacterium] marismortui"* originates from water of the Dead Sea.

Intrageneric Relationships of Species of *Deleya*

Data on the finer internal taxonomic relationships of the genus *Deleya* are scarce. Most of the well-established *Deleya* species have been delineated on the basis of numerical taxonomic studies of phenotypic features (Baumann et al., 1972; Marquez et al., 1987; Quesada et al., 1983). DNA-DNA hybridizations revealed that the following taxa are highly related (more than 75% homology): *D. aesta, D. aquamarina,* the lobster isolates (*[A. faecalis]* subsp. *homari*), and *"[Achromobacter] viscosum"* strain NCIB 9408 (P. Segers and B. Hoste, unpublished observations). They are all located in the top 1°C ΔTm(e) of the *Deleya* rRNA branch (Fig. 1), and the GC content of their DNA ranges from 57 to 61 mol%. No significant DNA homologies were detected between *D. aquamarina* and the type strains of the other *Deleya* species (Akagawa and Yamasato, 1989).

Habitats

One common characteristic of all the strains belonging to the *Deleya* rRNA branch is their isolation from salt-containing materials. Table 1 gives an overview of the various habitats where strains of the *Deleya* rRNA branch have been found. Many *Deleya* strains are from marine origin and grow optimally at salt concentrations of 0.4% to 1.2% NaCl (Baumann et al., 1983), but others such as *D. halophila* are moderate halophiles because they display optimal growth at 7.5% NaCl (Ferrer et al., 1987). *D. halophila* strains were usually isolated from hypersaline soils and solar salterns (Quesada et al., 1982; 1983). *Deleya* strains have also been isolated from ponds of an inland Spanish saltern which was supplied with water from a subterranean inland saline well (del Moral et al., 1987, 1988).

Some strains—previously assigned to the ill-defined genus *Achromobacter*—were isolated from various salt-containing foods (Table 1). Austin et al. (1981) isolated *[Alcaligenes faecalis]* subsp. *homari* strains from the hemolymph of moribund and dead lobsters. These lobsters were reared in tanks containing seawater; their death occurred after the water in the tanks accidently reached a temperature of 26°C for three days. The isolated bacteria were found to be pathogenic for lobsters. It has been proven (see above) that three of these isolates (including the type strain) were phenotypically and genomically highly related to *D. aesta* (type species) and *D. aquamarina* (Fig. 1).

Isolation

The following media and isolation procedures were used by several authors for the isolation of strains belonging to the *Deleya* rRNA branch.

More details can be found in Baumann and Baumann (1981).

Media For the Isolation of *Deleya* Species

Artificial Seawater (ASW) (MacLeod, 1968)

NaCl	400 mM
$MgSO_4 \cdot 7H_2O$	100 mM
KCl	20 mM
$CaCl_2 \cdot 2H_2O$	20 mM

Dissolve the salts separately in distilled water and combine.

Basal Medium (BM)

TrisHCl (pH 7.5)	50 or 100 mM
NH_4Cl	19 mM
$K_2HPO_4 \cdot 3H_2O$	0.33 mM
$FeSO_4 \cdot 7H_2O$	0.1 mM
Half-strength ASW	

The carbon source is usually supplied at a concentration of 0.1–0.2% (w/v for solids and v/v for liquids). Labile compounds should be filter-sterilized.

Basal Medium Agar (BMA)

Prepare equal volumes of double-strength BM and double-strength agar in distilled water (40 g agar per liter), sterilize by autoclaving, and combine before pouring plates. Carbon and energy sources can be added to double-strength BM prior to autoclaving. Labile compounds should be filter-sterilized and added to BMA, which has been cooled to about 42°C prior to pouring of plates.

Marine Agar (MA)

Marine agar 2216E is available from Difco Laboratories, Detroit, Mich., USA.

Medium for Moderately Halophilic Bacteria (MH Medium) (Quesada et al., 1983)

5 g proteose peptone, 10 g yeast extract, and 1 g glucose are dissolved in 10% (w/v) marine salts solution. Marine salts solution contains, in one liter of distilled water:

NaCl	81 g
$MgCl_2 \cdot 6H_2O$	7 g
$MgSO_4 \cdot 7H_2O$	9.6 g
$CaCl_2 \cdot 2H_2O$	0.36 g
KCl	2 g
$NaHCO_3$	0.06 g
NaBr	0.026 g

The pH is adjusted to 7.2 with 1 M KOH, and agar (20 g/liter) is added.

MH medium with e.g., 20% marine salts, is prepared by doubling the amount of inorganic salts in the above medium. Lowering the concentration of Mg^{2+} (to 1 g $MgSO_4 \cdot 7H_2O$/liter) increases the selectivity of the MH medium for moderate halophiles, because growth of extreme halophiles is suppressed (Marquez et al., 1987).

Isolation Procedures

ISOLATION FROM SEAWATER (BAUMANN ET AL., 1972). The enrichment culture methods have been described by Baumann et al. (1971) and Baumann and Baumann (1981). Various amino acids (L-lysine, L-glutamate, L-histidine), amines (e.g., histamine) and organic acids (glycolate, *o*-hydroxybenzoate) have been used as sole carbon source for enrichment of *Deleya* strains (Baumann et al., 1972). The strains are purified on BMA containing 0.1% of the same carbon compound as used in the enrichment, or on BMA containing 0.1% Na succinate, 0.1% Na lactate and 0.1% Na acetate.

For direct isolation, samples of sea water are filtered through 0.22- or 0.45-μm pore size nitrocellulose filters, which are placed in petri dishes containing Marine Agar or Basal Medium Agar with 0.1% organic substrates (e.g., *meso*-inositol, D-galactose, adipate, L-valine, etc.) (Baumann et al., 1972).

ISOLATION FROM HYPERSALINE SOILS (QUESADA ET AL., 1983, 1984). *D. halophila* was isolated from hypersaline soils in Spain by suspending one soil sample in 10 ml of 10% marine salts solution (see above); 10-fold dilutions were made (always keeping the same balanced salt concentrations), and 0.1 ml of each dilution was plated on MH medium.

ISOLATION FROM THE HEMOLYMPH OF LOBSTERS (AUSTIN ET AL., 1981). Drops of aseptically collected hemolymph from moribund lobsters *(Homarus americanus)* were inoculated on Marine Agar medium for the isolation of *[Alcaligenes faecalis]* subsp. *homari.* Plates were incubated at 19°C for 7 days.

Preservation of Cultures

Baumann and Baumann (1981) recommended maintenance of *Deleya* strains on MA slants. After each monthly transfer, cultures were allowed to grow at 25°C for 1–2 days and stored at 18°C. Quesada et al. (1983) maintained their strains on slants of MH medium. We maintained *Deleya* strains at 4°C on slants of the following medium: 1% peptone, 0.8% beef extract powder, 3% NaCl, and agar in tap water. Lyophilization is recommended for long-term preservation.

Identification

The range of GC content of the DNAs of members of the genus *Deleya* is very broad (52–68 mol%) and is reflected in 1) the fairly great span of ΔTm(e) values of their DNA-rRNA hybrids versus labeled rRNA of *D. aquamarina* (see Fig. 1); and 2) considerable phenotypic and geno-

Table 1. Sources and geographical distribution of taxa and strains belonging to the *Deleya* rRNA branch.

Species or strain[a]	Source of isolation	Geographical origin	Reference
Deleya aesta	Seawater (at 100–600 m deep)	Hawaii, USA	Baumann et al. (1972)
Deleya aquamarina (ex *[Alcaligenes] aquamarinus*)	Seawater		ZoBell and Upham (1944)
Deleya cupida	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
Deleya halophila	Hypersaline soil	Spain	Quesada et al. (1983, 1984)
Deleya marina	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
Deleya pacifica	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
Deleya venusta	Seawater (surface)	Hawaii, USA	Baumann et al. (1972)
Halomonas elongata	Solar saltern	Netherlands Antilles	Vreeland et al. (1980)
Halomonas subglaciescola	Hypersaline lake	Organic Lake, Antarctica	Franzmann et al. (1987)
Halomonas halmophila ATCC 19717^T (ex *"[Flavobacterium] halmophilum"*)	Hypersaline lake	Dead Sea, Israel	Elazari-Volcani (1940)
"[Achromobacter] halophilus" AHU 1333	Salted salmon	Japan	Bergey et al. (1930)
"[Achromobacter] turbidus" AHU 1337	Salted eggplant	Japan	
"[Achromobacter] viscosum" NCIB 9408			Bergey et al. (1925)
"Agarbacterium alginicum" NCMB 886	Mass of brown algae	Georgia, USA	Adams et al. (1961), Eller and Payne (1960)
[Alcaligenes faecalis] subsp. *homari*	Hemolymph of moribund lobsters	Mass., USA	Austin et al. (1981)
"[Chromobacterium] marismortui" ATCC 17056^T	Hypersaline lake	Dead Sea, Israel	Elazari-Volcani (1940)
"[Pseudomonas] bathycetes" ATCC 23597^T	Deep-sea sediments (at > 9,000 m)	Pacific Ocean	Quigley and Colwell (1968)
[Pseudomonas] beijerinckii ATCC 19372^T	Salted French beans		Hof (1935), Breed et al. (1957)
"[Pseudomonas] halosaccharolytica" CCM 2851	Rock salt	Japan?	Ohno et al. (1979), Yamada and Shiio (1953)

[a]Abbreviations: AHU, Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo, Japan; ATCC, American Type Culture Collection, Rockville, Md., USA; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; NCMB, National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

typic heterogeneity within the genus. An important common feature for all *Deleya* species is their absolute requirement for at least 75–200 mM NaCl for optimal growth. This feature differentiates *Deleya* from terrestrial *Pseudomonas* and *Alcaligenes* species (Baumann and Baumann, 1981).

Deleya species can be differentiated from other marine eubacteria by four important features: absence of fermentation of D-glucose, accumulation of poly-β-hydroxybutyrate, peritrichously flagellated cells (except *D. marina*), and by the parameters of their DNA-rRNA hybrids. Differentiation of *Deleya* from *Halomonas* spe-

cies is difficult because no comprehensive phenotypic or genomic studies have been performed using standardized procedures. Species of *Deleya* can best be differentiated from each other by carbon assimilation tests (Table 2).

Deleya strains are Gram-negative straight rods, 0.8 to 1.1 μm wide and 1.5 to 3.0 μm long. They accumulate poly-β-hydroxybutyrate as an intracellular reserve product. Involution forms may occur in old cultures or under adverse conditions of cultivation. Endospores are not formed. Motile by four to twelve peritrichous flagella, except *D. marina* which is motile by two to five polar flagella. They are obligately aerobic, possessing a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Colonies are nonpigmented. They do not denitrify or fix molecular nitrogen and do not grow chemolithotrophically with hydrogen gas and CO_2. All species are chemoorganotrophs able to grow on a mineral medium containing sea water base, D-glucose and NH_4Cl. Na^+ is an absolute requirement for all species; the minimal concentration for optimal growth ranges from 75 to 200 mM (or even 1.3 M for *D. halophila*). All species utilize acetate, succinate, DL-β-hydroxybutyrate, DL-lactate, glycerol, and L-α-alanine. All *Deleya* (and *Halomonas*) strains contain ubiquinone 9 as the major respiratory quinone with traces of Q8, and 16:1 *cis* 9, 16:0, 17:0 cyclo, 18:1 and 19:1 cyclo 11–12 as major fatty acids (Franzmann and Tindall, 1990). The GC content of the DNA ranges from 52 to 68 mol%. The type species is *D. aquamarina* (type strain ATCC 14400, NCMB 557, DSM 30161, LMG 2853, IAM 12550).

Table 2 lists the diagnostic features for the differentiation of species of the genus *Deleya*. The original publications should be consulted for full descriptions (see references in footnotes of Table 2). The type strains of the species are also listed at the bottom of Table 2. Akagawa and Yamasato (1989) give a few other biochemical and physiological features to differentiate the type strains of the *Deleya* species.

Physiological Properties

Species of *Deleya* have no organic growth factor requirements and utilize a great variety of organic compounds as sole sources of carbon and energy. They can assimilate pentoses, hexoses, disaccharides, sugar alcohols, sugar acids, fatty acids, dicarboxylic acids, tricarboxylic acid cycle intermediates, amino acids, amines, and some aromatic substances. The latter compounds are metabolized via the β-ketoadipate pathway involving the *ortho*-cleavage of diphenolic compounds (Baumann et al., 1972). D-Glucose and D-fructose are metabolized via the Entner-Doudoroff pathway (Baumann and Baumann, 1973; De Ley et al., 1970; Sawyer et al., 1977).

Growth characteristics of the type strain of *Deleya halophila* have been studied in more detail by the research group of A. Ramos-Cormanzana. *D. halophila* displays a specific requirement for Na^+, which cannot be replaced by other cations (Quesada et al., 1987). Optimal growth of *D. halophila* occurs at 7.5% total salts, when incubated at 32 or 42°C. However, when the incubation temperature is lowered to 22°C, its optimal growth occurs at 5% total salts. *D. halophila* is able to grow at salt concentrations varying from 2.5% to 25% (Ferrer et al., 1987). Such characteristics are typical of moderately halophilic bacteria isolated from hypersaline soils, where salt content can vary considerably in space and time.

Investigations have been performed on the effects of growth temperature and salt concentration on the cellular fatty acid composition of *D. halophila*, *"[Pseudomonas] halosaccharolytica,"* and *Halomonas halmophila* (ex *[Flavobacterium] halmophilum;* Franzmann et al., 1988) (Monteoliva-Sanchez and Ramos-Cormenzana, 1986, 1987; Monteoliva-Sanchez et al., 1988; Ohno et al., 1979). Increasing the salt concentration in the medium resulted in an increase of cyclopropanoic acids with a concomitant decrease in the monounsaturated fatty acids. *[Pseudomonas] beijerinckii* and *Halomonas halmophila* mainly contained ubiquinone Q-9 (Collins et al., 1981).

"Agarbacterium alginicum" hydrolyzes agar, a property which can be lost upon continued laboratory culture. The alginase of this bacterium has been studied in some detail (Williams and Eagon, 1962).

Calvo et al. (1988) were the first authors to report the existence of bacteriophages active against moderately halophilic bacteria. They demonstrated the existence of temperate phages in 52% of their *D. halophila* strains. Phage F9–11 (isolated from *D. halophila* strain F9–11) was studied in more detail and possessed an isometric head and a noncontractile tail. The phage could replicate at a wide range of marine salt concentrations, from 2.5% to 15%. Its stability seemed to be influenced by the osmolarity of the medium rather than by the NaCl concentration (Calvo et al., 1988).

Table 2. Features differentiating the species of *Deleya*.[a]

Feature	*D. aesta*[b] (n=6)	*D. aquamarina*[c] (n=1)	*D. cupida*[b] (n=5)	*D. halophila*[d] (n=38)	*D. marina*[b] (n=7)	*D. pacifica*[b] (n=6)	*D. venusta*[b] (n=14)
Flagellation	pr	pr	pr	pr	p	pr	pr
Oxidase	+	+	−	+	−	+	+
Growth at 4°C	−	−	−	+	[+]	−	+
Growth on:							
L-Arabinose	−	−	+	+	−	−	−
D-Ribose	−	+	[+]	+[e]	+	−	−
D-Mannose	−	−	+	+	−	−	−
Saccharate	−		+		−	−	−
Suberate	+	+	−	−[e]	−	−	−
Glycolate	−	−	+	+[e]	−	−	−
Aconitate	−	−	+	−[e]	+	[+]	[+]
Mannitol	+	+	+	[+]	+	−	[+]
δ-Aminovalerate	−	−	[+]	−	−	+	+
L-Histidine	−	−	[+]	−	−	+	[+]
L-Tyrosine	−	−	+	+[e]	+	+	+
DL-Kynurenine	−	−	−	−[e]	−	+	−
Ethanolamine	−	−	−	−[e]	−	−	+
Putrescine	−	−	+		−	+	+
Sarcosine	−	−	+	−[e]	[+]	+	+
Ring cleavage	−		o		−	o	o
GC content (mol%)	57–58	58	60–63	67	62–64	67–68	52–55
Type strain no.	ATCC 27128	ATCC 14400	ATCC 27124	CCM 3662	ATCC 25374	ATCC 27122	ATCC 27125

[a]Symbols: n, number of strains used; pr, peritrichous; p, polar; +, all strains positive; [+], more than 80% of strains positive; −, all strains negative; o, ortho-cleavage of aromatic ring for strains capable of growth on aromatic compounds.
[b]Data from Baumann et al. (1972, 1983).
[c]Data from Kersters and De Ley (1984).
[d]Data from Quesada et al. (1984).
[e]Determined with auxanographic API galleries (API System, Montalieu Vercieu, France) for the type strain only.

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CHAPTER 169

The Genus *Frateuria*

JEAN SWINGS

The genus *Frateuria* comprises bacteria formerly classified as *Acetobacter aurantius* (Kondo and Ameyama, 1958). These are strains isolated from the plant *Lilium auratum,* which are brown, pigment-producing, polarly-flagellated, acetic acid-like bacteria. They were thought by Asai (1968) to be an evolutionary "intermediate" between *Pseudomonas* and *Gluconobacter,* and the position of these "intermediate" strains was discussed in a separate chapter. Within the acetic acid bacteria, their assignment either to *Gluconobacter* or *Acetobacter* has been difficult (Swings et al., 1980; Yamada et al., 1976). Since DNA-rRNA hybridization studies showed that these isolates are quite removed from the family *Acetobacteraceae,* the new genus *Frateuria* was created for them (Swings et al., 1980). *Frateuria* belongs in the Proteobacteria in subclass gamma sensu Stackebrandt et al. (1988) (see Chapter 100). In *Bergey's Manual of Systematic Bacteriology, Frateuria* was treated as a separate genus (Swings et al., 1984).

Habitats

Only two habitats of *Frateuria* are known, *Lilium auratum* (Kondo and Ameyama, 1958) and the fruit of the raspberry *Rubus parvifolius* (Yamada et al., 1976). Only 14 strains are known and described in the literature.

Isolation

Isolation from Raspberries *(Rubus parvifolius)* (Yamada et al., 1976)

> The picked raspberries were incubated at 30°C in a medium containing glucose, 10 g; ethanol, 5 ml; yeast extract, 5 g; peptone, 3 g; and acetic acid 0.3 ml, per liter of 10% potato extract adjusted to pH 4.5. After the enrichment culture, typical acetic acid-like bacteria were selected on $CaCO_3$ plates, (see Chapter 111), where they dissolved the calcium carbonate.

Identification

As *Frateuria* possesses the phenotypical features of the family Acetobacteraceae (see Chapter 111), it was initially classified as an acetic acid bacterium. The minimal phenotypic description is as follows: Gram-negative, strictly aerobic, polarly flagellated rods when motile, requiring no growth factors, producing a water-soluble brown pigment on glucose-yeast extract-$CaCO_3$ agar, producing ubiquinone (menaquinone) with eight isoprenoid units (MK-8) and H_2S, able to grow at pH 3.6 and on Frateur's Hoyer mannitol medium; producing acid from ethanol, glucose, and xylose; lacking oxidase and gelatinase; not reducing nitrates; not hydrolyzing starch.

Frateuria presents another example of the unreliability of using solely phenotypic characterizations at the inter- and suprageneric levels in bacteriology. The production of acetic acid from ethanol and the growth at pH 3.6 suggest a relationship with the acetic acid bacteria which is not justified by more detailed phenotyphic analysis. Additional methods are necessary in order to identify the genus *Frateuria* unambiguously, e.g., the types of ubiquinones formed, cellular fatty acid composition and DNA-rRNA hybridization analysis. *Frateuria* typically produces the ubiquinone MK-8, whereas *Gluconobacter* produces MK-10 and *Acetobacter* MK-9 or MK-10 (Yamada et al., 1976). *Frateuria* has an unusual fatty acid profile, consisting of the iso-branched-chain acid of $C_{15:0}$, which is different from those of *Acetobacter* or *Gluconobacter* (Yamada et al., 1981) and which is more closely related to *Xanthomonas maltophilia.* The application of DNA-rRNA hybridizations constituted the ultimate proof that *Frateuria* is not related to the acetic acid bacteria (subclass alpha of the Proteobacteria), but constituted a separate genus within subclass gamma. The acid-tolerant, nitrogen-fixing bacterium isolates from sugarcane resembled *Frateuria* by its production of a brown diffusible pigment and of H_2S, its lack of growth factor requirements, and

its growth on 30% glucose (Cavalcante and Döbereiner, 1988), but it could be assigned unambiguously to the genus *Acetobacter* by DNA-rRNA hybridizations (Gillis et al., 1989) (see Chapter 111). The genus *Frateuria* currently comprises only one species, *Frateuria aurantia* (Swings et al., 1980, 1984).

Physiological Properties

Good growth occurs on glucose-yeast extract-$CaCO_3$ agar and in beer wort. In peptone broth, yeast extract broth, and Hoyer's mannitol medium, growth is faint. In Hoyer's medium, NH_4^+ is used as a nitrogen source in the presence of mannitol as a carbon source. All *Frateuria* strains grow well on casamino acids, in contrast with the acetic acid bacteria. Growth factors are not required.

The oxidation of lactate was demonstrated on oxydograms. In an oxydogram (Frateur, 1950), several strains are streaked on 2% agar plus 2% Ca lactate. Lactate oxidation is shown by the precipitation of $CaCO_3$. The ketogenic activity on polyalcohols was weak or doubtful. From glucose, 2-keto-, and 2,5-diketogluconic acids were formed but not 5-ketogluconic acid. The production of acid from D-glucose and D-xylose was marked, and the pH always dropped below 4. Ethanol, glycerol, D-arabinose, D-ribose, D-fructose, D-galactose, and D-mannose were also acidified (Swings et al., 1980). Ethanol concentrations above 5% were not tolerated. The majority of the strains still grew in 25 to 30% glucose concentrations, and, in this respect, they also resemble the acetic acid bacteria.

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The Family Chromatiaceae

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The Family Chromatiaceae (purple sulfur bacteria) comprises physiologically and genetically closely related species and genera (Fowler et al., 1984) that carry out anoxygenic photosynthesis. The most important and selective environmental factors in their aquatic habitats are anoxic conditions, the presence of hydrogen sulfide, and illumination. The only other groups of phototrophic bacteria that thrive under similar environmental conditions are the Ectothiorhodospiraceae (see Chapter 171) and the Chlorobiaceae (green sulfur bacteria; see Chapter 195). Because they live in the same types of habitats, some discussion of the Chlorobiaceae must be included in this chapter. However, since the Chlorobiaceae are not phylogenetically related to the other anoxygenic phototrophic bacteria (Stackebrandt et al., 1984), they are treated in a separate chapter, Chapter 195.

Ever since the first experimental studies of purple sulfur bacteria by Winogradsky (1888), it has been customary to grow these bacteria in the laboratory in raw enrichment cultures. These well-known Winogradsky columns are set up in tall glass cylinders with plant residues, $CaSO_4$, and anaerobic mud and water of a natural habitat and incubated in dim light at a north window. The preparation and development, as well as the advantages and limitations, of the different types of such long-lasting raw cultures are well described by Winogradsky (1888), Buder (1915), Schrammeck (1934), Schlegel and Pfennig (1961), and van Niel (1971). These raw-culture methods are not described in the present article, since nearly all the different genera and species of purple and green sulfur bacteria that thrive in Winogradsky columns can be grown and isolated today by direct methods in synthetic media.

The first rational enrichment and isolation procedures for certain species of purple and green sulfur bacteria were developed by van Niel (1931). Further attempts to grow in pure culture the large-celled purple sulfur bacteria *Chromatium okenii* and *Thiospirillum jenense* led to the elaboration of another synthetic medium that contains vitamin B_{12} (Pfennig, 1965; Pfennig and Lippert, 1966; also given by van Niel, 1971). With slight modification, this culture medium (see below, medium 1) provides rather nonselective growth conditions and allows the cultivation of most purple and green sulfur bacteria that occur in freshwater, estuarine, and marine habitats. Therefore, this medium will be described first in the present article. In addition, a second culture medium is given (medium 2; Biebl and Pfennig, 1978), which is more simple to prepare and which allows the cultivation of most common green and purple sulfur bacteria. A number of other media that were successfully used for the cultivation of certain species of phototrophic bacteria are described by Bose (1963).

Habitats

Freshwater Habitats

Pink to purple-red mass accumulations of purple sulfur bacteria in the water and on the mud of shallow, freshwater ponds or ditches were described during the last century by Ehrenberg (1838), Lankester (1873), Cohn (1875), Kützing (1883), Winogradsky (1888), and Miyoshi (1897). Winogradsky's publications marked a turning point; he gave the most comprehensive species and genus descriptions of the different morphological types of purple sulfur bacteria that he observed in samples from nature and raw cultures. As a result, later investigators of visible enrichments in natural habitats performed more detailed studies and used Winogradski's descriptions for identification (e.g., Bavendamm, 1924; Kolkwitz, 1909; Lauterborn, 1915; Molisch, 1907; Strzeszewski, 1913; Szafer, 1910). In his monograph on the colorless and purple sulfur bacteria, Bavendamm (1924) reviewed the older literature and gave, together with his own observations, the first detailed ac-

count on their ecology. More recent comprehensive reviews of the literature on the distribution of green and purple sulfur bacteria in nature have been given by Kondratieva (1965); Gorlenko et al. (1983); van Gemerden and Beeftink (1983); Tindall and Grant (1986); Lindholm (1987); and Pfennig (1989).

Sulfur springs create aquatic habitats with relatively constant sulfide concentrations (Winogradsky, 1888). Szafer (1910) and Strzeszewski (1913) in Poland first reported visible mass accumulations of green and purple sulfur bacteria in the effluents of such habitats exposed to light (40–100 mg H_2S/liter). Green sulfur bacteria, at that time often described as yellowish green forms of the cyanobacteria, occurred as the predominant forms at highest sulfide concentrations, followed downstream by different species of purple sulfur bacteria. Miyoshi (1897) reported the presence of purple sulfur bacteria in warm sulfur springs (35–44°C) in Japan. While growth temperatures up to 43°C are not uncommon for *Chromatium vinosum* strains, the optimum growth temperature of 48–50°C with *C. tepidum* is exceptional. This first moderately thermophilic purple sulfur bacterium was isolated from carbonaceous sinter in the Stygian Springs of Yellowstone Park (Madigan, 1986). No purple sulfur bacteria that tolerate higher temperatures of hot sulfur springs have been reported.

Visible accumulations of phototrophic sulfur bacteria occur temporarily in the anaerobic parts of small, freshwater environments, such as shallow ditches, ponds, and stagnant bodies of water in forests, botanical gardens, or near lakes or rivers. In such habitats, development usually proceeds during summer or fall when hydrogen sulfide is formed by sulfate-reducing bacteria from decaying plant material or from organic pollution. Pink to purple-red or yellowish-green blooms are often detected within the water itself or in the form of more-or-less profuse patches that cover the upper or lower side of dead leaves or the mud surface. Even if no accumulations are apparent, enrichment cultures usually yield positive results when hydrogen sulfide is present in the sediment (Bavendamm, 1924; Kaiser, 1966; Pfennig, 1967).

Certainly the largest and most significant freshwater environments of the phototrophic bacteria are lakes, because these habitats have remained more constant over longer periods of time. In these habitats, the blooms of green and purple sulfur bacteria are not directly visible; they are detected only by sampling the water from different depths. The most intensive development is usually found in summer and early fall. Depending on the location and depth of the anaerobic, sulfide-containing zone, three major types of lakes may be differentiated (Biebl and Pfennig, 1979):

1. Lakes with a maximum depth of 15 m, in which the anaerobic, sulfide-containing zone is primarily confined to the mud sediment and does not significantly extend into the adjacent water layers, show mass developments of purple sulfur bacteria on and above the mud. Examples of this type of lake are: the Kolksee and Edebergsee (northern Germany) with blooms of *Thiopedia* and *Pelochromatium* consortium (Utermöhl, 1925); Lunzer Mittersee (Austria) with *Chromatium okenii* and *Lamprocystis* blooms (Ruttner, 1962); Mulizne Lake (Poland) with *Thiopedia* bloom (Czeczuga, 1968a); and Haruna Lake (Japan) with a *Chromatium* bloom (Takahashi and Ichimura, 1968).
2. In holomictic lakes (lakes that undergo spring and fall mixing) during summer stratification, the anaerobic, sulfide-containing, stagnant water (hypolimnion) reaches the highest level and, hence, the best illumination condition. Blooms of phototrophic bacteria develop in the uppermost part of the hypolimnion in green- or red-colored water layers or plates. In certain lakes, the layer is dominated by green sulfur bacteria. In other lakes, green and purple bacteria occur either in mixed populations *(Ancalochloris, Pelochromatium, Lamprocystis),* as in the Pluss-See (northern Germany; Anagnostidis and Overbeck, 1966), or develop separated in different layers, as in Wintergreen Lake (USA; Caldwell and Tiedje, 1975), in which populations of gas-vacuolated, green sulfur bacteria thrive below the layers of *Thiopedia* and *Thiocystis.* The Lunzer Obersee (Austria; Ruttner, 1962), the Rotsee (Switzerland; Schegg, 1971), and Lake Vechten (Netherlands; Steenbergen and Korthals, 1982) are examples of holomictic lakes in which populations of *Chromatium okenii, Thiopedia rosea,* and *Lamprocystis roseopersicina* are the dominant species in purple-red layers. In the Banyoles karstic area of nontheastern Spain, several small lakes with mass developments of purple sulfur bacteria exist (Guerrero et al., 1987); Lake Cisó with blooms of *Chromatium minus* has been studied in detail (van Gemerden et al., 1985).
3. In the permanently stratified meromictic lakes, the anaerobic, sulfide-containing hypolimnion often consists of salt or sea water. Although the phototrophic bacteria can thrive in a layer at a more-or-less fixed depth,

major fluctuations in the population density occur in response to seasonal differences in the intensity of sunlight and the associated consequences. In 1913, the first purple-red layer (bloom of *Chromatium okenii*) was discovered at a depth of 12.6 m in the meromictic Ritomsee (Switzerland; Düggeli, 1924). In the famous, well-studied Belovod Lake (USSR; Kusnetzov, 1970; Sorokin, 1970), the purple-red layer also consisted predominantly of *Chromatium okenii*. Mixed populations of *Chromatium* and *Chlorobium* were reported for Suigetsu Lake and Kisaratsu Reservoir (Japan; Takahashi and Ichimura, 1968).

The last group of habitats for phototrophic sulfur bacteria are artificial: the anaerobic waste-stabilization ponds (Gloyna, 1971). Cooper et al. (1975) described an intensively pink to purple-red lagoon (2.17 ha) which received sulfide-containing fellmongery effluent in which *Thiocapsa roseopersicina* was the dominant sulfide-oxidizing bacterium. As a rule, conditions for the development of purple sulphur bacteria in waste-treatment lagoons are created when wastewater with a high organic load (e.g., sewage or slaughterhouse wastewater) is treated in fairly deep ponds (1–2 m in depth) which favor development of anaerobic conditions and the formation of sulfide by sulfate-reducing bacteria (Gloyna, 1971; Holm and Vennes, 1970; Sletten and Singer, 1971). In such ponds, blooms of purple sulfur bacteria are visible in spring and fall; during summer, the ponds are often turned green by unicellular algae. The most common purple sulfur bacteria in waste-treatment systems are *Thiocapsa roseopersicina, Amoebobacter roseus, A. pendens,* and *A. pedioformis* (Eichler and Pfennig, 1986). In addition to these, small *Chromatium* species and *Thiocystis violacea* were often present in small numbers.

Marine Habitats

The most abundant and conspicuous developments of phototrophic bacteria are found in seawater pools of salt marshes, in closed bays, and in estuaries (Baas Becking and Kaplan, 1956; Baas Becking and Wood, 1955), as well as on sand beaches ("Farbstreifensandwatt"; Schulz, 1937). These habitats have also been named "beach sulfureta" (Suckow, 1966), because they house a complete sulfur cycle that consists of bacterial sulfate reduction and the consequent anaerobic sulfide oxidation by phototrophic bacteria. Bacterial sulfate reduction in turn is facilitated by decaying plant and microbial materials that supply the sulfate-reducing bacteria see Chapter 24) with the necessary carbon sources (and electron donors). The food chain in such beach sulfureta has been the subject of a careful and excellent study by Fenchel (1969). Since Warming (1875) described mass developments of purple sulfur bacteria on the coasts of Denmark, they have been found and studied on the coasts of Germany (Gietzen, 1931; Hauser and Michaelis, 1975; Hoffmann, 1942; Schulz, 1937; Schulz and Meyer, 1939; Suckow, 1966; Stal et al., 1985), the Netherlands (Imhoff, 1976), the eastern United States (Trüper, 1970; Nicholson et al., 1987), mediterranean France (Matheron, 1976; Matheron and Baulaigue, 1972), and the Black Sea (Gorlenko, 1968; consult Kondratieva, 1965, for the older Russian literature; Yegunov, 1895).

"Red water" caused by phototrophic sulfur bacteria (Chromatiaceae as well as Chlorobiaceae) has been described and studied in many coastal lakes and lagoons: Lake Faro in Sicily (Genovese, 1963; Puchkova and Gorlenko, 1976; Trüper and Genovese, 1968); the Mar Piccolo near Tarento (Cerruti, 1938); Veliko and Malo Jezero on the Dalmatian island of Mljet (Cviic, 1955; 1960, Imhoff, 1976); the Bay of Tunis (Heldt, 1952; Stirn, 1971) and Bietri Bay of the Ebrie Lagoon, Ivory Coast (Caumette, 1984); the Etang du Prevost near Montpellier, France (Caumette 1986); lagoons at Messolonghi and Aitolikon in Greece (Hatzikakidis, 1952; 1953); the Solar Lake on the Sinai Peninsula (Cohen et al., 1977); and Lake Mogilnoye on the Arctic island of Kildin (Isachenko, 1914; Gorlenko et al. 1978). Typical splash water ponds on rocky coasts have been successfully studied in Japan (Taga, 1967), Helgoland, Germany, and Yugoslavia (Imhoff, 1976).

In contrast to estuarine or nearshore marine habitats, the oxygenated waters of the open seas have been found to be devoid of phototrophic sulfur bacteria. Only the Black Sea, a large, meromictic enclosed basin that is not part of the open ocean, contains phototrophic sulfur bacteria as was shown first by Kriss and Rukina (1953) and, after an initial unsuccessful attempt (Jannasch et al., 1974), as was confirmed by Hashwa and Trüper (1978). In recent studies, evidence was obtained for the development of brown Chlorobiaceae in areas where the chemocline is at depths of 68 to 90 m.

So far, there is only one type of habitat within well-oxygenated sea water where the anaerobic phototrophic sulfur bacteria can be regularly encountered: Eimhjellen (1967) found them in the North Sea sponge, *Halichondrium panicea*. Imhoff and Trüper (1976) investigated several Mediterranean sponges and isolated seven dif-

ferent species of Chromatiaceae and even some purple nonsulfur bacteria from *Euspongia officinalis* and *Ircinia* species. Marine sponges, although strictly aerobic, obviously contain anaerobic pockets that receive enough light to allow the growth of phototrophic bacteria.

Isolation

Preparation of Culture Media

The composition and preparation of two different culture media are described below for the cultivation of purple and green sulfur bacteria. Medium 1 is suitable for almost all green and purple bacteria presently in laboratory culture, including those species that are most difficult to grow (e.g., *Thiopedia rosea, Thiospirillum jenense, Chromatium okenii,* and *Thiodictyon elegans*). With minor modifications, this culture medium was published by Pfennig (1965), Pfennig and Lippert (1966), Trüper (1970), van Niel (1971), and Eichler and Pfennig (1988). Medium 2 (Biebl and Pfennig, 1978) is easier to prepare and can be successfully used for the cultivation of the most common purple and green sulfur bacteria.

Medium 1 for Cultivation of Purple and Green Sulfur Bacteria (Eichler and Pfennig, 1988)

The medium is prepared in a 5-liter bottle (or flask) with four openings at the top. Two openings for tubes are in the central, silicon rubber stopper: a short, gas-inlet tube with a sterile cotton filter; and an outlet tube for medium, which reaches the bottom of the vessel at one end and has, at the other end, a silicon rubber tube with a pinchcock and a bell for aseptic dispensing of the medium into bottles. The other two openings have gas-tight screw caps; one of these openings is for the addition of sterile solutions and the other serves as a gas outlet.

The composition of medium 1 is given below for a total of 5 liters of culture medium. A number of different solutions are required to prepare the medium.

Solution 1:

Distilled water	4,900 ml
KH_2PO_4	1.7 g
NH_4Cl	1.7 g
KCl	1.7 g
$MgSO_4 \cdot 7H_2O$	2.5 g
$CaCl_2 \cdot 2H_2O$	1.25 g

For enrichment cultures or pure cultures from marine or estuarine habitats, add 100 g NaCl to solution 1 and increase the $MgSO_4 \cdot 7H_2O$ to 15 g.

Solution 1 is autoclaved for 45 min at 121°C in the 5-liter bottle, together with a teflon-coated magnetic bar. It is cooled to room temperature under an N_2 atmosphere with a positive pressure of 0.05–0.1 atm (a manometer for low pressures is required). The cold medium is then saturated with CO_2 by magnetic stirring for 30 min under a CO_2 atmosphere of 0.05–0.1 atm. The sterile solutions 2 through 6 (see below) are then added through one of the screw-cap openings against a stream of either N_2 gas or, better, a mixture of 95% N_2 and 5% CO_2, while the medium is magnetically stirred.

Solution 2: Vitamin B_{12} solution
Prepare a sterile filtered stock solution containing 2 mg vitamin B_{12} in 100 ml distilled water. Add 5 ml to Solution 1.

Solution 3: Trace element solution SL 12
Prepare the following stock solution:

Distilled water	1 liter
Ethylenediamine-tetraacetate-di-Na-salt	3.0 g
$FeSO_4 \cdot 7H_2O$	1.1 g
H_3BO_3	300 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$MnCl_2 \cdot 4H_2O$	50 mg
$ZnCl_2$	42 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$Na_2MoO_4 \cdot 2H_2O$	18 mg
$CuCl_2 \cdot 2H_2O$	2 mg

The salts are dissolved in the order given and the solution is autoclaved in a screw-cap bottle. Add 5 ml to solution 1.

Solution 4: Na bicarbonate solution
Prepare a solution of 7.5% Na bicarbonate. The solution is saturated with CO_2 and autoclaved under a CO_2 atmosphere. Add 100 ml to solution 1.

Solution 5: Sodium sulfide solution
Add 20 ml of a sterile, 10% $Na_2S \cdot 9H_2O$ solution to solution 1 for purple sulfur bacteria, or 30 ml for green sulfur bacteria. The sulfide solution is prepared in a screw-cap bottle; after replacement of the air by N_2, the bottle is tightly closed and autoclaved.

After combining and mixing solutions 1 through 5, the pH of the medium is adjusted with sterile 2M HCl or Na_2CO_3 solution to pH 7.2 for purple sulfur bacteria or to pH 6.8 for green sulfur bacteria. The medium is then distributed aseptically through the medium outlet tube into sterile, 100-ml bottles with metal caps and autoclavable rubber seals, using the positive gas pressure (0.05–0.1 atm) of the N_2/CO_2 gas mixture. A small air bubble is left in each bottle to meet possible pressure changes. The tightly sealed, screw-cap bottles can be stored for several weeks and months in the dark. During the first 24 h, some iron of the medium precipitates in the form of black flocks. No other sediment should arise in the otherwise clear medium.

SUPPLEMENT SOLUTION. The amount of $Na_2S \cdot 9H_2O$ initially added to medium 1 (higher initial amounts may be inhibitory for some species) will only produce very limited growth. After the sulfide and sulfur are completely photooxidized, the bacteria stop growing and will be damaged by further illumination. In order

to keep the cultures growing and to obtain high cell yields it is necessary to feed the cultures several times with sterile, partially neutralized sulfide solution, which is prepared from solution 6.

Neutralized Sulfide Solution for Feeding Cultures of Green and Purple Sulfur Bacteria:

Solution 6:

Distilled water	100 ml
$Na_2S{\cdot}9H_2O$	3.6 g

The 0.15 M solution is prepared in a 250-ml screw-cap bottle; after replacement of the air by N_2, the bottle is tightly closed and autoclaved.

To prepare the neutralized feeding solution, a measured amount of sterile solution 6 is added to a sterile Erlenmeyer flask containing a magnetic bar. The solution is brought to about pH 7.3 by dropwise addition of sterile 2 M H_2SO_4 on a magnetic stirrer. If too much acid is added, the sulfide solution becomes turbid due to precipitation of elemental sulfur. The nearly neutralized solution is immediately used for feeding 100-ml-bottle cultures. Depending on the population density, 1–2 ml are used for Chromatiaceae. Before the addition, an equivalent amount of culture medium is aseptically removed from the bottle culture.

Alternatively the method of Siefert and Pfennig (1984) can be used to prepare neutralized feeding solution.

Cultures of purple and green sulfur bacteria that can use thiosulfate as an electron donor can be supplemented with 0.1% of this compound from stock solution 7.

Thiosulfate Solution for Cultivation of Purple and Green Sulfur Bacteria

Solution 7:

Distilled water	95 ml
$Na_2S_2O_3{\cdot}5H_2O$	10 g

The solution is prepared in a 200-ml screw-cap bottle and autoclaved. One ml of solution is aseptically added to 100 ml of culture medium.

Growth yields of purple and green sulfur bacteria can be increased by the addition of acetate as a readily assimilated carbon source; 0.03% or 0.05% acetate (from solution 8) is added to deep agar dilution cultures. Acetate can be added to liquid cultures only if they are free of purple nonsulfur bacteria. The ammonium and magnesium salts of acetate are used to avoid strong pH changes during growth.

Acetate Solution for Cultivation of Green and Purple Sulfur Bacteria

Solution 8:

Distilled water	100 ml
Ammonium acetate	2.5 g
Magnesium acetate	2.5 g

The solution is prepared in a 200 ml screw-cap bottle and autoclaved. Application is 1 ml added aseptically to 100 ml of culture medium.

Medium 2 for Cultivation of Purple and Green Sulfur Bacteria (Biebl and Pfennig, 1978)

This medium is prepared in a 2-liter Erlenmeyer flask with an outlet near the bottom. A silicon rubber tube (about 30 cm long) with a pinchcock and a bell for aseptic distribution of the medium into bottles is connected to the outlet. A magnetic bar is put into the flask.

Solution 1:

Distilled water	950 ml
Solution 2 (SL 12)	1 ml
KH_2PO_4	1 g
NH_4Cl	0.5 g
$MgSO_4{\cdot}7H_2O$	0.4 g
$CaCl_2{\cdot}2H_2O$	0.05 g

For marine strains, add 20 g NaCl to solution 1 and increase the $MgSO_47H_2O$ to 3 g. The solution is autoclaved in the cotton-plugged, 2-liter Erlenmeyer flask.

Solution 2:

Trace element solution SL 12 (see medium 1). Application is 1 ml for 1 liter of medium.

When the autoclaved solution 1 (including solution 2) is cold, the following sterile solutions 3 through 5 are aseptically added while magnetically stirring the medium:

Solution 3: Vitamin B_{12} solution

The solution is prepared as a sterile filtered stock solution containing 2 mg vitamin B_{12} in 100 ml distilled water. Add 1 ml of solution 1.

Solution 4: Na bicarbonate solution

The 5% solution is prepared in distilled water and is filter-sterilized. Add 30 ml to solution 1.

Solution 5: Sodium sulfide solution

A freshly autoclaved 6% solution is prepared in distilled water; 6 ml is added to medium for purple sulfur bacteria, or 10 ml to medium for green sulfur bacteria.

After additions from solutions 1 through 5, the pH of the medium is adjusted with sterile 2M H_2SO_4 or Na_2CO_3 solution to pH 7.3 for purple sulfur bacteria or to pH 6.8 for green sulfur bacteria. The medium is then dispensed aseptically into sterile, 50- or 100-ml bottles with metal screw caps containing autoclavable rubber seals. A small air bubble is left in each bottle to meet possible pressure changes.

Methods for Pure Culture Isolation

Irrespective of the source of the inoculum, e.g., sample from nature, enrichment culture, or suspension of a colony, the deep agar dilution method is the most convenient and reliable method for preparing pure cultures of phototrophic purple and green sulfur bacteria (Larsen, 1952; Pfennig, 1978; van Niel, 1931). For motile species that do not form colonies in agar media (e.g., *Thiospirillum*) Giesberger's (1947) "Pasteur pipette" method (see later) is recom-

mended. For nonmotile species, the well-known dilution method in liquid media should be applied.

Preparation of Pure Cultures of Phototrophic Purple and Green Sulfur Bacteria by Dilution in Deep Agar Tubes

Granular agar is thoroughly washed several times with distilled water, and a 3% solution is prepared in distilled water (for marine samples, 2% NaCl is added) and liquefied by autoclaving. While kept in a hot-water bath, the agar is dispensed in 3-ml portions into standard test tubes (16 or 18 mm × 200 mm), which are then plugged with cotton and autoclaved.

The molten agar is kept in a water bath at 60°C, and a 50- or 100-ml screw-cap bottle, containing complete medium 1 (or medium 2), is kept with a loosened screw cap in a water bath at 40°C. For one dilution series, eight tubes are supplied with 6 ml of the prewarmed medium and kept in the 40°C water bath. At the same time, the cotton plugs are replaced by rubber stoppers. No mixing is required at this stage.

One of the tubes is inoculated with one to three drops from the suspension of the phototrophic bacteria; the contents are immediately mixed by inverting the tube once. Of this culture, 0.5–1.0 ml are then transferred into a second tube that contains the agar medium, mixed immediately by inverting as with the first tube, and so on. This dilution series is continued over eight steps. After transfer to the next tube, each tube is set into a water bath with tap water (about +10°C) to harden the agar. After hardening, the air above the agar is replaced by gassing with sterile N_2 and 5% CO_2 and the tubes are tightly closed with the rubber stoppers. The agar dilution cultures are incubated at 20–28°C (depending on the previous treatment of the inoculum) at a light intensity of 200–500 lux.

After the cultures have developed, individual colonies are isolated from the highest dilution step that shows pigmented colonies. For this purpose, a single colony is removed through the open end of the test tube by suction, using a fine Pasteur pipette attached to a rubber tubing. The procedure is best carried out under a dissecting microscope. The contents of the colony are suspended in 0.5 ml of sterile medium in a test tube, the suspension is microscopically checked for purity, and the whole dilution series in deep agar tubes is repeated. In order to obtain a pure culture, it may be necessary to repeat the whole process again. When pure cultures and colonies are achieved, individual colonies are inoculated into liquid medium. It is advisable to start with small-sized bottles or screw-cap tubes (10 or 25 ml) and then to scale up to the regularly used sizes.

Purity is checked by use of A-C medium (Difco) and *Desulfovibrio* medium (after Postgate; See Chapter 183), adjusted to the respective salinities. As long as no growth in these media occurs and the culture is morphologically uniform, it can be considered pure.

Motile purple sulfur bacteria that do not grow well in agar media, e.g., *Thiospirillum jenense* (Pfennig, 1965), may be isolated by the method described by Giesberger (see below) for the isolation of heterotrophic spirilla or rhodospirilla (Giesberger, 1947).

Pasteur Pipette Technique for Isolating Motile Purple Sulfur Bacteria (Giesberger, 1947)

The wide part of a sterile Pasteur pipette is heated in a Bunsen burner not far from the onset of the capillary part; the softened glass is then squeezed to a flat tube using pliers with parallel movement of the jaws. The flat part is again heated and carefully pulled out to a flat capillary. The whole Pasteur pipette is then filled with sterile liquid medium and placed horizontally under a dissecting microscope so that the flat capillary part can be observed. The cell suspension with the thiospirilla is then introduced into the wide part of the Pasteur pipette not far from the capillary part. Some of the very fast and straight-moving thiospirilla soon leave their original position and swim through the flat capillary; this movement can be followed readily under the microscope. When a few cells have reached the other end, the capillary is aseptically cut in the middle of the capillary part, and the drop of medium with the cells is inoculated into a screw-cap test tube with sterile medium. When many parallel tubes of these capillary separations are set up, chances are good that one of the culture tubes will give rise to a pure culture.

Cultivation on Agar Plates in Anaerobic Jars

A useful method for cultivating phototrophic sulfur bacteria was introduced by Irgens (1983). It is based upon the release of hydrogen sulfide, ammonia, and acetic acid from decomposing thioacetamide.

The medium including agar is prepared without any sulfide and poured into Petri dishes. After inoculation, these are placed in anaerobic jars (for instance, the Gas-Pak system of Becton Dickinson, Cockeysville, MD. Before the jars are closed, they are supplemented with a test tube containing 0.05–0.1 g thioacetamide dissolved in 1.0 ml of 0.2 N or 0.5 N HCl. Irgens (1983) demonstrated that hydrogen sulfide is released over a period of at least one week. Also included in the jars are the methylene blue redox indicator and a strip of lead acetate-sulfide indicator. This method is also a convenient method for obtaining viable counts. The technique has been successfully used for the isolation of *Amoebobacter, Chromatium, Lamprocystis, Thiocapsa, Thiocystis* and *Ectothiorhodospira* strains with an illumination by a 60-W incandescent light bulb at a distance of 60 cm from the jars (Irgens, 1983).

Although less oxygen- tolerant species might not survive the short inoculation or transfer period between incubations, this is an easy way to grow the more oxygen-tolerant species. However, in general, it cannot replace the isolation by agar shake dilution series as described above.

Methods for Enrichment Culture

Van Niel's (1931) original observation that green sulfur bacteria have a lower pH optimum than purple sulfur bacteria has been corroborated by all later studies and forms the basis for the enrichment culture of the two groups. How-

ever, since the pH ranges of the groups overlap, the use of different pH values in the media is not sufficient to assure the selective enrichment of each group. In particular, enrichments for purple sulfur bacteria can readily be outgrown by Chlorobiaceae, in which case the use of light filters is helpful (see below, "Application of Light Filters in Enrichment of Purple Sulfur Bacteria").

The concentration of sulfide is critical for a number of species in both groups that are inhibited by higher concentrations (e.g., 0.1% $Na_2S \cdot 9H_2O$). Therefore, the sulfide concentrations for media 1 and 2 given earlier are low enough so that the cultivation of sensitive forms is not excluded. *Thiopedia rosea* is exceptional in being inhibited by sulfide concentrations above 0.6 mM. For cultivation of *Thiopedia*, the addition of sodium dithionite (50 mg for 1 liter) is required (see Overmann and Pfennig, 1989). High population densities can be achieved only by repeated addition of sulfide ("feeding") during growth (see the recipe for "Partially Neutralized Sulfide Solution").

The salinity of the culture medium is usually adjusted according to the salinity of the inoculum. For marine isolations, it is sufficient to raise the NaCl concentration to 2–3%. However, some isolates also require the addition of increased concentrations of magnesium (e.g., 0.3% $MgSO_4 \cdot 7H_2O$) and sometimes also of calcium (Biebl and Pfennig, 1978). It should be taken into account that the salinity of a natural habitat may not always be optimal for a particular isolate from that habitat. Therefore, use of enrichment media of different salinities may be useful for the isolation of a variety of species.

The incubation temperature is very important for the enrichment culture of several species of purple and green sulfur bacteria. As a rule, at high incubation temperatures (28–35°C) a wide variety of different species are outgrown by single, fast-growing species. At low temperatures (15–22°C), the enrichment cultures develop more slowly and a larger number of different species may be present simultaneously.

Selective Enrichment of Purple Sulfur Bacteria

For the successful enrichment of many characteristic species of purple sulfur bacteria, it is important to realize that they occur very irregularly in different aquatic habitats. The source and species composition of the inoculum is, therefore, of primary importance for the outcome of enrichment experiments. When the desired species is detected microscopically in reasonable numbers in a given sample, deep agar dilution series should be prepared directly from the sample without trying further enrichment in liquid culture. Even in this case, however, the incubation conditions for the agar cultures should closely resemble the conditions used for liquid enrichment cultures of the particular species.

APPLICATION OF LIGHT FILTERS IN ENRICHMENTS OF PURPLE SULFUR BACTERIA. Medium 1 with a pH value between 7.2 and 7.4 is used for the selective enrichment of purple sulfur bacteria from freshwater or marine habitats. The use of specific light filters can be of value in two cases: 1) in the presence of established populations of green sulfur bacteria (Pfennig, 1965, 1967); and 2) for the enrichment of purple sulfur bacteria that contain bacteriochlorophyll *b*, such as *Thiocapsa pfennigii* (Eimhjellen, 1970; Eimhjellen et al., 1967). Development of green sulfur bacteria (long-wavelength absorption maxima between 705 and 750 nm) can be prevented when the enrichments are illuminated behind an infrared filter that transmits light only above 800 nm (e.g., gelatin filter No. 530, Göttinger Farbfilter; or interference filter plus prefilter for light transmission at around 850 nm). The selective enrichment of *Thiocapsa pfennigii* is achieved with an infrared filter that transmits radiation only above 900–1,000 nm) (e.g., gelatin filter No. 533, Göttinger Farbfilter; or interference filter plus prefilter for light transmission at around 1,020 nm).

The various species of the purple sulfur bacteria differ with respect to the selective advantage they exhibit under different kinds of illumination. These differences can be exploited for the selective enrichment of certain groups of species in medium 1 (Pfennig, 1967). Two extremes of different illumination with incandescent light are recommended here.

CONTROL OF LIGHT INTENSITY AND ILLUMINATION IN SELECTIVE ENRICHMENT OF PURPLE SULFUR BACTERIA. If continuous illumination at high light intensities of 1,000–2,000 lux, and an incubation temperature of about 30°C is used, the small and fast-growing Chromatiaceae can be expected to enrich: *Chromatium vinosum, C. minus, C. violascens, C. gracile, C. minutissimum, Thiocystis violacea,* and *Thiocapsa roseopersicina.*

Results may be different with intermittent illumination at low light intensities of 50–300 lux and an incubation temperature of about 20°C. The light and dark phases may be either 12 h light and 12 h dark (or 6 h light and 6 h dark) or, according to van Gemerden (1974), 4 h light and 8 h dark. After inoculation from the natural

habitat, the bottle cultures should not be shaken until they are fed with more sulfide. Depending on the inoculum, the large and flagellated and the gas-vacuole-containing Chromatiaceae can be expected to enrich: *Thiospirillum jenense, Chromatium okenii, C. weissei, C. warmingii, C. buderi, Thiocystis gelatinosa, Lamprocystis roseopersicina, Thiodictyon elegans, Amoebobacter roseus,*, and *Thiopedia rosea.* The flagellated forms keep swarming in the whole bottle (Pfennig, 1962) and can be further enriched by carefully pipetting the inoculum for subsequent enrichment cultures from the upper part of the bottle culture. At first, the nonmotile forms enrich in the sediment at the bottom of the bottles; later, the gas-vacuole-containing cells tend to accumulate at the surface under the screw cap. This process can be accelerated by keeping the enrichment for a few days in a refrigerator at +4°C. For further enrichment, the floating cell mass is carefully pipetted from the surface and transferred to fresh medium.

Alternatively, enriched cell populations may be removed by a Pasteur pipette and directly used as inoculum for deep agar dilution series. Medium 1 for deep agar cultures is supplemented with 0.05% acetate (supplement solution 8). The deep agar cultures are incubated at room temperature and a light intensity of 200 lux. An illumination regimen of 16 h light and 8 h dark is the most favorable.

Pure cultures of most species of the purple and green sulfur bacteria have been successfully maintained by the authors for many years (30 year for some strains) in liquid cultures with medium 1, preferentially using 100-ml screw-cap bottles as culture vessels.

Maintenance and Preservation of Pure Cultures of Purple and Green Sulfur Bacteria

After transfer to fresh medium, stock cultures of green sulfur bacteria are incubated at room temperature and a light intensity of 200–500 lux; they are grown until the initially formed elemental sulfur has just disappeared. Then the stock cultures are stored in the dark in a refrigerator at +4°C to +6°C. Before transfer into fresh medium after 3–4 months of storage, the stock cultures are fed with 1 ml neutral sulfide solution (supplement solution 6) to a final sulfide concentration of 1.5 mM and incubated in dim incandescent light (100–200 lux) at room temperature. The stock cultures are transferred after the cells have formed elemental sulfur (milky appearance of the culture) and have started to grow.

Stock cultures of purple sulfur bacteria may be maintained in a similar way; however, the freshly grown cultures have to be fed with 1 ml neutral sulfide solution (supplement solution 6) and kept in the light for a few hours until the cells have formed intracellular globules of elemental sulfur. At this stage, the stock cultures can be stored in a refrigerator at +4°C to +6°C for several months. The cultures keep well when they are put back into dim light at room temperature after 4–6 weeks of storage in the dark. Cultures with sulfur-free cells are then fed with 1 ml neutral sulfide solution and, after formation of elemental sulfur, put back into the refrigerator.

Long-term preservation of purple and green sulfur bacteria is also possible by storage in liquid nitrogen (Biebl and Malik, 1976). Not all strains could be lyophilized (Malik 1990a, 1990b). For liquid-nitrogen preservation, heavy cell suspensions of liquid cultures with 5% dimethylsulfoxide (DMSO) as a protective agent were placed in 2-ml plastic ampules, sealed, and freeze-stored.

Most type strains of the Chromatiaceae are presently maintained in liquid nitrogen by the German Collection of Microorganisms and Cell Cultures, (DSM), Braunschweig, Germany (Claus and Schaab-Engels, 1977).

Identification

The properties of the species of the Chromatiaceae are listed in Table 1. Cells of all species multiply by binary fission. All motile cells carry polar or sub-polar flagella. Growth is influenced by environmental conditions; therefore, only pure cultures allow reliable identification. Fig. 1 shows the morphology of some typical species of the Chromatiaceae.

All species are able to photometabolize a number of simple organic carbon compounds, at least acetate and pyruvate. The large-cell species, *Chromatium okenii, C. weisseei, C. warmingii, C. buderi,* and *Thiospirillum jenense,* differ from the other species not only in size but in their requirement for vitamin B_{12}, inability to utilize organic compounds other than acetate and pyruvate, and lack of assimilatory sulfate reduction. Vitamin B_{12} is also required by *Amoebobacter roseus* and *C. salexigens.*

The majority of enrichment techniques for anoxygenic phototrophic bacteria select for certain physiological groups, but are not species specific. Therefore, reliable and rapid methods for identification are important.

A preliminary but relatively good differentiation between sulfur and nonsulfur bacteria is already given by the enrichment or isolation medium. Medium that contains H_2S and bi-

Table 1. Properties of the species of the Chromatiaceae.

Species	Shape, size (μm)	Motile	Slime[a]	Aggregate pattern[a]	Gas vacuoles	Predominant carotenoid[b]	Color of cell suspension	GC content (mol%)	Type or neotype strain	Special requirement
Amoebobacter pedioformis	Sphere, 2 × 2–3	–	+	Platelets	+	sp, rh	Pink-red	65.5	DSM 3802	Vit. B_{12} stimulates, opt. temp. 37°C
A. pendens	Sphere, 1.5–2.5	–	+	–	+	sp	Pink-red	65.3	DSM 236	Vit. B_{12} required
A. purpureus	Sphere, 3.3–3.8 × 3.5–4.5	–	+	Clumps	+	ok	Purple-red	63.4–64.1	DSM 4197	Opt. temp. 23–25°C
A. roseus	Sphere, 2.0–3.0	–	+	–	+	sp	Pink-red	64.3	DSM 235	Vit. B_{12} required
Chromatium buderi	Rod, 3.5–4.5 × 4.5–9	+	–	–	–	rl	Purple-violet	62.2–62.8	DSM 176 (= ATCC 25588)	2% NaCl, vit. B_{12} required
C. gracile	Rod, 1.0–1.3 × 2–6	+	+	–	–	sp, ly, rh	Brown-red	68.9–70.4	DSM 203	2% NaCl
C. minus	Rod, 2.0 × 2.5–6	+	–	–	–	ok	Purple-red	62.2	DSM 178	–
C. minutissimum	Rod, 1.0–1.2 × 2.0	+	–	–	–	sp, ly, rh	Brown-red	63.7	DSM 1376	–
C. okenii	Rod, 4.5–6.0 × 8–15	+	–	–	–	ok	Purple-red	48.0–50.0	DSM 169	Vit. B_{12} required
C. purpuratum	Rod, 1.2–1.7 × 3–4	+	+	–	–	ok	Purple-red	68.9	DSM 1591	5% NaCl
C. salexigens	Rod, 2–2.5 × 4–7.5	+	–	–	–	sp, ly, rh	Brown-red	64.6	DSM 4395	4–10% NaCl, vit. B_{12} required
C. tepidum	Rod, 1–2 × 2.8–3.2	+	–	–	–	sp, ly, rh, rv	Brown-red	61.5	ATCC 43061	Opt. temp. 48–50°C
C. vinosum	Rod, 2.0 × 2.5–6	+	–	–	–	sp, ly, rh	Brown-red	61.3–66.3	DSM 180 (= ATCC 17899)	–
C. violascens	Rod, 2.0 × 2.5–6	+	–	–	–	rl	Purple-violet	61.8–64.3	DSM 198 (= ATCC 17096)	–
C. warmingii	Rod, 3.5–4.0 × 5–11	+	–	–	–	rl	Purple-violet	55.1–60.2	DSM 173 (= ATCC 14959)	Vit. B_{12} required

(continued)

Table 1. *Continued*

Species	Shape, size (μm)	Motile	Slime[a]	Aggregate pattern[a]	Gas vacuoles	Predominant carotenoid[b]	Color of cell suspension	GC content (mol%)	Type or neotype strain	Special requirement
C. weissei	Rod, 3.5–4.5 × 7–9	+	–	–	–	ok	Purple-red	48.0–50.0	DSM 171	Vit. B_{12} required
Lamprobacter modestohalophilus	Rod, 2–2.5 × 4–5	+	+	Clumps	+	ok	Purple-red	64	RO-1	1–2% NaCl
Lamprocystis roseopersicina	Sphere, 3.0–3.5	+	–	– or Clumps	+	la, lo	Purple	63.8	DSM 229	–
Thiocapsa pfennigii	Sphere, 1.2–1.5	–	–	–	–	ts	Orange-brown	69.4–69.9	DSM 1375	0–3% NaCl
Thiocapsa roseopersicina	Sphere, 1.2–3.0	–	+	– or Tetrads	–	sp	Pink-red	63.3–66.3	DSM 217	0–3% NaCl
Thiocystis gelatinosa	Sphere, 3.0	+	+	–	–	ok	Purple-red	61.3	DSM 215	1% NaCl
Thiocystis violacea	Sphere, 2.5–3.0	+	+	Clumps	–	rl	Purple-violet	62.8–67.9	DSM 207	0–2% NaCl
Thiodictyon bacillosum	Rod, 1.5–2.0 × 3–6	–	–	Clumps	+	rl, rh	Purple-violet	66.3	DSM 234	–
Thiodictyon elegans	Rod, 1.5–2.0 × 3–8	–	–	Nets	+	rl, rh	Purple-violet	65.3	DSM 232	–
Thiopedia rosea	Ovoid, 1.0–2.0 × 1.2–2.5	–	–	Platelets	+	ok	Purple-red	62.5–63.5	DSM 1236	Vit. B_{12} required
Thiospirillum jenense	Spiral, 2.5–4.5 × 30–40	+	–	–	–	ly, rh	Orange-brown	45.5	DSM 216	Vit. B_{12} required

Abbreviations: +, present; –, absent.

[a]In pure culture.

[b]Abbreviations: la, lycopenal; lo, lycopenol; ly, lycopene; ok, okenone; rh, rhodopin; rl, rhodopinal; rv, rodovibrin; sp, spirilloxanthin; ts, tetrahydrospirilloxanthin.

Adapted from Caumette et al., 1988; Eichler and Pfennig, 1986, 1988; Gorlenko et al., 1979; Imhoff and Trüper, 1980; Madigan, 1986; Mandel et al., 1971; Pfennig and Trüper, 1971, 1974, 1977.

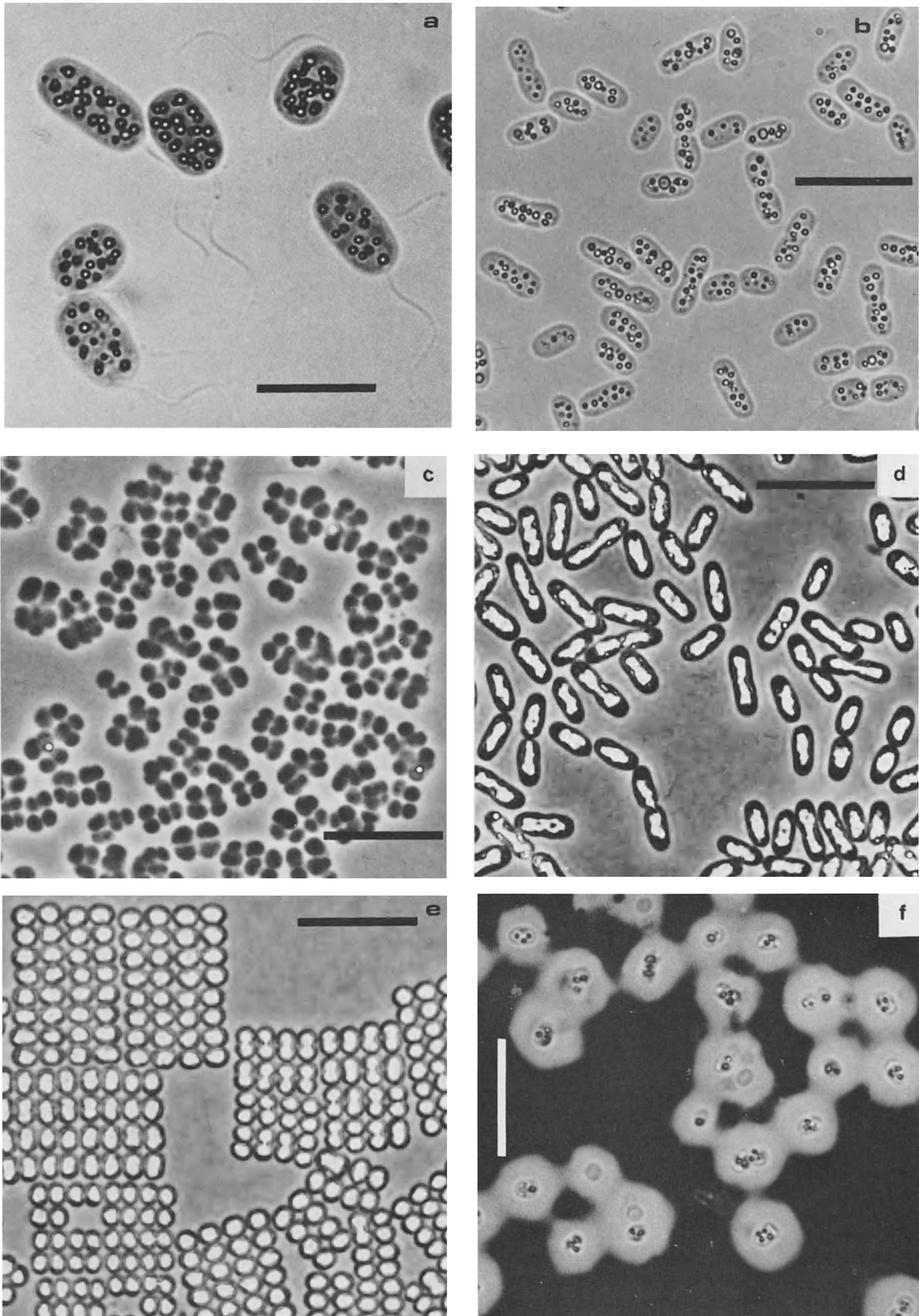


Fig. 1. Morphology of the Chromatiaceae. (a) *Chromatium okenii* (light-field micrograph). (b) *Chromatium vinosum* (light-field micrograph). (c) *Thiocapsa roseopersicina* (phase contrast micrograph). (d) *Thiodictyon elegans* (phase contrast micrograph). (e) *Thiopedia rosea* (phase contrast micrograph). (f) *Amoebobacter pendens* (light-field micrograph, India ink preparation). Bar = 10 μm.

carbonate is selective for sulfide-oxidizing bacteria, such as the Chromatiaceae and Chlorobiaceae, but *Rhodobacter sulfidophilus* (and perhaps others) will also grow (or survive) in sulfide-containing medium. If phototrophic bacteria are isolated from a sulfide-free medium with organic compounds as electron donors, no species of the Chlorobiaceae will grow, and only those species of the Chromatiaceae that are able to assimilate (and reduce) sulfate will grow.

For a quick differentiation of the Chromatiaceae from other purple bacteria that may prevail under these conditions, the following method is recommended:

Differentiation of Chromatiaceae from Ectothiorhodospiraceae and Purple Nonsulfur Bacteria

Place a drop of the cell suspension under the microscope and allow a drop of neutralized Na_2S solution to run in from besides the cover slip. If the bacteria form *intracellular sulfur globules* in the light (usually in less than 20 min), they belong to the Chromatiaceae.

Great attention should be paid to the microscopic appearance of a newly isolated strain. Width (diameter), length, shape of cells, type of flagellation, mode of division, and the presence or absence of slime capsules, sulfur globules, and gas vacuoles should be recorded (Fig. 1). Further, an absorption spectrum of a cell suspension should be taken to identify the predominant bacteriochlorophyll and the carotenoid composition. Some methods for the measurement of such absorption spectra follow.

Sucrose Method for Measuring Absorption Spectra

Mix 5 g of sucrose with 3.5 ml of cell suspension until the former is completely dissolved. Then record the spectrum against a blank of 5 g of sucrose in 3.5 ml of water (or medium) in an appropriate spectrophotometer.

Trüper and Yentsch (1967) described another method in which the cells are precipitated on glass fiber filters and the spectrum is measured through the wet filter. The most accurate method to date for directly measuring pure absorbance spectra of living phototrophic microorganisms was published by Göbel (1978). For the purpose of identification alone, however, the sucrose method given above is the simplest and quickest.

The characteristic absorption maxima of the bacteriochlorophyll (bchl) in living cells are: for bchl *a;* 375, 590, 805, and 830–890 nm; and for bchl *b;* 400, 605, 840, and 1020–1040 nm.

The only species of the Chromatiaceae containing bchl *b* rather than *a* is *Thiocapsa pfennigii.* This species also possesses a unique ultrastructure, i.e., bundles of tubes as the intracytoplasmic membrane system.

All other species of the Chromatiaceae contain membrane vesicles.

Fig. 2 shows a number of absorption spectra of living cell suspensions of Chromatiaceae. The in vivo absorption characteristics of bchls *a* and *b* as well as of the most common carotenoids are shown.

After the physiological group, morphology, and pigments of a newly isolated strain have been determined, identification with a known species is possible in most cases and, if not, further properties of the new strain must be studied. We recommend the determination of the GC content of the DNA and a study of ultrathin sections under the electron microscope in order to determine the fine structure, in particular the type of intracytoplasmic membranes present.

The description of a new species of the anoxygenic phototrophic bacteria should be based on pure cultures and, if possible, on more than one strain. Besides nomenclatural items, a new description should contain detailed information about cell morphology (color, size, shape, flagellation, mode of division, presence of gas vacuoles, slime capsules, sheaths, etc.); occurrence of cell aggregates; fine structure (membrane systems, storage materials, cell wall, flagellation); photosynthetic pigments (color of cell suspensions, types of bchl and carotenoids); and GC content. The following physiological data should be given: oxygen tolerance; sulfide tolerance; salinity requirements; optimum growth temperature and pH; sulfur, nitrogen, and carbon sources utilized; vitamins or other growth factors required; and capacity for: photo- versus chemotrophy, organo- versus lithotrophy, and hetero- versus autotrophy. An indication of natural habitats is also necessary.

Finally, a type strain must be designated and deposited in one of the recognized national type culture collections, preferably either ATCC, DSM, NCIB, or NTHC, as required by the International Code of Nomenclature of Bacteria (Lapage et al., 1975).

Physiology and Biochemistry

While the energy yield from lithotrophic metabolism is usually low, phototrophic organisms are able to produce large amounts of ATP as long as light is present. In their natural environments, however, this is only the case in the daytime. Therefore, all phototrophic organisms must in addition possess other energy-yielding

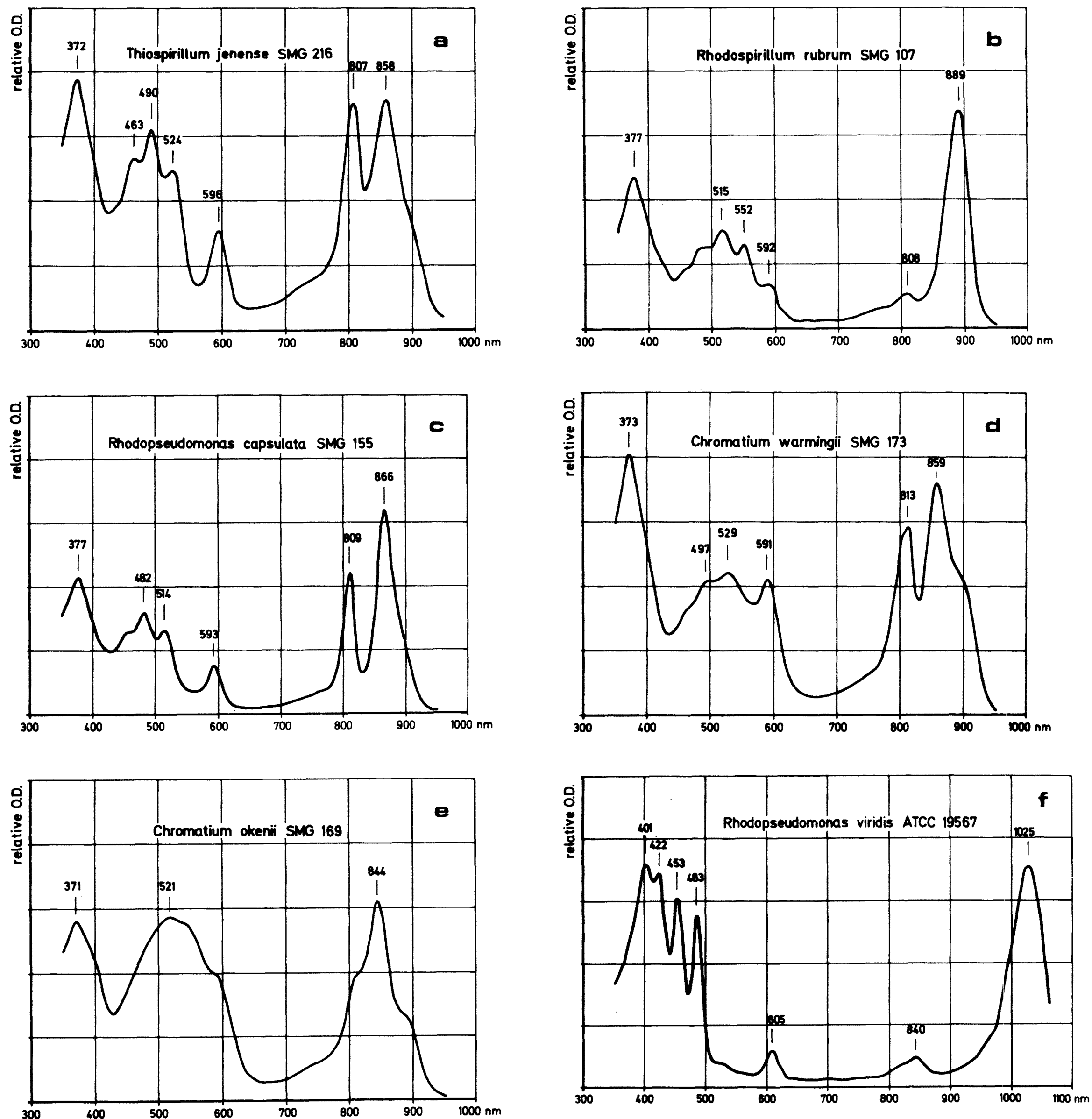


Fig. 2. Absorption spectra of living cell suspensions of Chromatiaceae (and some purple nonsulfur bacteria) containing bacteriochlorophyll *a* (a-e) or *b* (f). Typical carotenoids seen and their approximate absorption maxima: (a) lycopene and rhodopin (463, 490, 524 nm); (b) spirilloxanthin (486, 515, 552 nm); (c) spheroidene (450, 482, 514 nm) (this organism is now called *Rhodobacter capsulatus*); (d) rhodopinal (497, 529 nm); (e) okenone (521 nm); (f) 1,2-dihydro-derivatives of lycopene and neurosporene (422, 453, 483 nm).

metabolic processes, i.e., respiration and/or fermentation, which function during periods of darkness. In the light, ATP is produced by cyclic photophosphorylation. This term characterizes a process during which electrons are circled between reaction-center bacteriochlorophyll, a primary acceptor, and several electron carriers such as quinones, nonheme iron-sulfur proteins, and cytochromes, all associated with the photosynthetic membrane system. Light is harvested by antenna pigments (bacteriochlorophylls and carotenoids bound to proteins). From the light-harvesting pigments, the energy is transferred to the reaction centers. In purple bacteria, the reaction center consists of four molecules of bacteriochlorophyll, two of bacteriophaeophytin, and a protein consisting of several sub-units. Closely associated with the reaction center is an iron-ubiquinone complex which bridges the gap between the inner mem-

brane surface and the reaction center; the latter is located in the membrane toward the outer surface. Another biochemical complex stretching through the membrane contains a cytochrome *b*, a cytochrome c_1, and an iron-sulfur protein. Ubiquinone acts as a mobile carrier within the membrane, whereas cytochrome c_2 can change its site on the outside of the cytoplasmic membrane. When light energy is transferred from the antenna complex to the reaction center, one electron per light quantum is translocated from bacteriochlorophyll to bacteriopheophytin and further to the iron-ubiquinone complex. At the reaction center bacteriochlorophyll, the missing electron is immediately replaced by an electron from cytochrome c_2. From the reduced iron-ubiquinone complex, the electron enters the intramembraneous ubiquinone pool together with two protons (per quinone molecule and electron) from the cytoplasm. The reduced ubiquinone travels to the bc_1 complex, where the electron is donated to cytochrome c_2, and the two protons to the topological outside of the cell. Cytochrome c_2 acts as a mobile electron carrier between the bc_1 complex and the reaction center complex. The proton gradient created this way between outside and inside drives ATP synthase, thus forming ATP in the cytoplasm (For details, see Drews, 1989).

For the generation of NADH, anoxygenic lithoautotrophic bacteria require external electron donors such as H_2 or reduced sulfur compounds. In the Chromatiaceae, the Ectothiorhodaceae, and the facultatively photolithoautotrophic Rhodospirillaceae, NADH formation is not possible by direct reduction of NAD^+ via the primary acceptor but requires reverse electron flow. Reverse electron transfer is driven by the proton motive force provided by the light reaction mechanism described above, i.e., by cyclic photosynthetic electron flow. The electrons required for NAD^+ reduction are donated at less electronegative sites via *c*-type cytochromes. These electrons are derived from the oxidation of reduced sulfur compounds or molecular hydrogen.

Many phototrophic bacteria can grow using molecular hydrogen as the photosynthetic electron donor. This capability was detected more than 55 years ago in *Chromatium vinosum* strain D and other purple sulfur bacteria (Roelofsen, 1935; Gaffron, 1935).

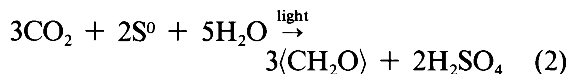
Hydrogen utilization depends upon the presence of the enzyme hydrogenase, and several "uptake" hydrogenases of phototrophic bacteria have been studied in much detail in *Chromatium vinosum* (Gitlitz and Krasna, 1975; Strekus et al., 1980) and *Thiocapsa roseopersicina* (Kondratieva and Gogotov, 1981, 1983; see also Vignais et al., 1985; Gogotov, 1978, 1984, 1986; Schlegel and Schneider, 1978). In general, the enzymes appear to be membrane-bound and probably contain nickel and iron-sulfur clusters. The natural electron acceptors of these hydrogenases are cytochromes of the *c*- or *b*-type in purple bacteria (Gogotov, 1986).

The use of reduced sulfur compounds as electron donors for anoxygenic photosynthesis has been found in all groups of phototrophic purple bacteria (Trüper, 1981a, 1984, 1989; Trüper and Fischer, 1982). In the Chromatiaceae, all species oxidize sulfide and elemental sulfur, and some also oxidize thiosulfate and sulfite.

As first shown by van Niel (1931), photosynthetic carbon dioxide fixation and sulfide (or thiosulfate) oxidation are stoichiometrically linked (in equations 1–6 below, $\langle CH_2O \rangle$ stands for organic matter at the oxidation level of carbohydrate).

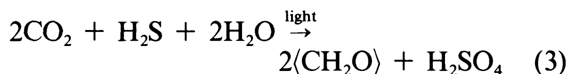
For sulfide:

$$CO_2 + 2H_2S \xrightarrow{\text{light}} \langle CH_2O \rangle + H_2O + 2S^0 \quad (1)$$



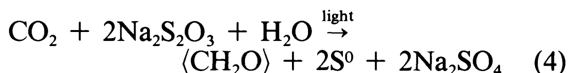
$$3CO_2 + 2S^0 + 5H_2O \xrightarrow{\text{light}} 3\langle CH_2O \rangle + 2H_2SO_4 \quad (2)$$

The sum of equations 1 and 2 gives the ratio of CO_2 to sulfide:

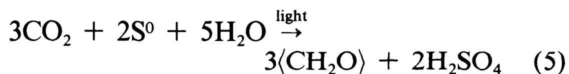


$$2CO_2 + H_2S + 2H_2O \xrightarrow{\text{light}} 2\langle CH_2O \rangle + H_2SO_4 \quad (3)$$

For thiosulfate:

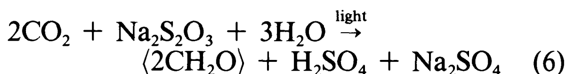


$$CO_2 + 2Na_2S_2O_3 + H_2O \xrightarrow{\text{light}} \langle CH_2O \rangle + 2S^0 + 2Na_2SO_4 \quad (4)$$



$$3CO_2 + 2S^0 + 5H_2O \xrightarrow{\text{light}} 3\langle CH_2O \rangle + 2H_2SO_4 \quad (5)$$

The sum of equations 4 and 5 gives the ratio of CO_2 to thiosulfate:



$$2CO_2 + Na_2S_2O_3 + 3H_2O \xrightarrow{\text{light}} \langle 2CH_2O \rangle + H_2SO_4 + Na_2SO_4 \quad (6)$$

A great deal of information about the action of reduced sulfur compounds as photosynthetic electron donors has been derived from experiments with whole cell suspensions, in contrast to work with cell extracts or purified enzymes.

Most species of phototrophic bacteria depend on or are at least capable of utilizing reduced sulfur compounds as electron donors. All organisms with this capacity utilize sulfide. Elemental sulfur is readily utilized by all Chromatiaceae. The utilization of thiosulfate is rather common in Chromatiaceae, but only

very few species oxidize sulfite or tetrathionate (Trüper, 1981a).

During oxidation of sulfide, sulfur usually appears in the form of globules inside the bacterial cells. Presumably, the elemental sulfur originating from sulfide reacts immediately with surplus sulfide, thus forming polysulfide. As soon as the sulfide is exhausted, the bacteria start to oxidize the polysulfides. Although the pH inside the cells and the optimal pH of media for the Chromatiaceae is rather close to 7.0, at which pH polysulfides are rather unstable, the formation of intracellular "sulfur globules" may follow this scheme. The formation of elemental sulfur is discussed in detail by Steudel (1989). Evidence is accumulating that these "sulfur globules" are not really elemental sulfur but rather are large globular agglomerates of long-chain polythionates. This idea is supported by activation energy measurements during cyanolysis (Then and Trüper, 1983, 1984; Then, 1984), the demonstration that only traces of sulfur ring molecules are present (Steudel, 1985), the hydrophilic properties of the globules (Then and Trüper, 1983, 1984), and density measurements (Guerrero et al., 1984). A detailed investigation of comparable "sulfur" globules of the chemolitho-autotrophic species *Thiobacillus ferrooxidans* by Steudel et al. (1987) revealed that these consisted of an inner core of S_8, S_7, S_9, and S_{12}, and an outer layer of long-chain polythionates (with more than 19 sulfur atoms) which render the globules hydrophilic, so that they are covered by a hydration shell. This structure has yet to be proven for the anaerobic phototrophic sulfur bacteria.

During growth of Chromatiaceae on thiosulfate, sulfur globules appear inside the cells, derived entirely from the sulfane group of thiosulfate (Smith, 1965; Trüper and Pfennig, 1966).

In Chromatiaceae, sulfate is the end product of sulfur oxidations. All sulfur compounds oxidized to sulfate have to pass through sulfite. The enzymatic pathways of dissimilatory sulfur metabolism in Chromatiaceae can be divided into three sections with a group of enzymes each:

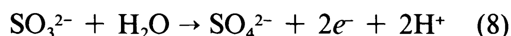
1. oxidation of sulfide and elemental sulfur;
2. utilization of thiosulfate; and
3. oxidation of sulfite.

Sulfide may be utilized by three different enzymes, leading to polysulfides, thiosulfate, or sulfite, respectively. The enzymatic step from sulfide to polysulfides and "sulfur" clearly involves cytochromes of the *c* type (Fischer and Trüper, 1977) as biocatalysts, and electron acceptors. The formation of thiosulfate from sulfide is catalyzed by flavocytochrome *c* as it occurs in *Chromatium* (Fischer, 1977). A "reverse" siroheme-containing sulfite reductase is responsible for sulfite formation from sulfide directly as well as from polysulfides and/or "elemental sulfur." This enzyme has been purified from *Chromatium vinosum* and characterized (Schedel et al., 1979). Thiosulfate is split to elemental sulfur and sulfite by an enzyme that may be measured by its thiosulfate-sulfur transferase (rhodanese) activity. *Chromatium vinosum* (Smith, 1966) contains thiosulfate:acceptor oxidoreductase, earlier called tetrathionase, which forms tetrathionate from thiosulfate. An enzyme oxidizing or splitting tetrathionate has not as yet been found in phototrophic bacteria. Under natural environmental conditions, however, a chemical reduction of tetrathionate by sulfide may readily occur. The enzyme adenylyl sulfate (APS) reductase, an essential enzyme in dissimilatory sulfate-reducing bacteria and in two *Thiobacillus* species was found to occur also in Chromatiaceae (Thiele, 1968; Trüper and Peck, 1970). The enzyme was purified from *Thiocapsa roseopersicina* (Trüper and Rogers, 1971). In *Chromatium vinosum* (Schwenn and Biere, 1979) and *Chromatium warmingii* (Leyendecker, 1983) it is firmly membrane-bound and cannot be solubilized. *Chromatium purpuratum* and *Chromatium gracile* lack this enzyme (Trüper and Fischer, 1982; Ulbricht, 1984). The intermediary formation of APS demands an additional enzyme to split off the sulfate moiety. This step allows conservation of the high-energy phosphate bond energy contained in APS. The enzyme ADP sulfurylase replaces the sulfate moiety of APS by inorganic phosphate, thus producing adenosine diphosphate (equation 7):

$$APS + (P_i) \rightarrow ADP + SO_4^{2-} \quad (7)$$

ADP then can be disproportionated by the enzyme adenylate kinase, leading to the formation of 1 ATP and 1 AMP per 2 ADP. This pathway has been found in six species of the Chromatiaceae (Trüper and Fischer, 1982; Ulbricht, 1984).

Another pathway that conserves the energy of APS is the replacement of its sulfate moiety by inorganic pyrophosphate, catalyzed by the action of ATP sulfurylase. Inorganic pyrophosphate is a common product of biosynthetic reactions, especially of protein biosynthesis, and therefore is readily available during the exponential growth phase of cells. An enzyme bypassing the formation of APS in phototrophic bacteria is sulfite:acceptor oxidoreductase (Trüper, 1981a; Trüper and Fischer, 1982):



$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2e^- + 2H^+ \quad (8)$$

This enzyme occurs in most Chromatiaceae in addition to the APS pathway; in a few cases, instead of the latter (Ulbricht, 1984): sulfite:acceptor oxidoreductase has been partly purified and characterized from *Chromatium vinosum* (Ulbricht, 1984). Dahl and Trüper (1989) showed that the activity considered by other authors in some strains as sulfite:acceptor oxidoreductase in *Thiocapsa roseopersicina* is a nonenzymatic property of cell-free extracts. Some strains of *T. roseopersicina* are devoid of this enzyme.

The flow of electrons from reduced sulfur compounds to the photosynthetic-reaction-center bacteriochlorophyll is, in all species of the Chromatiaceae studied, mediated by *c*-type cytochromes in its final step. The nature of the direct electron acceptors (and, perhaps intermediate electron carriers) of the sulfur-oxidizing enzymes is far from being resolved in all cases. The *c*- and *b*-type cytochromes, flavocytochromes *c*, and high-potential nonheme iron-sulfur proteins are all possible candidates (Fischer, 1984).

In the Chromatiaceae, autotrophic CO_2 fixation is performed by the reductive ribulosebisphosphate cycle (Calvin cycle) with ribulosebisphosphate carboxylase and phosphoribulokinase as the key enzymes. Although not all species have been studied in detail, there is enzymological proof for the Calvin cycle in *Chromatium vinosum* (Fuller et al., 1961), *C. okenii* (Trüper, 1964), *Thiocapsa roseopersicina* (Kondratieva et al., 1976; Zhukov, 1976), and *T. pfennigii* (Sahl and Trüper, 1977). Undoubtedly all species of the family posses the Calvin cycle when they are growing photolithoautotrophically (Kondratieva, 1979).

The enzymatic equipment necessary to make use of the assimilated carbon in phototrophic bacteria does not substantially differ from that of other prokaryotes. Versatility in carbon metabolism is higher in those Chromatiaceae with small cells (e.g., *C. vinosum, Thiocapsa roseopersicina*), than in the Chromatiaceae with large cells (e.g., *C. okenii, Thiospirillum jenense*) (Trüper, 1981b). The latter group is obligately photolithoautotrophic, however, with a certain mixotrophic potential, i.e., these species depend strictly on a supply of CO_2 and sulfide even when they are also utilizing acetate or pyruvate.

In principle, assimilatory nitrogen and sulfur metabolism is not different from that of nonphototrophic bacteria. The majority of photolithoautotrophic bacteria are able to fix dinitrogen, although in some species this capability is found only in certain strains. As dinitrogen fixation is linked with hydrogen (gas) production, this field has received much attention in research (for reviews, see Vignais et al., 1985; Hallenbeck, 1987).

Ammonia is used as the preferred nitrogen source by all species of phototrophic bacteria. As in other bacteria, it is assimilated via glutamine synthetase and further distributed via transamination (the "GOGAT" system). Nitrate is not utilized by Chromatiaceae.

In their natural environments, Chromatiaceae usually have access to abundant concentrations of reduced sulfur compounds—predominantly sulfide—for assimilation as well as for energy metabolism. Sulfur is bound to the carbon skeleton via *O*-acetylserine sulfhydrylase, forming cysteine (Hensel and Trüper, 1981). From cysteine, most of the other organic sulfur compounds needed can be synthesized. Assimilatory sulfate reduction is lacking in the large-cell species of Chromatiaceae, whereas all other species are able to assimilate sulfate and reduce it to the sulfhydryl level as nonphototrophic bacteria do.

Although several species of the Chromatiaceae are relatively oxygen tolerant, only *Thiocapsa roseopersicina* can also grow under fully aerobic conditions, either chemoorganoheterotrophically (Pfennig, 1970), or chemolithotrophically by the oxidation of reduced sulfur compounds (Kondratieva et al., 1976). Chemolithotrophic growth in the dark at low oxygen tension has been found in *Amoebobacter roseus* (Gorlenko, 1974), *Thiocapsa roseopersicina* (Kondratieva et al., 1975), *C. minus, C. vinosum, C. gracile, C. violascens,* and *Thiocystis violacea* (Kämpf and Pfennig, 1980). No growth under such conditions was found in *Thiospirillum jenense, C. okenii, C. weissei, C. warmingii, Thiocapsa pfennigii, Lamprocystis roseopersicina, Thiodictyon elegans, Thiodictyon bacillosum, Amoebobacter pendens,* and *Thiopedia rosea* (Kämpf and Pfennig, 1980). In *Thiocapsa roseopersicina,* the same pathway of sulfide oxidation to sulfate is used as under anaerobic conditions in the light (Dahl and Trüper, 1989).

Anaerobic growth in the dark is very poor in Chromatiaceae; several species have been found to possess a fermentative metabolism (Krasilnikova, 1976; Krasilnikova et al., 1975, 1983; van Gemerden 1968a, 1968b) that at least guarantees survival in the absence of light and oxygen. During fermentation of storage carbohydrate, the intracellular "sulfur" globules serve as an electron sink, and sulfide is excreted (van Gemerden, 1968a, 1968b, 1974).

Applications

The use of phototrophic sulfur bacteria in large-scale processes has the advantages that light is a clean energy source and oxygen is not required. The disadvantages are, however, the technical problems of illumination and corrosive effects of sulfide-containing media upon metal parts.

Nevertheless, these bacteria have been and are being used in a number of biotechnological processes (Mitsui, 1979), including:

production of single cell protein (biomass),
sewage and effluent treatment,
sulfide removal and S^0 production,
production of molecular hydrogen, and
production of organic molecules.

M. Kobayashi and coworkers (e.g., Kobayashi and Tchan, 1973; Kobayashi and Kurata, 1978, Kobayashi et al. 1971) developed a sewage-treatment plant working with mixed natural enrichments of phototrophic sulfur and nonsulfur bacteria. They showed that the biomass could be used as fish and chicken feed as well as fertilizer in citrus fruit cultivation. In addition to the organic and sulfide contents of a variety of sewages, phototrophic bacteria completely removed bad-smelling substances, such as putrescine, cadaverine, and mercaptans, as well as the carcinogen dimethylnitrosamine (Kobayashi and Tchan, 1978).

A laboratory-scale tube system for the degradation of poultry and cattle manure was described by Ensign (1977).

A simple but effective system for effluent treatment is lagooning, where liquid wastes from food industries or communities are fed into large shallow open lagoons. Such systems have been studied several times with respect to the abundance of phototrophic bacteria in them: *Chromatium* sp. in petroleum refinery waste, *Thiopedia rosea* in animal-fat-rendering waste (Cooper, 1963, 1965; Cooper et al., 1975), Chromatiaceae in municipal sewage (May and Stahl, 1967), *Thiocapsa roseopersicina* and *C. vinosum* in potato waste (Holm and Vennes, 1970), *Thiopedia rosea* in feedlot manure (Wenke and Vogt, 1981), and *Thiocapsa roseopersicina* in sugar-factory effluents (N. Pfennig and H. G. Trüper, unpublished observations).

Bharati et al. (1982) showed complete degradation of cellulose by mixed cultures of sulfate-reducing bacteria, anaerobic cellulose decomposers, and *C. vinosum.*

So far, species of the Chromatiaceae have not been used specifically for sulfide removal and sulfur production from H_2S-containing fluids and gases.

Bollinger et al. (1985) developed a system to produce hydrogen gas by phototrophic bacteria growing in sugar-refinery waste water. Immobilized purified hydrogenase of *Thiocapsa roseopersicina* has been used in biofuel cells (Yarapolov et al., 1982).

Chromatiaceae can be used for the production of vitamin B_{12} (Toohey, 1971; Koppenhagen, 1981; Koppenhagen et al., 1981) and biotin (Filippi and Vennes, 1971).

It is surprising that so far no use has been made of the wide spectrum of colorful carotenoids produced by purple sulfur bacteria.

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CHAPTER 171

The Family Ectothiorhodospiraceae

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The family Ectothiorhodospiraceae at present contains a single genus, *Ectothiorhodospira.* All species of this genus have rod- to spiral-shaped cells, with intracytoplasmic membranes as lamellar stacks (Cherni et al., 1969; Holt et al., 1968; Imhoff and Trüper, 1977, 1981; Imhoff et al., 1981; Raymond and Sistrom, 1967, 1969; Remsen et al., 1968; Oren et al., 1989). Species of this genus form two subgroups, one containing extremely halophilic species, the other containing species with lower salt requirements. The division into two subgroups is supported by the type of flagellation, the quinone content, and the similarity coefficients of the 16S rRNA (Imhoff, 1984b; Stackebrandt et al., 1984).

The position of *Ectothiorhodospira* species among the phototrophic purple bacteria has been disputed since their discovery by Pelsh (1936). He distinguished these bacteria, which he called "Ectothiorhodaceae," from those purple bacteria with elemental sulfur inside their cells, which he called "Endothiorhodaceae" (Pelsh, 1937). Pelsh's isolates were poorly characterized and were lost soon after their isolation. Trüper (1968) reisolated *Ectothiorhodospira mobilis,* and Pfennig and Trüper (1971) included *Ectothiorhodospira* as an exceptional genus into the Chromatiaceae because of its ability to perform a dissimilatory sulfur metabolism. With the isolation of new species and the availability of chemotaxonomic data, additional properties of *Ectothiorhodospira* species became apparent that clearly separate this genus from those of the Chromatiaceae. Therefore, the reassignment of the genus *Ectothiorhodospira* to the new family Ectothiorhodospiraceae has been proposed (Imhoff, 1984a).

Habitats

In comparison with Chromatiaceae and purple nonsulfur bacteria, Ectothiorhodospiraceae are more restricted in their natural distribution because of their growth requirements for alkalinity and salinity. Like other phototrophic sulfur bacteria, they may develop in the presence of anoxic conditions, soluble sulfide, light, and bicarbonate. A small number of simple organic compounds, such as certain fatty acids, markedly stimulate their growth. *Ectothiorhodospira* species have been isolated from marine sources and hypersaline lakes, such as estuaries, salt flats, salt lakes, and soda lakes, from many parts of the world (Grant et al., 1979; Imhoff and Trüper, 1976, 1977, 1981; Imhoff et al., 1978, 1979, 1981; Matheron and Baulaigue, 1972; Pelsh, 1936; Raymond and Sistrom, 1967; Tew, 1966; Tindall, 1980; Trüper, 1968, 1970; Ventura et al., 1988) (for review, see Imhoff, 1988a). More recently a new species, *Ectothiorhodospira marismortui* has been isolated from a hypersaline sulfur spring (Oren et al., 1989). Occasionally, certain *Ectothiorhodospira* species may be found in soil. Alkaline soda lakes show a natural abundance of *Ectothiorhodospira* species (Imhoff et al., 1978, 1979), which can be taken as proof of their successful adaptation to these environments. Because of their mass development in alkaline soda lakes, the extremely halophilic species may cause red or green coloration of these habitats. *E. abdelmalekii, E. halochloris,* and in particular *E. halophila* are among the most halophilic phototrophic bacteria (see Imhoff, 1988a). Isolates of *E. halophila* from soda lakes in the Wadi Natrun (Egypt), which have salt optima of 25% total salts and also can grow in saturated salt solutions, are the most halophilic eubacteria known to date.

Isolation

Selective Enrichment

For the extremely halophilic species, the dependence on and the tolerance to high salinity and alkalinity are strongly selective conditions for their enrichment. Marine strains of *Ectothiorhodospira* have been selectively enriched under photoautotrophic conditions in Pfennig's medium (Pfennig, 1965; Pfennig and Trüper,

1981) with sulfide as an electron donor, and in saline and alkaline media (3% NaCl and pH 8.5 to 9.0), even in the presence of high numbers of *Chromatium* cells in the natural sample (Trüper and Imhoff, 1981). Many *Ectothiorhodospira* strains have been isolated from various locations with a medium based on the mineral composition of the soda lakes of the Wadi Natrun (Imhoff and Trüper, 1977; Imhoff et al., 1979; Jannasch, 1957) and modifications thereof. Depending on the species composition of the sample and the salt concentration of the media (from 3 to 25%), various *Ectothiorhodospira* species will develop in enrichment cultures. Investigations of the vitamin requirements of a number of *Ectothiorhodospira* isolates have demonstrated that vitamins are not essential, although vitamin B_{12} stimulates growth in some strains.

Isolation Procedures

For enrichment, environmental samples are inoculated into suitable media in 20-ml screw-cap test tubes or 50-ml bottles, which are completely filled with medium. For isolation, agar dilution series are prepared either with enrichment cultures or with promising natural samples by direct inoculation from the environment. Selectivity is not necessary with media used for direct isolation without prior enrichment procedures.

Preparation of Agar Dilution Series

In a modification of the method of Pfennig (1965; see also Trüper, 1970), purified agar is dissolved (1.8%) and distributed in amounts of 3 ml into cotton-plugged test tubes. The agar is sterilized by autoclaving. The liquid agar is kept at 50°C in a water bath until use. A suitable medium is placed in the same water bath, and 6 ml of the prewarmed medium is added to each test tube. Medium and agar are mixed thoroughly by turning the tubes upside down and back and kept at 50°C. Six to eight tubes are sufficient for each dilution series. The first tube is inoculated with a natural sample or enrichment culture and mixed carefully; approximately 0.5 to 1.0 ml is transferred to a second tube, mixed carefully, and the procedure continued up to the last tube. The tubes are immediately placed into a cold water bath. After the agar has hardened, they are sealed with a paraffin mixture (3 parts paraffin oil and 1 part paraffin) and kept in the dark for several hours before incubating them in light (500–2,000 lux). After cells have grown to visible colonies, the paraffin layer is removed by melting, and well-separated colonies are picked with a Pasteur pipette (the tip drawn out to a thin capillary) and transferred to a second dilution series. In general, three to four such dilution series are necessary to obtain pure cultures. When pure cultures have been obtained, single colonies are inoculated into liquid medium.

Cultivation on Agar Plates

A convenient method for growing phototrophic sulfur bacteria on agar plates has been described by Irgens (1983). Medium without added sulfide is used with the GasPak system (Becton Dickinson and Co., Cockeysville, MD). As a source of sulfide, a test tube with 0.05 to 0.1 g of thioacetamide and 1 ml of 0.5 N HCl are placed in the anaerobic jar. Thioacetamide decomposes under acidic conditions to ammonia, hydrogen sulfide, and acetic acid. The H_2S gas is released over a period of at least one week. As indicators, methylene blue (for oxygen) and a strip with lead acetate (for H_2S) are included. This method is also a convenient method for performing viable counts of purple sulfur bacteria. It has been successfully used for the isolation of species of the genera *Amoebobacter, Chromatium, Lamprocystis, Thiocapsa, Thiocystis,* and *Ectothiorhodospira* (Irgens, 1983).

Media for *Ectothiorhodospira* Species

Enrichment and subsequent isolation of *E. mobilis* and *E. shaposhnikovii* have been achieved by using Pfennig's medium with 3% NaCl at alkaline pH 8.0 to 8.5 (Trüper, 1970). This medium, however, is not suitable for the extremely halophilic species *E. halochloris, E. halophila,* and *E. abdelmalekii.* Raymond and Sistrom (1967) described a medium for the cultivation of *E. halophila* (for recipe, see Trüper and Imhoff, 1981). Apparently, *E. marismortui* also does not grow on these media, and therefore a modified medium was used for isolation and cultivation of this new species (Oren et al., 1989).

A medium based on the mineral salts composition of the soda lakes of the Wadi Natrun has been used for isolation of many *Ectothiorhodospira* strains from this and other environments (Imhoff and Trüper, 1977, 1981; Imhoff et al., 1978, 1981). This medium is well suited for isolation and cultivation of most of the *Ectothiorhodospira* species. It is buffered by bicarbonate (200 mM, pH 9.0) and adjusted to the desired salinity by changing the concentration of NaCl. In our experience, Raymond and Sistrom's strain also grows better in the mineral salt-based medium described here as compared with the one described by Raymond and Sistrom (1967). The mineral composition of this

medium (Imhoff and Trüper, 1977) and its preparation method have been modified several times. Although trace element solutions and sulfide solutions were sterilized separately, the filtration procedures used formerly yielded less reproducible growth, presumably because of some interaction of medium constituents with the filters. The reproducibility of growth was much better with the preparation method described by Imhoff (1988b):

Medium for Extremely Halophilic *Ectothiorhodospira* Species

The basal medium has the following composition (amounts per liter for a medium with 15% salinity):

Trace element solution SLA (see below)	1 ml
KH_2PO_4	0.8 g
Na acetate	2.0 g
$Na_2S_2O_3 \cdot 5H_2O$	1.0 g
NaCl	130.0 g
1 M Na carbonate, pH 9.0	200 ml

Trace element solution SLA (Imhoff and Trüper, 1977):

$FeCl_2 \cdot 4H_2O$	1,800 mg
$CoCl_2 \cdot 6H_2O$	250 mg
$NiCl_2 \cdot 6H_2O$	10 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	70 mg
$ZnCl_2$	100 mg
H_3BO_3	500 mg
$Na_2MoO_4 \cdot 2H_2O$	30 mg
$Na_2SeO_3 \cdot 5H_2O$	10 mg

The salts are dissolved separately in a total of 900 ml of double-distilled water; the pH is adjusted with 1 N HCl to about 2–3, and the volume is brought to 1 liter.

The components are dissolved in 600 ml of distilled water and the carbonate buffer is added. The volume is then adjusted to 980 ml and the solution is autoclaved. The salinity is adjusted to the desired value by variation of the NaCl content (assuming a contribution of 2% by the other medium constituents). The remaining salts (see below) are sterilized separately and, after they have cooled down, are added under gentle stirring. The final pH is 9.0.

2% $MgCl_2 \cdot 7H_2O$ (5 ml/liter)
1% $CaCl_2 \cdot 2H_2O$ (5 ml/liter)
5% $Na_2S \cdot 9H_2O$ (5–10 ml/liter)
20% NH_4Cl (4 ml/liter)

The medium is then immediately placed in sterilized culture vessels, which are almost completely filled with only a small air bubble left. The vessels are preincubated at the incubation temperature (33 to 40°C) to achieve volume expansion and then inoculated with 10% of a fresh preculture.

The addition of $Na_2S_2O_3$ is not necessary for the two green-colored *Ectothiorhodospira* species, as these can not use thiosulfate. In the red-colored species, it is suitable as an additional electron donor, and makes feeding with sulfide unnecessary. Acetate is a suitable carbon source for all species and may also be used as an additional electron donor.

In standard media for *E. halochloris* and *E. abdelmalekii* 10% of the total salinity is added as Na_2SO_4, and the NaCl is reduced by the equivalent amount (in g per liter). If *E. mobilis* is grown photoheterotrophically, reduced sulfur compounds are omitted and sodium ascorbate (0.1%) is added to achieve anoxic conditions. Under these conditions, sulfate is used as sole sulfur source.

Medium for *Ectothiorhodospira marismortui* (Oren et al., 1989)

For the preparation of 1 liter of medium, in a volume of 900 ml are dissolved:

NaCl	100 g
Na_2SO_4	0.5 g
Yeast extract (Bacto)	0.1 g

The following components were sterilized separately and added as sterile solutions to the medium to final concentrations of:

KH_2PO_4	0.33 g
NH_4Cl	0.33 g
KCl	0.33 g
$MgCl_2 \cdot 6H_2O$	0.33 g
$CaCl_2 \cdot 2H_2O$	0.33 g
Na acetate·3 H_2O	0.5 g
Na_2CO_3	1.5 g
Trace element solution (Pfennig and Lippert, 1966)	10 ml

The pH is adjusted with 1 M HCl to a final value of 6.5–6.8. Immediately before use of the medium, $Na_2S_2O_4$ was added to a final concentration of 0.08 g/liter from a freshly prepared filter-sterilized solution. Sulfide may be used as an electron donor and is added to a final concentration of 1 mM.

Cultivation

In general, cultivation of *Ectothiorhodospira* is possible in any size of bottle and in glass fermenters as long as the illumination is sufficient and the temperature is controlled. Cultures are incubated at 1,000 to 20,000 lux (approximately 100 to 2,000 foot candles) and at temperatures between 33 to 40°C. The pH optima found with different strains and species (and also in different media) are between 7.4 and 9.1. Salt optima of the different species are shown in Table 1. For routine cultivation with the medium for extremely halophilic *Ectothiorhodospira* species, we use salinities of 15% for *E. abdelmalekii, E. halochloris,* and some strains of *E. halophila* (including the type strain), 25% for the most halophilic strains of *E. halophila,* 5% for *E. vacuolata* and some strains of *E. mobilis,* and 2% for *E. shaposhnikovii.*

Table 1. Some characteristic properties of the *Ectothiorhodospira* species.

Species	Cell shape	Cell diameter (μm)	Flagellation	Color of culture	Major carotenoid	Major bchl	Major quinone	Gas vacuoles	Optimal salinity range	GC content (mol%)
Ectothiorhodospira										
mobilis	Rod—spiral	0.7–1.0	Polar	Red	sp	*a*	Q-7,MK-7	−	3–10%	62.0–69.9
shaposhnikovii	Rod—spiral	0.8–0.9	Polar	Red	sp	*a*	Q-7,MK-7	−	1–3%	61.2–62.8
vacuolata	Rod	1.5	Polar	Red	sp	*a*	Q-7,MK-7	+	1–6%	61.4–63.6
marismortui	Rod	0.9–1.3	Polar	Red	(sp)	*a*	ND	−	3–8%	65
halophila	Spiral	0.8–0.9	Bipolar	Red	sp	*a*	Q-8,MK-8	−	12–30%	64.3–69.7
halochloris	Spiral	0.5–0.6	Bipolar	Green	rhg,rh	*b*	Q-8,MK-8	−	14–20%	50.5–52.9
abdelmalekii	Spiral	0.9–1.2	Bipolar	Green	*	*b*	Q-8,MK-8	−	12–20%	63.3–63.8

Abbreviations. Carotenoids: rh = rhodopin; rhg = rhodopin glucoside and derivatives; sp = spirilloxanthin; (sp) = most probably spirilloxanthin; * = most probably similar to *E. halochloris*. bchl = bacteriochlorophyll; Q = ubiquinone; MK = menaquinone; ND = no data available.

Based on Imhoff (1984a), Imhoff and Trüper (1981), Imhoff et al. (1981), Oren et al. (1989), Schmidt and Trüper (1971), and Trüper and Imhoff (1981).

The less halophilic isolates may be grown on Pfennig's medium for purple sulfur bacteria (Pfennig, 1965) adjusted to the desired pH and salinity.

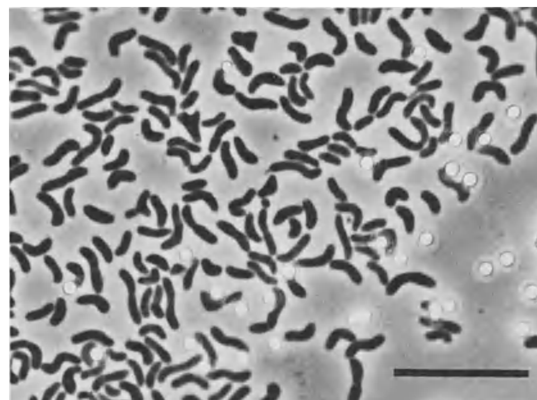
Preservation of Cultures

Well-grown cultures in screw-cap bottles may be kept at 4°C in a refrigerator or at room temperature. Screw-cap bottles with strains of *E. mobilis* and *E. halophila* that have been kept on a laboratory desk for several years contained numerous viable cells that grew well after transfer into fresh medium. Maintenance transfer of cultures should be every 2 to 6 months. In particular, cultures of *E. halochloris* and *E. abdelmalekii* lose viability much faster than do cultures of the other species. *Ectothiorhodospira* strains have also been stored in closed ampules kept in liquid nitrogen with good viability after more than 20 years.

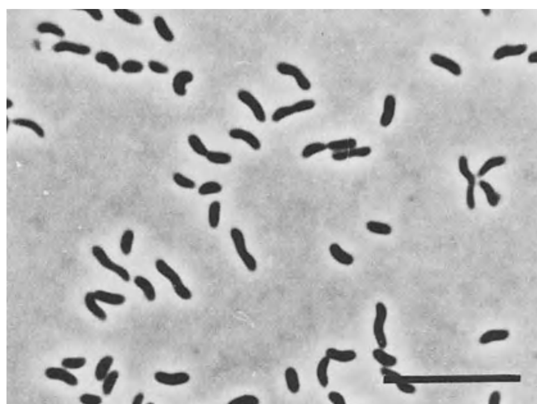
Identification

Morphological and physiological properties are both very important for the identification of phototrophic bacteria. The Ectothiorhodospiraceae are distinguished from the Chromatiaceae by the intermediate deposition of elemental sulfur outside the cells. Their dependence on saline and alkaline growth conditions and the presence of intracytoplasmic membranes as lamellar stacks are characteristic.

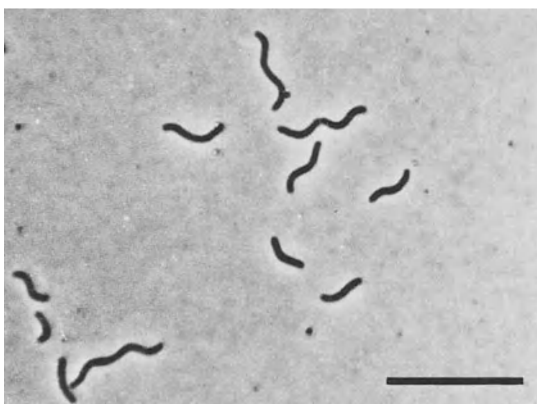
Cells are rod- to spiral-shaped and are motile by means of mono- or bipolar tufts of flagella (Fig. 1, Table 1). They form red- or green-colored colonies. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series, with spirilloxanthin as the predominant component in the red-colored species (Schmidt and Trüper, 1971). Two green-colored species, *E. halochloris* and *E. abdelmalekii,* contain bacteriochlorophyll *b* esterified with Δ-2,10-phytadienol, not with phytol as is the bacteriochlorophyll *a* of the other *Ectothiorhodospira* species (Steiner et al., 1981; R. Steiner, personal communication). The carotenoid content is low in *E. halochloris* and *E. abdelmalekii.* The carotenoid composition of both of these species is apparently quite similar. Mainly methoxyrhodopin glucoside (major), rhodopin glucoside, and rhodopin have been found in *E. halochloris* (K. Schmidt, personal communication). Absorption spectra of living cells or chromatophore suspensions allow the identification of the bacteriochlorophyll present and provide some information on the types of carotenoids present (Fig. 2).



a



b



c

Fig. 1. Comparison of *Ectothiorhodospira* species. (*a*) *E. mobilis* DSM 237; (*b*) *E. halophila* BN 9621; (*c*) *E. halochloris* ATCC 35916. Note large and small sulfur globules in (*a*) and (*c*). Bar = 10 μm.

Ectothiorhodospira species have a characteristic composition of polar lipids (Asselineau and Trüper, 1982; Imhoff et al., 1982), fatty acids, and quinones (Imhoff, 1984a, 1984b), which distinguishes this group from Chromatiaceae and purple nonsulfur bacteria (Rhodospirillaceae).

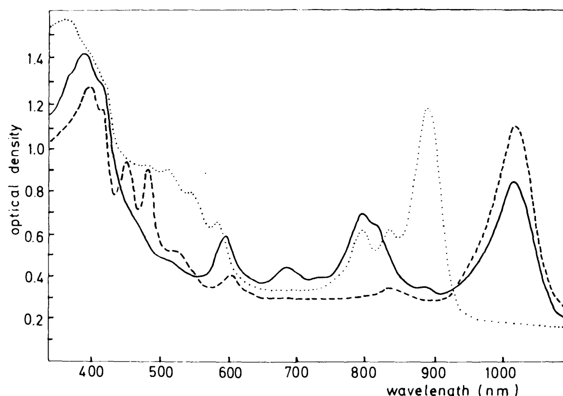


Fig. 2. Absorption spectra of chromatophore suspensions of: *Ectothiorhodospira halochloris* (———), *Ectothiorhodospira halophila* (.........), and, as reference, the bacteriochlorophyll *b*-containing *Rhodopseudomonas sulfoviridis* (---------).

DNA hybridization studies demonstrated the close similarity of *E. mobilis* and *E. shaposhnikovii* (Ivanova et al., 1985; Turova et al., 1982), although analysis of 16S rRNA clearly revealed a larger distance between these two species (Stackebrandt et al., 1984). The currently known species are well characterized by their content of pigments, salinity requirement, GC content of the DNA, major quinones, pigments, and cell sizes (Table 1).

Verification of enriched and isolated *Ectothiorhodospira* species is best obtained by comparison with the type strains deposited in and available from culture collections such as the American Type Culture Collection (ATCC, Rockville, Maryland) and the Deutsche Sammlung von Mikroorganismen (DSM, Göttingen, FRG). The type strains are: *E. mobilis* (DSM 237), *E. shaposhnikovii* (DSM 243), *E. vacuolata* (ATCC 43036, DSM 2111), *E. halophila* (DSM 244), *E. halochloris* (ATCC 35916), *E. abdelmalekii* (ATCC 35917), and *E. marismortui* (DSM 4180).

Physiological Properties

All *Ectothiorhodospira* species grow well under anaerobic conditions in the light, with reduced sulfur compounds as photosynthetic electron donors and in the presence of organic carbon sources and inorganic carbonate. *Ectothiorhodospira mobilis* and *E. shaposhnikovii* also grow microaerobically in the dark if sulfide is present. During phototrophic growth with sulfide as an electron donor, sulfide is first oxidized to elemental sulfur which is then oxidized to sulfate, as has been shown for *E. mobilis* (see Trüper, 1978). Under alkaline growth conditions that are favorable for *Ectothiorhodospira* species, polysulfides are stable intermediates in sulfide oxidation. As a result, polysulfides (probably formed by the chemical reaction between elemental sulfur and sulfide) and elemental sulfur are the first measurable oxidation products, and the medium becomes yellow-translucent at this stage. After sulfide depletion, elemental sulfur droplets are rapidly formed and the medium becomes cloudy to opaque. During further growth, cultures of red-colored species become pinkish and finally red if elemental sulfur disappears. These color changes are best observed with the red-colored species under photoautotrophic growth conditions. Our knowledge about the enzymes involved in the oxidation of reduced sulfur compounds by *Ectothiorhodospira* species has recently been summarized by Trüper and Fischer (1982).

Under autotrophic growth conditions, the fixation of carbon dioxide via the ribulose-bisphosphate pathway is apparently the major route of carbon assimilation in *E. shaposhnikovii* (Firsov et al., 1974). High activities of ribulose-bisphosphate carboxylase have been found in *E. shaposhnikovii* (Firsov et al., 1974), *E. mobilis* (Sahl and Trüper, 1977), and *E. halophila* (Tabita and McFadden, 1972). Under photoheterotrophic growth conditions, considerable proportions of cellular carbon are derived from carbon dioxide, which is assimilated by pathways not involving ribulose-bisphosphate pathway. The assimilation of several organic carbon sources (such as acetate and propionate) depends on the presence of carbon dioxide and proceeds via several carboxylation reactions (Firsov and Ivanovsky, 1974, 1975). Phosphoenolpyruvate carboxylase, ferredoxin-dependent pyruvate synthase, and α-ketoglutarate synthase were found in *E. shaposhnikovii* (Firsov et al., 1974); phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase were found in *E. mobilis* (Sahl and Trüper, 1977). All enzymes of the glycolytic pathway and the tricarboxylic acid cycle, with the exception of α-ketoglutarate dehydrogenase, are present in *E. shaposhnikovii* (Krasilnikova, 1975). Cells grown on acetate had increased activities of isocitrate lyase, indicative of the function of the glyoxylic acid pathway. Poly-β-hydroxybutyric acid is the principal reserve material formed from acetate and butyrate in the absence of carbon dioxide. In the presence of carbon dioxide, carbohydrates are preferentially formed from acetate and butyrate as well as from other carbon sources (Novikova, 1971).

One of the most prominent properties of *Ectothiorhodospira* species is their ability to adapt to the most extreme salt concentrations tolerated by eubacteria. They not only tolerate these high concentrations, but optimally thrive. The structural and physiological basis of this adaptation is currently under investigation. To adapt to these high and sometimes varying concentrations of salts, *Ectothiorhodospira* species accumulate high concentrations of organic solutes that balance the outside osmotic pressure. In *Ectothiorhodospira halochloris,* glycine betaine has been found to be the main osmotically active cytoplasmic component (Galinski and Trüper, 1982), but by ^{13}C-NMR techniques, ectoine, a new cyclic amino acid, and trehalose were found in *E. halochloris* (Galinski et al., 1985) and in other species of *Ectothiorhodospira* (Imhoff and Galinski, unpublished observations; see also Imhoff, 1988a).

Applications

Like other phototrophic sulfur bacteria, *Ectothiorhodospira* species (in particular *E. mobilis* and *E. shaposhnikovii*) are suited for the removal of toxic sulfide from waste waters. Their growth requirements call for their application under alkaline and saline conditions. Further studies are necessary to show whether the isolation of ectoine from *Ectothiorhodospira* deserves applicability. At present, *Ectothiorhodospira* and products thereof are not used in commercial and technical processes.

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CHAPTER 172

The Genus *Oceanospirillum*

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Although *Oceanospirillum* (Hylemon et al., 1973) is a genus that has been only recently described, some of its members have a very long history as former species of the genus *Spirillum* (Ehrenberg, 1832).

Members of *Oceanospirillum* consist of rigid, helical, Gram-negative cells that have a clockwise helix. The cells have a diameter of 0.4 to 1.4 μm; the length of the helix is 1.2 to 75 μm. Cells are motile by bipolar tufts of flagella. In all species examined so far by electron microscopy, a polar membrane underlies the cytoplasmic membrane at the cell poles. Intracellular poly-β-hydroxybutyrate (PHB) is formed. The strains of most species form thin-walled coccoid bodies, which predominate in old cultures. They are chemoorganotrophic with a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Nitrate respiration does not occur and nitrate is not reduced to nitrite or beyond this stage. The optimum temperature for growth is 25° to 32°C. Cells are oxidase-positive, indole-negative, and casein, starch, hippurate, and esculin are not hydrolyzed. Carbohydrates are neither fermented nor oxidized and amino acids or organic acids serve as carbon sources. Seawater is required for growth. Growth factors are usually not required. The organism is isolated from coastal seawater, from decaying seaweed, and from putrid infusions of marine mussels. The GC content of the DNA ranges from 45 to 50 mol%, as determined by the thermal denaturation method. The type species is *Oceanospirillum linum* (Hylemon et al., 1973) and the type strain is *O. linum* ATCC 11336.

During the last 15 years, the classification of both freshwater and marine spirilla has changed considerably. In the original description (Hylemon et al., 1973), *Oceanospirillum* comprised five species: *O. linum, O. minutulum, O. beijerinckii, O. maris,* and *O. japonicum* (Table 1). Four other species, *O. hiroshimense, O. pelagicum, O. pusillum,* and *O. multiglobuliferum* (Table 1) were created later by Terasaki (1973, 1979). These nine species are described by Krieg (1984) in *Bergey's Manual of Systematic Bacteriology.*

Bowditch et al. (1984) and DeLong et al. (1984) reported a close immunological relationship among *O. linum, O. beijerinckii, Marinomonas vaga, M. communis* (Van Landschoot and De Ley, 1983), and two unnamed groups (groups H-1 and I-1) of marine bacteria. Although these relationships were not confirmed by other methods, Bowditch et al. (1984) concluded that *M. communis* and *M. vaga* should be assigned to the genus *Oceanospirillum* as *O. vagum* and *O. commune,* and they created the species *O. jannaschii* and *O. kriegii* for the two unnamed groups H-1 and I-1 respectively (Table 1). An unfortunate consequence of this extension of the genus *Oceanospirillum* was the loss of most of the readily determinable phenotypic features of the genus definition and the extension of the upper GC values for the genus from 51 to 57 mol% (Bowditch et al., 1984). Using DNA:rRNA hybridizations, Pot et al. (1989) showed that *O. vagum, O. commune, O. jannaschii,* and *O. kriegii* were erroneously included in the genus *Oceanospirillum.* Moreover, it was shown that *O. minutulum* and *O. pusillum* were likewise generically misnamed and ought to be removed from the genus *Oceanospirillum.* Using DNA-DNA hybridizations and protein gel electrophoresis, it was further shown that *O. hiroshimense* was very closely related to, and should be considered a subspecies of, *O. maris,* and that *O. pelagicum* is a subspecies of *O. beijerinckii.* Table 2 lists the differential characteristics for the five species of the emended genus *Oceanospirillum* (Pot et al., 1989).

As representatives of the genus *Oceanospirillum* and *Aquaspirillum* have a long common history as members of the genus *Spirillum,* many methods mentioned in the present chapter are described in more detail in Chapters 130 and 131.

Table 1. List of strains previously included in the genus *Oceanospirillum*.

Species name as in Krieg, 1984 and Bowditch et al., 1985[a]	Type strain		Source, place, and year of isolation	Reference
	Strain no. as received[b]	LMG no.[c]		
O. beijerinckii	NCMB 52	LMG 5405	Coastal water, United States, 1957	Williams and Rittenberg, 1957; Hylemon et al., 1973
[O.] commune	ATCC 27118	LMG 2864	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al., 1972; Van Landschoot and De Ley, 1983; Bowditch et al., 1984
O. [hiroshimense]	IFO 13616	LMG 7371	Marine shellfish, 1963	Terasaki, 1973, 1979
[O.] jannaschii	ATCC 27135	LMG 6239	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al., 1972; Bowditch et al., 1984
O. japonicum	ATCC 19191	LMG 5215	Marine shellfish, 1959	Watanabe, 1959; Hylemon et al., 1973
[O.] kriegii	ATCC 27133	LMG 6238	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al., 1972; Bowditch et al., 1984
O. linum	ATCC 11336	LMG 5214	Coastal water, United States, 1957	Williams and Rittenberg, 1957; Hylemon et al., 1973
O. maris subsp. *maris*	ATCC 27509	LMG 5213	Seawater, 1973	Hylemon et al., 1973
O. maris subsp. *williamsae*	ATCC 29547	LMG 5210	From mixed culture, 1978	Linn and Krieg, 1978
[O.] minutulum	ATCC 19193	LMG 5334	Marine shellfish, 1959	Watanabe, 1959; Hylemon et al., 1973
O. multiglobuliferum	IFO 13614	LMG 5306	Marine shellfish, 1960	Terasaki, 1973, 1979
O. [pelagicum]	IFO 13612	LMG 5307	Marine shellfish, 1966	Terasaki, 1973, 1979
[O.] pusillum	IFO 13613	LMG 5308	Marine shellfish, 1961	Terasaki, 1973, 1979
[O.] vagum	ATCC 27119	LMG 2845	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al., 1972; Van Landschoot and De Ley, 1983; Bowditch et al., 1984

[a]Brackets enclose the name of a generically or specifically misnamed taxon (Pot et al., 1989).
[b]Abbreviations: NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; ATCC, American Type Culture Collection, Rockville, Md.; IFO, Institute for Fermentation-Osaka, Osaka, Japan.
[c]LMG, Bacterial Culture Collection of the Laboratorium voor Microbiologie, Gent, Belgium.

Habitats

In a search for spirochaetes in Baltimore market oysters, Dimitroff (1926a, 1926b) found and isolated a spirillum *("Spirillum virginianum")* from the mud adhering to the outside of an oyster shell. It has been proven since that marine and freshwater shellfish are a source of spirilla. Although Watanabe (1959) isolated marine spirilla from the viscera of marine shellfish and Terasaki (1963, 1970) observed spirilla in the alimentary tracts of marine shellfish, it is more likely that the adherent mud is the source of the spirilla (Terasaki, 1963, 1970). Williams and Rittenberg (1957) isolated many strains from seawater samples taken from the intertidal zone and Jannasch (1963a, 1963b) isolated a marine spirillum from decaying seaweed. Whether *Oceanospirillum* occurs in the open sea is unknown. There is only one report, by Oppenheimer and Jannasch (1962), in which direct microscopic counts of bacteria present in clear and turbid seawaters near Port Aransas, Tex., revealed that 0.1 to 2.5% of the total bacterial population consisted of spirilla, compared to 0.9 to 4.8% vibrios and 90.0 to 99.5% cocci. The same study demonstrated that the turbid water along the shore of Redfish Bay contained 2.9×10^7 bacteria per ml, compared to 9×10^5 per ml outside the Gulf surf where the water was clear. This possible effect of nutrient concentration was further investigated by Jannasch (1963a, 1963b) with a marine spirillum isolated from decaying seaweed, using a chemostat with extremely low concentrations of asparagine as a nitrogen-limiting nutrient. With a medium composed of 3% commercial sea salt, phosphate buffer, lactate as carbon source, and asparagine as the limiting source of nitrogen, Jannasch maintained a steady state population of about

Table 2. Differential features of the species in the redefined genus *Oceanospirillum*.[a]

Feature	*O. linum*	*O. maris*	*O. beijerinckii*	*O. multiglobuliferum*	*O. japonicum*
Length of helix (μm)	4.0–30.0	2.5–40.0	2.0–15.5	2.0–10.0	5.0–75.0
Cell diameter (μm)	0.4–0.6	0.6–1.1	0.6–1.2	0.5–0.9	0.8–1.4
Coccoid bodies predominant					
After 3–4 weeks	+	+	+	+	−
After 24–48 h	−	−	−	+	−
Maximum salt tolerance not higher than 4% NaCl	−	−	−	+	−
Optimum temperature 25°C rather than 30–32°C	−	d[b]	−	−	−
Phosphatase	+	d[b]	+	+	W
Catalase	+ or W	d[b]	+ or W	+	W or −
Auxotrophic growth requirement	+[c]	d[d]	−	−	−
GC content (mol%)	48–50	45–47	47–49	46	45

Symbols: +, present in all strains; −, lacking in all strains; d, differs among strains; W, weak reaction.
[a]The phenotypic data are from Krieg (1984).
[b]*O. maris* strains can have an optimum temperature of 25°C; catalase can be present, weak or lacking, or weak; phosphatase may be present or lacking. For detailed information see Hylemon et al., 1973; Terasaki, 1972 and 1979.
[c]*O. linum* grows poorly or not at all in defined media with single carbon sources and ammonium ions as the nitrogen source; however, abundant growth occurs in a defined medium containing succinate plus malate as carbon sources and methionine as the nitrogen source.
[d]*O. maris* subsp. *williamsae* ATCC 29547 fails to grow in vitamin-free defined media and requires a growth factor that has not yet been identified.

4×10^5 cells per ml at 0.28 mg of asparagine-N per liter. Experiments with unsupplemented natural seawater (even extremely polluted water from Naples Harbor) and high dilution rates (to obtain higher growth rates) indicated that the spirillum was unable to multiply faster than one generation per 24 h and was therefore washed out of the chemostat. Addition of lactate and asparagine at the concentrations established in Jannasch's previous experiments stimulated growth. Jannasch therefore suggested that the growth of the spirillum might be restricted to environments of higher nutrient concentrations than found in ordinary seawater, such as in zones surrounding decaying particulate plant matter. In support of this, he found that all enrichments from seawater were unsuccessful unless pieces of decaying seaweed were added.

Isolation

For the isolation of marine spirilla, enrichment is necessary, since these bacteria are relatively slow-growing and are never the predominant organism in natural samples. The chance of isolating spirilla is increased by sampling water in environments with higher nutrient concentrations (Jannasch, 1963a, 1963b).

Isolation by Dilution

Harold and Stanier enriched for marine spirilla in 1955 by placing pieces of thalli of marine algae in flasks almost completely filled with seawater and by incubating these infusions for several days at 25°C. Williams and Rittenberg (1957), however, found it extremely difficult to use this method to enrich the seawater samples they collected from the intertidal zone along the coast of Long Island Sound. Sufficient enrichment was finally accomplished by mixing the seawater samples with an equal volume of doublestrength Giesberger's medium (Giesberger, 1936) containing per liter:

NH_4Cl	1 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g

and by adding 1% of calcium lactate to the mixture. Calcium malate, an excellent substrate for the enrichment of freshwater forms, was not successful for the marine spirilla, although these organisms all utilized malate when grown in pure culture. After incubation, spirilla appeared and a portion of the initial culture was sterilized and mixed with an equal volume of sterile Giesberger's medium lacking ammonium chloride. This sterile mixture was then inoculated from the unsterilized portion of the initial culture. After one to three subcultures in the transfer medium, spirilla predominated. Isolation was finally accomplished by diluting the enriched culture 1:100 to 1:100,000 times (depending on the relative abundance of contaminating forms) with sterile seawater in large dilution bottles. These were shaken vigorously and allowed to stand at room temperature for 20 min to allow spirilla to migrate to the surface of the dilution mixture, where they accumulated just beneath the surface scum that generally forms in

marine cultures. A loopful of this surface water was then streaked onto nutrient agar prepared with seawater and containing 0.3% yeast autolysate. Plates were incubated at 30°C and after 24 h examined for distinctive granular, umbonate, or pulvinate colonies with ground-glass appearance. After another 24 h, wet mounts were prepared from typical colonies to confirm the presence of spirilla. Samples of spirilla yielding colonies could then be restreaked serially until all colonies developing on a plate showed cells with a uniform spirillar morphology.

The effectiveness of the limitation of the available nutrients for the isolation of marine (and freshwater) spirilla (in particular, by reuse of a partially spent medium without the addition of supplementary nitrogen source) was also demonstrated by Jannasch (1967), who found that *Oceanospirillum maris* grew faster than a *Pseudomonas* species in a continuous culture with diluted media containing lactate as the limiting nutrient. The spirilla were outgrown, however, in more concentrated media. Williams and Rittenberg (1957) were convinced that the dilution method resulted in reduced growth of the undesired types rather than stimulating the development of the spirilla.

Isolation from Putrid Infusions

Terasaki (1963, 1970, 1972) and Watanabe (1959) obtained excellent results using undiluted enrichment media. The method used by Terasaki (1970) is comparable to the one he used before (Terasaki, 1961a, 1961b) for the isolation of freshwater spirilla (see Chapter 131).

A handful of marine mussels *(Tapes philippinarum)* were washed with distilled water and smashed with a hammer. The mussels were boiled for several minutes and after cooling they were placed in a petri dish with a teaspoon of mud. The dish is filled with sterilized seawater until the mussels sink completely in the solution. This infusion is incubated at 27–28°C and examined for the development of spirilla after 1, 2, 4, and 7 days. Isolation is accomplished by streaking dilutions on suitable agar media.

Maintenance of Marine Spirilla

The cultivation of isolates has been accomplished in media containing natural or synthetic seawater. Williams and Rittenberg (1957) found that growth in synthetic seawater media, when compared with media prepared with natural seawater, 1) required larger inocula, 2) initiated growth more slowly; and 3) after repeated transfers finally resulted in failure to grow.

Terasaki (1972) maintained cultures on seawater nutrient agar stabs at room temperature with monthly transfers.

Hylemon et al. (1973) maintained strains of *Oceanospirillum* in semisolid peptone-succinate-salt medium (PSS), containing per liter of synthetic seawater:

Peptone	10 g
Succinic acid	1 g
$(NH_4)_2SO_4$	1 g
$MgSO_4 \cdot 7H_2O$	1 g
$FeCl_3 \cdot 6H_2O$	0.002 g
$MnSO_4 \cdot H_2O$	0.002 g
Agar	1.5 g

Adjust to pH 6.8. The synthetic seawater contained per liter:

NaCl	27.5 g
$MgCl_2$	5.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
$CaCl_2$	0.5 g
KCl	1.0 g
$FeSO_4$	0.01 g

Using this synthetic seawater, Hylemon et al. (1973) could not detect any loss of viability. In our laboratory we have used the same medium with only 0.5% peptone (marine MPSS).

Long-term Preservation

Long-term preservation can be accomplished by suspending a dense concentration of cells in seawater nutrient broth containing 10% (v/v) dimethyl sulfoxide, with subsequent freezing in liquid nitrogen (Krieg, 1984).

Freeze-drying can be performed with cells grown on the appropriate medium, as described for the aquaspirilla. (see Chapter 131).

Identification and Taxonomy

The taxonomy of the genus has been investigated on several occasions (McElroy and Krieg, 1972; Terasaki, 1972, 1973; Hylemon et al., 1973; Carney et al., 1975; Krieg and Hylemon, 1976) and all investigators found the genus to be quite heterogeneous and difficult to classify in relation to other Gram-negative bacteria. Krieg (1976) has pointed out that, at least on the basis of phenotypic characters, a continuum of species may interconnect spirilla with various other genera of Gram-negative, oxidative bacteria. The genera that were recognized were useful, but may prove to be inadequate for expressing taxonomic relationships. Krieg has suggested that DNA-DNA or DNA-rRNA homology studies might be useful in clarifying these relationships.

Due to the scarcity of strains, the degree of variation within the species and genus has not been defined. Hylemon et al. (1973) mentioned

Table 3. List of phenotypic features of all the species of the genus *Oceanospirillum*.

Feature	*O. linum*	*O. maris* ssp. *maris*	*O. maris* ssp. *hiroshimense*	*O. maris* ssp. *williamsae*	*O. beijerinckii* ssp. *beijerinckii*	*O. beijerinckii* ssp. *pelagicum*	*O. multiglobuliferum*	*O. japonicum*
Cell diameter (μm)	0.4–0.6	0.7–1.0	0.6–1.1	0.7–1.0	0.7–1.0	0.6–1.2	0.5–0.9	0.8–1.4
Type of helix	C	C	C	C	C	C	C	C
Wavelength of helix (μm)	1.8–4.0	3.5–7.0	3.0–4.0	4.4–6.0	6.3–7.2	3.0–6.0	3.5–5.0	7.0–20.0
Helix diameter (μm)	0.8–1.4	1.4–2.8	1.2–2.5	1.5–2.0	1.5–3.0	1.0–2.0	1.0–2.0	2.0–5.0
Length of helix (μm)	4.0–30.0	7.0–21.0	2.5–40.0	4.4–20.5	7.0–15.5	2.0–14.0	2.0–10.0	5.0–75.0
Polar membrane present		+			+			+
Flagellar arrangement	BT	BT	BT	BT	BT	BT	BT	BT
Intracellular PHB formed	+	+	+	+	+	+	+	+
Coccoid bodies predominant after 3–4 weeks	+	+	+	+	+	+	+	−
Coccoid bodies predominant after 24–48 h	−	−	−	−	−	−	+	−
Maximum salt tolerance is low (4% NaCl)	−	−	−	−	−	−	+	−
Optimum temperature 25°C rather than 30–32°C	−	−	+	−	−	−	−	−
Oxidase	+	+	+	+	+	+	+	+
Catalase	+ or W	+	W or −	W	+ or W	+	+	W or −
Phosphatase	+	−	+	−	+	+	+	W
Nitrate reduced to nitrite	−	−	−	−	−	−	−	−
Auxotrophic growth requirement	+[a]	−	−	+[b]	−	−	−	−
Anaerobic growth with nitrate	−	−	−	−	−	−	−	−
Denitrification	−	−	−	−	−	−	−	−
Acid from sugars	−	−	−	−	−	−	−	−
Hydrolysis of esculine, hippurate	−	−	−	−	−	−	−	
Indole reaction	−	−	−	−	−	−	−	−
Temperature range for growth	11–39		2–35		14–37	8–41	6–37	10–43
Range of NaCl (%) for growth in peptone water	0.5–8.0		0.5–8.0		0.5–6.0	0.5–8.0	0.5–4.0	0.5–8.0
Gelatine liquefaction, 20°C								
after 7 days	−		−		+	d	−	d
after 28 days	+		−		+	d	−	d
after 42 days	+		−		+	d	−	d
Gelatine hydrolysis, 30°C, after 4 days	−	−			−			−
Growth in the presence of 1% oxgall	+	+		+	+			+
Growth in the presence of 1% glycine	+	+		−	−			−
Deoxyribonuclease	−	−		+	+			−
Ribonuclease	d	−		+	+			−
GC content (mol%)	48–50	46	47	45	47	49	46	45

Symbols: +, present in all strains; −, lacking in all strains; blank space, not determined; C, clockwise or right-handed helix (indicated by the pattern //// when focusing on the bottom of the cells); BT, bipolar tufts; W, weak reaction; d, differs among strains; PHB, poly-β-hydroxybutyrate.

[a] *O. linum* grows poorly or not at all in defined media with single carbon sources and ammonium ions as the nitrogen source: however, abundant growth occurs in a defined medium containing succinate plus malate as carbon sources and methionine as the nitrogen source.

[b] *O. maris* subsp. *williamsae* fails to grow in vitamin-free media and requires a growth factor that has not yet been identified.

From Linn and Krieg, 1973; Hylemon et al., 1973; Krieg, 1984; Pot et al., 1989; and Terasaki, 1972, 1979.

that the descriptions they provided for members of the genera *Aquaspirillum* and *Oceanospirillum* were in many cases based upon only a single strain. A number of new species have since been described, mostly based on a limited number of strains. Although Krieg (1984) recognized nine species in the genus *Oceanospirillum,* he pointed out again that no DNA-DNA hybridization studies were done to substantiate this subdivision.

Woese et al. (1982, 1985) measured the deeper relationships of the genus using the oligonucleotide cataloging method and provided information on the intrageneric relationships of five *Oceanospirillum* strains (*O. beijerinckii* ATCC 12754, *O. japonicum* ATCC 19191, *O. linum* ATCC 11336, *O. maris* ATCC 27649 and *[O.] minutulum* ATCC 19193; brackets enclose a generically or specifically misnamed taxon name). These studies indicated that these strains should be classified in the gamma group of the Proteobacteria (Stackebrandt et al., 1988).

Pot et al. (1989) included all named *Oceanospirillum* species in a polyphasic study in which DNA-rRNA hybridizations played an important role. They found that *O. beijerinckii, O. hiroshimense, O. japonicum, O. linum, O. pelagicum, O. maris,* and *O. multiglobuliferum* constitute one separate rRNA branch in the rRNA superfamily II (gamma group of the *Proteobacteria*) and concluded that *Oceanospirillum* consequently should be restricted to members of this rRNA branch. Therefore, the six other species are generically misnamed and ought to be removed from the genus *Oceanospirillum. [O.] vagum* and *[O.] commune* should be relegated to their previous generic positions as *Marinomonas vaga* and *M. communis,* respectively (Van Landschoot and De Ley, 1983); *[O.] jannaschii, [O.] kriegii,* and *[O.] minutulum* constitute three separate rRNA branches in rRNA superfamily II that are not related more closely to *Oceanospirillum* than to any other rRNA branch of this rRNA superfamily (see Chapter 100); *[O.] pusillum* belongs to rRNA superfamily IV, where it constitutes a separate rRNA branch, closest to and equidistantly removed from *Azospirillum, Rhodospirillum rubrum,* and some misnamed *Aquaspirillum* species.

Using DNA-DNA hybridizations and comparative protein gel electrophoresis, some species were found to be too closely related to be kept as separate species (Pot et al., 1989), but the differences in morphological and nutritional characteristics warranted the description of a number of subspecies (Table 3).

With a DNA binding of at least 84%, *Oceanospirillum [hiroshimense]* and *O. maris* should be included in one species, *O. maris.* From Table 3 it can be concluded that, due to the presence of phosphatase activity, a weak or negative catalase reaction and an optimum temperature for growth of 25°C rather than 30° to 32°C, it is appropriate to create a new subspecies, *O. maris* subsp. *hiroshimense,* in addition to the two existing subspecies *(O. maris* subsp. *maris* and *O. maris* subsp. *williamsae).* For other differentiating characteristics, see Pot et al. (1989) and Krieg (1984).

With a DNA binding of 77%, *O. [pelagicum]* should be included in *O. beijerinckii.* Table 3 shows that the difference in temperature range for growth and the range of NaCl for growth in peptone water justifies the description of the subspecies *O. beijerinckii* subsp. *pelagicum.* For other differentiating characteristics, see Pot et al. (1989) and Krieg (1984).

The inclusion of *O. japonicum* in the genus *Oceanospirillum* can be considered to be provisional because of its large $T_{m(e)}$ difference (8.2°C) with the homologous $T_{m(e)}$ value of the type strain for the genus *Oceanospirillum.* It has been described before that a $T_{m(e)}$ difference of 6°C or more between homologous and heterologous DNA-rRNA hybrids, usually represents differences on the generic level (see also Chapter 100).Phenotypically, *O. japonicum* is quite different from the other species of the genus (Table 3), but as long as there are no other strains of this species available, it is not appropriate to create a new genus for this single species.

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CHAPTER 173

Serpens flexibilis: An Unusually Flexible Bacterium

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Habitats

Many eutrophic aquatic environments as typified by ponds or sewage lagoons harbor a wide variety of aerobic bacteria in the upper water layers. Thin, flexible bacteria such as spirilla, spirochetes, and *Serpens flexibilis* constitute a small fraction of the total microorganisms in these environments. The use of enrichment procedures to isolate these flexible bacteria usually is not successful because they are rapidly overgrown by the other bacteria, even when low-nutrient-containing media are used. Presumably, these flexible bacteria can compete in their natural environments because of factors such as high cell-surface to cell-volume ratios that allow for maximizing transport of nutrients, possession of chemotaxis mechanisms, and unusual motility properties that allow for movement through viscous solutions (Greenberg and Canale-Parola, 1977).

Isolation

Selective isolation

S. flexibilis can be selectively isolated by using procedures originally developed for isolation of thin spirilla and spirochetes (Canale-Parola et al., 1966). A small amount of pond water or mud slurry is placed in the center of a sterile cellulose filter disc (0.3 to 0.45 μm pore diameter) which has been placed on the surface of a petri dish containing isolation medium made with 1.0% agar. The disc is removed after aerobic incubation of the plate at 30°C for 6 to 12 h. After subsequent incubation of the plate for 2 to 4 days, a subsurface, whitish veil of growth develops. The organism can then be isolated by picking from the outer edge of the veil and streaking onto a second plate. Restreaking several times may be necessary to obtain pure, cloned cultures. An isolation medium that has been routinely used (Hespell, 1977, 1984) includes: yeast extract, 0.2 g; peptone, 0.1 g; 10 ml hay extract; and 90 ml distilled water. The pH of the medium is adjusted to pH 7.0 with KOH prior to autoclaving. The hay extract is prepared by boiling 1.0 g of dried wheat or barley straw in 100 ml of distilled water for 15 min., cooling the mixture to room temperature, and decanting the fluid which is then clarified by centrifugation (8,000 × g, 10 min).

Cultivation

Optimal growth yields (10^9 to 10^{10} cells/ml) and growth rates (20 to 30 min doubling times) of *S. flexibilis* can be obtained in a nutrient-rich medium such as LYPP medium (Hespell, 1977): 60% sodium lactate syrup, 1.0 ml; yeast extract, 0.3 g; peptone, 0.2 g; 0.2 M potassium phosphate buffer (pH 7.4), 10 ml; and 90 ml distilled water. Typical cultures consist of 250 ml of medium in 1-liter Erlenmeyer flasks shaken at 150 to 250 rpm at 30°C. The organism also can be grown on chemically defined media such as LCH, but the growth yields and growth rates are considerably lower. LCH medium consists of: 60% sodium lactate syrup, 1.0 ml; 0.4 g ammonium chloride; trace minerals (Hespell and Canale-Parola, 1970), 1.0 ml; 0.2 M potassium phosphate buffer (pH 7.4), 20 ml; and 78 ml of distilled water. Replacement of the ammonium chloride with an equivalent amount of peptone or Trypticase markedly stimulates growth. *S. flexibilis* will not grow on media having an initial pH of 5.8 or less, and optimal growth occurs when the initial pH is 6.8 to 7.2. During growth the pH of the medium rises to 9 to 11, and increased cell yields can be obtained by periodically lowering the pH by additions of weak HCl. Highest growth yields are obtained at 28° to 33°C, but no growth occurs at 10° or at 45°C.

Preservation of Cultures

Long-term storage of *S. flexibilis* can be accomplished by routine lyophilization of cultures. Alternatively, cultures can be stored in liquid ni-

trogen or ultracold (−75 to −85°C) freezers (Hespell and Canale-Parola, 1970).

Identification

S. flexibilis forms a subsurface veil of growth similar to that commonly observed with spirochetes. Microscopic examination of agar pieces removed from the leading edge of the veil show extremely flexible rods measuring about 0.3 by 10 μm long. Often several cells are seen together and appear as "a pit of microbial snakes." The cells display furious lashing motions and often tend to twist in and out of knot formations (Fig. 1a). In liquid media, the movement is less dramatic. The long, slender cells usually move in straight lines displaying an overall flexing of the cell with the trailing cell tip having a vibrating motion (Fig. 1b). No tumbling type of movement is observed. In stationary phase cultures, clumps of entwined cells are common, and often partially lysed cells, spherical cell bodies, are present (Fig. 1c). Electron microscopy shows that cells typically possess a cluster of 4 to 10 flagella that are present at the tips of both cell ends; in addition, the presence of several lateral flagella randomly distributed along the cell body is not uncommon. (Fig. 1d). The flagella are quite long (15 to 30 μm), have a constant waveform with a 0.3 μm amplitude, and possess the hook end and disk structure commonly observed with other bacteria.

In addition to its unusual motility characteristics, another key feature of *S. flexibilis* is the rather limited range of substrates that support its growth. Essentially, only lactate is catabolized and supports good growth. Limited growth can be obtained with α-ketoglutarate, acetate, or pyruvate. In the presence of peptone, the addition of glucose and a few other disaccharides results in about a doubling of cell yields over that observed in the absence of added substrates (2.0 to 6.0 × 10^7 cells/ml).

Colonies can be formed under aerobic conditions on media containing 2% agar. These are usually 3 to 4 mm in diameter, off-white to cream colored, and round with filamentous edges. The cells from colonies tend to be thicker (0.5 to 1.0 μm) and shorter (4 to 6 μm). Cell pellets from liquid cultures often are light pink to reddish in color, probably due to the presence of the cytochrome proteins; the pigments cannot be extracted with chloroform, methanol, or other solvents used to remove carotenoids. Whether taken from liquid cultures or colonies, cells are always Gram negative. Electron microscopy of thin sections of *S. flexibilis* shows that the cells have a classical double-track, Gram-negative cell structure (Hespell, 1977, 1984).

The phylogenetic status of *S. flexibilis* has been examined with the 16S rRNA oligonucleotide-cataloging technique. The results indicated that *S. flexibilis* has an S_{AB} of 0.9 with *Pseudomonas pseudoalcaligenes* (Woese et al., 1982). Woese et al. (1982) concluded that *S. flexibilis* was a close relative to *P. pseudoalcaligenes* and that it might be a variant pseudomonad that developed a defect in septum formation because *S. flexibilis* forms long cells with multiple cytoplasmic invaginations (Hespell, 1977, 1984). This 16S RNA phylogenetic placement of *S. flexibilis* has been confirmed by analysis of aromatic amino acid biosynthesis enzymes of *S. flexibilis* (Ahmad and Jensen, 1987). These studies indicate this organism belongs with subgroup Ia pseudomonads. While *S. flexibilis* shares a number of common features with *P. pseudoalcaligenes*, there are a number of distinct differences. Glucose and many other sugars do not support the growth of either organism, and both organisms can grow with lactate, acetate, or pyruvate as energy sources. However, *S. flexibilis* does not grow with succinate, citrate, ethanol, fructose, or glycerol. The GC content of the DNA of *S. flexibilis* is 66 mol% (Hespell, 1977) and that of *P. pseudoalcaligenes* strains is 62 to 64 mol% (Palleroni, 1984). *S. flexibilis* differs from many *Pseudomonas* species in having multiple flagella and in lacking poly-B-hydroxybutyrate formation. At present, it would seem reasonable to consider *S. flexibilis* to be related to the pseudomonads. Because of its distinct features, this organism should probably be classified in its own genus (*Serpens*) and species (*flexibilis*) within the family Pseudomonadaceae. Although *S. flexibilis* strains have been isolated from several geographical locations (Hespell, 1977), detailed studies have not been done to determine whether enough differences exist to justify multiple species of *Serpens*.

Physiological Properties

S. flexibilis is a strictly aerobic bacterium and cannot grow anaerobically even in the presence of electron acceptors such as nitrate or nitrite. The organism displays an endogenous respiratory quotient (CO_2 released O_2 consumed) of 0.7 to 0.8, and this value increases to 1.56 in the presence of sodium lactate. Approximately 60% of the lactate catabolized is converted to carbon dioxide and about 30% is incorporated into cell material (Hespell, 1977). Growth on lactate-containing media results in production

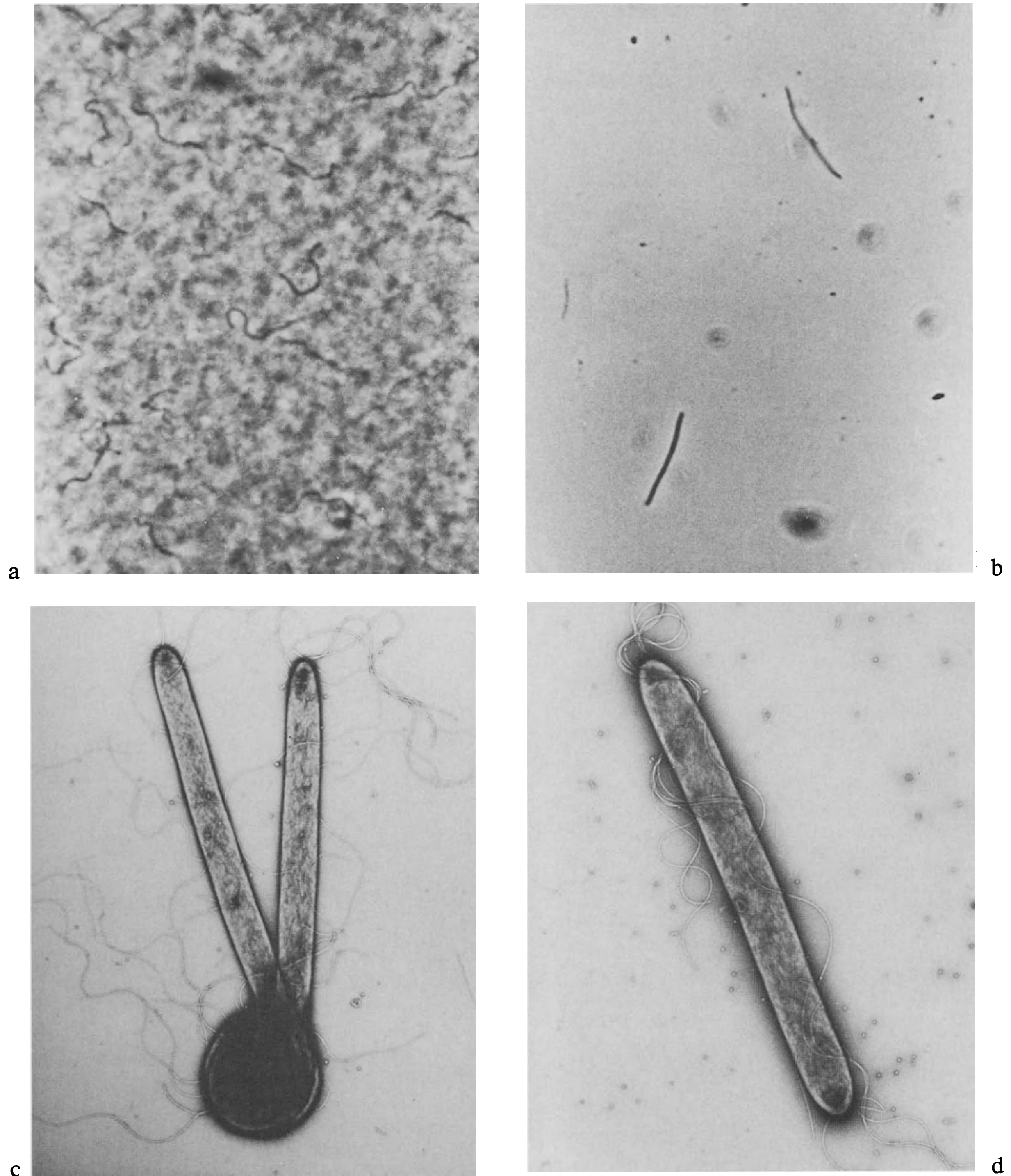

Fig. 1. Phase contrast micrographs (Fig. 1a and b) and transmission electron micrographs (Fig. 1c and d) of *S. flexibilis* strain PFR-1. Cells from subsurface growth in agar media show rapid coiling movements (1a), whereas in liquid media (1b), cells display straight-line movement of flexing rods. Cells are quite flexible and are able to coil up (1c). They are motile by means of lateral flagella (1c) and polar tufts of flagella (1d). Cells are about 0.3 μm in diameter and 10 μm in length.

of trace amounts of formate and acetate. Cell extracts contain high levels of lactic acid dehydrogenase and enzyme activities associated with the tricarboxylic acid cycle. However, only trace levels of enzymes of the Embden-Meyerhof and hexose monophosphate pathways are

present. Analysis of the peptidoglycan layer indicates that diaminopimelic acid is the main cross-linking compound, and the other constituents include alanine, glutamate, muramic acid, and glucosamine (Hespell, 1977). The peptidoglycan appears to be typical of that found in most Gram-negative bacteria.

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CHAPTER 174

The Genus *Psychrobacter*

ELLIOT JUNI

In early studies, all saprophytic, nonpigmented Gram-negative rods were classified together in the genus *Achromobacter* (Bergey et al., 1923; Ingram and Shewan, 1960). Although the original *Achromobacter liquefaciens* type strain (no longer available) was motile, the genus was said to include both motile and nonmotile organisms. Brisou and Prévot (1954) suggested that the nonmotile achromobacters be separated from the motile species and grouped in the genus *Acinetobacter*. When the oxidase test, an assay for the presence of cytochrome *c* (Baumann et al., 1968a), first became widely used (Buttiaux and Gagnon, 1959), it was recognized that the genus *Acinetobacter* included both oxidase-positive and oxidase-negative members. Further studies resulted in classification of only the oxidase-negative organisms as strains of *Acinetobacter* (Lessel, 1971) because of similarities in their phenotypic properties (Baumann et al., 1968b), genetic interaction of all strains with a competent strain (Juni, 1972), and also from the results of DNA-DNA homology studies (Johnson et al., 1970).

Until recently, the large group of nonmotile, oxidase-positive, nonpigmented, chiefly psychrotrophic, Gram-negative rods or coccobacilli isolated from the skin of fish and chickens and also from a variety of processed foods were recognized and frequently referred to as "*Moraxella*-like" organisms (Shaw and Shewan, 1968), but remained unclassified. The morphological and physiological characteristics of these organisms were not sufficiently unique to permit the definition of a new genus based solely on the available phenotypic properties. Studies of a number of these organisms revealed that many of them were naturally competent for genetic transformation (Juni and Heym, 1980). This finding made it possible to devise a transformation assay in which only DNA from related strains could transform an auxotroph of a competent strain for ability to grow on a defined medium on which the auxotroph could not grow (Juni and Heym, 1980). It soon became clear that the large number of genetically interacting strains examined constituted a distinct genospecies and these were then grouped together as members of the genus *Psychrobacter* (Juni and Heym, 1986). The name *Psychrobacter* was chosen because most members of this group are psychrotrophic, i.e., they grow best at low temperatures.

Relationship to *Moraxella* and *Acinetobacter*

Psychrobacters are typically cocci or coccobacilli and resemble strains of *Moraxella* and *Acinetobacter* microscopically. These three genera have been shown to be fairly closely related. Since strains of each genus have been shown to be competent for genetic transformation, it has been possible to demonstrate weak, but distinct, genetic interactions between strains of these genera (Bøvre, 1967; Juni, 1972; Bøvre and Hagen, 1981). Because of qualitative similarity in fatty acid compositions, it was "suggested that the generic similarity among *Acinetobacter* species, *Moraxella* species and *Moraxella*-like species is fairly close" (Nishimura et al., 1979). In a study of similarities of ribosomal RNA cistrons of the family Neisseriaceae, it was shown that strains known to be psychrobacters cluster with the *Moraxella* group (Rossau et al., 1986). Because of a variety of findings regarding their relatively close relationship, it has been suggested that the genera *Acinetobacter, Moraxella,* and *Psychrobacter* be grouped together in a new, and as yet unnamed, family (Rossau et al., 1986). Although these genera are presently classified as members of the Neisseriaceae, it is clear that there is no longer any reason for such association. The original proposal that the moraxellae be included in the family Neisseriaceae was based primarily on similarities in phenotypic properties (Henriksen, 1952) and also because of the relatively strong genetic interactions between *Neisseria catarrhalis* and some moraxellae (Henriksen and Bøvre, 1968). The

organism orginally called *Neisseria catarrhalis* is currently designated *Moraxella (Branhamella) catarrhalis* (see Chapter 177) the subgenus *Branhamella* being used for the coccal species of the moraxellae (Bøvre, 1979).

Habitats

Psychrobacters have been found to occur normally on the skins and gills of fish (Scholes and Shewan, 1964) and on the skins of poultry (Barnes and Impey, 1968; Lahellec et al., 1975). Since many of these organisms apppear to be radiation resistant, in contrast to the more common food-spoilage bacteria, they have been found surviving in foods following irradiation for preservation purposes (Firstenberg-Eden et al., 1980; Ito et al., 1976; Laycock and Regier, 1970; Welch and Maxcy, 1975). Psychrobacters have been isolated from the open sea and have also been found as air contaminants on complex media plates (Juni and Heym, 1986). It has also been demonstrated that approximately 30% of the strains that were isolated from a variety of human sources and assigned to group EO-2 by the Center for Disease Control (Clark et al., 1984) were actually psychrobacters (Hudson et al., 1987; Moss et al., 1988).

Isolation

At the present time there is no selective medium for isolation of psychrobacters. Numerous psychrobacters have been isolated by immersing fish heads in saline solution, stirring for a short period of time, and streaking loopfuls of the wash solution on heart infusion agar (Difco) plates. After incubation at room temperature for 2 days, portions of nonpigmented colonies were placed on the surface of a dry oxidase test paper. Oxidase test paper consists of an 8-cm disk of Whatman no. 1 qualitative filter paper, which had previously been soaked thoroughly in a 1% solution of *N,N,N',N'*-tetramethyl-*P*-phenylendiamine dihydrochloride, placed in a 9-cm petri dish and dried at 35°C. The appearance of a deep purple color in a few seconds signifies a positive oxidase reaction. Wet mount suspensions of oxidase-positive colonies were examined for the presence of nonmotile rods or coccobacilli, and those meeting this criterion were then Gram-stained. All Gram-negative, oxidase-positive, nonpigmented rods or coccobacilli were then subject to the transformation assay for psychrobacters (Juni and Heym, 1980). More than half of these, presumptive psychrobacters, gave positive results in the transformation assay (E. Juni, unpublished observations).

Media

Psychrobacters usually grow well, from 5 to 30°C, on standard complex media such as heart infusion agar (Difco), tryptone-yeast extract agar (LB agar), and trypticase soy agar (BBL), exhibiting lower growth rates at the extremes of the temperature range. These organisms may be found growing slowly as contaminants on plates stored in the refrigerator. For many of them, 20–25°C is an optimal temperature. A complex medium containing vitamin-free casein hydrolysate (medium M9A) has been found useful for performing the *Psychrobacter* transformation assay (Juni and Heym, 1980) but the simpler defined medium (medium P96), described below, is equally effective.

Most psychrobacters can grow on a mineral-agar plate containing either (or both) sodium lactate and (or) monosodium glutamate as the carbon source(s) and ammonium salts as the nitrogen source. Strain ATCC 43116, the *Psychrobacter* type strain, has been demonstrated to have a generation time of approximately 100 min on such media when grown at 25°C (E. Juni, unpublished observations). Although not absolutely required, the addition of certain amino acids and Tween 80 to the lactate plus glutamate mineral medium serves to shorten the lag period for growth of liquid cultures in this medium. Double-strength medium P96 contains the following components dissolved in distilled water to a final volume of 1 liter:

Medium P96	
Na_2HPO_4	11.2 g
KH_2PO_4	4.0 g
NH_4Cl	2.0 g
$MgSO_4 \cdot 7H_2O$	0.4 g
$CaCl_2$ (1.0% solution)	1.0 ml
$FeSO_4 \cdot 7H_2O$ (freshly prepared 0.1%	0.5 ml
solution)	0.5 ml
Sodium DL-lactate (60% solution)	13.0 ml
Monosodium L-glutamate	10.0 g
Tween 80	8.0 ml
L-Methionine	0.4 g
L-Isoleucine	0.4 g
L-Leucine	0.4 g
L-Valine	0.4 g

This medium, which has a pH of 7.2, is sterilized by membrane filtration and may be stored in the refrigerator for longer than one year. Semisolid medium P96 plates are prepared by pouring 200 ml of double-strength medium P96 (warmed to room temperature, or higher) into 200 ml of freshly melted 3.0% agar (previously

sterilized by autoclaving), mixing thoroughly, followed by addition of 2.0 ml of sterile 2.0% ferric ammonium citrate and a final thorough mixing. After cooling to a suitable temperature (estimated by touching the bottle), approximately 20 plates are poured. Failure to include ferric ammonium citrate results in inability of single cells to give rise to colonies but confluent growth does take place in such media.

Carbon and Nitrogen Sources for *Psychrobacter*

Strain ATCC 43116 can grow in medium P96 lacking sodium lactate, monosodium glutamate, Tween 80, and all amino acids when supplied with any of the following carbon sources: sodium DL-lactate, sodium pyruvate, L-glutamate, L-serine, L-proline, L-alanine, sodium succinate, sodium n-butyrate, sodium caproate, sodium oleate, and Tween 80. Of these carbon sources, the combination of sodium lactate and monosodium glutamate was found to result in the best rate of growth (E. Juni, unpublished observations). In studies of possible carbon sources for 22 isolates of psychrobacters (Shaw and Latty, 1988), it was reported that 25% or more of these strains can utilize propionate, n-butyrate, isobutyrate, n-valerate, caproate, caprylate, pelargonate, caprate, fumarate, glutarate, suberate, azelate, D-malate, L-malate, DL-β-hydroxybutyrate, benzoate, phenylacetate, L-alanine, L-aspartate, L-glutamate, γ-aminobutyrate, δ-aminovalerate, histidine, proline, tyrosine, phenylalanine, α-ketoglutarate, ethanol, n-propanol, n-butanol, ethanolamine, and putrescine.

Ammonium salts can serve as the nitrogen source in a defined medium. Glutamate can also fuction as a nitrogen source but is not as effective as ammonium chloride (E. Juni, unpublished observations).

Identification

Phenotypic Properties

Pure culture isolates are tentatively identified as psychrobacters if they are fairly plump (0.9 to 1.3 μm in diameter), nonmotile, Gram-negative, aerobic, oxidase-positive, catalase-positive rods or coccobacilli, and form nonpigmented, smooth, opaque colonies on complex semisolid media. Fig. 1 illustrates the typical microscopic appearances of the two most common morphological types encountered. Diplo forms are common. Rod-shaped cells are frequently swollen, and the Gram stain often tends to be retained. Most psychrobacters grow well at room temperature and fail to grow at 35°C. They grow slowly at refrigerator temperatures (5–10°C). Growth takes place in media containing 6% NaCl, with some strains able to grow in the presence of twice this salt concentration. Most strains are sensitive to penicillin.

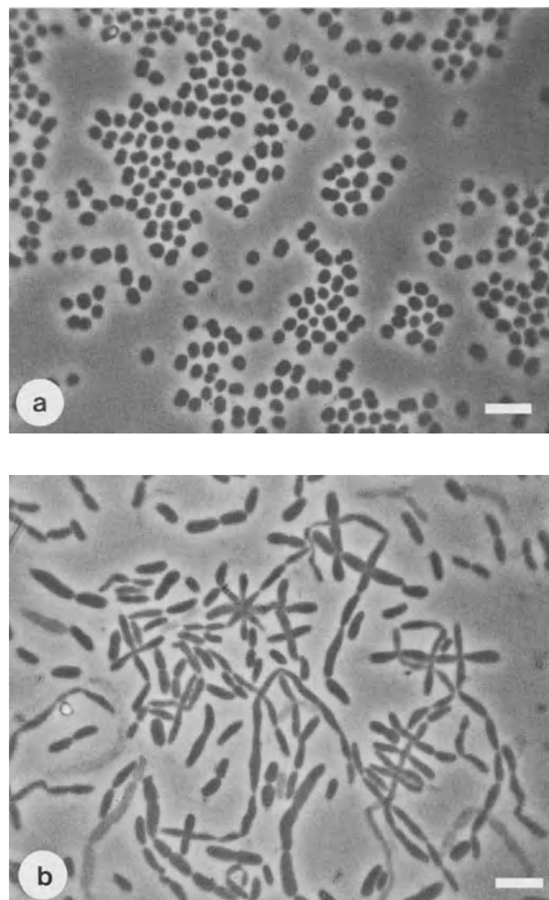

Fig. 1. Typical microscopic morphology of *Psychrobacter* grown overnight at room temperature on heart infusion agar. (a) strain ATCC 15174; (b) strain A103. Bar = 5 μm.

Unlike the oxidase-positive moraxellae, whose species fail to oxidize sugars, most psychrobacters form acid aerobically from sugars such as glucose, mannose, galactose, arabinose, xylose, and rhamnose, and thus resemble the strains of *Acinetobacter* that are capable of forming acid from sugars aerobically. Psychrobacters fail to form acid from fructose, maltose, or sucrose. Nitrate is reduced. Starch, gelatin, and serum are not hydrolyzed, and indole and H_2S are not produced. Urease activity is present. Phenylalanine and tryptophan are deaminated. Tween 80 is hydrolyzed. Phenethyl alcohol is formed from L-phenylalanine and ethanol (Chen and Levin, 1974). In this connection, it was noted that during growth (pre-

sumably on heart infusion agar plates) of certain human-derived psychrobacters, previously designated as EO-2 strains, the cultures "had an odor which resembled the odor of a phenethyl alcohol blood agar plate" (Hudson et al., 1987).

Identification by Transformation

A transformation assay has been devised for definitive identification of suspected strains of *Psychrobacter* (Juni and Heym, 1980). In this procedure crude DNA, prepared by lysing small quantities of cells in a saline-citrate solution containing a low concentration of sodium dodecyl sulfate, is mixed with a hypoxanthine and thiamine-requiring mutant *Psychrobacter* strain (ATCC 43117) and the mixture permitted to grow overnight. If the DNA was derived from an authentic strain of *Psychrobacter,* some of the mutant cells will be transformed to prototrophy and will be capable of giving rise to colonies when the overnight mixture is streaked on a medium upon which the mutant strain itself is unable to grow. The appearance of transformant colonies on a medium M9A plate (Juni and Heym, 1980), or on a medium P96 plate, described above, confirms unequivocally that the isolate is a *Psychrobacter.*

Chemical Characterization

Several investigators have analyzed the fatty acid composition of *Psychrobacter* strains (Thorne et al., 1973; Nishimura et al., 1979; Moss et al., 1988). Oleic acid ($C_{18:1}$) constitutes more than 50% of the total fatty acids with moderate amounts of $C_{16:0}$, $C_{16:1}$, $C_{17:1}$, and $C_{18:2}$ being detected. Based upon the proportions of the latter fatty acids in a group of six psychrobacters, it was concluded that these could be separated into two groups each containing three strains (Nishimura et al., 1979).

Psychrobacters have been demonstrated to contain waxes (Gallagher, 1971; Bryn et al., 1977; Russell and Volkman, 1980), which may be constituent parts of their cell membranes, since waxes have been found to be associated with these structures. Analysis of isoprenoid quinones of *Psychrobacter* has shown that the major ubiquinone contains eight isoprene units (Q_8) (Moss et al., 1988). The GC content of *Psychrobacter* DNA is 44–46 mol%.

Genetics

Since many psychrobacters are naturally competent for genetic transformation, the possibility for genetic studies of single strains, or for genetic interaction between different strains, is clearly evident. To date, the most extensive genetic study making use of transformation has concerned the establishment of *Psychrobacter* as a unique genospecies (Juni and Heym, 1986). In a preliminary report, transformation of a *Psychrobacter* strain (ATCC 15174) to grow at 30°C when incubated with DNA preparations from mutant strains able to grow at 30°C was described (Tai and Jackson, 1969a). Using DNA from radiation-resistant psychrobacters to transform two auxotrophs of a radiation-sensitive competent strain to prototrophy, it was demonstrated that prototrophic transformants remained radiations sensitive (Ito and Iizuka, 1983). Since the genetic loci determining radiation resistance are most probably unlinked to the nutritional loci that were transformed, the results obtained by Ito and Iizuka are to be expected.

Physiology

All psychrobacters are aerobic and their oxidase-positive characteristic indicates that they produce cytochrome *c* (Baumann et al., 1968a). Oxidase-negative *Psychrobacter* mutants have been reported (Tai and Jackson, 1969b; Juni and Heym, 1980). Although most psychrobacters have temperature optima from 20 to 25°C, mutants able to grow at higher temperatures have been reported (Tai and Jackson, 1969b), and some strains, such as ATCC 17955 (misnamed *Moraxella phenylpyruvica* in the ATCC catalog), are able to grow at 35°C. The physiological basis for these differences in optimal growth temperature is not known.

One strain of *Psychrobacter* (ATCC 15174), isolated from pork sausage, and originally designated *Micrococcus cryophilus* (McLean et al., 1951), has been studied in some detail because it was considered that the relatively large size of this bacterium made it particularly suitable for cytological studies. Enzymatic assays of cell-free extracts of strain ATCC 15174 have revealed the presence of all the enzymes of the tricarboxylic acid cycle (Tai and Jackson, 1969b). Studies have been reported of wax ester synthesis (Lloyd and Russell, 1983) and phospholipid synthesis (McGibbon and Russell, 1985) by strain ATCC 15174.

Cell membranes of *Psychrobacter* appear to resemble those of other Gram-negative bacteria (Thorne et al., 1973). The cell surface layer of one particular strain (199A) has been studied in considerable detail. An array made up of a single protein has been shown to be attached to and to cover the outer membrane (Thorne, 1977). When excreted into the medium, this

protein has phospholipase activity but such activity is lacking when the protein is arranged in the layered array (Thorne et al., 1976). The protein outer layer appears to be linked to the outer membrane by a salt bridge involving calcium or magnesium ions. It has been suggested that this additional surface layer, which is similar to surface layers in other Gram-negative bacteria, may serve to protect cells from hostile environments (Thorne, 1977).

Now that it is possible to identify *Psychrobacter* strains with certainty, it may be expected that in the near future the combined efforts of different laboratories concerned with various aspects of *Psychrobacter* physiology will serve to increase our understanding of this group of bacteria.

Taxonomic Considerations

Thornley (1967) studied the taxonomy of *Acinetobacter,* using the definition of this genus originally proposed by Brisou and Prévot (1954). On the basis of morphological properties and biochemical tests, she divided a large collection of strains into five phenones, most of which were grouped at 82.5% similarity. Some strains in Thornley's phenones 2, 3, and 4 were subsequently shown to be psychrobacters (Juni and Heym, 1980). Most of the oxidase-positive strains had been grouped into phenones 3 and 4. The results of this study serve to emphasize the difficulties in classifying the Gram-negative coccobacilli only on the basis of their phenotypic properties.

Since all known strains of *Psychrobacter* interact genetically, they are clearly members of a genospecies (Ravin, 1963). Because of their isolation from a variety of natural sources, it might be expected that different strains may have evolved independently and may be somewhat genetically heterogeneous. It has been shown, for example, that the streptomycin-resistance marker in some strains can transform certain competent psychrobacters poorly, or occasionally fail to transform a particular competent strain, in contrast to the efficient transformation observed when the DNA used was derived from a streptomycin-resistant mutant of the competent strain being transformed (Juni and Heym, 1980). Certain auxotrophic mutants of competent strains also display a similar range of transformation efficiencies when tested with DNA samples from a large number of psychrobacters (Juni and Heym, 1980). In spite of such findings, the apparent conserved nature of certain genetic markers has made it possible to demonstrate the relatedness of all *Psychrobacter* strains, since DNA from each of them is capable of transforming a conserved marker in a particular competent strain. Even with conserved markers, however, the efficiency of their transformation has been found to depend upon the strain from which the transforming DNA was derived (Juni and Heym, 1980).

It remains to be demonstrated whether the psychrobacters can be grouped into clearly defined species. Strains of the related genus *Acinetobacter* have been divided into more than 13 species based upon the results of DNA-DNA hybridization studies (Bouvet and Grimont, 1986). Unlike *Psychrobacter* and *Acinetobacter,* the moraxellae, which normally live in fairly constant environments associated with animal tissues, have been shown to occur in clearly defined species with all members of a given species being very nearly genetically identical (Bøvre and Hagen, 1981). At the present time all psychrobacters are considered to be members of a single genospecies, *Psychrobacter immobilis.* Further studies are required to explore the possibilities of more detailed speciation of *Psychrobacter.*

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CHAPTER 175

The Genus *Leucothrix*

THOMAS D. BROCK

Leucothrix, a large-diameter, morphologically distinct, marine gliding bacterium, has been known in natural material since the time of Oersted in 1844, but its modern history began with the remarkable study Harold and Stanier published in 1955. In the introduction to their paper, Harold and Stanier state: "*Leucothrix* may be characterized succinctly as a chemoheterotrophic counterpart of the colorless sulfur-oxidizing organism *Thiothrix.* It has been observed on a few occasions . . . in the century since its original description, but the existing accounts of its morphology and development, based entirely on the examination of crude cultures, are either incomplete or inaccurate. Thanks to the ease with which it can be grown in pure culture, we have been able to determine its complete cycle of development, which includes a unique and hitherto undescribed process of gonidial aggregation to form many-celled rosettes."

Leucothrix is fascinating not only because of its morphological distinctiveness, but also because of its large size and the ease with which it can be recognized in natural material. The organism seems to be entirely marine and is widespread as an epiphyte of marine algae (Fig. 1) (Brock, 1966). It also causes an extensive infestation of benthic crustacea and fish eggs (Johnson et al., 1971), and has become a problem in the field of aquaculture, especially in the artificial cultivation of lobsters. *Leucothrix* appears to be related to the cyanobacteria (Pringsheim, 1957; Raj, 1977), as determined by morphological similarities. However, no detailed studies of molecular relationships between *Leucothrix* and any cyanobacteria have been carried out, although the DNA base composition of *Leucothrix mucor* isolates, 47–49 mol% GC (Brock and Mandel, 1966), is similar to that of a number of filamentous cyanobacteria (Edelman et al., 1967), which cluster in the range 42–51 mol% GC. This similarity is made even more striking when it is compared with the wide variation in DNA base compositions of the unicellular cyanobacteria (Edelman et al., 1967; Stanier et al., 1971) and with the wide variation found in the narrow-diameter gliding bacteria (Edelman et al., 1967).

Characteristics of *Leucothrix*

A simplified life cycle of *Leucothrix mucor* is given in Fig. 2. *Leucothrix* filaments are usually 2–3 μm in diameter and may reach lengths of 0.1–0.5 cm. The filaments have clearly visible cross-walls, and cell division is not restricted to either end but occurs throughout the length of the filament, as shown by autoradiography with tritiated thymidine (Brock, 1967). The free filaments never glide (thus distinguishing them from many other filamentous gliding bacteria, such as *Beggiatoa* and *Vitreoscilla*), although they occasionally wave back and forth in a jerky fashion. Under environmental conditions unfavorable to rapid growth, such as low temperature or low nutrient concentration, individual cells of the filaments become round and form ovoid structures called "gonidia," which are released individually, often from the tips of the filaments (Fig. 3). The gonidia are able to glide in a jerky manner when they come into contact with a solid surface. They settle down on solid surfaces, synthesize a holdfast, and form new filaments through growth and successive cell divisions. In nature, the gonidia are presumably elements of dispersal and enable the organism to spread to other areas. "Gonidia," first used by Winogradsky (1888) for *Thiothrix,* is an unfortunate term because it implies some sort of unique structure. Actually, the gonidium of *Leucothrix* and *Thiothrix* is functionally and structurally equivalent to the hormogonium of the cyanobacteria, which is a motile structure of dispersal formed by the rounding up of a vegetative cell or group of cells in a filament.

Both Winogradsky (1888) for *Thiothrix* and Harold and Stanier (1955) for *Leucothrix* con-

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This paper is taken directly from the 1st edition of *The Prokaryotes.*

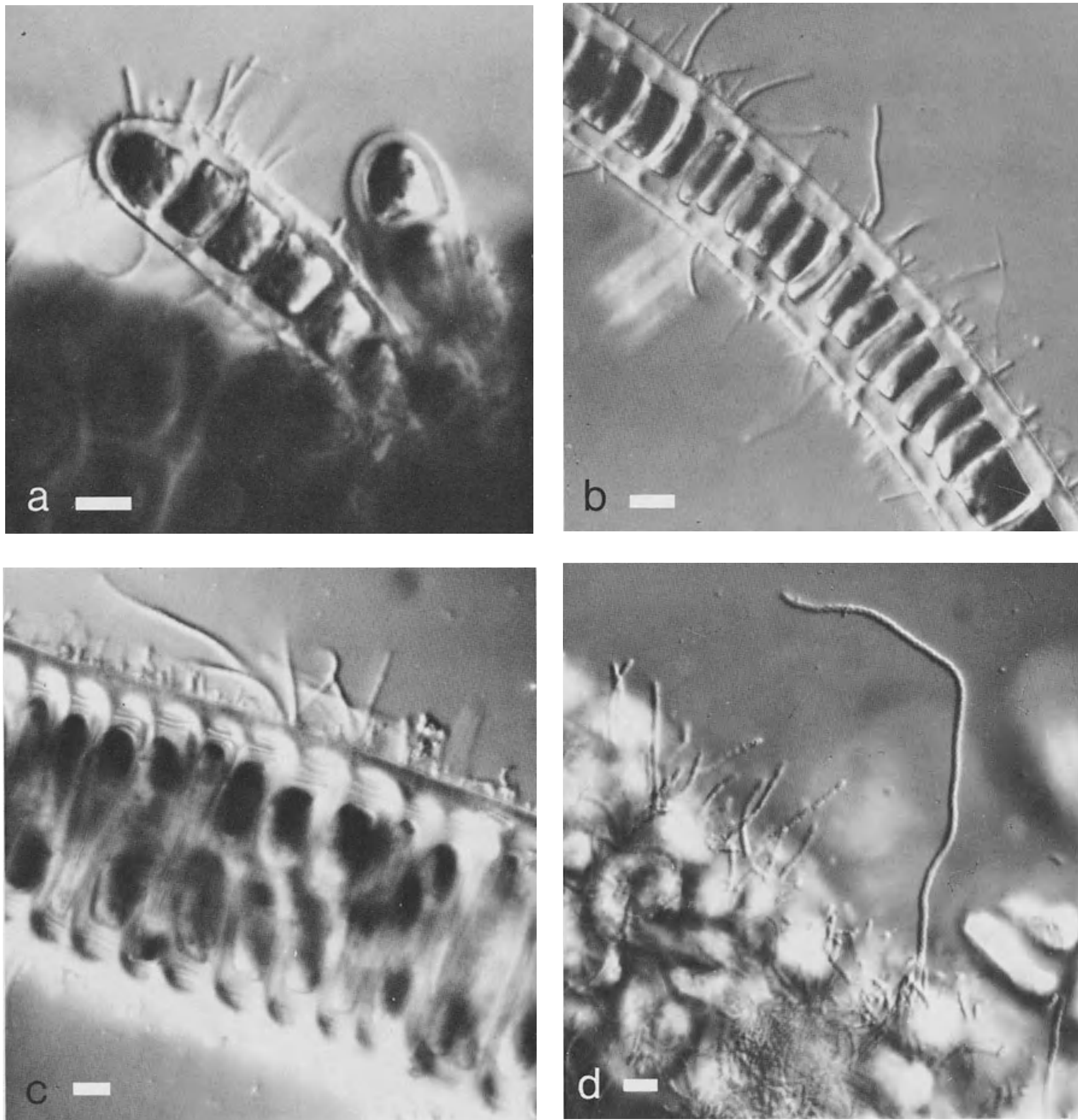

Fig. 1. Filaments of *Leucothrix mucor* attached to fronds of the seaweed *Bangia fuscopurpurea* of various ages. (a) Very young, (b) young, (c) mature, (d) old. Nomarski interference contrast photomicrograph. Bars = 10 μm. (From Bland and Brock, 1973.)

cluded that an apical-basal differentiation of filaments existed and that gonidia were formed primarily at the tips of filaments. Actually, such an apical-basal differentiation does not exist, and filaments do not taper. Careful examination of even long filaments has never revealed narrower cell diameters at the ends than in the middle. Also, although gonidia may form primarily at the filament tips, this is by no means always the case. I have frequently seen whole filaments convert to a series of gonidia, with every cell in the filament rounding up.

Gonidia do not have a holdfast when first formed, but make it only in response to the proper environmental conditions. If there are high concentrations of gonidia, individual cells may aggregate, probably because of reciprocal attraction (chemotaxis?). They then synthesize a holdfast and the gonidia become adherent to each other by the holdfast and form a small rosette. A new filament grows out from each gonidium, eventually resulting in a large and striking structure (Fig. 4). Rosette formation is found in both *Leucothrix* and *Thiothrix* and is

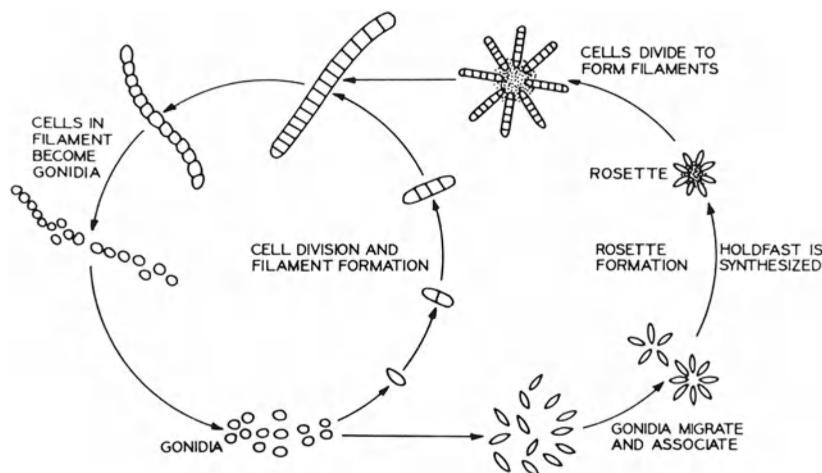

Fig. 2. Life cycle of *Leucothrix mucor.*

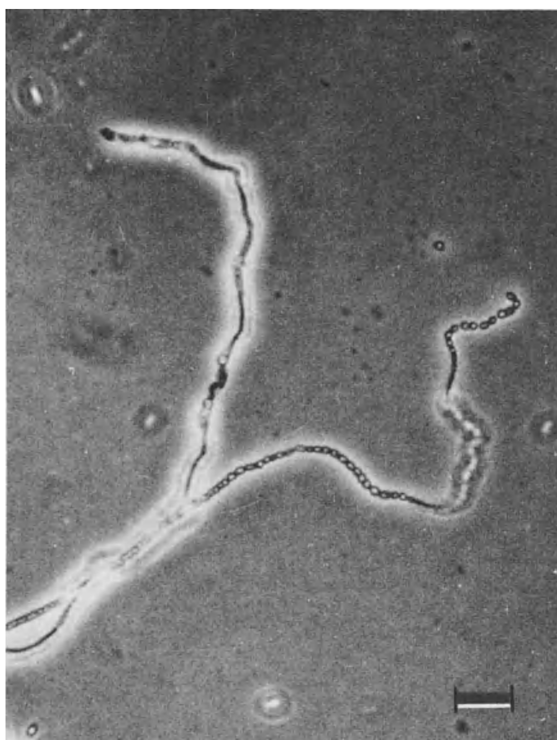

Fig. 3. Release of gonidia from filaments of *Leucothrix mucor.* Phase-contrast photomicrograph. Bar = 10 μm.

an important means of distinguishing these organisms from many other filamentous bacteria.

Another interesting characteristic of *Leucothrix* is the ability of filaments to grow in such a way that knots are formed (Fig. 5) (Brock, 1964). Knots occur mainly when the organism is growing in rich liquid culture media, where filamentous growth is rapid. Knot formation is frequent enough in *Leucothrix* cultures to be used as a taxonomic characteristic. Since individual filaments do not flex or glide, a preformed filament cannot form a knot. Actually, knots seem to be formed as part of the growth process, probably because growth occurs faster on one side of the filament than on the other, causing the filament to form a loop through which the tip of the filament can pass. Once the knot is formed it cannot be untied; rather, the cells in the region of the knot eventually fuse and form a bulb that is later released from the rest of the filament. As a result, the long filament is separated into two shorter filaments. Knots are also seen occasionally in *Thiothrix* filaments in sulfur springs, where large accumulations of these organisms appear, and in *Leucothrix* populations in nature (T. D. Brock, unpublished observations). All of the structural features of the *Leucothrix* life cycle can be observed not only in culture but also in natural material, especially when Nomarski optics are used to examine the surfaces of algal fronds (J. A. Bland, personal communication).

An electron microscopic study of *Leucothrix* was carried out by Brock and Conti (1969), and some of the structural features of the organism are seen in Fig. 6.

Taxonomy of *Leucothrix*

The question of the physiological or taxonomic relationship between *Leucothrix* and *Thiothrix* has not been resolved. A *Thiothrix* filament or rosette that has lost its sulfur granules cannot be distinguished from *Leucothrix.* Harold and Stanier (1955) placed pure cultures of *Leucothrix* in sulfide-containing sea water and examined them at intervals for the presence of intracellular sulfur granules. No evidence of sulfur accumulation was obtained; this was also my experience with one pure culture. However, Kjell Eimhjellen of the Technical University in

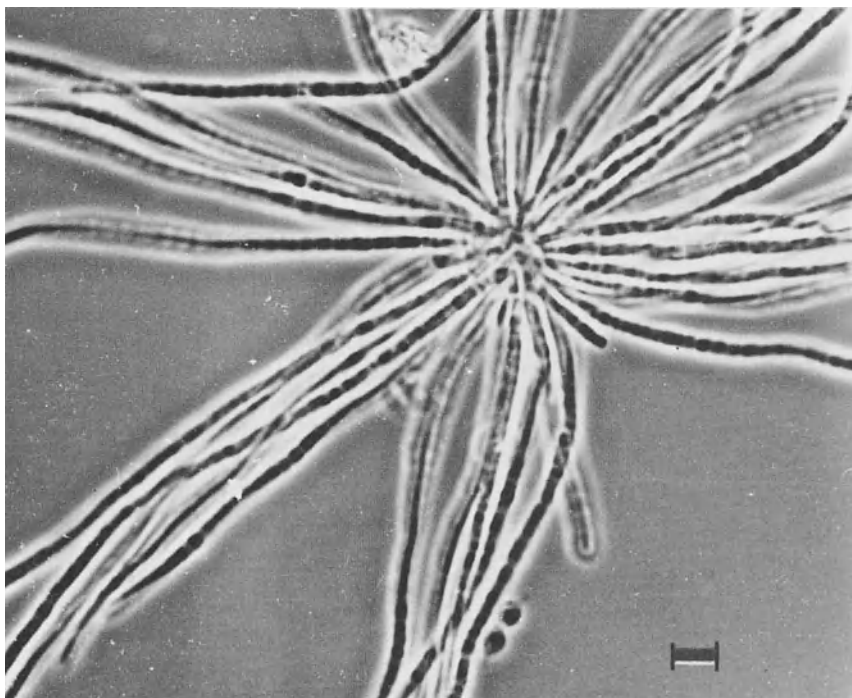

Fig. 4. Rosette composed of several *Leucothrix* filaments. Phase-contrast photomicrograph. Bar = 10 μm.

Trondheim, while working in C. B. van Niel's laboratory at Pacific Grove, California, was successful in attempts to show oxidation of hydrogen sulfide by *Leucothrix*. In his words (personal communication, 26 March 1970):

Leucothrix mucor (the strains I isolated all corresponded very well to the description of Harold and Stanier) pregrown on the usual media, can be made to oxidize sulphide when exposed to an atmosphere of hydrogen sulphide and oxygen, very much in the manner of *Sphaerotilus* observed by Skerman. Based on comparison of the pictures published by Skerman, *Leucothrix,* however, seemed to have a greater ability to oxidize sulphide. The filaments of *Leucothrix* often had a very heavy accumulation of internal S-globules, making the filaments resemble the very best rosettes of *Thiothrix* you can find in nature. After extensive washing of such cells to remove chemically produced sulphur, resuspension in low-sulphate media and incubation by gentle shaking, the internal S-globules disappeared and copious amounts of sulphate could be isolated from the supernatant. The sulphide oxidation was never followed manometrically, but I did a great many measurements of the ability of *L. mucor* to oxidize thiosulphate. Every time the oxidation would proceed quantitatively to tetrathionate and in a fair number of experiments the oxidation went further to a complete oxidation to sulphate. I could never clear up the reasons for this inconsistency. In separate experiments, the presence or absence of carbon dioxide seemed to make no difference on the total oxygen uptake, indicating no use of thiosulphate as electron donor for CO_2 fixation. . . . My conclusion is that the strain of *L. mucor* I used had the enzymatic capability to oxidize sulphide, sulfur and thiosulphate, all compounds being oxidized to sulphate, but no evidence for any energy link has been found.

It seems likely from a comparison of the results of Eimhjellen with those of Harold and Stanier (1955) that strain differences exist and that some strains of *Leucothrix* are able to deposit elemental sulfur. Further work on this problem would be of considerable interest.

Only a single species of *Leucothrix* is currently recognized, *Leucothrix mucor* (Brock, 1974). Pringsheim (1957) described *L. cohaerens* as a second species, based on cell diameter and filament length, but there is considerable variation in these properties even in a single pure culture, and since Pringsheim's isolates are no longer available, it seems preferable to maintain only a single species. Kelly and Brock (1969b) showed by physiological and molecular techniques that a wide variety of *Leucothrix* isolates, obtained from coastal areas around the world, were very similar. Of 35 strains characterized, all had a pH optimum of 7.6 and a salinity optimum of 30 parts per thousand. (The GC content of the DNA varied only over the narrow range of 48–51 mol% GC. Temperature optima of all strains were in the range 25–30°C, with a maximum of 33°C. Most strains showed a minimum temperature for growth of 0–2°C, although one strain, isolated from a tropical area, had a minimum temperature for growth of 13°C and hence was more stenothermal.

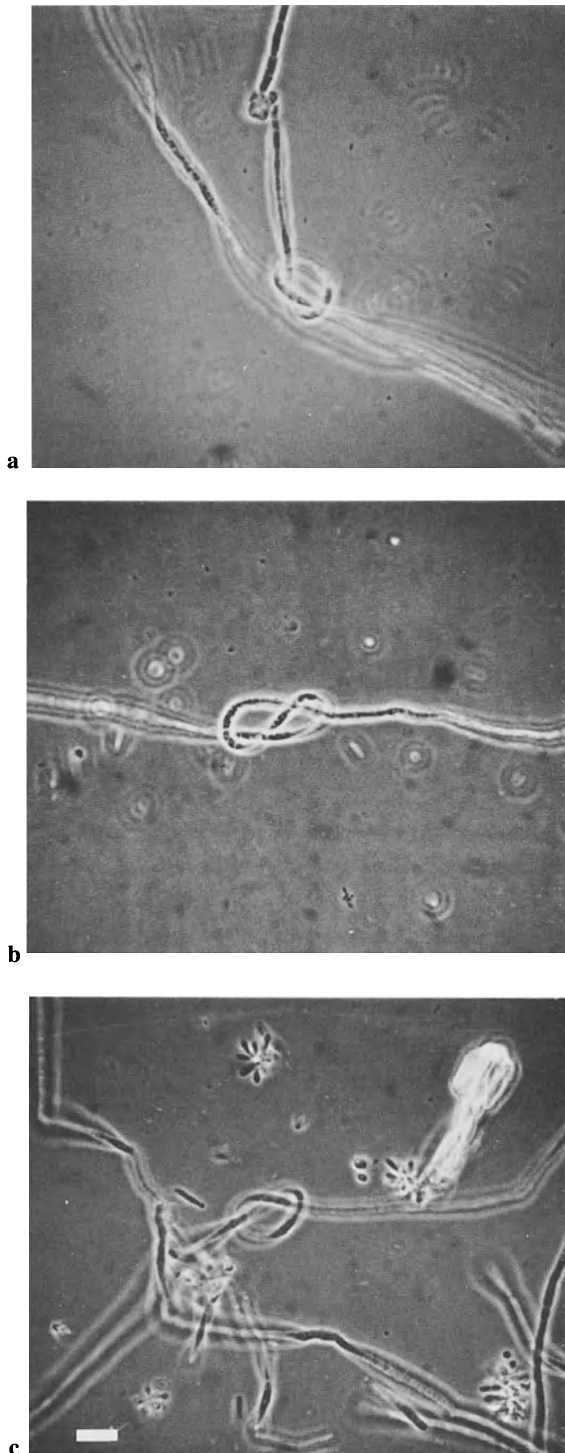

Fig. 5. Knots formed by filaments of *Leucothrix mucor*. Phase-contrast photomicrograph. Bar = 5 μm. (From Brock, 1964.)

Growth factor requirements were determined for all 35 strains. Most had no growth factor requirements and were able to grow heterotrophically on a simple glutamate culture medium, but 2 strains required thiamine, 1 strain (the warm water isolate) required vitamin B_{12}, and 3 strains showed a partial aspartate requirement. All the strains possessed the array of morphological characteristics typical of the species: filaments, gonidia, rosettes, knots, and bulbs.

Brock described a neotype strain of *Leucothrix mucor* and listed its characteristics as follows:

Morphology. Filaments are of variable length, often much greater than 100 μm, with a diameter of 2–3 μm. Sulfur granules are not formed. True knots are usually formed by pure cultures when growing in organically rich media. Filaments are colorless, unbranched, and nonmotile (although occasionally waving back and forth) and lack a sheath, although cells in regions of a filament may become emptied of their contents and give the appearance of a sheath. Filaments often grow intertwined or in dense tangles. Swollen cells often form at random along filaments. Larger structures (bulbs) usually form in knotty cultures, probably as a result of fusion of cells in the region of the knots. Filaments are attached to solid substrates by means of an inconspicuous holdfast that can be seen by staining with primulin and viewing with blue light in a fluorescent microscope; the holdfast fluoresces red. Individual filament cells round up and form ovoid to spherical gonidia that acquire a jerky gliding motility when released. Gonidia frequently aggregate in cultures, probably chemotactically, to form rosettes.

Nutrition. Most strains do not require growth factors. They grow on glutamate as sole source of carbon, nitrogen, and energy. They also use sugars, organic acids, and other amino acids as carbon and energy sources and NH_4^+ as nitrogen source. *Leucothrix* requires Na^+ for growth (optimum 1.5% NaCl, minimum 0.3%, maximum 7%).

Relation to temperature. Optimum 25–28°C; maximum 32–35°C. *Leucothrix* grows at 0°C to form visible colonies within 1–2 weeks.

Relation to O_2. Obligately aerobic.

Habitats

As noted in the Introduction, *Leucothrix* appears to be strictly a marine organism. It is widespread as an epiphyte on marine algae and also occurs as an infestation of benthic crustacea (Johnson et al., 1971). On seaweeds, it appears most commonly in temperate waters

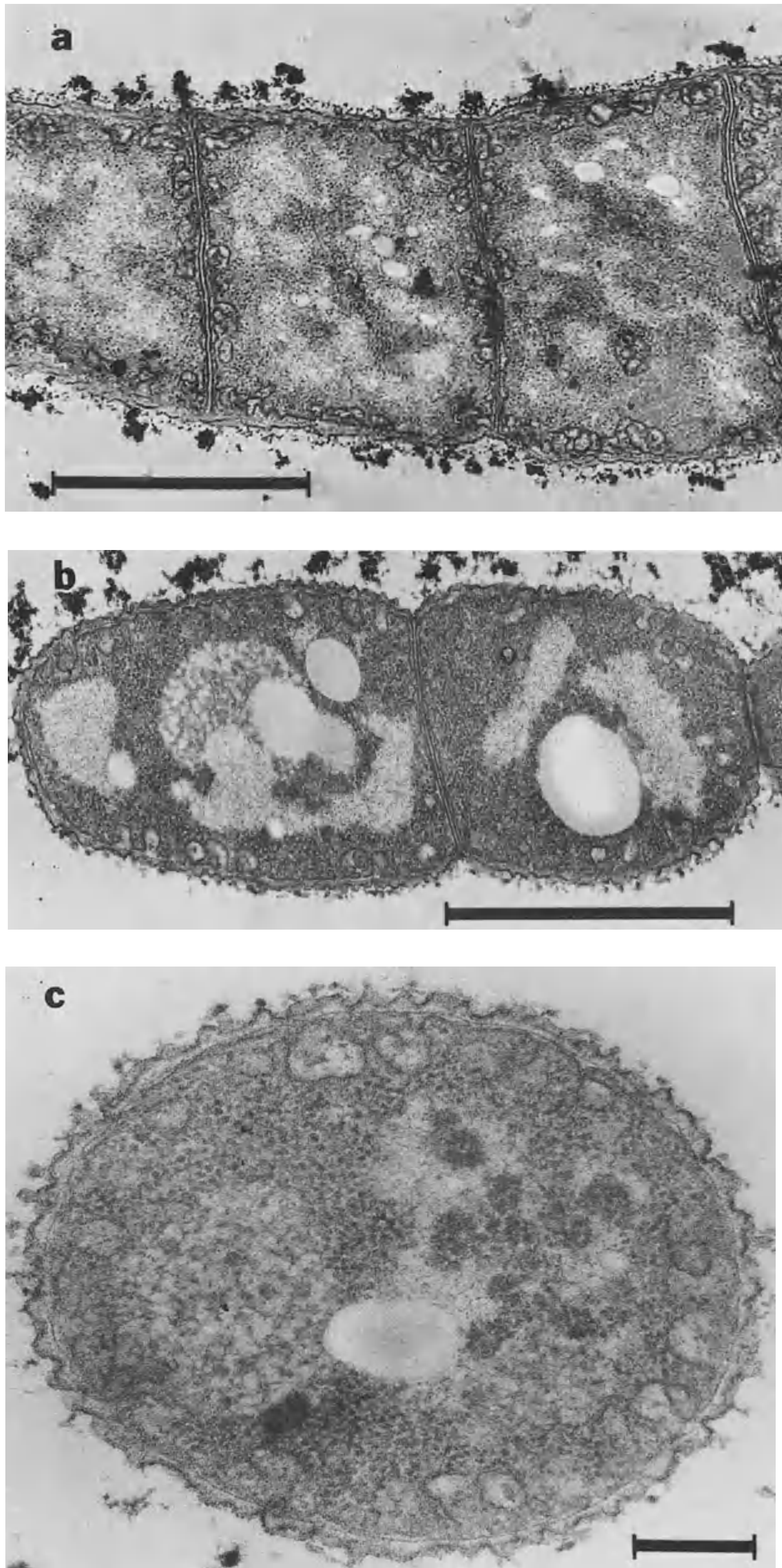

Fig. 6. Electron micrographs of thin sections of *Leucothrix mucor*. (a) Portion of a multicellular filament. (b) Filament in early stage of gonidial formation, showing rounding up of the cells. Note that there is no change in the ultrastructure of the wall. (c) A single gonidium. The gonidia retain all of the envelope structures observed in filaments. (Continued on next page.)

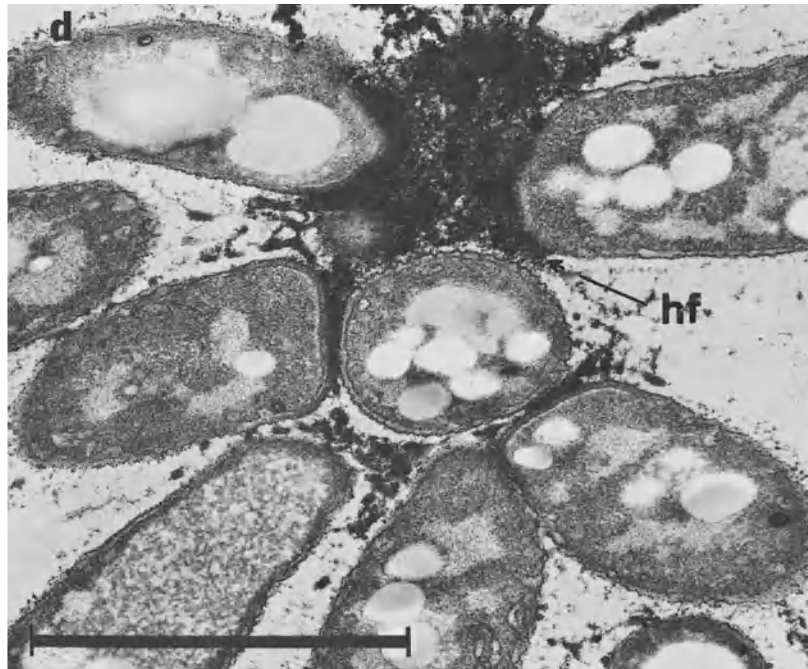

Fig. 6 (continued). (d) Thin section through a rosette. The holdfast material (hf) is an electron-dense material surrounding the cells. (a, b, d) Bars = 1.0 μm; (c) bar = 0.2 μm. (From Brock and Conti, 1969.)

(Kelly and Brock, 1969a), where densities are often quite high. In a detailed study at Friday Harbor, Washington, Bland and Brock (1973) made quantitative microscopic determinations of *Leucothrix* densities on a number of seaweeds. The organism was found on a variety of red, green, and brown algae, but was much more abundant on intertidal than on subtidal algae. The red alga *Bangia fuscopurpurea,* a filamentous species living in the high intertidal region, was unusually heavily colonized, with *Leucothrix* populations 10–30 times larger than those on other algal species in the same locations. Because of its high intertidal location, *B. fuscopurpurea* is exposed to air for fairly long periods during each tidal cycle. *B. fuscopurpurea* mats, dried during low tide, showed greater *Leucothrix* populations on the underside of the mats than on the top, presumably because the underside of the mats retained moisture longer. Examination of *B. fuscopurpurea* filaments of various ages showed that there was a continuous increase in the *Leucothrix* density with algal age, a finding that suggested growth on the alga itself. Using axenic two-membered cultures in an autotrophic medium with several red algae (including *B. fuscopurpurea*) and *L. mucor,* Brock (1966) showed that *Leucothrix* could grow on nutrients produced or liberated from the alga. Bland and Brock (1973) used artificial substrates and a variety of ecological studies to demonstrate that in nature *Leucothrix* obtained most of its nutrients from the alga and not from the sea water. Details of these ecological experiments and a review of other literature on the ecology of *Leucothrix* should be sought in the original paper (Bland and Brock, 1973).

Brock (1966) thought that *Leucothrix* was more abundant on seaweeds in temperate than in tropical waters (see also Kelly and Brock, 1969c), but Johnson et al. (1971) found *Leucothrix* to be abundant on tropical algae in the Pacific. Brock (1966) also concluded that *Leucothrix* was more abundant on algae in habitats of high aeration, such as open, exposed coasts and areas with heavy tidal currents. Since *Leucothrix* is an obligate aerobe, the preference for highly aerated habitats is not surprising. The finding that *Leucothrix* prefers algae in the intertidal rather than the subtidal zone is in keeping with this hypothesis, although there are, of course, factors other than aeration that differ in these two kinds of habitats. A survey of marine habitats showed that *Leucothrix* was worldwide in distribution (Kelly and Brock, 1969b), including the southern hemisphere (Brock, unpublished observations in New Zealand).

An authentic freshwater strain of *Leucothrix* has never been isolated, although *Thiothrix* occurs in both fresh and marine waters. Microscopic surveys of freshwater algae, even freshwater red algae, have never shown any indication of the presence of *Leucothrix* (Brock, unpublished observations), although Eikelboom (1975) found a number of *Leucothrix*-like organisms in activated sludge (see also van Veen, 1973; Sladka and Ottova, 1973). However, because there are various other filamen-

tous septate bacteria, positive confirmation of freshwater *Leucothrix* will require isolation in pure culture and demonstration of the various stages of the life cycle, especially gonidia and rosettes.

Isolation

Harold and Stanier (1955) first isolated *Leucothrix* from crude enrichment cultures of rotting seaweeds, but such cultures are generally grossly contaminated and purification of *Leucothrix* is often difficult. A more effective way of isolation (true for many other gliding filamentous bacteria as well) is to avoid enrichments completely and carry out a direct, single-colony isolation from natural material. This operation is made especially easy with *Leucothrix* when it is growing on relatively clean seaweeds, because on such samples, motile unicellular contaminants (the organisms presenting the greatest difficulty in isolation) are relatively small in number.

Any marine-type seawater base can be used in the formation of a culture medium; natural sea water (filter-sterilized) itself can be used, but pH control and avoidance of metal precipitation are easier in a Provasoli-type culture medium (Provasoli, 1963). The following salts formulation (Brock, 1966) proved quite effective:

NaCl	11.75 g
$MgCl_2 \cdot 6H_2O$	5.35 g
Na_2SO_4	2.0 g
$CaCl_2 \cdot 2H_2O$	0.75 g
KCl	0.35 g
Tris(hydroxymethyl) aminomethane	0.5 g
Na_2HPO_4	0.05 g
Deionized water	1 liter
Adjust to pH 7.6.	

The low phosphate concentration of this salts base is critical, as media of normal phosphate levels are inhibitory. Most strains have no vitamin requirements and use glutamate as the sole source of carbon, nitrogen, and energy, so that this salts medium with 0.1% monosodium glutamate (MSG) and 2% agar (to inhibit swarming organisms) will permit the isolation of *Leucothrix* from most materials. Other useful media include 0.1% MSG plus a vitamin mixture, 0.1% MSG plus 0.01% yeast extract, and 0.1% tryptone plus 0.1% yeast extract. In the initial isolation step, it is best to keep the organic concentration of the medium low to avoid problems with overgrowth by unicellular bacteria.

For isolation, single algal filaments are streaked directly (or after washing in sterile salts) onto agar plates, which are incubated at 20–25°C overnight. Within 12–18 h after streaking, the plates are examined under 125× magnification (10× eyepiece with a long working distance, phase-contrast objective is best) for the presence of characteristic *L. mucor* colonies, which have a coiled rope or thumbprint morphology. These colonies are picked by touching them with a sterile insect pin and transferring them to fresh agar plates of the same composition. It is important that colonies be identified early after inoculation, before the plates are overgrown. Although a dissecting microscope can also be used to locate *Leucothrix* colonies (Harold and Stanier, 1955), I have found that a 10× microscope objective (12.5× eyepiece) is better for locating colonies when they are very small (before they are overgrown). By using these procedures, it has been possible not only to isolate *Leucothrix* from seaweed from a wide variety of habitats, but also, with very clean material, to obtain growth of *Leucothrix* colonies directly from filaments still attached to seaweed fronds. In this way, the precise habitat from which the isolate was obtained is known; such information is of considerable value in studies on the molecular evolution of *Leucothrix* (Kelly and Brock, 1969b).

During transfer of colonies to liquid culture, it has often been observed that only very slight growth, if any, occurred after several days when an inoculum was transferred from agar to 100-ml liquid cultures; but when the inoculum was placed in a small volume of medium, such as 1–2 ml, heavy growth occurred overnight. Such small cultures could be easily used as the inoculum for large flasks and permitted the buildup of large-volume cultures. In liquid medium, growth is best when the flasks are shaken gently, such as on a wrist-action shaker or slowly on a rotary shaker. With the latter kind of shaker, growth rate is increased if the flasks contain small internal baffles, made by pushing in the sides of the flasks during heating with an oxygen flame.

For growing high-density cultures, a medium containing 1% MSG, 0.2% sodium lactate, and 0.01% yeast extract has proved quite suitable, the yeast extract providing growth factors needed by some strains. The sodium lactate was found to substantially increase the yield of most cultures.

Identification

Leucothrix is identified simply on the basis of morphological examination with the light microscope. The filaments are of large diameter

(greater than 2 μm; average, 2–3 μm), and each filament is composed of short cylindrical or ovoid cells, with cross-walls clearly visible. The filaments are colorless, unbranched, and of variable length, often very long. The filaments do not taper, but there may be variation in diameter along the length of the filaments. The filaments do not glide, although they may wave sporadically from side to side. Rosette formation is a key diagnostic characteristic. Without observation of the presence of rosettes, it is not possible to easily identify an isolate as *Leucothrix.* Conversion of cells in filaments into gonidia may result in the formation of chains of spherical cells, or gonidia may form only at the ends. The gonidia exhibit gliding motility, although the rate of motility is generally so slow that gliding itself is not a good diagnostic characteristic. To study gonidia formation, gliding, and aggregation, slide cultures are preferable; chambers that permit examination with 10–25× objectives should be used. Growth in such chambers is better if a considerable air space is left along one side, since *Leucothrix* is an obligate aerobe.

Filaments in culture often form true knots, and the presence of knots in a culture may be considered indicative of *Leucothrix* even without the formation of rosettes. However, the density of knots is never high, and a number of microscopic fields must be searched to ascertain if knots are present. Knot formation is most frequent when growth occurs to a high cell density in a relatively rich culture medium.

All strains that have been isolated are marine and require NaCl for growth; but, since freshwater strains could theoretically exist, NaCl requirement should not be considered a diagnostic characteristic. The GC content of the more than 30 strains examined ranged from 46 to 51 mol%, a relatively narrow range. Only one species is recognized (Brock, 1974), *Leucothrix mucor.*

Acknowledgments

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CHAPTER 176

The Genus *Lysobacter*

HANS REICHENBACH

The species classified in the genus *Lysobacter* are Gram-negative rods that move by gliding. The cells are slender and cylindrical, with rounded ends (Figs. 1 and 2). They typically measure 0.4–0.6 × 2–5 μm, but in the population there are also always long to very long (up to 70 μm) cells and filaments. The cell shape and the occurrence of long cells are both very characteristic for the genus. *Lysobacter* cells resemble the vegetative cells of certain myxobacteria, specifically of the genera *Polyangium* and *Sorangium,* with which the lysobacters were confused for many years. They also share with the myxobacteria a high GC content of their DNA of 65 to 70 mol%. Due to the gliding movements of the cells, the colonies of *Lysobacter* are spreading or swarming on solid media and may become very large and extremely thin (Figs. 3 and 4). Sometimes the organisms produce copious amounts of slime, and the colonies then become thick and deliquescent, but colonies with a wrinkled and dry surface also occur. *Lysobacter* colonies may be white or cream-colored but often they are greenish-yellow, purplish-red, or brown, although their color is often rather pale. Some strains produce an unpleasant odor reminiscent of certain pseudomonads or of pyridine. In agitated liquid cultures, the lysobacters grow as homogeneous cell suspensions, but, as with all gliding bacteria, the suspended cells are unable to translocate. The *Lysobacter* species live in soil, decaying organic matter, and fresh water, sometimes in large populations. Many strains are of considerable ecological and biotechnological interest as producers of exoenzymes and of antibiotics.

The genus *Lysobacter* was defined by Christensen and Cook (1978) who also described the presently recognized species and created a new family, Lysobacteraceae, and a new order, Lysobacterales. The organisms thus classified had already been known, however, for a long time under various names, such as *Cytophaga, Sorangium,* and *Myxobacter* (the latter an obsolete myxobacterial genus), which were usually presented with some doubts of the investigators concerning the classification of their strains. The first lysobacter in the scientific literature may have been *Flexibacter albuminosus* (Soriano, 1945, 1947), which had the cell size and shape of a lysobacter and formed thick dirty-white colonies and a diffusible dark pigment. But the description is not accurate enough and the strains are no longer available so that the question cannot be decided. The first unequivocal *Lysobacter* strain was a chitinolytic strain first tentatively identified as *Cytophaga johnsonae* (Veldkamp, 1955). It is deposited at the National Collection of Industrial Bacteria (NCIB no. 8501) and was originally listed as a *Polyangium* species. The strain has a GC content of 71 mol% (T_m) and was noted as an unusual case of a cytophaga with a high GC content (Mitchell et al., 1969). Other early strains that later turned out to be lysobacters are: 1) "myxobacter" or *"Sorangium"* strain 495, which was studied because it attacks nematodes (Katznelson et al., 1964) and various bacteria (Gillespie and Cook, 1965) and contains very interesting proteases (for details, see "Practical Aspects," this chapter); the strain also produces two peptide antibiotics, the myxosidins (Clapin and Whitaker, 1976, 1978); 2) "Myxobacter" AL-1, which became of interest because it digested cells and cell walls of *Arthrobacter crystallopoietes* (Ensign and Wolfe, 1965) and which was later found to excrete two unusual proteases; 3) *"Sorangium"* 3C, producer of the wide-spectrum phenazine antibiotic myxin (Peterson et al., 1966); 4) *"Cytophaga"* L1, (NCIB 9497) for which a patent was filed for a number of unusual enzymes of practical interest, e.g., keratinase, laminarinase, and chitinase (Brit. Pat. 1,048,887, 23 November 1966), and 5) *"Cytophaga johnsonae"* (ATCC 21123), originally isolated because of its lytic enzymes at Kyowa Hakko in Japan (Jap. Pat. 06624, 1969), and from which the new quinoline antibiotic G1499-2 was obtained (Evans et al., 1978). In addition a number of lysobacters, usually labeled "myxobacters," were isolated because

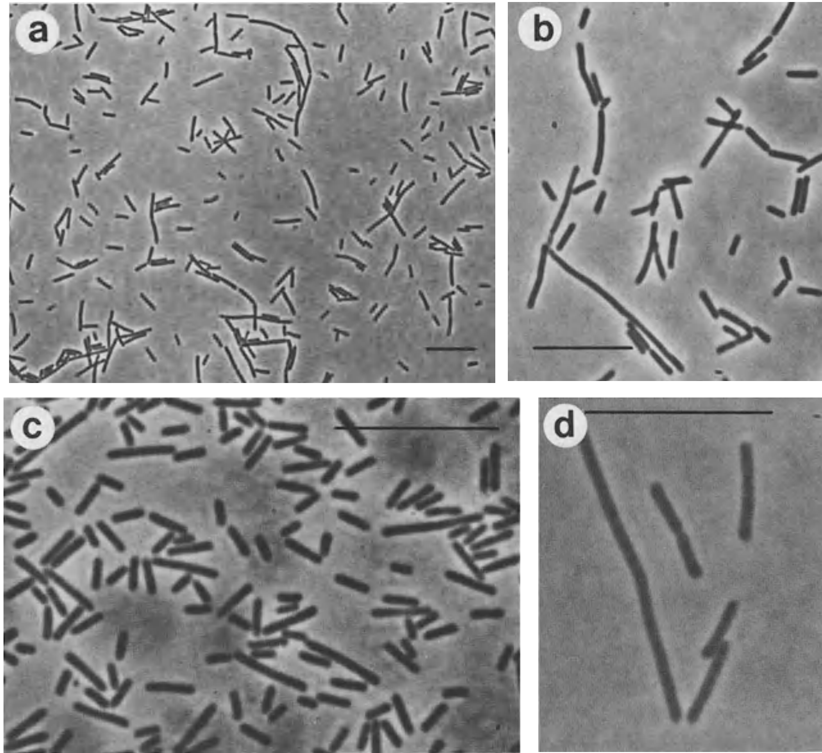

Fig. 1. Phase contrast photomicrographs of *Lysobacter*. (a), (b), and (d) *L. antibioticus* strain UASM L17 (= ATCC 29480) grown on CY agar (0.3% Casitone, 0.1% yeast extract) for 3 days at 20°C. Typical is a fairly regular cylindrical cell shape and a substantial variation in cell length. (c) *L. enzymogenes* type strain UASM 495 (= ATCC 29487), grown on VY/2 (yeast) agar for 13 days at 30°C. (a) Bar = 20 μm. (b), (c), and (d) Bars = 10 μm.

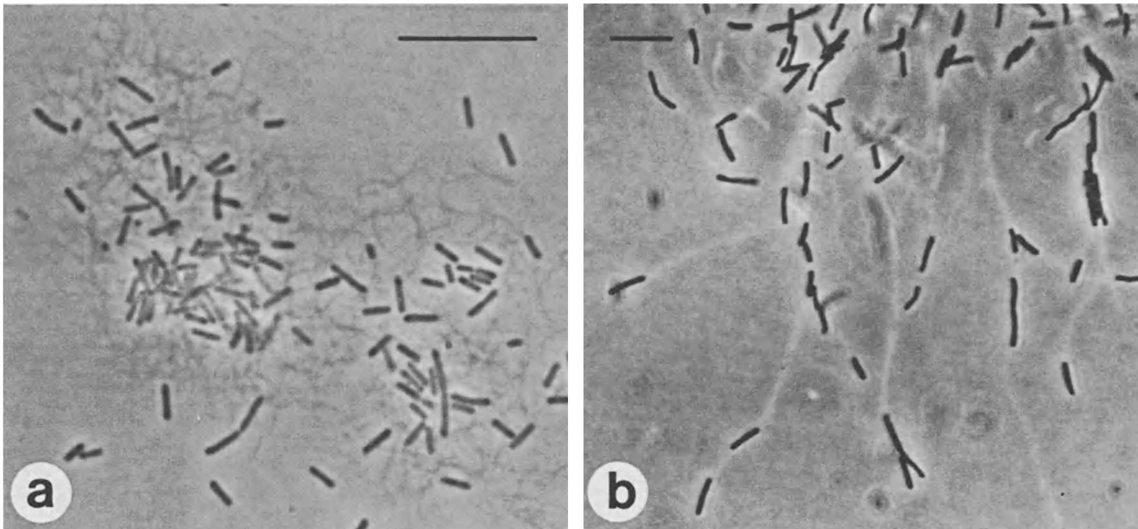

Fig. 2. Phase contrast photomicrographs of slime structures produced by *Lysobacter*. (a) *L. antibioticus* strain UASM L17 grown on CYG2 agar (CY agar plus 0.3% glucose) for 3 days at 20°C. For photography, the cells were transferred to a thin film of water agar. A network of slime threads is clearly visible. Bar = 10 μm. (b) The cyanobacteriolytic lysobacter of Shilo (1970). Edge of a colony on Shilo's (2% Casitone) agar in a chamber culture. Slime trails have been laid by the cells during their gliding movements. Bar = 10 μm.

they attacked cyanobacteria and green algae and multiplied spectacularly during algal blooms. Thus, "myxobacter" FP-1 specialized on cyanobacteria (Shilo, 1967, 1970); *"Cytophaga"* N-5, later renamed "myxobacter" 44, lysed cyanobacteria and green algae (Stewart and Brown, 1969); "myxobacters" 45 and 46, which with "myxobacter" 44, *"Sorangium"* 3C, and "myxobacter" AL-1, have an uncommonly high GC content of around 70 mol% (Stewart and Brown, 1971); and the cyanobacterium-lysing bacteria with a high GC content isolated from

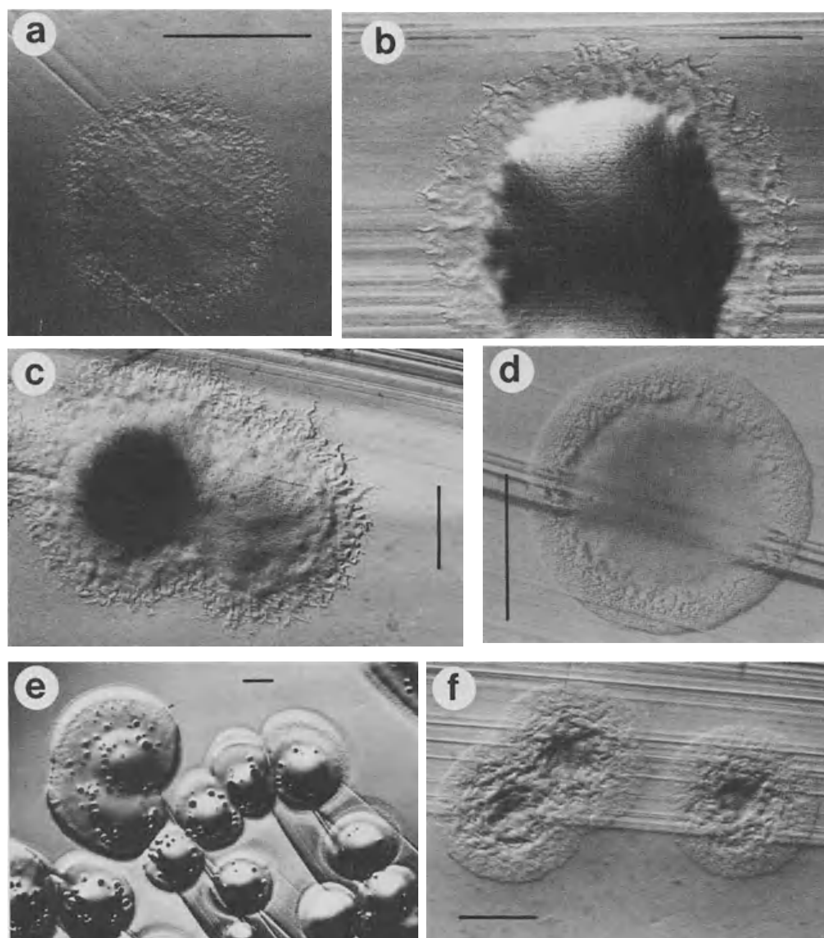

Fig. 3. The *Lysobacter* colony. Bars = 1 mm. (a), (b), and (c) *L. antibioticus* strain UASM L17 grown on different media for 7 days at 20°C: (a), on CA2 agar (0.075% KNO_3, 1% glucose); (b), on MYX agar (0.5% Na glutamate, 0.1% yeast extract, 0.2% glucose); and (c), on CY agar. (d) and (e) the chitinoclastic strain (NCIB 8501) of Veldkamp (1955): (d) grown on CA2 agar; (e) grown on CY agar; both 7 days old (20°C). (f) *L. enzymogenes* strain UASM 495 grown on CA2 agar for 7 days at 20°C. In all cases, the colonies are spreading, but they still are rather small after 1 week. On diluted CA2 agar containing nitrate as the sole nitrogen source, the swarm colonies of all three strains remain rather delicate. In (e), small crystals can be seen within the colonies, as is often the case with lysobacters.

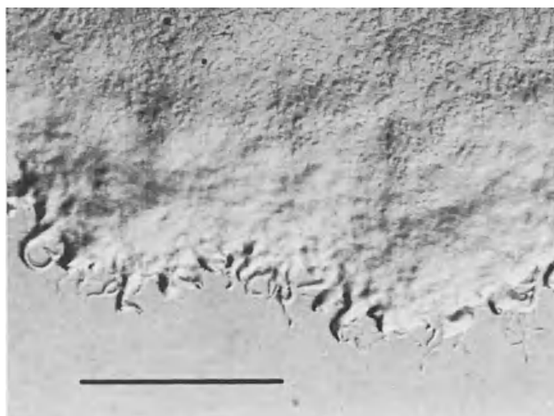

Fig. 4. *Lysobacter antibioticus* strain UASM L17. Edge of a swarm colony showing the typical flamelike protrusions. CY agar, 7 days at 20°C. Bar = 1 mm.

British waters, e.g., strains CP-1, -2, -3, and -4 (Daft and Stewart, 1971; Daft et al., 1975).

The phylogenetic position of the genus *Lysobacter* remained obscure until recently. To the early investigators, gliding motility suggested some relationship with other, existing groups of unicellular gliding prokaryotes, specifically the myxobacteria and the cytophagas (Reichenbach, 1981). This is reflected by the names given to the strains isolated at that time. But as was already correctly anticipated in the taxonomic description of the new organisms, these bacteria form a group of their own (Christensen and Cook, 1978). Later, 16S RNA studies demonstrated that *Lysobacter* is relatively closely related with the xanthomonads and belongs to the gamma-3 branch of the purple bacteria (Woese et al., 1985) known today as the class Proteobacteria (Stackebrandt et al., 1988).

Habitats

The lysobacters live in soil and fresh water and appear to be wide spread and in many places are rather abundant. They have been isolated from soils in the Netherlands (Veldkamp, 1955), Canada (Christensen and Cook, 1978; Katznelson et al., 1964; Peterson et al., 1966), the United States (Ensign and Wolfe, 1965), and

Scotland (Daft et al., 1975). In Scotland, they have been found in agricultural soils and sand dunes where the pH was neutral to slightly alkaline (pH 8.8) and not below pH 6. Population densities of up to 500 plaque-forming-units (PFU) per cm^3 were observed. This may seem very low, but the isolation method was designed only to detect organisms that lyse cyanobacteria (forming plaques in the cyanobacterial lawns), which may not be the case with all lysobacters. Also, the counts would be much reduced if most of the lysobacters are attached to particles. In soil, the lysobacters probably subsist by degrading various biomacromolecules and microorganisms other than cyanobacteria. In fact, lysobacters are known to decompose nematodes and different kinds of bacteria, as already mentioned. So, while their ecological niche is not really identified, the enzymatic equipment and lytic capabilities of the lysobacters suggest that they preferentially live in places rich in (recalcitrant) organic matter and microbial life.

Much more is known about the freshwater habitats of the lysobacters because there strains exist that can lyse living and healthy cyanobacteria and for this reason aroused much interest (see reviews by Stewart and Daft, 1976, 1977). The bacteria were found in fish ponds in Israel (Shilo, 1967; Shilo, 1970), in sewage plants in the United States (Stewart and Brown, 1969, 1971) and Scotland (Daft et al., 1975), and in lakes, reservoirs, and rivers in Great Britain (Daft et al., 1973, 1975). This, of course, does not mean that those lytic strains are the only lysobacters living in fresh water, as lysobacters were also isolated from that source by other techniques, and the ability of these strains to lyse cyanobacteria was never specifically demonstrated (in Canada: Christensen and Cook, 1978; in Germany: H. Reichenbach and coworkers, unpublished observations).

In contrast to cyanobacterial viruses, the lytic lysobacters have a wide activity spectrum with respect to the cyanobacterial species attacked. Thus, strain FP-1 lysed 9 out of 11 species of unicellular and filamentous cyanobacteria whereas the green alga *Chlorella pyrenoidosa* and the chrysophyte *Prymnesium parvum* were completely resistant (Shilo, 1970). All Gram-negative bacteria tested were also lysed, but Gram-positive bacteria were not. Strain CP-15 disintegrated 21 out of 23 strains of cyanobacteria (Daft et al., 1973), and strains CP-1 to -4 among themselves lysed 29 out of 42 cyanobacteria from nine different genera (Daft and Stewart, 1971). In the latter case, there were slight differences among the four strains with respect to the spectra of cyanobacteria attacked, and also different strains of the same cyanobacterial species could vary in their sensitivity to lysis. All 16 British *Lysobacter* strains used in these studies were serologically related and may have belonged to one species (Stewart and Daft, 1976).

The population densities of lysobacters observed in British waters are usually low (Daft et al., 1975), but since lysobacters attach themselves to plankton and other particles, the counts may not reflect the true numbers. In lakes in summer and at the water surface, between 0 and 400 PFU/ml were found, and the numbers decreased rapidly with depth (to 1 m). But 94% of all samples contained lytic bacteria. At the height of cyanobacterial water blooms, 0.001 to 0.05% of all cultivatable bacteria were lytic, and in general the population densities of cyanobacteria and lytic bacteria correlated well, with a slight shift in the time of the maxima. Large differences in the cell numbers could occur in simultaneous samples taken from different sites in the same lake. This apparently was simply due to wind drift which caused the plankton to accumulate at certain places. In samples with particularly high densities of cyanobacteria, the numbers of lysobacters could drop substantially. As the lysobacters are strict aerobes, their decline in number under such conditions was explained by oxygen depletion during the night, but perhaps the bacteria only seem to disappear because there was now an increased opportunity for attachment. In February, after 3 months of low phytoplankton density (and low temperature), the count of lytic bacteria fell to zero at the water surface, but some lysobacters still survived in the depth at the sediment-water interface. From there and from soils around the lakes, the repopulation of the water bodies may take place in spring. Another source for lytic bacteria was found in sewage works where in summer the counts in the final effluents were around 700 PFU/ml, and in the effluents from filter beds even as high as 1,300 PFU/ml. Also, in sewage plants, the numbers dropped during winter, in February to about 1% of the summer counts (Daft et al., 1975). Dense populations of lytic bacteria were found in the sand filters of a waterworks at a reservoir with blooming cyanobacteria. Up to 2,500 PFU/ml were found in the effluent, and 59,000 PFU/ml on the filter sand. The maximum in the open reservoir was 92 PFU/ml (Daft et al., 1973). No lysobacters were detected in water from underground springs (Daft et al., 1975).

While the lysobacters are able to lyse bloom-forming cyanobacteria, their low population densities make it questionable whether they really play a role in the control of natural water

blooms. In field trials, at least 10^5 cells/ml were required to induce rapid lysis in cyanobacterial populations. In small bays at the edge of a reservoir, which were separated artifically from the main body and inoculated to a density of 10^6 bacteria/ml, there was clear evidence for lysis within 24 h at 13°C, and within 60 h, the *Microcystis* population was completely destroyed. In the lysing cell suspension the number of lytic bacteria rapidly declined, which again was explained by a lack of oxygen (Daft et al., 1973; 1975). In summary, it appears that cyanobacteria and lysobacters have similar growth requirements and simply coexist rather than prey upon one another. As the lysobacters can grow perfectly well as saprophytes, they may utilize material, including biomacromolecules, excreted by the cyanobacteria. In fact, they appear to interfere in a subtle way with the photosynthesis of the cyanobacteria and their excretion of dissolved organic carbon, reducing the rate of the former and stimulating the rate of the latter, and are able to rapidly assimilate excreted material, particularly certain amino acids, and to grow exclusively on it with a generation time of about 10 hrs (20°C). In a similar way they also exploit bloom-forming green algae like *Scenedesmus quadricauda,* which they cannot lyse at all (Fallowfield and Daft, 1988). Under conditions unfavorable for the cyanobacteria, the lysobacters also may lyse some of them, but they probably have little to do with the cyclic break down of water blooms in nature.

Isolation

Two properties of the lysobacters may be used for their enrichment: their efficient hydrolytic exoenzymes and their gliding motility. As both attributes are also found with other organisms, the enrichment techniques are not entirely specific.

Christensen and Cook (1978) recommend enriching soil samples with chitin, ground mushrooms, or *Arthrobacter* cells, and then leaving them for at least one month. After this period, the soil is suspended in water and appropriate dilutions are spread on yeast agar.

Yeast Agar

Bakers' yeast	0.5 %
Agar	1.5%

Adjust to pH 7.2 and autoclave.

After incubation, colonies arise that are surrounded by lysis zones, and their number increases substantially during the enrichment phase. Two types are usually observed: 1) pink-colored *(Lysobacter antibioticus)* and 2) cream-colored *(L. enzymogenes).* Rarely, also, an off-white gummy colony may appear *(L. gummosus).* On subcultivation, most of the cream colonies produce two colony variants: 1) dirty-white mucoid, and 2) yellowish nonmucoid. When water samples from lakes and rivers are used, yellow-brown colonies are obtained *(L. brunescens).*

As all lysobacters appear to degrade chitin (Christensen, 1989), one also could try to isolate them on chitin agar as described by Veldkamp (1955). He used 1% of finely powdered chitin suspended in a medium containing 0.1% K_2HPO_4, 0.1% $MgSO_4$, and 2% agar. He purified the chitin from shrimp shells by a very cumbersome procedure, so a simpler method is given here as well as two chitin media, which are used in our laboratory with excellent results in enriching for cytophagas and myxobacteria.

Preparation of the Chitin Stock Suspension (Modified from Hsu and Lockwood, 1975)

Finely divided commercial chitin (e.g., from Fluka or Sigma) is suspended in concentrated (32%) HCl. For 40 g of chitin, approximately 400 ml of HCl is required, but sometimes more HCl (600–800 ml) has to be used. Within about 30 min, a relatively thin, blackish collodial solution is obtained. The hydrochloric acid solution should never stand for more than 1 h at room temperature to avoid degradation of the chitin. The solution is poured into 2 liters of ice-cold water, upon which the chitin precipitates immediately as a pure white, fluffy material. It is collected on a separating funnel under suction and extensively washed. During these manipulations the material should never become dry, because drying makes it very difficult to resuspend. The washed precipitate is dialyzed against tap water for 12 to 24 h until the pH of the water remains above at least 4.5. Then enough distilled water is added to the chitin suspension to give a slurry which is sufficiently thin to be pipetted. The pH is adjusted to 7.2 with KOH. The exact volume is determined, and the approximate chitin content of the suspension calculated from the amount employed in the beginning. The material is distributed in convenient aliquots in bottles, autoclaved, and stored at 6°C. If the precipitated chitin appears too coarse, which may happen if the starting material was not well ground, it can be further homogenized with a blender.

Chitin agars are prepared best as overlay media, which saves chitin and gives clearer results, because the layer to be hydrolyzed is thinner and the material remains at the top of the plate, so that the bacteria have an easier access to it.

CT6 Agar

Top layer:

$MgSO_4 \cdot 7H_2O$	0.1%
K_2HPO_4	0.02%
Agar	1.5%

Adjust the pH to 7.5. After autoclaving, enough of the sterile chitin suspension is added to give a good turbidity, which should be achieved with about 0.5% chitin and not more than 30% (by volume) of the suspension. The medium is poured as a thin layer on top of the following base agar:

Base agar:

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.2%

Adjust the pH to 7.2 and autoclave.

CT7 Agar

The top layer is as above; the base agar, however, is water (WAT) agar, which is as follows:

WAT agar:

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Adjust the pH to 7.2 and autoclave.

In CT7 agar, chitin is the only nitrogen, carbon, and energy source besides agar, and thus the medium is more selective. But not all chitin degraders grow on it. On CT6 agar, on the other hand, chitin degradation is sometimes suppressed by the peptone, and organisms that are not chitinovorous also grow on it.

Lysobacters decompose many bacteria, living and dead, and therefore may be isolated on such substrates. Strain AL-1 was obtained from lysis zones in lawns of *Arthrobacter crystallopoietes* (Ensign and Wolfe, 1965). Aqueous soil extracts were streaked on plates of medium A and incubated at 30°C.

Medium A

A basal agar with 0.5% peptone and 1% agar was overlaid with the same medium containing 10^9 cells/ml of *Arthrobacter.*

The bacteria from the plaques that developed in the lawn were purified by plating diluted suspensions on medium B.

Medium B

Agar, 1.5%, containing 10^9 cells/ml of washed *Arthrobacter.*

Presumably the *Arthrobacter* cells were living in the isolation medium and dead in the purification medium. Strain AL-1 was also maintained on medium B (at 30°C, with weekly transfers).

Similar isolation techniques may be devised with other bacteria and in fact have been used repeatedly to obtain lysobacters that lyse cyanobacteria. Stewart and Brown (1969) and Shilo (1970) isolated their organisms from water collected from a waste stabilization pond and a fish pond with a water bloom, respectively. Both groups used the soft-agar overlay technique developed for the isolation of cyanophages, and living *Nostoc muscorum* or *Plectonema boryanum* as the indicator organisms. Shilo (1970) first enriched the lytic agent by inoculating the sample into a *Plectonema* culture. While viral plaques appear after just 2–3 days, the bacterial plaques need 5–7 days to develop, and in addition are slightly sunken into the agar. Further, the bacteria also produce lysis zones in layers of autoclaved cyanobacteria. Shilo (1970) separated her strain from the cyanobacteria by transfer to a mineral salts liquid medium with 0.2% Casitone and incubation in the dark. The overlay technique may also be used for counting the lytic bacteria. Stewart and Brown (1969) determined for strain N-5 (myxobacter 44) a ratio of 1.3 for the number of viable cells to plaque-forming units.

A slightly different method was applied by Daft and Stewart (1971) and Daft et al. (1973). The cyanobacteria were grown on a suitable mineral salts medium with 0.6% agar. On the lawns produced after 7 days at 22–25°C, 1-ml water samples from the top-most layer of lakes or other surface waters, preferentially such with a cyanobacterial water bloom, were spread and the lysobacters isolated from the lysis zones that arose in the lawns. As indicator organisms, *Nostoc ellipsosporum, Anabaena catenula, A. flos-aquae,* or *Phormidium foveolarum* were used, and *N. ellipsosporum* (strain 1453/19 Cambridge University) proved particularly sensitive to lysis. The CP strains could be maintained without loss of lytic activity on CP agar.

CP Agar

K_2HPO_4	17 mg
$MgCl_2 \cdot 6H_2O$	49 mg
$CaCl_2 \cdot 6H_2O$	15 mg
NaCl	60 mg
$FeCl_3$	0.3 mg
EDTA	7.4 mg
Standard trace element solution*	
Casitone (Difco)	2 g
Agar	6 g
Distilled water	1 liter

The mineral part is that of ASM medium (Daft and Stewart, 1971) for the cultivation of algae (and probably dispensable for the lysobacters).

*See, for example, Drews (1974), p. 6.

Lysobacter colonies may be recognized by their spreading growth due to gliding motility, and colony morphology can thus be used as a lead to isolate the organisms. Thus, on plates

designed for the isolation of myxobacteria and consisting of water agar (WAT agar) with streaks of living *Escherichia coli* that are inoculated with soil, we occasionally also observe swarming lysobacters.

The purification of *Lysobacter* strains is easy and is done either by plating of diluted cell suspensions or by making transfers from the advancing edge of swarm colonies. As swarming is much reduced or completely suppressed on rich media, the latter possibility exists only when the bacterium is grown on a lean medium like yeast agar or CY agar (see below). To avoid confluence of the arising colonies, plating is done best on a rich medium such as standard nutrient agar (Difco).

Cultivation

The lysobacters are robust, adaptable organisms, and their cultivation is no problem. They actually grow on any bacteriological standard medium. Christensen and Cook (1978) recommend the three following media:

PC Agar (Plate Count Agar, Difco)

Yeast extract	0.25%
Tryptone	0.5%
Glucose	0.1%
Agar	1.5%

CC Agar (Cook's *Cytophaga* Agar; Christensen and Cook, 1972)

Tryptone	0.2%
Agar	1.0%

SA Agar (Skim-Milk Acetate Agar; Christensen and Cook, 1972)

Skim milk powder	0.5%
Yeast extract	0.05%
Sodium acetate	0.02%
Agar	1.5%

The pH for all of these media is adjusted to about 7.0 and the medium is sterilized by autoclaving. On PC agar, the colonies remain small and compact like those of nongliding bacteria, whereas on CC and SA agar, spreading swarms are obtained. Another good medium for lysobacters, including stock cultures, is the Yeast Agar mentioned above. On this medium the organisms form thin, spreading swarms, and because no or much less ammonia is produced than on the peptone media, the cultures tend to remain viable for longer time.

SA medium may also be used as a broth for liquid cultures, but a nonturbid medium is more convenient, e.g., 1% yeast extract medium (Ensign and Wolfe, 1966). We often use PEP medium.

PEP Medium

Peptone from casein, tryptically digested	1%
$MgSO_4 \cdot 7H_2O$	0.1%
Adjust the pH to 7.2 and autoclave.	

Other peptones can be used as well, e.g., Casitone (Difco), which is a pancreatic digest of casein, and casamino acids have also been employed successfully (Gillespie and Cook, 1965). *L. brunescens* appears not to grow well or to lyse early in pure peptone media. It could, however, be cultivated with a generation time of 4 h at 20–22°C in tryptone-starch medium.

Tryptone-Starch Medium (von Tigerstrom and Stelmaschuk, 1987b)

Tryptone	0.8%
Yeast extract	0.2%
Starch	0.3%
$MgCl_2$	0.02%

A fully defined medium for *L. enzymogenes* strain AL-1 was developed by Ensign and is mentioned in Tan et al. (1974):

Defined Liquid Medium (for Strain AL-1)

Aspartic acid	0.2%
$K_2HPO_4 \cdot 3H_2O$	0.34% (0.03 M)
$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.01%
Tap water	1%
Glucose	0.5%
Adjust to pH 7.0 and autoclave.	

Although lysobacters tolerate relatively high pH values and can grow above pH 10 (Christensen and Cook, 1978), their optimum is between pH 7 and 9. While all appear to grow at 30°C, their temperature optimum is sometimes lower, and cultivation at 25°C or even 22°C may give better results. The organisms are strict aerobes, and liquid cultures have to be shaken.

The generation times are typically around 2.5 h (2.3 h for *L. enzymogenes* strain 495 in 0.8% yeast extract broth at 22°C: von Tigerstrom and Stelmaschuk, 1989; and 2.6 h for strain myxobacter 44 in a mineral salts medium with 0.2% starch and 0.25% peptone, 30°C: Stewart and Brown, 1971. Much longer generation times of 10.7 and 12.6 h are reported for growth under quasi-natural conditions in nutrient-poor culture filtrates from cyanobacteria and green algae, respectively (strain CP-1, 20°C: Fallowfield and Daft, 1988). A maximum cell yield of 2.5 g/l (dry weight) was obtained with strain 495 in condensed fish solubles with 2% glucose (Wah-On et al., 1980).

Large-scale fermentations with lysobacters have been performed for the production of the antibiotic myxin, and of an enzyme complex capable of lysing living yeast cells. While apparently nothing has been published about the industrial process developed by Hoffmann-La Roche (Nutley, NJ) for the manufacture of myxin with strain *"Sorangium"* 3C, some data on 10-liter fermentations are given in a Canadian patent (Can. Pat. 784,213; 30 April 1968). The fermentation was performed in the following medium: Tryptone, 0.1%; glucose, 0.1%; K_2HPO_4, 0.1%; $MgSO_4$, 0.02%; $CaCl_2$, 0.01%; and $FeCl_3$, 0.001%; at pH 7.5 and 25°C. The aeration rate was 0.1 liter air per liter medium and min; the stirring rate, 300 rpm; the harvest time, 20 h with a 2% (v/v) inoculum, and 12 h with a 10% inoculum.

Production of yeast-lytic enzymes by *"Cytophaga"* NCIB 9497 was studied at up to a 900–1 liter scale (Asenjo et al., 1981). The medium consisted of 1% yeast extract and 1% glucose and permitted a maximum growth rate (μ_{max}) of 0.36 h^{-1}. The inoculum size was 3.8%, the initial pH 7.2, and the temperature 26°C. The aeration rate was set at 0.2 liter air per liter medium and min, and the impeller tip speed between 1.21 and 1.57 $m \cdot sec^{-1}$ (around 100 rpm). It appeared that a relatively low level of dissolved oxygen around 20–30% was favorable, particularly in the beginning. Polypropylene glycol 2000 was added as an antifoam agent and was well tolerated at 2 ml/1 but not higher. Still, after 24 h, the broth began to foam heavily. Harvest was at 30 h with a good yield of active enzyme, part of which was set free only by cell lysis at the end of the fermentation. Early cell lysis may be a serious problem. Obviously it is essential to start with young, vigorous seed cultures, e.g., from freeze-dried material.

Preservation

Stock cultures may be kept on yeast agar at room temperature (21°C) and should be transferred every 1 to 2 weeks. With stock cultures on peptone media, production of toxic levels of ammonia can be a problem. Storage of cultures in the cold is not recommended. In one reported case, all cells were dead after 3 weeks at 5°C (Asenjo et al., 1981). It seems understandable that the lytic enzymes produced by lysobacters also destabilize resting cultures.

Lysobacters survive freezing very well, either at −80°C or in liquid nitrogen. Cells from young plate cultures are suspended in PEP medium, or young shake cultures in the same medium are taken, and 1-ml samples are frozen without further precautions. The preserved cultures are reactivated by immersing them in tap water for a quick thawing and transferring the content as soon as it is liquefied. The longest storage period we have tested so far was 13 years at −80°C, which was reliably tolerated by all strains.

Lysobacters also can be lyophilized in skim milk by the standard procedure. It is reported that reactivated freeze-dried cultures may show a slightly different colony morphology and reduced bacteriolytic and proteolytic activity (Christensen, 1989). We dry the organisms with good results at room temperature, which may be less harmful to them. A few drops of a cell suspension in sterile skim milk are applied to a plug of lyophilized skim milk in an ampule. The wetted plug is then dried in vacuo, the ampule filled with nitrogen gas, and sealed.

Characterization

Morphologically, the various lysobacter species resemble one another closely (Christensen and Cook, 1978; Christensen, 1989). The cells are slender cylinders with rounded ends, and, at least in young healthy cultures, are rather regular in shape (Figs. 1 and 2). They typically measure 0.3–0.6 × 2–6 μm, but much longer threads, up to 70 μm, are usually also present. Those filaments obviously are incompletely divided cells and cell chains. A mixed population of short and long cells is very characteristic for the lysobacters and distinguishes them at once from any myxobacterium. To avert confusion, it should be mentioned that the morphological description given by Veldkamp (1955) of his chitinolytic strain does not fit the present NCIB 8501 strain, which indeed is a typical lysobacter. Instead, Veldkamp has given the morphology and cyclic-shape change of *Flexibacter filiformis,* which also is strongly chitinolytic.

Electron micrographs of thin sections show a typical Gram-negative cell wall, large mesosome-like structures in the center or at the poles, and granules interpreted as poly-β-hydroxybutyrate and polyphosphate. On the surface there may be ruthenium-red-positive material, presumably slime (Shilo, 1970; Stewart and Brown, 1971; Stewart and Daft, 1977); in other cases no such material was seen ("myxobacter" AL-1: Pate and Ordal, 1967). True rhapidosomes, i.e., the contracted tails of a defective phage (Reichenbach, 1967), were found in cell lysates of *"Sorangium"* 495 (Pate et al., 1967).

In contact with an interface, the lysobacters may move by gliding. The movements usually are rather slow but observable under the mi-

croscope. An average speed of 1 μm per min (30°C) has been reported (Veldkamp, 1955). When suspended in liquid the cells are nonmotile.

On lean media like CC, SA, and yeast agar, most lysobacters produce thin, filmlike swarms with little flamelike extensions at the edge (Figs. 3 and 4). Only *L. gummosus* appears not to spread on any medium, and its gummy colonies always show an entire edge. In general, the spreading of lysobacter swarms is not too fast, and it may take 2–3 weeks before they cover the entire plate. The swarm sheet itself may be somewhat slimy, but otherwise it is rather unstructured and smooth. This distinguishes the lysobacter swarms from myxobacterial swarms, which almost always are morphologically differentiated with radial veins, concentric ridges, and oscillating waves or ripples, and which in addition may etch themselves deeply into the agar surface (degenerate strains may produce homogeneous, slimy swarms). On rich media like PC agar, the colonies tend to remain compact, small, with a smooth convex surface and an entire edge. Those colonies often are highly mucoid. Enough slime may be produced to drop from the colony into the lid of the inverted dish (Ensign and Wolfe, 1965). As briefly mentioned, *L. enzymogenes* produces two different colonies, one dirty-white and mucoid, the other yellowish and nonmucoid. While both colony types are always obtained when starting from white colonies, the yellow ones yield only the yellow type. Physiologically and biochemically, the two organisms are identical (Christensen and Cook, 1978). Often, small, colorless crystals are seen within the colonies (Fig. 3e). In the case of *L. gummosus,* the colonies are of a rubbery consistency.

The color of colonies of *Lysobacter* may be off-white; cream; pale to deep yellow, sometimes with a greenish hue; pinkish; salmon; or orange-brown, depending on the species and on the medium. The chemical nature of those pigments is not known. The published absorption spectra of crude extracts show maxima or shoulders at 455, 482, and 516 nm (*n*-hexane) for salmon-colored strain FP-1; 448, 466, and 470 nm (ethanol) for three yellow "myxobacters" (Stewart and Brown, 1971); and 424, 442, and 464 nm (in methanol) for the yellow strains CP-1 and CP-15 (Daft and Stewart, 1971; Daft et al., 1973). While those spectra suggest a polyene chromophore, perhaps a carotenoid, they may be spectra of a mixture of pigments and thus cannot be reliably interpreted. Many strains produce a water-soluble, dark-brown, probably melanoid pigment, which is particularly prominent in old cultures and on media containing amino acids or peptones. Color reactions of phenolic compounds indicate the presence of a mono- and diphenol oxidase (Stewart and Brown, 1971). Deep-red crystals of myxin may appear within the colonies of *L. antibioticus.* Also, many cultures give off an unpleasant *Pseudomonas*- or pyridine-like odor.

In agitated liquid media, the lysobacters grow as homogeneous cell suspensions. When gently rotated, those cultures appear silky. Depending on the medium, liquid cultures may become somewhat viscous, and those of *L. gummosus* virtually solid.

Relatively little has been published about the composition of the lysobacter cell. A typical Gram-negative peptidoglycan was demonstrated in *L. enzymogenes* strain AL-1, with *meso*-diaminopimelic acid and D-alanine crossbridges (Harcke et al., 1975). The inner and outer membrane could be separated starting from osmotically shocked cells. The outer membrane had a density of 1.30 $g \cdot cm^{-3}$ (CsCl), the inner one of 1.23 $g \cdot cm^{-3}$ (Hartmann et al., 1977). Ubiquinone Q-8 is the only respiratory quinone of *L. antibioticus* and *L. enzymogenes* (M. D. Collins, personal communication). This allows one to quickly distinguish lysobacters from myxobacteria as well as from organisms of the *Cytophaga* group (including high GC strains of *Taxeobacter*) which all contain menaquinones exclusively. The ribosomes of "myxobacter" 495 *(L. enzymogenes)* were difficult to isolate because tightly bound nucleases and proteases were attached to them and could not be removed by the usual methods (Sendecki et al., 1971). Otherwise, lysobacter ribosomes have the typical prokaryotic composition. The rRNA has the same base ratio as that of *E. coli* (GC/AU = 1.20). The presence of poly-β-hydroxybutyrate in the lytic "myxobacters" was substantiated by its conversion into crotonic acid (Stewart and Brown, 1971). The compound accumulates in the course of cultivation, with a maximum on the third day. Capnoids, a new type of sulfonolipids found in bacteria of the *Cytophaga* group (Godchaux and Leadbetter, 1983), and multicopy single-stranded DNA common in myxobacteria (Dhundale et al., 1985), are both absent in lysobacters. The lysobacter DNA has a high GC content of 65–71 mol% (Christensen and Cook, 1978; Daft and Stewart, 1971; Mitchell et al., 1969; Shilo, 1970; Stewart and Brown, 1971).

A number of metabolic enzymes have been studied in *L. enzymogenes* strain AL-1 (Guntermann et al., 1975; Hartmann et al., 1977). In the cytoplasm there is an α- and a β-glucosidase, a β-galactosidase, and an isocitrate dehydrogenase; on the cytoplasmic membrane, a

succinate dehydrogenase; and bound to the outer membrane, an alkaline phosphatase and a *N*-acetyl glucosaminidase. The activities of those enzymes change during the cell cycle. Two distinct patterns were noted, one for the five hydrolases, the other for the two dehydrogenases. In the case of the α-glucosidase, the activity increase was shown to be due to de novo enzyme synthesis. While β-glucosidase and β-galactosidase are constitutive, α-glucosidase is inducible by maltose. In a study on the phylogenetic implications of the isozyme pattern of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (important for the synthesis of aromatic amino acids) in superfamily B (to which *Lysobacter* belongs according to 16S rRNA data), *L. enzymogenes* was found to possess only one enzyme variant, the one that is sensitive to feedback inhibition by tryptophan and, unique for *Lysobacter,* ultrasensitive to chorismate (Ahmad et al., 1986). This pattern is shared only by group V pseudomonads, which fits its classification based on 16S rRNA studies. In addition, the lysobacters produce a host of exoenzymes. As some of those are of a more general interest and have found applications, they will be discussed under "Practical Aspects," this chapter.

For a large number of strains, comparative physiological and biochemical tests were performed and used for the differentiation of species (Christensen, 1989; Christensen and Cook, 1978). The strains studied for their ability to destroy cyanobacteria were not characterized in the same scheme as those able to destroy other bacteria, so that the following generalizations may not always apply to them.

The lysobacters are aerobic organisms, although many strains appear to grow best at a reduced oxygen level (10% O_2). In the oxidation-fermentation (OF) test with glucose, practically all strains show oxidative growth but many also show fermentative growth. Growth of the lytic freshwater organisms stops immediately when the culture is flooded with N_2 gas; no growth is obtained in paraffin-sealed tubes, and the generation time increases to 9 h (30°C) when the oxygen tension is reduced to 20% of the normal atmospheric pressure (Daft et al., 1975; Stewart and Brown, 1971). Catalase and oxidase are positive, but the catalase reaction is very sensitive to the culture conditions and sometimes is very weak or even absent (H. Reichenbach, unpublished observations).

The pH range is 4.5 to over 10, the optimum between 7 and 9. There is almost always a remarkable tolerance to alkaline conditions. Even the freshwater strains usually grow well at pH 9 (Stewart and Brown, 1971; Daft et al., 1975). The ability to grow at an acid pH varies with the different strains; many do not grow below pH 6. During growth on peptones, the pH may rise substantially due to ammonia production.

The temperature optimum is usually around 30°C, even for the freshwater organisms, but it varies substantially from isolate to isolate. It may be as low as 25°C or as high as 40°C. A few strains can grow at 2°C and at 50°C, but the limits often are 4 and 40°C.

The salt tolerance is usually limited to 1% NaCl or less, and no strain was found to grow in the presence of 3% NaCl.

The lysobacters are chemoorganotrophs. For many strains, NO_3^-, NH_4^+, glutamate, or asparagine serve as the sole nitrogen source whereas urea appears to be utilized only by a few strains. Some organisms, however, are more fastidious. Several of the strains that lyse cyanobacteria do not grow on inorganic nitrogen (Daft et al., 1973; Shilo, 1970), while others do (Stewart and Brown, 1971). All lysobacters grow on peptone as the only organic substrate, but the type and concentration of the peptone may be critical. Casitone (Difco) seems to be suitable in all cases, but strain FP-1 has a low optimum of 0.2% for Casitone and does not grow at 0.5%. The same strain also does not accept yeast extract, tryptone, or casamino acids, and does not grow on nutrient broth or nutrient agar (Shilo, 1970). In general, peptides appear to be the preferred carbon and energy source, and monosaccharides may only be used slowly (von Tigerstrom and Stelmaschuk, 1987b).

Glucose is probably utilized by all strains. Growth may take place on a wide spectrum of carbohydrates, e.g., in one case on: glycerol, mannitol, arabinose (weak), glucose, galactose, fructose (weak), mannose, lactose, sucrose, raffinose, and rhamnose (Veldkamp, 1955). Acid is produced from glucose and from several other sugars in a varying pattern that may be useful for the differentiation of species. Many strains grow on citrate as the sole carbon source.

All strains are strongly proteolytic as can be seen, e.g., by cultivation on skim milk agar. They liquefy gelatin, peptonize milk, and produce α-, β-, or γ-type hemolysis on sheep blood agar, including strain AL-1, which originally was reported to be negative for hemolysis (Ensign and Wolfe, 1965). Almost all lysobacters hydrolyze chitin. Veldkamp (1955) observed *N*-acetylglucosamine, glucosamine, acetic acid, and NH_4^+ as products. Several of the isolates that decompose cyanobacteria did not attack chitin (the failure was perhaps due to the method used; Stewart and Brown, 1969; Daft et al., 1975), while others do (Stewart and Brown, 1971). Crystalline cellulose (filter paper) is not

decomposed, but many strains degrade carboxymethyl cellulose, and at least some strains can even grow on it as the only organic substrate (Stewart and Brown, 1971). While agar is not liquefied or softened, gelase fields (uncolored areas) may appear upon flooding with iodine solution (Shilo, 1970). Starch, pectate, and alginate may or may not be decomposed. Many strains produce lipases that cleave Tweens.

Ammonia is produced from proteins and peptones. Nitrate is only rarely reduced to nitrite. Some strains generate H_2S. The indole, methyl red, and Voges-Proskauer tests are always negative, and the phosphatase test is always positive.

Sodium dodecyl sulfate usually diminishes growth at 0.01% and completely inhibits it at 0.1%. All lysobacters appear to be sensitive to polymyxin B, although the result may vary somewhat with the way the test is done (Christensen and Cook, 1978; Shilo, 1970; Stewart and Brown, 1971). This is a valuable distinguishing characteristic in so far as most *Cytophaga*-like bacteria are resistant to polymyxin B (Mitchell et al., 1969). All strains of *L. brunescens* as well as the freshwater lysobacters (Shilo, 1970; Stewart and Brown, 1971) also are rather sensitive to actinomycin D (around 2 μg/ml), which is in contrast to most Gram-negative bacteria but in accordance with the behavior of most other gliding bacteria (Dworkin, 1969). All *L. brunescens* strains are inhibited by chloramphenicol, penicillin G, and usually also streptomycin, but most other lysobacters are not. Strain FP-1 was also resistant to erythromycin and tetracycline, but inhibited by kanamycin and neomycin (Shilo, 1970). The strains of Stewart and Brown (1971) and of Daft et al. (1975) were sensitive to all these and several more antibiotics.

As already mentioned, the lysobacters are able to kill and disintegrate many living and healthy microorganisms, such as both Gram-positive and Gram-negative bacteria, including actinomycetes and cyanobacteria; filamentous fungi; algae; and even nematodes. Gram-negative strains are usually less readily killed and lysed than Gram-positive ones. The first signs of lysis may be observed within minutes after the culture broth has been added to a suspension of sensitive bacteria (Gillespie and Cook, 1965). The technique by which the lysobacters destroy cyanobacteria is particularly well documented (Daft and Stewart, 1971, 1973; Daft et al., 1973, 1975; Shilo, 1970; Stewart and Brown, 1970). They use two different mechanisms: 1) One strain, strain N-5 (myxobacter 44) attacks the cyanobacteria with free exoenzymes (Stewart and Brown, 1969, 1970, 1971). The first assault seems to be on the cell wall with lysozyme-like enzymes, but later the whole cell with the exception of the membranes is digested. Heterocysts and akinetes are more resistant but finally also destroyed. Only their walls remain. 2) All other strains need to be in contact with the cyanobacteria before they can lyse them (Daft and Stewart, 1973; Shilo, 1970). Since neither culture supernatants nor cell extracts are sufficient to disintegrate cyanobacteria on solid or in liquid media, enzymes bound to the surface of the lysobacter cell are probably involved. It appears that the lysobacters move actively toward their prey, perhaps attracted by the oxygen produced by photosynthesizing cells. They attach themselves with one cell pole often close to the cross-septa of the filaments, and then become fixed perpendicularly on the surface of the target cells. No special organelle for attachment is recognizable. The need for contact explains why lysis does not take place in shaken cultures. In unshaken liquid cultures, the lysobacters are found fixed to the cyanobacteria within 20 min after mixing. A single lysobacter cell may lyse a *Nostoc* cell within 20 min. It then moves on and attacks another cell in the filament. There is no specific order in which the cells along a filament are assaulted. When a lysobacter culture of a sufficiently high cell density is added to a cyanobacterial population, lysis may be complete within 1 to 3 h. The time required depends, of course, on environmental factors, notably oxygen and temperature. Thus, in a well-aerated culture at 26°C, *Nostoc ellipsosporum* is completely destroyed within 60 min. On ultrathin sections in the electron microscope, it can be seen that the first structure to disappear is the peptidoglycan layer. The cell contents are then gradually broken down, and only the cell membranes, lipid droplets adhering to them and, if present, the gas vacuoles remain. The latter rise to the surface where they can be collected. The membranes often coil up, looking in cross-sections like scrolls. Protoplasts may be released from the filaments. If there is an outer sheath, it also may disintegrate. In the case of *Oscillatoria redekei,* amorphous material disappears from the sheath and a fibrous layer is left. Heterocysts initially resist lysis but eventually also disintegrate, either under the impact of the bacterial enzymes or by autolytic processes started after the cells are released from the filaments. In infected cyanobacterial cultures, the chlorophyll *a* content and the nitrogenase activity go down concomitantly with lysis, so that either parameters can be used to measure its progress (Daft et al., 1973).

Tan et al. (1974) have devised a method to establish synchronized cultures of myxobacter AL-1: Cells of different sizes, equivalent to different stages of the growth cycle, were separated by centrifugation in a sucrose density gradient. As already mentioned, in this way, changes of enzyme activities during growth could be determined.

Taxonomy and Identification

The first step in the identification procedure is to make sure that an isolate belongs to the genus *Lysobacter.* An organism qualifies as a lysobacter if it is a chemoorganotrophic, aerobic, unicellular, gliding, Gram-negative bacterium with a GC content of 65 to 71 mol%. Those characteristics are, however, shared by the myxobacteria and by certain organisms of the *Cytophaga* group (such as *Taxeobacter*). Some myxobacteria have a very similar cell shape (for instance, *Polyangium, Sorangium,* and *Chondromyces*) but the populations never show the variation in cell length typical for lysobacters. The morphology and structure of myxobacterial swarms is usually completely different, myxobacteria produce fruiting bodies and myxospores, and they contain menaquinones instead of ubiquinones as respiratory quinones. The organisms of the *Cytophaga* group, as far as they are gliding, may form very similar soft, slimy swarm colonies, but their cells usually look much different, they often contain flexirubin-type pigments and always menaquinones. The colonies of most *Cytophaga-* like bacteria are more-or-less yellow to orange, often brightly colored, particularly on peptone agar, and turn red if covered with 20% KOH (flexirubin reaction), which is never observed with lysobacter strains. Unfortunately, *Taxeobacter,* the only genus that in its GC content comes close to *Lysobacter,* is red. It has a similar cell shape to *Lysobacter,* but its cells are much stouter and tend to arrange themselves side-by-side in a palisade like fashion. Also, *Taxeobacter* lacks the unpleasant smell of *Lysobacter.*

Presently, four *Lysobacter* species are recognized. Their differential characteristics are listed in Table 1. The separation of the species rests entirely on physiological and biochemical data and is based on phenotypic analysis of a large number of strains (Christensen and Cook, 1978). However, the relatively wide GC range of the strains of two species (65–70 mol% in *L. enzymogenes* and 66–69 mol% in *L. antibioticus*) and the substantial differences in the temperature maxima of strains of *L. brunescens* (37–50°C) suggest that more than one taxon may be hidden among the strains presently allocated to one species. First attempts to subdivide one of the species have been made (*L. enzymogenes* subsp. *enzymogenes* and subsp. *cookii;* Christensen and Cook, 1978), but before that can reasonably be done, the methods of molecular taxonomy, notably DNA-DNA hybridization, need to be used. It seems doubtful whether the occurrence of myxin can be used as a characteristic of the species *L. antibioticus,* because production of an antibiotic usually is strain- and not species-specific.

As there is a free exchange of organisms between soil and fresh water, the habitats given in Table 1 for the various species should not be taken as absolute. In fact, the "freshwater" lysobacters attacking cyanobacteria have also been found in soil (Daft et al., 1975), and we have isolated myxin-producing lysobacters from freshwater (H. Reichenbach, unpublished observations).

Two more species not shown in Table 1 also have been proposed *L. lactamgenus* (Ono et al., 1984) was studied because of its new cephem antibiotics, the cephabacins. While several characteristics of the strain are in accordance with a lysobacter, it differs substantially in its extremely high GC content (76 mol%, T_m) and in its inability to produce acid from any sugar. *L. albus* (Nozaki et al., 1987) synthesizes the novel antibiotic lactivicin. The description of this isolate is very preliminary and lacks essential data, e.g., on gliding motility and GC content. Acceptance of the new species will require that the strains be made freely accessible to others in the field.

Practical Aspects

The spectacular lytic capabilities of the lysobacters suggest that they may play an important role in the control of microbial populations in nature, although their population densities appear never to become high enough to cause a breakdown of a cyanobacterial water bloom (Daft et al., 1975). Still, it may be possible to eliminate blooming organisms in a limited area, or pathogenic or otherwise undesirable bacteria in sewage plants, like the filamentous organisms responsible for the bulking of sludge, by introducing lysobacter strains with strong, lytic enzymes. The potential is probably limited by the requirement for cell-to-cell contact as a prerequisite for lysis, which would restrict any application to nonagitated environments, such as trickling filters or dense, floating mats of the target organisms.

Table 1. Characteristics distinguishing the four species of *Lysobacter*.

Character	*L. enzymogenes*[a]	*L. antibioticus*	*L. brunescens*	*L. gummosus*
Colony morphology	Sheetlike, soft slimy	Sheetlike, soft slimy	Thin, filmlike	Compact, gummy
Colony color	Cream to greenish-yellow	Pink to greenish-yellow	Yellow to brown	Yellowish-gray
Viscosity of broth cultures	+	+	−	+++
Urea as sole N-source	V	−	−	+
Citrate as sole C-source	+	+	+	−
Acid from cellobiose	+	+	−	+
Acid from sucrose	+	+	−	+
Acid from lactose	+	V	−	+
Hydrolysis of carboxymethyl cellulose	+	+	−	+
Hydrolysis of pectate	+	−	+	+
Hydrolysis of starch	V	−	+	−
Hemolysis (sheep blood)	α, β, or γ	β or α	γ or −	α
Sensitive to chloramphenicol	V	−	+	−
Sensitive to actinomycin D	V	−	+	V
GC content, Tm (mol%)	65–70	66–69	67	66
Habitat	Soil	Soil	Fresh water	Soil
ATCC no. of type strain	29487[b]	29479[c]	29482	29489

Symbols: +, present; −, absent; V, variable.
[a]Type species of the genus *Lysobacter*.
[b]Formerly "myxobacter" or *"Sorangium"* 495.
[c]Formerly *"Sorangium"* 3C.
Adapted from Christensen (1989) and Christensen and Cook (1978).

The ability of lysobacters to synthesize new secondary metabolites is of particular importance. The following antibiotics have been isolated from lysobacters (and *Lysobacter*-like organisms): the phenazine-*N*-oxide, myxin (Peterson et al., 1966; Weigele and Leimgruber, 1967); two cyclic decapeptides, myxosidin A and B (Clapin and Whitaker, 1976, 1978; Monahan and Whitaker, 1976); the quinoline compound, G 1499-2 (Evans et al., 1978); new cephem antibiotics, the cephabacins (Harada et al., 1984; Ono et al., 1984); the acyltetramic acids, catacandin A and B (Meyers et al., 1985); a dicyclic dipeptide with lactam-like properties, lactivicin (Harada et al., 1986, 1988; Nozaki et al., 1987, 1989); and a dibasic macrocyclic peptide lactone, lysobactin (Bonner et al., 1988; O'Sullivan et al., 1988; Tymiak et al., 1989).

Of those antibiotics, myxin has been produced industrially by Hoffmann-La Roche in Nutley for some time. It was applied in the form of a copper chelate called cuprimyxin and sold under the trade name Unitrop®. In contrast to free myxin, the chelate is very stable and practically insoluble. In contact with animal tissue, myxin is gradually released, so that the complex guarantees a sustained activity. Cuprimyxin was applied topically, mainly against skin infections including dermatophytes, and its use was restricted to veterinary medicine. The antibiotic is produced by strains of *L. antibioticus*, which was originally named *"Sorangium"* 3C (in the chemical literature, it is often further mislabelled as *"Sporangium"*). Myxin acts on DNA. There exists a voluminous literature on the various aspects of myxin, which can, however, not be reviewed here (see, e.g., on the chemistry: Sigg and Toth, 1967; Weigele and Leimgruber, 1967; Weigele et al., 1971; on the mechanism of action: Behki and Lesley, 1972; Lesley and Behki, 1967; on the biological activity and application: Grunberg et al., 1967; Maestrone and Brandt, 1980; Maestrone and Mitrovic, 1974; Maestrone et al., 1972; McDonald et al., 1980; Snyder and Imhoff, 1975).

The main reason why the lysobacters initially were noticed was their rich stock of exoenzymes. Several of those enzymes have a potential for an application in research or industry. One of the *Lysobacter* proteases has been studied in great detail for theoretical reasons and is one of the best-understood enzymes. Again, the literature on this topic is by far too extensive to be reviewed here comprehensively (see below).

Strain NCIB 8501 (if the present strain is really identical with the one first described, see above) was the first lysobacter to be studied for its exoenzymes, a chitinase complex (Veldkamp, 1955). The products of the enzymatic activity are *N*-acetylglucosamine, acetate, and ammonia, but not glucose. While chitin decomposition is a common feature of the lysobacters, it appears that so far no other study has been performed on the subject. An extracellular enzyme with both β-1,4-glucanase and chitosanase activity was purified from *L. enzymogenes* strain AL-1 (Hedges and Wolfe, 1974). It does not attack chitin. Enzymes of this type are probably responsible for the often-observed hydrolysis of carboxymethyl cellulose. They could also be useful for the study of the structure of fungal cell walls. Strain "Cytophaga" L1 (= NCIB 9497) was patented for its impressive combination of fast-working hydrolytic exoenzymes (Brit. Pat. 1,048,887, 23 November 1966). The enzyme complex includes chitinase, laminarinase, lipase, elastase, keratinase, and other proteolytic activity and may be useful for the decomposition of fungal mycelia from fermentations. Using the same strain, a process was later worked out for the degradation of living yeast cells (Andrews and Asenjo, 1984; Asenjo, 1980; Asenjo and Dunnill, 1981; Asenjo et al., 1981; Hunter and Asenjo, 1987a, 1987b). The yeast lytic enzymes are produced constitutively and consist of β-(1–6)-glucanase, mannanase, and high protease activities. The breakdown of the yeast cells is connected mainly with the protease and the glucanase activities, which have to act in sequence because a specific protease must remove the mannoprotein layer in the outer cell wall before the glucanase can become active. As the glucanase is repressed if more than 0.8 g of glucose is present per liter of medium, a 20-fold-higher enzyme yield is obtained in continuous cultures with optimal dilution rates compared with batch cultures. The lysobacter glucanase appears not to adsorb to the yeast glucan, attacks the fibrillar and the amorphous glucan fractions equally well, and shows no inhibition by its hydrolysis products. If crude enzyme preparations are applied in higher concentrations, the rate of lysis may decline. This may be due to competitive inhibition by carbohydrates present in the enzyme solution. The lysobacter enzyme mixture solubilizes the yeast cells almost completely and thus appears particularly useful for the production of yeast extract and of animal feed. A two-step model has been proposed to describe the kinetics of the process of lysis. Also, the separation of the enzyme complex from the culture broth and its application in an immobilized form has been studied. It appears, however, that the free enzyme is more promising for technical purposes.

Two alkaline phosphatases were demonstrated in *L. enzymogenes* strain 495 (von Tigerstrom, 1983; 1984; von Tigerstrom and Stelmaschuk, 1986, 1987a). The appearance of both activities is repressed by inorganic phosphate. One of those enzymes is excreted into the medium, has a molecular weight of about 25 kDa, and does not contain a metal ion, which is rather unusual for a bacterial phosphatase. As the enzyme is easy to recover and purify, it might be a useful tool in nucleic acid research. The other enzyme appears to be bound to the outer membrane, has a molecular weight of about 69 kDa, and seems to be composed of at least two subunits. It is a metal enzyme probably containing Zn^{2+}, hydrolyzes a wide variety of 5′-,3′-, and 2′-ribose and -deoxyribose nucleotides, as well as sugar phosphates, and shows a remarkably high specific activity. A corresponding cell-bound phosphatase was also found in the three other *Lysobacter* species, and all four enzymes are immunologically related among themselves but completely different from cell-associated phosphatases from other gliding bacteria. The extracellular and the cell-bound phosphatase also differ in their amino acid composition.

The same strain produces two extracellular endonucleases (von Tigerstrom, 1980, 1981). One is a nonspecific nuclease (22–28 kDa) preferentially cleaving double-stranded DNA and, with reduced efficiency, single-stranded DNA and RNA, but not poly(A) and poly(C). It produces large oligonucleotides with 5′-phosphate groups. The synthesis of this enzyme is inhibited by RNA in the medium. The second enzyme is an RNAse (46–47 kDa) that also cleaves poly(A) and poly(C) but not double-and single-stranded DNA. It appears to have no base specificity. The enzyme is unusual in that it generates short oligonucleotides with 5′-phosphate ends (instead of the more common 3′-phosphate groups). The production of the RNAse is inhibited by phosphate in the medium. During its purification, a contaminating enzyme was discovered that degraded the dialysis bags. The extracellular enzymes of strain 495 are obtained with good yields in tryptone (0.4%) broth at 25°C (von Tigerstrom, 1983). While the production of nuclease, RNAse, and phosphatase is inhibited by Mg^{2+} and by Mn^{2+} above 0.1 and 0.01 mM, respectively, that of protease is stimulated by Mg^{2+} and neutral to Mn^{2+}. The ions appear to interact specifically with the synthesis of the enzymes and not with their release from the cells.

Other hydrolases excreted by lysobacters are: endoamylases found in all four species (von Tigerstrom and Stelmaschuk, 1987b), which seems surprising because the characterization of the lysobacter species excludes starch hydrolysis in almost all strains of three species (see Table 1); the paradox may be a result of the test conditions. The enzyme of *L. brunescens,* the most potent organism, has a molecular weight of 47–49 kDa and also decomposes amylopectin, amylose, and glycogen, but not dextran. The enzyme probably has no commercial interest because of its enzymatic properties and low yield. Further, all species produce two extracellular lipolytic esterases (von Tigerstrom and Stelmaschuk, 1989). One is excreted into the medium and is inducible by olive oil. As it is synthesized only after exponential growth, it appears to be under catabolite repression. The other enzyme is associated with the outer membrane and is constitutive. The two esterases differ in their substrate specificity. Both are very active on Tween 20, but only the free enzyme is able to attack olive oil. In addition, there is a third, cytoplasmic esterase which, in contrast to the extracellular enzymes, is very active on tributyrin. Finally, there is a periplasmic β-lactamase.

The most fascinating *Lysobacter* enzymes are their proteases. Two proteases were isolated from the culture supernatant of *L. enzymogenes* strain (*"Sorangium"* or "myxobacter") 495; one of them α-lytic protease, became a very important enzyme for scientific reasons. The strain was originally noted for its ability to rapidly lyse a wide spectrum of bacteria (Gillespie and Cook, 1965) and nematodes (Katznelson et al., 1964). Soon it was realized that the lytic activity was due to at least two different proteases, α- and β-lytic proteases (Whitaker, 1965), and procedures were worked out to produce and purify those enzymes (Whitaker et al., 1965a; Whitaker, 1967a). Later it was found that there are at least two more proteases in the culture broth.

A major research effort has been made to characterize the α-lytic protease. This enzyme is an alkaline serine protease (for a summary of the early work see: Whitaker, 1970) that aroused great interest because it was the first bacterial serine protease with the same amino acid sequence in its active center, Gly-Asp-Ser-Gly-Gly, as the mammalian pancreatic serine proteases (Olson et al., 1970; Whitaker et al., 1966; Whitaker and Roy, 1967), and not Thr-Ser-Met as in most other bacterial serine proteases. This suggested a common ancestor for both enzymes and invited studies on the evolution of protein structure and function. The similarity to porcine pancreatic elastase is particularly notable, a similarity which also extends to substrate specificity and kinetic properties (Kaplan and Whitaker, 1969; Kaplan et al., 1970). While the homology in the amino acid sequence is only high in certain critical regions (overall homology 18%), long sections appear conserved in the three-dimensional structure as revealed by high-resolution X-ray analyses and molecular models (55% topological equivalence). There is an even higher correspondence with *Streptomyces griseus* proteases A and B, with 35% and 36% sequence homology and 80% topological equivalence (e.g., Brayer et al., 1979; Fujinaga et al., 1985; McLachlan and Shotton, 1971). The α-lytic protease (EC 3.4.21.12; 19.8 kDa) consists of 198 amino acids and contains three disulfide bridges. It cleaves polypeptides at the carbonyl side of short neutral aliphatic amino acids, preferentially alanine (Kaplan and Whitaker, 1969). It also attacks bacterial peptidoglycans, mainly at the cross-linkages (Tsai et al., 1965). Like other serine proteases, α-lytic protease has several subsites (in this case, six) for substrate binding, with different amino acid specificities (Bauer et al., 1981). This explains why the enzyme prefers long substrate molecules over short ones. While the pH optimum for catalytic activity is reached at pH 8, substrate binding is independent of pH between pH 5 and 10.5 (Kaplan and Whitaker, 1969; Paterson and Whitaker, 1969). The enzyme shows a high stability unparalleled in any other protease (Kaplan et al., 1970), which appears to arise from a high structural rigidity (Fujinaga et al., 1985). As with other serine proteases, a strong H-isotope effect is observed: the catalytic rate drops to one-third in $^{2}H_2O$ (Hunkapiller et al., 1973). The discovery that α-lytic protease contains but one histidine (His) residue (Jurášek and Whitaker, 1967) proved particularly momentous for the elucidation of the catalytic mechanism of serine proteases, for the other enzymes all contain more than one His. This finding at once excluded a requirement of several His residues for the catalytic process, as was postulated before. The histidine in α-lytic protease is homologous with the His-57 in α-chymotrypsin and is part of the catalytic triad, Asp-102/His-57/Ser-195 (the adopted numbering is that of α-chymotrypsin). By the use of a His auxotrophic mutant, ^{13}C and ^{15}N could substantially be enriched in His-57 (Westler et al., 1982), which allowed refined NMR studies of the dissociation behavior and hydrogen bonding in the active center (Bachovkin, 1986; Bachovkin et al., 1981; Hunkapiller et al., 1973). Another approach was the use of peptide-boronic acid inhibitors forming rather stable enzyme-substrate complexes, which can be analyzed as models of

the otherwise-not-accessible transition state (Bone et al., 1987; 1989). Those studies provided deep insights into the mechanism of catalysis and substrate specificity. The gene of α-lytic protease (the progene) has also been cloned in *Escherichia coli* (Epstein and Wensink, 1988; Silen et al., 1988). At the amino end of the protease domain, the gene has an open reading frame that extends 198 (or 199: the two articles do not completely agree in the details) amino acid codons upstream and represents a very large prepro sequence. The latter consists of a typical bacterial pre (or signal) peptide of 24 (or 33) amino acids ending with a Ala-Leu-Ala-Ala cleavage site, followed by a pro region of 174 (or 166) amino acids, i.e., more than twice the size of other known bacterial propeptides. The high sequence homology observed between α-lytic protease and proteases A and B of *Streptomyces griseus* continues for 49 amino acids into the pro region (21% and 38% homology, respectively), and then falls suddenly to a low value of 7% and 3%. This suggests that the *Lysobacter* pro region is a composite of two parts, perhaps of different origin and with different functions. In fact the amino acid sequence around the cleavage site of the signal peptide shows a unique homology to a trypsin inhibitor of maize, and it was speculated that this part of the peptide may block the protease activity, while the conservative part may serve an identical function as the (much shorter) propeptides of proteinases A and B, namely, arranging the correct folding of the protease domain (Epstein and Wensink, 1988). The *Lysobacter* ribosomal binding site (AGGAG) is homologous to known *E. coli* binding sites. When the *Lysobacter* gene is fused with an inducible *E. coli* promoter and signal sequence (e.g., pho A), α-lytic protease is expressed in *E. coli* and (probably unspecifically) exported into the medium (Silen and Agard, 1989; Silen et al., 1989). Deletion of the pro region results in inactive enzyme, which is immunologically and electrophoretically indistinguishable from α-lytic protease, but presumably improperly folded. Site-specific mutation of the Ser-195 in the catalytic pocket leads to the production of a double-sized inactive protease molecule, which suggests that the removal of the propeptide is an autocatalytic process. Surprisingly, the activation (not the production) of α-lytic protease proved temperature sensitive and did not take place above 30°C. If the pro region and the protease domain were cloned separately with independently inducible *E. coli* promoters, active enzyme was obtained when both were induced concomitantly, in spite of the fact that in this case, the two peptide were not covalently connected. Obviously, the propeptide functions as a chaperonin-like, though specific, folding template. It appears that α-lytic protease is produced exclusively by strain 495.

Much less is known about the β-lytic protease, a second protease from strain 495. The enzyme is a nonserine protease and resembles mammalian carboxypeptidase and particularly the bacterial neutral proteases (Oza, 1973). Its specificity is quite distinct from that of the pancreatopeptidases. It cleaves very selectively at the carbonyl side of neutral amino acids. Thus, only the B chain of (denatured) insulin is cleaved, and that between glycine-23 and phenylalanine-24 and, much more slowly, between valine-18 and cysteine-19 (Whitaker et al., 1965b). A free α-amino group or a free terminal carboxyl next to the cleavage site is not accepted, but an amido group on the carboxyl already eliminates the blocking effect of the latter. The β-enzyme is more active than the α-enzyme on bacterial peptidoglycan rapidly hydrolyzing the muramic-acid alanine bond (Tsai et al., 1965). The β-lytic protease (19 kDa) appears to be composed of 177 amino acids and to contain two disulfide bridges and one atom of zinc (Jurášek and Whitaker, 1967). The zinc can be removed and restored again with reconstitution of enzyme activity (Whitaker, 1967b). While the apoenzyme is no longer proteolytic (Oza, 1973), the cell-wall-lytic activity was still observed (Whitaker and Roy, 1967). But conceivably the protease was simply restored by a contamination of the peptidoglycan with zinc. The pH optimum of β-lytic protease is 6.5 (Oza, 1973). The proteases of strain 495 can be produced with good yields (4–4.5 g of α- and 2 g of β-enzyme from 100 liters) on casamino acid/glucose media (Whitaker, 1967a). Also, production on media with condensed fish solubles, a by-product of the salmon canneries, seems feasible (Wah-On et al., 1980).

Finally, the proteases of *L. enzymogenes* strain ("myxobacter") AL-1 (ATCC 27796) should briefly be mentioned. The organism was isolated because it lysed cells and cell walls of *Arthrobacter crystallopoietes,* and enzymes with that property were of much interest at that time for studies on the cell wall structure of bacteria (Ensign and Wolfe, 1965, 1966). In fact, one of the AL-1 enzymes was soon successfully applied for that purpose (for a review, see Ghuysen, 1968). The enzyme is particularly useful because it cleaves the bond between muramic acid and L-alanine, making it possible to isolate the pure polysaccharide backbones of peptidoglycan. The enzyme could thus be used to demonstrate a shortening of the polysaccharide chain length during the rod-to-sphere conversion of *Arthrobacter* (Krulwich et al., 1967). At

first, only the enzyme just mentioned, AL-1 protease or, later, AL-1 protease I, was recognized and characterized (Ensign and Wolfe, 1966; Jackson and Wolfe, 1968; Jackson and Matsueda, 1970). The enzyme can be produced by fermentation in 1% yeast extract medium at 30°C for 36 h, and is recovered from the culture supernatant with a yield of 350 mg of pure enzyme from 100 liters. The AL-1 protease I (13.5 kDa) is an alkaline endopeptidase with a sharp pH optimum at 9.0. The molecule consists of 136 amino acids, with Ser, Asp, Gly, Thr, and Ala accounting for more than 50% of the residues. It contains one disulfide bridge, and does not contain a hexose molecule, as was previously suggested. The enzyme is spherical, compact, and very stable. Within minutes, it completely lyses whole cells of many Gram-positive and some Gram-negative bacteria, as well as purified peptidoglycan (Ensign and Wolfe, 1965). It cleaves about 30% of the bonds in casein and albumin, and 15% in gelatin. In the B chain of insulin only the bonds between Ala and Leu, Gly and Phe, and Val and Cys are attacked. Thus it appears that a hydrophobic residue is required at the cleavage site, but the exact specificity is not yet known. In peptidoglycan, besides the connection of the peptide side chain to the polysaccharide backbone, only the bonds between terminal D-Ala and the pentaglycine or L-Ala bridges, and bonds within the pentaglycine bridge are cleaved. The enzyme therefore became a useful tool for the elucidation of the structure of bacterial cell walls (e.g., Ghuysen, 1968; Jarvis and Strominger, 1967; Katz and Strominger, 1967; Tipper, 1969).

Also, the second protease from strain AL-1, AL-1 protease II (17 kDa), is a small, stable, alkaline endopeptidase, but it does not attack bacterial cell walls (Wingard et al., 1972). It is obtained from the culture supernatant with a yield of 10% of protease I. The enzyme is very unusual in two respects: it readily crystallizes from the column fractions, and it cleaves exclusively at the amino side of lysine. It does not remove terminal lysine residues. The enzyme consists of 157 amino acids, 50% of which are Asp, Ala, Thr, and Gly, and has a broad pH optimum between 8.5 and 9.0.

A third unusual protease from AL-1 is called endoproteinase Lys-C. This enzyme has a high although not absolute specificity for peptide and ester bonds at the carboxyl end of lysine (information leaflet Boehringer Mannheim, Biochemica-Dienst no. 42, April 1980), so that it can be used for sequencing proteins and for peptide mapping (Au et al., 1989; Boileau et al., 1982; Hofsteenge et al., 1983; Jekel et al., 1983; Jolles et al., 1983). The enzyme (30 kDa unreduced, 33 kDa reduced) is an alkaline serine protease, is very stable even in the presence of 0.1% sodium dodecylsulfate or 5 M urea, and is commercially available from Boehringer Mannheim (Mannheim, Germany).

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The *Moraxella* and *Branhamella* Subgenera of the Genus *Moraxella*

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Introduction

The *Moraxella* genus is comprised of two subgenera, *Moraxella* and *Branhamella* (Bøvre, 1984). Within the *Moraxella* subgenus are found six clearly delineated species: *M. (M.) lacunata,* the type species of this subgenus, *M. (M.) nonliquefaciens, M. (M.) atlantae, M. (M.) phenylpyruvica,* and *M. (M.) osloensis.* In addition, there is one species of uncertain taxonomic status, *M. urethralis* (species incertae sedis). The *Branhamella* subgenus consists of four species: *M. (B.) catarrhalis,* the type species of this subgenus, *M. (B.) caviae, M. (B.) ovis,* and *M. (B.) cuniculi.*

Characteristics common to both subgenera of *Moraxella* include Gram-negative staining reactivity, lack of pigmentation, a positive oxidase test using either the tetra- or dimethyl-*p*-phenylenediamine reagent, catalase reactivity, and lack of motility. In addition, organisms within this genus are uniformly asacharolytic and chemoorganotrophic. Optimum growth occurs under aerobic conditions at a temperature of 33–35°C. With the exception of *M. (B.) catarrhalis* and *M. (M.) nonliquefaciens,* most strains are inhibited by low concentrations of penicillin. The GC content of the DNA varies between 40.0 and 47.5 mol% for all species of the *Moraxella* genus.

The principal distinguishing feature between the *Moraxella* and *Branhamella* subgenera of the *Moraxella* genus is cellular morphology. Species within the *Moraxella* subgenus typically produce large rods that are often blunted and plump, frequently appearing almost coccid in shape. In contrast, *Moraxella (Branhamella)* species form small cocci, usually occurring in pairs with flattened adjacent sides. In this respect, they closely resemble *Neisseria* species. Characteristics useful in identifying the species of both the *Moraxella* and *Branhamella* subgenera of the *Moraxella* genus are presented in detail in the most recent edition of *Bergey's Manual of Systematic Bacteriology* (Bøvre, 1984).

The *Moraxella* Subgenus

With the exception of *M. (M.) bovis,* all species of the *Moraxella* subgenus are found in association with mucosal surfaces of humans. They are uncommonly recovered as causes of disease. However, when found as etiologic agents of infection, the most frequent disease associations are as follows: *M. (M.) lacunata,* conjunctivitis; *M. (M.) nonliquefaciens,* acute tracheobronchitis; *M. (M.) atlantae, M. (M.) phenylpyruvica,* and *M. (M.) osloensis,* opportunistic systemic pathogens in profoundly immunocompromised individuals; and *M. urethralis,* a possible cause of acute urethritis and cystitis in women. *Moraxella (M.) bovis* on the other hand, is an important veterinary pathogen and will, therefore, be discussed further.

Moraxella (Moraxella) bovis

Moraxella (M.) bovis is a common cause of infectious bovine keratoconjunctivitis (IBK) (Gil-Turnes and Ribeiro, 1985). This disease most frequently affects calves and is often seen in the setting of an epidemic. The causative agent probably resides on the ocular and nasal mucosa of asymptomatic immune cattle as part of the normal commensal bacterial flora (Pugh and MacDonald, 1986). One phenotypic characteristic that has been most strongly associated with disease-producing strains of *M. (M.) bovis* is the presence of fimbriae on the cell surface (Moore and Rutter, 1987). Fimbriae appear to be a primary virulence determinant. Nonfimbrinated strains are avirulent in experimental infections of the corneas of cattle and laboratory mice (Marrs et al., 1988). Furthermore, antibody reactive with fimbrial antigens administered passively or acquired following vaccination with purified fimbriae is protective (Ostle and Rosenbusch, 1985).

The fimbriae of *M. (M.) bovis* can be divided into seven serogroups based on patterns of enzyme-linked immunsassay (ELISA) reactivity, tandem two-dimensional crossed-immunoelec-

trophoresis, and slide agglutination (Moore and Rutter, 1987). Humoral immune protection is fimbrial antigen-specific. The fimbriae mediate attachment to cultured bovine corneal cells in vitro; the attachment is abrogated by specific homologous antiserum (Moore and Rutter, 1989). There is no evidence that strains of one particular fimbrial serogroup are more virulent than others.

Expression of fimbrial proteins has been extensively studies in one strain (Epp 63) of *M. (M.) bovis.* This strain can express either of two different fimbrial proteins, alpha and beta (Marrs et al., 1988). The phase variation noted between the expression of alpha and beta fimbrial proteins appears to be under control of an approximate 2-kb region of DNA within the fimbrial gene. The amino acid sequences of both the alpha and beta fimbrial proteins have been determined and are approximately 70% homologous (Ruehl et al., 1988). Interestingly, beta-fimbrinated strains of *M. (M.) bovis* were significantly more virulent than alpha-fimbrinated strains when inoculated into calves eyes (Ruehl et al., 1988). Initial studies support the notion that other strains of *M. (M.) bovis* also produce two types of fimbriae and that this might be a characteristic of all fimbrinated strains within the species (Ruehl et al., 1988).

A second characteristic associated with virulence of *M. (M.) bovis* is hemolytic activity when grown on blood-containing medium (Kagonyera et al., 1989). Hemolytic strains appear to be more infectious for calves and laboratory mice and also demonstrate greater cytotoxicity for bovine neutrophils. In addition, cattle that have experienced IBK typically are found to have high titers of antihemolysin antibody (Ostle and Rosenbusch, 1985).

The *Branhamella* Subgenus

Among the four recognized species of the *Branhamella* subgenus of *Moraxella, M. (B.) catarrhalis* is clearly the most important. The other three species, *M. (B.) caviae, M. (B.) ovis,* and *M. (B.) cuniculi,* have been found in association with guinea pigs, sheep, and rabbits, respectively, although their significance in health or disease is unknown. *M. (Branhamella) catarrhalis* is, on the other hand, a well-documented commensal and pathogen of humans and thus, will be further discussed.

Moraxella (Branhamella) catarrhalis

First known as *Micrococcus catarrhalis* in the early 1900s, this organism came to be referred to as *Neisseria catarrhalis* during the 1950s due largely to its morphologic resemblance to members of the *Neisseria* genus (i.e., it consists of Gram-negative, kidney bean-shaped diplococci) and because of its positive oxidase reactivity, another characteristic of *Neisseria* species. Catlin (1970) proposed that this organism be renamed *Branhamella catarrhalis* in honor of the noted microbiologist Sarah Branham. Separation of *B. catarrhalis* from the *Neisseria* genus was predicated on a demonstrated lack of chromosomal DNA homology between the two, genetic transformation experiments which indicated unrelatedness, and widely varying DNA base composition. The taxon *Branhamella catarrhalis* was quickly adopted during the 1970s and has become widely applied since then.

Subsequent to the work of Catlin, however, Bøvre and colleagues provided additional information demonstrating a clear genetic and physiologic relatedness between *Branhamella catarrhalis* and species of the *Moraxella* genus, in particular *M. nonliquefaciens* (Bøvre, 1979). Based on their studies, Bøvre advocated assignment of the *Branhamella* genus to one of two subsgenera of the *Moraxella* genus; the other subgenus was designated *Moraxella* and contained all species of the previously designated genus *Moraxella* (Bøvre, 1984). As noted above, this taxonomy has now been adopted.

M. (B.) catarrhalis exists as part of the commensal flora of the upper respiratory tract. It is also recognized as a cause of a variety of human infectious diseases (Doern, 1986). The most important of these, at least with respect to incidence and morbidity, are acute otitis media, sinusitis, and infections of the lower respiratory tract. Otitis media and sinusitis due to *M. (B.) catarrhalis* are primarily found to occur in infants and young children. By contrast, *M. (B.) catarrhalis* lung infections usually occur in adults, in particular the elderly with some predisposing condition. The most common predisposition is chronic obstructive pulmonary disease.

MORPHOLOGY. *M. (B.) catarrhalis* may be visualized directly in clinical material obtained from infected patients by use of the Gram stain or by cultivation on suitable growth media with resultant biochemical identification. The Gram-stain morphology of *M. (B.) catarrhalis* noted previously is characteristic, but does not serve to differentiate this organism from *Neisseria* species which may look identical.

ISOLATION. *M. (B.) catarrhalis* grows readily on standard nonenriched laboratory media such as brain heart infusion and tryptic soy digest. Sup-

plementation of media with blood (e.g., 5% sheep blood agar) or boiled blood plus vitamin/amino acid enrichments (e.g., chocolate agar) yields enhanced growth (Doern and Morse, 1980). *M. (B.) catarrhalis* grows in ambient air at a wide range of temperatures, (i.e., 20–42°C), but optimum growth is achieved in 5% CO_2 at 35–37°C. A defined medium has been described for propagation of *M. (B.) catarrhalis* in which sodium lactate, proline, aspartate, arginine, glycine, and methionine suffice as source of carbon, nitrogen, and growth cofactors (Juni et al., 1986).

A variety of selective media have been described for use in recovering *M. (B.) catarrhalis* from sources likely to be contaminated with other bacteria or with fungi (Corkill and Makin, 1982; Soto-Hernandez et al., 1988; Vaneechoutte et al., 1988a). A common feature of these media is the inclusion of vancomycin, trimethoprim, and an antifungal agent to inhibit growth of Gram-positive bacteria, Gram-negative bacteria, and fungi, respectively. *M. (B.) catarrhalis* is resistant to all three antibiotics. Enhanced recovery of *M. (B.) catarrhalis* has been described when a fourth inhibitory substance, i.e., acetazolamide, a synthetic sulfonamide, was added to selective media already containing vancomycin, trimethoprim, and amphotericin B (Vaneechoutte et al., 1988a).

IDENTIFICATION. *M. (B.) catarrhalis* is catalase- and oxidase-positive but fails to ferment glucose, maltose, sucrose, and lactose (Doern and Morse, 1980). Proline aminopeptidase and gamma glutamylaminopeptidase are lacking. All strains produce extracellular DNAse and reduce nitrates to nitrites and ammonia. In addition, butyric acid esterase is produced (Riley, 1987). This enzyme can be detected using colorimetric assays with tributyrin or idoxyl butyrate as substrates or fluorimetrically using 4-methylumbelliferyl-butyrate as a substrate (Dealler et al., 1989; Janda and Ruther, 1989; Vaneechoutte et al., 1988b).

Taking advantage of the DNAse reactivity of *M. (B.) catarrhalis*, a selective and differential medium has been devised for the recovery and presumptive identification of this organism (Soto-Hernandez et al., 1988). Vancomycin, trimethoprim, and amphotericin B are added to DNAse test agar. Colonies of Gram-negative diplococci which grow on this medium are tested for DNAse production by exposure to a drop of toluidine blue O solution. Development of a purple color in the area adjacent to colonies is taken as a positive reaction.

CELL ENVELOPE CHARACTERISTICS. *M. (B.) catarrhalis* has been found to possess eight outer membrane proteins when analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Bartos and Murphy, 1988). These proteins range in molecular weight from 21–98 kDa and are remarkably conserved since there is only minimum variation in the outer membrane protein profiles among different strains of this species.

The lipopolysaccharide (LPS) of *M. (B.) catarrhalis* is also extremely homogeneous (Murphy, 1989). Its polysaccharide moeity is comprised of a small and simple seven-residue oligosaccharide consisting of D-glucose (4 mol) and D-galactose, 2-amino-2-deoxy-D-glucose, and 3-deoxy-D-manno-octulosonic acid (1 mol each) (Johnson et al., 1976). This oligosaccharide appears to be common to all strains of *M. (B.) catarrhalis*, although minor epitope differences in the antigenic composition have been noted (Murphy, 1989). The specific LPS of *M. (B.) catarrhalis* has not been observed in any other bacteria.

Preliminary observations also indicate the presence of fimbriae on the surface of at least some strains of *M. (B.) catarrhalis* (Murphy, 1989). It is possible, but yet unproven, that these surface appendages participate in the attachment of fimbrionated strains to receptors on epithelial cells of mucosal surfaces of the respiratory tract in humans who are colonized.

OTHER PHENOTYPIC CHARACTERISTICS. Restriction endonuclease digestion of chromosomal DNA has proven to be a useful tool for determining strain relatedness among different isolates of *M. (B.) catarrhalis* (Patterson et al., 1989). Four of eight restriction endonucleases, Hae III, Hind III, Pst I, and Cla I, produce distinguishable digestion patterns. Digests obtained from Hae III are the most definitive.

Other phenotypic characteristics that have been investigated include susceptibility to normal human serum-mediated bacteriolysis, ability to agglutinate human group O erythrocytes and trypsin sensitivity (Soto-Hernandez et al., 1989). Although a variety of interesting relationships among these attributes have been described for apparently unrelated strains of *M. (B.) catarrhalis*, no correlation with virulence has been proven.

The large majority of clinically significant strains of *M. (B.) catarrhalis* produce β-lactamase (Jorgensen et al., 1989). The enzyme is produced constitutively, is encoded for by chromosomal genes, and is produced in very small amounts and remains tightly cell-associated (Farmer and Reading, 1986; Labia et al., 1986).

It probably confers resistance to penicillin and ampicillin on strains that produce it (Doern and Tubert, 1987). Based on isoelectric profiles, the β-lactamase of *M. (B.) catarrhalis* exists in numerous different forms (Labia et al., 1985; Stobberingh et al., 1986). Two of them, designated Ravasio and 1908, or BRO-1 and BRO-2, respectively, appear to be most common (Farmer and Reading, 1986; Nash et al., 1986). BRO-1-type β-lactamase-producing strains can be distinguished from strains that produce the BRO-2-type enzyme by a number of different characteristics. These include the specific pattern obtained when β-lactamase isoelectric profiles are determined, the substrate affinity profile of the two β-lactamases, the relative prevalence of these strains (i.e., BRO-1-type strains are much more common among clinical isolates) and the absolute amount of enzyme produced. BRO-1-type strains produce significantly more enzyme than do BRO-2-type strains. Finally, BRO-1-type strains not surprisingly appear to be more resistant to penicillin and ampicillin than are BRO-2-type strains (Nash et al., 1986).

Aside from the variable activity of penicillin and ampicillin, the pattern of antimicrobial susceptibility of strains of *M. (B.) catarrhalis* is strikingly homogeneous (Doern and Tubert, 1988). Vancomycin, trimethoprim, and clindamycin are uniformly inactive. Colistin or polymycin B and the isoxazoly penicillins (methicillin, nafcillin, and oxacillin) have variable activity. All other agents are highly active.

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CHAPTER 178

The Genus *Legionella*

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Legionnaires' Disease and Legionellosis

Legionnaires' disease is the commonly accepted name for an acute purulent bacterial pneumonia caused by *Legionella pneumophila.* The name resulted from the investigation of an explosive outbreak of pneumonia (189 cases with 29 deaths) among the approximately 3,000 delegates to the annual convention of the Pennsylvania Division of the American Legion in July 1976. Although the exact source of the infection was never determined, epidemiologic investigations demonstrated a strong association between disease acquisition and the amount of time spent in or near the headquarters hotel (Fraser et al., 1977). Initial attempts at isolation of the causative agent were unsuccessful, but subsequent attempts resulted in the isolation of a novel bacterium, *L. pneumophila,* from lung tissue removed at the autopsies of several of the patients (McDade et al., 1977). The new bacterium was not related to any recognized at that time and was placed into a new family, the Legionellaceae (Brenner et al., 1979).

The recognition of *L. pneumophila* as an important new etiologic agent was quickly followed by the discovery of additional outbreaks of Legionnaires' disease and the realization that Legionnaires' disease was in no way a new infection. Epidemics have been documented to have occurred as early as 1957 (Osterholm et al., 1983). The earliest known isolation of *L. pneumophila* occurred in 1947 from a guinea pig, which had been inoculated with blood from a patient with an unknown febrile illness (Bozeman et al., 1968). It is now well recognized that Legionnaires' disease occurs world-wide and that members of the family Legionellaceae are ubiquitous in distribution.

Additional organisms were also quickly recognized as belonging to the genus *Legionella.* An organism isolated by Tatlock (1944) from the blood of a patient who was involved in an outbreak of febrile illness among soldiers at Fort Bragg, NC, was subsequently shown to be a member of the new family and named *L. micdadei* (Hebert et al., 1980). Interestingly, the febrile illness in the patient from whom Tatlock recovered this *Legionella* isolate was subsequently shown to be caused not by *L. micdadei* but by *Leptospira interogans* serotype autumnalis and the role of the *L. micdadei* isolate in the patient's original illness is unknown (Gocehnour et al., 1952; Tatlock, 1982). A third historical isolate, WIGA, also isolated by Bozeman et al., (1968), was subsequently shown to be a member of the new family and named *Legionella bozemanii* in her honor (Brenner et al., 1980). The decade following the discovery of these important bacteria was marked by a rapid identification of additional members of the genus. At the current time, there are 29 named species and two subspecies in the genus *Legionella* which together account for 47 serovars (Table 1). There are also a number of isolates representing additional species that have been partially characterized but remain to be named (Brenner, 1987).

Clinical and Epidemiologic Features

Most of what is known about *Legionella* infections is derived in large part from the study of infections caused by *L. pneumophila.* The data that are available from the study of other *Legionella* infections, however, indicate that they are substantially similar. Collectively, these infections are referred to as legionellosis. Legionella infections have occurred in both sporadic and epidemic distributions. A second form of the disease, called Pontiac fever, was first recognized among visitors to a health department building in Pontiac, MI. Pontiac fever is a self-limiting nonpneumonic disease consisting of fever and vague constitutional symptoms but does not include pneumonia (Glick et al., 1978).

Table 1. Phenotypic characteristics of *Legionella* species.

Species (serovar)	Oxidase	β-Lactamase	Motility	Hippurate hydrolysis	Gelatin liquefaction	Autofluorescence
L. pneumophila (14)[a]	+[b] or V[c]	+	+	+	+	−[d]
L. feeleii (2)	−	−	+	V	−	−
L. micdadei	+	−	+	−	−	−
L. spiritensis (2)	+	+	+	−(w)	+	−
L. longbeacheae (2)	+	V	+	−	+	−
L. jordanis	+	+	+	−	+	−
L. oakridgensis	−	+(w)[e]	−	−	+	−
L. wadsworthii	−	+	+	−	+	−
L. sainthelensi	+	+	+	−	+	−
L. hackeliae (2)	+	+	+	−	+	−
L. maceachernii	+	−	+	−	+	−
L. jamestownensis	−	+	+	−	+	−
L. santicrucis	+	+	+	−	+	−
L. israelensis	−	NR	+	−	+(w)	−
L. cincinnatiensis	+	−	+	−	+	−
L. brunensis	−	+	+	−	+	−
L. moravica	−(w)	+	+	−	+	−
L. quinlivanii	−	−	+	−	+	−
L. bozemanii (2)	V	V	+	−	+	+(BW)[g]
L. dumoffii	−	+	+	−	+	+(BW)
L. gormanii	−	+	+	−	+	+(BW)
L. anisa	+	+	+	−	+	V(BW)
L. cherii	−	+	+	−	+	+(BW)
L. steigerwaltii	−	+	+	−	+	+(BW)
L. parisiensis	+	+	+	−	+	+(BW)
L. tucsonensis	−	+	+	−	+	+(BW)
L. birminghamensis	V	+	+	−	+	+(YG)[h]
L. rubrilucens	−	+	+	−	+	+(R)[i]
L. erythra	+	+	+	−	+	+(R)

[a]Includes subsp. *pneumophila*, subsp. *fraseri*, and subsp. *pascullei*. The numbers in parentheses indicate the number of serovars.
[b]Positive.
[c]Variable.
[d]Negative.
[e]Weak reaction.
[f]Not reported.
[g]Blue-white.
[h]Yellow-green.
[i]Red.
Adapted from Brenner (1987).

The reasons why patients develop one or the other form of infection are not known, but clearly Legionnaires' disease and Pontiac fever represent opposite ends of a clinical spectrum. Different persons exposed simultaneously to the same environmental source of *Legionella* have developed either Pontiac fever or Legionnaires' disease (Girod et al., 1982). In addition, guinea pigs exposed to the air-conditioning system in the Pontiac Health Department building developed pneumonias rather than Pontiac fever (Kaufmann et al., 1981). Pontiac fever-like illness has also been caused by *L. feeleii* (Herwaldt et. al., 1984) and *L. micdadei* (Goldberg et al., 1989).

Legionellosis is an acute purulent pneumonia that appears to begin with the inhalation of bacteria-laden aerosols. Following an incubation period of 2–10 days, patients often develop a prodromal illness, which is often described as "flu-like" and consists of malaise, lethargy, myalgia, and headache. As the disease progresses, patients develop high fever, chills, and a nonproductive cough accompanied by shortness of breath and pleuritic chest pain. Slightly fewer than 50% of patients with Legionnaires' disease suffer nausea, vomiting, and a watery diarrhea. Neurologic symptoms are also not uncommon and include headache, mental confusion, and even seizures (Kirby et al., 1980; Edelstein and Meyer, 1984; Yu et al., 1982; Strampfer and Cunha, 1987). The radiographic features of legionellosis are not sufficiently specific to differentiate legionellosis from other in-

fections. Most frequently, patchy alveolar infiltrates, which may eventually progress to consolidation, can be demonstrated. Spread of the pneumonia to another lobe of the lung is not uncommonly seen and pleural effusions may also be present.

Legionellosis has occurred among all age groups (range 2–84 years), but occurs most predominantly among persons over the age of about 50 (England et al., 1981). Various studies have attempted to calculate the incidence of legionellosis in various populations. Such studies are difficult to compare because of differing study populations and criteria for infection (Reingold, 1988). The reported incidence has been as low as 1% among members of a prepaid health group and as high as 26% among patients hospitalized with community-acquired pneumonia (Foy et al., 1979; Marrie et al., 1981). In addition to age, a number of other important risk factors have also been identified. The incidence of disease is almost three times greater in males than in females and the risk of infection is also increased in smokers, those with underlying chronic diseases, and other forms of immunosuppression (Fraser et al., 1977; England et al., 1981; Kirby et al., 1980). Renal and heart transplant patients appear to have increased risk of legionellosis as well (Dowling et al., 1984; Fuller et al., 1985).

Person-to-person spread of legionellosis is not known to occur (Marrie et al., 1986). Humans also appear to be the only natural hosts. Although horses have been found to have antibodies against legionellae, attempts to produce legionellosis experimentally in horses produced only asymptomatic seroconversion (Cho et al., 1983; Cho et al., 1984.)

Pathology and Pathogenesis

Pathologic Features

It is suspected that legionellosis arises after inhalation of aerosols or droplet nuclei that contain the bacteria. Most of the pathologic changes present in the lung of humans occur only in the alveoli, suggesting that the infecting agent is inhaled. A pathologically similar disease has been produced in guinea pigs following exposure to aerosolized legionellae (Baskerville et al., 1981). It has also been suggested that legionellosis might arise from aspiration of organisms from the oropharynx (Johnson et al., 1985). This indeed is possible and legionellosis has been produced in experimental animals by instillation of bacteria directly into the trachea, a technique that mimics aspiration. In animals so infected, the pneumonia uncharacteristically is in the portions of the lung nearest the major bronchi rather than in the peripheral segments seen in animals (and humans) infected via inhalation of aerosols (Winn et al., 1982).

Histologically, legionella infection is confined to the alveoli and bronchioles whereas the larger bronchi are spared. The exudate contained within the alveoli is composed of both polymorphonuclear leukocytes and alveolar macrophages. Early in the disease, the infiltrate is composed mostly of polymorphonuclear cells, while later in the infection, alveolar macrophage tend to predominate. Smaller lesions tend to coalesce into larger ones as the disease progresses, but there is very little destruction of the lung architecture and frank abscess formation only occurs in the most severe cases. In very late infections, lysis of the cells in the center of the infected area often occurs. Fibrin and edema fluid may be present in the alveoli but there is little or no blood. (Blackmon et al., 1981; Winn and Myerowitz, 1981). Other changes that are sometimes present, such as hyaline membrane formation and interstitial edema, are more likely related to the supportive care of the patient than to the infection.

Extrapulmonary infections with legionellae have also been infrequently reported and can occur in the absence of clinical Legionnaires' disease. Legionellae have been identified from hemodialysis fistulae (Kalweit et al., 1982), pericarditis (Nelson, 1984; Mayock et al., 1983), wound and skin infections (Brabender et al., 1983; Ampel et al., 1985), and even a perirectal abscess (Arnow et al., 1983). Bacterial endocarditis has been documented on prosthetic heart valves (McCabe et al., 1984; Tomkins et al., 1988).

Early investigations of legionellosis were hampered because the stains commonly applied to lung tissue (Brown-Brenn, Fite-Faraco, etc.) failed to detect bacteria in lung tissue that was obviously compromised by a purulent process. The bacterium was first visualized by Chandler et al. (1977), who employed the Dieterle silver impregnation stain on human lung tissue. Use of this stain enabled the demonstration that the bacteria are highly associated with the inflammatory cells in infected tissues. Extracellular bacteria are present primarily in areas of the lung where lysis of the inflammatory exudate (leukocytoclasis) has occurred. Large numbers of apparently multiplying bacteria are found in the cytoplasm of alveolar macrophage.

Bacteremia may occur in many patients with *Legionella* infection. Up to one-third of patients with culture-proven legionellosis in one series (Rihs et al., 1985) had positive blood cultures.

The bacteremia most likely leads to the seeding of other organs remote from the lung and is probably responsible for the extrapulmonary infections already mentioned. Soluble antigens of legionellae are detectable in the urine of many infected patients. In a small number of patients, this antigen may actually be present during the prodromal phase of the illness (Sathapatayavongs et al., 1982) and may continue to be shed for several months after illness.

As previously mentioned, many patients with legionellosis also display one or more symptom referable to organ systems other than the lung, such as mental confusion, diarrhea, abnormalities of liver function, and hyponatremia. These observations suggest that *Legionella* may indirectly affect other organs, perhaps by secreting one or more toxins (Friedman, 1978). The short incubation period (36 h) and lack of symptoms referable to the respiratory system also suggest that the symptoms of Pontiac fever may also result largely from intoxication rather than infection.

Pathogenesis and Virulence

Legionellae are facultative intracellular bacteria with pathologic properties that are intimately related to their ability to disarm and multiply within professional phagocytes. In the lung of both humans and experimental animals, large numbers of apparently multiplying bacteria can be visualized in the cytoplasm of alveolar macrophages. Extracellular bacteria are mainly present only in those areas of the lung where lysis of the inflammatory cells has taken place (Winn and Myerowitz, 1981). Multiplication of the legionellae has been demonstrated in vitro in peripheral blood monocytes (Horwitz and Silverstein, 1980) and in these cells, the doubling time of the bacteria may be as short as 2 h.

Studies in guinea pigs suggest that the polymorphonuclear leukocyte (PMN) is important in host defense against the legionellae. Although the first cells to encounter legionellae in the lung are the resident alveolar macrophage, the first cells recruited into the lung following infection are PMNs, which are present by the end of the first day. The numbers of alveolar macrophages present do not increase for about 3 days following infection. When the cellular infiltrate from infected guinea pigs was fractionated by cell type, the numerous viable bacteria were found to be associated with the macrophage fraction. In contrast, few bacteria were recovered on culture of the PMN fraction, but light and electron microscopy demonstrated that large numbers of dead bacteria were present in the PMNs (Davis et al., 1983). Similarly, Fitzgeorge et al. (1988) have demonstrated that guinea pigs that are made neutropenic are more susceptible to infection with *L. pneumophila* and suffer higher mortality than normal guinea pigs.

In vitro studies of the PMN, in contrast, do not demonstrate a protective role for these cells. Phagocytosis of *L. pneumophila* in vitro by PMNs requires the presence of antibody and complement, but few of the infecting bacilli are killed. PMN cultures that are co-infected with *Legionella* and *Escherichia coli* kill the *E. coli* while the legionellae survive (Horwitz and Silverstein, 1981). The reason for the apparent dichotomy between the in vivo and in vitro observations is not clear.

In vitro observations of the interaction between macrophages and legionellae complement those made in vivo and are outlined schematically in Fig. 1. Phagocytosis of legionellae by macrophages is enhanced in the presence of antibody plus complement and attachment of the bacteria to these cells is mediated by complement receptors, which fix complement via the alternate pathway (Payne and Horwitz, 1987). Phagocytosis of *L. pneumophila* has been reported to occur by a novel process, termed "coiling phagocytosis," whereby a pseudopod coils around the bacterium during its internalization by the phagocyte (Horwitz, 1984). The significance of this unusual phagocytic process is not understood. It has been demonstrated that phagocytosis of *Legionella* can be inhibited by cytocholasin D, suggesting that the phagocytic process may be only slightly different from that employed by other microorganisms (Elliot and Winn, 1986). Rechnitzer and Blom (1989) have also reported that phagocytosis of the Knoxville 1 strain of *L. pneumophila* and of *L. micdadei* did not involve coiling phagocytosis and that only the Philadelphia 1 strain appeared to be taken up by this mechanism.

The intracellular behavior of the legionellae is most fascinating. The internalized bacteria are surrounded by a membrane-bound phagolysosome, which eventually becomes associated with smooth vesicles. Later, these phagosomes become surrounded by mitochondria, which later are replaced by what appear by microscopic observation to be mitochondria (Fig. 2) (Horwitz, 1983a). The normal fusion of lysosomal granules and the phagosome does not occur in *L. pneumophila*-infected monocytes. Acidification of the phagosome also does not occur (Horwitz, 1983b; Horwitz and Maxfield, 1984), permitting the bacteria to multiply within and ultimately cause the rupture of the infected phagocyte. Monocytes that have phag-

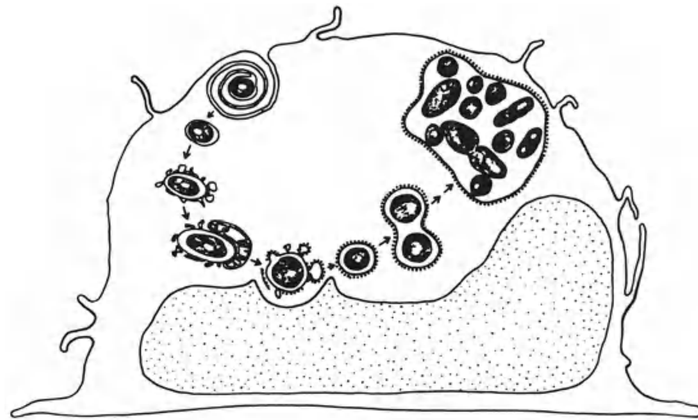

Fig. 1. Diagram of the sequence of cytoplasmic events involved in the intracellular multiplication of *L. pneumophila.* (a) Phagocytosis occurs by engulfment of the bacterium in a coiling pseudopod. (b) The internalized bacterium is surrounded in a membrane-bound vacuole, which is associated first with smooth vesicles within 15 min after ingestion. (c) Within 1 h after infection, the vesicles become surrounded by one or more mitochondria in addition. (d) At about 4 h after ingestion, the vesicles become surrounded by rough vesicles and ribosomes and by 8 h, the vesicle is studded entirely by ribosomes. (e) Multiplication of the bacteria occurs within these ribosome-studded vacuoles, eventually resulting in the rupture of the cell. (From Horwitz, 1983a.)

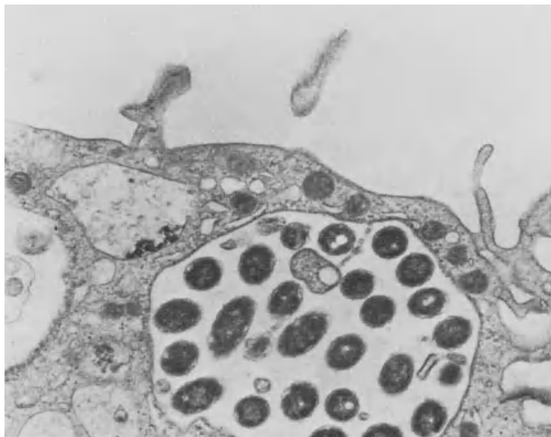

Fig. 2. *L. pneumophila* multiplying in a guinea pig alveolar macrophage. The bacteria are contained in a vacuole, which is studded with ribosomes.

ocytosed avirulent legionellae do not contain ribosome-studded phagosomes. In these cells, phagosome-lysosome fusion and phagosome acidification occur in normal fashion. While the legionellae are still not killed by these phagocytes, at the same time they neither replicate within nor kill the phagocyte (Horwitz, 1987).

Virulence Factors

While much is known about the intracellular behavior of the legionellae, the search for the virulence factors that underlie this behavior has to date been frustrating. The unique intracellular life cycle of these bacteria suggests that virulence must be multifactorial. Virulence must involve at least four attributes, as demonstrated in vitro. 1) Bacteria must be recognized and phagocytosed in order to gain entrance into the favored site for multiplication. This, as previously discussed, is known to be mediated by complement receptors on the bacterial cell (Payne and Horwitz, 1987). 2) Once within the bacterial cell, the organisms must inhibit phagosome-lysosome fusion, and also resist the oxidative killing mechanisms of the phagocyte. 3) Evasion of the oxidative killing mechanisms must be required because it has been shown that an avirulent mutant is not killed even in the presence of phagosome-lysosome fusion (Horwitz, 1987). 4) Having entered the cell and resisted or abrogated its defenses, a virulent bacterium must be capable of multiplication within the cell. How the legionellae effect such a life cycle continues to be the subject of much investigation.

Most of the legionellae, with the exception of *Legionella micdadei,* produce a 39-kDa metalloprotease that can be recovered from culture filtrates (Thompson Miller and Iglewski, 1981). The enzyme is cytotoxic for cultured chinese hamster ovary cells and causes the hydrolysis of canine erythrocytes (Keen and Hoffman, 1989). Conlan et al. (1986) reported that the instillation of this protease into the lungs of guinea pigs produced a pattern of cellular destruction which resembled that seen in Legionnaires' disease, but it was not demonstrated that such destruction in natural infections is caused by this enzyme. Blander and Horwitz (1989) demonstrated that animals immunized with the purified protease produced antibody against the molecule and displayed evidence of cell-mediated immunity to the protease. Such vaccinated animals were also resistant to aerosol challenge with virulent *L. pneumophila.* Later studies, however, showed that vaccinated animals were not immune to infection by a pro-

tease-deficient mutant, suggesting that while the enzyme may modulate the immune response, it is probably not a virulence factor.

A highly basic protein on the surface of *L. pneumophila* has been identified by Cianciotto et al. (1989). Mutants made deficient in the genetic sequence coding for this protein were found to be less capable of penetrating a macrophage-like cell line than was the isogenec parent strain. However, once internalized, the mutant strain appeared to be as capable of multiplication as the parent strain. These workers named this protein and the gene coding for it MIP (for macrophage infectivity promoter).

Saha et al. (1988) identified an acid phosphatase in *L. micdadei* that interferes with superoxide formation by stimulated human PMNs. The purified enzyme has been shown to hydrolyze phosphatidyl inositol 4,5-bisphosphate, an important intermediary molecule in the production of reactive oxygen intermediates by human PMNs. Since most legionellae contain such enzymes, they also may be an important virulence factor. At this time, however, the relationship of this property to virulence remains indeterminate since the enzyme can be isolated from highly passaged and hence avirulent organisms and a similar biochemical "lesion" has not been detected in PMNs infected with legionellae.

L. pneumophila, L. micdadei, and several other species produce novel cytotoxin, which might play some role in virulence. The molecules are small (300–3,400 kDa) peptides, which are heat and acid-resistant. The toxin from *L. pneumophila* has been shown to be cytotoxic for chinese hamster ovary cells (Friedman et al., 1980). The toxin also inhibits the respiratory burst and subsequent production of reactive oxygen intermediates by PMNs (Friedman et al., 1982), as does a similar product from *L. micdadei* (Hedlund, 1981). What role if any these molecules may play in virulence remains to be determined since these compounds have been isolated from laboratory strains of unconfirmed virulence.

Studies of immunity against legionellosis have also uncovered another potential virulence factor for the legionellae; the ability to scavenge iron from the cytoplasm of macrophages. Byrd and Horwitz (1989) discovered that gamma-interferon-activated human monocytes could limit the intracellular growth of *L. pneumophila* by decreasing the number of transferrin receptors present on the cell surface thereby limiting the availability of iron to the intracellular bacteria. Treatment of the immune cells with iron transferrin restored their ability to support growth of the legionellae. Likewise, an iron chelating agent, deferoxamine, prevented the multiplication of *L. pneumophila* in nonimmune cells. How legionellae are able to obtain needed iron from the intracellular milieu is not yet known. Siderophores have not been detected in *Legionella* to date (Reeves et al., 1983).

Therapy

The epidemiologic investigation of the Philadelphia outbreak of Legionnaires' disease suggested that erythromycin was an effective therapeutic agent and that β-lactam and aminoglycoside agents were not (Fraser at al., 1977). Subsequent studies in animals (Fraser, 1978) confirmed the efficacy of erythromycin against the legionellae. In addition, rifampin, the tetracyclines, and the combination of sulfamethoxazole with trimethoprim have been demonstrated to be efficacious in infected animals (Edelstein Calarco and Yasui, 1984; Pasculle, et al., 1985). The susceptibility of legionellae to antibiotics can be measured by in vitro susceptibility tests (Pasculle et al., 1981), but these tests do not predict which agents will be therapeutically efficacious because in vitro tests do not take into account whether or not these agents can penetrate cells. Most species of *Legionella* produce a broad spectrum β-lactamase and are not susceptible to penicillins and cephalosporins. A relatively new class of agents, the fluoroquinolones (such as ciprofloxacin) also appear to have both in vitro and in vivo activity against the legionellae and may also find clinical use (Saito et al., 1986).

On a weight basis, rifampin appears to be the agent most active against the legionellae. Rifampin, however, is never used alone for the treatment of legionellosis because it rapidly selects for resistant subpopulations of legionellae (Dowling et al., 1982). Most patients are treated with erythromycin alone or in combination with rifampin for 3 weeks. Tetracyclines and sulfamethoxazole/trimethoprim have also been reported to be therapeutically effective. The response of patients to therapy is variable. In some patients the clinical response to antimicrobial therapy may appear delayed and the patient may even seem to worsen after therapy begins. Legionellae may be detected by immunofluorescence or culture after up to 10 days of therapy, but this observation also does not correlate with the patient's ultimate response to therapy (Kirby et al., 1980).

Diagnosis

Culture

Culture remains the most sensitive test for the laboratory diagnosis of legionellosis (Edelstein

Meyer and Finegold, 1980; Zuravleff et al., 1983.; Edelstein, 1987; Pasculle et al., 1989). The sensitivity of culture methods varies not only with the type of specimen but also from laboratory to laboratory. Whether this phenomenon reflects variation in intra-laboratory proficiency or the varying quality of commercially prepared media is not known. Culture is most sensitive (about 90%) when applied to specimens such as those obtained by transtracheal aspirates or bronco-alveolar lavage, since these are not usually contaminated with oral bacteria. Culture of sputum specimens is much less sensitive and the sensitivity of culture in hospitalized patients may be somewhat lower, about 65–75%. The sensitivity will vary primarily because of variations in the prevalence of cefamandole-resistant bacteria (especially *Pseudomonas* and enterococci) in various hospitals.

The legionellae are not known to colonize humans (Bridge and Edelstein, 1983; Baumgardner et al., 1988.). Thus, the specificity of culture methods appears to be close to 100% and any patient from whom the organism is isolated should be presumed to have legionellosis. A variety of ancillary procedures are used to enhance cultural diagnosis of legionellosis. A discussion of these may be found elsewhere in this chapter.

Direct Antigen Detection

IMMUNOFLUORESCENCE. Direct fluorescent antibody staining (DFA) has been employed for the rapid detection of legionellae in both tissue and respiratory secretions (Broome et al., 1979). The major benefit of this technique is that it can be performed rapidly with results usually available within 2 h. The sensitivity of DFA in various laboratories has not been constant. Some laboratories have reported a sensitivity of 25–30% for DFA (Zuravleff et al., 1983; Buesching et al., 1983) while other have found the sensitivity of the test to be in the order of 70% (Edelstein, 1987; Pasculle et al., 1989). The reasons for the widely varying estimates of sensitivity are not fully understood but may include differing patient populations, methodological differences such as volume of specimen examined, or even laboratory proficiency. The above not withstanding, all laboratories find to a greater or lesser extent that culture is more sensitive than DFA.

Direct immunofluorescence has additional limitations as well. As the number of serovars has increased, the number of fluorescent conjugates required to detect all possible organisms has become unmanageable. At present, there are 47 serovars in the genus. Even more problematic is that conjugated antisera are not available commercially for most *Legionella* serotypes, making complete examination of specimens possible in only a few reference laboratories. On the other hand, since over 90% of all legionella infections are caused by *L. pneumophila* serogroups 1 through 6 and *L. micdadei* (Reingold et al., 1984), detection of the greatest majority of cases is possible using commercially available antiserum pools that contain antibodies for these organisms. Many American laboratories limit their immunofluorescent testing to these seven serovars and rely on culture (which should always be performed in parallel with DFA) to detect other serovars.

The specificity of DFA in most laboratories has been high (93–99%) (Edelstein, 1987; Pasculle et al., 1989). However, in areas of low disease prevalence, this level of specificity considerably lowers the predictive value of a positive test. A number of bacterial species are known to be stained by *Legionella* DFA conjugates. These include *Pseudomonas fluorescens, P. aeruginosa, P. putida,* and *Bacteroides species* (Tenover et al., 1986; Edelstein et al., 1980). There are also reported cross-reactions between *Legionella* and *Bordetella pertussis* (Benson et al., 1987; Ng et al., 1989).

Since legionellae are often present in water distribution systems, the buffers and other aqueous reagents used in the DFA procedure can become contaminated with these bacteria, producing false positive tests (Ristagno and Sarovolatz, 1985). This type of false positive reaction is easily prevented by membrane filteration of all the aqueous reagents used for the DFA test.

DFA staining is not usually applied to environmental specimens because of unsuitably low specificity. In some studies involving environmental specimens, fewer than 50% of samples that tested positive by DFA yielded *Legionella* upon culture (Fliermans et al., 1981.). Whether this is the result of cross-reactivity or of the presence of dead or dormant legionellae in these specimens is not actually known.

The first DFA reagents available were produced in rabbits and it is these polyclonal reagents that have been associated with most of the false positive reactions. Monoclonal antibody (MAb) reagents are now commercially available. One MAb recognizes all serotypes of *L. pneumophila* while additional reagents are also available for *L. micdadei* and *L. dumoffii.* These monoclonal reagents have been demonstrated to be of equivalent sensitivity to the polyclonal reagents and to have higher specificity (Edelstein et al., 1985; Cercenado et al., 1987.). In addition, the polyclonal reagents produce less

nonspecific florescence of the background material. The *L. pneumophila* MAb appears to be directed against a species-specific protein antigen, probably in the outer membrane. Because of this, the MAb does not react with organisms in formalin-fixed specimens (Edelstein et al., 1985). One false positive reaction caused by spores of *Bacillus cereus* spores in an environmental specimen has been reported (Flournoy et al., 1988).

Nucleic Acid Probes

A DNA probe which hybridizes with the rRNA of *Legionella* has been commercially produced. Initial studies using bacterial isolates indicated that the probe could detect all 22 *Legionella* species tested and did not react with other common and uncommon bacterial species (Edlestein, 1986; Wilkinson et al., 1986). A retrospective premarket study of the probe using frozen respiratory tract specimens demonstrated that the sensitivity of the DNA probe was about 75% compared to culture and the specificity was 99% (Edelstein et al., 1986). Two prospective studies of the commercial product have been performed. One (Doebbeling et al., 1988) found the sensitivity and specificity to be 50 and 99% respectively, while the other (Pasculle et al., 1989.) reported values of 71 and 99%. The probe appears to be equivalent to DFA testing, but not as sensitive as culture. The major advantage of such a reagent is the ability to detect all species and serogroups with a single reagent. The probe has two major disadvantages: its somewhat higher cost limits its use to high-volume laboratories; and the test probe employs an ^{125}I label and thus has a relatively short shelf life of 6 weeks.

Two other DNA probes have been prepared experimentally. Grimont et al. (1985) used radiolabeled chromosomal restriction endonuclease fragments to demonstrate in situ hybridization on nitrocellulose filters. Another group used cloned DNA, which codes for the major outer membrane protein of *Legionella* as a probe to detect the organism in lung tissue of mice (Engelberg et al., 1986).

Despite their differing compositions, all the above DNA probes appear to be about equal in sensitivity and are reported to detect between 10^4 and 10^5 colony-forming units/ml. Amplification of *Legionella* DNA in artificially seeded tap water has been accomplished using the polymerase chain reaction (Starnbach et al., 1989). It was reported that as few as 35 colony-forming units of *L. pneumophila* could be detected.

Urinary Antigen Detection

Legionella urinary antigen can be detected in the urine of patients with Legionnaires' disease by a number of immunologic methods. Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) appear to be more sensitive than latex agglutination. The sensitivity of the test has ranged from 70–100% (Sathapatayvayongs et al., 1982.; Aguero-Rosenfield and Edelstein, 1988) and the specificity appears to be in the 99–100% range as well. One study found that false positive ELISA tests could be eliminated by boiling the urine prior to testing (Sathapatayvayongs et al., 1982).

The RIA test has recently become commercially available. A retrospective evaluation of that reagent found it to be 100% sensitive and specific (Aguero-Rosenfield and Edelstein, 1988.). The major limitation of the tests for antigenuria is that the antibody employed to date detects the antigen of only *L. pneumophila* serogroup 1. Thus, in certain areas where other *Legionella* serotypes and/or species are more prevalent, the predictive value of a negative test will be unacceptably low. Also, the use of a radioisotope (^{125}I) in the RIA method will make the method undesirable for many laboratories. Finally, antigen may be present in the urine of some patients for several months after recovery, slightly diminishing the predictive value of a positive test.

Antigens of other *Legionella* serogroups also appear to be detectable in the urine (Kohler et al. 1987), suggesting that a polyvalent screening system might be possible. Alternatively, the production of a broadly reactive antibody that detects multiple serotypes and species has been demonstrated. Its sensitivity appears to be equivalent to the monovalent serogroup 1 reagent (Tang and Toma, 1986). If multivalent or panvalent antibody reagents become commercially available, the urinary antigen test may very likely become the method of choice for rapid screening for legionellosis.

Antibody Detection

Serological diagnosis is still often used despite a number of significant problems. Antibodies against the legionellae can be detected by a number of methods, including indirect immunofluorescence (IFA), ELISA, and microagglutination tests. The IFA test continues to be most often employed for the serological diagnosis of legionellosis. The sensitivity of the IFA test appears to be about 75% when paired sera are tested, while the specificity is above 99% in most series (Wilkinson et al., 1981). A number of factors reduce the diagnostic utility of serological testing. Most importantly, the antibody response following infection is variable. In patients with legionellosis documented by culture,

only about 25% of patients may have demonstrable antibody during the first 2 weeks following infection. Some patients do not appear to develop antibody for up to 6 weeks following infection documented by cultures and 15–20% of such patients fail to develop antibody at all (Kirby et al., 1980). Thus, for maximal diagnostic yield, paired sera, often collected 6 weeks apart, must be tested. Thus, serological testing can do little to establish the diagnosis of legionellosis in a critically ill patient within a clinically relevant time frame.

The varying prevalence of antibody (1–20%) among various populations and the probable lifelong persistence of antibody also make the interpretation of single antibody titers unreliable. Single IFA antibody titers of ≥256 appear to be associated with both recent and remote *Legionella* infection and cannot reliably establish a diagnosis of legionellosis in a critically ill patient. This is particularly true in areas where the antibody prevalence is known to be high. The most reliable diagnostic yields result from the demonstration of fourfold rises in antibody titer in paired sera with a titer of ≥128 in the convalescent serum (Wilkinson et al., 1981) These interpretive criteria are also often applied to the serological diagnosis of infections caused by other *Legionella* species and serotypes, but criteria for the interpretation of serologic titers for these organisms has not been conclusively established (Wilkinson et al., 1983). Finally, while antibody of the IgG, IgM, and IgA classes are produced in response to *Legionella* infection, they appear to be produced concomitantly in many patients and remain elevated. Thus, it is important to employ reagents that can detect both IgG and IgM. The detection of IgM alone does not always demonstrate recent infection (Wilkinson, 1987).

Serological testing also cannot determine the identity of the *Legionella* serovar causing disease in a particular patient. Patients with culturally proven legionellosis may produce antibody to another serovar or even to multiple serovars. The nature of the serological response seems to be influenced more by the patient than by the identity of the infecting organism (Wilkinson et al., 1983; Fallon and Johnston, 1987). Because of this, the interpretation of many published studies of the prevalence of legionellosis is subject to some skepticism (Reingold, 1988). In addition, serological cross-reactions have been reported in the sera of patients with *Bacteroides* infections and in patients with cystic fibrosis, presumably due to *Pseudomonas aeruginosa.* (Edelstein et al., 1980; Collins et al., 1984).

Isolation

L. pneumophila was initially isolated in guinea pigs and in embryonated eggs (McDade et al., 1977), as were some of the other early species in the genus. A number of artificial media have been developed that support growth of the legionellae. Artificial media are superior to inoculation of guinea pigs (Edelstein et al., 1982) because specimens with low numbers of organisms produce only subclinical infection of the animals. The legionellae require iron and cysteine in order to initiate growth on artificial media, and the first medium upon which *L. pneumophila* was cultivated consisted of Mueller-Hinton agar to which hemoglobin and the supplement IsoVitaleX were added (Feeley et al., 1978). Ferric pyrophosphate was found to be an acceptable substitute for hemoglobin, and L-cysteine was found to be the active component in IsoVitaleX. The addition of these compounds to a basal medium consisting of casein, beef extract, and starch produced F-G agar (Feeley et al., 1978) upon which many isolations of *L. pneumophila* were made. Feeley et al. (1979) subsequently developed a more sensitive semisynthetic charcoal-based medium, charcoal yeast extract agar. Good control of the pH of the medium was necessary since the legionellae initiated growth over a very narrow range of pH (6.85–6.95) and the addition of a zwitterionic buffer, ACES, to charcoal yeast extract agar by Feeley's group (Pasculle et al., 1980) resulted in buffered charcoal yeast extract agar (BCYE), which remains the medium of choice for the isolation of the legionellae. Edelstein (1982) added α-ketoglutarate to BCYE agar and demonstrated a marked increase in both the speed of growth and in the plating efficiency of the medium. It is this version, commonly referred to as BCYE-α, which is most widely used.

BCYE agar is very rich and in no way selective for legionellae. A number of modifications have been made to BCYE agar to produce selective and differential media for specialized use. Edelstein (1982) added cefamandole, polymyxin B, and the antifungal agent anisomycin to produce a semiselective medium that is often employed for clinical specimens such as sputum, which may contain other bacterial species. Wadowsky and Yee (1981) added glycine (to inhibit Gram-negative bacteria) vancomycin, and polymyxin B to produce a medium that they found highly useful for culturing environmental sites heavily contaminated with other bacteria. Vickers et al. (1981) found that the addition of bromcresol purple and bromthymol blue dye to these media enabled *L. micdadei* to be differentiated from other legionellae by the blue color

of their colonies on the agar. Selective media should not be used alone for clinical specimens since they are somewhat inhibitory for small numbers of legionellae (Roberts et al., 1987).

The pH of the medium is exceedingly critical and should be held between 6.85 and 6.95 (Feeley et al., 1979; Pasculle et al., 1980). Media should probably be poured in a darkened room to prevent the photochemical oxidation of yeast extract and the light-catalyzed autoxidation of cysteine. These oxidations produce inhibitory superoxide anion and peroxides (Hoffman et al., 1983). Adequate quality control of the media is necessary to ensure that the media are of good quality. Legionella isolates that have been subcultured several times on agar no longer constitute an adequate quality control challenge for these media and a virulent strain of bacteria maintained in homogenates of human or guinea pig lung should be used for this purpose (Feeley et al., 1979; Kealthly and Winn, 1984). All the media previously discussed are currently available from commercial vendors but the quality can be highly variable (Kealthly and Winn, 1984).

Additional techniques are often used to increase the yield of legionellae from clinical and environmental specimens. Treating specimens with an HCl-KCl buffer (pH 2.2) prior to inoculation on agar media kills many contaminating bacteria more rapidly than the legionellae in environmental specimens (Bopp et al., 1981; Roberts et al., 1987). This procedure also results in about a 10% greater yield of positive specimens when used with clinical samples (Buesching et al., 1983). Tissue specimens may harbor substances that are inhibitory for the legionellae. Dilution of such specimens at least 10—fold in bacteriological broth also may increase the yield of positive cultures from such specimens (Lattimer et al., 1980; Wilkinson, 1987). Other specimens that are dilute or otherwise contain small numbers of bacteria should be concentrated by centrifugation before inoculation onto media. Such specimens include pleural fluids, trans-tracheal aspirates, bronchoalveolar lavage fluids, and pericardial fluids.

Several methods have been employed to collect and process environmental specimens, which frequently contain small numbers of legionellae. Some method of concentration is often employed. These include centrifugation (Voss et al., 1984) and membrane filtration (Orrison et al., 1981). Both techniques appear to be of equal efficiency (Brindle et al., 1987).

Several liquid media have been developed for propagation of the legionellae for antigenic, metabolic, and other studies. Two chemically defined media contain complex mixtures of amino acids, vitamins, and trace elements (Pine et al., 1979; Warren and Miller, 1979). Ristroph et al., (1980) developed a novel semisynthetic broth medium, which consists of filter-sterilized yeast extract with cysteine and ferric pyrophosphate. Sterilized agar may be added to produce a semisynthetic agar and ACES buffer has also been added to this medium (Dowling et al., 1982) to produce more reliable growth. Wild type strains of *Legionella* do not appear to initiate growth well in liquid media. Growth of small inocula is variable and the lag phase may last over 24 h. Strains that have been transferred in broth culture several times appear to become somewhat adapted to growth in these media. Whether this poor growth of legionellae in liquid media is a characteristic of the genus or reflects remaining deficiencies in the available media is currently not known. Nevertheless liquid media are never relied on for the isolation of these bacteria from clinical or environmental sources.

Legionella has been isolated from blood by a variety of techniques including biphasic blood culture bottles (Edelstein et al., 1979), lysis centrifugation (Dorn and Barnes, 1979), and conventional radiometric methods (Rihs et al., 1985). However the diagnostic utility of routinely performing such blood cultures remains to be established.

Buffered Charcoal Yeast Extract Agar

N-(2-acetamido)-2-aminomethane sulfonic acid (ACES)	10 g
Yeast extract (Difco)	10 g
Charcoal (Norit SG)	2 g
Agar (Difco)	17 g
α-Ketoglutarate (monopotassium salt)	1 g
L-Cysteine $HCl \cdot H_2O$ (in 10 ml distilled water)	0.4 g
Ferric pyrophosphate (in 10 ml distilled water)	0.25 g
Distilled/deionized water	950 ml

Dissolve ACES completely in water, warming water if necessary. Add α-ketoglutarate and yeast extract. Adjust pH to 6.95 with 1 N KOH (40–45 ml) and then bring volume to 1 liter with water. Add charcoal, agar, and dissolve by boiling. Autoclave for 15 min at 121°C. Allow to cool to 50°C and add filter-sterilized cysteine and ferric pyrophosphate solutions, always adding the cysteine first. Check pH and adjust with 1 N KOH if necessary.

Swirl the flask or use a magnetic stirring bar while pouring plates to keep the charcoal particles suspended in the medium. Pour 25 ml per plate to resist drying out during prolonged incubation. It may be beneficial to pour media in a darkened room to prevent generation of peroxides.

Precautions: The ferric pyrophosphate must be kept dry (desiccator) and the solution must be freshly made just prior to use. If the powder changes from its green

color (yellow or brown), it is no longer usable. This solution must be made fresh each time it is required. Ferric pyrophosphate can be readily dissolved in water by heating it to 50°C.

Identification

Colonial Morphology

Colonies of legionellae appear on BCYE agar within 36–48 h of incubation. Recognition and preliminary identification of the bacteria are facilitated by the use of a dissecting microscope for the examination of the media. *Legionella* colonies display a characteristic appearance resembling "ground glass" when viewed under obliquely transmitted light. The colonies are gray, convex with an entire edge that usually displays a birefringent band, which is either greenish blue or pink depending on the species. As the colonies become several days older, the ground-glass appearance and the birefringent edge tend to disappear and the colonies appear more like those of other bacteria. When the colonies are illuminated with a Woods' lamp the colonies of most species will exhibit a dull yellow fluorescence (Table 1). The colonies of *L. bozemanii, L. gormanii, L. dumoffii,* and several other species display a very striking blue-white autofluorescence under similar conditions of illumination while colonies of *L. rubrilucens* and *L. erythra* produce a red fluorescence. (Feeley et al., 1978; Brenner et al., 1985).

Cellular Morphology and Biochemical Characteristics

The legionellae are fastidious bacteria that require iron and cysteine for primary isolation. Growth does not occur on common laboratory media such as blood or chocolate agar. Despite their apparent acid resistance (Bopp et al., 1981), the legionellae appear only to initiate growth on artificial media over a very narrow pH range of 6.8–7.0. The optimum temperature for human isolates appears to be 35°C, but isolates from warmer environments, such as hot water systems, may grow at higher temperatures. Growth of the legionellae on the agar media mentioned earlier, such as FG agar, appear to be stimulated by incubation in a 5–10% CO_2 atmosphere. In contrast, growth on yeast extract-based media (CYE and BCYE) appears not to be stimulated by incubation in an atmosphere enchriched with CO_2 (Feeley et al., 1978; Pasculle et al., 1980.). They do not grow appreciably under anaerobic conditions.

Legionella cells are small, Gram-negative rods, 1–2 μm in length and 0.5 μm wide. Filamentous forms up to 20 μm may also be present in some cultures, particularly after prolonged passage in artificial media (Pine et al., 1979). Their ultrastructural arrangement is typical for Gram-negative rods, with typical trilaminar inner and outer membranes. *L. micdadei* has a unique electron dense layer in the periplasmic space that is not seen in *L. pneumophila* (Gress and Myerowitz, 1980). Several species, except *L. micdadei,* appear to have an extracellular polysaccharide capsule or slime layer that can be vizualized by ruthenium red staining (Hebert et al., 1984), even though the bacteria appear unencapsulated by other means. Staining characteristics depend to some degree on the source of the bacteria. Typically, the legionellae in clinical specimens are not reliably stained by the Gram stain, although bacterial cells from artificial media stain somewhat better. The substitution of carbol fuchsin for the saffranin counterstain in the Gram technique makes the legionellae more readily visible. Staining of the legionellae can also be accomplished using crystal violet (de Freitas et al., 1979) and methylene blue (Pasculle et al., 1980). *L. micdadei* is unusual in that the cells are weakly acid fast when present in tissue but readily lose this property after a single passage on artificial media. The reason for this is unknown (Pasculle et al., 1980). The legionellae can also be readily stained by the Gieminez stain (McDade, 1978) and the Dieterle silver (Chandler et al., 1977) stains, but neither technique is specific for these bacteria.

The legionellae are chemoorganotropic and they do not possess a glucose transport system and do not appear to ferment any other carbohydrates as well. Early studies using several defined media suggested that methionine, arginine, threonine, serine, isoleucine, leucine, and valine are required by the legionellae, in addition to cysteine. Serine, glutamate, and perhaps threonine appear to serve as the primary carbon and energy sources for the legionellae and are catabolized via the Krebs cycle. Carbohydrate synthesis occurs through gluconeogenesis via the Embden-Meyerhof pathway (Pine et al., 1979; George et al., 1980; Keen and Hoffman, 1984; Tesh et al., 1983).

Members of the genus *Legionella* cannot be identified with any degree of certainty using traditional biochemical testing. The few phenotypic tests that can be used to separate these organisms into groups are given in Table 1. All species are motile via one to three polar or subpolar flagella and the flagella of all legionella species appear to be antigenically identical. Only *L. oakridgensis* appears to be nonmotile (Thomason et al., 1979; Orrison et al., 1983).

Tests for nitrate reductase and urease are negative for all species. Most strains are reported to be catalase positive when whole cells are tested for the ability to decompose hydrogen peroxide. Studies using cell-free extracts, however, have demonstrated the *L. pneumophila* and *L. gormanii* have only peroxidase activity whereas other *Legionella* species in actuality do possess catalase (Pine et al., 1984). Most species, except *L. feeleii* and *L. micdadei,* liquify gelatin. Most strains of *L. pneumophila* and some strains of *L. Feeleii* hydrolyze hippurate. Most strains produce a beta-lactamase that is active against cephalosporins and can readily be demonstrated using nitrocefin (Marre' et al., 1982.).

Cellular Lipids

The cell wall of the legionellae is somewhat unique among Gram-negative bacteria in that it contains large amounts of branched chain fatty acids and only minor amounts of hydroxy acids (Moss et al., 1977). When there were few species in the genus, the determination of the presence of these unusual fatty acids was a rapid means for the presumptive identification of these bacteria. With the subsequent recognition of multiple species in the genus, the determination of fatty acid profiles is useful primarily for the initial examination of an isolate. Both quantitative and qualitative measurements of fatty acid content are made by gas-liquid chromatography. Using such quantitative measurements, Lambert and Moss (1989) were able to divide the first 23 *Legionella* species into three major groups.

The legionellae also are unusual in that their isoprenoid quinones are ubiquinones with side chains containing greater than nine isoprenoid units (Karr et al., 1982; Lambert and Moss, 1989). Lambert and Moss (1989) were able to divide 23 *Legionella* species into four groups and differentiate *L. feeleii* from all the other legionellae by quantitative estimation of their ubiquinone content. The determination of fatty acid and ubiquinone profiles is generally too complex for routine laboratory use. These procedures are, however, very helpful in the preliminary characterization of isolates by reference laboratories.

Identification Techniques

The identification of *Legionella* isolates ranges from a simple task for common species to a very complicated task for new species. Traditionally, the first step is to demonstrate the requirement of an isolate for cysteine, which is characteristic of the genus *Legionella* (Brenner et al., 1988). In most laboratories, this is done by inoculating the isolate onto a BCYE agar plate from which the cysteine has been omitted (Wilkinson, 1988). Other cysteine-deficient media, such as sheep blood agar, may be used for this purpose, but occasional strains of other heterotrophic environmental bacteria may be encountered that will grow on BCYE and not on blood agar (Thacker et al., 1981). Isolates can also be preliminarily assigned to the genus by reacting them with a commercially available DNA probe, which appears to be highly specific for members of the genus (Wilkinson et al., 1986; Edelstein, 1986).

Other methods may eventually come into use to assign organisms to the genus *Legionella.* Most of the antibody in patients' sera appears to be specific for serogroup-dependent determinants in the lipopolysaccharide of the legionellae (Ciesielski et al., 1986.). There are, however, protein antigens associated with the cell surface that may be common to some or all species (Hindahl and Iglewski, 1986; Nolte and Conlin, 1986). The genetic locus for the major outer membrane protein of *Legionella* pneumophila appears to contain both genus- and species-specific regions. An experimental DNA probe for this locus has already been prepared (Engelberg et al., 1986). Butler et al. (1985) demonstrated extensive cross-reactions by immunoblotting between the major outer membrane proteins of 10 *Legionella* species. Similarly, Sampson et al. (1986) were able to demonstrate a common 58-kDa protein antigen in the outer membrane of the first nine *Legionella* species. The use of a genetic or immunological probe to detect one or more of these common antigenic determinants could also be used to assign isolates to the genus.

The identification of the various recognized species and serovars of *Legionella* is most readily achieved by serological methods. Fluorescein-conjugated antisera for direct immunofluorescence testing can be used for the rapid presumptive identification of isolates (Cherry et al., 1978; Wilkinson, 1987). Since *L. pneumophila* accounts for the greatest preponderance of clinical isolates (Reingold et al., 1984), most clinical isolates can indeed be identified using such commercially available antisera. Antibody conjugates are available commercially for *L. pneumophila* serogroups 1–6, *L. bozemanii, L. dumoffii, L. gormanii, L. micdadei,* and *L. longbeachae* serotypes 1 and 2. A monoclonal antibody conjugate that reacts with at least 10 serogroups of *L. pneumophila* is also available, as are similar conjugates for *L. micdadei* and *L. dumoffii* (Edelstein et al., 1985; Cercenado et al., 1987).

A simple slide agglutination test for the identification of the legionellae has also been developed. Extensive crossreaction among the legionellae requires that the polyclonal antisera used for this procedure and for immunofluorescence be subjected to reciprocal adsorption to remove cross-reacting antibodies. Thacker et al. (1985) were able to identify 22 *Legionella* species using slide agglutination reagents. The only organisms not identifiable were *L. bozemanii* and *L. parisiensis,* whose cross-reactions cannot be removed. Despite its rapidity and simplicity, slide agglutination reagents are not available commercially.

Identification of Strains

Epidemiologic studies are greatly enhanced by the recognition of common clones or strains among isolates. The earliest studies employed simple serotyping to recognize isolates that were different from each other. However, the preponderance of isolates belonging to *L. pneumophila* serogroups 1 and 6 limits the use of this technique to those situations involving other species and serotypes. Plasmid profiling has been used to characterize environmental and clinical isolates in several outbreaks. A large majority of isolates appear not to contain plasmids, but when present the identification of plasmid types has been useful (Mikesell et al., 1981; Brown et al., 1982). In addition to comparing plasmids by molecular weight, it is also possible to further characterize plasmids of similar size by analyzing the fragments produced after treatment of the plasmids with restriction endonucleases (Nolte et al., 1984.)

Panels of monoclonal antibody reagents have been developed for typing isolates of *L. pneumophila* serogroup 1. (Joly et al., 1983; McKinney et al., 1983; Watkins et al., 1985). A panel of standardized reagents has been developed that could separate *L. pneumophila* serogroup 1 into 10 major subgroups (Joly et al., 1986). A panel of two monoclonal antibodies for *L. pneumophila* serogroup 6 has also been developed, which could divide these isolates into two groups. In conjunction with other typing methods, monoclonal antibody typing of serogroup 6 isolates is also useful for epidemiologic purposes (McKinney et al., 1989).

Analysis of restriction endonuclease fragments of chromosomal DNA has also been employed to evaluate the relationships between isolates of *L. pneumophila, L. micdadei,* and *L. dumoffii.* The advantage of this technique is that it permits comparison of species and serotypes that do not contain plasmids and/or for which monoclonal antibodies are not available (Tompkins at al., 1987).

Multilocus enzyme electrophoresis (MEE) has also been applied to the study of the legionellae at both the strain and species level. In this analysis, as applied to *Legionella,* the electrophoretic mobility of a panel of 22 bacterial enzymes is measured. Differences in electrophoretic mobility of a particular enzyme result from small genetic changes in the structure of the enzyme, which do not affect enzyme function. Selander et al. (1985) could divide *L. pneumophila* into 62 distinct electrophoretic types and identified two groups of strains that were actually new species of *Legionella* (see below). Edelstein et al. (1986) compared MEE with monoclonal antibody testing and plasmid analysis of strains involved in a large outbreak and found that MEE was the most sensitive typing system in that it separated the strains into the largest number of types. Tompkins et al. (1987) found MEE and analysis of chromosomal restriction fragments of approximately equal sensitivity. McKinney et al. (1989) identified 11 MEE types among isolates of *L. pneumophila* serogroup 6, and in conjunction with two monoclonal antibodies were able to divide this group of organisms into 12 separate types. Two-thirds of the isolates fell into a single group.

Taxonomy

Isolates that cannot be identified by the above methods must be submitted to a reference laboratory for identification. Definitive identification requires the examination of cellular lipids, as described above, for preliminary characterization and also genetic analysis. The genus *Legionella* was initially defined by Brenner et al. (1979), who examined several isolates of the newly discovered Legionnaires' disease bacillus and concluded that they represented a new family, genus, and species. Since then, the genus has been redefined twice to take into account the ever-increasing number of species and subspecies. The creation of a single genus to hold this collection of species has been challenged by a small group of investigators.

Oligonucleotide cataloging of *Legionella* 16S rRNA has demonstrated that the legionellae are not closely related to other groups of organisms, but are closely related to each other at the ribosomal level. They are distantly related to the purple sulfur bacteria and their nonphotosynthetic relatives (Ludwig and Stackebrandt, 1983; Brenner, 1987). Other taxonomic studies have also demonstrated the uniqueness of the genus *Legionella.* Brenner (1987) reported that hybridization studies using the DNA which

specifies rRNA demonstrated that the legionellae were most closely related (approximately 60%) to common members of the *Enterobacteriaceae* and to *Pseudomonas,* all of which are also related at the ribosomal level to the purple photosynthetic bacteria.

The GC content of the DNA of species in the genus is 38 to 52 mol% (Brenner et al., 1985, 1988). The various *Legionella* species share up to 67% DNA relatedness at optimal hybridization temperature while strains within a given species have 70% or greater relatedness and <5% divergence in related sequences. It is upon this basis that the 29 species have been created within the genus (Brenner, 1987; Brenner et al., 1988; see also Table 1).

Garrity et al. (1982) recovered a number of isolates belonging to *L. pneumophila* serogroup 5, which were 15–42% related to the reference strain of *L. pneumophila* serogroup 5. They concluded that these isolates represented a species closely related to but separate from *L. pneumophila.* A taxonomic study of *L. pneumophila* isolates, using multilocus enzyme analysis, identified two clones among a collection of isolates with closely related, but not identical allotypes. Isolates belonging to these clones belonged to *L. pneumophila* serogroups 1, 4, and 5 (including the strains studied by Garrity) and an unnumbered serotype, Lansing 3. The analysis suggested that these two clones represented two species closely related but not identical to *L. pneumophila* (Selander et al., 1985). Upon further examination of their DNA, Brenner et al. 1988) also concluded that these two groups of clones form two distinct DNA hybridization groups. Organisms within each group are highly related (88–98%) to each other but were only distantly related (40–50% under stringent conditions) to *L. pneumophila,* with which most isolates share common antigens. Since these organisms can only be differentiated from each other in most cases by DNA hybridization studies rather than phenotypic testing, it was proposed that the species *L. pneumophila* be divided into three subspecies. The species formerly known as *L. pneumophila* was changed to *L. pneumophila* subsp. *pneumophila.* The second hybridization group was named *L. pneumophila* subsp. *fraseri,* and the third group, *L. pneumophila* subsp. *pascullei* (Brenner et al., 1988). While the creation of these subspecies is of taxonomic importance, the use of the subspecific epithets will be limited to those few laboratories that can identify *L. pneumophila* to this level.

The taxonomy of the legionellae has been challenged at the genus level by Garrity et al. (1980) and Brown et al. (1981), who examined *L. micdadei, L. bozemanii, L. dumoffii,* and *L. gormanii* by DNA hybridization. Their reported hybridization values were virtually identical to those reported by others (Brenner et al., 1980) and showed that these species are less than 25% related to *L. pneumophila.* These workers suggested that organisms that were less than 25% related to each other should be placed in separate genera and proposed the genus *Tatlockia* for *L. micdadei (T. micdadei),* and *Fluoribacter* for the autofluorescent species *L. bozemanii (F. bozemanae)* (sic), *L. dumoffii (F. dumoffii),* and *L. gormanii (F. gormanii).*

The controversy over the classification of the legionellae centers not on the degree of relatedness between the species tested, since both research groups have produced identical values, but lies in the interpretation of the data relative to what constitutes a genetic definition of a genus. Brenner (1987) has stated that there is no accepted definition of a species at the genetic level and that a genus should include organisms that are phenotypically related and show some degree of genetic relatedness. He suggests that the common ecological niche, metabolic pathways, pathologic features, antimicrobial susceptibility, and antigens shared by these organisms should also be taken into account along with their degree of genetic relatedness in the practical definition of a genus. It would appear that most agree with the proposal of Brenner et al (1979) because the generic designation *Legionella* is used almost exclusively in the scientific literature.

Habitat and Reservoirs

Natural Habitats

The legionellae are widely distributed in nature and seem to prefer moist environments. In addition to their isolation from cooling towers, water distribution systems, and various medical devices already mentioned, they have been isolated form a wide range of natural and artificial habitats, which include rivers, lakes, streams, and riparian soil (Fliermans et al., 1981). They have been isolated from water at temperatures ranging from 5–65°C and survive and multiply (in water) over a pH range of 5.5–9 (Fliermans et al., 1981; States et al., 1987b).

Reservoirs

One of the earliest recognized outbreaks of Legionnaires' disease occurred among patients in a psychiatric hospital in Washington, DC (Thacker et al., 1978) and nosocomial Legionnaires' disease outbreaks were recognized at

many institutions during the 1980s. It is through the investigation of such outbreaks that we have come to gain an appreciation of the ubiquitous distribution of the legionellae and their relationship to our natural and inanimate environment. The first association between *L. pneumophila* and water distribution systems occurred when the organism was isolated from the shower fixtures in the rooms of two renal transplant patients who had contracted Legionnaires' disease (Tobin et al., 1980). This observation was followed by many others, which also indicated that *Legionella* could be recovered from the potable water supply of large buildings and from equipment that came in contact with tap water (Shands et al., 1985; Edelstein et al., 1986; Helms et al., 1983). Legionellae have been found in numerous locations, such as showers (Tobin et al., 1980; Cordes et al., 1981) and faucets (Stout et al., 1985; Wadowsky et al., 1982), and ice machines (Stout et al., 1985). It is not surprising, then, that the legionellae are found in water-containing devices such as respiratory therapy equipment (Arnow et al., 1982; Gorman et al., 1980) and even a room humidifier (Zuravleff et al., 1983). They have also been found in both therapeutic and recreational whirlpool baths (Mangione et al., 1985; Brabender et al., 1983). The major source of legionellae that contaminate these devices appears to be the hotwater distribution system (FischerHoch et al., 1981; Wadowsky et al., 1982; Helms et al., 1983).

The source of the bacteria that ultimately colonize water distribution systems appears to be the municipal water distribution system, which may harbor the organism in small numbers (States et al., 1987). Reservoirs of warm water, such as hotwater tanks, then serve as efficient bacterial "amplifiers," allowing small numbers of the bacteria to multiply and subsequently colonize the water distribution system. Multiplication of the legionellae under such circumstances in water distribution systems appears to be enhanced by the presence of sediment and other microorganisms (Wadowsky and yee, 1985) and even by some components of the system such as faucet washers made from certain types of rubber (Colbourne et al., 1984). Legionellae have been detected in the home water systems of some, but not all, patients with community-acquired Legionnaires' disease (Stout et al., 1987).

Air conditioning cooling towers, which usually harbor a bacterial flora, also often contain legionellae (Dondero et al., 1980; Orrison et al., 1981; Girod et al., 1982). The source of legionellae that colonize cooling towers is also most likely the municipal water supply used to fill such systems. Again, because of their heat-exchanging function, cooling towers serve as amplifiers of legionellae. Bacteria-laden water vapor from cooling towers can travel long distances (Shands et al., 1985) and has even entered a building by traveling down an open chimney (Band et al., 1981).

While the epidemiologic associations between environmental contamination and Legionnaires' disease are strong, these organisms have also been isolated without any disease association (Bartlett et al., 1983). The reasons why Legionnaires' disease does not always occur in the presence of a contaminated water supply or cooling tower is not yet known. One very interesting hypothesis is that not all legionellae are of equal virulence. Plouffe et al. (1983) described the isolation of two different strains of *L. pneumophila* serogroup 1 from separate hospital buildings. Legionnaires' disease occurred in only one of the buildings and was caused by the strain associated with that building. Subsequent studies of the two strains in animals indeed suggested that one was more virulent than the other (Bolin et al., 1985).

Methods for the Control of Legionellae

A number of methods have been used for the control of legionellae in mechanical systems. Chlorination of building water systems has been shown to be very effective in eliminating the legionellae from potable water distribution systems (Fisher-Hoch et al., 1981; Shands et al., 1985; Helms et al., 1983). Most workers have employed initial shock chlorination of the water supply, followed by maintenance treatment with 1–5 ppm of free available chlorine. The major drawbacks of this method appear to be corrosion of the plumbing system and the potential for formation of toxic and/or carcinogenic trihalomethanes (Helms et al., 1988).

Elevation of the temperature of building hotwater systems to temperatures between 60 and 80°C has also been used to eliminate legionellae from plumbing systems both alone and in combination with chlorination (Fisher-Hoch et al. 1981; Best et al., 1983). This method is effective in reducing the numbers of legionellae in the plumbing system, but also increases the risk of scalding. Intermittent raising of the temperature followed by flushing of the hot water through all building fixtures is effective, but requires a highly coordinated staff to carry out. Treatment of a hospital water supply with ultraviolet radiation has also been used to elim-

inate legionellae (Farr et al., 1988). Ozone treatment also appeared to be effective in reducing the numbers of legionellae in a single study (Edelstein et al., 1982). No controlled comparisons of the various disinfection methods have been carried out. Each of the methods has been shown to be efficacious in the studies mentioned above and also under more controlled circumstances in a model plumbing system (Muraca et al., 1987).

Control of legionellae in cooling towers has been much more difficult, both because of their mechanical and biological complexity. In vitro studies have demonstrated that the legionellae are susceptible to a number of commonly used colling tower biocides (Grace et al., 1981; Skaliy et al., 1980). In use, however, biocides alone have failed to prevent multiplication of legionellae in cooling towers. Many factors are undoubtedly responsible for this, including inactivation of biocides by aeration and/or light, the presence of large amounts of biofilm, and the presence of other microorganisms that may protect the bacteria from inactivation (England et al., 1982; Kurtz et al., 1982). Chlorine has also been the most reliable decontaminating agent used to date, but its use is not simple because of corrosion problems (Fliermans et al., 1982; States et al., 1987b). Cooling tower maintenance programs are further complicated by the need to add antifouling and anticorrosion agents.

Relationships with Other Microorganisms

Symbiosis with Prokaryotes

A novel and probably important aspect of the ecology of the legionellae is their apparent symbiotic relationship with other members of their ecosystem. Early studies (Wadowsky et al., 1982) suggested that legionellae were found in greatest numbers at the botton of hotwater tanks and were often found in association with the sediment at the bottom of the tank. A strain of *Flavobacterium breve* present in one water system when cocultivated with *L. pneumophila* eliminated the need to supplement the medium with cysteine (Wadowsky and Yee, 1983). Other heterotrophic bacteria present in tap water are also capable of stimulating the growth of legionellae in cysteine-deficient media, but do not appear to stimulate the growth of these bacteria in water cultures (Wadowsky and Yee, 1985). Photosynthetic cyanobacteria (*Fischerella* sp.), which were recovered from an algal mat that also contained *L. pneumophila,* have also been shown to stimulate multiplication of *L. pneumohila* in mineral salts medium (Tison et al., 1980). The exact role of these organisms in the ecology of legionellae is not clear since these photosynthetic cyanobacteria require light and would not be expected to be present in mechanical systems such as water distribution systems.

Symbiosis with Eukaryotes

Studies of legionellae growth in tap water cultures demonstrated that a factor that was retained by 1-μm membrane filters supported the growth of *L. pneumophila. Hartmanella vermiformis* and two other species of hartmanellid amoebae were isolated from the filter retentates and shown to be capable of supporting the growth of *L. pneumophila* in tap water (Fig. 3). A number of other free-living eukaryotes were also shown to be capable of supporting the growth of the legionellae. Rowbotham et al. (1980) demonstrated that the free-living amoebae *Acanthamoeba* and *Naegleria* can become infected with legionellae. A similar growth-promoting ability has been demonstrated for the ciliate *Tetrahymena pyiriformis* (Fields et al., 1984). Clear proof of the role of these organisms in the multiplication or dissemination of legionellae to humans is still lacking, probably due in large part to the difficulty in reliably cultivating protozoa from water sources. One group (Barbaree et al., 1986) recovered ciliated protozoa from an air conditioning tower that was associated with an outbreak of legionnaires disease. *L. pneumophila* could infect and multiply within the ciliate in vitro. More reliable methods for determining the presence of various protozoal species in water systems are required.

Given that the intracellular milieu of the human monocyte is remarkably similar (see Figs. 2 and 3) to that of these one-celled organisms, many speculate that the legionellae have adapted to multiplication in human monocytes by co-evolving in the natural environment with simple protozoa. Indeed, one study has suggested that virulent *L. pneumophila* multiply more rapidly in ciliated protozoa than avirulent strains (Fields et al., 1986). Likewise, just as their intracellular location protects the legionellae from the action of many antimicrobial agents in humans, it is equally possible that residence within these protozoa provides the legionellae with nutritional support while at the same time protecting legionellae from environmental stress, including disinfecting agents added by humans to control the bacteria in mechanical systems.

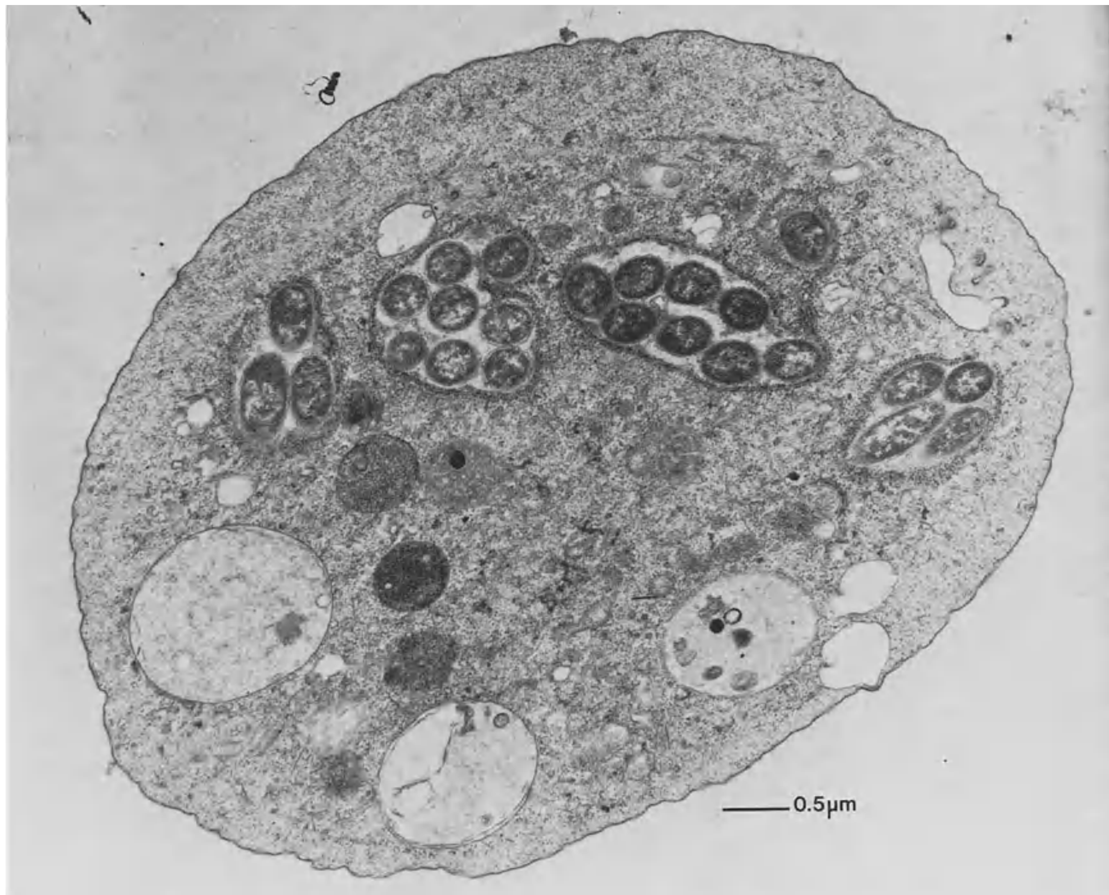

Fig. 3. Transmission electron micrograph of the free-living amoeba, *Hartmannella vermiformis,* infected with *Legionella pneumophila* serogroup 1. *Legionella* cells are multiplying within vesicles studded with ribosomes. (Courtesy of E. H. White, C. H. King, and B. S. Fields, Centers for Disease Control, Atlanta, GA.)

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CHAPTER 179

The Genus *Haemophilus*

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The genus *Haemophilus* is traditionally defined as being comprised of small, pleomorphic, Gram-negative rods (often coccobacilli) with growth requirements for one or more factors present in blood (NAD and/or hemin); hence the name *Haemophilus* (blood-loving). This is a diverse genus, comprised of both animal and human pathogens, as well as a number of species that are primarily commensals of the mucous membranes. The organisms are not free-living in nature.

The type species, *H. influenzae,* was described by Pfeiffer in 1892 and was erroneously thought to be the cause of influenza. The reason for this error probably lies in the fact that even though it is not the cause of influenza, *H. influenzae* can often be isolated in high numbers from the respiratory tract of individuals infected with influenza virus. *H. influenzae* is, however, a pathogen in its own right and is the leading cause of bacterial meningitis in the United States (Schlech et al., 1985). It also causes other invasive diseases (epiglottitis, arthritis, pneumonia, cellulitis, septicemia), primarily of children (see below).

The taxonomy of this genus has been subject to many changes and is still undergoing considerable revision. The genus *Haemophilus* was at one time included in the family Brucellaceae (Breed et al., 1957); it was included with "Genera of Uncertain Affiliation" in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Zinnemann and Biberstein, 1974) and was only recently included (along with *Actinobacillus* and *Pasteurella*) in the family Pasteurellaceae (Kilian and Biberstein, 1984). In contrast to the nonfermentative Brucellaceae, the haemophili are heterofermentative (with the possible exception of *H. ducreyi,* see below). All reduce nitrate to nitrite or beyond and are facultative anaerobes.

The haemophili are closely related to the actinobacilli and pasteurellae, and some investigators feel that based on DNA hybridization studies and rRNA sequences, *Actinobacillus actinomycetemcomitans* is more closely related to members of the genus *Haemophilus* than to other actinobacilli and should be placed in the genus *Haemophilus* (Potts et al., 1985; Chuba et al., 1988). Conversely, it has been suggested (and fairly well accepted) that *Haemophilus pleuropneumoniae* is more closely related to the actinobacilli than to other species of *Haemophilus* and should be placed in the genus *Actinobacillus* (Pohl et al., 1983). There are significant relationships between members of all three genera, and some species could be placed in one or more of these genera. Some of the taxonomic difficulties stem from the traditional definition of the genus *Haemophilus* based on requirements for NAD and/or hemin. *H. aphrophilus* has been reported to require hemin for primary isolation, but not usually for subsequent subculture (Kilian and Biberstein, 1984); it has the enzymes necessary to convert δ-aminolevulinic acid to protoporphyrin (White and Granick, 1963) and is therefore different from other hemin-requiring *Haemophilus* species. Since it also does not require NAD, the taxonomic position of *H. aphrophilus* has been questioned. *H. ducreyi* requires hemin, but based on a number of other criteria, may not be a valid *Haemophilus* species (Casin et al., 1985). Strains that were traditionally defined as *Haemophilus pleuropneumoniae* require NAD; there are however, strains associated with pleuropneumonia of pigs that resemble *H. pleuropneumoniae* but which do not require NAD. *H. avium* requires NAD, but may be more closely related to *Pasteurella multocida* (the type species of the genus *Pasteurella*) than to *H. influenzae,* and Mutters et al. (1985) have proposed transfer of *H. avium* to the genus *Pasteurella.*

Because the structure of the family Pasteurellaceae is undergoing considerable realignment, and the position of many species is still unsettled, I will for the purposes of this chapter discuss the traditionally defined *Haemophilus* species as they were listed in *Bergey's Manual of Systematic Bacteriology* (Kilian and Biberstein, 1984). I will note, where appropriate, the changes that have been proposed since 1984.

One of the most significant observations may be that of Niven and O'Reilly (1990), who suggest that inability to amidate the carbonyl group of nicotinic acid may be a characteristic of the entire family Pasteurellaceae; thus, all members of the Pasteurellaceae may require as a minimum, preformed nicotinamide (which is present in most complex media). Only certain species, however, are unable to add a phosphoribosyl group to nicotinamide, and therefore have a requirement for intact nicotinamide adenine dinucleotide (or nicotinamide mononucleotide or nicotinamide riboside, all of which are heat labile and/or otherwise lacking in most culture media). Although most members of the genus *Haemophilus* require NAD and/or hemin, these requirements may also appear in other members of the family Pasteurellaceae. A redefinition of the individual genera within the family is thus needed.

Bergey's Manual of Systematic Bacteriology (Kilian and Biberstein, 1984) included 16 species in the genus *Haemophilus* and listed three additional organisms as "species incertae sedis." Since the animal species rarely infect humans and vice versa, the individual *Haemophilus* species will be discussed in terms of their host origin.

Human Isolates

The human isolates include *H. influenzae, H. aegyptius, H. haemolyticus, H. ducreyi, H. parainfluenzae, H. parahaemolyticus, H. paraphrohaemolyticus, H. aphrophilus, H. paraphrophilus,* and *H. segnis* (Table 1). Of these, *H. influenzae, H. aegyptius,* and *H. haemolyticus* require both hemin and NAD; *H. ducreyi* only requires hemin (X factor), and *H. parainfluenzae, H. parahaemolyticus, H. paraphrohaemolyticus, H. paraphrophilus,* and *H. segnis* only require NAD (V factor). As mentioned earlier, *H. aphrophilus* sometimes requires hemin for primary isolation but not usually thereafter.

Animal Isolates

Haemophilus species isolated from animals include *H. parasuis* and *H. pleuropneumoniae* (from pigs), *H. paragallinarum* and *H. avium* (from poultry), *H. haemoglobinophilus* (from dogs), and *H. paracuniculus* (from rabbits). Of the three species listed as "incertae sedis," *"H. somnus"* is isolated from cases of bovine septicemia and meningoencephalitis (Kirkham et al., 1989); *"H. agni"* causes septicemia of lambs (Kirkham et al., 1989), and *"H. equigenitalis"* is the causative agent of contagious equine metritis (Taylor et al., 1978).

H. equigenitalis is not a "good" *Haemophilus* species; it does not require NAD or hemin (Sugimoto et al., 1983), does not reduce nitrate (Rossau et al., 1987), and does not ferment sugars (Taylor et al., 1978). Sugimoto et al. (1983) have proposed transfer of this organism to a new genus, *Taylorella,* as *Taylorella equigenitalis* comb. nov. This organism is not related to any of the Pasteurellaceae and the International Committee on Systematic Bacteriology (ICSB), Subcommittee on Pasteurellaceae has rejected it from the family (Frederiksen, 1987). The closest relatives of *T. equigenitalis* appear to be the family Alcaligenaceae and the newly proposed genus *Oligella* (Rossau et al., 1987); this organism will not be discussed further.

The remaining "species incertae sedis," *"H. somnus"* (isolated from cattle) and *"H. agni"* (isolated from lambs), are closely related and may represent host-specific subgroups of the same species (Kirkham et al., 1989). Both are related to a third group of organisms named *"Histophilus ovis." "H. ovis"* has been associ-

Table 1. The Genus *Haemophilus*.[a]

Host origin	NAD- and hemin-dependent species	NAD-dependent species	Hemin-dependent species	NAD- and hemin-independent species
Humans	*H. influenzae* *H. aegyptius* *H. haemolyticus*	*H. parainfluenzae* *H. parahaemolyticus* *H. paraphrohaemolyticus* *H. paraphrophilus* *H. segnis*	*H. ducreyi*	*H. aphrophilus*
Pigs		*H. parasuis* *H. pleuropneumoniae*		
Poultry		*H. paragallinarum* *H. avium*		
Dogs			*H. haemoglobinophilus*	
Rabbits		*H. paracuniculus*		

[a]Members of the genus according to Kilian and Biberstein, 1984.

ated with suppurative mastitis of sheep and epididymitis of rams (Walker et al., 1985). All three organisms are small, pleomorphic rods or coccobacilli and have fastidious growth requirements (optimal growth on chocolate or enriched blood agar with 5–10% CO_2); in this sense they resemble the haemophili. There is however, no specific growth response to NAD and/or hemin (Kilian and Biberstein, 1984). Based on DNA hybridization analyses, all three organisms appear highly related (Walker et al., 1985; Piechulla et al., 1986); they do not, however, appear to be significantly related to *H. influenzae* or *H. parainfluenzae*. Piechulla et al. (1986) found some binding to *H. haemoglobinophilus* DNA but did not recommend inclusion in the genus *Haemophilus* via *H. haemoglobinophilus*. There have been suggestions that the genus *Histophilus* be revived to accommodate these organisms (Piechulla et al., 1986) and the Subcommittee on Pasteurellaceae felt that such a genus could be accommodated in the family Pasteurellaceae (Frederiksen, 1987). These organisms will not be discussed further.

In addition to the above-named species, it has been reported that *Haemophilus*-like organisms can be isolated from the mucous membranes of the respiratory tract, mouth, or genitals of a wide variety of animals (Kilian and Frederiksen, 1981, and references therein). Most of these isolates have not been well characterized, although Nicklas (1989) has published a detailed description of NAD-dependent organisms isolated from rats.

Habitats/Pathogenesis

NAD- and Hemin-Dependent Human Isolates

H. HAEMOLYTICUS. *H. haemolyticus* is an occasional member of the normal pharyngeal flora (Kawakami et al., 1982; Kuklinska and Kilian, 1984); it rarely, if ever, causes disease (Albritton, 1982).

H. INFLUENZAE. The habitat for *H. influenzae* is mainly the upper respiratory tract (oro- and nasopharynx), where it is frequently present as part of the normal flora. The species contains both encapsulated and nonencapsulated forms (Pittman, 1931). As many as 50–80% of individuals may be colonized with nonencapsulated strains, whereas carriage rates for encapsulated strains are much lower (2–4% for type b strains and 1–2% for other capsule types; Turk, 1984). Disease, however, does occur in some individuals. The spectrum of disease caused by encapsulated and nonencapsulated strains is quite distinct. More than 95% of invasive *H. influenzae* disease (meningitis, epiglottitis, cellulitis, pneumonia, septicemia, septic arthritis) is caused by encapsulated type b strains; the majority of cases occur in children less than five years of age (peak age incidence six months to two years; Cochi et al., 1985). Antibody to the capsular polysaccharide is known to be protective (Peltola et al., 1977), and most adults have protective antibody levels. What is not known is why some infants succumb to disease whereas other infants (even though susceptible) become colonized but do not become ill (Moxon, 1986). The other *H. influenzae* capsule types (a, c–f) are only rarely isolated from cases of systemic disease. Nonencapsulated strains, while common members of the normal upper respiratory flora, are only rarely isolated from cases of systemic disease (and then patients are usually neonates, elderly, or have other underlying disease; see below). Nonencapsulated strains are, however, frequently isolated from localized diseases, especially otitis media (Turk, 1982); they also cause sinusitis and are implicated in exacerbations of chronic bronchitis (Turk, 1982).

Epidemiological studies suggest that there are approximately 12,000 cases of *H. influenzae* type b meningitis per year in the United States; an additional 7,500 cases of other invasive diseases due to type b strains also occur (Cochi et al., 1985). Because of the severity of the disease, and the fact that a significant number of survivors are left with neurologic deficits (Ferry et al., 1982), a great deal of work has been focused on the development of an *H. influenzae* type b vaccine. Antibody directed against the type b capsule is protective (Peltola et al., 1977), and vaccines aimed at eliciting anticapsular antibodies have recently been licensed. The first-generation vaccine, licensed in the USA in 1985, contained purified capsular polysaccharide and was recommended for use in children at 24 months of age or greater (Immunization Practices Advisory Committee, 1985). The problem with this vaccine is that the purified capsular polysaccharide is not a good immunogen in infants less than 24 months of age (the age group with the highest incidence of disease). Progress has been made in improving the immunogenicity of the vaccine by coupling the polysaccharide to protein carriers (Schneerson et al., 1980; Anderson et al., 1985; Weinberg and Granoff, 1988). A conjugate vaccine was originally approved for use in infants at 18 months of age (Immunization Practices Advisory Committee, 1988), and field trials are underway to determine the efficacy in younger age groups. Data from a large trial in Finland are encour-

aging (Eskola et al., 1987), and a conjugate vaccine was licensed in the USA in October of 1990 for use at two months of age.

Although viewed primarily as an inhabitant of the upper respiratory mucosa, *H. influenzae* is also capable of colonizing the genital mucosa. It is occasionally implicated in genital tract infections (Wallace et al., 1983; Quentin et al., 1989), and like other organisms capable of colonizing the vagina and cervix, it is occasionally associated with neonatal and obstetrical infections (Khuri-Bulos and McIntosh, 1975; Wallace et al., 1983; Friesen and Cho, 1986; Milne et al., 1988). Although only a small percentage (<1%, Khuri-Bulos and McIntosh, 1975; 0.3 to 0.5%, Albritton et al., 1982) of pregnant women were found to carry *H. influenzae* in their cervix or vagina, a number of studies have suggested that transmission to the infant during or prior to delivery can occur. In their study of eight cases of *H. influenzae* neonatal infection, Khuri-Bulos and McIntosh (1975) found evidence of genitourinary colonization with *H. influenzae* in all eight mothers. A number of more recent reports support this observation (Wallace et al., 1983; Campognone and Singer, 1986; Quentin et al., 1989). Although still moderately low (0.14 to 0.59 per 1,000 live births), the reported incidence of neonatal septicemia due to *H. influenzae* appears to be increasing (Wallace et al., 1983; Milne et al., 1988; Quentin et al., 1989). In contrast to *H. influenzae* meningitis in the pediatric patient (where most strains are encapsulated type b), many of the neonatal isolates are nonencapsulated (Wallace et al., 1983; Campognone and Singer, 1986; Friesen and Cho, 1986; Quentin et al., 1989). Vaccines directed against the type b capsular polysaccharide will not be effective in preventing disease due to nonencapsulated strains.

It is often stated that *H. influenzae* has no nonhuman hosts. This is not completely true, as *H. influenzae* (including encapsulated type b) has been isolated from the respiratory tract of naturally infected chimpanzees (Dochez et al., 1932). *H. influenzae* has also been reported to cause bacteremic pneumonia in monkeys (Good and May, 1971), and McClure (1980) reported a variety of naturally occurring *H. influenzae* infections in nonhuman primates. Dawkins (1982) reports a case of septic arthritis in a cynomolgus monkey. It is not known whether primates are colonized with *H. influenzae* in their native environment or whether they are accidentally infected via humans.

Infant monkeys have been used as an experimental model to study *H. influenzae* pathogenesis (Scheifele et al., 1980), although an infant rat model is more commonly used (Moxon et al., 1974; Smith et al., 1973). Rats are not naturally colonized with *H. influenzae,* but can be infected experimentally. The ability to infect rats decreases with the age of the rat, and unlike the human situation, resistance to infection in older rats is not due to anticapsular antibody (Smith et al., 1973). Infant rats are however, a good model in which to study the progression from nasopharyngeal colonization, to bacteremia, to meningitis.

H. AEGYPTIUS. *H. aegyptius* has traditionally been associated with acute conjunctivitis, particularly in warm climates (Pittman and Davis, 1950; Mazloum et al., 1982). The taxonomic status of this "species," however, is controversial (Pittman and Davis, 1950; Kilian et al., 1976; Martel et al., 1986; Casin et al., 1986; Mazloum et al., 1982). Koch (1883) first observed small bacilli in smears of exudates from the eyes of Egyptian children suffering from conjunctivitis. Weeks (1886) cultured what was assumed to be a similar organism from cases of conjunctivitis in New York. For many years, *Haemophilus*-like organisms, isolated from cases of conjunctivitis, were referred to as the Koch-Weeks bacillus; it was not clear how (or if) they differed from *H. influenzae;* some investigators attempted to make a distinction between *H. influenzae* and the Koch-Weeks bacillus, whereas others did not. Pittman and Davis (1950) proposed use of the name *H. aegyptius* for certain of the conjunctivitis-associated isolates. They suggested that *H. aegyptius* could be distinguished from nonencapsulated *H. influenzae* on the basis of its slightly more elongated cellular morphology, its inability to ferment xylose, the more fluffy, cometlike appearance of its colonies in semisolid agar, and its ability to agglutinate red blood cells. They also detected differences in serology between *H. aegyptius* and nonencapsulated *H. influenzae.* Their criteria have not, however, in subsequent studies allowed unambiguous distinction between *H. influenzae* and *H. aegyptius;* some isolates of *H. influenzae* also fail to ferment xylose (Brenner et al., 1988; Hollis et al., 1980). The hemagglutinating property is unstable, and can be lost on subculture (Kilian et al., 1976); furthermore, other *Haemophilus* isolates can exhibit hemagglutinating properties. Mazloum et al., (1982) proposed that *H. aegyptius* could be separated from *H. influenzae* on the basis of its inability to grow on tryptic soy agar with NAD and hemin, and by its susceptibility to troleandomycin. These tests have also not allowed unambiguous distinction between the two "species" (Carlone et al., 1985; Martel et al., 1986). Despite the confusion, the clinical picture sug-

gests that certain strains may be more likely than others to cause the highly contagious, epidemic form of conjunctivitis, especially in warm climates. Unfortunately, the characteristics that could be used to unambiguously identify such strains are not yet established. What is clear is that strains of *H. influenzae* that do *not* fit the classical description of *H. aegyptius* can also cause conjunctivitis (Pittman and Davis, 1950; Kilian et al., 1976); *H. parainfluenzae* is also occasionally isolated (Kilian et al., 1976).

The problems in distinguishing *H. aegyptius* from *H. influenzae* are not surprising in view of the high level of genetic relatedness between the two organisms. Pohl (1981), in his DNA relatedness study, reported 80% binding between *H. aegyptius* and *H. influenzae* DNA (three strains of *H. influenzae,* including the type strain, NCTC 8143, and two strains of *H. aegyptius,* including the type strain ATCC 11116, were included in his analysis). Albritton et al. (1984) reported 90% binding between the type strains of *H. aegyptius* and *H. influenzae.* Except for one strain of *H. aegyptius* (which bound *H. influenzae* DNA at levels of only 63–66%), Casin et al. (1986) also found high (80–90%) levels of binding between the type strains of *H. influenzae* and *H. aegyptius* and several other strains of each "species." Included in this study were three of the *H. aegyptius* isolates described by Pittman and Davis (1950). DNA binding levels of 70% or greater are generally taken as indicative of species identity (Brenner et al., 1982), suggesting that a distinction between *H. aegyptius* and *H. influenzae* may be unwarranted.

Interest in distinguishing these organisms, however, has recently been spurred by the finding that *"H. aegyptius"* is responsible for the newly described disease, Brazilian Purpuric Fever (BPF) (Brazilian Purpuric Fever Study Group, 1987a). This disease is characterized by a rapid onset of fever, often accompanied by vomiting and abdominal pain; in the absence of treatment there is a rapid progression to petechiae, purpura, vascular collapse, hypotensive shock, and death, usually within 48 hours of onset (Brazilian Purpuric Fever Study Group, 1987a, 1987b). Epidemiological investigations revealed an association with a preceding purulent conjunctivitis. An organism labelled *H. influenzae* biogroup aegyptius (to emphasize its relatedness to *H. influenzae*) was isolated from cases of conjunctivitis, and eventually (once proper blood culture techniques were initiated), from the blood of individuals during the purpuric stage (Brazilian Purpuric Fever Study Group, 1987a, 1987b). This is the first time *"H. aegyptius"* has been reported to cause anything more than conjunctivitis. Fortunately, cases have been limited to certain regions of Brazil, with two additional cases reported in Australia; the patients are young children, with a median age of 30 months (Harrison et al., 1989).

Extensive characterization (including plasmid profiles, outer membrane protein profiles, and multilocus enzyme typing) revealed that the BPF case isolates constituted a single clone of *H. aegyptius* (Brenner et al., 1988), and that there was a high degree of DNA homology between case isolates and reference strains of both *H. aegyptius* (including three of the Pittman and Davis isolates) and *H. influenzae* (averages of 88 and 85%, respectively). These results again confirmed the high level of genetic relatedness between isolates labeled *"H. aegyptius"* and *H. influenzae;* these investigators proposed use of the name *H. influenzae* biogroup aegyptius to emphasize this fact.

The occurrence of Brazilian Purpuric Fever raises a number of interesting questions concerning the evolutionary history of the organism involved. The factors responsible for the unique virulence potential of this organism are not known, although a number of potential factors have been examined (Carlone et al., 1989). The case clone differs from other *H. influenzae* biogroup aegyptius strains in several respects and may represent a "new" clone with unique pathogenic properties. Musser and Selander (1990) compared the relatedness of the case clone to other isolates of *H. influenzae* and *H. aegyptius* by the technique of multilocus enzyme typing (ET) and found that the closest relatives of the BPF clone are type c strains of *H. influenzae.* Although the case isolates do not appear to be encapsulated (Brenner et al., 1988), they do have some homology to an *H. influenzae* capsular gene probe (S. Hoiseth, unpublished observations; Carlone et al., 1989), and unlike other isolates of *"H. aegyptius"* (which produce a type 1 IgA protease), the BPF clone produces a type 2 IgA protease (as do type c *H. influenzae;* Carlone et al., 1989). Non-BPF isolates of *"H. aegyptius"* fell into two additional clusters on the *H. influenzae* ET dendrogram (Musser and Selander, 1990), again confirming the close relationship of *"H. aegyptius"* and *H. influenzae.* Clearly, additional work needs to be done to define the genetic relationships and pathogenic potential of the BPF isolates and established *Haemophilus* species.

NAD-Dependent Human Isolates

H. PARAINFLUENZAE, H. PARAHAEMOLYTICUS, H. PARAPHROHAEMOLYTICUS, H. SEGNIS, AND *H. PARAPHROPHILUS.* These organisms are part of

the normal flora of the oral cavity and pharynx. Unlike *H. influenzae,* which seems to be confined to the pharyngeal mucosa, *H. parainfluenzae* is frequently found in both the pharynx (Kuklinska and Kilian, 1984) and the oral cavity (mean counts in saliva of approximately 4 $\times 10^7$/ml, representing approximately 10% of the cultivable flora; Kilian and Schiott, 1975). It is also found in dental plaque (Kilian and Schiott, 1975; Liljemark et al., 1984). *H. segnis* and *H. paraphrophilus* are also found in the normal pharynx (Kuklinska and Kilian, 1984) and saliva (Kilian and Schiott, 1975) but much less frequently than *H. parainfluenzae. H. segnis* and *H. paraphrophilus* are more likely to be found in dental plaque than in saliva (Kilian and Schiott, 1975). Liljemark et al., (1984) found that a large percentage (98%) of individuals have *Haemophilus* species in their dental plaque; the total number of haemophili relative to other organisms, however, is low (in only 20% of the sites sampled did the proportion of *Haemophilus* exceed 5.9% of the total cultivable organisms; 50% of the sites had 1.5% or fewer *Haemophilus* organisms).

The oral haemophili are only rarely associated with disease. Like other members of the oral flora, they occasionally cause endocarditis; even more rarely, they are associated with brain abscesses, pneumonia, osteomyelitis, septicemia, and other miscellaneous infections (Albritton, 1982). *H. parainfluenzae* has been reported as a rare cause of meningitis (Albritton, 1982, cited 22 cases reported in the English literature between 1966 and 1980). *H. parainfluenzae* has also been implicated in genital tract infections (Sturm, 1986; Quentin et al., 1989) although it probably acts mainly as an opportunistic pathogen (e.g., many of the cases reported by Quentin et al. were associated with the use of an intrauterine device). The most recently described species, *H. segnis* (Kilian, 1976), has been isolated from several cases of appendicitis (Welch et al., 1986), from a pancreatic abscess in a 29-year-old alcoholic (Bullock and Devitt, 1981), and from a case of endocarditis (Bangsborg et al., 1988).

In his review of "Infections due to *Haemophilus* species other than *H. influenzae,*" Albritton (1982) emphasized (and rightly so) that many of the organisms reported may have been improperly speciated. This is especially true for the distinction between *H. parainfluenzae* and *H. paraphrophilus.* The tests to distinguish *H. parainfluenzae* from *H. paraphrophilus* are few (primarily lactose fermentation; see "Identification") and are often not done. The rationale for the distinction between *H. parainfluenzae, H. parahaemolyticus,* and *H. paraphrohaemolyticus* has been questioned (Kilian, 1976). *H. parahaemolyticus* is distinguished from *H. parainfluenzae* by its hemolytic activity (Pittman, 1953); this trait can, however, be lost on subculture (Kilian, 1976). *H. paraphrohaemolyticus* is distinguished from *H. parahaemolyticus* on the basis of its CO_2 dependence (Zinnemann et al., 1971), another trait that can be lost on subculture. Kilian (1976) has suggested that *H. parahaemolyticus* and *H. paraphrohaemolyticus* do not warrant species status, and he included them with *H. parainfluenzae. H. parahaemolyticus* and *H. paraphrohaemolyticus* were, however, included as separate species in *Bergey's Manual of Systematic Bacteriology* (Kilian and Biberstein, 1984), and the type strain of *H. parahaemolyticus* (NCTC 8479) does not appear to be related to strains of *H. parainfluenzae* when tested by DNA hybridization analysis (Pohl, 1981). In practice, the tests to distinguish these "species" are often not done. Of the 55 reports of *H. parainfluenzae* infection reviewed by Black et al. (1988), hemolysis testing was done for only 31. Many of the reports would also not have distinguished *H. parainfluenzae* from *H. paraphrophilus.* The distinction of *H. segnis* may also be problematic. This species was proposed by Kilian (1976) on the basis of its relatively high GC content and weak fermentation activity. It is difficult to distinguish from the other NAD-dependent haemophili, and as pointed out by Albritton (1982), will very likely be reported as *H. parainfluenzae* by most clinical laboratories.

In reviewing the literature on infections due to NAD-dependent *Haemophilus* species, one must keep in mind that there is often insufficient information to assign a species name (though one is generally reported). One must also keep in mind that the tests to distinguish these organisms are few, that some species distinctions may be unwarranted, and that additional taxonomic studies are needed.

Hemin-Dependent Human Isolates

H. DUCREYI. *H. ducreyi* is the causative agent of the sexually transmitted disease, chancroid (soft chancre). The disease is common in tropical or subtropical areas, but has been considered uncommon in developed countries such as the USA (Morse, 1989). The number of cases in the USA, however, has risen considerably over the past several years. There were an average of just under 1,000 cases per year throughout much of the 1960s, 1970s, and early 1980s; by 1987 there were over 5,000 cases reported per year (Morse, 1989).

H. ducreyi produces genital ulcers and localized adenitis, but has not been reported to cause

systemic infection; untreated ulcers can, however, become superinfected with other microorganisms, resulting in extensive tissue destruction (Morse, 1989). D'Costa et al. (1986) state that they have been unable to culture *H. ducreyi* from asymptomatic men; the possibility of asymptomatic carriage, however, remains controversial, especially in females (Morse, 1989). Although ulcers on the external genitalia are more common, females may occasionally have cervical lesions. These are often painless, and the woman may be unaware of the infection (D'Costa et al., 1986; Morse, 1989); this is probably important in transmission of the disease. In addition to the above situation, occasional women may be culture-positive for *H. ducreyi* but have no symptoms or cervical lesions (D'Costa et al., 1986); whether these are true asymptomatic carriers or whether they are still in the incubation period for disease is not yet established. Unlike the oral haemophili, *H. ducreyi* can rarely, if ever, be considered normal flora.

The history, biology, and epidemiology of chancroid has been hindered by difficulty in cultivating the organism (Deacon et al., 1954, 1956) and by the fact that many different organisms have at one time or another been labeled *"H. ducreyi."* Kilian and Theilade (1975) reported that several of the strains studied by Reymann (1947, 1949) had cell walls typical of Gram-positive bacteria when examined by electron microscopy. The strains studied by Deacon et al. (1954) were later found to be *Corynebacterium* (Deacon et al., 1956). Part of the problem stems from the fact that *H. ducreyi* is extremely fastidious, and the lesions are often contaminated with other organisms. Progress has been made, however, with the introduction of enriched, selective media. Hammond et al. (1978) used an Isovitalex-enriched chocolate agar containing 3 μg/ml vancomycin and were able to enhance the recovery of *H. ducreyi* by suppression of many of the contaminating organisms. Inclusion of fetal bovine serum may further enhance recovery (see "Isolation" for additional comments).

Although improvements have been made in the cultivation of *H. ducreyi,* there is still a great deal that is not known concerning the basic physiology and taxonomy of this species. *H. ducreyi* requires hemin for growth, and has a GC content (38%) within the range for the genus *Haemophilus* (37–44%); it differs from other haemophili, however, in a number of respects, and its position within the genus has been questioned (Casin et al., 1985; Albritton, 1989). Casin et al. (1985) found very little homology between DNA from *H. ducreyi* and DNA from other *Haemophilus* species (1–4%, S1 nuclease method, 60°C). Albritton (1989) found binding levels of 13% between the DNA of *H. influenzae* and *H. ducreyi* (hydroxyapatite method, 55°C), but found higher binding ratios (31%) for *H. ducreyi* and *Actinobacillus (Haemophilus) pleuropneumoniae* DNAs, and for *H. ducreyi* and *Pasteurella ureae* (27%), suggesting that *H. ducreyi* is at least moderately related to some members of the family Pasteurellaceae.

There also appear to be basic differences between *H. ducreyi* and the other haemophili in terms of carbohydrate metabolism, with *H. ducreyi* generally regarded as inert in sugar fermentation tests (Kilian, 1976; Kilian and Biberstein, 1984). Sng et al. (1982), however, reported positive reactions for glucose, fructose, and mannose, although the only reference strain tested, Institut Pasteur strain CIP 542, was negative. Sottnek et al. (1980) reported that occasional strains gave weakly positive results with glucose or maltose, but the reactions could not be consistently repeated. Hammond et al. (1978) apparently found negative sugar fermentation for 19 clinical isolates and 4 reference strains, and S. Spinola and S. Misra (personal communication) reported negative sugar reactions for five reference strains of *H. ducreyi* (CIP 542, Winnipeg strain 35,000, and CDC strains 82–029362, 84–018676, and 85–023233). These results suggest that *H. ducreyi* is either not fermentative, or at a very minimum, only weakly fermentative. Additional studies are needed to clarify these and other problems pertaining to the taxonomic status of *H. ducreyi.*

NAD- and Hemin-Independent Human Isolates

H. APHROPHILUS. *H. aphrophilus* is found primarily in the oral cavity and in dental plaque. Using a selective medium containing bacitracin, vancomycin, and sodium fluoride, Tempro and Slots (1986) detected *H. aphrophilus* in the saliva of 6 of 14 subjects; *H. aphrophilus* was detected in subgingival dental plaque of 11 of the 14 subjects. The proportion of *H. aphrophilus* relative to other organisms, however, was low. In healthy subjects, *H. aphrophilus* comprised only 0.13% of the cultivable plaque flora, and in periodontitis patients, the percentage was even lower. They concluded that *H. aphrophilus* is frequently found as part of the normal oral flora; it comprises only a small proportion of the total oral flora and apparently plays no role in periodontal lesions.

Like other members of the oral flora, *H. aphrophilus* occasionally causes endocarditis, brain abscesses, and other miscellaneous infections (Albritton, 1982; King and Tatum, 1962).

The taxonomic position of this species is also somewhat of a problem. *H. aprophilus* was originally included in the genus *Haemophilus* because it is a small, Gram-negative coccobacillus that reportedly required hemin (Khairat, 1940; Boyce et al., 1969). Both Khairat (1940) and Boyce et al. (1969) noted that the hemin requirement could be lost after repeated subculture, but felt it was required for primary isolation. Kraut et al. (1972), however, reported that *H. aphrophilus* could be recovered from the oral cavity by direct plating on trypticase soy agar with bacitracin but without added hemin. Tempro and Slots (1986) performed primary isolations on hemin-containing media, but found that none of 12 *H. aphrophilus* isolates required hemin when tested on subculture. White and Granick (1963) showed that one of Khairat's original strains (NCTC 5886) contains the enzymes necessary for hemin biosynthesis; analysis of additional *H. aphrophilus* isolates has shown that they give positive (but often weak) porphyrin tests (Kilian and Biberstein, 1984; see "Identification" for details of the porphyrin test). Thus, although it is possible that some *H. aphrophilus* isolates require hemin for primary isolation, *H. aphrophilus* does not appear to be a true hemin-dependent species. Since it also does not require NAD, the taxonomic position of this species has been questioned. *H. aphrophilus* is, however, closely related (both biochemically and at the DNA level) to the NAD-dependent species, *H. paraphrophilus* (Kilian, 1976; Potts and Berry, 1983; Potts et al., 1985; Tanner et al., 1982). Both species are also related to *Actinobacillus actinomycetemcomitans,* although *H. aphrophilus* and *H. paraphrophilus* are more closely related to each other (~70% DNA binding; Potts and Berry, 1983; Tanner et al., 1982) than to *A. actinomycetemcomitans* (~20 to 40% DNA binding; Potts and Berry, 1983; Tanner et al., 1982). Because of the significant relationships between *H. aphrophilus, H. paraphrophilus,* and *A. actinomycetemcomitans,* Potts et al. (1985) proposed transfer of *A. actinomycetemcomitans* to the genus *Haemophilus.* Assessment of the DNA relatedness between *H. aphrophilus, A. actinomycetemcomitans,* and the type species of the genera *Haemophilus* and *Actinobacillus* has, however, yielded conflicting results. Potts and Berry (1983) found that neither *H. aphrophilus* or *A. actinomycetemcomitans* showed significant binding to *H. influenzae* or *A. ligniersii* DNA (<10% binding, S1 nuclease method, 63°C). Pohl (1981), however, using the renaturation rate method, found *A. actinomycetemcomitans* to be 40% related to *A. ligniersii* and 30% related to *H. influenzae;* he found *H. aphrophilus* to be 30% related to *H. influenzae.* Albritton et al. (1984) found relative binding ratios of 29% for *H. aphrophilus* and *H. influenzae* DNAs (hydroxyapatite method, 60°C). Additional studies are needed to resolve the taxonomic status of *H. aphrophilus* and related organisms.

Additional Comments: Human Isolates

As can be seen from the above descriptions, the human haemophili primarily inhabit the oral and pharyngeal mucosa. *H. influenzae* and *H. parainfluenzae* may occasionally colonize the genital tract (Albritton et al., 1982; Sturm, 1986) and with appropriate selection, haemophili can even be recovered from feces. Using selective media, Palmer (1981) detected *H. influenzae* in 1.5% of the fecal specimens he examined; NAD-dependent haemophili *(H. parainfluenzae, H. segnis,* and *H. paraphrophilus)* were detected in 20% of the specimens. Using the *Campylobacter* filtration technique, Megraud et al. (1988) detected *H. influenzae* in 0.4% of fecal specimens and *H. parainfluenzae* in 1.9%; whether this reflects active colonization, or simply survival from the nasopharynx through the alimentary canal is not known.

NAD-Dependent Avian Isolates

H. PARAGALLINARUM AND *H.* AVIUM. *H. paragallinarum* causes infectious coryza of fowl. This acute respiratory disease causes economic loss to poultry growers through reduced weight gain and lowered egg production (Blackall, 1989). The organism responsible for infectious coryza was originally named *H. gallinarum* and was thought to require both hemin and NAD. Subsequent studies have not confirmed a requirement for hemin and all currently existing strains only require NAD (Blackall, 1989). The name *H. paragallinarum* was proposed by Biberstein and White (1969) for strains requiring NAD only. *H. gallinarum* is no longer a recognized species name (Skerman et al., 1980; Kilian and Biberstein, 1984).

The apparent hemin dependency of the original isolates was probably due to inadequacy of the methods used by early investigators for growth factor testing (Blackall and Yamamoto, 1989). *H. paragallinarum* is somewhat finicky in its growth characteristics, and many strains require chicken serum and/or CO_2 for growth (Blackall, 1989).

The species *H. avium* was proposed by Hinz and Kunjara (1977) to accommodate avian isolates that are catalase-positive and nonpathogenic (in contrast to *H. paragallinarum,* which is catalase-negative and pathogenic). *H. avium*

is considered to be a member of the normal respiratory flora of poultry (Blackall, 1989).

Mutters et al. (1985) have suggested that *H. avium* is more closely related to *Pasteurella* species than to other members of the genus *Haemophilus* and should be transferred to the genus *Pasteurella.* Based on DNA homology, Mutters et al. (1985) further divided the catalase-positive avian isolates into three species, *P. avium, P. volantium,* and *Pasteurella* species A. The number of isolates examined, however, was small. Blackall (1988), using the biochemical criteria proposed by Mutters et al. (1985), was able to assign only 25 of 39 strains of *"H. avium"* to one of the three proposed species, and suggested that additional studies are needed to resolve the taxonomic status of the biochemically heterogeneous, catalase-positive, avian haemophili.

NAD-Dependent Porcine Isolates

H. PARASUIS AND *H.* PLEUROPNEUMONIAE. *H. parasuis* is responsible for the disease syndrome of pigs referred to as Glässer's disease. This disease is characterized by polyserositis, arthritis, and/or meningitis (Nicolet, 1986). In addition to its role in Glässer's disease, *H. parasuis* may also cause pneumonia, probably as a secondary invader (Nicolet, 1986); it is frequently found in the normal upper respiratory flora of healthy pigs (Biberstein et al., 1977; Nicolet, 1986).

H. parasuis requires NAD but not hemin. Controversy exists, however, concerning organisms originally labeled *"H. suis."* Lewis and Shope (1931) observed hemophilic bacilli in the respiratory tract of pigs infected with swine influenza virus, and proposed the name *H. influenzae suis* (which was later shortened to *H. suis*). *H. suis* was originally thought to require both hemin and NAD. Existing *H. suis* strains, however (including two of Shope's original isolates), require NAD but not hemin (Kilian, 1976), and Biberstein and White (1969) proposed use of the name *H. parasuis* to indicate this fact. *"H. suis"* was not included in the *Approved Lists of Bacterial Names* (Skerman et al., 1980), although occasional hemin- and NAD-requiring strains can apparently be isolated from pigs (Biberstein et al., 1977; Biberstein and White, 1969). The relationship of these hemin- and NAD-dependent isolates to strains originally described as *H. suis* is not known, nor is much known about their pathogenic potential.

H. pleuropneumoniae is the etiological agent of porcine necrotizing pleuropneumonia. The history of this species has also been somewhat complicated. The disease was first described in the 1960s (Matthews and Pattison, 1961; Shope, 1964), and the NAD-dependent organisms isolated were called *H. parainfluenzae* or *H. parahaemolyticus* by some investigators, and *H. pleuropneumoniae* by Shope (1964). Kilian et al. (1978) demonstrated that the porcine isolates were significantly different from the NAD-dependent human isolates, and recommended adoption of Shope's proposal (Shope, 1964) that they be given specific status as *H. pleuropneumoniae.* In addition to being distinct from the human isolates, these strains also differed from *H. parasuis,* particularly in their more luxuriant growth characteristics (*H. parasuis* grows quite poorly, even on chocolate agar; Matthews and Pattison, 1961; see "Identification" for additional tests) and by differences in disease pathology.

No sooner had *H. pleuropneumoniae* been accepted as the etiologic agent of porcine pleuropneumonia, when the situation was once again complicated by the isolation of *Pasteurella haemolytica*-like organisms from cases of porcine pleuropneumonia. These NAD-independent isolates were otherwise biochemically very similar to *H. pleuropneumoniae,* and DNA hybridization analysis indicated that both groups were highly related (Pohl et al., 1983). Furthermore, both groups were found to be much more closely related to the type species of the genus *Actinobacillus* (56 to 75% binding) than to *H. influenzae* (6% binding), and Pohl et al. (1983) proposed that both the NAD-dependent and NAD-independent porcine pleuropneumonia isolates be renamed *Actinobacillus pleuropneumoniae.*

The majority of *Actinobacillus (Haemophilus) pleuropneumoniae* isolates appear to be encapsulated (Sebunya et al., 1982; Sebunya and Saunders, 1983; Jacques et al., 1988), and there is apparently some homology to an *H. influenzae* capsular gene probe (J. S. Kroll, unpublished observations, cited in Musser et al., 1988). Unlike the situation with many other encapsulated organisms, antibody to the *H. pleuropneumoniae* capsule does not appear to be fully protective (Inzana et al., 1988); hemolysins and/or other toxins may contribute to the organism's pathogenic potential.

In addition to the named species, other *Haemophilus*-like organisms can be isolated from pigs. These include the "minor group" strains (Kilian et al., 1978) and taxon C (Kilian, 1976). Minor group strains are similar to *H. pleuropneumoniae* in that they are urease positive, but are negative in several sugar fermentations for which *H. pleuropneumoniae* is positive (see Identification Section). Minor group strains are commonly isolated from porcine lungs at slaughter (Pijoan et al., 1983), but are thought

to be less virulent than *H. pleuropneumoniae* (Rosendal et al., 1985).

NAD-Dependent Isolates from Rabbits

H. PARACUNICULUS. NAD-dependent coccobacilli have been isolated from the gastrointestinal tract of rabbits with mucoid enteritis (Targowski and Targowski, 1979). The isolate examined had a GC content of 40%, was nitrate reductase positive, and differed from most other NAD-dependent isolates in being indole positive (though occasional *H. parainfluenzae* strains may also be indole positive; Bruun et al., 1984); the name, *H. paracuniculus* was proposed (Targowski and Targowski, 1979). The pathogenic potential of this organism and its relationship to other NAD-dependent haemophili have not been established, and the biochemical description of the species appears to have been based on the analysis of a single isolate (Kilian and Biberstein, 1984; Targowski and Targowski, 1979).

Hemin-Requiring Animal Isolates

H. HAEMOGLOBINOPHILUS. *H. haemoglobinophilus* forms part of the normal flora of the preputial sac of dogs. Its pathogenic potential is probably low. It has, however, been isolated from dogs with suppurative inflammation of the prepuce (Rivers, 1922), and reportedly from two human infections (otitis media in a 10-year-old agammaglobulinemic and from a case of osteomyelitis following a dog bite in a 12-year-old boy; Frazer and Rogers, 1972; Lavine et al., 1974). This organism was originally referred to as *H. haemoglobinophilus canis* and later as *H. canis; H. haemoglobinophilus* has priority and is the taxonomically correct name (Kilian, 1976).

Additional Comments: Habitats

As indicated in the introduction, *Haemophilus*-like organisms have been isolated from many additional animal species (Kilian and Frederiksen, 1981); these isolates have not, however, been well characterized. It is generally held that animal isolates do not colonize humans and vice versa, and for the most part, this is probably true. Occasional exceptions may, however, occur, and perhaps some of the heterogeneity found for the NAD-dependent human isolates will someday be explained as the occasional isolation of animal species from humans (e.g., *"H. parainfluenzae"* strain NCTC 4101, isolated from human tongue, has been shown by DNA hybridization to be highly related to *H. avium;* Mutters et al., 1985; Pohl, 1981).

Isolation

Haemophili are fastidious organisms and require enriched media for their isolation. The most commonly used is chocolate agar, which provides both hemin and NAD. Although unheated blood also contains both hemin and NAD, strains requiring NAD do not grow on ordinary sheep blood agar (the type of blood used in most blood agar media). This is because sheep blood contains enzymes that degrade NAD, and unless the enzymes are destroyed by heating, the NAD is rapidly inactivated (Holt, 1961). Other sources of blood (e.g., rabbit or horse) provide more NAD, but the amounts can vary, and these types of blood are more expensive and not as readily available. What is commonly used, therefore, is chocolate agar. Originally, chocolate agar was prepared by adding 5 to 10% defibrinated blood to sterilized blood agar base media and holding the mixture at 80°C for 15 to 20 min, until the medium turned a dark chocolate brown. This served to release NAD from the red cells, as well as to inactivate NAD-degrading enzymes. However, NAD itself is heat labile, and this procedure also destroys some of the NAD; individual lots of chocolate agar must be carefully checked for their ability to support the growth of NAD-requiring strains. More commonly used is a somewhat artificial "enriched" chocolate agar, prepared by adding "hemoglobin" and either supplement B (Difco) or Isovitalex (BBL) to the autoclaved base medium. Supplement B and Isovitalex are chemically defined supplements that contain NAD as well as other nutrients, and are added (at a final concentration of 1%) after the autoclaved base media has cooled to 50°C. The "hemoglobin" is not purified hemoglobin, but rather a dried preparation consisting of washed and hemolyzed blood cells (Spray, 1930; available from Difco). The "hemoglobin" preparation is dissolved in water at a concentration of 2% and autoclaved separately; it is cooled to 45 to 50°C, and one volume is added to one volume of double-strength base media. The base medium can be one of several (GC agar base, brain heart infusion (BHI), blood agar base, etc.); the final agar concentration is usually 1%. Commercially prepared chocolate agar is available from a number of vendors and is also useful for cultivation of pathogenic *Neisseria.*

Because of its transparent nature, supplemented brain heart infusion agar (sBHI) is useful for distinguishing encapsulated from nonencapsulated organisms. This medium is prepared by adding purified NAD and hemin to autoclaved BHI agar or by adding Fildes extract (a commercially available peptic digest of blood)

to BHI. The latter method is preferable, as the extra nutrients present in the Fildes extract generally result in slightly larger colonies, and plates made with Fildes extract can usually be stored for longer periods than plates made with NAD and hemin only. Plates should be stored in sealed plastic bags (to prevent loss of moisture) and can generally be kept for about a month. Occasional isolates may not grow on sBHI, and sBHI cannot be recommended for *H. ducreyi* (see below).

Methods for Preparation of Supplemented BHI (sBHI) Agar

This medium can be made in either of the following ways.

sBHI made with NAD and hemin:

1. Prepare Difco brain heart infusion agar according to the manufacturer's instructions; autoclave at 121°C for 15 min; cool to 45–50°C.
2. Add 10 ml of a 1 mg/ml hemin stock solution (see below) per liter of BHI (final concentration 10 μg/ml).
3. Add 5 ml of a 2 mg/ml NAD stock solution (see below) per liter of BHI (final concentration 10 μg/ml).

sBHI made with Fildes extract:

1. Prepare BHI agar as in step 1 above.
2. Add Fildes extract to a final concentration of 2%.
3. Fildes extract contains some NAD, but we recommend adding more (2 ml of a 2 mg/ml NAD stock solution per liter).

NAD stock solution:

1. Dissolve β-NAD powder in water (2 mg/ml).
2. Sterilize by filtration.
3. Aliquot and store at −20°C.

Hemin stock solution:

1. To a sterile bottle, add 100 mg hemin powder and 4 ml triethanolamine (Kodak).
2. Add 96 ml sterile water.
3. Heat at 70°C for 10 min; test sterility.
4. Store at 4°C in a dark bottle; use within one month (generation times will increase with increasing length of storage).

Alternatively, hemin can be prepared in 0.2 M KOH in 50% ethanol (Kilian, 1981).

Other methods that have been used in the preparation of transparent media are based on the method of Levinthal (1918). This method is similar in concept to that of chocolate agar (i.e., a blood-containing medium is heated), but the medium is made transparent by removal of particulate material. This is done by filtration through sterile filter paper or by centrifugation. Horse blood is added to autoclaved BHI broth at a concentration of 35%, the mixture is boiled with stirring (use of a magnetic stirrer is helpful) until a clotted brown suspension forms. This is then filtered through sterile filter paper or centrifuged, and the clear filtrate referred to as "Levinthal" stock. This can be stored frozen and added to autoclaved (and cooled) BHI agar at a 1 to 10 ratio. The original formulas did not add additional NAD; however, as with the original chocolate agars, the amounts can vary. We recommend addition of extra NAD to a final concentration of 10 μg/ml.

BHI supplemented with Fildes extract gives satisfactory results and is much simpler to prepare than Levinthal's medium. Although use of sBHI as a sole medium for recovery of haemophili from clinical specimens is not advisable, its use along with chocolate agar might solve many problems concerning capsule status and/or mixed populations. The iridescence of encapsulated strains can be detected very readily by examination of sBHI plates with a strong, obliquely transmitted light source. Capsule production by *H. influenzae* is genetically unstable (Hoiseth et al., 1985, 1986) and in vitro passage tends to select out nonencapsulated variants. The ATCC reference culture of type b *H. influenzae* (strain 9795), as received from the ATCC, consists predominantly of capsule-deficient variants (S. Hoiseth, unpublished observations). Unless sBHI is used, most investigators will be unaware of this problem.

Liquid media may be prepared as per sBHI agar but the agar is omitted; less NAD can be used for liquid media (3 μg/ml) than for agar plates (10 μg/ml).

Care should be taken to avoid excessive autoclaving; even a slight excess of time or temperature can affect doubling times and ability of the media to support growth from small inocula. Evans and Smith (1974) suggest that dithionite or sodium oleate may be useful for neutralizing potential inhibitors; they found that some strains of *H. parainfluenzae* failed to grow on proteose peptone agar with NAD unless sodium oleate was also added. Additionally, some strains may require more thiamine than is available in certain nutrient agars (Evans and Smith, 1972). These and other problems may occasionally affect the ability to determine hemin and NAD requirements on such media. Doern and Chapin (1984) compared four different base media (all from Scott Laboratories, Inc.) for their suitability in growth factor tests. They found that BHI agar (plus strips containing hemin and NAD) supported growth of 185 of 187 strains of *H. influenzae;* tryptic soy agar (plus strips) supported growth of 181 of the 187 strains. Nutrient agar (plus strips), however, failed to support growth of 80 of the 187 strains, and Mueller-Hinton (plus strips) failed for 51 strains.

Tebbutt (1983) compared proteose peptone, Columbia agar, nutrient broth, nutrient broth No. 2, BHI, and blood agar base No. 2, all from Oxoid, and found that with the exception of

proteose peptone, all (when supplemented with NAD and hemin at 10 mg/l each) supported growth of 93 to 99% of 438 strains of *H. influenzae.* Several of these media, however, failed to support the growth of a significant number of NAD-dependent strains (only 75% of NAD-dependent strains grew on BHI plus NAD, 68% grew on nutrient broth No. 2 plus NAD, and 49% grew on proteose peptone plus NAD). In contrast, both Columbia agar plus NAD and blood agar base No. 2 plus NAD supported growth of 93 to 95% of the V-dependent isolates. The above studies suggest that some NAD-dependent isolates may be more exacting in their growth requirements, and that base media from various manufacturers may not always give similar results. (See "Identification" for further discussion of hemin and NAD tests, and for problems with hemin contamination of the base media.)

Minimal Media

Several minimal media have been developed, primarily for genetic studies (see below). In addition to requirements for NAD and hemin, *H. influenzae* has been shown to have a number of other growth factor requirements. The defined medium of Michalka and Goodgal (1969) contains 15 different components and that of Herriott et al. (1970b) contains even more. Not all strains of *H. influenzae* will grow on such media (according to Klein and Luginbuhl, 1979, 11 of 43 clinical isolates failed to grow even on the medium of Herriott et al., 1970b). Klein and Luginbuhl (1979) developed a somewhat less complicated defined medium, but even so, it contained a minimum of 13 compounds. Although not required, Tween 80, polyvinylalcohol, and bovine serum albumin (BSA) were found to improve growth (probably by adsorption of toxic products) and were generally included in addition to the 13 basic ingredients. Requirements for glutamic acid, glutathione, inosine, and citrulline (or arginine and uracil in place of citrulline) were common among the strains examined (Klein and Luginbuhl, 1979); thiamine and pantothenate are also apparently required (Holt, 1961; Klein and Luginbuhl, 1979).

Selective Media

Isolation of haemophili from nonsterile body sites is enhanced by inclusion of bacitracin (and/or vancomycin) in the culture media. This is particularly important in studies attempting to determine the frequency of *Haemophilus* species in the normal flora of the oral, pharyngeal, and genital mucosa, for isolation of haemophili from patients with upper respiratory tract infections, and for isolation of *H. ducreyi* from genital lesions. In these settings, other microorganisms may be more numerous and overgrow the haemophili, or in some instances, produce products that are inhibitory to haemophili. Haemophili are generally resistant to bacitracin, and Hovig and Aandahl (1969) recommended use of 300 μg/ml bacitracin to inhibit many of the contaminating organisms; this method has been used extensively by Kilian and coworkers in their surveys of normal oral and pharyngeal haemophili (Kilian and Schiott, 1975; Kuklinska and Kilian, 1984). Chapin and Doern (1983) observed break-through growth of normal upper respiratory tract flora on chocolate agar containing only bacitracin, and they devised a selective medium containing bacitracin (300 μg/ml) plus 5 μg/ml vancomycin and 1 μg/ml clindamycin. Although they did not compare their formulation to that of chocolate agar with bacitracin alone, they did find significantly improved recovery of *H. influenzae* on their medium compared to chocolate agar without antibiotics or to chocolate agar containing only vancomycin. Tempro and Slots (1986) found that a number of strains of *H. aphrophilus* were inhibited by 300 μg/ml bacitracin, and they devised a medium containing 75 μg/ml bacitracin, 5 μg/ml vancomycin, and 50 μg/ml sodium fluoride. Although *H. aphrophilus* was inhibited by 300 μg/ml bacitracin, they found that reduction of the bacitracin concentration to 75 μg/ml also failed to inhibit many of the contaminants (especially streptococci) and that these organisms significantly inhibited recovery of *H. aphrophilus* from clinical specimens. Addition of vancomycin (5 μg/ml) inhibited the streptococci and greatly improved recovery of *H. aphrophilus.* The sodium fluoride was added to inhibit *Actinobacillus actinomycetemcomitans.* Since this medium was designed to be selective for *H. aphrophilus,* it was deliberately lacking in NAD and would not have supported the growth of other *Haemophilus* species. The usefulness of this selective combination (particularly the use of sodium fluoride) for isolation of other *Haemophilus* species is not known.

The above studies all suggest that unless selective media are used, haemophili may often be missed. This is important in the interpretation of studies in which selective media were not used. Furthermore, some early studies of normal flora did not include media that would be capable of supporting the growth of haemophili.

H. ducreyi has traditionally been difficult to isolate. Introduction of Isovitalex-enriched chocolate agar containing 5 μg/ml vancomycin

(Hammond et al., 1978) has greatly improved recovery of *H. ducreyi* from chancroidal lesions. Inclusion of 5 to 10% fetal bovine serum has been reported to further enhance recovery (Sottnek et al., 1980; Nsanze et al., 1984), and the use of two different media may also improve chances of recovery. For a review of the various formulations that have been tested, see Morse (1989).

Growth Environment

It is often stated that *H. influenzae* does not require CO_2, but that some strains may grow better with CO_2. The author's experience, however, has been that CO_2 does not affect the growth of most strains, but occasional strains may not grow at all unless incubated in a candle jar. This includes some encapsulated strains as well as certain nontypables. It is not always clear from the literature, however, whether "CO_2 requirements" reflect a requirement for CO_2 per se, or a requirement for the increased humidity that is present in a candle jar or CO_2 incubator. Three "CO_2-requiring" strains tested in our laboratory grew in a closed jar with moistened paper towels (without a candle) but did not grow on parallel plates placed on the incubator shelf. Kraut et al. (1972) also found that moisture could substitute for the "CO_2 requirement" of *H. aphrophilus.*

The species *H. aphrophilus, H. paraphrophilus,* and *H. paraphrohaemolyticus* all supposedly require CO_2. Indeed, the original species definitions were based on a CO_2 requirement (Khairat, 1940; Zinnemann et al., 1968, 1971), and the names are derived from "aphros," the term used in classical times to describe CO_2 bubbles escaping from fermenting wine (Khairat, 1940). CO_2 dependence can, however, be lost on subculture (Zinnemann et al., 1971; von Essen et al., 1987). As already mentioned, the validity of CO_2 requirements for species distinction has been questioned (Kilian, 1976), especially as pertains to the distinction between *H. parahaemolyticus* and *H. paraphrohaemolyticus.* What is clear, however, is that many of these strains do require a "CO_2 environment" for optimal growth (particularly on primary isolation). Again, it is not clear whether it is CO_2 per se or increased humidity. According to Kilian (1976), occasional strains of *H. parainfluenzae* also need CO_2 for optimal growth (including certain nonhemolytic, biotype I strains that do not fit the classical definition of *H. paraphrohaemolyticus*). *H. ducreyi* also grows better under increased humidity and 5% CO_2 (Albritton, 1989). Incubation in a moist environment containing 5–10% CO_2 is thus recommended for all specimens potentially containing haemophili; this is true for the animal species as well (especially *H. paracuniculus, H. parasuis,* and *H. paragallinarum*).

Optimal growth for most *Haemophilus* species occurs between 35–37°C. *H. ducreyi* prefers a slightly lower temperature (33°C was reported as optimal; Sturm and Zanen, 1984).

Growth Rates

Haemophili are fastidious organisms, but contrary to what is often stated, they are not necessarily slow growing. In rich media, free of inhibitory substances, *H. influenzae* can double with a generation time similar to that of *E. coli* (26 min; Rubin, 1986). Not all species are rapid growers, however, and for *H. ducreyi,* it may take 48 h or more for colonies to appear (Morse, 1989). Some conjunctival isolates may also be slow growing *("H. aegyptius"?),* and *H. parasuis* is reported to grow "feebly," even after 48 h on chocolate agar (Kilian and Biberstein, 1984). *H. segnis, H. pleuropneumoniae,* and *H. paragallinarum* may also be somewhat slower growing.

Identification

Identification of the various *Haemophilus* species has been based primarily on hemin and NAD requirements, host origin, and a few additional biochemical tests (Kilian, 1976, 1985; Kilian and Biberstein, 1984). Haemophili are small, pleomorphic, Gram-negative rods (sometimes with filamentous forms); all reduce nitrate.

Hemin-and NAD-Requiring Human Isolates

Human isolates requiring both hemin and NAD are *H. influenzae, H. haemolyticus,* and *H. aegyptius. H. haemolyticus* is identified by its hemolytic activity, whereas *H. influenzae* and *H. aegyptius* are nonhemolytic. The problems in distinguishing *H. influenzae* and *H. aegyptius* have already been discussed (see description of the individual species under "Habitats"). *H. influenzae* is further identified on the basis of capsular antigens, with most invasive isolates being serotype b. The serotype b capsule is composed of a linear, repeating unit of ribose-ribitol-phosphate (Crisel et al., 1975) and is considered to be a major virulence determinant (Turk, 1982; Zwahlen et al., 1989). The structures of the other capsule types (a, c-f) are listed in Table 2. Serotyping reagents for both traditional slide agglutinations, and for latex or coagglutination

Table 2. Structures of the capsular polysaccharides of *H. influenzae.*

Serotype	Structure	Reference[a]
a	4)-β-D-Glc-(1→4)-D-ribitol-5-(PO_4→	1
b	3)-β-D-Rib-(1→1)-D-ribitol-5-(PO_4→	2, 3
c	4)-β-D-GlcNAc-(1→3)-α-D-Gal-1-(PO_4→ 3 ↑ OAc	4, 5
d	4)-β-D-GlcNAc-(1→3)-β-D-ManANAc-(1→ 6 ↑ R[b]	6, 7
e	3)-β-D-GlcNAc-(1→4)-β-D-ManANAc-(1→	8, 9
e′	3)-β-D-GlcNAc-(1→4)-β-D-ManANAc-(1→ 3 ↑ 2 β-D-fructose	9
f	3)-β-DGalNAc-(1→4)-α-D-GalNAc-(1→PO_4→ 3 ↑ OAc	10, 11

[a]Citations are: 1, Branefors-Helander et al. (1977); 2, Crisel et al. (1975); 3, Branefors-Helander et al. (1976); 4, Branefors-Helander et al. (1979); 5, Egan et al. (1980a); 6, Branefors-Helander et al. (1981a); 7, Tsui et al. (1981a); 8, Tsui et al. (1981b); 9, Branefors-Helander et al. (1981b); 10, Egan et al. (1980b); 11, Branefors-Helander et al. (1980).
[b]R = seronine, threonine, or alanine.

tests are available from a number of commercial suppliers (Difco, Wellcome Diagnostics, Pharmacia) and have been evaluated by Himmelreich et al. (1985). Nonencapsulated strains are nonagglutinable with capsular typing sera, although some strains are autoagglutinable, and saline controls should always be performed. Other factors may also cause occasional false positive reactions, and as emphasized earlier, iridescence on sBHI agar is an extremely useful aid in distinguishing encapsulated and nonencapsulated strains. Note, however, that iridescence begins to fade after approximately 20 to 24 h incubation.

Kilian (1976) introduced a biotyping scheme for *H. influenzae* based on the production of indole, urease, and ornithine decarboxylase (see Table 3). Biotyping is not mandatory for routine identification of *H. influenzae,* but has been of some use in surveys of normal flora versus disease isolates. It has limited value as an epidemiological tool for studying transmission of type b strains, since most invasive isolates tend to be biotype I (Barenkamp et al., 1981; Kilian et al., 1979; Oberhofer and Back, 1979). Biotype IV may be more common among genital isolates (Albritton et al., 1982; Wallace et al., 1983), and *"H. aegyptius"* is biotype III (though not all nonencapsulated, biotype III strains are *"H. aegyptius"*).

NAD-Dependent Human Isolates

As already mentioned (see "Habitats"), identification of NAD-dependent species is often problematic, and in practice, often not even attempted. Many labs simply report NAD-dependent isolates as *H. parainfluenzae.* Although the original description of *H. paraphrophilus* was based on a CO_2 requirement, this alone is not a reliable criterion for species designation (see "Habitats" and "Isolation"). Lactose fermentation, however, has been reported to be useful for separating *H. parainfluenzae* (lactose negative) from *H. paraphrophilus* (lactose positive; Albritton, 1988; Kilian, 1976; Kilian and Biberstein, 1984). Additional sugars that may be useful for distinguishing the NAD-dependent isolates are listed in Table 4. The biotyping scheme described above for *H. influenzae* has also been applied to *H. parainfluenzae,* although the numerical designations for a given combination of plus and minus test results are not the same for *H. influenzae* and *H. parainfluenzae* (Table 3). The situation is further complicated by the fact that two different definitions have been given for biotype IV *H. parainfluenzae.* Oberhofer and Back (1979) designated strains that were negative for all three tests as biotype IV, whereas Bruun et al. (1984) designated strains that were positive for all three tests as biotype IV. Some investigators have used the

Table 3. Characterization of the biotypes of *H. influenzae* and *H. parainfluenzae*.

Biotype designation		Production of		
H. influenzae	*H. parainfluenzae*	Indole	Urease	Ornithine decarboxylase
I[a]	V[b]	+	+	+
II[a]	VII[e]	+	+	−
III[a]	III[a]	−	+	−
IV[a]	II[a]	−	+	+
V[a]	VI[e]	+	−	+
VI[b]	I[a]	−	−	+
VII[c]	VIII[f]	+	−	−
VIII[d]	IV[b,g]	−	−	−

[a]Kilian, 1976.
[b]Oberhofer and Back, 1979.
[c]Gratten, 1983.
[d]Sottnek and Albritton, 1984.
[e]Sturm, 1986.
[f]Doern and Chapin, 1987.
[g]Bruun et al., 1984, used a different designation for biotype IV strains of *H. parainfluenzae;* they designated strains that were *positive* for all three reactions as biotype IV.

Table 4. Fermentation tests reported to be useful for distinguishing the NAD-dependent human species of *Haemophilus*.

	Acid production from						
Species	Lactose	Ribose	Sorbose	Melibiose	Mannitol[a]	Glucose	Sucrose[b]
H. parainfluenzae[c]	−[d]	−[d]	−[d]	−[d]	−[d]	+[d]	+[d]
	(0/121)[e]	(0/121)[e]		(0/83)[f]	(0/121)[e]	(121/121)[e]	(118/121)[e]
	(0/83)[f]				(0/83)[f]	(83/83)[f]	(83/83)[f]
H. paraphrophilus	+[d]	+[d]	+[d]	+[d]	−[d]	+[d]	+[d]
	(11/11)[e]	(11/11)[e]				(11/11)[e]	(11/11)[e]
H. segnis	−[d]	−[d]	−[d]	−[d]	−[d]	Weak[d]	Weak[d]
	(0/22)[g]	(0/22)[g]	(0/22)[g]			(22/22 weak)[g]	(22/22 weak)[g]

[a]Mannitol fermentation is a characteristic of the porcine species, *H. pleuropneumoniae* (see Table 6), and some avian isolates (most *H. paragallinarum* and some *H. avium;* Blackall and Reid, 1982).
[b]Acid from sucrose may be useful for distinguishing NAD-dependent species from *H. influenzae;* Kilian (1976) found that none of 185 strains of *H. influenzae* fermented sucrose and Oberhofer and Back (1979) reported negative sucrose reactions for 464 of 464 isolates of *H. influenzae*.
[c]This includes *H. parahaemolyticus* and *H. paraphrohaemolyticus;* see text for comments on these species.
[d]Kilian and Biberstein, 1984 (number of isolates tested not stated; + means > 90% positive; − means < 10% positive).
[e]Kilian, 1976.
[f]Oberhofer and Back, 1979.
[g]Kilian and Theilade, 1978.
Numbers in parentheses indicate the number of strains giving a positive test out of the total number of isolates tested.

Oberhofer and Back (1979) designation (Sturm, 1986; Doern and Chapin, 1987) whereas others have used the Bruun et al. (1984) designation (Kilian, 1985). Fortunately, a large percentage of *H. parainfluenzae* isolates fall into biotypes I–III (Oberhofer and Back, 1979), so this is only occasionally a problem. It becomes important, however, when the results of the biotyping tests are used as an aid in distinguishing *H. parainfluenzae* from *H. segnis* and *H. paraphrophilus,* both of which are reported to be negative for all three tests (Kilian, 1976; Kilian and Biberstein, 1984; Kilian and Theilade, 1978). A strain that is positive for one or more of the biotyping tests is probably not *H. segnis* or *H. paraphrophilus,* but a strain that is negative for all three tests should be carefully evaluated if a definitive species identification is desired. It should, however, be kept in mind that the above statement is based on data from only a limited number of strains of *H. segnis* and *H. paraphrophilus.*

The problems in distinguishing *H. parahaemolyticus, H. paraphrohaemolyticus,* and *H. parainfluenzae* have already been discussed (see "Habitats"). *H. parahaemolyticus* was defined on the basis of NAD-dependence and hemolysis, while *H. paraphrohaemolyticus* was de-

fined as NAD-dependent, CO_2-requiring, and hemolytic. These traits can, however, be lost on subculture, and/or appear in other species. *H. parahaemolyticus* and *H. paraphrohaemolyticus* are otherwise biochemically similar to *H. parainfluenzae* (Kilian, 1976), although there may be an inverse correlation between hemolysin production and mannose fermentation (Kilian and Biberstein, 1984; Oberhofer and Back, 1979). The type strain of *H. parahaemolyticus* (NCTC 8479) does not, however, appear to be related to *H. parainfluenzae* when tested by DNA hybridization (Pohl, 1981). The type strain of *H. paraphrohaemolyticus* (NCTC 10670 = ATCC 29237) also appears to be unrelated to *H. parainfluenzae;* it does, however, appear to be closely related to *H. aphrophilus* and *H. paraphrophilus* (Pohl, 1981). It is thus not surprising that there is considerable confusion concerning the identification of the NAD-dependent haemophili. Other discrepancies concerning NAD-dependent strains include ATCC strain 7901, which was deposited as *H. parainfluenzae,* but which by DNA hybridization appears to be *H. paraphrophilus* (Pohl, 1981; Potts et al., 1986). Conversely, *H. paraphrophilus* strain NCTC 10558 (= ATCC 29242) was considered to be *H. parainfluenzae* by Kilian (1976); DNA hybridization studies with this strain, however, have yielded conflicting results (Tanner et al., 1982; Potts and Berry, 1983). The DNA hybridization studies of Roberts et al. (1986) also suggest considerable heterogeneity among strains labeled *H. parainfluenzae,* although in this study ATCC strain 7901 showed significant homology to a number of strains labeled *H. parainfluenzae.* Clearly, additional DNA hybridization studies are needed in order to clarify some of the above-mentioned problems, as well as to establish the appropriateness of the biochemical tests used for speciation of the NAD-dependent haemophili.

The DNA hybridization studies of Potts et al. (1986) appear to have justified Kilian's proposal (1976) for creation of the species *H. segnis.* This species was proposed on the basis of a high GC content, and weak fermentation capacity. *H. segnis* is reportedly negative for all three biotyping reactions (urease, indole, and ornithine decarboxylase), and gives weak acid reactions for glucose and sucrose, but is negative for lactose, ribose, and melibiose (Kilian and Theilade, 1978; see Table 4).

Hemin-Dependent Human Isolates

H. ducreyi is identified partially on the basis of its clinical circumstance (i.e., from genital lesions), combined with Gram stain, hemin dependence, and negative sugar reactions. Gram stain alone should not be used to make a definitive diagnosis, as other (sometimes Gram-variable) organisms can resemble *H. ducreyi.* The "school of fish" pattern (arrangement of organisms in long parallel chains) observed by early investigators is not always observed and is more commonly observed with plate-grown organisms than in direct smears (Morse, 1989). Hemin dependence cannot be demonstrated by the traditional disk or strip methods (see below) because *H. ducreyi* does not grow on the routine media used for these tests (Hammond et al., 1978; Oberhofer and Back, 1982). Hemin dependence has, however, been demonstrated using BBL strips on GC agar base supplemented with 0.1% glucose and 0.01% glutamine (Hammond et al., 1978), or on GC agar base supplemented with glucose, glutamine and cysteine (Sottnek et al., 1980). *H. ducreyi* requires much higher hemin concentrations than do other hemin-dependent haemophili (Hammond et al., 1978), and while the hemin strips from BBL contain sufficient hemin to initiate growth of *H. ducreyi* (Hammond et al., 1978; Sottnek et al., 1980), the Difco disks apparently do not (Difco Laboratories, personal communication). The recommended method for determining the hemin requirement is the porphyrin test (see below).

Hemin- and NAD-Independent Human Isolates

H. aphrophilus does not require NAD, and as indicated earlier, generally does not require hemin either (although it may on primary isolation); it is porphyrin positive. *H. aphrophilus* can be distinguished from other haemophili, since with the exception of *H. ducreyi* (and the animal species, *H. haemoglobinophilus*), all other haemophili require NAD, or both hemin and NAD. The major problem, therefore, lies in distinguishing *H. aphrophilus* from other small, Gram-negative coccobacilli, including *Actinobacillus actinomycetemcomitans, Cardiobacterium hominis,* and *Eikenella corrodens,* all of which share a similar habitat with *H. aphrophilus,* and which like *H. aphrophilus,* may occasionally cause endocarditis or brain abscesses. Like *H. aphrophilus,* these organisms also grow best with increased CO_2. Tests useful for distinguishing these organisms are shown in Table 5.

NAD-Dependent Porcine Isolates

The porcine isolates *H. pleuropneumoniae* and *H. parasuis* are distinguished from each other primarily on the basis of the urease test and the

Table 5. Tests useful for distinguishing *Haemophilus aphrophilus* from other Gram-negative coccobacilli or small rods.

Species	Glucose fermentation	Indole	Catalase	Lactose fermentation	Sucrose fermentation	Nitrate reduction
H. aphrophilus[a]	+ (16/16)	− (0/16)	− (1/16)	+ (16/16)	+ (16/16)	+ (16/16)
Eikenella corrodens[b]	− (0/595)	− (0/595)	(+/−) (54/595 weakly positive)	− (0/595)	− (0/595)	+ (593/595)
Cardiobacterium hominis[c]	+ (32/32; but some take > 3 days)	+ (31/32)	− (1/32)	− (0/32)	+ (32/32; but some take > 3 days)	− (0/32)
Actinobacillus actinomycetemcomitans[c]	+ (119/120; but some take > 3 days)	− (0/120)	+ (119/120)	− (0/120)	− (0/120)	+ (120/120)

Symbols: +, >90% positive; −, <10% positive; numbers in parentheses indicate number of strains giving a positive test out of the total number of isolates tested.

[a]Based on Kilian, 1976. *H. aphrophilus* is resistant to 50 μg/ml sodium fluoride, whereas *A. actinomycetemcomitans* is reported to be sensitive (Tempro and Slots, 1986).

[b]Rubin et al., 1985.

[c]Weaver et al., 1985.

CAMP reaction, with *H. pleuropneumoniae* positive, and *H. parasuis* negative for both tests (Table 6). The CAMP reaction detects a hemolysin that acts synergistically with the *Staphylococcus aureus* β- toxin; *H. pleuropneumoniae* is the only *Haemophilus* species to give a positive CAMP reaction. Mannitol fermentation also serves to distinguish *H. pleuropneumoniae* from the other porcine isolates, as well as the human NAD-dependent species, all of which are negative for mannitol fermentation (See Tables 4 and 6). *H. parasuis* grows much more feebly than *H. pleuropneumoniae*, and this is also a useful distinction.

NAD-Dependent Avian Isolates

The avian isolates *H. paragallinarum* and *H. avium* are distinguished from each other primarily on the basis of the catalase test and galactose fermentation, with *H. avium* being positive for both tests and *H. paragallinarum* negative (Blackall, 1988; Blackall and Reid, 1982). As mentioned earlier, *"H. avium"* isolates are genetically and biochemically heterogeneous, and Mutters et al. (1985) have proposed that *H. avium* be split into three species in the genus *Pasteurella* (as *Pasteurella avium, Pasteurella volantium,* and *Pasteurella* species A). Not all isolates from chickens fit the criteria for these three species (Blackall, 1988), and additional studies are needed.

Of the 39 catalase-positive strains studied by Blackall (1988), all were found to produce acid from glucose, galactose, fructose, and mannose; 37 of 39 strains also fermented trehalose. Of the 19 catalase-positive strains studied by Mutters et al. (1985), all were found to ferment glucose, galactose, fructose, mannose, sucrose, and trehalose. In both studies, the variable profiles for arabinose, maltose, mannitol, sorbitol, and xylose indicated considerable heterogeneity among strains. The nearly universal fermentation of trehalose, however, helps distinguish *"H. avium"* from human isolates of *H. parainfluenzae* (Oberhofer and Back, 1979, found that none of 83 *H. parainfluenzae* strains fermented trehalose). Readers should consult Blackall (1988) and Mutters et al. (1985) for the biochemical profiles of individual *"H. avium"* isolates. Biochemical profiles for 39 strains of *H. paragallinarum* can be found in Blackall and Reid (1982). Most *H. paragallinarum* isolates require chicken serum and CO_2 for growth (Blackall and Reid, 1982).

The animal isolates are generally assumed to be relatively host-species adapted, and this is weighted quite heavily in most identification schemes. It is interesting to note, however, that one strain considered by Mutters et al. (1985) to be *Pasteurella (Haemophilus) avium,* was isolated from a calf; this calf isolate showed 88% homology to the type strain of *Pasteurella (Haemophilus) avium.* As already mentioned, an organism highly homologous to *H. avium* has also been isolated from a human tongue (e.g., NCTC 4101, originally considered *H. parainfluenzae,* shows 80% homology to *H. avium* DNA; Mut-

Table 6. Differentiation of NAD-dependent porcine isolates.

	Acid from					
	Mannitol	Lactose	Xylose	Ribose	Urease	CAMP reaction
H. pleuropneumoniae	+[a] (43/43)[b]	V[a] (4/43)[b]	+[a] (43/43)[b]	+[a] (43/43)[b]	+[a] (43/43)[b]	+[a] (43/43)[b]
"Minor group"[b]	– (0/4)	+ (4/4)	– (0/4)	– (0/4)	+ (4/4)	– (0/4)
H. parasuis[d]	–[a] (0/9)[c]	V[a] (0/9)[c]	–[a] (0/9)[c]	+[a] (9/9)[c]	–[a] (0/9)[c]	–[a]

[a]Kilian and Biberstein, 1984 (number of isolates tested not stated; + means >90% positive; – means < 10% positive; and V means variable). Numbers in parentheses indicate the number of strains giving a positive test out of the total number of isolates tested.
[b]Kilian et al., 1978.
[c]Kilian, 1976.
[d]Some urease-negative, CAMP-negative strains isolated by Pijoan et al. (1983) were positive for xylose and/or mannitol, suggesting that the situation might not be as straightforward as this table would indicate.

ters et al., 1985; Pohl, 1981). In addition to these *"H. avium"* isolates from nonavian hosts, organisms considered to be *H. pleuropneumoniae* have been isolated from a lamb and a steer (Biberstein et al., 1977).

Laboratory Tests for the Identification of *Haemophilus* Species

One of the most important tests for identifying *Haemophilus* species is the determination of the hemin and NAD requirements. While conceptually simple, this step can cause problems unless carefully performed. Originally, NAD requirements were demonstrated by satellite growth on blood agar plates containing a contaminant NAD-secreting organism, or on blood agar plates that had been deliberately cross-streaked with an NAD-secreting strain of *Staphylococcus aureus.* The presence of satellite colonies around such NAD-producing organisms, and absence of growth on sheep blood agar plates without a feeder culture, is suggestive evidence that one is dealing with an NAD-dependent *Haemophilus* species. This evidence should not, however, be taken as definitive proof, since other microorganisms can occasionally show satellite growth for reasons other than NAD-dependency. Determination of hemin and NAD requirements has long been performed by placing hemin and NAD-impregnated paper disks or strips on blood-free media. The problem with this method is that haemophili have nutritional requirements in addition to NAD and hemin (see "Isolation"), and the media used for testing must satisfy all the other requirements, but be free of NAD and hemin. Since NAD is destroyed by autoclaving, this is not a problem; hemin contamination of base media, however, is sometimes a problem, and individual lots should be carefully checked (i.e., should be incapable of supporting growth of known strains of *H. influenzae* unless both factors are added). Another problem concerning hemin testing may be carry-over of hemin from the original culture plate. Colonies should be picked carefully and resuspended in saline (to reduce carry-over) before swabbing to the plate for factor testing. Factor-containing disks or strips are available from a number of commercial suppliers (BBL, Difco, Remel); brain heart infusion, heart infusion, or tryptic soy agar are commonly used as base media (Doern and Chapin, 1984; Lund and Blazevic, 1977).

Because of the problems associated with the above method, more definitive proof of a hemin requirement can be obtained by the porphyrin test. This test, based on the observations of Biberstein et al. (1963), and modified by Kilian (1974), detects intermediates in the hemin biosynthetic pathway. When supplied with the hemin precursor, δ-aminolevulinic acid (ALA), hemin-independent species can convert ALA to porphyrin. Because they lack the enzymes of the hemin pathway, hemin-dependent species are unable to convert ALA to porphyrin. Porphyrin production by hemin-independent species can be readily detected by the orange-red fluorescence produced following exposure of ALA-exposed cultures to long-wave ultraviolet light (360 nm). A variety of formats have been used to detect porphyrin production from ALA, including a tube test (Kilian, 1974), disk tests, and incorporation of ALA into the agar growth medium (Zadik, 1982; Gadberry and Amos, 1986). The disk method is probably not as sensitive as the tube test; the tube test, however, requires a large inoculum and may require subculture from the primary isolation plate before inoculation. Porphyrin test agar is relatively new, and has not been extensively compared to the other methods; it may be more sensitive than the disk

method (Gadberry and Amos, 1986), but the number of isolates tested was small.

Porphyrin Tube Test (Based on Kilian, 1974)

1. Prepare 2 mM ALA (Sigma), 0.8 mM $MgSO_4$ in 0.1 M phosphate buffer, pH 6.9. Distribute in 0.5-ml quantities in small glass tubes (solution can be frozen at −20°).
2. Resuspend a *heavy* loopful of bacteria in the above solution (culture should be <24 h old and suspension should appear milky; Lund and Blazevic, 1977).
3. Incubate 4 h at 37°C.
4. Expose tube to 360-nm UV light; red fluorescence indicates porphyrin production (i.e., hemin not required for growth).

Other tests of importance for identifying *Haemophilus* species include sugar fermentations and the production of indole, urease, and ornithine decarboxylase. For determination of sugar reactions, Kilian (1976; 1985) recommended use of 1% sugar in phenol red broth base (Difco), supplemented with 10 μg/ml each NAD and hemin. This method generally requires incubation for 24 h or longer. Workers at the U.S. Centers for Disease Control prefer a rapid (4 h), nongrowth method, utilizing small volumes and heavy inocula (Hollis et al., 1980; Miller and Sottnek, 1987). A number of kits containing miniaturized, dehydrated reagents are available commercially, although these sometimes give discrepant sugar reactions. Doern and Chapin (1984) found that the RapID NH System (Innovative Diagnostics Systems, Inc.) correctly identified only 89.8% of 187 strains of *H. influenzae;* most of the incorrect identifications were due to false-negative glucose reactions. Oberhofer and Back (1979), using the BBL Minitek system, however, reported very consistent sugar reactions for a large number of strains (461 of 464 strains of *H. influenzae,* and 83 of 83 strains of *H. parainfluenzae* gave positive glucose reactions; none of 464 *H. influenzae* fermented sucrose, whereas 83 of 83 strains of *H. parainfluenzae* gave positive reactions with sucrose). Kawakami et al. (1981) compared the Minitek system to that of the conventional phenol red broth method and found extremely good agreement between the two methods.

Several of the commercially available kits also contain reagents for the detection of urease, tryptophanase (indole production), and ornithine decarboxylase. Traditional methods (Kilian, 1976, 1985) for determination of these reactions are presented below. All substrates are dispensed in 0.5-ml volumes.

Indole Test

1. Prepare 0.1% tryptophan in 0.05 M phosphate buffer, pH 6.8.
2. Inoculate heavily and incubate at 37°C for 4 h.
3. Add an equal volume of Kovac's reagent and shake; red color in the upper layer indicates the presence of indole.

Urease Test

1. Prepare a 20% solution of urea; filter sterilize.
2. Prepare 1:500 phenol red (0.2 g phenol red, 8 ml NaOH, 92 ml water).
3. Add 0.1g KH_2PO_4, 0.1g K_2HPO_4, 0.5g NaCl, and 0.5 ml of the phenol red solution to 100 ml water. Adjust to pH 7.0 and autoclave. After autoclaving, add 10.4 ml of the filter-sterilized urea.
4. Inoculate heavily and incubate at 37°C for 4 h. Red color indicates urease activity.

Ornithine Decarboxylase Test

Use Moeller's decarboxylase medium (Difco) and inoculate heavily. Incubate at 37°C for 4–24 h; purple color indicates decarboxylase activity.

Genetics

Although *H. influenzae* is one of the naturally transformable microorganisms (Kahn and Smith, 1984), it has not been as amenable to genetic analysis as some of the more robust organisms like *E. coli* and *Salmonella.* The complex nutritional requirements of the haemophili have made analysis of auxotrophic mutants difficult, and only a limited number of auxotrophic markers and antibiotic resistance genes have been mapped by transformation (Michalka and Goodgal, 1969; Stuy, 1985). Transducing phages have not been described, and there is but a single report of a conjugal system for the ordered transfer of chromosomal genes (Deich and Green, 1987). This system utilized an F′ from *E. coli* and transfer of *H. influenzae* chromosomal genes occurred only at very low frequency. Until recently (see below for a discussion of mapping by pulse-field electrophoresis), transformation has been the mainstay for genetic analysis in this genus, with work limited mainly to *H. influenzae* and *H. parainfluenzae.*

Transformation

During exponential growth, *H. influenzae* and *H. parainfluenzae* transform poorly, as only about one cell in ten thousand is competent for DNA uptake. Under conditions of slowed growth, however, competence can be induced in the entire population, and transformation of chromosomal markers can occur with frequencies of 1–5% (Herriot et al., 1970a; Kahn and

Smith, 1984). The protocols for induction of competence in *H. influenzae* and *H. parainfluenzae* are different. *H. parainfluenzae* undergoes a significant loss of viability in the protocol giving optimal transformability of *H. influenzae,* whereas *H. influenzae* undergoes severe loss of viability in the protocol giving optimal transformation of *H. parainfluenzae* (Barany and Kahn, 1985). The protocol for *H. influenzae* involves an incubation period in a nongrowth minimal medium (the MIV medium of Herriott et al., 1970a), whereas the protocol for *H. parainfluenzae* utilizes overnight incubation as a thin layer in a stationary petri dish (Gromkova and Goodgal, 1979). The latter method has, however, been used by Gromkova et al. (1989) for transformation of *H. influenzae,* but the transformation frequencies were significantly lower than those obtained by other investigators using the MIV protocol. The stationary method is easier to perform, so depending on the transformation frequency required, this method may be sufficient (e.g., to move a chromosomal streptomycin-resistance marker into a new strain, etc.). Growth of cells (with aeration) to a density of 1×10^9/ml, followed by a shift to more anaerobic conditions (e.g., by tipping the culture into the arm of a side-arm flask and holding without agitation for 90 min) has also been used with some success (Goodgal and Herriott, 1961) but this method is not as reliable as the MIV method of Herriott et al. (1970a).

Transformation of both *H. influenzae* and *H. parainfluenzae* involves binding of DNA (via specific uptake sequences—see below) to membranous blebs that appear on the surface of competent cells (Barany et al., 1983; Kahn et al., 1983). These membranous blebs have been termed "transformasomes." The mechanism by which DNA passes from the transformasomes into the cell also differs between *H. influenzae* and *H. parainfluenzae* (Barany and Kahn, 1985).

Unlike the artificial transformation of *E. coli* with plasmid DNA (which utilizes calcium chloride plus a temperature shock and which produces a leaky membrane), transformation of naturally transformable organisms like *Haemophilus* occurs via a genetically determined system for the active uptake and integration of exogenous DNA. DNA is bound by specific receptors that appear on the surface of competent cells, and for *H. influenzae* and *H. parainfluenzae,* these receptors recognize specific sequences that occur on *Haemophilus* DNA and which are generally absent or rare on foreign DNA; transformation of *Haemophilus* is thus relatively specific for uptake of *Haemophilus* DNA (Danner et al., 1982; Deich and Smith, 1980; Goodgal and Mitchell, 1984; Kahn and Smith, 1984; Scocca et al., 1974; Sisco and Smith, 1979).

During transformation of competent *H. influenzae,* DNA passes from the transformasome into the cell via a mechanism that results in degradation of one complete strand of DNA, as well as degradation of the entering strand from the 3′ end (Barany et al., 1983). The entering single strand searches for its homolog in the chromosome and is incorporated via *rec*-dependent homologous recombination. Although highly efficient for introduction of chromosomal markers, the degradative step makes this a poor method by which to introduce plasmid DNA into *H. influenzae.* Plasmid transformation of competent *H. influenzae* occurs only at very low frequency, and is thought to occur via accidental escape of intact double-stranded plasmid DNA from the transformasome (Pifer, 1986). The efficiency of plasmid transformation can be improved by adding glycerol to competent cells (Stuy and Walter, 1986) or by ligating linearized plasmid DNA to form concatamers (Pifer, 1986).

As indicated above, *H. influenzae* and *H. parainfluenzae* are considered to be naturally transformable microorganisms, and their mechanisms of transformation have been extensively studied. It was thus somewhat surprising to learn that a significant number of clinical isolates of *H. influenzae* appear to be nontransformable (Cope et al., 1989; Rowji et al., 1989). The evolutionary significance of this observation is not yet understood. Transformation of other *Haemophilus* species has not been well studied, but Leidy et al. (1956) and White et al. (1964) reported transformation of *H. parasuis* by *H. parasuis* DNA; the efficiency, however, was considerably lower than they observed for transformation of *H. influenzae* with *H. influenzae* DNA. White et al. (1964) also attempted to transform *H. pleuropneumoniae* but found it to be "virtually incompetent as a receptor in transformation studies." Additional studies, however, are needed. As expected (by virtue of its close relationship to *H. influenzae*), *"H. aegyptius"* is also naturally transformable (White et al. 1964).

Transposon Mutagenesis

A system for direct transposon mutagenesis of *Haemophilus* species has only recently been described (Kauc and Goodgal, 1989a). This system utilizes the 16.4-kb, tetracycline-resistance element, Tn*916,* derived originally from *Streptococcus.* Cloned *Haemophilus* genes can also

be mutagenized with transposons in *E. coli* and the insertion mutations returned to the *H. influenzae* chromosome by transformation (Grundy et al., 1987; Tomb et al., 1989). The kanamycin-resistance gene of Tn*5* is apparently not expressed well in *Haemophilus* and selection on even low concentrations of kanamycin leads to amplifications and other chromosomal rearrangements (Grundy et al., 1987; S. Hoiseth, unpublished observations). The kanamycin-resistance gene of Tn*903*, however, appears to function well in *H. influenzae* and mini-Tn*10 kan* (which contains the kanamycin-resistance gene from Tn*903*) has been used successfully (Tomb et al., 1989).

Haemophilus Cloning Vectors

The tetracycline-resistance gene (non-Tn*10*) of the *E. coli* cloning vector pBR322 does not express in *Haemophilus,* and the plasmid does not replicate in *Haemophilus.* Shuttle vectors capable of replicating in both *E. coli* and *Haemophilus* have therefore been developed for cloning *Haemophilus* DNA in *H. influenzae* (Danner and Pifer, 1982; McCarthy et al., 1982; Tomb et al., 1989). A number of *Haemophilus* genes have also been cloned in *E. coli* (see Lee et al., 1989, for a partial list); at least some of these genes appear to be expressed in *E. coli* using their own promoters (Holmans et al., 1985; Nelson et al., 1988).

Chromosome Mapping by Pulse-Field Gel Electrophoresis

The technique of pulse-field gel electrophoresis has been used to study the organization of the *H. influenzae* and *H. parainfluenzae* chromosomes, and ordered restriction maps of these species have recently been published (Kauc and Goodgal, 1989b; Kauc et al., 1989; Lee et al., 1989). By this technique, the size of the *H. influenzae* chromosome was estimated to be 1834 (Lee and Smith, 1988) to 1980 kb (Kauc et al., 1989) while that for *H. parainfluenzae* was estimated at 2340 kb (Kauc and Goodgal, 1989b). A number of cloned *Haemophilus* genes have been used as hybridization probes to localize known gene products to specific chromosomal restriction fragments (Lee et al., 1989). This technique should very rapidly increase our understanding of the genetics of *Haemophilus* species and should be useful for comparing the relationships between isolates.

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CHAPTER 180

The Genus *Pasteurella*

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The genus *Pasteurella* is the type genus of the family Pasteurellaceae which comprises the recognized genera *Actinobacillus, Haemophilus,* and *Pasteurella* (Mannheim, 1984). The organization of *Actinobacillus, Haemophilus,* and *Pasteurella* in a family has been established from phenotypic comparisons (Kilian and Frederiksen, 1981a, 1981b) as well as DNA-DNA hybridization analysis (Christian et al., 1981; Mannheim, 1981; Pohl, 1981) and 16S rRNA sequence analysis (Chuba et al., 1988). The members of the family Pasteurellaceae have had various taxonomic placements over the past 50 years. Some of these include Parvobacteriaceae in 1934 (Bergey, 1934) and Brucellaceae in 1957. The latter family included other genera such as *Yersinia, Francisella, Moraxella, Bordetella,* and *Brucella* in addition to *Actinobacillus, Haemophilus,* and *Pasteurella* (Breed et al., 1957). In the 1984 *Bergey's Manual of Systematic Bacteriology,* the family Pasteurellaceae is listed in Section 5 under "Facultatively Anaerobic Gram-Negative Rods" (Manheim, 1984). A detailed taxonomic analysis of the genus *Pasteurella* is described by Mutters et al. (1989). Using 16S RNA cataloging analysis, Woese et al. (1985) proposed that the family Pasteurellaceae be placed in the gamma subgroup of the purple bacteria together with the Enterobacteriaceae and the Vibrionaceae as the three families of Gram-negative facultative anaerobic bacteria (Woese, 1987).

The genus *Pasteurella* was established by Trevisan in 1887 and named after Louis Pasteur. The type species for the genus is *P. multocida,* which was previously named variously as *P. cholerae-gallinarum* and *P. gallicida.* Bacteria in the genus *Pasteurella* are commensal parasites on mucous membranes of vertebrates, particularly mammals and birds. Many species are potential pathogens, acting opportunistically when the host's defenses are compromised. The 1984 *Bergey's Manual of Systematic Bacteriology* recognized the following species: *P. multocida, P. pneumotropica, P. haemolytica, P. ureae, P. aerogenes,* and *P. gallinarum* (Carter, 1984).

The species *P. anatipestifer* has been placed under the name of *Moraxella anatipestifer* in the same edition of *Bergey's Manual* (Carter, 1984) even though there is considerable doubt about its proper placement. Due to the extensive use of its name, some of the properties of *P. anatipestifer* will also be described in this article. Although the species *P. ureae* has been proposed to be placed under the genus *Actinobacillus* (Bercovier et al., 1984; Escande et al., 1984), this reclassification is still under debate.

Using DNA-DNA hybridization studies under stringent reassociation conditions, a genus-like group *P. sensu stricto* was also established which includes a group of bacteria having a DNA binding level of 50% or higher with the rest of the genus of *Pasteurella* (Mutters and Mannheim, 1984; Mutters et al., 1985). The genetic relationship of the bacteria in this group remains to be clarified.

Biochemical Characteristics

The genus *Pasteurella* may be characterized as: small, Gram-negative cocci or rods of dimensions 0.3–1.0 by 1.0–2.0 μm, bipolar staining, nonmotile, facultatively anaerobic, catalase and oxidase positive, fermenting glucose and other carbohydrates and producing acid but no gas. Temperature range for growth is 22–44°C, with the optimum at 37°C. They are negative in the methyl-red, Voges-Proskauer, and gelatinase tests. The GC content of the DNA is between 40–45 mol%. Only *P. haemolytica* produces a zone of β-hemolysis on blood agar plates. Most strains of *P. haemolytica* and *P. multocida* also produce a neuraminidase.

Habitats

Members of this genus are commonly found as commensals on the mucous membranes of the upper respiratory and digestive tracts of healthy

mammals and birds. *P. multocida* has been recovered from many species including domesticated and wild, as well as healthy and diseased animals (Rimler and Rhoades, 1989b; Snipes et al., 1988a). Some of the major sources of *P. multocida* include cattle, buffalo, swine, goats, turkeys, chicken, ducks, geese, wild fowl, cats, and dogs. As an animal pathogen, *P. multocida* causes fowl cholera in chickens, turkeys, and other avian species (Rhoades and Rimler, 1989b); hemorrhagic septicemia of cattle and buffalo (Carter and de Alwis, 1989); and atrophic rhinitis of pigs (Chanter and Rutter, 1989). *P. multocida* is also the major cause of bacterial infections in humans resulting from animal bites (Arons et al., 1982).

P. haemolytica has been recovered frequently from cattle, sheep, and goats and occasionally from the upper respiratory tract of chickens and turkeys. It is the principal microorganism involved in pneumonic pasteurellosis in cattle in North America (Frank, 1989; Yates, 1982) and pneumonia and septicemia in sheep in the United Kingdom (Gilmour and Gilmour, 1989). It has also been reported to cause fatal pneumonia in bighorn sheep in the wild (Foreyt, 1989).

P. pneumotropica can be recovered from guinea pigs, hamsters, mice, rats, cats, and dogs (Kunstyr and Hartmann, 1983; Lesher et al., 1985). Most reported isolations of *P. pneumotropica* have been from laboratory animals where it is a potential respiratory pathogen (Kunstyr and Hartmann, 1983). Additionally, *P. pneumotropica* has also been shown to be one of the dominant microorganisms in the vagina of healthy adult rats (Carthero and Aldred, 1988; Ward et al., 1978; Yamada et al., 1986). In rare incidences, *P. pneumotropica* has been found to cause infections in humans (Gadberry et al., 1984; Sammarco and Leist, 1986).

The natural host of *P. ureae* appears to be humans, the bacterium being located mainly in the upper respiratory tract (Bigel et al., 1988). Reports of *P. ureae* isolation from rats or mice may be the result of misidentification (Mutters et al., 1984). In the compromised host, *P. ureae* may cause peritonitis (Nobel et al., 1987), septicemia and meningitis (Grewal et al., 1983; Marriott and Brady, 1983; Verhaegan et al., 1988; Yagupsky et al., 1985).

P. anatipestifer is an opportunistic pathogen in domestic ducks and turkeys as well as in waterfowl such as geese and swans in the wild (Donahue and Olson, 1969; Munday et al., 1981). It affects primarily the respiratory tract and the nervous system and results in pericarditis and perihepatitis as well as meningitis in a variety of avian species (Brogden, 1989). The bacterium may be transmitted among young avian species under favorable conditions and then be spread to other birds. The mode of transmission among waterfowl in the wild is not known, although the bacterium appears to be carried by healthy geese and swans in a subclinical form. Under conditions of environmental stress, overcrowding and predisposing viral or other bacterial infections (Mo and Burgess, 1987), *P. anatipestifer* can result in disease outbreaks in dense natural bird flocks. Juvenile birds appear to be more susceptible to infection than mature adults (Wobeser and Ward, 1974). Once an enzootic outbreak occurs, transmission to domestic duck and turkey populations may take place via contaminated drinking water. Mortality may reach 12% in turkey flocks. The route of infection is thought to be the respiratory tract or puncture wounds. Acute clinical signs include lacrimation, nasal discharge, and diarrhea, as well as mild coughing and sneezing in the early stages (Chaudbury and Mahanta, 1985). Later stages of infection result in leg weakness, tremors, loss of equilibrium, and eventually death (Heddleston, 1975). As mentioned above, the taxonomic placement of *P. anatipestifer* is uncertain. This bacterium has been placed in the genus *Pfeifferella* (Bergey, 1934), and recently *Moraxella* (Carter, 1984). However, based on DNA hybridization studies and biochemical characterization of fatty acids, it has been suggested that *P. anatipestifer* should be transferred to the family Cytophagaceae together with other members of *Flavobacterium* and *Cytophaga* (Piechulle et al., 1986).

P. gallinarum is usually recovered from the upper respiratory tract in chickens and turkeys. It is a weak pathogen and is often associated with secondary respiratory infections (Heddleston, 1975). *P. aerogenes* is found in the intestinal tracts of pigs and appears to be part of the normal flora and nonpathogenic (McAllister and Carter, 1974). Little is known about the characteristics of these two species of *Pasteurella* since they appear to be of minor importance in veterinary medicine.

Pathogenesis and Virulence

Most of these bacteria coexist peacefully with the animal hosts as obligate parasites. However, under conditions which compromise the host defenses, clinical infections are manifested. Such conditions include environmental stress as well as concurrent or prior infection by other bacterial or viral agents. As successful pathogens, bacteria of the *Pasteurella* genus possess and express a number of virulence factors that

contribute to the manifestations of disease. To date, most of the research on the pathogenesis and virulence factors of these bacteria has been focussed on *P. multocida* and *P. haemolytica* due to the significant economic loss resulting from diseases caused by these two microorganisms.

Virulence Factors of *Pasteurella multocida*

Based on specific antigens associated with the capsule, there are five serotypes of *P. multocida* (Rimler and Rhoades, 1989b). Recently, a sixth serotype has been proposed (Rhoades and Rimler, 1989a). In pigs, and sometimes goats, *P. multocida* serotypes A and D cause atrophic rhinitis and pneumonia. Atrophic rhinitis is a contagious disease with clinical symptoms of sneezing, nasal discharge, shortening and twisting of the snout, pneumonia, and reduced growth rates. Death does not necessarily occur. Pathogenic strains of serotypes A and D produce a similar toxin which is a major virulence factor in atrophic rhinitis. *P. multocida* strains of serotype A have also been shown to be the primary cause of fowl cholera in poultry. The common symptoms of fowl cholera include ruffled feathers, fever, anorexia, and diarrhea, leading to death. *P. multocida* also infects cattle and water buffaloes and causes a disease called haemorrhagic septicemia (Carter and de Alwis, 1989). Hemorrhagic septicemia is a fatal disease caused by serotype B (and occasionally serotype E) of *P. multocida,* resulting in respiratory stress, edematous swelling of the head-throat-brisket region, and hemorrhage in many organs and tissue.

P. multocida isolates recovered from atrophic rhinitis of pigs produce a protein toxin which has been shown to be a major virulence factor of the bacterium (Chanter and Rutter, 1989). Comparison of experimental infection of pigs with toxigenic and nontoxigenic *P. multocida* isolates further demonstrates the importance of this toxin in pathogenesis (Elling and Pederson, 1985; Rutter, 1985). Intranasal inoculation of cell-free culture supernatant from toxigenic *P. multocida* results in atrophy of the turbinate bones in rabbits and pigs (Dominick and Rimler, 1986; Rutter and Mackenzie, 1984).

The toxin is produced in stationary-phase cultures and appears to be released on cell lysis (Nakai et al., 1985). The purified toxin has a molecular weight varying from 112–160 kDa. The toxin contains 2–8% carbohydrate and can be inactivated by formaldehyde, heat, or proteolytic enzymes. Mild treatment with trypsin and partial denaturation with dithiotheritol dissociate the toxin into three fragments of molecular weights 23 kDa, 67 kDa, and 74 kDa (Nakai and Kume, 1987a). The fragments may reassociate to form an active protein (Nakai and Kume, 1987b). The primary histopathological features of turbinate atrophy produced by the purified toxin were progressive degeneration of osteoblasts, increase in osteoclasts, and osteoclastic bone resorption (Chanter et al., 1986; Dominick and Rimler, 1986). The mode of action of the toxin is currently unknown; no ultrastructural changes are apparent in embryonic bovine lung cells after treatment with the toxin in vitro. In addition, there are no detectable changes in the levels or synthesis of protein or DNA in the treated cells nor any alteration in the intracellular concentrations of ATP and cAMP (Chanter et al., 1986). The gene encoding the toxin has been cloned and expressed in *E. coli* (Chanter, 1989).

P. multocida capsular type A isolates from rabbits produce fimbriae which mediate adhesion to isolated pharangeal cells (Trigo and Pijuan, 1988). Porcine type A isolates adhere more readily than type D isolates to porcine epithelial cells. However, there is no evidence to demonstrate adhesion of toxigenic *P. multocida* isolates to turbinate mucosal cells, indicating that these adhesins may only be accessory factors.

Colonization of toxigenic *P. multocida* is greatly enhanced in combined infection with *Bordetella bronchiseptica* (Cowart et al., 1989). It appears that a protein factor produced by *B. bronchiseptica* acts to facilitate colonization of *P. multocida.* Similarily, toxigenic *P. multocida* also enhance colonization of *B. bronchiseptica,* suggesting a synergistic action of the two bacteria in pathogenesis.

The capsule of type A *P. multocida* is largely made of hyaluronic acid. An antiphagocytic protein of 50 kDa was recently purified from an avian isolate of serotype A3 (Truscott and Hirsh, 1988). Other virulence factors of *P. multocida* may include neuraminidase and a hyaluronidase, but there is no evidence for their role in pathogenicity (Drzeniek et al., 1972).

In addition, the outer-membrane proteins of *P. multocida* have also been suggested to be virulence factors (Truscott and Hirsh, 1988). Some of the outer-membrane proteins may be involved in an iron-scavaging role to promote growth in vivo (Snipes et al., 1988b).

Virulence Factors of *P. haemolytica*

There are 16 serotypes of *P. haemolytica* based on agglutination using specific typing sera (Biberstein et al. 1960; Frank and Wessman, 1978; Fodor et al., 1988; Fraser et al., 1982). Two bio-

types, A and T, are also established. Serotype A1 of *P. haemolytica* is the most common isolate from bovine pneumonic pasteurellosis, a fibrinous pneumonia of feedlot cattle (Wessman and Hilker, 1968). Other serotypes, serotype A2 in particular, have been shown to cause ovine pneumonic pasteurellosis (Gilmour and Gilmore, 1989). The bacterium produces a number of virulence factors which contribute to pathogenesis of the diseases (Adlam, 1989; Moiser et al., 1989). However, environmental and physical stress and concurrent or prior infection with respiratory viruses predispose the animals to the bacterial infection.

One of the major virulence factors produced by pathogenic isolates of *P. haemolytica* is a heat-labile toxin specific for ruminant leukocytes (Kaehler et al., 1980; Shewen and Wilkie, 1982). This toxin may contribute to pathogenesis either by imparing the primary defense of the lungs and subsequent immune response or by the induction of inflammation as a consequence of leukocyte lysis. The genetic determinant for the leukotoxin has been cloned and its nucleotide sequence determined (Chang et al., 1987; Lo et al., 1985, 1987). The data were used to predict that the size of the active toxin was 102 kDa (Lo et al., 1987). In addition to a structural gene which encodes the toxin, an activation protein was also identified, as well as two secretory proteins which participate in the secretion of the toxin out of the cell (Strathdee and Lo, 1989). The leukotoxin has been found to share extensive homology with the *E. coli* alpha-haemolysin as well as with many other haemolysins in pathogenic bacteria (Strathdee and Lo, 1987). The leukotoxin has been suggested to act by the formation of a transmembrane pore in the target cells which leads to cell swelling, loss of electrolytes, and eventually cell lysis by osmotic pressure (Clinkenbeard et al., 1989).

At least two types of fimbriae have been detected on *P. haemolytica* serotype one grown in vitro or recovered in lavage fluid from experimentally infected animals (Morck et al., 1987). Transmission electron microscopy revealed that fimbrial structures on *P. haemolytica* adhered to tracheal epithelium (Morck et al., 1988). Although role for fimbriae in pathogenesis of *P. haemolytica* pneumonia has not been demonstrated, fimbriae may be important in colonization of the upper respiratory tract in the early stages leading to pneumonic pasteurellosis (Morck et al., 1989). Recently, Potter et al. (1988) reported the purification of the larger rigid fimbriae from *P. haemolytica* A1, and these purified fimbriae does not seem to have hemagglutinating activity.

A polysaccharide capsule has been observed on *P. haemolytica* cells recovered from the trachea or the alveoli of infected animals (Adlam et al., 1984; Morck et al., 1987, 1988, 1989). The capsule may enhance attachment of the bacterium to alveolar and bronchiolar surfaces, inhibit the phagocytic and bactericidal activities of neutrophils, or impair complement-mediated serum killing of the bacteria (Brogden et al., 1989; Corstvet et al., 1982; Emau et al., 1986, 1987).

Although the lipopolysaccharide (LPS) of *P. haemolytica* was not lethal for bovine leukocytes in vitro, it does affect many biological activities of these cells (Confer and Simons, 1986), including altered migration of peripheral blood leukocytes as well as enhancement of phagocytosis of polymorphonuclear leukocytes (Confer and Simons, 1986). However, the actual contribution of LPS to the pathogenesis of lung lesions is not known.

Other potential virulence factors of *P. haemolytica* have been identified, including a glycoprotease and a neuraminidase (Otulakowski et al., 1983). These two proteins may act to cleave surface components off the target cells, rendering the cells more susceptible to attachment or lysis by the leukotoxin. In addition, many cell-surface serotype-specific antigens may also be important in adherence or colonization of the bacterium (Donachie et al., 1984; Durham et al., 1986; Gonzalez-Rayos et al., 1986).

Future Studies on the Genus *Pasteurella*

The advent of recent techniques in molecular biology will continue to allow further understanding of the bacteria in the genus *Pasteurella*. The use of nucleic acid analysis, both DNA-DNA hybridization and 16S rRNA comparison, has already changed the taxonomic organization of the genus, and its relationship to other genera within the same family. A number of bacteria will no doubt be reassigned to more appropriate placements. For example, it has been proposed that the biotype A species of *P. haemolytica* should more appropriately be included in the genus *Actinobacillus* (Pohl, 1981). Such a change from the traditional may be faced with resistance, although at least new isolates will be properly named and catagorized appropriately.

In addition to redefining the taxonomic placement of these bacteria, the use of molecular approaches enable a stepwise dissection of their

many virulence factors. As well, the genetic determinants of the encoded virulence factor(s) could be used for detail characterization of their function and activities during an infection. An understanding of the role(s) of the various virulence factors, singularly and in concert, is important in the design and development of efficacious vaccines to protect animals against infection by these pathogenic bacteria (Confer et al., 1988; Kobish and Pennings, 1989; Mosier et al., 1989; Rimler, 1987; Shewen and Wilkie, 1987; Sutherland et al., 1989).

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CHAPTER 181

The Genus *Cardiobacterium*

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The genus *Cardiobacterium* consists of one species, *Cardiobacterium hominis,* originally designated "group II D organisms" until further described and named by Slotnick and Dougherty (1964). This organism is a fastidious, facultatively anaerobic, nonmotile, pleomorphic Gram-negative rod with a fermentative type of metabolism.

The first known report of these organisms was by Tucker et al. (1962). This paper described four cases of endocarditis caused by a *Pasteurella*-like organism. These isolates and two subsequent isolates, also from cases of endocarditis, were designated as "group II D" by the Centers for Disease Control, Atlanta, GA, until the name C. *hominis* was proposed by Slotnick and Daugherty (1964). The current assumption is that infectious endocarditis is the only disease caused by *C. hominis* in humans. This assumption may be valid or it may be based on a lack of more complete knowledge of the biology of the organism. Although past isolation of *C. hominis* has been almost exclusively limited to blood cultures, there have been at least two isolations from cerebral spinal fluid (Slotnick, 1968; Francioli et al., 1983).

Slotnick (1964, 1968), in an attempt to extend our knowledge of the human occurrence of this organism, used fluorescent antibody smears and culture techniques to sample other sites. He and his colleagues isolated *C. hominis* from nose and throat specimens of 68 of 100 persons sampled, from cervical and vaginal cultures of 2 among 159 studied, and obtained positive fluorescent smears from stool specimens of 14 of 20 individuals. No positive stool cultures were obtained because of a lack of a selective medium and the overgrowth of *C. hominis* by the enteric bacilli. No isolations or positive smears were obtained from any urine specimens collected (no sample size was given for the urine specimens). These investigators concluded that *C. hominis* is part of the indigenous commensal respiratory flora and supported this conclusion with the following observations: 1) None of the individuals harboring the organism had any signs or symptoms of disease. 2) Injection of viable suspensions of representative strains into laboratory animals elicited no visible sign of disease. 3) In relation to the high proportion of individuals who harbor the organism, few clinical cases occur. With the exception of these studies, there is a paucity of information concerning the ecology and pathogenicity of these organisms. All recently published information is limited to descriptive case reports of isolation of *C. hominis* from bacterial endocarditis, with the exception of one case report of *C. hominis* meningitis (Francioli et al., 1983). There appear to be no published reports on pathogenicity or virulence mechanisms.

Even though *C. hominis* was originally called a *Pasteurella*-like organism (Tucker et al., 1962), it was soon discovered that it was antigenically unrelated to members of the genus *Pasteurella* as well as to the genera *Brucella, Bordetella, Moraxella, Hemophilus, Streptobacillus, Corynebacterium, Bacteroides, Neisseria, Escherichia, Aerobacter (Enterobacter)* and *Lactobacillus* (Slotnick et al., 1964). It was compared to both Gram-negative and Gram-positive organisms because of the Gram-variable characteristics noted by early investigators. Because of this anomalous Gram-stain reaction, the fine structure of *C. hominis* was studied by Reyn et al. (1971). These investigators found that the cell wall was of the Gram-negative type, but an unusual feature of all strains examined was a 20–40-nm thick polar cap. This material was strictly limited to the terminal portion of the cell, adhering to the outside of the cell wall. Profile sections of this cap indicated that it was formed by radial arrangements of tufts of material but subsequent studies employing freeze-etching and negative-staining techniques were unable to further elucidate the nature of this cap material. The other notable feature of the *C. hominis* cell wall was an unusually dense outer layer. This layer was composed of a repeating structure which consisted of units exhibiting tetragonal or rectangular packing. The periodicity of these arrays measured 5.5 nm; the

average diameter of the units was 3.4 nm and the space between them was 2 nm. Surface arrays are more typical of Gram-positive bacteria than of Gram-negative bacteria but are not unknown in the latter (Reyn et al., 1971).

Limited genetic studies have demonstrated that the GC content of the DNA for *C. hominis* is quite different from the other Gram-negative rod-shaped organisms which have similar physiological characteristics. *C. hominis* GC is 59–60%, *Haemophilus aphrophilus* is 42%, *Kingella* sp. is 47.3–54.8%, *Actinobacillus actinomycetemcomitans* is 42.7%, *Pasteurella* sp. is 40–45%, *Eikenella corrodens* is 56.2–58.2%, and *Capnocytophaga* sp. is 33–41% (Weaver, 1984).

Isolation

Except for the studies of Slotnick and colleagues, all published isolation attempts have been from normally sterile sites, predominantly blood. The one recorded isolation from cerebral spinal fluid does not give cultural information (Francioli et al., 1983). *C. hominis,* although a slow-growing organism, appears to be able to initiate growth on a variety of blood culture media under both anaerobic and aerobic conditions. Only two types of basal media were reported not to support primary blood culture isolation attempts—thioglycollate and Schaedler broths (Bruun et al., 1983; Midgley et al., 1970; Piot et al., 1978). Since it has been reported that *C. hominis* growth may not be noted until after 23 days of incubation, the failure to see growth in the above two media may be due to the fact that the cultures were discarded as negative after 14 days of incubation (Geraci et al., 1978). More typically, growth of the organism is seen after 5 to 8 days of incubation. For subculture from primary cultures, 5% sheep blood or chocolate agar are most frequently used although *C. hominis* will grow on media without blood (Midgley et al., 1970). Neither CO_2 or humidity is an absolute growth requirement for all strains, but both supplemental CO_2 and increased humidity enhance growth (Savage et al., 1977).

For isolation from nonsterile sites, no selective medium is presently available. Earlier studies on throat and nose specimens used typticase-soy agar slants or plates enriched with 5% human blood incubated at 37°C for 48–72 h (Slotnick et al., 1964). Slotnick's subsequent screen of vaginal and cervical swabs for *C. hominis* employed a slightly different method. Swabs were placed in tubes containing 2 ml of Trypticase Soy Broth (BBL) for transport to the laboratory where they were plated on a Casman's blood agar plate and a chocolate agar plate (BBL). Incubation was at 37°C for 4 days in a candle jar (Slotnick, 1968).

Identification

Differential characteristics of *C. hominis* which distinguish it from other similar Gram-negative organisms are given in Table 1. General biochemical characteristics of *C. hominis* are given in Table 2.

Microscopically, cells of *C. hominis* are pleomorphic Gram-negative rods which may show retention of crystal violet in the central portion or in the enlarged ends. Teardrop cells and filaments of varying lengths may occur. One study attributes this extreme pleomorphism and the irregular staining to growth on media without

Table 1. Biochemical differentiation of *Cardiobacterium hominis* and other fastidious Gram-negative rods.

					Fermentation of:					
	CAT	OXI	IND	NIT	GLU	LAC	MAL	MAN	SUC	XYL
Cardiobacterium hominis	−	+	+	−	+	−	+	+	+	−
Kingella species	−	+	D	D	+	−	D	−	D	−
Capnocytophaga species (DF-1, DF-2)	D	D	−	D	+	+	D	−	D	−
Actinobacillus actinomycetemcometans	+	D	−	+	+	−	+	+	−	D
Pasteurella species	+	+	D	+	+	D	D	D	+	D
Eikenella corrodens	−	+	−	+	−	−	−	−	−	−
Haemophilus aphrophilus	−	D	−	+	+	+	+	−	+	−
Streptobacillus moniliformis	−	−	−	−	+	D	+	−	−	−
Brucella species	+	+	−	+	−	−	−	−	−	−
Bordetella species	+	−	−	+	−	−	−	−	−	−

[a]CAT = catalase; OXI = oxidase; IND = indole; NIT = nitrate reduction; GLU = glucose; LAC = lactose; MAL = maltose; MAN = mannitol; SUC = sucrose; XYL = xylose; D = different biotypes.

Table 2. Biochemical characteristics of *Cardiobacterium hominis.*

Characteristic	Reaction
Oxidase	+
Catalase	−
Motility	−
Nitrate reduction	−
Indole	+
Urease	−
Citrate	−
Esculin hydrolysis	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Arginine dihydrolase	−
o-Nitrophenyl-β-D-galactopyranoside	−
(ONPG)	−
Growth on MacConkey agar	−
Gelatin liquefaction	D[a]
Litmus milk acidification	D
Methyl red	D
Voges-Proskauer	−
Tween 20 hydrolysis	−
Tween 40 hydrolysis	
Acid from:	+
Glucose	−
Adonitol	−
Arabinose	−
Cellobiose	−
Dulcitol	−
Erythritol	+
Fructose	−
Galactose	−
Inositol	−
Lactose	+
Maltose	+
Mannitol	+
Mannose	−
Melezitose	−
Melibiose	−
Rhamnose	−
Salicin	+
Sorbitol	+
Sucrose	−
Trehalose	−
Xylose	

[a]D = different biotypes.

yeast extract. This report states that when *C. hominis* was grown on a medium containing yeast extract, the organisms appeared mainly as uniform Gram-negative rods in contrast to the pleomorphism and Gram-variable characteristics exhibited on media without yeast (Savage et al., 1977).

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CHAPTER 182

The Genus *Actinobacillus*

JAMES E. PHILLIPS

Organisms of the genus *Actinobacillus* were first described by Lignières and Spitz (1902) in actinomycotic lesions in cattle in Argentina. The authors referred to this organism as "l'actinobacille" based on its morphology and its association with the pathological lesion. In 1910, Brumpt proposed the name of *Actinobacillus lignieresii.*

This genus has also been used as a repository for "species for which no obvious home can be found" (Cowan, 1974). In *Bergey's Manual of Systematic Bacteriology* (Phillips, 1984) five species are listed as comprising the genus; *Actinobacillus lignieresii, A. equuli, A. suis, A. capsulatus,* and *A. actinomycetemcomitans.* Each of these species is discussed in detail in this chapter. Various other organisms have been described as actinobacilli, but here they have not been included in (nor excluded from) the genus. Ross et al. (1972) isolated actinobacilli from sows that were related to, but not identical with, the generally accepted species of the genus. Hacking and Sileo (1977) have recovered a hemolytic actinobacillus from waterfowl and have shown it to have close similarities with *A. lignieresii* and *Pasteurella ureae,* an organism already reported by Jones (1962) as closely resembling the actinobacilli and considered by Frederiksen (1973) to be an *Actinobacillus* of human origin.

The close relationship of organisms of the genus *Actinobacillus* and those of the *Pasteurella* group may give rise to difficulties in identification and has certainly presented taxonomic problems. Genetic studies of these two groups together with the genus *Haemophilus* by Mannheim et al. (1980) have given support to the inclusion of the three genera within a single family, the Pasteurellaceae. Mráz (1969), in a comparative study of *A. lignieresii* and *P. haemolytica,* has drawn attention to the close similarity between these two species (similarity index, 95% on 60 characters) and has proposed that *P. haemolytica* should be renamed *Actinobacillus haemolyticus.* Mráz et al. (1976) have also examined strains of another organism originally described by Kohlert (1968) under the name *Pasteurella salpingitidis* and have proposed that this be renamed *Actinobacillus salpingitidis.* The rearrangement of organisms within these three genera has also been supported by genetic studies by Pohl (1981), who noted a close relationship between members of the *Actinobacillus* group and *Haemophilus pleuropneumoniae.* Pohl et al. (1983) later concluded that an organism recovered from cases of necrotizing pleuropneumonia in pigs and designated *Pasteurella haemolytica*-like organism (Bertschinger and Seifert, 1978) is a biotype of *Haemophilus pleuropneumoniae* and that both should be transferred to the genus *Actinobacillus* under the name *Actinobacillus pleuropneumoniae.* This organism will be discussed in this chapter.

There are also some species that have previously been included in the genus but which should now be excluded: *A. actinoides* (Smith, 1918), a cause of pneumonia in calves, bears great similarities to *Streptobacillus moniliformis* and should probably be placed in the same genus. *A. mallei* has been removed to the genus *Pseudomonas.* The organism described by Baynes and Simmons (1960) from cases of ovine epididymitis, *A. seminis,* should not be included in the genus *Actinobacillus.*

Habitats

Members of the genus *Actinobacillus* are encountered most often as pathogens causing a variety of conditions in animals, especially domesticated stock. They also occur as commensal organisms in the alimentary, respiratory, and genital tracts of normal animals. They are to be regarded as opportunistic pathogens, there usually being some factor present that assists entry of the organism into susceptible tissues. The cases of disease caused by the actinobacilli are usually sporadic, but where the trigger factor is common to a group of animals, several individuals in that group may be affected.

Actinobacillus lignieresii

The classical disease in cattle caused by *Actinobacillus lignieresii* is wooden tongue, a chronic granulomatous lesion affecting the tongue and other soft tissues of the head and upper alimentary tract together with the associated lymph nodes (Bosworth, 1923; Davies and Torrance, 1930; Thompson, 1933; Till and Palmer, 1960). Lesions have also been reported in the lungs and liver (Davies and Torrance, 1930), in the pleura (Misdorp. 1963), and in the heart (Thornton, 1976). Subcutaneous lesions affecting the skin of various areas of the body have been recorded (Hebeler et al., 1961; Mawditt and Greenham, 1962).

In sheep, the lesions are more usually suppurative in character, often with involvement of the skin or lungs (pyobacillosis). The infecting organism was first described by Christiansen (1917) as *Bacterium purifaciens,* but the identity of this organism with *A. lignieresii* was established by Tunnicliff (1941) and Taylor (1944). The organism has been reported to cause epididymo-orchitis (Laws and Elder, 1969a) and mastitis (Laws and Elder, 1969b) in sheep.

Infection of animals other than cattle and sheep with *A. lignieresii* is not common, but lingual lesions have been reported in the dog by Fletcher et al. (1956) and by Kemenes and Markói (1959) and epidural abscess in a horse by Chladek and Ruth (1976). In ducks the organism has been recovered from cases of salpingitis (Bisgaard, 1975). The presence of *A. lignieresii* associated with disease in humans has also been reported (Dibb et al., 1981; Orda and Wiznitzer, 1980; Pathak and Ristic, 1962; Thompson and Willius, 1932).

A. lignieresii has been demonstrated as a commensal organism in the mouth of healthy cattle (Phillips, 1964), in normal bovine rumen (Phillips, 1961), and in the rumen of healthy sheep (Phillips, 1966). It is likely that such organisms constitute the source of infection, with entry occurring through minor wounds produced in the epithelial surfaces of the upper alimentary tract. The presence of agents that may cause mechanical damage of the epithelium has been found associated with multiple cases of actinobacillosis in herds of cattle (Campbell et al., 1975; Gerring, 1947; Hebeler et al., 1961; Nakazawa et al., 1977) and in sheep (Davis and Stiles, 1939; Hayston, 1948; Thomas, 1931).

A. lignieresii is worldwide in distribution, and clinical infections have been reported from all continents.

Actinobacillus equuli

Actinobacillus equuli is a pathogen of horses found in association with various clinical conditions, especially in young animals. The most usual syndrome in foals, occurring within the first few days of life, is an acute septicemic infection ("sleepy foal disease") that may become chronic ("joint ill") with lesions of purulent nephritis and purulent arthritis (Dimock et al., 1947). In adult horses it may be found associated with septicemia (Magnusson, 1919; Mráz et al., 1968; Zakopal and Nesvadba, 1968), purulent nephritis (Meyer, 1910), endocarditis (Innes et al., 1950; Svenkerud and Iversen, 1949; Vallée et al., 1974), meningitis (Weidlich, 1955), chronic alveolar emphysema (Larsen, 1974), and abortion (Webb et al., 1976).

The organism is also pathogenic for swine. Piglets are most often affected, but older animals may show lesions. The lesions that may be found include abortion (Werdin et al., 1976), arthritis (Pedersen, 1977), endocarditis (Ashford and Shirlaw, 1962; Jones and Simmons, 1971), meningoencephalitis (Terpstra and Akkermans, 1955), metritis (Edwards and Taylor, 1941), and septicemia (Magnusson, 1931; Windsor, 1973).

A. equuli has been recognized in horses as part of the normal bacterial flora of the intestinal tract (Cottew and Francis, 1954; Laudien, 1923) and has been isolated from both the tonsillar region (Dimock et al., 1947; Jarmai, 1929) and the tracheal mucus (Kim et al., 1976) of healthy horses. The occurrence of *A. equuli* in normal swine, however, has not been documented.

A. equuli has not been widely recognized as a pathogen in other animal species, but Moon et al. (1969) isolated it from monkeys, and du Plessis et al. (1967) and Osbaldiston and Walker (1972) described outbreaks of enteritis in calves in which *A. equuli* predominated in the intestinal flora. Vallée (1959) reported *A. equuli* causing disease in rabbits and Vallée et al. (1960) recorded its association with skin lesions in a dog.

The opportunistic character of *A. equuli* as a pathogen is recognized, especially in the case of young animals, where it is often the weak individual that is liable to succumb to infection.

Actinobacillus suis

The porcine actinobacillus, *Actinobacillus suis,* is found as a pathogen of all ages of swine; there is both an acute septicemic form (Mair et al., 1974; Zimmermann, 1964), sometimes with pneumonia (van Dorssen and Jaartsveld, 1962) or nephritis (Bouley, 1966), and a more chronic

form with arthritis (van Dorssen and Jaartsveld, 1962). It has also been recognized as a pathogen of horses (Veterinary Investigation Service, 1975). In a number of cases of disease in horses reported to be due to *A. equuli,* the infecting organism has undoubtedly been *A. suis* (Bell, 1973; Carter et al., 1971; Cottew and Francis, 1954), and it is likely that other unidentified actinobacilli from horses (Larsen, 1974) are also strains of this species. An organism identified as *A. suis* was isolated from conjunctivitis in a free-living Canada goose (Maddux et al., 1987), and previously reported, unnamed actinobacilli from ducks and swans (Hacking and Sileo, 1977; Onderka and Kierstead, 1979) have characteristics which indicate they also belong to this species.

The presence of *A. suis* in normal swine has not been reported, but the hemolytic actinobacilli recovered from irradiated swine by Wetmore et al. (1963) may have been commensal strains of *A. suis* that had assumed the pathogenic role in the stressed hosts. Cutlip et al. (1972) isolated actinobacilli from the tonsils of normal pigs in a herd in which a case of septic embolic actinobacillosis had occurred.

The presence of *A. suis* in normal horses has also been reported (Kim et al., 1976).

Actinobacillus capsulatus

For many years after its first isolation from lesions of arthritis in laboratory rabbits (Arseculeratne, 1961, 1962), *Actinobacillus capsulatus* was not recovered from any other animal species; however, Zarnke and Schlater (1988) have recently reported the isolation of *A. capsulatus* from a generalized infection in free-ranging snowshoe hares in Alaska. In rabbits the lesions of arthritis show characteristic granules consisting of a central mass of organisms with radiating club-like structures similar to those seen in lesions in cattle. The organism has not been recognized in the commensal state in the rabbit nor any other host species.

Actinobacillus actinomycetemcomitans

Actinobacillus actinomycetemcomitans was first described by Klinger (1912) in actinomycotic lesions in humans. Heinrich and Pulverer (1959) considered that it played a distinct part in the pathogenesis of actinomycosis. More recently it has been recognized as a commensal organism in the human oral cavity, where it was present in 36% of adults cultured (Slots et al., 1980). However, its role as a human pathogen has been firmly established in cases of juvenile periodontitis in which it is regarded as the major etiological agent (Zambon, 1985). It occurs also as the sole infecting organism in other lesions in humans, such as endocarditis and abscesses (Page and King, 1966; Blair et al., 1982), and it may give rise to chronic skin lesions in drug abusers arising from infection of injection sites (Fenichel et al., 1985).

A. actinomycetemcomitans may also be responsible for infections in animals. It is found associated with epididymitis in rams (DeLong et al., 1979; Bulgin and Anderson, 1983).

Actinobacillus (Haemophilus) pleuropneumoniae

Actinobacillus (Haemophilus) pleuropneumoniae is a primary bacterial respiratory pathogen of pigs, causing porcine contagious pleuropneumonia. It was named by Shope (1964) but some early authors classified it as *H. parahaemolyticus,* along with isolates from human beings (Nicolet, 1968). Kilian (1976a), however, was strongly of the opinion that the porcine strains constituted a species distinct from the human isolates.

The organism is often the only infective agent present in the lungs of affected pigs, although other secondary organisms may be present. In very young piglets the disease may be septicemic in form, often resulting in death, but in older pigs a subclinical form of the disease often results in high economic loss but no overt clinical disease.

A. pleuropneumoniae can be detected in the tonsillar crypts of clinically normal pigs (Brandreth and Smith, 1985) and also in the nasal cavity (Kume et al., 1984).

Isolation of *Actinobacillus*

Representatives of the genus *Actinobacillus* grow readily on the enriched media usually employed for the isolation of pathogens from animal tissues. In most fresh tissues they will usually be found as the sole or predominant organism. The following procedure can be used to isolate actinobacilli from lesions in the tongue and associated lymph nodes of cattle:

Isolation of *A. lignieresii*

To prepare Hartley's digest broth, the following ingredients are used:

Ox heart (minced)	3,000 g
Water	5 liters
Anhydrous sodium carbonate (0.8% solution)	5 liters
Pancreatin	50 g
Concentrated hydrochloric acid	80 ml

Mix the minced meat and water and heat to 80°C. Add the sodium carbonate and cool to 45°C. Add pancreatin and incubate at 45°C for 4 h, stirring frequently. When digestion is complete, add the hydrochloric acid and steam at 100°C for 30 min. Cool to room temperature and add 1 N caustic soda to bring the pH to 8.0. Boil for 25 min to precipitate phosphates and filter while hot. Allow to cool and adjust to pH 7.5. Sterilize by autoclaving at 121°C for 15 min.

Prepare horse blood agar by adding 1% agar (Oxiod no.1) to the digest broth, autoclave to sterilize, cool to 50°C and add 5% oxalated horse blood. Distribute into sterile petri plates.

Preparation of diseased tissue: Incise to the center of the lesion using aseptic technique and withdraw samples of the scanty volumes of pus with an inoculating loop or capillary pipette.

Inoculation of medium: Spread the inoculum so as to give well-isolated colonies at one side of the plate.

Incubation: Incubate at 37°C for 18–24 h. The addition of 5–10% carbon dioxide to the atmosphere usually improves the growth for primary isolation, but most strains will grow adequately without this addition.

The ability of most strains of actinobacilli to grow on MacConkey agar (Mrâz, 1975) suggests that it also might be of use for isolation, especially if cultures on this medium are set up in parallel with those on blood or serum agar. When working with *A. suis,* the use of sheep blood agar has the advantage that colonies of this species, being hemolytic, are more easily selected.

The isolation of actinobacilli from mixed populations (e.g., from the surface of the tongue and from the contents and the epithelial and mucosal surfaces of the alimentary tract) may prove difficult because of the overgrowth of the actinobacillus colonies by other more rapidly growing bacteria. In these situations selective media may be of advantage. Two selective media have been described, that of Till and Palmer (1960) for the isolation of *A. lignieresii* from the mouth of normal cattle and that of Phillips (1961, 1964) used to recover *A. lignieresii* from the rumen and mouth of normal cattle.

Selective Medium for *Actinobacillus lignieresii* (Till and Palmer, 1960)

Prepare Filde's peptic digest of blood according to the method of Cruickshank (1965) as follows:

Sodium chloride, 0.85% aqueous solution	150 ml
Hydrochloric acid	6 ml
Defibrinated sheep blood	50 ml
Pepsin	1 g
Sodium hydroxide, 20% aqueous solution	about 12 ml
Chloroform	0.5 ml

Mix the saline, acid, blood, and pepsin in a stoppered bottle and heat at 55°C for 2–24 h. Add sodium hydroxide until a sample of the mixture diluted with water gives a permanganate red color with cresol red indicator. Add pure hydrochloric acid drop by drop until a sample of the mixture shows almost no change of color with cresol red but a definite red tint with phenol red. It is important to avoid an acid excess. Add chloroform and shake the mixture vigorously.

The complete medium consists of:

Hartley's digest broth	900 ml
Agar	10 g
Filde's peptic digest*	100 ml
Oleandomycin phosphate*	20 mg
Neomycin sulfate*	1.5 mg

The medium is dispensed in petri plates.

Swabs taken from the surface of the bovine tongue are inoculated on to the medium and the inoculum is spread. Incubate the plates overnight at 37°C and then select colonies of the correct morphology for further examination.

*These components are added after the basic nutrient agar has been sterilized and cooled to 50°C.

Although Till and Palmer (1960) did not succeed in isolating *A. lignieresii* from the surface of the tongues of normal cattle, they showed that their medium would support the growth of known strains of *A. lignieresii* while providing a high degree of inhibition of contaminating organisms.

Selective Medium for *Actinobacillus lignieresii* (Phillips, 1961, 1964)

Phillips' selective medium incorporates an antifungal agent and is prepared as follows:

Prepare a stock solution of oleandomycin phosphate (5 mg/ml) in sterile distilled water, distribute it in 0.2 ml amounts, and store it frozen at −20°C. After thawing, prepare a working solution by adding 9.8 ml sterile distilled water. Prepare a stock suspension of nystatin (200 units/ml) in sterile distilled water, distribute in 1-ml amounts, and store frozen at −20°C. Both these stocks will store satisfactorily for at least 2 months. The final medium is prepared by adding the antibiotics to horse blood agar, to give final concentrations of oleandomycin (1 μg/ml) and nystatin (200 units/ml) as follows:

Hartley's digest agar	93 ml
Horse blood (oxalated)	5 ml
Oleandomycin phosphate working solution	1 ml
Nystatin stock suspension	1 ml

Melt the agar base and cool to 50°C. Add the blood and antibiotics and pour into sterile petri plates.

The ability to recover *A. actinomycetemcomitans* from environments with mixed bacterial populations has become increasingly important with the recognition of the association of this organism with human periodontal disease, especially as some of the normal oral organisms

may exert an inhibitory effect upon the actinobacilli (Yamamoto et al., 1981).

Selective Medium for *Actinobacillus actinomycetemcomitans* (Slots, 1982)

This medium is tryptic-soy-serum-bacitracin-vancomycin agar. Vancomycin suppresses the growth of streptococcal species, including *Streptococcus mitis* which has been shown (Yamamoto et al., 1981) to produce in vitro a substance inhibitory to *A. actinomycetemcomitans* and to other Gram-negative organisms found in the mouth.

The medium consists of:

Tryptic soy agar (Difco)	1 liter
Yeast extract (BBL)	1 g

Adjust pH to 7.2 and autoclave at 121°C for 15 min. Cool to 50°C and add the following (which have been sterilized by filtration) to give the respective final concentrations:

Horse serum	10%
Bacitracin	75 μg/ml
Vancomycin	5 μg/ml

Pour into sterile petri plates: Inoculated plates should be incubated either with 10% carbon dioxide in air, or under anaerobic conditions with 5% carbon dioxide added.

The isolation of *A. pleuropneumoniae* from infected tissues can be done using blood agar prepared from Hartley's digest broth. Although horse blood agar will support the growth of the organism, the use of bovine blood or sheep blood has the advantage that the colonies of freshly isolated strains of *A. pleuropneumoniae* will lyse the red cells of these two species, thus making selection of colonies easier especially when secondary bacteria such as *P. multocida* are present. Most strains of this organism require the V factor for growth and this is provided by using a "blood agar satellite plate," i.e., by inoculating a streak of a β-toxigenic staphylococcus strain across the plate after the tissue has been inoculated (Morgan and Phillips, 1978). This provides an additional identification character, the CAMP reaction (potentiation of the β-lysin of the staphylococcus) shown by all strains of *A. pleuropneumoniae* (Kilian, 1976b).

The presence of other organisms may make the isolation of *A. pleuropneumoniae* difficult, but selective media can be used to inhibit such organisms.

Selective Medium for *Actinobacillus pleuropneumoniae:* Modification of Sims' (1970) Medium (Morgan and Phillips, 1978)

Sims' medium consists of chocolate agar with bacitracin and cloxacillin added. The medium is prepared by cooling Hartley's digest agar to 80°C after autoclaving, adding 5% horse blood and heating in a steamer until it is a rich chocolate color. This is then cooled to 56°C and 10% yeast extract (Hers' method described by Marmion, 1967) added together with bacitracin and cloxacillin solutions to give final concentrations of 10 units/ml and 5μg/ml respectively. Yeast extract and antibiotic solutions are previously sterilized by filtration.

Best results are obtained by incubating inoculated plates in 10% carbon dioxide in air at 37°C for up to 48 h.

Heavy suspensions of actinobacilli may be prepared by growing the organisms on Hartley's digest agar in Roux flasks at 37°C for 24 h and washing off the growth into sterile saline or other suitable medium using sterile glass beads to dislodge the growth. An alternative method is to grow the organism in a flask of Hartley's digest broth in a shallow layer and incubate at 37°C for 24 h in a shaking bath (120 strokes/min).

Actinobacilli rapidly lose their viability when stored on solid media (e.g., blood agar) for more than 4 days. Cultures suspended in sterile rabbit serum or sterile 20% peptone solution and dried from the frozen state will remain viable in sealed ampules for many years. An alternative method of keeping stock cultures is as heavy suspensions in either rabbit serum or 20% peptone solution, stored at −70°C in a low-temperature cabinet.

Identification

All members of the genus *Actinobacillus* are markedly pleomorphic, having short bacillary or coccobacillary forms interspersed with coccal elements lying in close association with the rods and having the "Morse code" form described for *A. lignieresii* (Phillips, 1960). On media containing glucose or maltose, longer rods and almost filamentous forms may be seen, and often chains of short bacillary elements are present. In the case of *A. equuli* and *A. suis,* extracellular material staining faintly pink with Gram's stain is often seen. *A. capsulatus* and *A. pleuropneumoniae* both form capsules that can be demonstrated in wet india ink preparations.

Colonies of all species are sticky, especially on primary isolation, but those of *A. lignieresii* lose this characteristic on subculture. *A. suis* and *A. equuli* are very sticky (*A. suis* less so than *A. equuli*), and colonies are firmly adherent to the underlying medium. In broth cultures, the sticky nature of these two species is also apparent. The colonies of *A. actinomycetemcomitans* are not so sticky, but they characteristically adhere firmly to the underlying medium.

Table 1. Characteristics of the genus *Actinobacillus*.

Positive:	Reduction of nitrate to nitrite
	Production of β-galactosidase and urease
	Fermentation (acid only) of glucose, levulose, mannose, galactose, xylose, and maltose
Negative:	Production of indole
	Fermentation of inulin, inositol, dulcitol, adonitol, rhamnose, and sorbose

This adherence to the medium is also seen with the "waxy" colonies of *A. pleuropneumoniae,* but not with the soft, glistening colony form.

All members of the genus share the characteristics shown in Table 1. These characteristics would not exclude some members of the *Pasteurella* group, and, indeed, differentiation between the two genera may be difficult, so many isolates do not fall clearly into the recognized species of either genus. Close similarities of the two genera have been pointed out (Hacking and Sileo, 1977; Mráz, 1969, 1975; Smith, 1974).

The differential characteristics of the species of *Actinobacillus* are set out in Table 2. There is evidence, however, that a degree of overlapping between species occurs, especially between *A. equuli* and *A. suis.* The occurrence of strains isolated from horses having characters similar to those of *A. suis* has been reported (Kim et al., 1976); these differ from *A. equuli* mainly in their hydrolysis of esculin, fermentation of cellobiose and salicin, and nonfermentation of mannitol, and in being hemolytic on sheep blood agar. However, Mráz et al. (1968) drew attention to the existence of strains that, while being strongly hemolytic on blood agar, are similar in all other respects to *A. equuli,* and this possibility should be borne in mind in identification. The equine strains examined by Kim et al. (1976) have, on further investigation (Kim, 1976), shown one difference from those of porcine origin: unlike the porcine strains, they do not produce pigment. The pigment, creamy-yellow in color, can best be demonstrated by washing centrifuged broth cultures and observing the color of the deposit.

Gelatinase activity was considered by Phillips (1974) to be a characteristic feature of *A. equuli,* based upon the examination of a small number of strains from the National Collection of Type Cultures, using the gelatin agar method of Frazier (1926). Examination of a wider selection of strains (Kim, 1976) has shown that many are gelatinase negative, although positive strains are encountered from time to time (Frederiksen, 1973; Meyer, 1910; Vallée et al., 1974).

The determination of DNA base ratios does not give useful differentiation among species. Boháček and Mráz (1967) reported GC values lying within the range 40.0–42.6 mol% for *A. lignieresii* and *A. equuli,* while Mannheim et al. (1980) obtained values between 42.1 and 47.1 mol% for the strains of actinobacilli they investigated.

Most of the organisms included in this genus can be separated into a number of antigenic types or serotypes. In *A. lignieresii,* at least six such types have been described (Phillips, 1967), based upon heat-stable somatic antigens, and *A. equuli* presents at least 28 types (Kim, 1976). *A. actinomycetemcomitans* strains can be di-

Table 2. Differential characteristics of *Actinobacillus* species.

Characteristic	*A. lignieresii*	*A. equuli*	*A. suis*	*A. capsulatus*	*A. actinomycetemcomitans*	*A. pleuropneumoniae*
Fermentation of						
Lactose	+[a]	+	+	+	−	−
Mannitol	+	+	−	+	V	+
Salicin	−	−	+	+	−	−
Cellobiose	−	−	+	ND	−	ND
Melibiose	−	+	+	ND	−	ND
Trehalose	−	+	+	+	−	−
Sucrose	+	+	+	+	−	+
Hydrolysis of						
Hippurate	−	+	+	ND	ND	ND
Esculin	−	−	+	+[a]	−	−
Hemolysis on sheep blood agar	−	−	+	−	−	+
CAMP reaction	−	−	−	−	−	+
Pigment production	−	−	+	−	−	−
V factor required	−	−	−	−	−	+[b]

Symbols: +, positive; −, negative; V, variable; ND, no data.
[a]Response is slow.
[b]Some strains are negative.

vided into three distinct serotypes based on their immunodiffusion and indirect immunofluorescence properties (Zambon et al., 1983), and there is evidence that the serotype might be related to the pathogenicity of the organism. In *A. pleuropneumoniae* the indirect haemagglutination test, together with immunodiffusion, makes possible the recognition of 12 serotypes (Nielsen, 1986).

Some minor antigenic cross relationships between members of the genus *Actinobacillus* and those of the *Pasteurella*, especially *P. haemolytica,* have been noted, but in general there are no strong antigenic cross-reactions between these two genera (Mráz, 1977; Ross et al., 1972).

Within the genus, however, antigenic cross relationships have been recognized. Such cross-reactions may occur between *A. equuli* and *A. suis* (Kim, 1976; Vallée et al., 1974), between *A. lignieresii* and *A. suis,* (Ross et al., 1972; Vallée et al., 1974) and between *A. lignieresii* and *A. equuli* (Wetmore et al., 1963).

MacInnes and Rosendal (1987), using polyacrylamide gel electrophoresis (PAGE) and immunoblotting to investigate the major antigens of *A. pleuropneumoniae,* were able to show similarities in the protein profiles of all the actinobacilli and also of *Pasteurella haemolytica.* This supports the earlier work suggesting that PAGE could be of value in differentiation (Ross et al., 1972; Vallée et al., 1974).

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Gram-Negative Mesophilic Sulfate-Reducing Bacteria

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An overview of the sulfate-reduction process is given in Chapter 24. Most types of dissimilatory sulfate-reducing bacteria that have been isolated from nature and described so far are mesophilic, nonsporeforming anaerobes. They are members of the delta subdivision of the proteobacteria. The earliest known representative of this category is *Desulfovibrio* (Beijerinck, 1895). Further investigations have revealed a great morphological and nutritional diversity within this group. Various cell types have been described including cocci; oval or long straight rods; more or less curved rods or spirilla; cell packets; cells with gas vesicles; and gliding, multicellular filaments (Figs. 7–9). Electron donors used for sulfate reduction include H_2, alcohols, fatty acids, other monocarboxylic acids, dicarboxylic acids, some amino acids, a few sugars, phenyl-substituted acids, and some other aromatic compounds (Table 2). Even long-chain alkanes can be anaerobically oxidized by a particular type of sulfate-reducing bacterium (Aeckersberg et al., 1991). The utilization of polysaccharides or polypeptides, such as has been observed with the extremely thermophilic sulfate-reducing archaebacterium *Archaeoglobus* (Stetter, 1988; Stetter et al., 1987), has not been reported for mesophilic sulfate reducers.

All mesophilic, nonsporeforming, sulfate-reducing bacteria in which 16S rRNA has been sequenced form a phylogenetically distinct group of Gram-negative bacteria (Devereux et al., 1989, 1990; Fowler et al., 1986). The closest relatives are the phototrophic purple bacteria and their nonphototrophic relatives assigned to the alpha, beta, or gamma subdivision (Woese, 1987). The mesophilic, Gram-negative, sulfate reducers are thus grouped with the purple phototrophs and are termed the delta subdivision, even though no phototroph has been detected in this subdivision until now. However, the delta subdivision does contain chemotrophic nonsulfate-reducing bacteria; these are the obligately anaerobic *Pelobacter* species (Stackebrandt et al., 1989), sulfur-reducing bacteria of the genus *Desulfuromonas,* and the obligately aerobic *Bdellovibrio* and *Myxococcus* species (Fowler et al., 1986; Woese, 1987).

Habitats

Mesophilic nonsporeforming types appear to be the most wide-spread sulfate reducers in nature. The sulfur cycle in aquatic habitats depends on sulfate reduction as a primary process and is mainly governed by members of this subdivision of sulfate-reducing bacteria. The typical habitats of sulfate reducers are the subsurface parts of aquatic environments like sediments or bottom waters that have turned anoxic. The greatest variety of species has been isolated from marine sediments (Widdel, 1988) where sulfate, due to its high concentration in sea water (28 mM), is seldom a growth-limiting factor. Sulfate reducers are also found in rice paddies (Jacq and Dommergues, 1971; Takai and Kamura, 1966; Watanabe and Furusaka, 1980) and in anaerobic digestors of sewage plants (Badziong et al., 1978; Widdel, 1980). From anoxic sediments and sludge, these bacteria may spread into oil production plants (Cord-Ruwisch et al., 1986, 1987) and occasionally other industrial water systems such as paper production plants (Soimajärvi et al., 1978). Dissemination from anaerobic aquatic sediments may occur via oxic water in which sulfate reducers may survive for a while, probably in a metabolically inactive state (Cypionka et al., 1985). Significant activities and cell densities of sulfate-reducing bacteria have been observed even in the upper oxic zones of fresh water and marine sediments (Bak and Pfennig 1991a, 1991b; Jørgensen and Bak, 1991); growth of sulfate reducers at these sites may be explained by the presence of anoxic microniches (Jørgensen, 1977). Furthermore, sulfate reducers have been detected in the sheep rumen (Howard and Hungate, 1976) and in human intestines (Beerens and Romond, 1977; Moore et al., 1976).

Members of the genera *Desulfovibrio* and *Desulfobulbus* appear to be equally common in

fresh water and marine or other saline habitats (Bak and Pfennig, 1991b; Postgate, 1984a; Widdel, 1988), even though particular isolates are often adapted to a certain salt concentration. The growth of freshwater strains may be inhibited in salt media, whereas marine strains often require NaCl and Mg^{2+} ions. There are also salt-tolerant sulfate reducers such as *Desulfovibrio desulfuricans* and *Desulfococcus multivorans* that grow almost equally well in freshwater and saltwater medium. *Desulfobacter, Desulfobacterium, Desulfosarcina,* and *Desulfonema* may be regarded as primarily marine or brackish; most of their strains require media with $\geq$100 mM NaCl and $\geq$5 mM $MgCl_2$. Some *Desulfobacter* species have been isolated from freshwater sediments far away from the sea; however, growth was stimulated in saline media (Widdel and Pfennig, 1981; Widdel, 1987).

With H_2 as electron donor, *Desulfovibrio* species have a high maximum growth rate (μ_{max} = 0.23 h^{-1}) and a low half-saturation constant (K_s = 3.3 μM; for overview and literature, see Widdel, 1988). The μ_{max}/K_s value, which indicates the effectiveness of substrate utilization at limiting concentration, is higher than in the case of H_2-consuming methanogenic bacteria. Therefore, *Desulfovibrio* species and probably also the physiologically similar *Desulfomicrobium* species seem to be important H_2 scavengers in the anaerobic degradation of organic matter in sediments. High numbers of *Desulfovibrio* have been also found in a methane-producing whey fermenter without added sulfate (Zellner et al., 1987; Zellner and Winter, 1987). This suggests that even in the absence of sulfate, *Desulfovibrio* is involved in anaerobic degradation, namely by channeling lactate into methanogenesis. In aquatic sediments, lactate may be formed after rapid high-carbohydrate input. *Desulfobacter* is probably the main utilizer of acetate in brackish or marine sediments, as shown by growth kinetic data of this sulfate reducer in comparison to those of the important acetate-utilizing methanogenic bacteria (for overview, see Widdel, 1988). The role of other more versatile sulfate reducers, e.g., *Desulfobacterium* species, may be a simultaneous utilization of various electron donors produced during the primary breakdown of dead biomass in sediments.

Cultivation Techniques and Media

Sulfate-reducing bacteria grow under anoxic, reducing conditions. If inoculated into oxygen-containing (air-saturated) media, only fresh cultures of a few robust species may continue to grow. If the size of the inoculum is not too small, enough sulfide is transferred to scavenge oxygen. However, sulfide may also increase the toxicity of O_2, especially if the latter is present in excess (Cypionka et al., 1985). Removal of O_2, e.g., by sparging with N_2 prior to inoculation, eliminates the danger of cell damage by O_2 and always facilitates initiation of growth. Still, removal of O_2 merely by physical means is not sufficient for most slowly growing or fastidious strains, or in case of small inocula such as in dilution series. For these, addition of reductants is usually necessary. Reductants* that have often been used for the cultivation of sulfate-reducing bacteria are ascorbate (dehydroascorbate/ascorbate, $E^{0\prime}$ = +0.058 V); thioglycollate (dithioglycollate disulfide/thioglycollate, $E^{0\prime}$ = −0.14 V); sulfide (S^0/H_2S, $E^{0\prime}$ = −0.243 V; for 1 mM H_2S, E' = −0.198 V); and dithionite ($2SO_3^{2-}/S_2O_4^{2-}$, $E^{0\prime}$ = −0.29 [−0.46] V; for 1 mM, E' = −0.38 [−0.55] V). Dithionite is relatively toxic and can be added only at low concentrations (0.1–0.2 mM), usually in addition to other reductants; such low concentrations were clearly growth-promoting for many sulfate reducers (e.g., Bak and Widdel, 1986b; Widdel and Pfennig, 1981; Widdel et al., 1983). Sulfite, a rather strong reductant (SO_4^{2-}/SO_3^{2-}, $E^{0\prime}$ = −0.516 V), is part of the commercially available "iron-sulfite agar" (Postgate, 1984a), but has otherwise not been used for many media. One disadvantage of sulfite is a certain toxicity; in serial dilutions for viable counts, as little as 0.5 mM inhibited growth of certain sulfate reducers (F. Bak, unpublished observations). Also, below pH 7, sulfite tends to react with sulfide to yield thiosulfate, which by further reaction with sulfite yields polythionates and sulfur (Heunisch, 1976). Cysteine (cystine/cysteine, $E^{0\prime}$ = −0.325 V), which is frequently used for the cultivation of methanogens (Balch et al., 1979), may be also useful for sulfate reducers. As an indicator of reducing conditions, resazurine (resorufin/dihydroresofin, $E^{0\prime}$ = −0.03 to −0.04 V; Veldkamp, 1970) may be added at a final concentration of 1 mg/liter compatibility should be tested first.

All Gram-negative mesophilic sulfate reducers known so far grow in defined medium

*The redox potentials given refer to pH 7.0; values were taken or calculated from Dawson et al. (1986), Loach (1970), Thauer et al. (1977), and Weast (1989). Values for dithionite calculated from literature values given for alkaline solution (pH = 14) and for acidic solution (pH = 0) are not consistent. The latter yielded the values given in brackets; the dissociation constants of the corresponding acids (Greenwood and Earnshaw, 1984; Hollemann et al., 1985) were considered in these calculations.

without complex nutrients such as yeast extract or peptone. Complex nutrients, however, may be stimulatory for a number of species. Defined media are supplemented with growth factors only; many sulfate reducers require *p*-aminobenzoate, biotin, and/or some other vitamins.

It is recommended that media be prepared in big batches and then dispensed into smaller culture vessels. The expense of equipment for media preparation and cultivation largely depends on how strict the demand for anaerobic conditions is.

In case of robust, rapidly growing species, it may not be necessary to prepare media under anoxic conditions; it is sufficient to exclude air from the cultivation vessel during growth. A device for preparation of media in such simple way is shown in Fig. 1. After addition of reductants, the completed medium is distributed to tubes or bottles via a dispenser made of a piece of glass tubing and a small glass hood ("bell," e.g., a bottle with cut bottom) for protection against contamination from the air. Useful cultivation vessels are tubes (20 to 25 ml) or bottles (50 or 100 ml) with aluminum caps fitted with rubber disks. They are autoclaved with loosely attached caps or with a drop of water and tightly closed caps. Autoclaved tubes and bottles are completely filled with medium to exclude air. If incubated at elevated temperature, the tightness of the screw caps must be released somewhat (without loosening them) for about one hour because of liquid expansion. A small drop will be squeezed out between rubber seal and orifice. Then, the screw caps are tightened. Before being opened again for transfer, the aluminum caps are briefly heated in a flame to minimize the danger of contamination; therefore plastic caps are unsatisfactory.

For the cultivation of most sulfate-reducing bacteria, it is necessary to exclude air as far as possible, even during preparation of the me-

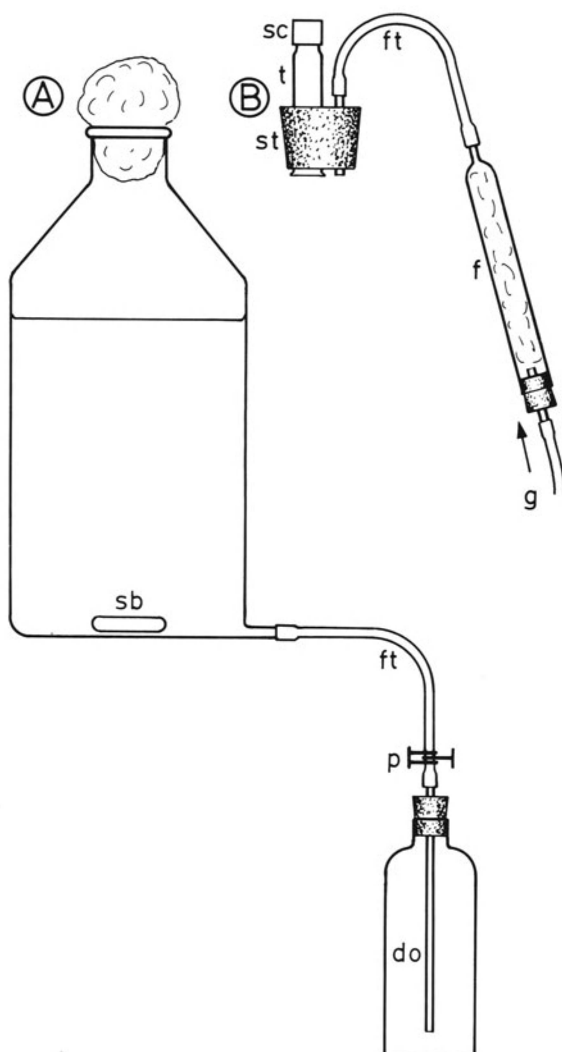

Fig. 1. Bottle for batch preparation of sterile medium and dispensing to culture tubes or bottles. Components that are not autoclaved with the medium are added afterwards from separately sterilized stock solutions. (A) For the preparation of media for some robust strains, exclusion of air during preparation may not be necessary so that a cotton stopper (or metal cap) is sufficient. Nevertheless, the completed medium should be dispensed into the culture vessels soon after addition of the reductants. (B) For more strict exclusion of air, the bottle is autoclaved with a stopper (st) containing a protruding flanged tube (t) with a screw cap (sc); the stopper is also connected to a cotton filter (f) for aseptic gassing. Before autoclaving, the screw cap is loosened and the dispenser is wrapped in aluminum foil. After autoclaving, the filter is connected to a gas supply (g) of N_2 or an N_2/CO_2 mixture which is flushed for some minutes through the head space to replace air while the screw cap is kept open. The gas pressure should be below 20 kPa (0.2 atm); this allows tightening of the screw cap after flushing or addition of stock solutions without blowing off the stopper. If insoluble iron compounds are formed (as in medium B of Postgate, 1984), the medium is gently stirred during dispensing to guarantee equal distribution of the precipitate. The end of the dispenser for open culture vessels (do) is always kept below the meniscus of the bottled medium to minimize contact with air. Other abbreviations: (ft) flexible tubing; (p) pinch clamp; (sb) stirring bar.

dium. The batch is therefore prepared under an anoxic gas phase. Some useful devices are shown in Fig. 1B and Fig. 2. The device in Fig. 2 is made from a pressure-proof Erlenmeyer flask. This is advantageous especially for media containing sulfide as a reductant. Since the flask is inverted when the media is dispensed, the surface area of the liquid gets smaller as its level decreases; this minimizes the loss of H_2S to the gas space towards the end of dispensing. Again, the medium may be dispensed into open tubes or bottles via a piece of glass tubing with a protective hood. During this procedure, the medium is in brief contact with air; however, if the exposure is minimized by keeping the tip of the dispenser below the miniscus (Fig. 2B), only

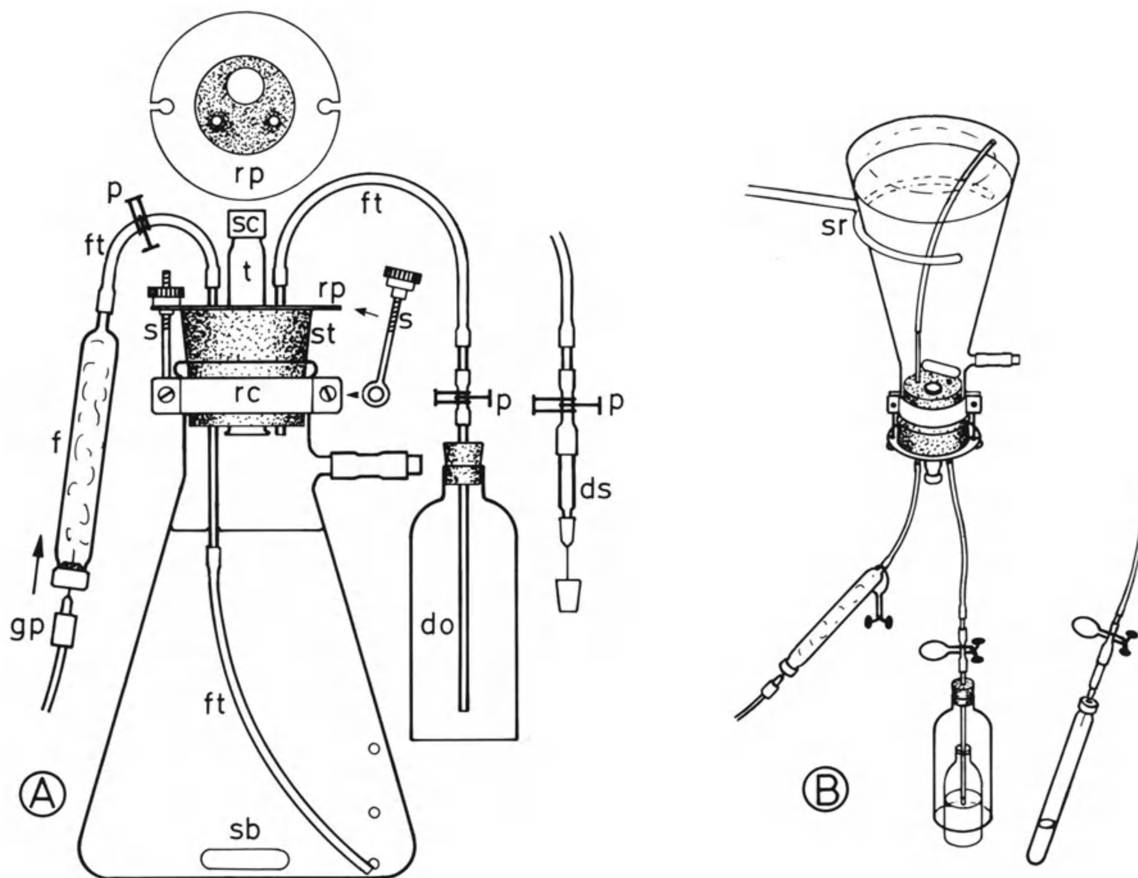

Fig. 2. Pressure-proof device for batch preparation of anoxic medium and dispensation into culture tubes or bottles. A suction pipe on the pressure-proof (vacuum-proof) flask is closed by means of a piece of rubber tubing with a glass rod. (A) Detailed view. A stopper (st) is provided with a cut and flanged tube (t) with screw cap (sc) and connected to a cotton filter (f) and dispenser (do, ds). The stopper is fixed by means of a ring clamp (rc) and a ring plate (rp) held together with long screws (s) and nuts. The ring clamp is made up of two half-circles that are fixed around the neck of the flask by means of short screws; the latter also hold the long screws. The upper part of the figure shows a view of the top of the stopper with the holes and the ring plate. Instead of the dispenser for open culture vessels (do), a different one for stoppered vessels (ds) may be used to allow injection. The latter is made of a cut narrow syringe barrel (or autoclavable needle adaptor) with a needle. For the flexible tubings (ft) outside the flask, rather gas-impermeable material (e.g., Viton, butyl) should be used. However, a short piece of tubing close to the dispenser should be made of silicon since this allows easy, rapid closing and opening with the pinch clamp (p). Since silicon is relatively gas-permeable, this piece is kept as short as possible. The medium with heat-stable components is autoclaved in the flask with loosened screw cap and pinched tubings. Dispensers are wrapped in aluminum foil before autoclaving. After autoclaving, the needle of a gassing probe (gp) is inserted in the stopper of the filter to gas the medium and the head space with an N_2/CO_2 mixture. At the same time, the flask may be cooled in a water bath. Stock solutions are added through the open tube (t) while the escaping anoxic gas prevents the entry of air. Between and after the additions, the screw cap is tightened. After anoxic gassing of the dispenser (ds) by brief opening of the pinch clamp, the needle may be closed with a sterile rubber stopper if not immediately used for injection. (B) The medium is dispensed either into open or stoppered culture vessels. In the latter case, an overpressure of 1 to 1.5 atm may be applied from the gas supply. For safety, use of a plexiglas shield is recommended. Other abbreviations: (sb) stirring bar; (sr) support ring.

traces of oxygen get into the medium, and they are soon scavenged by the reductant.

For very strict exclusion of air, the dispenser is made of a small syringe (or autoclavable needle adaptor) which allows injection into stoppered, anoxically gassed culture vessels (Fig. 2). Tubes or bottles with black rubber or butyl stoppers are used (Balch et al., 1979). Desorption of O_2 from stoppers and glass should be allowed. This may be achieved by pregassing the stoppered vessels with N_2, and waiting for one day. If available, an anoxic hood (with an N_2/H_2 mixture in the presence of a palladium catalyst) is preferred for desorption of O_2. After incubation in the hood for one day, a drop of water is placed in the culture vessels, and they are sealed inside the chamber with stoppers that are fixed with crimps or open-top screw caps. Tubes and bottles pregassed with N_2 or taken from the chamber are repeatedly evacuated and flushed with an N_2/CO_2 mixture. A device for evacuation and gassing is shown in Fig. 3. For the described medium with 30 mM $NaHCO_3$ and a pH of 7.0–7.4, the CO_2 content in the mixture should be 5–10%. For buffering at lower pH, the CO_2 content is increased, e.g., to 20% for a pH of around 6.8. At the end of the gassing procedure, tubes or bottles are brought to ambient pressure. Before dispensing the medium, the gas space of the dispenser with the needle is briefly flushed with the gas from the head space of the pressure-proof flask. The medium is injected by pressure (101 kPa, 1 atm) from the inverted flask into the anoxic tubes or bottles so as to fill approximately ⅓ to ½ of the volume. Anoxic gas bubbles inside the dispensor can be removed by inverting it during dispensing. The overpressure created in the culture vessels by addition of the medium is favorable for maintaining anoxic conditions. All further additions or withdrawals are carried out through the stoppers by means of syringes. Syringes are gassed immediately before use by repeated suction and release of sterile N_2. This is done by inserting the needle into another needle (of larger diameter) attached to a sterile gassing syringe, such as the one shown in Fig. 5. Stopper surfaces are sterilized with ethanol (that may be burned) before injection. Sterile stoppers should be covered with flamed aluminum foil or caps.

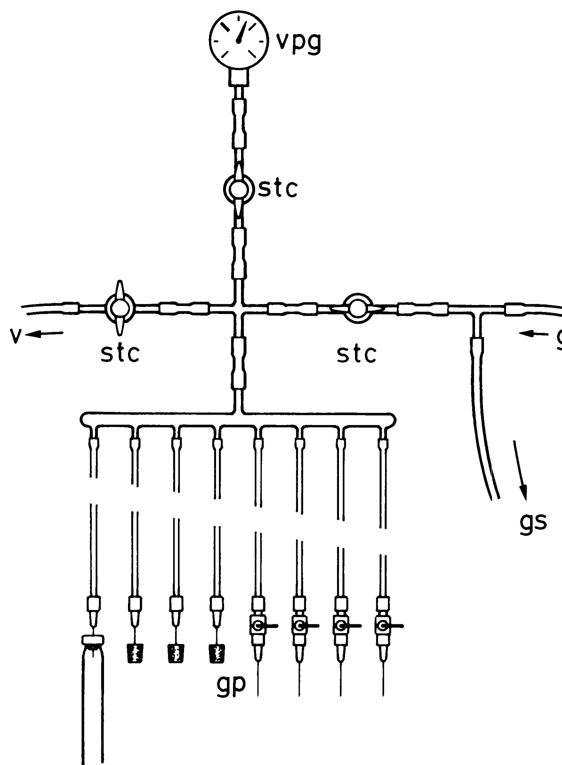

Fig. 3. Gassing manifold, modified from Balch et al. (1979). A glass tube with side arms and gassing probes (gp) can be alternately connected via stopcocks (stc) to a gas supply (g) or a vacuum line (v). A separate line branches off to a sterile gassing syringe (gs) as shown in Fig. 5. Gassing probes (gp) not inserted into culture vessels are closed with stoppers or stopcocks. Culture tubes (one shown on the left gassing probe) or bottles are gassed by alternate opening and closing of the vacuum line and gas supply. The process is controlled by means of the vacuum-pressure gauge (vpg). The applied overpressure may be 100 to 200 kPa (1 to 2 atm), depending on the thickness of the glass wall. In any case, for safety, the use of a plexiglas shield is recommended. Culture vessels to be filled as described in Fig. 2B are finally brought to ambient pressure by briefly opening a free gassing probe or the vacuum line. If cultures rich in sulfide have to be evacuated and gassed, the connection to the gauge via the stopcock is closed to avoid corrosion of metal parts by H_2S.

Culture Media

Many different types of media have been used for the cultivation of sulfate-reducing bacteria; however, no one medium is well suited for all species. In the following, two media frequently employed are described.

A lactate medium that can be prepared rather simply has been used successfully for decades for cultivation of the "classical" sulfate reducers *Desulfovibrio* and *Desulfotomaculum*. The medium has not been used so far for other sulfate reducers. Lactate (or pyruvate) serves as both the electron donor and carbon source. The medium is buffered with phosphate and contains iron. Further trace elements or vitamins are not added; they may be present in the yeast extract or as impurities in chemicals or water.

Lactate Medium for *Desulfovibrio*

Preparation of stock solutions:

1. $FeSO_4$ solution

Distilled water	10 ml
$FeSO_4 \cdot 7H_2O$	0.5 g

It is advantageous to autoclave the solution under a head space of N_2 in tubes with fixed stoppers to prevent oxidation. Also acidification, e.g., with 1 ml of 1 M H_2SO_4, counteracts oxidation.

2. Reductants solution

Distilled water	10 ml
Sodium thioglycollate*	0.1 g
Ascorbic acid	0.1 g

Autoclaving under N_2 as described for the $FeSO_4$ solution is recommended.

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*Stocks of sodium thioglycollate should not be old since the compound easily undergoes oxidation even as a dry salt. Its reducing capacity may be checked by iodometric titration.

Preparation of media:

Of several reported versions of lactate medium, two are given here using the designations of Postgate (1984a, 1984b); suggestions by Lapage et al. (1971) are included. The simple device depicted in Fig. 1 may be used for preparing and dispensing these media.

	Medium B	Medium C
Distilled or tap water	1 liter	1 liter
Sodium lactate (50% solution)	7.0 g (5.5 ml)	12.0 g (9.5 ml)
Na_2SO_4	–	4.5 g
$CaSO_4$	1.0 g	–
$CaCl_2 \cdot 2H_2O$	–	0.06 g
Sodium citrate	–	0.3 g
NH_4Cl	1.0 g	1.0 g
KH_2PO_4	0.5 g	0.5 g
$MgSO_4 \cdot 7H_2O$	2.0 g	2.0 g
NaCl (only for marine strains)	(2.5 g)	(2.5 g)
Yeast extract	1.0 g	1.0 g

To avoid formation of precipitates, dry salts should not be mixed together before dissolution. They are added successively to the water during stirring. The pH is adjusted to approx. 7.2 and the solution is autoclaved. Further components are added from the sterile stock solutions after autoclaving:

$FeSO_4$ solution	10 ml	0.08 ml
Reductants solution	10 ml	10 ml

In several cases, the $FeSO_4$ and reductants solutions were added to the medium before autoclaving. However, it is better to autoclave the solutions separately. Since the additions are somewhat acidic, the pH may be controlled and readjusted using aseptic techniques. The completed medium is aseptically distributed into bottles or tubes as described. For storage of medium B or C in batches, the $FeSO_4$ and reductants are omitted; they are added only directly before use, after the medium has been boiled again. Alternatively, the complete medium with $FeSO_4$ and reductants may be stored as batch under N_2.

For plates, media B and C are prepared with 10 g agar per liter. Before autoclaving, the agar should be dissolved and distributed in the medium by stirring in a boiling water bath.

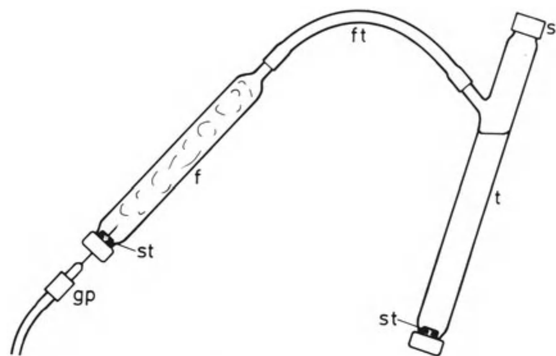

Fig. 4. Tube (t) with side arm and cotton filter (f) connected by flexible tubing (ft) for preparation or maintenance of a dithionite solution. Sterile distilled H_2O in the tube is deaerated by evacuation and repressurized with N_2 via the gassing probe (gp) inserted in the stopper (st). $Na_2S_2O_4$ weighed on a flamed, dry piece of aluminum foil is added while a slow stream of the anoxic gas prevents access of air. The tube is closed with the screw cap (sc). After dissolution by gentle shaking, samples are taken by means of pipettes via the upper opening under a stream of the anoxic gas. Alternatively, samples may be taken by means of N_2-flushed syringes via the bottom stopper (st) that is fixed with an open-top screw cap. For refrigeration of the dithionite solution, the gassing probe is disconnected.

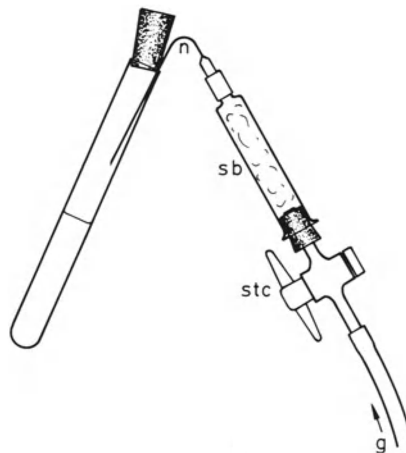

Fig. 5. Anoxic gassing of an agar tube with a gassing syringe according to the Hungate technique (Bryant et al., 1972). A syringe barrel (sb) filled with cotton and a bent needle (n) is connected to a gas supply (g) via a stopcock (stc). Before gassing, the needle is sterilized in a flame; the needle fixture should therefore be of metal. Tubes or bottles with liquid medium or stock solutions may be gassed in the same way.

Medium B turns black upon bacterial sulfide production (precipitation of FeS) and can thus be used for diagnostic purposes, e.g., for detection of sulfate reducers in counting series. Also for maintenance of stock cultures, the medium is useful since the abundant precip-

itate apparently provides a favorable microenvironment for the preservation of viable cells. Medium C is almost clear; it is suitable for mass culture of *Desulfovibrio*.

For cultivation of *Desulfovibrio* with H_2, media B and C are supplemented with about 0.2 to 0.4 g sodium acetate per liter; the H_2 gas phase should contain some CO_2. Acetate and CO_2 are the main carbon sources required for lithotrophic growth (Badziong et al., 1979; Brandis and Thauer, 1981). The added yeast extract usually contains only limited amounts of acetate. Acidification by CO_2 can be counteracted by addition of $NaHCO_3$ (see the next recipe).

A second major type of medium is a defined one that contains all the minerals, trace elements, and vitamins that may be required by sulfate-reducing bacteria. The medium has been developed for sulfate reducers of the newer genera (Widdel, 1980; Pfennig et al., 1981; Widdel et al., 1983; Widdel and Pfennig, 1984) but may be used for the classical *Desulfovibrio* species as well. The main buffer is HCO_3^-/CO_2, which may also serve for carboxylation reactions. Sulfide is added as a reductant. The phosphate concentration is low to allow addition of high Ca^{2+} concentrations if necessary (Widdel et al., 1983). Precipitates other than very small amounts of dark metal sulfides (mainly FeS) are not formed. Compounds that are volatilized, precipitated, or destroyed by heat are sterilized separately and added to the autoclaved, cold medium. The medium is first prepared as batch without organic electron donors and carbon sources and dispensed into tubes or bottles. Electron donors, carbon sources, and eventually other supplements are added to each culture vessel from sterile concentrated stock solutions as required for individual species and growth experiments.

Defined Multipurpose Medium

Preparation of basal stock solutions:

1. Nonchelated trace element mixture (Widdel et al., 1983)

Distilled water	987 ml
HCl (25% = 7.7 M)	12.5 ml (100 mM)
$FeSO_4 \cdot 7H_2O$*	2100 mg (7.5 mM)
H_3BO_3	30 mg (0.5 mM)
$MnCl_2 \cdot 4H_2O$	100 mg (0.5 mM)
$CoCl_2 \cdot 6H_2O$	190 mg (0.8 mM)
$NiCl_2 \cdot 6H_2O$	24 mg (0.1 mM)
$CuCl_2 \cdot 2H_2O$	2 mg (0.01 mM)
$ZnSO_4 \cdot 7H_2O$*	144 mg (0.5 mM)
$Na_2MoO_4 \cdot 2H_2O$	36 mg (0.15 mM)

The trace element mixture is autoclaved in bottles tightly closed with rubber-fitted screw caps or fixed stoppers; a head space of approx. ⅓ of the volume must be left (air for common use; N_2 for strictly anoxic procedures).

*Sulfates of ferrous iron and zinc are easier to handle than the corresponding chlorides, which, in air, undergo weathering or deliquesce, respectively; chlorides are only used if strictly sulfate-free media are required for special experiments.

2. Trace element mixture with EDTA (modified from Pfennig and Trüper, 1981)
The solution has the same composition as trace element mixture 1 except that HCl is replaced by 5.2 g (15.5 mM) sodium EDTA; the pH is adjusted to 6.0 with NaOH. The solution is autoclaved as described for solution 1.
3. Trace element mixture with NTA (Badziong et al., 1978)

Distilled water	800 ml
Nitrilotriacetic acid (NTA)	12.8 g (190 mM)

The pH is adjusted to 6.5 with dilute NaOH.

$FeSO_4 \cdot 7H_2O$	420 mg (1.5 mM)
H_3BO_3	10 mg (0.16 mM)
$MnCl_2 \cdot 4H_2O$	100 mg (0.5 mM)
$CoCl_2 \cdot 6H_2O$	170 mg (0.7 mM)
$CuCl_2 \cdot 2H_2O$	20 mg (0.12 mM)
$ZnSO_4 \cdot 7H_2O$	210 mg (0.73 mM)
$Na_2MoO_4 \cdot 2H_2O$	10 mg (0.04 mM)

Add distilled water to a final volume of 1000 ml. The solution is autoclaved as described for solution 1.
4. Selenite-tungstate solution

Distilled water	1 liter
NaOH	0.4 g (10 mM)
$Na_2SeO_3 \cdot 5H_2O$	6 mg (0.02 mM)
$Na_2WO_4 \cdot 2H_2O$	8 mg (0.02 mM)

The solution is autoclaved as described for solution 1. A slight turbidity and flocs may be formed by reaction of the alkaline solution with the glass surface, but the solution is still useful.
5. Bicarbonate solution (1.0 M)
84 g $NaHCO_3$ are dissolved in distilled water to a final volume of 1 liter. The solution is dispensed in the required portions (30 ml for 1 liter of medium) into bottles, leaving approx. ⅓ as head space. Gassing of the head space with CO_2 and saturation of the solution by repeated flushing and shaking is recommended. Bottles are tightly closed with rubber stoppers and autoclaved. Stoppers must be fixed before autoclaving with aluminum crimps or screw caps. Alternatively, bottles may be mounted between two parallel metal sheets that are held together by screws.
6. Vitamin mixture

Sodium phosphate buffer (10 mM; pH 7.1)	100 ml
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
Calcium D(+)-pantothenate	5 mg
Pyridoxine dihydrochloride	15 mg

The solution is filter-sterilized (pore size, 0.2 μm). The solution is kept at 4°C in the dark, preferentially in bottles of brown glass. For strictly anoxic procedures, the solution may be kept under N_2 as described later for phenol solution (solution 17).
7. Thiamine solution
10 mg thiamine chloride dihydrochloride are dissolved in 100 ml of 25 mM sodium phosphate buffer, pH 3.4. The solution is filter-sterilized and stored as described for solution 5.

8. Vitamin B_{12} solution
   5 mg cyanocobalamine are dissolved in 100 ml distilled water. The solution is filter-sterilized and stored as described for solution 5.
9. Sulfide solution, 0.20 M
   Clear, colorless, large crystals of $Na_2S \cdot 9H_2O$ from a fresh and not deliquesced stock* are weighed and dissolved in dist. H_2O so as to yield a solution of 48 g/liter. Crystals from old stocks, which usually contain oxidation products on the surface, should be washed on a plastic sieve by briefly rinsing with distilled water. Crystals are dissolved in distilled water under N_2. The solution is dispensed in the required portions (7.5 ml for 1 liter of medium) into tubes that are stoppered under N_2; stoppers are fixed with screw caps or aluminum crimps. The tubes are autoclaved. Formed precipitates from etched glass do not affect usability (see solution 4).

*Sodium sulfide is autooxidizable and hygroscopic. The chemical should be refrigerated or, preferably, be kept under N_2.

10. Acid or carbonate solution
    1 M H_2SO_4, 2 M HCl (for sulfate-free media), or 1 M Na_2CO_3 solution are autoclaved as described for solution 1. Autoclaving in closed bottles is necessary, especially for the heat-volatile HCl solution.

Preparation of basal media:

	Freshwater medium	Brackish medium	Saltwater medium
Distilled water	1.0 liter	1.0 liter	1.0 liter
NaCl	1.0 g	7.0 g	20.0 g
$MgCl_2 \cdot 6H_2O$	0.4 g	1.2 g	3.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g	0.1 g	0.15 g
Na_2SO_4*	4.0 g	4.0 g	4.0 g
NH_4Cl	0.25 g	0.25 g	0.25 g
KH_2PO_4	0.2 g	0.2 g	0.2 g
KCl	0.5 g	0.5 g	0.5 g

*If fermentative growth or syntrophic growth with methanogens is tested, sulfate is omitted.

To avoid formation of precipitates, dry salts should not be mixed before dissolution. They are added successively to the stirred water. The solution is autoclaved in one of the devices shown in Figs. 1B or 2. After autoclaving, redissolution of oxygen is prevented by cooling under N_2/CO_2. Then, the aforementioned stock solutions or aliquots therefrom are aseptically added (amounts per liter of medium):

Trace element mixture 1*	1.0 ml
Selenite-tungstate solution	1.0 ml
$NaHCO_3$ solution	30.0 ml
Vitamin mixture	1.0 ml
Thiamin solution	1.0 ml
Vitamin B_{12} solution	1.0 ml
Na_2S solution	7.5 ml

*Alternatively, 1 ml of trace element mixture 2 or 10 ml of trace element mixture 3 are added. Trace element mixture without chelator is tolerated by all sulfate-reducing bacteria and is therefore used for enrichment of new types and most pure cultures. The possibility of growth stimulation by one of the chelated trace element mixtures has to be tested for each species; chelators can also impede growth.

The pH is controlled in aliquots aseptically withdrawn by means of a sterile pipettes; aliquots are collected in small test tubes. To avoid loss of CO_2 from these samples and increase of the pH, the tip of the pipette is kept below the meniscus of the added sample. A pH value of 7.0 to 7.3 (or other pH desired) is obtained using sterile 1 M H_2SO_4 (for sulfate-free medium, with 2 M HCl) or Na_2CO_3 solution (see below). Ordinary pH probes may gradually deteriorate if exposed to sulfide. Therefore, sulfide-resistant probes with bridged reference electrodes (e.g., Ross™, Orion) are recommended. Electrode damage may be also avoided by adjusting the pH before the addition of Na_2S to the medium (i.e., after addition of vitamin B_{12}). Then, an additional, calculated amount of acid is added that exactly compensates the increase in pH caused by the following addition of the alkaline Na_2S. Per ml of Na_2S solution (0.2 M) to be added, approx. 0.13 ml of 1.0 M H_2SO_4 or 2 M HCl are added in advance. Thereafter, the sulfide solution is added, and the medium is dispensed into sterile bottles or tubes (Fig. 2) that are stored in the dark.

Organic substrates and further additions:
Depending on the type of sulfate reducer to be enriched or cultivated, aliquots from the following stock solutions are added to tubes or bottles with basal media.

11. Acetate solution, 2.0 M
    27.2 g $CH_3COONa \cdot 3H_2O$ are dissolved in distilled water to a final volume of 100 ml and autoclaved in a closed bottle as described for solution 1.
    Application: 5 to 10 ml per liter of medium.
12. Propionate solution, 2.0 M
    Unlike acetate, propionate (and also salts of most other acids) is prepared from the free acid, which is usually of higher purity than the commercially available sodium salt.
    Distilled water 20 ml
    Propionic acid 14.9 ml (14.8 g)
    NaOH (4.0 M) 48 ml
    The NaOH is added slowly with stirring. The pH is then adjusted between 8 and 9 by dropwise addition of further NaOH (preferentially more dilute). To remove propionic acid ester that is often present, the slightly alkaline solution is gently boiled until the ester smell has disappeared. Distilled water is added to a final volume of 100 ml. The solution is autoclaved in a closed bottle as described for solution 1.
    Application: 5 to 10 ml per liter of medium.
13. Butyrate or isobutyrate solution, 1.0 M
    Distilled water 40 ml
    Butyric or isobutyric acid 9.20 ml (8.81 g)
    NaOH (4.0 M) 24 ml
    Adjustment of pH between 8 and 9 and removal of ester is carried out as described for solution 11. Distilled water is added to a final volume of 100 ml. The solution is autoclaved as described for solution 1.
    Application: 5 to 15 ml per liter of medium.
14. Palmitate or stearate solution, 0.10 M
    Water 80 ml

Palmitic acid 2.54 g
NaOH (4.0 M) 2.50 ml

Stearate solution is prepared with 2.85 g of stearic acid. The suspension is heated in a closed bottle (with head space) in a boiling water bath. The bottle is vigorously shaken until the solution has become clear and all particles and droplets have disappeared. Distilled water is added to a final volume of 100 ml. The solution is autoclaved as described for solution 1. The solution solidifies when cooled and has to be remelted in a boiling water bath before use. Gradually formed precipitates from glass do not affect the usability (see solution 4).

Application: 8 to 15 ml per liter of medium.

15. Ethanol solution, 2.0 M

11.6 ml (9.21 g) ethanol are dissolved in water to a final volume of 100 ml and autoclaved in a tightly closed bottle as described for solution 1.

Application: 5 to 20 ml per liter of medium.

16. Lactate solution

The commercially available 50% (5.7 M) sodium lactate solution is autoclaved as described for solution 1. More dilute solutions, e.g., 2.0 M, may be also used.

Application: 2 to 7 ml (50%) per liter of medium.

17. Benzoate or nicotinate solution, 0.5 M

Distilled water 40 ml
Benzoic acid 6.11 g
NaOH (4.0 M) 24 ml

Nicotinate solution is prepared with 6.16 g of nicotinic acid. The NaOH is added under stirring. The poorly soluble acids dissolve gradually. Heating in a water bath and breaking up the clumps that form using a flat-ended glass rod facilitates dissolution. The pH is adjusted to 7 to 8 with further NaOH. Distilled water is added to a final volume of 100 ml. The solution is autoclaved as described for solution 1.

Application: 4 to 10 ml per liter of medium.

18. Phenol, catechol, or aniline solution, 0.25 M

2.4 g phenol, 2.75 g catechol, or 2.3 ml (2.3 g) aniline* are dissolved in distilled water in a final volume of 100 ml. Anoxic storage is important especially for catechol and aniline. Solutions are filter-sterilized (pore size, 0.2 μm) into autoclaved bottles with crimped rubber stoppers; the filter holder is connected with a needle to pierce through the stopper. The bottles are repeatedly evacuated and aseptically gassed with N_2 via a cotton-filled syringe according to Balch et al. (1979). The solutions are kept in the dark. Aliquots are withdrawn by means of syringes.

Application: 2 to 4 ml per liter of medium.

*Caution: aniline is highly toxic.

19. Indole solution, 0.025 M

0.29 g indole are added either to 100 ml distilled water or, in case of marine strains, to 100 ml of an aqueous solution containing 2 g NaCl and 0.3 g $MgCl_2 \cdot 6H_2O$. The suspension is heated in a closed bottle (with head space) in a boiling water bath. The hot bottle is vigorously shaken until droplets of indole have disappeared. The solution is autoclaved and kept in the dark. Indole recrystallizes after cooling. Before use, the indole is redissolved as described.

Application: 10 to 20 ml per liter of medium.

20. Sulfite solution, 1.0 M

An aqueous solution of 12.6 g Na_2SO_3 per 100 ml is filter-sterilized and stored under N_2 at 4°C as described for solution 17.

Application: 0.5 to 3 ml per liter of medium.

21. Thiosulfate solution, 1.0 M

An aqueous solutions of 24.8 g $Na_2S_2O_3 \cdot 5H_2O$ in 100 ml is filter-sterilized and stored at 4°C. For maintenance over many months, storage under N_2 is recommended.

Application: 5 to 15 ml per liter of medium.

22. Salt concentrate

NaCl 286.4 g
$MgCl_2 \cdot 6H_2O$ 44.7 g
$CaCl_2 \cdot 2H_2O$ 2.2 g

The salts are dissolved in water to a final volume of 1 liter and is autoclaved as described for solution 1. Applications are described in the section on "Special Procedures and Modifications."

23. Dithionite solution

Stocks of solid $Na_2S_2O_4$ are stored in dry, sterile glass bottles closed with screw caps. A tube with a side arm connected to a sterile cotton filter as shown in Fig. 4 is recommended for preparation of solutions. 10 ml of autoclaved distilled water is deaerated by evacuation and repressurization with N_2. 0.2 g of dry $Na_2S_2O_4$ are weighed on a sterile (flamed) piece of aluminium foil placed in a petri dish; the amount is poured into the deaerated water while a slow stream of N_2 is flushed through the tube to avoid entrance of air. Dithionite is dissolved by gentle shaking. The tube is wrapped against light. If dithionite samples have to be taken with syringes, a more dilute solution may be prepared to facilitate withdrawal. If refrigerated, the solution is stable for about 5 days.

Solid $Na_2S_2O_4$ is usually sterile, as can be demonstrated by adding aliquots into complex test media (e.g., AC medium, Difco). If doubts exist about sterility, a solution prepared under N_2 is sucked into an anoxic syringe and filter-sterilized into the storage tube shown in Fig. 4.

Applications are described in the section on "Special Procedures and Modifications."

24. Yeast extract or peptone solution

An aqueous solution of 10 g yeast extract or 10 g peptone in a final volume of 100 ml is autoclaved as described for solution 1, or, preferably, under anoxic conditions as described for solution 17.

Application: 5 to 10 ml per liter of medium.

25. Fermented peptone broth

2 g peptone, 0.2 ml trace element solution 1, 0.1 g KH_2PO_4, and 0.05 g $MgSO_4 \cdot 7H_2O$ are added to 100 ml water, adjusted to pH 7.0, and deaerated by shaking under N_2 in a stoppered bottle. As a source of fermentative bacteria, approximately 1 ml of anoxic dark mud from a ditch, pond, or sewage digestor is added. The enrichment is incubated at 30°C. The pH is measured from time to time and readjusted to 7.0. After 10 days, insoluble matter is removed by centrifugation (40,000× *g*). The supernatant is

filter-sterilized and stored under N_2 as described for solution 17.

Application: 25 to 50 ml per liter of medium.

Inoculation procedures:

For inoculation of completely filled bottles or tubes, a certain amount of medium is withdrawn by means of a sterile pipette. Required organic or inorganic substrates (see Table 2, later) are added from one of the stock solutions 10 to 20 or other solutions of choice not listed here. Furthermore, approximately 1 ml dithionite solution (solution 22) is added per liter of medium, which significantly favors initiation of growth of many sulfate reducers. The tip of the pipette is dipped into the medium to avoid direct contact of dithionite with air. Instead of the dithionite solution, an estimated amount of dry $Na_2S_2O_4$ may be added by means of a microspatula, e.g., a flattened platinum wire. Finally, the inoculum is added by means of a pipette so as to fill the bottle completely, and it is immediately sealed with the screw cap.

If strictly anoxic procedures are employed throughout, additions are made via the stoppers of the rubber-sealed bottles or tubes by means of N_2-flushed syringes.

Special Procedures and Modifications

CULTIVATION ON H_2. For cultivation with H_2 as the electron donor, a mixture of H_2/CO_2 (90/10 to 80/20, v/v) in a head space of 2/3 to 3/4 of the culture volume is provided. A slight overpressure of 50 kPa (0.5 atm) may be applied; higher overpressures (200 kPa) may be applied after growth has started. Nonautotrophic sulfate reducers require acetate (1–4 mM) as a carbon source in addition to CO_2 if growing with H_2. Vessels are incubated horizontally or shaken to facilitate dissolution of gas.

CULTIVATION ON LONG-CHAIN FATTY ACIDS IN SALTWATER MEDIA. Addition of melted sodium palmitate or sodium stearate to saltwater media yields compact clumps of insoluble magnesium and calcium alkanoates that are poorly utilized by bacteria. To avoid this, the melted palmitate or stearate stock solution is first added to freshwater medium. Thereafter, 22 or 67 ml of salt concentrate (solution 21) are added per liter which yields brackish or saltwater medium, respectively; the alkanoates will precipitate as loose flocs.

CULTIVATION ON LIQUID ALKANES. If sulfate-reducing bacteria are grown with liquid alkanes (e.g., hexadecane), tubes or bottles with medium and a head space (approximately 1/5 of the volume) are used. After inoculation, 5–10 ml of autoclaved or filter-sterilized deaerated alkane is added per liter while the head space is gassed with an N_2/CO_2 mixture. After sealing with stoppers, tubes or bottles are inverted and intensely shaken for some seconds to remove alkane droplets adhering to the stopper. Inverted bottles are incubated at a slightly inclined angle to avoid permanent contact of the water-insoluble, buoyant alkanes with the stopper. More detailed information about anoxic cultivation on alkanes is given in the original literature (Aeckersberg et al., 1991).

ARTIFICIAL SEDIMENT FOR GLIDING SPECIES. Filamentous sulfate reducers of the genus *Desulfonema* require an insoluble substratum for optimal gliding movement and growth. Precipitated aluminum phosphate and sloppy agar (0.2%, w/v) have both been used as substrata (Widdel 1983). The former is precipitated by adding per liter of medium, 5 ml from an autoclaved solution of 48 g $AlCl_3 \cdot 6H_2O$ per liter; the pH is readjusted with 1.6 ml from a solution of 106 g Na_2CO_3 per liter.

GROWTH-STIMULATING ADDITIONS. Complex nutrients such as yeast extract or peptone may stimulate growth of *Desulfovibrio* and some other species (Postgate, 1984a) or promote their development after transfer from old cultures. Several sulfate reducers degrading fatty acids or aromatic compounds are not stimulated by complex nutrients; for a few, yeast extract may be even inhibitory (see e.g., Aeckersberg et al., 1991; Bak and Widdel, 1986b).

For a number of sulfate reducers, fermented peptone (solution 24) is a better stimulant than yeast extract or fresh peptone.

Growth of *Desulfonema* species on acetate is stimulated by low concentrations of additional, defined electron donors and carbon sources. For instance, succinate at a final concentration of 0.5 mM and a mixture of straight- and branched-chain volatile fatty acids at final concentrations between 0.02 and 0.05 mM have been used (Widdel et al., 1983; Widdel, 1989).

MEDIA WITH MODIFIED MINERAL COMPOSITION. During isolation and cultivation of sulfate-reducing bacteria from lake sediment, best growth was observed in a low-salt version of the multipurpose freshwater medium given earlier (Bak and Pfennig, 1991b). The amounts per liter were: 0.25 g NaCl, 0.2 g $MgCl_2 \cdot 2H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, and 0.1 g KCl. Concentrations of the other compounds were unchanged.

Desulfonema magnum requires high concentrations of Ca^{2+} ions. Good growth was observed in saltwater medium with 5 g $MgCl_2 \cdot 6H_2O$ and 1.4 g $CaCl_2 \cdot 2H_2O$ per liter. Other additions were as described.

If a medium with the same concentrations of major salts as in natural seawater has to be pre-

pared, Na_2SO_4 in the given saltwater medium is replaced by 6.8 g $MgSO_4 \cdot 7H_2O$ per liter, and 26 g NaCl, 5.6 g $MgCl_2 \cdot 6H_2O$, 0.72 g KCl, and 1.4 g $CaCl_2 \cdot 2H_2O$ are added per liter instead of the indicated amounts. Concentrations of other minerals in this artificial seawater medium are as described.

Further media with somewhat different content of major salts, trace elements, and organic substrates have been described by Gibson et al. (1987) and Tanner (1989).

Enrichment, Isolation, and Maintenance

Selective Enrichment

Many species of the Gram-negative mesophilic sulfate reducers can be selectively enriched by adding a particular electron donor (Table 1). Other important selective factors may be the salt concentration and the incubation temperature.

Most new types of sulfate-reducing bacteria have been enriched in batch cultures. Such cultures enrich for bacteria with the highest maximum growth rate under the given conditions. However, such species are not necessarily the most important degraders of the respective substrate in the natural habitat. Types growing more slowly in batch enrichments may occur in higher numbers in the natural habitat, due to a better adaptation to the in situ conditions. Of the many factors that are decisive for adaptation to the habitat, two important ones are effective competition at limiting substrate concentration (high μ_{max}/K_s value) and an advantage as a result of mixed substrate utilization. Enrichment of sulfate reducers at low, limiting substrate concentrations may be attempted in chemostat cultures. So far, however, chemostat enrichments have only yielded known types of sulfate reducers. Still, an interesting result from chemostat cultures was that *Desulfobulbus propionicus* can be selectively enriched not only with propionate and sulfate, but also in sulfate-free medium with ethanol as the limiting substrate; ethanol and CO_2 were fermented to propionate and acetate (Laanbroek et al., 1982). A simple method to isolate sulfate reducers that may be outcompeted in batch enrichments is the direct dilution of natural samples in agar or liquid media, without preceding enrichment. Bacteria predominating in the habitat will also be the most abundant in such dilution series, provided the artificial media are compatible.

Natural samples such as mud for enrichment and isolation of sulfate reducers should be kept under anoxic conditions. This can best be achieved by filling the vessels completely or by gassing the head space with nitrogen and sealing with a stopper. If not used immediately, samples are refrigerated but not frozen. The size of the inoculum should be based on the type of sulfate reducers to be enriched. For widespread, rapidly growing ones using common fermentation products (e.g., H_2, acetate, ethanol, lactate), an inoculum of $\leq 1\%$ (v/v) is usually enough for fast enrichment. For enrichment of slowly growing species, especially on less common electron donors (e.g., some aromatic compounds, alkanes), inocula of 5 to 10% are recommended. During incubation, the enrichments are briefly shaken once a day.

Enrichments should be transferred two to five times into new medium before pure culture isolation is attempted. In this way, sludge particles and the bulk of indigeneous non-sulfate-reducing bacteria are gradually diluted away. The transferred volume should be 1 to 10% of the culture volume. Many sulfate reducers, especially those from marine and brackish habitats, tend to form clumps or to stick to the sediment. Enrichments are therefore shaken immediately before inoculation of new medium to guarantee a transfer of some sediment also. More and more free bacteria will appear in the medium as the particulate fraction disappears. Enrichments are kept in the dark to prevent the appearance of photosynthetic, especially green sulfur, bacteria; by oxidation of sulfide to elemental sulfur, such phototrophs alter the redox conditions to the disadvantage of many sulfate reducers. Also, certain sulfate reducers are sensitive to light (Widdel, 1980).

The time for growth of sulfate-reducing bacteria in the first enrichment depends on the type of electron donor. For instance, enrichments on H_2 may grow within a few days, whereas sulfate reducers using phenolic compounds or alkanes may appear only after 1 to 4 months. The best proof of the positive growth of sulfate reducers is the production of H_2S. This can be detected by a simple chemical test. From the enrichment, 0.2 ml (or another defined amount) is added by means of a pipette or syringe to 1.0 ml of a reagent containing 5 mM $CuSO_4$ and 50 mM HCl in distilled water. The tip of the pipette or syringe should dip into the reagent to avoid loss of volatile H_2S. The reagent is gently shaken while the sample is added. The intensity of the brown color of the (initially) colloidal CuS gives a good estimate of sulfide production*; an enrichment without electron donor serves as

*Thiosulfate interferes with the test.

Table 1. Substrates and conditions for selective enrichment of representatives of Gram-negative sulfate reducers.

Species	Electron donor and carbon source (mM)	Medium used[a]	Comments[b]
Desulfovibrio species	H_2 plus CO_2 and acetate (2); lactate (20); ethanol (15)	F, B, S	
sulfodismutans	Thiosulfate (10) or sulfite (5) plus acetate (2)	F	Growth by sulfite or thiosulfate dismutation
Desulfomicrobium species	H_2 plus CO_2 and acetate (2); lactate (20)	F	No special selection versus *Desulfovibrio* known
Desulfobulbus species	Propionate (10); ethanol (10) without sulfate in a chemostat	F, B, S	
Desulfobacter			
Oval type	Acetate (10–20)	B, S	*D. hydrogenophilus* and *D. latus* isolated as acetate scavengers from a marine isovalerate enrichment
Curved type	Acetate (10–20)	B, S	May be promoted in NH_4^+-free medium under N_2; some strains obtained from naphthenic acid[c] enrichments, or by enrichment around 10°C
Desulfobacterium			
autotrophicum	H_2 plus CO_2	S	25°C; selective enrichment unlikely; direct dilution of marine mud in agar medium recommended
anilini	Aniline (0.5)	F, B	Lag phase of some months; portions of 0.5 mM aniline may be repeatedly added
indolicum	Indole (0.5–1)	S	Portions of 0.5 mM indole may be repeatedly added
phenolicum	Phenol (0.5–1)	S	Portions of 0.5 mM phenol may be repeatedly added
niacini	Nicotinate (4)	S	
vacuolatum	Isobutyrate (10)	S	May also yield other complete oxidizers
Desulfococcus			
biacutus	Acetone (10)	F	May also yield *D. multivorans*
multivorans	Benzoate (4), cyclohexanecarboxylate (3), acetone (10), 2-propanol (10)	F, B	35°C; acetone may also yield *D. biacutus*
Desulfosarcina			
variabilis	Benzoate (4)	S	
Desulfomonile			
tiedjei	No selective substrate reported	F	Isolated from a methanogenic 3-Cl-benzoate enrichment; 1,4-naphthoquinone required as special growth factor
Desulfonema			
limicola	Acetate (10) plus small amounts of additional substrates[d]; isobutyrate (5)	S	Not very selective; other sulfate reducers may become dominant
magnum	Benzoate (4)	S	≥5 mM Ca^{2+} to be added; enrichment may also yield other complete oxidizers
Desulfobotulus			
sapovorans	Palmitate (1.5), caproate (3), caprylate (2)	F	May yield other vibrioid or rod-shaped complete oxidizers. Further types obtained in saline medium
Desulfoarculus			
baarsii	Stearate (1)	F	36°C

[a]F, freshwater medium; B, brackish medium; S, saltwater medium.
[b]Unless indicated otherwise, the incubation temperature is 28–30°C.
[c]Organic acid fraction from oil (petroleum); is usually growth inhibitory, but some species seem to be resistant.
[d]Fermented peptone broth (20 ml/l) may be used (see text).

blank. If the substrates are relatively toxic, e.g., indole or phenol, only rather low concentrations can be used, which do not permit much growth or H_2S production (Bak and Widdel, 1986a, 1986b). In such cases, small portions of the substrate may be added a number of times during growth. An analytical test (UV absorption, gas chromatography, liquid chromatography, etc.) to prove substrate consumption can be useful. Sometimes, enrichments suffer from grazing by protozoa. They may be controlled by adding cycloheximide at a concentration of 10 to 50 μg/ml, which inhibits eukaryotic protein synthesis. Also, early transfer at the end of bacterial growth is recommended in such case.

Addition of complex nutrients such as yeast extract or peptone is not recommended for enrichments. These nutrients cause rapid development of various fermentative bacteria.

Isolation

ISOLATION USING AN AGAR DILUTION SERIES. The most frequently used method for the isolation of sulfate-reducing bacteria is serial dilution in anoxic agar medium.

Agar powder is first washed to remove soluble substances that may be inhibitory or favor fermentative bacteria. 3.3 g agar is suspended in approximately 300 ml distilled water in a calibrated flask or beaker and stirred for 10 min. The agar is then allowed to settle. After removal of the supernatant by slow decanting or suction with an aspirator, fresh water is added. The washing procedure is repeated four times. At the end, water is added to a final volume of 100 ml. The suspension is melted to homogeneity by stirring in a boiling water bath. The melted agar is dispensed in portions of 3 ml to test tubes that are sealed, e.g., with aluminum foil. The tubes are autoclaved and stored in plastic bags or sealed cans at 4°C to avoid drying.

For preparation of a dilution series, 6 to 8 agar tubes are heated in a boiling water bath to remelt the agar, which is then kept liquid in a bath at about 60°C. The aluminum seals are replaced by rubber stoppers using flamed tweezers; the stoppers are not fixed too tightly. Medium with substrate is prewarmed in another water bath at 41°C. From the prewarmed medium, 6 ml are added by means of a pipette to each tube to yield a 1% agar medium; to minimize contact with air during addition, the tip of the pipette is inserted into the agar. The mixture of agar and medium is kept liquid at 41°C. To each agar tube (liquid volume, 9 ml), 12 to 18 μliter of dithionite solution are added by means of a pipette which is used at the same time for gentle mixing. A sample of about 0.1 ml from the enrichment culture is added to the first tube; for mixing, the tube is slowly turned once or gently stirred with a pipette. A small, estimated amount of about 0.5 ml is transferred to the next tube by direct pouring or by means of a pipette and mixed again. The dilution is continued to the last tube. After each transfer, the previous tube is placed in a cold water bath. Immediately after solidification of the agar, the tubes are aseptically gassed with an N_2/CO_2 mixture, as shown in Fig. 5.

For isolation of brackish or marine sulfate reducers, the concentration of NaCl and other salts may become critical due to addition to the aqueous agar. This dilution can be compensated by addition of 13 or 35 ml of salt concentrate (solution 21) per liter of brackish or saltwater medium, respectively. After addition to the agar, the medium will regain its normal salt concentration. Addition of the salts to the agar before autoclaving is not recommended since hydrolysis may be promoted.

The method described allows slight contact with oxygen during dilution. However, this is not critical for most species of sulfate reducers; the exposure to air is brief and its dissolution is slowed down by the viscosity of the agar. However, for a completely anoxic procedure, the 3-ml portions of the concentrated agar are dispensed into tubes that have been provided with a 9 ml mark; the agar is allowed to solidify in an almost horizontal position. Tubes are then incubated under N_2 (in stoppered tubes or an anaerobic chamber) to allow desorption of O_2. Thereafter, stoppers are fixed with crimps or screw caps and tubes are autoclaved. The further procedure is in principal analogous to the aforementioned method. Medium is added to the melted agar by injection from the dispensing device shown in Fig. 2; the medium should be prewarmed in the device to avoid early solidification of the agar. Anoxically gassed syringes are used for addition of dithionite from the tube shown in Fig. 5 and also for transfers. For isolation of H_2-utilizing bacteria, an H_2/CO_2 mixture is used for gassing. In such case, the agar is allowed to solidify in an almost horizontal position.

Strictly anoxic dilution series may be also carried out with open tubes inside an anoxic chamber.

All tubes are inverted during incubation so that water exudate from the agar accumulates on the stopper; this water is removed when tubes are opened for isolation of colonies.

Colonies of sulfate-reducing bacteria are usually recognized by a yellowish, reddish, or brownish pigmentation. For isolation, the end of a Pasteur pipette is softened in a small flame,

taken out, and immediately drawn to a fine capillary by means of tweezers. The end is broken off and the capillary is filled with some medium. For isolation of colonies from tubes with horizontally solidified agar, the drawn Pasteur pipette should have a bent tip. Single, separated colonies are sucked into the medium-containing pipette by using a flexible tube connected to a mouth piece. The isolated bacteria usually need further purification via a second dilution series from which pure cultures may be picked and transferred into liquid medium. Part of the colonies may be used for microscopic control.

ISOLATION ON AGAR PLATES. Sulfate reducers may be also purified by streaking on agar plates that are incubated in anoxic jars containing some H_2 and a palladium catalyst. Preparation of plates, streaking, and transfer to jars is best carried out inside an anoxic chamber. If plates are handled in air, the reductant may be oxidized due to the large surface, and oxygen-sensitive cells may die off. If CO_2 is required as a carbon source, this is added to the atmosphere of the jar; the agar plates in such case should contain $NaHCO_3$.

Instead of plates and jars, anoxic flat bottles (100 ml) with an agar layer (10 ml) may be used ("bottle plates"). Problems caused by water exudate from the agar were solved with improved bottle plates (Hermann et al., 1986). Enrichments are streaked on the agar while the bottle is anoxically gassed. Some H_2S (approx. 1%, v/v) should be injected into the stoppered bottle. H_2S may be provided from a small steel bottle with a pressure regulator and a septum for withdrawal of gas samples by means of a syringe. The device has to be installed inside a fume hood with vigorous ventilation. Use of a gas mask is recommended.

ISOLATION IN LIQUID MEDIA. Instead of agar media, liquid media may be used for purification of sulfate-reducing bacteria via serial dilutions. The method is analogous to the dilution technique for determination of most probable numbers (MPN technique).

An alkane-utilizing sulfate-reducing isolate was purified in liquid medium under insoluble hexadecane by transfer of samples from both phases. In this way, alkane degraders attached to the hydrocarbon phase were gradually separated from free commensals (Aeckersberg et al., 1991).

ISOLATION OF FILAMENTOUS SULFATE-REDUCING BACTERIA. Isolation of the long, filamentous *Desulfonema* species by dilution or streaking is difficult since the numbers of the unicellular commensals or competitors are usually higher. For purification of *D. limicola* and *D. magnum,* the bulk of unicellular bacteria was removed by anoxic washing of the filaments (Widdel, 1983). This was achieved in a piece of glass tubing containing a fine-mesh copper grid (as used in electron microscopy); the latter acted as a sieve that allowed unicellular bacteria to pass through whereas filaments were retained. Washed filaments of *D. limicola* were further purified via dilution series in soft (0.8%, w/v) agar. For *D. magnum,* a pure culture was finally obtained from the washed filaments by transferring one of the relatively thick, visible filaments through a series of small portions of sterile anoxic medium (Widdel, 1983).

Maintenance

For short-term preservation, stock cultures are usually kept at 2–6°C. Since fully grown cultures easily die off or lyse if further incubated at optimal growth temperature, strains should be refrigerated before or right at the end of growth. However, *Desulfonema magnum* is very sensitive to refrigeration and is maintained at around 20°C. Also, sulfate reducers grown on alkanes may die off rapidly if refrigerated.

The interval for transfer depends on the tendency of stored cells to undergo lysis. Lysis is usually indicated by the presence of slime and a decrease in turbidity. For most species, transfer every 6 to 12 weeks is recommended.

For long-term preservation, cultures containing 5–10% (v/v) dimethylsulfoxide (DMSO) are kept in liquid N_2.

Taxonomy and Identification

Ribosomal 16S RNA sequences are generally accepted as decisive criteria for the definition of taxa at all levels and for the affiliation of new isolates to these taxa (Woese, 1987). This method has been applied to many mesophilic nonsporeforming sulfate-reducing bacteria. Derived relationships among the bacteria known as the delta subdivision of the proteobacteria are illustrated by the unrooted tree of Fig. 6. Wide-range sequencing of 16S rRNA of *Desulfonema* species has not yet been carried out. The older method of oligonucleotide cataloging indicated that *Desulfonema limicola* is closely related to *Desulfosarcina,* and thus it also is a member of the delta subdivision. Based on 16S rRNA analyses, *Desulfomonas* (Moore et al., 1976) groups with the genus *Desulfovibrio* (Devereux et al., 1989) and is therefore assigned to

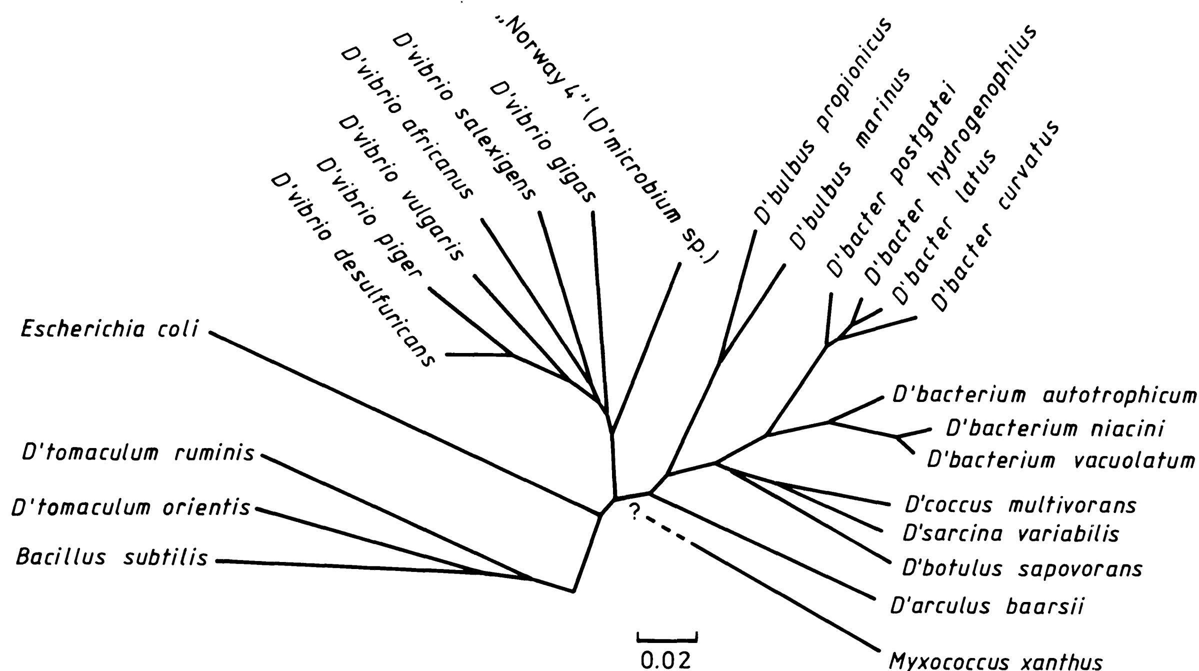

Fig. 6. Relationships of mesophilic nonsporeforming sulfate-reducing bacteria (delta-group) among themselves and to some other bacteria, as derived from 16S rRNA sequences. The unrooted tree of Devereux et al. (1990) and more recent, revised data (R. Devereux, personal communication) were combined into one figure. The scale bar is in units of fixed nucleotide substitutions per sequence position. Species with new names are: *Desulfobulbus marinus,* first described as *Desulfobulbus* strain 3pr10 (Widdel and Pfennig, 1982); *Desulfobacterium niacini,* formerly *Desulfococcus* (Imhoff-Stuckle and Pfennig, 1983); *Desulfobotulus sapovorans,* formerly *Desulfovibrio* (Widdel, 1980); *Desulfoarculus baarsii,* formerly *Desulfovibrio* (Widdel, 1980). The combining form *Desulfo-* is abbreviated *D'-*.

this genus.* The former *"Desulfovibrio" sapovorans* and *"Desulfovibrio" baarsii* are now listed as *Desulfobotulus sapovorans* and *Desulfoarculus baarsii,* respectively; establishment of these new genera appears appropiate because they resemble *Desulfovibrio* species only morphologically but not by other properties. By these rearrangements, genera of sulfate reducers become not only phylogenetically homogeneous, but also physiologically. A genus consists either of incompletely or completely oxidizing sulfate reducers. Morphology is not necessarily a good criterion for taxonomic assignments and it may not always agree with the 16S RNA groupings.

In view of the genealogy it seems justified to also define taxa above the genus level to the family level. The obvious separation of *Desulfovibrio* and *Desulfomicrobium* from the other members of the delta subdivision suggests the establishment of two families (Devereux et al., 1990); the designations Desulfovibrionaceae and Desulfobacteriaceae are proposed.

*This reclassification is done with permission from W. E. C. Moore (personal communication).

For determination of relationships at the species and genus level, other simpler methods based on nucleic acids may be useful. Competitive hybridization was measured between DNA from *Desulfovibrio vulgaris* and rRNAs from a number of other bacteria. The results confirmed that *Desulfovibrio* species were most closely related to each other, but less closely to *Desulfotomaculum* and non-sulfate-reducing Gram-positive and Gram-negative bacteria (Pace and Campbell, 1971). DNA-DNA hybridization revealed genotypic differences between *Desulfovibrio* species and nutritionally similar, but rod-shaped mesophilic or thermophilic sulfate reducers assigned with the genus. This led to the removal of *D. baculatus* and *D. thermophilus* from the genus *Desulfovibrio* (Nazina et al., 1987). The new genus *Desulfomicrobium* (Rozanova et al., 1988) was established for the mesophile, whereas the thermophile was affiliated with the formerly established genus *Thermodesulfobacterium* (Rozanova and Pivovarova, 1988; Zeikus et al., 1983). The exact relationship of *Thermodesulfobacterium* is not known. Preliminary sequencing indicated branching near the root of the eubacterial tree, at a point remote from the delta group (C.R. Woese, personal communication).

Since phylogenetic groupings were in good agreement with nutritional and biochemical traits (Fowler et al., 1986; Devereux et al., 1989), such characteristics are often useful for the identification and affiliation of sulfate-reducing bacteria to genera. Phenotypic properties of the classified mesophilic nonsporeforming sulfate reducers are listed in Table 2. The cell morphology of a number of strains is shown in Fig. 7 to 9 and the ultrastructure of some isolates is shown in Fig. 10. Besides nutritional and morphological characteristics, biochemical markers can be useful for the identification or affiliation of isolates to genera. Such markers are menaquinones (Collins and Widdel, 1986), lipid fatty acids (Boon et al., 1977; Dowling et al., 1986; Taylor and Parkes, 1983; Ueki and Suto, 1979), and pigments such as desulfoviridin. Most genera possess menaquinones MK-7 or MK-7(H_2); other different, characteristic menaquinones were found in *Desulfovibrio* species, *Desulfobulbus* species, and *Desulfonema magnum.* Since the presence or absence of biochemical markers may not always coincide with genera, they can only provide complementary criteria in addition to other characteristics.

The genera *Desulfovibrio, Desulfobulbus,* and *Desulfobacter* are phenotypically rather homogeneous, well-defined groups of Gram-negative sulfate-reducing bacteria. Affiliation to these solely by phenotypic characteristics is rather unproblematic. In the case of *Desulfonema,* the striking morphology allows easy identification.

The following brief description may help to identify sulfate-reducers of the delta group.

Descriptions of Genera and Families

Desulfovibrionaceae

DESULFOVIBRIO. Cells of *Desulfovibrio* are more or less curved and often motile. *Desulfovibrio piger* (formerly *Desulfomonas pigra*) has nonmotile, somewhat irregular, rod-shaped cells. The most commonly utilized organic substrates are lactate, pyruvate, ethanol, and in many cases also malate and fumarate. Electron donors are incompletely oxidized to acetate. The use of H_2 as electron donor is common; lithotrophic growth requires acetate as a carbon source in addition to CO_2. Oxidation of long-chain fatty acids is generally not observed. Growth in the absence of an external electron acceptor is possible by fermentation of pyruvate and in several cases also of malate or fumarate. Metabolism of lactate with concomitant formation of H_2 in the absence of sulfate has been observed (Pankhania et al., 1988). However, growth due to this reaction apparently does not take place unless hydrogen is kept at very low partial pressure, e.g., in co-cultures with methanogenic bacteria (Bryant et al., 1977). Such syntrophic growth is also possible with ethanol (Bryant et al., 1977) and choline (Fiebig and Gottschalk, 1983). Syntrophic growth has been observed so far only with *Desulfovibrio* species; *Desulfobacterium, Desulfosarcina* and *Desulfomaculum* species did not form active co-cultures with methanogens even though hydrogenase was present in these sulfate reducers (F. Widdel, unpublished observations). All species presently affiliated with the genus contain the bisulfite reductase desulfoviridin. Sulfate reducers that possess desulfoviridin but do not belong to the genus *Desulfovibrio* are the coccoid *Desulfococcus* species, *Desulfomonile,* and the filamentous *Desulfonema limicola.* The predominant menaquinone in *Desulfovibrio* species is MK-6; the isoprenoid side chain of the molecule is sometimes saturated in the terminal position (Collins and Widdel, 1986). Lipid fatty acids with an odd number of C atoms (mainly C_{15} and C_{17}) and iso- or anteiso branching* are characteristic for many *Desulfovibrio* species (Boon et al., 1977; Taylor and Parkes, 1983; Ueki and Suto, 1979).

DESULFOMICROBIUM. This genus is nutritionally very similar to *Desulfovibrio.* However, cells are rod-shaped and desulfoviridin is lacking. The bisulfite reductase desulforubidin (Lee et al., 1973) and MK-6 (Collins and Widdel, 1986) have both been detected in a strain that probably belongs to *Desulfomicrobium baculatum.*

Desulfobacteriaceae

DESULFOBULBUS. Most species have oval to lemon-shaped or onion-shaped (i.e., with pointed ends) motile or nonmotile cells. Some types form slender rods. A characteristic substrate is propionate, which is incompletely oxidized to acetate. Other commonly used electron donors are lactate, ethanol, or H_2; growth on dicarboxylic acids has not been observed. In contrast to *Desulfovibrio, Desulfobulbus* can grow by fermentation of lactate or ethanol and CO_2 in a sulfate-free medium. As in *Pelobacter* (Schink, 1984; Schink et al., 1987), propionate and acetate are formed in a ratio of 2:1 (Laanbroek et al., 1982; Widdel, 1980; Widdel and Pfennig, 1982) via a randomizing pathway (Stams et al., 1984). Fermentative growth of *D.*

*Methyl-branched, one or two C atoms, respectively, from the ω-end.

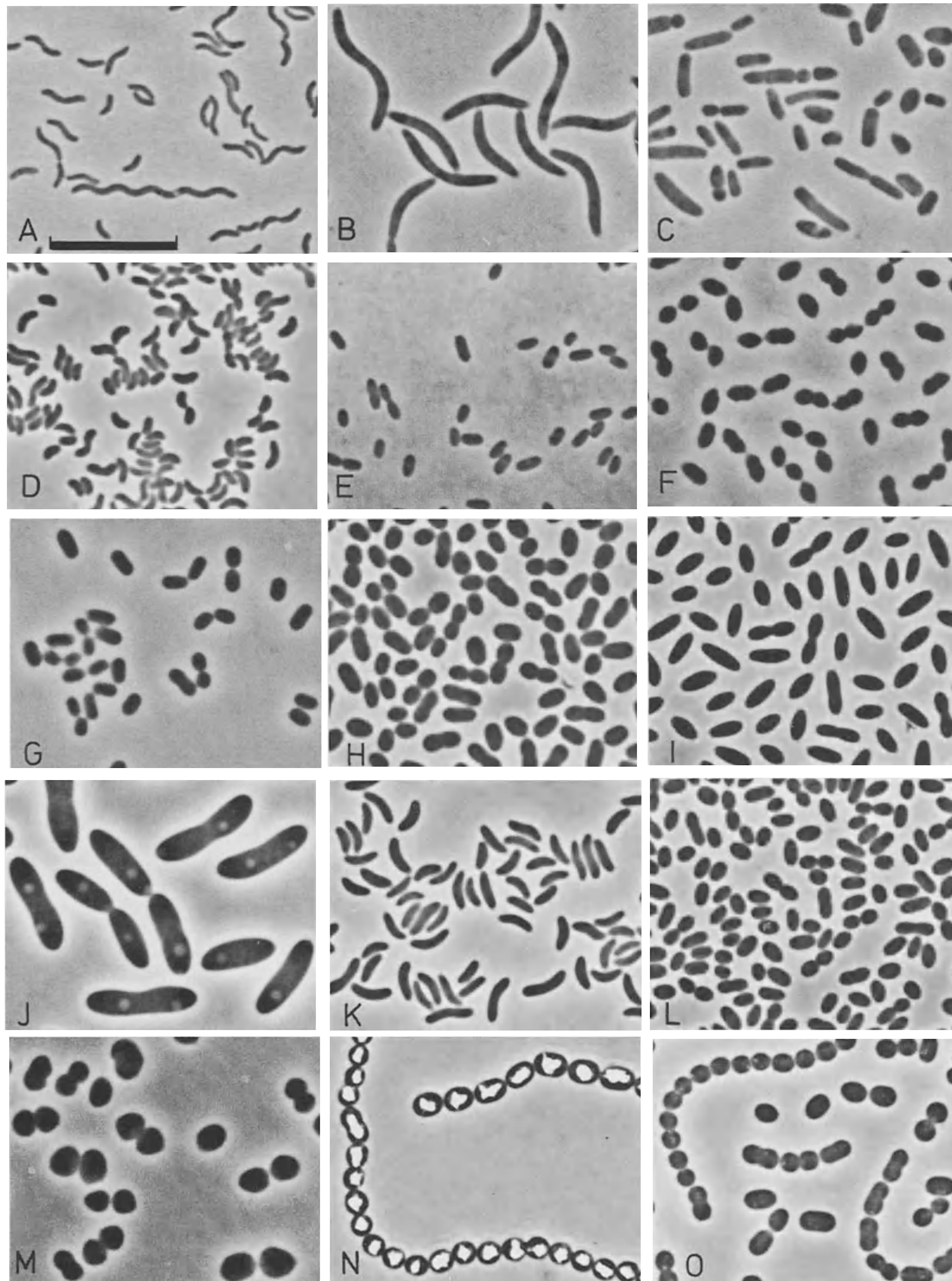

Fig. 7. Light micrographs of viable cells of some sulfate-reducing bacteria. (A) *Desulfovibrio desulfuricans.* (B) *Desulfovibrio gigas.* (C) *Desulfovibrio piger* (formerly *Desulfomonas pigra*). (D) *Desulfovibrio salexigens.* (E) Strain "Norway 4," probably a *Desulfomicrobium* species (formerly a *Desulfovibrio* species). (F) *Desulfobulbus propionicus.* (G) *Desulfobulbus marinus.* (H) *Desulfobacter postgatei.* (I) *Desulfobacter hydrogenophilus.* (J) *Desulfobacter latus,* with unidentified inclusions. (K) *Desulfobacter curvatus.* (L) *Desulfobacterium autotrophicum.* (M) *Desulfobacterium* (formerly *Desulfococcus*) *niacini.* (N) *Desulfobacterium vacuolatum.* (O) *Desulfobacterium vacuolatum,* after application of pressure which caused the collapse of gas vesicles. All photographs were taken by phase contrast. Bar in (A) = 10 μm; applicable to all photographs.

marinus is poor. Syntrophic growth with methanogens does not occur. The sulfite reductase desulforubidin has been detected in *D. elongatus* (Samain et al., 1984). The predominant, characteristic menaquinone is MK-5(V-H_2). Lipid fatty acids with straight, unbranched C chains are common (Taylor and Parkes, 1983).

DESULFOBACTER. Most species have oval, motile, or nonmotile cells. Motility may be lost after isolation. A few species with curved cells are known which morphologically resemble *Desulfovibrio* (Widdel, 1987). The most common and characteristic electron donor is acetate, which appears to be utilized by *Desulfobacter* species more effectively than by any other mesophilic, completely oxidizing sulfate reducer. The *Desulfobacter* species so far investigated are the only completely oxidizing sulfate reducers that possess an operative citric acid cycle (Chapter 24). The enzyme 2-ketoglutarate:ferredoxin (2-ketoglutarate:methyl viologen) oxidoreductase may be regarded as a key enzyme and indicator of the cycle. This enzyme has not been observed in completely oxidizing sulfate reducers of other genera, which cleave acetyl-CoA by means of carbon monoxide dehydrogenase; the latter is absent in *Desulfobacter. Desulfobacter* species seem to be typical brackish or marine organisms; they require NaCl (at $>$100 mM) and $MgCl_2$ (at $>$5 mM) for optimal growth. However, isolation of *D. postgatei* and some vibrio-shaped species was also possible from fresh water habitats; these isolates also required NaCl and $MgCl_2$ for optimal growth (Widdel and Pfennig, 1981; Widdel, 1987). Detection of desulforubidin has been reported (Odom and Peck, 1984). Lipids contain palmitic acid, 10-methylpalmitic acid, and cyclopropyl fatty acids as major characteristic components (Dowling et al., 1986; Taylor and Parkes, 1983).

DESULFOBACTERIUM. A number of completely oxidizing sulfate reducers that are nutritionally rather versatile and/or exhibit special degradative capacities, e.g., the oxidation of aromatic compounds) have been combined in the genus *Desulfobacterium.* Sequencing of 16S rRNA from *D. autotrophicum, D. vacuolatum,* and *D. niacini* (formerly *Desulfococcus*) indeed revealed close relationships among these three species and separate clustering from other genera. 16S rRNA analyses of other sulfate reducers described as *Desulfobacterium* species have not been carried out so far. *Desulfobacterium* species have rod-shaped to oval or almost spherical cells. Fatty acids, dicarboxylic acids, alcohols, or H_2 serve as electron donors for many species. Some may use lactate or aromatic compounds, e.g., benzoate, phenol, or indole. *D. indolicum* isolated on indole is less versatile than other species classified with the genus (Table 2). Autotrophic growth of H_2-utilizing *Desulfobacterium* species is common (Brysch et al., 1987). Like *Desulfobacter, Desulfobacterium* species are mainly brackish or marine organisms that require elevated NaCl ($>$150 mM) and $MgCl_2$ ($>$ 5 mM) concentrations.

DESULFOCOCCUS AND DESULFOSARCINA. Species of these two completely oxidizing genera are not only phylogenetically but also nutritionally related to each other. There are also striking nutritional and morphological similarities to many *Desulfobacterium* species. Obviously, neither phenotypic characteristics nor the GC content of the DNA permit clear distinctions among these three genera; hence, affiliation of sulfate reducers with these genera requires 16S rRNA sequencing. *Desulfosarcina* sometimes grows in dense cell packets; free cells are oval to rod-shaped. *Desulfococcus* contains desulfoviridin. *Desulfococcus* has been isolated from freshwater mud, but it will grow just as well or even slightly better in brackish or marine medium. *Desulfosarcina* is a marine organism.

DESULFOMONILE. A rod-shaped sulfate reducer that had originally been isolated as a pyruvate-utilizing organism capable of reductive dechlorination of 3-chlorobenzoate (Shelton and Tiedje, 1984) was later classified as *Desulfomonile tiedjei* (DeWeerd et al., 1990). According to 16S rRNA sequences compared so far, its closest relative is the sulfur reducer *Desulfuromonas acetoxidans,* followed by *Desulfosarcina variabilis. Desulfomonile* contains desulfoviridin, but otherwise it differs from *Desulfovibrio.* Experiments with defined mixed cultures suggested that dehalogenation is associated with energy conservation (Dolfing and Tiedje, 1987). The ability for the utilization of acetate and its complete oxidation has been demonstrated so far only with thiosulfate as electron acceptor, but not with sulfate. *Desulfomonile* was reported to utilize benzoate, which has been observed before only in case of completely oxidizing sulfate reducers. A special nutritional feature is the demand for 1,4-naphthoquinone as growth factor.

DESULFONEMA. Filamentous, gliding sulfate reducers have been classified in the genus *Desulfonema.* Their striking morphology rather than their other properties has been decisive for classification of these sulfate reducers. The relationship between *Desulfonema limicola* and *D.*

Table 2. Properties of the classified species of Gram-negative sulfate-reducing bacteria.[a]

Species[b]	Morphology	Width×length (μm)	Motility[c]	GC content (mol%)	Sulfite reductase[d] DV	Sulfite reductase[d] O	Major menaquinone[e]	Opt. temp. (°C)	Oxid.[f]
Desulfovibrionaceae									
Desulfovibrio									
africanus	Vibrio	0.5–0.6×2–3	+ (lo)	65	+	nr	MK-6(H$_2$)	30–36	I
carbionolicus	Rod	0.6–1.1×1.5–5	−	65	+	nr	nr	37–38	I
desulfuricans[T]	Vibrio	0.5–0.8×1.5–4	+ (sp)	59	+	nr	MK-6	30–36	I
fructosovorans	Vibrio	0.5–0.7×2–4	+ (sp)	64	+	nr	nr	35	I
furfuralis	Vibrio	0.3–1.2×0.8–3	+ (sp)	61	+	nr	nr	38	I
giganteus	Vibrio or rod	1×5–10	+ (sp)	56	+	nr	nr	35	I
gigas	Large vibrio	0.8–1×6–11	+ (lo)	65	+	nr	MK-6	30–36	I
piger[k]	Rod	0.8–1.3×1.2–5	−	66	+	nr	MK-6	37	I
salexigens	Vibrio	0.5–0.8×1.3–2.5	+ (sp)	49	+	nr	MK-6(H$_2$)	30–36	I
simplex	Vibrio	0.5–1×1.5–3	+ (sp)	48	+	nr	nr	37	I
sulfodismutans	Vibrio	0.5–1×3–5	+	64	+	nr	nr	30–35	I
vulgaris	Vibrio	0.5–0.8×1.5–4	+ (sp)	65	+	2	MK-6	30–36	I
Desulfomicrobium									
apsheronum	Rod	0.7–0.9×1.4–2.9	+ (sp)	52	−	nr	nr	25–30	I
baculatum[T]	Rod	0.6×1.3	+ (sp)	57	−[m]	nr	nr	28–37	I
Desulfobacteriaceae									
Desulfobulbus									
elongatus	Rod	0.6–0.7×1.5–2.5	+ (sp)	59	−	DR	MK-5(H$_2$)	35	I
marinus[n]	Oval	1–1.3×1.8–2.5	+ (sp)	nd	−	nr	MK-5(H$_2$)	29	I
propionicus[T]	Oval or lemon shape	1–1.3×1.8–2	−[p]	60	−	DR	MK-5(H$_2$)	28–39	I
Desulfobacter									
curvatus	Vibrio	0.5–1×1.7–3.5	+	46	−	nr	MK-7(H$_2$)	28–30	C
hydrogenophilus	Rod	1–1.3×2–3	−	45	−	nr	MK-7(H$_2$)	28–30	C
latus	Large oval	1.6–2.4×5–7	+/−[q]	44	−	nr	MK-7	28–32	C
postgatei[T]	Oval	1–1.5×1.7–2.5	+/−[q] (sp)	46	−	DR	MK-7	28–32	C
Desulfobacterium									
anilini	Oval	1.3×1.5–3	−	59	−	P582	nr	35	C
autotrophicum[T]	Oval	0.9–1.3×1.5–3	+ (sp)	48	−	nr	MK-7(H$_2$)	20–26	C
catecholicum	Lemon shape	1.3–1.8×2.2–2.8	−	52	−	nr	nr	28	C
indolicum	Oval	0.7–1.5×2–2.5	+ (sp)	47	−	nr	MK-7(H$_2$)	28	C
macestii	Rod	0.7×1.9–2	+ (sp)	58	−	nr	nr	35	nr
niacini[r]	Irregular sphere	1.5–3	+ (sp)	46	−	nr	MK-7	29	C
phenolicum	Oval to curved rod	1–1.5×2–3	+ (sp)	41	−	nr	MK-7(H$_2$)	28	C
vacuolatum	Oval or sphere	1.5–2×2–2.5	−	45	−	nr	MK-7(H$_2$)	25–30	C
Desulfococcus									
biacutus	Lemon shape	1.4×2.3	−	57	+	nr	nr	28–30	C
multivorans[T]	Sphere	1.5–2.2	−	57	+	nr	MK-7	35	C
Desulfosarcina									
variabilis[T]	Oval rod, packages	1–1.5×1.5–2.5	+/− (sp)	51	−	nr	MK-7	33	C
Desulfomonile									
tiedjei[T]	Rod	0.8–1×5–10	−	49	+	nr	nr	37	nr
Desulfonema									
limicola[T]	Multicellular filament	2.5–3×2.5–3; 10–400 cells	Gliding	35	+	nr	MK-7	30	C
magnum	Multicellular filament	6–8×9–13; 10–200 cells	Gliding	42	−	P582	MK-9	32	C
Desulfobotulus									
sapovorans[T v]	Vibrio	1.5×3–5.5	+ (sp)	53	−	P582	MK-7	34	I
Desulfoarculus									
baarsii[T v]	Vibrio	0.5–0.7×1.5–4	+ (sp)	66	−	nr	MK-7(H$_2$)	35–39	C

[a]nr, Not reported or not determined.
[b]A [T] following the species name indicates this is the type species.
[c]Flagellation type is given in parentheses: lo, lophotrichous; sp, single, polar.
[d]DV, desulfoviridin; O, other; 2, two further are known; DR, desulforubidin. The compound P582 is proposed from absorption spectra data.
[e]Terminal saturation in the isoprenoid side chain is indicated by (H$_2$).
[f]Oxid., extent of oxidation of organic substrates; I, incomplete; C, complete.
[g]Symbols: +, utilized; +*, autotrophic growth; ( ), poorly utilized; −, not utilized.
[h]This is not a complete list; for further substrates, see references.
[i]Symbols: bi, biotin; ni, nicotinate; nq, 1,4-naphthoquinone; pa, *p*-aminobenzoate; pt, pantothenate; th, thiamine.
[j]Also utilized by several other species of sulfate reducers (Folkerts et al., 1989).
[k]Formerly *Desulfomonas pigra;* reclassified with permission of W. E. C. Moore, Blacksburg, Virginia.
[l]Grows by dismutation of sulfite or thiosulfate.

H_2	Formate	Acetate	Fatty acids: C atoms	Isobutyrate	2-Methylbutyrate	3-Methylbutyrate	Ethanol	Lactate	Fumarate	Malate	Benzoate	Others[h]	Growth factor requirement[i]	NaCl requirement (g/l)	References
Electron donors for sulfate reduction[g]															
+	+	−	−	nr	nr	nr	+	+	−	+	nr	nr	−	−	Postgate, 1984a, 1984b
+	+	−	−	nr	nr	nr	+	+	+	+	nr	Methanol, glycerol	Unknown	−	Nanninga and Gottschal, 1987
+	+	−	−	nr	nr	nr	+	+	+	+	nr	Choline	−	−	Postgate, 1984a, 1984b
+	+	−	−	nr	nr	nr	(+)	+	+	+	nr	Glycerol, fructose	−	−	Ollivier et al., 1988
nr	nr	−	−	nr	nr	nr	+	+	+	nr	nr	Furfural[j]	nr	−	Folkerts et al., 1989
+	−	−	−	nr	nr	nr	+	+	−	−	−	Glycerol, cysteine	bi	>2	Esnault et al., 1988
+	+	−	−	nr	nr	nr	(+)	+	+	+	nr	nr	bi	−	Postgate, 1984a, 1984b
+	−	−	−	nr	nr	nr	+	+	−	nr	nr	nr	pa	−	Moore et al., 1976
+	+	−	−	nr	nr	nr	+	+	−	+	nr	nr	−	20	Postgate, 1984a, 1984b
+	+	−	−	nr	nr	nr	+	+	+	+	nr	nr	Unknown	−	Zellner et al., 1989
(+)	−	−	−	nr	nr	nr	+	+	−	−	−	nr[l]	bi, pt		Bak and Pfennig, 1987
+	+	−	−	nr	nr	nr	(+)	+	+	+	nr	Subsp.: oxamate	−		Postgate, 1984a, 1984b
+*	+	−	−	nr	nr	nr	+	+	−	+	nr	nr	Unknown	−	Rozanova et al., 1988
(+)	+	−	−	nr	nr	nr	−	+	−	+	nr	nr	Unknown	−	Rozanova et al., 1988
+	−	−	3	nr	nr	nr	+	+	−	−	nr	nr	Unknown	−	Samain et al., 1984
+	+	−	3	nr	nr	nr	+	+	−	−	−	nr	pa	20[o]	Widdel and Pfennig, 1982
+	−	−	3	−	−	−	+	+	−	−	−	nr	pa	−	Widdel and Pfennig, 1982
(+)	−	+	−	−	−	−	+	−	−	−	−	nr	bi	10[o]	Widdel, 1987
+*	−	+	−	−	−	−	−	−	−	−	−	nr	bi, pa	20[o]	Widdel, 1987
−	−	+	−	−	−	−	−	−	−	−	−	nr	bi, th	20[o]	Widdel, 1987
−	−	+	−	−	−	−	−	−	−	−	−	nr	bi, pa	7[o]	Widdel and Pfennig, 1981
(+)	(+)	(+)	(3–18)	nr	(+)	(+)	−	−	−	−	+	Phenol, (aniline)	B_{12}	15[s]	Schnell et al., 1989
+*	+*	(+)	(3)–16	+	+	−	+	+	+	+	−	Succinate	bi, ni, th	20[o]	Brysch et al., 1987
(+)	(+)	(+)	(3–20)	nr	nr	nr	(+)	(+)	(+)	(+)	+	Catechol	Unknown	−	Szewzyk and Pfennig, 1987
−	(+)	(+)	(3)	−	−	−	(+)	(+)	(+)	(+)	−	Indole	B_{12}	20[o]	Bak and Widdel, 1986a
+*	+*	−	−	nr	nr	nr	+	+	nr	−	−	nr	Unknown		Gogotova and Vainshtein, 1989
+*	+*	(+)	(3)–16	−	−	−	(+)	−	+	+	−	Nicotinate, succinate, glutarate	bi, th	15[o]	Imhoff-Stuckle and Pfennig, 1983
−	(+)	(+)	(4)	−	−	−	(+)	−	(+)	(+)	+	Phenol, p-cresol, glutarate	−	20[o]	Bak and Widdel, 1986b
+*	+*	(+)	(3)–16	+	(+)	(+)	(+)	+	+	+	−	Succinate	−	20[o]	Widdel 1988
nr	nr	(+)	3–nr	nr	+	+	+	−	−	−	−	Acetone, butanone	Unknown	−	Platen et al., 1990
−	+*	(+)	3–16	+	+	+	+	+	−	−	+	Phenylacetate, some strains: acetone	bi, pa, th	5[o,s]	Widdel, 1980
+*	+*	(+)	3–14	−	(+)	(+)	+	+	+	−	+	Phenylacetate	−	15[o]	Widdel, 1980
+*	+*	−[t]	(4)	nr	nr	nr	−	−	−	nr	+	3- or 4-anisate	ni, nq, th	−	DeWeerd et al., 1990
+*	+*	(+)	3–14	+	+	+	−	+	+	−	−	Succinate	bi	15[o]	Widdel et al., 1983
−	+	(+)	3–10	+	(+)	+	−	−	+	(+)	+	Succinate	bi, pa, B_{12}	20[u]	Widdel et al., 1983
−	−	−	4–16	−	+	−	−	+	−	−	−	nr	−	−	Widdel, 1980
−	+*	(+)	(3)–18	+	(+)	+	−	−	−	−	−	nr	−	−	Widdel, 1980

[m]Desulforubidin was found in a similar isolate (strain "Norway 4", formerly regarded as a *Desulfovibrio* species) probably belonging to the same genus or species (Lee et al., 1973).

[n]Suggested new name of a marine isolate (Widdel and Pfennig, 1982).

[o]In addition, 1.2 to 3 g of $MgCl_2 \cdot 6H_2O$ per liter of medium required or routinely added.

[p]Type strain is nonmotile; other strains may be motile (single, polar flagellum).

[q]Motility may disappear after isolation.

[r]Formerly *Desulfococcus*.

[s]Addition is stimulatory, but not absolutely required.

[t]May be used with thiosulfate as electron acceptor.

[u]In addition, 5 g of $MgCl_2 \cdot 6H_2O$ and 1.3 g of $CaCl_2 \cdot 2H_2O$ per liter of medium are required.

[v]Formerly *Desulfovibrio*.

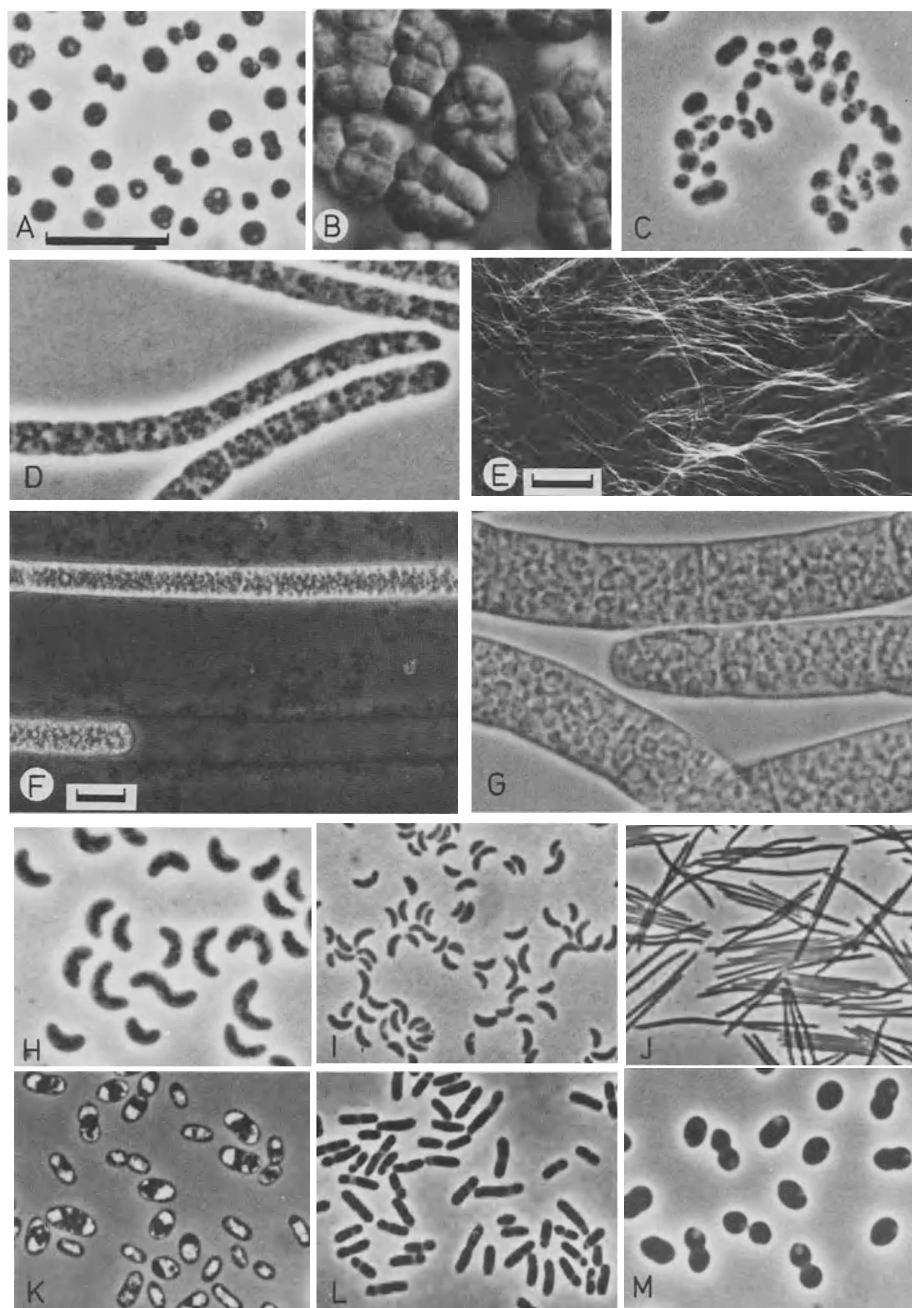

Fig. 8. Light micrographs of viable cells of some sulfate-reducing bacteria. (A) *Desulfococcus multivorans,* with granules of poly-β-hydroxyalkanoic acid. (B) *Desulfosarcina variabilis,* cell packets from an agar colony. (C) *Desulfosarcina variabilis,* single cells from liquid medium. (D) *Desulfonema limicola,* with granules of poly-β-hydroxyalkanoic acid. (E) *Desulfonema magnum,* layers of filaments at the wall of a culture bottle. (F) *Desulfonema magnum,* with granules of poly-β-hydroxyalkanoic acid; filaments glide in a synthetic sediment of aluminum phosphate which makes slime trails visible. (G) *Desulfonema magnum,* with granules of poly-β-hydroxyalkanoic acid. (H) *Desulfobotulus* (formerly *Desulfovibrio*) *sapovorans,* with granules of poly-β-hydroxyalkanoic acid. (I) *Desulfoarculus* (formerly *Desulfovibrio*) *baarsii.* (J) through (M) are unnamed isolates: (J) Long, thin cells nutritionally resembling *Desulfovibrio* species; the strain was isolated from an oil tank with H_2. (K) Cells with gas vesicles of a sulfate reducer nutritionally resembling *Desulfovibrio* species; the strain was isolated from marine sediment with H_2. (L) Cells of a species that preferentially grows by sulfite or thiosulfate dismutation. (M) Isolate nutritionally similar to *Desulfobulbus* species; the strain was isolated from freshwater sediment with 2-methylbutyrate. Photographs were taken by phase contrast, except for (B), (E), and (G). (B) Interference contrast; (E) dark field; (G) bright field. Bar in (A) = 10 μm; applicable to all photographs except for (E) and (F). Bar in (E) = 1 mm; bar in (F) = 10 μm.

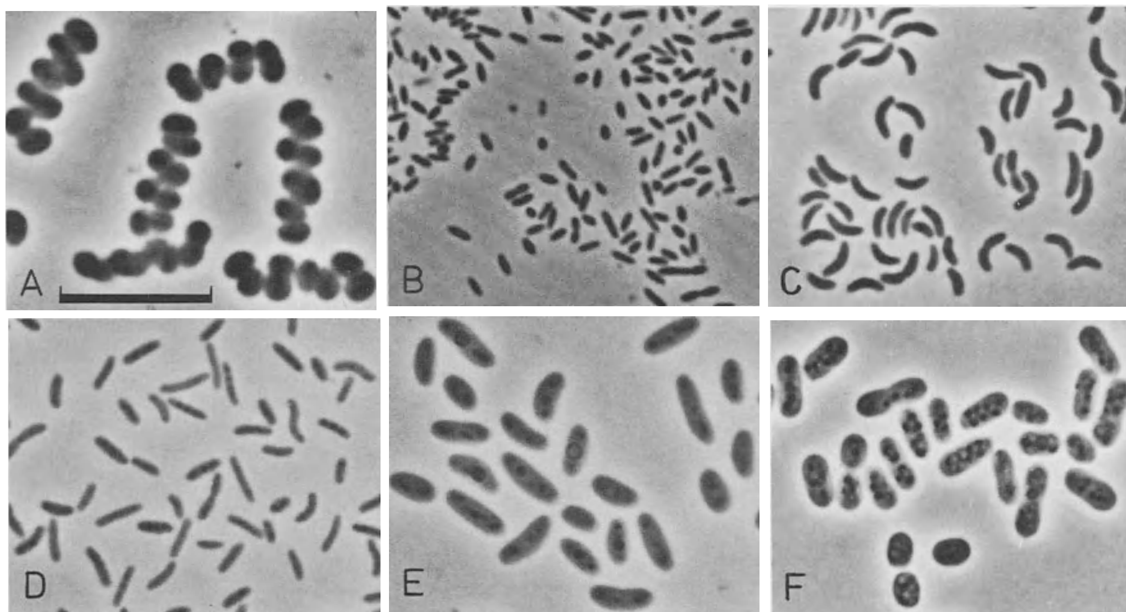

Fig. 9. Light micrographs of viable cells of some unnamed sulfate-reducing isolates. (A) Spirilloid, markedly screwed cells of an isolate nutritionally resembling *Desulfobacter* species; the species was isolated with acetate from marine sediment. (B) Hexadecane-degrading sulfate reducer, tentative name *Desulfobacterium oleovorans.* (C) through (F) are sulfate reducers nutritionally resembling *Desulfobotulus sapovorans.* (C) Vibrioid cells from marine sediment. (D) Rod-shaped to slightly curved cells from marine sediment. (E) Oval to slightly curved cells from marine sediment; inclusions are probably poly-β-hydroxyalkanoic acid. (F) Oval cells from freshwater sediment, with granules of poly-β-hydroxyalkanoic acid. All photographs were taken by phase contrast. Bar in (A) = 10 μm; applicable to all photographs.

magnum has not been confirmed yet by 16S rRNA analyses. Based on oligonucleotide catalogs, the closest relative of the former is *Desulfosarcina* (Fowler et al., 1986). Both *Desulfonema* species are versatile complete oxidizers; *D. magnum* is able to grow on benzoate. The mechanism of terminal oxidation has not been examined. *D. limicola* contains desulfoviridin. *Desulfonema magnum* contains MK-9, the menaquinone with the longest side chain found in sulfate reducers.

DESULFOBOTULUS. An incompletely oxidizing species originally named *Desulfovibrio sapovorans* (Widdel, 1980) branches off among completely oxidizing sulfate reducers, according to 16S rRNA sequences (see Fig. 6). The new genus *Desulfobotulus* is proposed. *Desulfobotulus sapovorans* utilizes lactate like *Desulfovibrio* species, but differs from them by the ability to use fatty acids from butyrate through palmitate, the inability to use H_2 or ethanol, and the absence of desulfoviridin. The curved cells of *D. sapovorans* are thicker than those of *Desulfovibrio* and usually contain granules of poly-β-hydroxyalkanoic acid.

DESULFOARCULUS. A completely oxidizing sulfate reducer originally named *Desulfovibrio baarsii* (Widdel, 1980) appears to represent a separate line within the delta group (Devereux et al., 1989), for which the new genus *Desulfoarculus* is suggested. Morphologically, *D. baarsii* is almost indistinguishable from the nutritionally completely different *Desulfovibrio desulfuricans* and other species of this genus. *Desulfoarculus baarsii* oxidizes monocarboxylic acids from C_1 to C_{18}, but no other compounds and thus differs nutritionally from the more versatile *Desulfobacterium, Desulfococcus,* and *Desulfosarcina* species.

Unclassified Types

A variety of nonsporeforming sulfate-reducing bacteria have been isolated and characterized but not definitely classified. Only a number of representatives can be briefly described in the following.

Two striking cell types physiologically resembled *Desulfovibrio* species, but were morphologically completely different. One of these types had long, thin cells (Cord-Ruwisch et al., 1986; Jones, 1971; Fig. 8J). The other type that was the predominant H_2-consuming sulfate reducer in a marine sediment consisted of ovals with gas vesicles (F. Bak, unpublished observations; Fig. 8K).

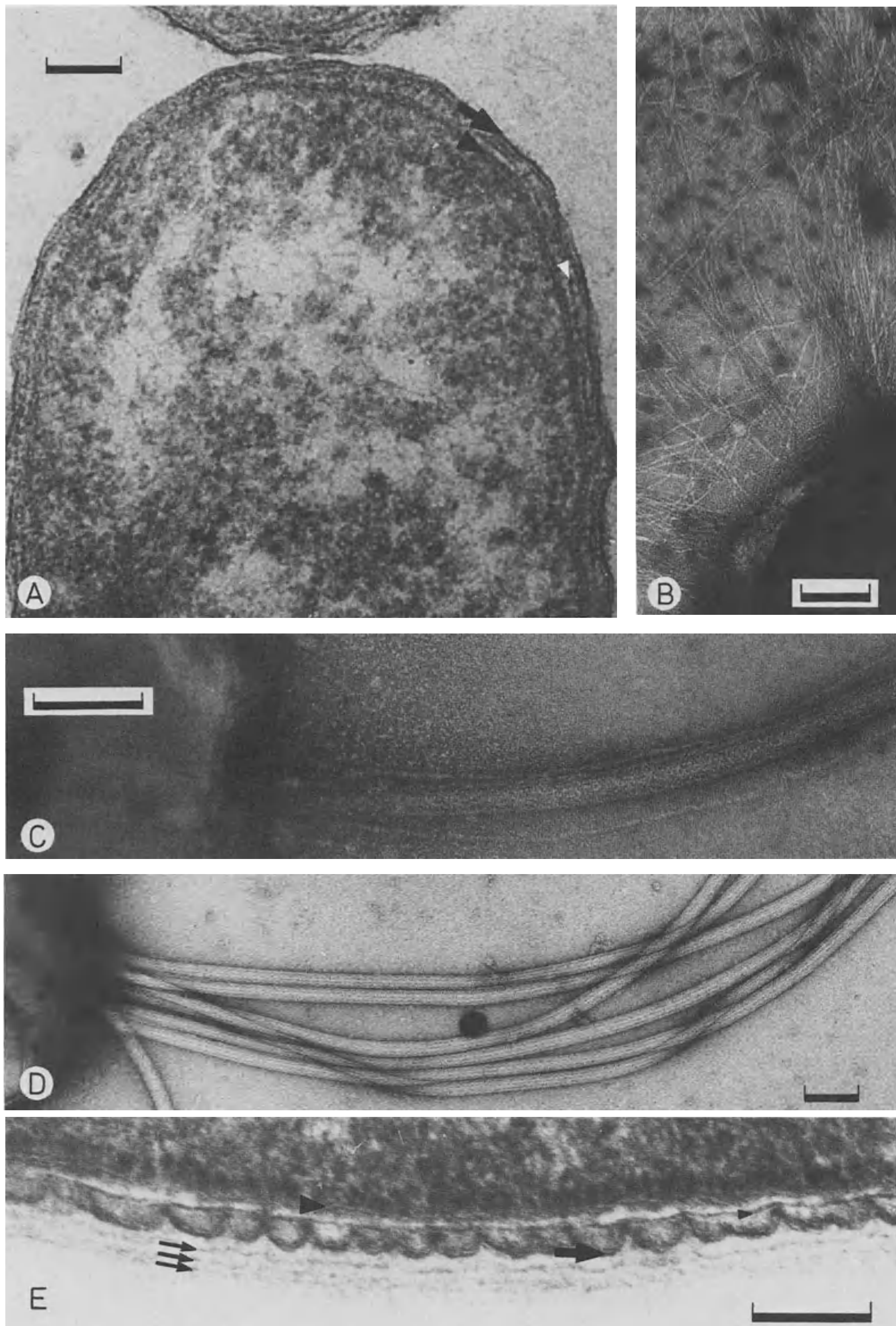

Fig. 10. Electron micrographs of some sulfate-reducing bacteria. (A) *Desulfomicrobium apsheronum;* ultrathin section. (B) Fimbria of *Desulfobulbus propionicus;* negative staining. (C) Sheathed flagellum of *Desulfobotulus sapovorans;* negative staining. (D) Polar bundle of flagella of an unnamed vibrio-shaped isolate that oxidized fatty acids incompletely; negative staining. (E) *Desulfonema magnum,* with waved outer membrane. Large arrowhead, cytoplasmic membrane; small arrowhead, peptidoglycan layer; large arrow, outer membrane; small arrows, outer wall layers. All bars = 0.1 μm. Photographs were kindly provided by the following authors: (A) T. N. Nazina and T. A. Pivovarova, Moscow; (B) and (C) F. Mayer, Göttingen; (D) E. Spiess, Heidelberg; (E) G.-W. Kohring and F. Mayer, Göttingen.

A great variety of incompletely oxidizing sulfate reducers have been isolated with palmitate or fatty acids of shorter chain lengths (Widdel, 1980). All of these vibrioid to rod-shaped isolates, except for one type, utilized fatty acids from C_4 through C_{16}, but not H_2, and did not contain desulfoviridin. Most motile species had single, polar flagella; a bundle of polar flagella in one species in shown in Fig. 10C. Due to their similarity to *Desulfobotulus sapovorans,* all these isolates were tentatively comprised as the "sapovorans group" (Pfennig et al., 1981). An exception was one type isolated on butyrate or 2-methylbutyrate that did not use fatty acids with chain lengths longer than C_5, but grew on H_2 and contained desulfoviridin (Widdel, 1980, 1988).

A rod-shaped sulfate reducer growing preferentially by dismutation of thiosulfate and sulfite was isolated from marine sediment (Bak and Pfennig, 1987; Fig. 8L).

An incompletely oxidizing sulfate-reducing bacterium with oval cells (Fig. 8M) that was enriched with 2-methylbutyrate from freshwater sediment also grew on H_2, ethanol, propionate, butyrate, and lactate and thus shared a number of nutritional properties with *Desulfobulbus* species (F. Widdel, unpublished observations).

A number of sulfate-reducing bacteria isolated with acetate nutritionally resembled described *Desulfobacter* species but were morphologically different (Widdel, 1987). A striking cell type with pronounced, narrow, spiral turns (Fig. 9A) was isolated from sediment from the Kattegat, Denmark (F. Bak, unpublished observations).

Completely oxidizing sulfate reducers differing morphologically and nutritionally from described species have also been isolated on H_2 (Brysch et al., 1987), benzoate (Cord-Ruwisch et al., 1986), catechol, and resorcinol (Schnell et al., 1989).

From an enrichment with hexadecane, a sulfate reducer with vey tiny cells (Fig. 9B) has been isolated (Aeckersberg et al., 1991). Complete oxidation of hexadecane was demonstrated by growth experiments in sealed (fused) glass ampules. The isolate grew on long-chain alkanes, hexadecane, long-chain alcohols, and fatty acids, but not on H_2, ethanol, or lactate. Its tentative name is *Desulfobacterium oleovorans.*

Acknowledgments

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The Genus *Desulfuromonas* and Other Gram-Negative Sulfur-Reducing Eubacteria

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The ability to gain energy for growth by dissimilatory reduction of elemental sulfur in a respiratory type of metabolism (with the formation of sulfide) is found in several genera of eubacteria and archaebacteria. H_2 or organic substrates, mainly simple organic acids, serve as electron donors. There are, however, other organisms, including some eukaryotes, that reduce sulfur in a nonrespiratory manner; in this case, sulfur acts merely as a hydrogen sink in a "facilitated fermentation," or is reduced in a by-reaction without obvious bioenergetic significance. An overview and details of physiology and biochemistry of sulfur reduction are given in Chapter 24.

The concept of the sulfur-reducing bacteria as a physiological group was first advanced with the description of *Desulfuromonas acetoxidans*, an obligately anaerobic mesophile which grows by sulfur respiration (Pfennig and Biebl, 1976). Further well-characterized, sulfur-reducing eubacteria besides *Desulfuromonas* species include the obligately anaerobic moderate thermophile *Desulfurella acetivorans* (Bonch-Osmolovskaya et al., 1990), as well as facultatively microaerobic, spirilloid mesophiles such as the "spirillum 5175" of Wolfe and Pfennig (1977), the "free-living *Campylobacter* species" of Laanbroek et al. (1978), a similar strain of Zinder and Brock (1978), and *Wolinella* (formerly *Vibrio*) *succinogenes* (Macy et al., 1986; Wolin et al., 1961). Dissimilatory sulfur reduction has been observed even in bacteria that otherwise grow aerobically at normal O_2 tension, namely in a *Pseudomonas mendonica* subspecies (Balashova, 1985) and in *Alteromonas putrefaciens* (Loveley et al., 1989; Myers and Nealson, 1988). The latter two organisms have been studied mainly because of their ability to reduce ferric iron or manganese (IV). All of the above-mentioned sulfur reducers cannot reduce sulfate. In addition, there are a few sulfate-reducing bacteria that are able to use sulfur as alternative electron acceptor. These are *Desulfovibrio gigas*, another *Desulfovibrio* strain (tentatively named *Desulfovibrio multispirans*; He et al., 1986), and rod-shaped species most likely belonging to the genus *Desulfomicrobium* (Biebl and Pfennig, 1977; Rozanova et al., 1988), which is physiologically similar to *Desulfovibrio*. In the following, these will be briefly referred to as thiophilic sulfate reducers. The extremely thermophilic sulfur-reducing archaebacteria that can use H_2, most of which are obligate lithoautotrophs, are described in Chapter 24 and 28.

Analyses of 16S rRNA revealed that *Desulfuromonas* and the fermentative *Pelobacter* form a branch among the sulfate-reducing bacteria and are thus members of the delta subdivision and distant relatives of the purple bacteria (Fowler et al., 1986; Stackebrandt et al., 1989). *Wolinella* and the closely related *Campylobacter* species form their own branch, which on a deep level is also related to the purple bacteria (Lau et al., 1987; Romaniuk et al., 1987). *Alteromonas putrefaciens* is a member of the gamma subdivision of the purple bacteria (Schleifer and Ludwig, 1989). Genealogical relationships of *Desulfurella* and the facultatively sulfur-reducing bacterium assigned to *Pseudomonas mendonica* have not been elucidated. A summary of the eubacteria able to grow by sulfur respiration is given in Table 1. (For more details see "Taxonomy and Identification," this chapter).

Habitats and Cocultures

Desulfuromonas species are abundant and widespread in anoxic marine or brackish water sediments but occur less frequently in sediments of freshwater habitats (Pfennig and Biebl, 1981). Marine species usually require certain minimum concentrations of NaCl and Mg^{2+} ions. The predominance in marine or brackish habitats is understood in view of the intense sulfide production by sulfate-reducing bacteria, due to the high sulfate concentration (28 mM) in seawater (Chapter 183). Close to the sediment surface, sulfide is oxidized with O_2 (in a

Table 1. Eubacteria able to grow by respiratory sulfur reduction.

Species	Morphology	Width (μm)	Length (μm)	Motility[a]	GC content of DNA (mol%)	Major menaquinone[b]	Temperature optimum (°C)	Anaerobic oxidation of organic substrates
Desulfuromonas								
acetexigens[c]	Rod	0.8–1.2	1–2	+ (sp)	61–63	MK-8	30	Complete
acetoxidans[d]	Rod	0.4–0.7	1–3	+ (sl)	50–52	MK-8	30	Complete
succinoxidans[c]	Rod; some strains curved	0.5	1–2	+ (sl)	44–46	MK-8	30	Complete
thiophila[c]	Oval or rod	0.8–1	1–2	+ (sl)	59–61	ND	30	Complete
Desulfurella								
acetivorans	Oval	0.5–0.7	1–2	+ (sp)	31	ND	52–57	Complete
Desulfovibrio								
gigas	Large vibrio	0.8–1	6–11	+ (sp)	66	MK-6	30–36	Incomplete
Desulfomicrobium								
species	Oval or rod	0.6–0.9	1.3–2.9	+ (sp)	52–57	MK-6	25–37	Incomplete
Spirillum 5175 and other strains	Vibrio or spirillum	0.5	1.5–4.5	+ (sp)	38–42	MK-6 + TPQ[e]	30–37	Incomplete
Wolinella								
succinogenes	Vibrio or spirillum	0.5	1.5–4.5	+ (sp)	42–48	MK-6 + TPQ[e]	35	Incomplete
Pseudomonas								
mendonica-like isolate	Oval or rod	0.7–0.8[f]	1.4–2.8[f]	+ (sp)	63–64[f]	ND	35–36	ND
Alteromonas								
putrefaciens	Straight or curved rod	ND	ND	+ (sp)[g]	45–55[g]	ND	35	Incomplete

[a]Flagellation pattern is shown in parentheses; sl, single, lateral; sp, single and polar.
[b]See Collins and Widdel (1986); ND, not determined or not reported.
[c]Tentative names (R. Bache and N. Pfennig, unpublished observations).
[d]Type species.
[e]Thermoplasmaquinone-6 (2,[5 or 8]-dimethyl-3-hexaprenyl-1,4-napthoquinone).
[f]From Palleroni, 1984.

Electron donors for sulfur reduction[h]						Electron acceptors[h]										
H_2	Formate	Acetate	Ethanol	Lactate	Others	Sulfur	Thiosulfate	Sulfite	Sulfate	Fumarate or malate	Nitrate	O_2	Others	Growth factor requirement	NaCl requirement (g/l)	References
−	−	+	−	−	−	+	−	−	−	+	−	−	−	Biotin	—	Pfennig and Biebl, 1981
−	−	+	+	−	Pyruvate, propanol	+	−	−	−	+	−	−	−	Biotin	20[i]	Pfennig and Biebl, 1976
−	−	+	−	±	Succinate, glutamate	+	−	−	−	+	+	−	−	Biotin	Some: 20[i]	N. Pfennig, unpublished observations
−	−	+	−	−	Pyruvate	+	−	−	−	−	−	−	−	Biotin	Some: 20[i]	N. Pfennig, unpublished observations
−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	—	Bonch-Osmolovskaya et al., 1990
+[j]	+[j]	−	+	+	Pyruvate	+	+	+	+	−	−	−	−	Biotin	—	Postgate, 1984a, 1984b; Biebl and Pfennig, 1977
+[j]	+[j]	−	+	+	Pyruvate	+	+	+	+	−	−	−	−	Unknown	—	Biebl and Pfennig, 1977
+	+	−	−	±	Pyruvate	+	+	+	−	+	+	+[k]	DMSO[l]	−	—	Laanbroek et al., 1978; Wolfe and Pfennig, 1977; Zinder and Brock, 1978
+	+	−	−	ND	−	+	+	+	−	+	+	+[k]	N_2O	−	—	Macy et al., 1986; Wolin et al., 1961; Yoshinari, 1980
+	ND	ND	ND	ND	Propionate, succinate, malate, glutamate	+	+	+	−	ND	+	+	$Fe(OH)_3$	−	—	Balashova, 1985
+[j]	+[j]	−	−	+	Pyruvate	+	+	+	−	ND	+	+	$Fe(OH)_3$, MnO_2	Unknown	—	Loveley et al., 1989; Myers and Nealson, 1988

[g]From Baumann et al., 1984.
[h]Symbols: +, utilized; ±, utilized by some strains; −, not utilized; ND, not determined.
[i]In addition, 1.2 to 3 g of $MgCl_2 \cdot 6H_2O$ per liter of medium required or routinely added.
[j]Has not been tested with sulfur, but appears likely due to utilization with other electron acceptors.
[k]Microaerobic growth with 1 to 5 kPa (0.01 to 0.05 atm) O_2.
[l]Dimethylsulfoxide.

chemical reaction or by thiobacilli) to yield free sulfur and more oxidized products. If light is available, phototrophic green sulfur bacteria in the upper zone of sediments excrete elemental sulfur as the primary oxidation product of H_2S. In fact, *Desulfuromonas* species do form well-growing, robust cocultures with green sulfur bacteria in the presence of acetate or another organic electron donor. Green sulfur bacteria of marine origin that tend to oxidize H_2S primarily to the level of sulfur (forming very little sulfate) have been shown to be ideal partners for *Desulfuromonas.* Green sulfur bacteria can only assimilate organic compounds (usually acetate) in the presence of an inorganic electron donor and CO_2 and therefore depend on H_2S formed by the sulfur reducer; and vice versa, the sulfur excreted by the green bacterium is immediately reduced back to sulfide by *Desulfuromonas* at the expense of the organic substrate, which is oxidized to CO_2. In such a syntrophism, the sulfur serves as an electron-carrying catalyst between both types of bacteria (Pfennig and Biebl, 1976; Fig. 1). As little as 0.25 mmol sulfide or sulfur per liter is sufficient to maintain optimal growth of both partners (Biebl and Pfennig, 1978).

The moderately thermophilic species *Desulfurella acetivorans* has been isolated from a cyanobacterial mat of a hot water pool in a solfataric field (Bonch-Osmolovskaya et al., 1990).

Thiophilic sulfate reducers occur in the same habitats as other sulfate reducers that cannot grow with sulfur (see Chapter 183). Thiophilic sulfate reducers have been enriched together with *Desulfuromonas* species in cocultures with green sulfur bacteria on ethanol. Syntrophism between such sulfate reducers and green sulfur bacteria has been verified with defined binary cocultures growing on ethanol, that was incompletely oxidized to acetate (Biebl and Pfennig, 1978). Lactate may function as well in these cocultures.

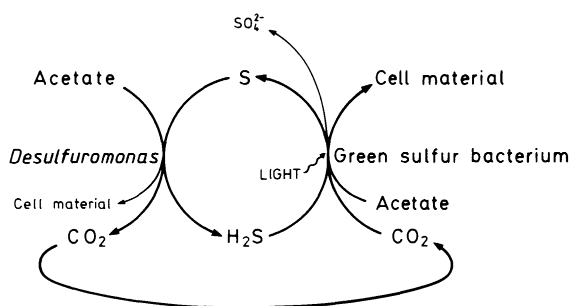

Fig. 1. The H_2S/sulfur cycle in a syntrophic coculture of acetate-oxidizing *Desulfuromonas* and a green sulfur bacterium that assimilates acetate only together with H_2S and CO_2 in the light. *Desulfuromonas* acting as a chemotroph forms less cell material in the coculture than the phototroph. If sulfur is also converted to sulfate, this cannot be recycled by the binary coculture; in sediments, however, sulfate is reduced by sulfate-reducing bacteria.

The spirilloid, facultatively microaerobic, sulfur-reducing bacteria are found in virtually all small or large accumulations of dirty fresh or brackish water (N. Pfennig, unpublished observations). So far, unnamed strains have been isolated from freshwater sediment (Wolfe and Pfennig, 1977; Zinder and Brock, 1978) and from an anaerobic digester fed with potato refuse (Laanbroek et al., 1977). *Wolinella* (originally *Vibrio*) *succinogenes* was obtained from bovine rumen (Wolin et al., 1961). The ubiquitous occurrence of spirilloid sulfur reducers may be due to their versatile respiratory metabolism (Table 1). In addition to *Desulfuromonas,* spirilloid sulfur reducers have also been shown to form robust cocultures with phototrophic green sulfur bacteria; formate was used as electron donor since these sulfur reducers cannot oxidize acetate.

A facultative sulfur-reducing subspecies of *Pseudomonas mendonica* was isolated from activated sludge, from an anaerobic sewage digester, and from river and swamp sediments with H_2 as electron donor and ferric hydroxide as electron acceptor (Balashova, 1985; Balashova and Zavarzin, 1979). Another facultative sulfur reducer, *Alteromonas putrefaciens,* was isolated as a manganese (IV)-reducing bacterium from lake sediment (Myers and Nealson, 1988).

Cultivation Techniques and Media

For cultivation of sulfur-reducing bacteria, the same anoxic media, cultivation vessels, equipment, and techniques may be applied as described in detail for sulfate-reducing bacteria (see Chapter 183). Occasional diffusion of some air into media during their preparation may be less critical than in case of sulfate-reducing bacteria, as long as sulfide is present as reducing agent. Polysulfide and sulfur formed by reaction of sulfide with oxygen may inhibit sulfate reducers, but are readily reduced by sulfur reducers. Spirilloid sulfur reducers may even be grown in nonreduced media; since they are able to grow microaerobically, growth may be started in media from which only the bulk of oxygen has been removed by sparging or flushing with anoxic gas.

For growth on H_2, sulfur-reducing eubacteria require CO_2 in addition to acetate (1 to 2 mM) as carbon source for cell synthesis. For growth of complete oxidizers on acetate or ethanol as electron donors, CO_2 should also be present,

even though it is formed during growth. Media are therefore prepared with $NaHCO_3$. If H_2 or another gas is used in the head space, this should contain 5 to 10% CO_2, depending on the required pH (see Chapter 183). With C_4-dicarboxylic acids or lactate as substrates, bicarbonate and CO_2 may be omitted.

Concentrations of NaCl and $MgCl_2$ should be varied, depending on whether the inoculum is of freshwater or marine origin.

Desulfuromonas, Desulfurella, thiophilic sulfate reducers, and spirilloid sulfur reducers grow in defined media without complex substrates such as yeast extract. *Desulfurella* may be stimulated by low concentrations of yeast extract (0.1 g/liter Schmitz et al., 1990). *Pseudomonas mendonica* exhibited significant reduction of sulfur with H_2 only in the presence of yeast extract (0.2 g/liter Balashova, 1985). For *Alteromonas putrefaciens,* a requirement for arginine, glutamine, and serine was reported (Loveley et al., 1989).

For preparation of media, NaCl, $MgCl_2$, $CaCl_2$, NH_4Cl, KH_2PO_4, and KCl are dissolved and autoclaved at concentrations indicated in Chapter 183 (see "Defined Multipurpose Medium"). Sulfate is omitted. Trace elements, $NaHCO_3$, vitamins, and Na_2S are added from separately sterilized stock solutions to the cold medium as described. Trace elements chelated with EDTA (ethylenediaminetetraacetic acid, used as the sodium salt) have been used most frequently for cultivation of sulfur reducers. Of the vitamins, isolated strains of sulfur reducers require, if at all, only biotin and B_{12}. In addition to the medium given in Chapter 183, two modifications have been used (Pfennig and Biebl, 1976, 1981). In the modified media, the concentration of KH_2PO_4 was 1 g/liter the concentration of $NaHCO_3$ was 2 or 4 g/liter for freshwater or marine species, respectively (corresponding to 24 and 48 ml, respectively, of the given 1 M $NaHCO_3$ solution per liter). Growth of completely oxidizing sulfur reducers with sulfur and acetate, ethanol, or other organic compounds causes a significant decrease of the pH. Media for growth on these substrates should therefore be adjusted to a pH between 7.6 and 7.8. Medium for the moderately thermophilic *Desulfurella,* which has a rather low pH optimum (6.8 to 7.0), is adjusted to a pH between 7.2 and 7.4. For growth of sulfate reducers on formate as electron donor when sulfur, fumarate, or malate is used as the electron acceptor, or for growth in cocultures with green sulfur bacteria, the pH is adjusted between 7.0 and 7.2. The medium is dispensed into 20-ml tubes or into 50- or 100-ml bottles, which are completely filled and sealed with screw caps fitted with soft rubber discs. Alternatively, culture vessels are filled so as to leave a head space which is gassed with an N_2/CO_2 mixture and sealed with rubber stoppers according to the Hungate technique (see Chapter 183, Fig. 5).

Depending on the type of sulfur reducer to be enriched and cultivated, organic substrates are added individually to tubes or bottles from the following, separately autoclaved stock solutions.

Medium Supplements for Sulfur Reducers

1. Stock solutions of acetate, ethanol, lactate, or other substrates of interest are prepared as described in Chapter 183 ("Defined Multipurpose Medium").
2. Formate solution, 2.0 M:
   13.6 g HCOONa are dissolved in distilled water to a final volume of 100 ml and autoclaved in a closed bottle with head space. Application: 10 ml per liter of medium.
3. Succinate, fumarate, or malate solution, 1.0 M:
   Sodium salts of the dicarboxylic acids are prepared from the free acids which are usually of higher purity than the commercial salts.

Distilled water	20 ml
Succinic acid (or fumaric acid, 11.6 g; or malic acid, 13.4 g)	11.8 g
NaOH (4.0 M)	48 ml

   The NaOH solution is added slowly under stirring. The pH is then adjusted to between 7 and 8 by dropwise addition of 1 M NaOH.
   Distilled water is added to a final volume of 100 ml. The solution is autoclaved in a closed bottle with head space.
   Application: 10 to 20 ml per liter of medium.

The electron acceptor, sulfur, is either added as a sulfur slurry or as polysulfide from the separately autoclaved stocks described in the following.

4. Sulfur slurry:
   Only highly pure (e.g., sublimized) flowers of sulfur should be used. This hydrophobic sulfur, which cannot readily be suspended in water, should be moistened by thoroughly grinding in a mortar with distilled water to give a slurry.
   Alternatively, flowers of sulfur can be moistened by stirring with water in a bottle that is repeatedly evacuated and reflushed with air or N_2. The resulting slurry is distributed in approximately 30- or 60-ml amounts into 50- or 100-ml screw-capped bottles, respectively, and excess water is decanted. Sulfur melts at 119.6°C, yielding an unusable compact clump, so the slurry should be autoclaved for 30 min at only 112 to 115°C.
   Application: Samples are taken with a flamed, cooled spatula and added individually to each culture tube or bottle. A pea-sized amount of sulfur in a very small volume of water is added per 50-ml bottle. A sterile glass bead should be also added to the culture bottle. If the culture is then incubated on a shaker, the bead keeps the sulfur partially in suspension.
5. Polysulfide solution:
   24 g $Na_2S{\cdot}9H_2O$ and 7.2 g flowers of sulfur are added

to water to yield a final volume of 100 ml. The mixture and a magnetic stirring bar are kept in a bottle under N_2 gas, with a tight rubber seal (see Chapter 183) to avoid autoxidation. The suspension is heated in a boiling water bath and stirred until the sulfur has dissolved (yielding an intensely yellowish-brown solution). In this way, a polysulfide solution with the average composition of $Na_2S_{3.25}$ at a concentration of 1 M is obtained. The solution is autoclaved under N_2 in the closed bottle.
Application: 6 ml per liter of medium.
Adding polysulfide increases the pH of the medium. If this is critical, medium with a relatively low pH (7.0 to 7.2) may be used. Alternatively, the pH is readjusted by addition of about 4 ml of sterile 2 M HCl or 1 M H_2SO_4 per liter of medium (see Chapter 183, "Defined Multipurpose Medium").

Enrichment, Isolation, and Maintenance

Selective Enrichment

Sulfur-reducing eubacteria can be selectively enriched in sulfate-free batch cultures with sulfur and a particular electron donor. Other important selective factors may be the salt concentration and the incubation temperature.

Samples from sediments or other types of sludge are kept under anoxic conditions (see Chapter 183). If not used immediately, samples should be refrigerated but not frozen. The size of the inoculum should be 1 to 2% of the culture volume.

There are essentially two methods for selective enrichment of sulfur-reducing bacteria:

1. The first and most direct method is the use of a sulfate-free, sulfide-reduced mineral medium with the electron donor of choice and an excess amount of moistened flowers of sulfur. An unequivocal proof of positive enrichment is H_2S production. This can be detected by testing with acidic $CuSO_4$ solution as described in Chapter 183 ("Selective Enrichment").
2. For the second method, the same culture medium and electron donor is used, but no sulfur is added. Instead, the medium is inoculated with the sediment sample and a pure culture of a green sulfur bacterium, e.g., *Prothecochloris aestuarii,* and incubated in dim light. Under these conditions, the *Prosthecochloris* forms elemental sulfur from sulfide; the sulfur is used directly by the sulfur reducers to be enriched. The successful enrichment culture is readily recognized by the abundant growth of the green sulfur bacterium as compared with a parallel culture that has not received the sediment sample as inoculum.

Enrichments should be subcultured two to five times before isolation is attempted. For inoculation, 1 to 2% of the culture volume is transferred from the previous positive enrichment.

ENRICHMENT OF *DESULFUROMONAS.* A generally suitable electron donor for enrichment of *Desulfuromonas* with sulfur is acetate, added at a concentration of 5 to 10 mM. Alternatively, 5 to 10 mM ethanol may be used. Enrichment of *Desulfuromonas succinoxidans* may be attempted with 5 to 10 mM succinate as sole electron donor. The concentration of NaCl and $MgCl_2 \cdot 6H_2O$ in the enrichment medium for freshwater types is 1 and 0.4 g, respectively, and for marine types 20 and 3.0 g, respectively. Intermediate salt concentrations have not been used so far. The incubation temperature is 30°C.

ENRICHMENT OF *DESULFURELLA. Desulfurella* is enriched on sulfur and acetate in freshwater medium at a temperature of 55°C. Thermophilic lithotrophic green sulfur bacteria that obligately depend on H_2S are not known and can therefore not be used to establish cocultures at this temperature.

ENRICHMENT OF SULFATE-REDUCING BACTERIA UTILIZING ELEMENTAL SULFUR. In addition to non-sulfate-reducing sulfur reducers, thiophilic sulfate reducers may appear in enrichments with lactate or ethanol and sulfur. The concentration of the organic substrate should be about 10 mM. Cultures are incubated at 28 to 35°C.

ENRICHMENT OF SPIRILLOID SULFUR-REDUCING BACTERIA. Successful enrichment of the spirilloid, facultatively microaerobic, sulfur-reducing bacteria is achieved with sulfur in the presence of 20 mM formate or under an atmosphere of H_2/CO_2 (90/10, vol/vol); acetate at a concentration of 1 to 2 mM has to be added as a carbon source. The incubation temperature may be around 30°C. Freshwater medium has been used most frequently. Tiny, highly motile vibrioid-to-spirilloid cells of the desired sulfur reducers usually appear within a few days. Enrichments may also be attempted with 10 mM dimethylsulfoxide (DMSO) as electron acceptor and formate, H_2/CO_2, or 10 mM lactate as electron donors (Zinder and Brock, 1978); with the former two electron donors, 1 to 2 mM acetate has to be added as the carbon source. Spirilloid sulfur reducers have also been enriched in a che-

mostat with aspartate as sole, growth-limiting carbon and energy source (Laanbroek et al., 1977).

Isolation

A simple, ideally suited method for the isolation of sulfur-reducing bacteria from enrichments is a serial dilution in agar deeps as described for sulfate-reducing bacteria (see Chapter 183). The agar dilutions with sulfide-reduced media may be carried out in open tubes. The brief contact with air does not affect the sulfur-reducing bacteria. Since flowers of sulfur are too coarse and inhomogeneous for agar dilutions, 0.15 ml polysulfide solution (see above) is added to a 50-ml bottle containing medium with the same electron donor as has been used for enrichment. Before the gradually precipitating sulfur forms flocs, the medium is distributed in 6-ml portions to tubes with the 3-ml portions of melted agar, as described in Chapter 183. Dithionite is not added. After serial dilution of an inoculum from the enrichment and gelling of the agar, tubes are anoxically sealed under N_2/CO_2 and incubated at the same temperature as the enrichment culture.

In case of obligate sulfur-reducing bacteria such as *Desulfuromonas thiophila* or *Desulfurella acetivorans,* elemental sulfur cannot be replaced by soluble electron acceptors. Isolation of facultative sulfur-reducing bacteria, however, may be attempted alternatively in agar dilution series with 10 to 20 mM fumarate or malate. Preferably, both electron acceptors are used in a parallel series. Acetate or ethanol is added as the electron donor in the case of *Desulfuromonas* species, and formate in the case of the spirilloid sulfur reducers. In this way, the turbidity caused by elemental sulfur is avoided, and the recognition of colonies at high dilution is facilitated.

Isolation of thiophilic sulfate reducers should be carried out in an agar dilution series with 10 to 20 mM sulfate instead of sulfur, while the electron donors are the same as in the enrichment. Such a counter-selection eliminates non-sulfate-reducing bacteria enriched together with the sulfate-reducing bacteria. After isolation, the pure cultures are tested for their capacity to use elemental sulfur in the sulfate-free culture medium.

In dilution series with sulfur precipitated from polysulfide, positive development is indicated by the disappearance of turbidity. At higher dilution, sulfur may only disappear around the colonies. Sulfur-grown colonies are usually rather small. Agar tubes are inspected under a dissecting microscope. In a dilution series with a soluble electron acceptor, colonies are bigger and easier to recognize. *Desulfuromonas* colonies are pink or intensely yellowish-brown (Pfennig and Biebl, 1976). *Desulfurella* does not possess cytochromes and forms whitish colonies (Bonch-Osmolovskaya et al., 1990; Schmitz et al., 1990). Colonies of sulfate reducers are brownish. Spirilloid sulfur reducers form yellowish-to-ochre colonies. With malate or fumarate as electron acceptor, these colonies appear chalky since they are interspersed with and surrounded by tiny whitish crystals of sulfur; this is produced by the oxidation of sulfide added as reductant, with fumarate or malate acting as electron acceptor (Macy et al., 1986; Wolfe and Pfennig, 1977).

Maintenance

For short-term preservation, stock cultures are kept at 2 to 6°C. Since grown cultures easily die off or lyse if further incubated at optimal growth temperature, strains should be refrigerated during or right after the end of active growth.

The interval of transfer depends on the tendency of stored cells to undergo lysis, which may be indicated by the presence of slimy layers or threads and a decrease in turbidity. For most species, a transfer every 6 to 10 weeks is recommended.

For long-term preservation, cultures containing 5 to 10% (v/v) DMSO are kept in liquid N_2.

Taxonomy and Identification

Relationships of a number of sulfur-reducing eubacteria among themselves and to other bacteria have been examined by oligonucleotide cataloging and by sequencing of 16S rRNA. As far as they have been analyzed, the phylogenetic groups coincide with those originally based on nutritional and morphological characteristics. Sulfur reducers assigned to the genus *Desulfuromonas,* mainly because of their ability to oxidize acetate, are also phylogenetically closely related to each other (Fig. 2; Fowler et al., 1986). However, there is the same degree of relatedness to fermentative, non-sulfur-reducing bacteria assigned to the genus *Pelobacter* (Stackebrandt et al., 1989). This interweaving of physiologically rather different groups at the genus and species level is difficult to interprete in terms of a biochemical evolution. Nevertheless, the genus designations *Desulfuromonas* and *Pelobacter* should be kept since they are in agreement with the physiological properties. The next relatives of this group are the completely oxidizing sulfate-reducing bacteria, *Desulfonema limi-*

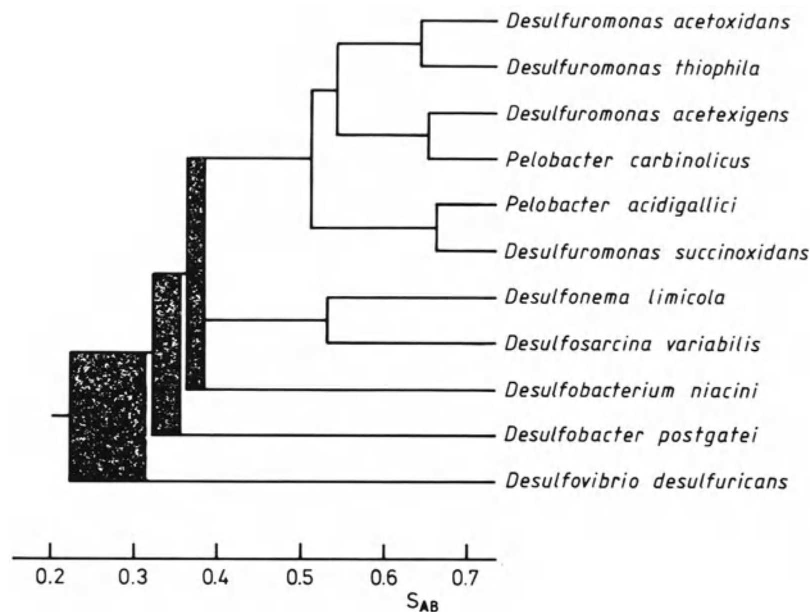

Fig. 2. Relationships of *Desulfuromonas* species among themselves and to other bacteria as revealed by 16S rRNA oligonucleotide cataloging. *D. acetexigens* and *D. thiophila* are tentative names. (Based on Fowler et al., 1986; Stackebrandt et al., 1989, and E. Stackebrandt, personal communication.)

cola, Desulfosarcina variabilis, and *Desulfobacterium niacini.* Desulfuromonas, Pelobacter, and the mesophilic sulfate-reducing bacteria together with *Myxococcus* and *Bdellovibrio* form the delta subdivision.

Nothing is known about the phylogenetic position of *Desulfurella acetivorans.* Its rather low GC content of 31.4 mol% distinguishes it from that of *Desulfuromonas* species (44 to 63 mol%).

Thiophilic sulfate reducers belong to the *Desulfovibrio* and *Desulfomicrobium* branch (tentatively comprised as Desulfovibrionaceae; see Chapter 183).

Spirilloid sulfur-reducing bacteria—originally isolated with formate and sulfur (Wolfe and Pfennig, 1977), aspartate (Laanbroek et al., 1977, 1978), or DMSO and lactate (Zinder and Brock, 1978)—and *Wolinella succinogenes* (Macy et al., 1986; Myers and Nealson, 1988) are physiologically very similar. Besides the latter, only the strain obtained with aspartate had been tentatively classified as a "free-living *Campylobacter* species." A genealogical relationship of this and the unnamed spirilloid isolates to authenticated *Campylobacter* species has not been demonstrated thus far. However, sequencing of 16S rRNA from *Wolinella* revealed that this species is closely related to the pathogenic *Campylobacter pylori,* which has a somewhat exceptional position in the genus. On a deeper level, a specific relationship exists to other pathogenic *Campylobacter* species (Fig. 3; Lau et al., 1987; Romaniuk et al., 1987). It is unknown whether any of the pathogenic *Campylobacter* species can grow by dissimilatory sulfur reduction, but it appears likely that the *Campylobacter* branch not only includes *Wolinella* but also some other hitherto-unnamed spirilloid sulfur reducers. The *Campylobacter* branch, with its presently known species, is related to the purple bacteria, but does not show any specific relationship to a particular member of the alpha, beta, gamma, or delta subdivisions (Lau et al., 1987; Romaniuk et al., 1987). *Campylobacter* and the spirilloid sulfur reducers probably form a fifth subdivision besides the ones mentioned (K. H. Schleifer, personal communication). The sulfur reducers await proper naming and classification.

Identification of sulfur-reducing bacteria by physiological characteristics, given in the following and in Table 1, is rather straightforward. Morphological features are shown in Fig. 4 and 5.

Dissimilatory Sulfur-Reducing Eubacteria (Physiological Group)

To be considered as a sulfur-reducing bacterium, an isolate must be able to grow with sulfur and produce sulfide over an indefinite number of subcultures. An unequivocal proof of a respiratory sulfur reduction is growth with H_2 or formate as sole electron donor, since neither of

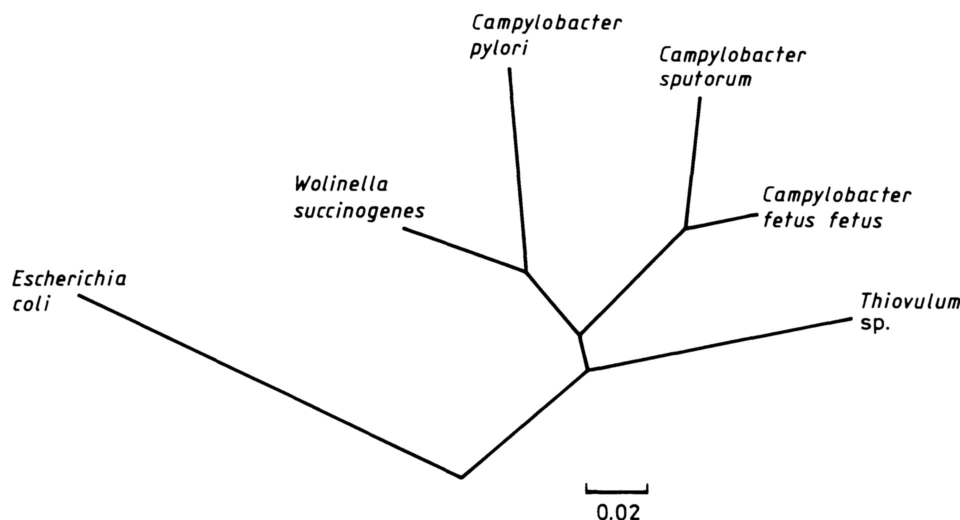

Fig. 3. Relationships of the sulfur-reducing bacterium *Wolinella succinogenes* to the pathogenic *Campylobacter* species as revealed by 16S rRNA sequencing. The scale bar represents the evolutionary distance measured as fixed nucleotide changes per sequence position. (Based on Romaniuk et al., 1987.)

these energy sources allows substrate level phosphorylation (see Chapter 24). Growth on H_2 or formate may require the addition of 1 to 2 mM acetate as carbon source for cell synthesis. According to present biochemical knowledge, oxidation of acetate with sulfur is also only possible in a respiratory metabolism (see Chapter 24). Sulfur reduction with sugars or lactate is not necessarily a respiration but may be only a facilitated fermentation or by-reaction.

DESCRIPTION OF *DESULFUROMONAS*. Cells are ovals or straight to slightly curved rods (Fig. 4A and B). Motility is common. Most species possess a single lateral (Fig. 5) or subpolar flagellum that cause a propellerlike movement. A few species have a single polar flagellum. Colonies and cell pellets are pink or yellowish-brown to reddish, due to a high content of cytochromes and other pigments (Bache et al., 1983; Pfennig and Biebl, 1976; Probst et al., 1977). *Desulfuromonas* species are obligately anaerobic, mesophilic bacteria. Acetate is the common electron donor and carbon source. Oxidation occurs via the citric acid cycle (see Chapter 24). Some species use, in addition, ethanol, 1-propanol, pyruvate, succinate, or some other simple organic acids. H_2 or formate are not oxidized. In the absence of sulfur, several species can grow with acetate as electron donor and malate or fumarate as electron acceptor, which are converted to succinate. After a certain adaptation period, especially in media with low bicarbonate and CO_2 concentrations, growth may occur on malate or fumarate alone. A few species obligately depend on elemental sulfur as electron acceptor. Sulfate, sulfite, or thiosulfate are never reduced. Nitrate is reduced in a few instances, nitrite being an intermediate and ammonia the end product. Growth with sulfur is usually rather rapid: *Desulfuromonas acetoxidans* has a doubling time of about 2.5 h on acetate and sulfur, while the doubling time on acetate and malate is around 7 h (Pfennig and Biebl, 1976, 1981).

DESCRIPTION OF *DESULFURELLA*. Cells are short rods that are motile by a single polar flagellum. Colonies and cell pellets are whitish, due to the absence of cytochromes (Bonch-Osmolovskaya et al., 1990; Schmitz et al., 1990). *Desulfurella* is an obligately anaerobic, moderately thermophilic bacterium. The optimum temperature for growth is 52 to 57°C. Growth occurs only on acetate and sulfur, the former being oxidized via the citric acid cycle. Growth of *Desulfurella acetivorans* can be rather rapid, with a doubling time of about 2 h (Bonch-Osmolovskaya et al., 1990).

DESCRIPTION OF SULFATE-REDUCING BACTERIA THAT REDUCE SULFUR. Cells are rod-shaped or vibrioid and are motile by polar flagella. Colonies or cell pellets are brownish. Growth occurs on lactate and in many cases also on ethanol in the presence of elemental sulfur. The capacity for indefinite growth on H_2 and sulfur has not been demonstrated, but is very likely, since H_2 is an excellent electron donor for growth on sulfate. Organic substrates are incompletely oxidized to acetate. Rod-shaped types usually do not contain desulfoviridin, but desulforubidin. They are considered as members of the genus

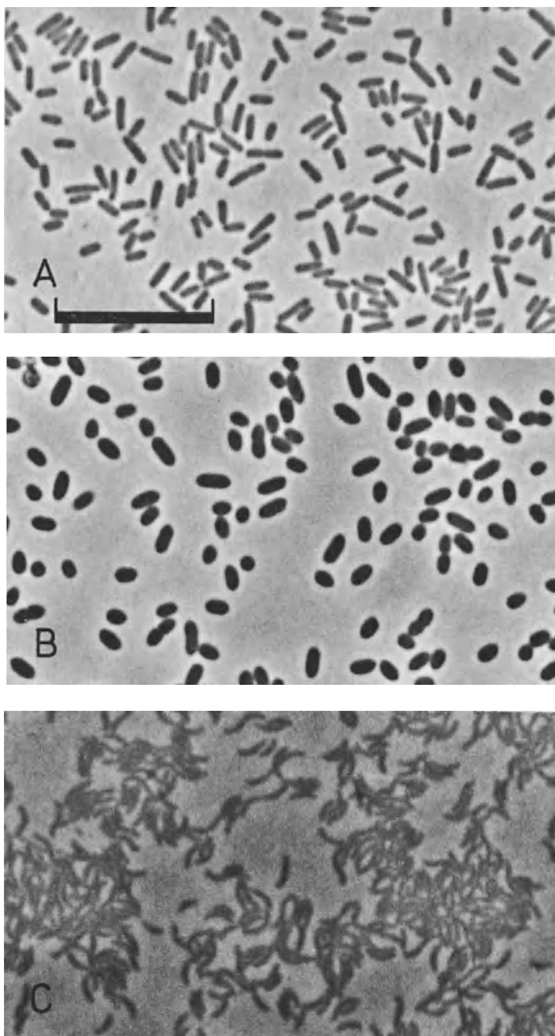

Fig. 4. Phase contrast micrographs of viable cells of sulfur-reducing bacteria. Bar = 10 μm. (A) *Desulfuromonas acetoxidans*. (B) *Desulfuromonas thiophila* (tentative name). (C) Spirilloid, facultatively microaerobic sulfur-reducing bacterium strain "spirillum 5175" (Wolfe and Pfennig, 1977).

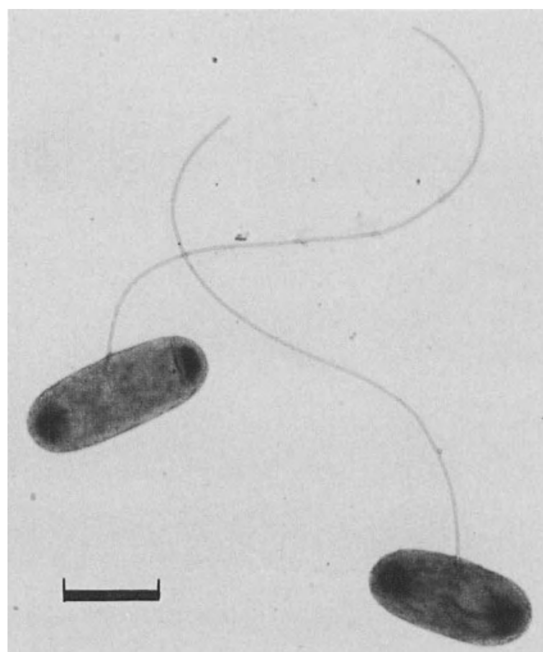

Fig. 5. Electron micrograph of negatively stained cells of *Desulfuromonas acetoxidans*, showing lateral flagella. Bar = 0.1 μm. (Courtesy of F. Mayer.)

Desulfomicrobium. Vibrioid types contain desulfoviridin and belong to the genus *Desulfovibrio*. Further descriptions are given in Chapter 183.

DESCRIPTION OF SPIRILLOID SULFUR REDUCERS INCLUDING *WOLINELLA*. Cells are vibrioid to spirilloid (Fig. 4C) and highly motile by a single polar flagellum. Colonies are yellowish to ochre. Growth occurs anaerobically or microaerobically (O_2 partial pressure, 1 to 5 kPa). The optimum temperature is between 30 and 37°C, depending on the type of isolate. Anaerobic growth occurs by oxidation of H_2 or formate with sulfur in the presence of 1 to 2 mM acetate as carbon source. Further electron acceptors are listed in Table 1. Dimethylsulfoxide is reduced to dimethylsulfide, nitrate is reduced to ammonia with nitrite as an intermediate. Reduction by *Wolinella* of N_2O to N_2 has been reported (Yoshinari, 1980). In the absence of an electron donor, spirilloid sulfur reducers, with the exception of *Wolinella*, are able to grow by fermentation of malate or fumarate; some species may ferment aspartate (Laanbroek et al., 1977).

OTHER SULFUR-REDUCING EUBACTERIA. A *Pseudomonas mendonica* subspecies and strains of *Alteromonas putrefaciens* were originally enriched as ferric iron- or manganese (IV)-reducing bacteria, respectively. Such types have never been enriched directly with sulfur as electron acceptor, probably because they utilize this electron acceptor more slowly and less effectively than the aforementioned sulfur reducers. For further descriptive information, the original literature should be consulted (Balashova, 1985; Loveley et al., 1989; Myers and Nealson, 1988).

Acknowledgments

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The Genus *Thermodesulfobacterium*

FRIEDRICH WIDDEL

The genus *Thermodesulfobacterium* was established for a thermophilic sulfate-reducing eubacterium that grew optimally at 70°C (Zeikus et al., 1983) and possessed unusual nonisoprenoid ether-linked lipids (Langworthy et al., 1983). Later a sulfate reducer originally described as *Desulfovibrio thermophilus* (Rozanova and Khudyakova, 1974) was transferred to the same genus as *Thermodesulfobacterium mobile* (Rozanova and Pivovarova, 1988). The two *Thermodesulfobacterium* species are nutritionally relatively restricted, incomplete oxidizers using H_2, formate, lactate, or pyruvate as electron donors for sulfate reduction to H_2S. The morphology of *T. mobile* is shown in Fig. 1.

Preliminary sequencing of 16S rRNA of *T. commune* and *T. mobile* indicated that both species are related to each other and branch near the root of the eubacterial tree (C. R. Woese, personal communication; Chapter 24, Fig. 1). The genus is therefore phylogenetically entirely separate from other eubacteria and hence also from the delta group of Gram-negative sulfate reducers or from the genus *Desulfotomaculum.*

Habitats

T. commune was isolated from sediment of a hot spring (70°C) in Yellowstone National Park, USA. Morphologically identical thermophilic sulfate reducers were enriched from other hot pools or springs of this area (Zeikus et al., 1983). *T. mobile* (Rozanova and Khudyakova, 1974) and a similar isolate that could only use H_2 (Cord-Ruwisch et al., 1986) were isolated from warm-oil-field water.

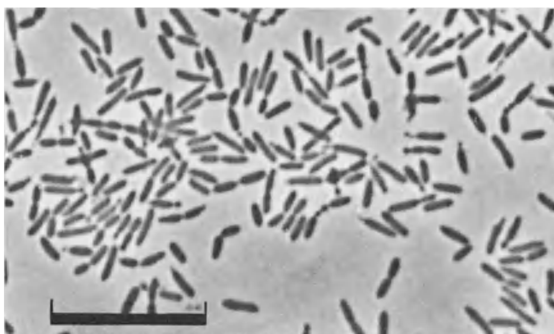

Fig. 1. Phase contrast photomicrograph of viable cells of *Thermodesulfobacterium mobile* (formerly *Desulfovibrio thermophilus*). Bar = 10 μm.

In their natural habitat, *Thermodesulfobacterium* species probably grow with H_2 derived from thermophilic fermentation processes or from geothermal reactions. Habitats of *Thermodesulfobacterium* probably also contain some acetate, since this organic compound is required for cell synthesis during growth on H_2 (Rozanova and Pivovarova, 1988; F. Widdel, unpublished observations).

Even though *Thermodesulfobacterium* strains were isolated from saline oil-field water, they do not appear to have a pronounced salt requirement or a significant salt tolerance.

Cultivation Techniques and Media

Thermodesulfobacterium species are grown under anoxic, reducing conditions in the lactate medium or the defined multipurpose medium described in Chapter 183; the latter is provided with lactate. Instead of lactate, pyruvate may be used for both media, the latter yielding less inhibitory H_2S than lactate and therefore allowing higher final cell densities. A pyruvate stock solution is prepared and applied as described in Chapter 80. For growth on H_2, the multipurpose medium is supplemented with 2 mM sodium acetate as carbon source for cell synthesis, and the culture is incubated under a head space of H_2/CO_2 (80/20, v/v).

The trace elements used (see Chapter 183, "Defined Multipurpose Medium") were either unchelated according to solution 1, with a threefold-increased $FeSO_4$ content (F. Widdel, unpublished observations), or they were chelated with NTA (nitrilotriacetic acid, neutralized), according to solution 3 (Zeikus et al., 1983). Growth occurs in a defined medium; however,

yeast extract (0.5 to 1 g/liter) is stimulatory, especially for *T. mobile.*

Enrichment, Isolation, and Maintenance

Selective Enrichment

For enrichment of *Thermodesulfobacterium* species, media with lactate are inoculated with sediment or mud samples from hot springs or oil production plants and incubated at 60 to 70°C. Parallel enrichments are carried out under a head space of H_2/CO_2 in the presence of 2 mM acetate; for this, the bicarbonate-buffered multipurpose medium should be used. Enrichments under H_2/CO_2 are carried out in tubes that are horizontally incubated, or in bottles that are gently shaken. Successful enrichment is recognized by sulfide formation (for test, see Chapter 183), the appearance of relatively small rod-shaped cells and, in the case of H_2 as electron donor, by the underpressure which develops. The underpressure is detected by piercing the stopper with a glass syringe lubricated with water and filled with H_2/CO_2 and observing retraction of the syringe barrel. Before isolation is attempted, two to five subcultures should be made.

Isolation

Thermodesulfobacterium species are isolated by the dilution techniques described in Chapter 183. Media with agar at the concentration used for mesophiles may be too soft at the relatively high incubation temperature, and the agar concentration should therefore be increased to 2% (this can be achieved by adding 3 ml of medium to tubes with 6 ml of 3% melted agar). The concentration of salts, reductants, and organic substrates in the medium to be added are increased by a factor of 3 to account for the dilution by the melted agar. Also, yeast extract (final concentration, 0.5 to 1 g/liter) is added to promote development of colonies. The dilution is carried out with tubes placed in a water bath at about 50°C. Before incubation at 60 to 70°C, the anaerobically sealed tubes are incubated for 30 min in a cold water bath to guarantee complete gelling of the agar.

Isolation is also possible via serial dilutions in liquid medium (see Chapter 183).

Maintenance

For short-term preservation, stock cultures are refrigerated or kept at room temperature. Since fully grown cultures easily die off or lyse if further incubated at 60 to 70°C, they should be cooled near to or at the end of active growth.

For most species, transfer every 3 to 5 weeks is recommended.

For long-term preservation, cultures with an addition of 5 to 10% (v/v) dimethylsulfoxide (DMSO) are kept in liquid N_2.

Taxonomy and Identification

Detailed studies on genealogical relationships among *Thermodesulfobacterium* species and of these species to other sulfate-reducing bacteria are lacking. Sequencing of 16S rRNA via reverse transcriptase could not be completed, probably due to modified bases, but the partial sequences obtained indicated a deep branching near the eubacterial tree, somewhere next to the branching point of *Thermotoga* (C.R. Woese, personal communication). Apart from these investigations, DNA-DNA hybridization studies were carried out with *T. mobile* and other sulfate reducers (Nazina et al., 1987). As might be expected, there was no hybridization with DNA from *Desulfovibrio* or *Desulfomicrobium.*

Thermodesulfobacterium species only resemble *Desulfovibrio* species nutritionally.

Thermodesulfobacterium species are identified by their high temperature optimum (65 to 70°C), rod-shaped cells that do not form spores, and the utilization of H_2, formate, lactate, or pyruvate as electron donors for sulfate reduction. However, some strains may use only H_2. Utilization of alcohols or fatty acids, as in case of thermophilic *Desulfotomaculum* species, has not been observed.

Thermodesulfobacterium species do not contain desulfoviridin. A new bisulfite reductase has been detected in *T. commune* (Hatchikian and Zeikus, 1983) and *T. mobile* (Fauque et al., 1986); the enzyme is named desulfofuscidin. Furthermore, cytochrome c_3 has been identified in this genus (Fauque et al., 1991; Hatchikian et al., 1984). The major menaquinone is MK-7 (Collins and Widdel, 1986).

A special biochemical feature of *Thermodesulfobacterium* species is the presence of ether-linked lipids, mainly phospholipids (Fig. 2). These were found in *T. commune* (Langworthy et al., 1983) and *T. mobile* (T.A. Langworthy, personal communication). However, in contrast to the isoprenoid lipids of archaebacteria, lipids of *Thermodesulfobacterium* contain alkyl chains with one subterminal methyl branch (anteiso branching). The lipid fraction also contains a certain portion of fatty acids and hydrocarbons (for details, see Langworthy et al., 1983).

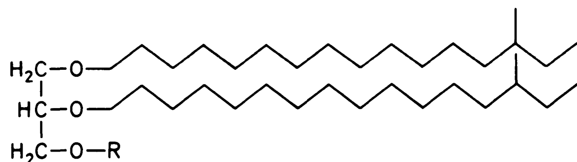

Fig. 2. Structure of the most frequently occurring lipophilic moiety (1,2-di-O-anteisoheptadecyl-*sn*-glycerol) in lipids of *Thermodesulfobacterium commune* (Langworthy et al., 1983). R, hydrophilic residue.

Thermodesulfobacterium commune, the type strain, has nonmotile cells, which are 0.3 μm in diameter and 0.9 μm in length. The optimum temperature is 70°C, the maximum 82°C. The GC content of the DNA is 34 mol%.

Thermodesulfobacterium mobile has motile cells with polar flagella. The cell diameter is 0.3 μm, the length 0.9 μm. The optimum temperature is 65°C, the maximum 82°C. The GC content of the DNA is 38 mol%.

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CHAPTER 186

The Genus *Pelobacter*

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The genus *Pelobacter* was proposed as a taxonomic entity consisting of strictly anaerobic, Gram-negative, nonsporeforming, rod-shaped bacteria that use only a very limited number of substrates. The members of the genus are all unable to ferment sugars and therefore cannot be grouped with any other genus in the family Bacteroidaceae (Krieg and Holt, 1984). The genus comprises five different species, *P. acidigallici* (Schink and Pfennig, 1982), *P. venetianus* (Schink and Stieb, 1983), *P. carbinolicus* (Schink, 1984), *P. propionicus* (Schink, 1984), and *P. acetylenicus* (Schink, 1985), which all are based on 3–5 described strains.

Comparisons of the various *Pelobacter* species by DNA-DNA hybridization experiments revealed that the genus is rather inhomogenous; therefore, a reorganization may perhaps be necessary in the future (J. P. Touzel and B. Schink, unpublished observations). Whereas the species *P. venetianus, P. carbinolicus,* and *P. acetylenicus* form a homogenous cluster, *P. acidigallici* and *P. propionicus* appear to be only distantly related to the others. These findings are consistent to some extent with the fermentation patterns of these species (see below). Comparison of three *Pelobacter* species with other anaerobes on the basis of 16S rRNA structure analysis supports this view. Whereas *P. venetianus* and *P. carbinolicus* exhibit a rather high similarity, with an S_{AB} of 0.70, *P. acidigallici* is related to both at an S_{AB} of only 0.53 (Stackebrandt et al., 1989). It is interesting to note that these three Pelobacter strains did not show any resemblance to other fermenting Gram-negative strict anaerobes; instead, they appeared to be highly related to several strains of sulfur-reducing anaerobes, namely, *Desulfuromonas succinoxidans, D. acetexigens,* and *D. acetoxidans,* to which they are even more closely related than *P. acidigallici* is to the other two *Pelobacter* species. Since *Pelobacter* species and the obligately sulfur-respiring bacteria are quite diverse metabolically and the latter are supposed to have derived directly from phototrophic ancestors, it has been suggested that the genus *Pelobacter* represents a group of fermenting bacteria that developed a fermentative metabolism as a "secondary" evolutionary event, and that they are separate from the first fermentative bacteria (Stackebrandt et al., 1989).

Habitat

All *Pelobacter* strains have been isolated so far from marine or freshwater sediments. The name *Pelobacter* was based on this origin (Greek *pelos* meaning mud, sediment). Enrichments from sewage sludges led to similar isolates as well. Viable counts using the characteristic substrates gallic acid, acetoin, polyethylene glycol, and acetylene showed that there were approximately 100 cells/ml of each of the *Pelobacter* species in sediment and up to 2,500 cells/ml in sewage sludge. Since their substrate ranges are comparably small, their ecological niche in such sediments can be understood rather well in most cases. *P. acidigallici* is restricted to the utilization of trihydroxybenzenoids, which are probably their only energy source in their natural habitat. *P. venetianus, P. carbinolicus, P. propionicus,* and *P. acetylenicus* were enriched and isolated with polyethylene glycol, 2,3-butanediol, and acetylene, respectively, but the ecological importance of these substrates in the respective environments is questionable. Since all these species can also ferment ethanol, either in syntrophic cooperation with hydrogen scavengers or in pure culture, it appears more probable that degradation of this important fermentation intermediate is their predominant function in these environments. *P. carbinolicus* has been identified as the dominant ethanol-degrading bacterium in digesting industrial sewage sludge (Dubourguier et al., 1986), and high numbers (10^6 - 10^7 cells/ml) of syntrophically ethanol-oxidizing anaerobes were detected also in other sewage sludges (Schink et al., 1985) and in freshwater creek

sediments (Eichler and Schink, 1985). *Pelobacter* has to compete for ethanol with certain homoacetogenic bacteria, e.g., *Clostridium aceticum* (Wieringa, 1940) or *Acetobacterium carbinolicum* (Eichler and Schink, 1984), which appear to be at least as successful in freshwater sediments (Schink et al., 1985), especially if the sediment is slightly acidic (Schink et al., 1985) or the temperature is low (Conrad et al., 1989). The ecological importance of ethanol fermentation to propionate by *P. propionicus* has been elucidated by enumerations and by tracer experiments (Schink et al., 1985). These studies revealed that up to 20% of the total ethanol turnover can go through propionate, and that bacteria forming propionate from ethanol contribute significantly to the total ethanol-metabolizing microbial community.

It has to be concluded that bacteria of the metabolic types represented by the various *Pelobacter* species make up a significant part of the anaerobic microbial population in sediments and sewage sludge. No *Pelobacter*-like bacteria have so far been isolated from the rumen. The numerically predominant, syntrophically ethanol-oxidizing *Pelobacter* species represent new isolates of the metabolic type of the S-strain in the mixed culture *"Methanobacillus omelianskii"* (Bryant et al., 1967). These *Pelobocter* species have become accessible to pure culture growth in our laboratory by the use of unusual substrates that all can be converted easily into acetaldehyde, the key intermediate in the energy metabolism of these bacteria (see next section).

Isolation

Growth Media

All *Pelobacter* strains have been enriched and isolated in a carbonate-buffered, sulfide-reduced mineral medium that contained only one organic energy and carbon source. Since they grow with substrates that yield 2-carbon intermediates exclusively, they have to form pyruvate and sugars via reductive carboxylation of acetyl coenzyme A and need carbon dioxide for this reaction. Use of a bicarbonate-buffered medium is therefore recommended for enrichment, isolation, and maintenance. Three different versions of this medium are described below for the isolation of *Pelobacter* from freshwater, estuarine, and marine sediment, respectively (after Widdel and Pfennig, 1981; Schink and Pfennig, 1982):

Pelobacter Growth Media

Dissolve in 1 liter of distilled water:

	Freshwater	Estuarine	Marine
KH_2PO_4	0.2 g	0.2 g	0.2 g
NH_4Cl	0.5 g	0.5 g	0.5 g
NaCl	1.0 g	8.0 g	20.0 g
$MgCl_2 \cdot 6H_2O$	0.4 g	1.2 g	3.0 g
KCl	0.5 g	0.5 g	0.5 g
$CaCl_2 \cdot 2H_2O$	0.15 g	0.15 g	0.15 g

Autoclave the complete mineral medium in a vessel equipped with 1) a filter inlet to allow flushing of the headspace with sterile oxygen-free gas; 2) screw-cap inlets for addition of thermally unstable additives after autoclaving; 3) a silicon tubing connection from the bottom of the vessel out to a dispensing tap (if possible with a protecting bell) for sterile dispensing of the medium (do not use latex tubing; it releases compounds which are highly toxic to many anaerobes); and 4) a stirring bar.

After autoclaving, connect the vessel with the still-hot medium to a line of oxygen-free nitrogen/carbon dioxide mixture (90%: 10%) at low pressure (< 100 mbar), flush the headspace and cool it under this atmosphere to room temperature, perhaps with the help of a cooling water bath.

The mineral medium is amended with the following additions from stock solutions that have been sterilized separately (amounts/l of medium): a) 30 ml of 1 M $NaHCO_3$ solution (autoclaved in a *tightly closed* screw-cap bottle with about 30% headspace; the bottle should be autoclaved inside another protective vessel, e.g., a polypropylene beaker, to avoid spills of carbonates if the bottle breaks in the autoclave); b) 2 ml of 0.5 M Na_2S9H_2O solution (autoclaved separately under oxygen-free gas atmosphere as above); c) 1 ml of trace element solution, e. g., SL 10 (Widdel et al., 1983); d) 0.5 ml of 10-fold concentrated, filter-sterilized vitamin solution (Pfennig, 1978); and e) adequate amounts of sterile 1 M HCl or 1 M Na_2CO_3 to adjust the pH to 7.1–7.3.

The complete medium is dispensed into either screw-cap bottles or screw-cap tubes which are filled completely to the top, leaving a lentil-sized air bubble for pressure equilibration. Enrichment cultures usually produce gas in the first enrichment stages and are better cultivated in half-filled serum bottles (50–100 ml volume) under a headspace of nitrogen: carbon dioxide mixture (90%:10%).

This mineral medium is amended with the respective organic substrates for enrichment and cultivation of pure cultures. The vitamin mixture is not really needed by all strains.

Selective Enrichment

All enrichment cultures were set up in our laboratory at 27–30°C in 50-ml fluid cultures inoculated with about 5 ml of sediment or sludge. Smaller inocula may also lead to isolation of the same bacteria, but this has not been evaluated in our lab. *P. acidigallici* can be selectively enriched with either one of its growth substrates (see Table 1) at 5–10 mM concentration. For enrichment of *P. venetianus,* either polyethylene glycol mol wt 106–20,000; 0.1% w/v) or meth-

oxyethanol (10 mM; Tanaka and Pfennig, 1988) is recommended. In this medium, 10 mM 2,3-butanediol enriches for *P. carbinolicus* from marine sediments, and for *P. propionicus* from freshwater sediments. *P. acetylenicus* is successfully enriched with mineral medium under the above-mentioned nitrogen: carbon dioxide atmosphere containing 5–10% acetylene in addition.

Isolation

After 3–4 transfers in liquid medium, a homogeneous microbial population should have become established in liquid enrichment cultures. Purification of *Pelobacter* species is most easily done by serial dilution in agar deep cultures ("agar shakes"; Pfennig, 1978). Roll tubes have not been used and are not necessary since these bacteria neither consume nor produce insoluble gaseous compounds. *P. acetylenicus* can be easily purified with 10 mM acetoin as substrate. Other procedures (streaking on agar plates in an anoxic glove box or on agar surfaces in flat agar bottles) have not been tried yet, but there is no reason why such methods would not be successful as well, if the solid media are incubated under a nitrogen/carbon dioxide atmosphere.

Preservation

Liquid cultures were maintained in our laboratory in 50-ml bottles at 4°C for 4–12 weeks between transfers. Longer storage intervals may be possible as well. Long-term preservation is easily accomplished by storage of dense cell suspensions in glass capillaries kept in liquid nitrogen.

Identification

Morphological and Cytological Properties

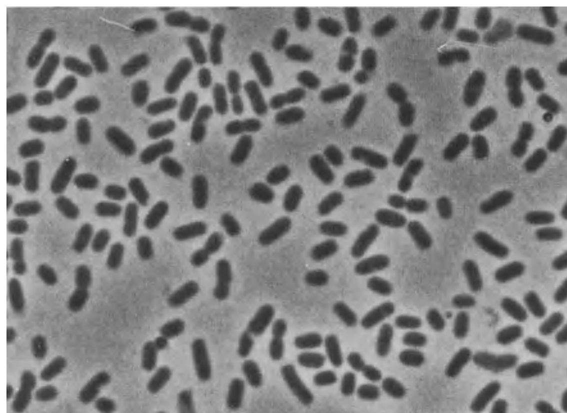
Cells of all Pelobacter species are Gram-negative, short rods that do not form spores (Fig. 1). The cell ends are usually rounded; however, *P. acetylenicus* cells can be slightly pointed (Fig. 1e). The cell sizes vary from 0.5 to 1.0 μm in width and from 1.2 to 6.0 μm in length. More exact cell sizes of the different type strains are listed in Table 1. The temperature optima for growth are in the range of 28–35°C; the pH optima are 6.5–7.5. On the basis of the GC content of the DNA, two clusters can be defined; the one *(P. acidigallici, P. venetianus, P. carbinolicus)* has a GC value at 50–53 mol%, the other one *(P. acetylenicus, P. propionicus)* at 57–58 mol%. It should be noted that this clustering does not agree with the subgroupings based on DNA and RNA homologies (see "Introduction," this chapter) or with physiological similarities.

Cytochromes have been found only in *P. propionicus*. A *b*-type cytochrome was detected at a very low level (Schink et al., 1987), but there is no evidence that this cytochrome plays any role in ATP-generating electron transport (see below).

Physiological Properties and Biochemistry

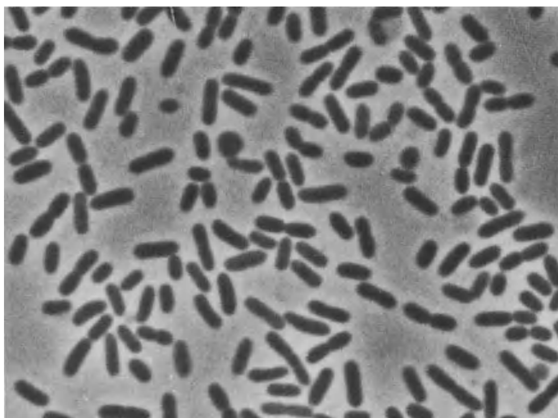
Table 1 presents a listing of all substrates used by the various *Pelobacter* species described so far. It is obvious from this table that *Pelobacter acidigallici* differs in its substrate spectrum from all other *Pelobacter* species. It utilizes only trihydroxybenzenoids for growth, and ferments them to acetate as sole fermentation product. With this, it resembles *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986) to some extent, but does not depend on formate as an external co-substrate for degradation of these compounds. Growth experiments with increased substrate concentrations have revealed that the pathway of gallic acid fermentation leads via pyrogallol and phloroglucinol (Samain et al., 1986), and has nothing in common with the pathway of anaerobic benzoate degradation (Evans, 1977). Obviously, three hydroxyl groups in alternating position at the ring polarize the π-electron system sufficiently to permit selective reduction to dihydrophloroglucinol and ring opening by a thiolytic or hydrolytic mechanism. The isomerization of pyrogallol to phloroglucinol has recently been characterized as a unique transhydroxylase reaction involving a tetrahydroxybenzene as co-substrate (Brune and Schink, 1990). The third trihydroxybenzene isomer, hydroxyhydroquinone, is not metabolized by these bacteria, but it is fermented by other new anaerobic bacteria, also via phloroglucinol (A. Brune, S. Schnell and B. Schink, unpublished observations).

P. venetianus, P. carbinolicus, and *P. acetylenicus* have been enriched and isolated with polyethylene glycol, 2,3-butanediol, or acetylene, respectively. They can all grow with acetoin, some also with ethylene glycol or ethanolamine. All these substrates are converted to acetaldehyde, which is further dismutated to acetate and ethanol as final products. ATP is formed exclusively by substrate level phosphorylation via the acetate kinase reaction. The higher homologs of ethylene glycol, 1,2-propanediol, and 1,2-butanediol, are dismutated to propanol and propionate or butanol and butyrate, respectively, provided that the medium

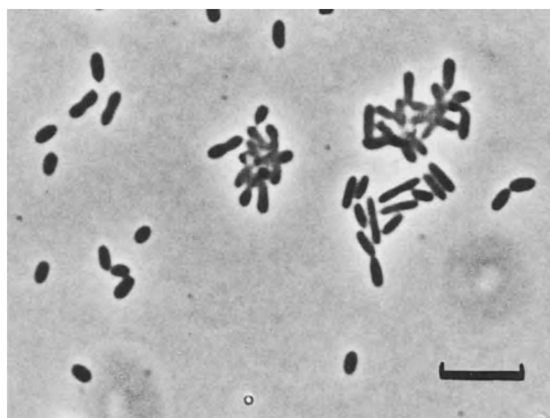


(a)

Fig. 1. Phase contrast photomicrographs of cells of *Pelobacter* species. Bar = 5 μm for all figures. (a) *P. acidigallici;* (b) *P. venetianus;* (c) *P. carbinolicus;* (d) *P. acetylenicus;* (e) *P. propionicus.*



(b)



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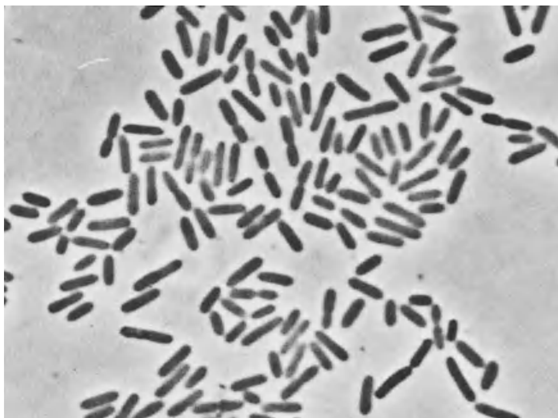
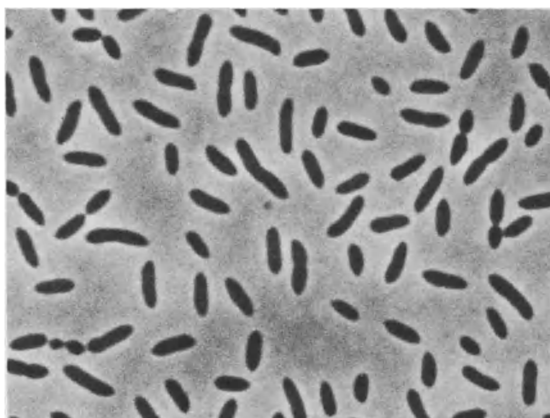


(d)



(e)

contains some acetate (2–3 mM) for synthesis of cell material. Glycerol undergoes a similar dismutation to 3-hydroxypropionate and 1,3-propanediol. It is interesting to note that glycerol fermentation also requires the presence of acetate, and that this substrate cannot itself be assimilated.

In the presence of hydrogen-scavenging anaerobes, e.g., homoacetogens or methanogens, *Pelobacter* oxidizes primary aliphatic alcohols to the corresponding acids; with propanol and butanol, acetate is again required for cell matter synthesis. This syntrophic oxidation of ethanol via "interspecies hydrogen transfer" was first observed with the S-strain isolated from the syntrophic mixed culture *"Methanobacillus omelianskii"* (Bryant et al., 1967), which was lost many years ago. *Pelobacter* strains are the only representatives of this type of metabolism that are available today in pure cultures. Pure and mixed culture experiments have been carried out recently to understand the energetics

Table 1. Properties of the five *Pelobacter* species.

	P. acidigallici	*P. venetianus*	*P. carbinolicus*	*P. acetylenicus*	*P. propionicus*
Width (μm)	0.5–0.8	0.5–1.0	0.5–0.7	0.6–0.8	0.5–0.7
Length (μm)	1.5–3.5	2.5	1.2–3.0	1.5–4.0	1.2–6.0
GC content (mol%)	51.8	52.2	52.3	57.1	57.4
Substrate metabolized					
Gallic acid	+	−	−	−	−
Pyrogallol	+	−	−	−	−
Phloroglucinol	+	−	−	−	−
Phloroglucinolcarboxylate	+	−	−	−	−
Acetoin	−	+	+	+	+
2,3-Butanediol	−	+	+	+	+
Ethylene glycol	−	+[a]	+	−	−
Polyethylene glycols	−	+	−	−	−
Ethanol	−	+[b]	+[b]	+[b]	+
n-Propanol	−	+[b,c]	+[b,c]	+[b,c]	+[c]
n-Butanol	−	+[b,c]	+[b,c]	+[b,c]	+[c]
1,2-Propanediol	−	+[c]	−	+[c]	−
Acetylene	−	−	−	+	−
Lactate	−	−	−	−	+
Pyruvate	−	−	−	−	+
Glycerol	−	+[c]	−	+[c]	−
Typical Products	Acetate (CO_2)	Acetate, ethanol	Acetate, ethanol	Acetate, ethanol	Acetate, propionate

Symbols: +, growth; −, no growth.
[a]Growth is possible only at very low concentration (<1 mM) or in continuous culture.
[b]Growth is possible only in the presence of a hydrogen-scavenging anaerobe, e.g., a methanogenic bacterium.
[c]Growth is possible only in the presence of small amounts of acetate for cell carbon synthesis.

and kinetics of interspecies hydrogen transfer in model cultures of *P. acetylenicus* and hydrogen-scavenging homoacetogenic and methanogenic partners (Seitz et al., 1988; Seitz et al., in preparation).

The biochemistry of polyethylene glycol degradation is not yet understood, neither in *P. venetianus* nor in other polyethylene glycol-degrading anaerobes (Dwyer and Tiedje, 1986; Wagener and Schink, 1988). *P. venetianus* degrades all polymers from the dimer up to a molecular weight of 40,000. Growth experiments in batch and continuous culture have shown that ethylene glycol can also support growth if it is provided at limiting amounts, and that acetaldehyde is the first free intermediate in both polymer and monomer degradation (Strass and Schink, 1986). Perhaps the polymer is attacked by a diol dehydratase-like reaction that transforms the terminal ether linkage into an unstable half-acetal linkage yielding acetaldehyde as product. Unfortunately, the cleavage reaction is very difficult to demonstrate in cell-free extracts, and a B_{12} compound of atypical structure appears to be involved (E. Schramm and B. Schink, unpublished observations). This cleavage reaction occurs inside the cells, and it is not clear how high-molecular-weight polyethylene glycols are able to cross the cytoplasmic membrane at sufficiently high transport rates.

Anaerobic degradation of polyethylene glycols is of major ecological concern because many industrially produced nonionic surfactants contain polyethylene glycols as hydrophilic moieties that maybe subject to anaerobic degradation in anoxic sediments and sludge (Wagener and Schink, 1987, 1988).

P. carbinolicus is related to *P. venetianus* and has basically the same biochemistry and physiology. It degrades ethylene glycol rather than polyethylene glycols, and it was originally enriched and isolated with acetoin or 2,3-butanediol as substrate. 2,3-Butanediol is oxidized to acetoin, which undergoes oxidative cleavage to acetyl CoA and acetaldehyde by a dichlorophenol indophenol-dependent enzyme analogous to pyruvate dehydrogenase (Oppermann et al., 1988). The physiological electron acceptor of this enzyme is not yet known. Acetaldehyde is either oxidized by a benzyl viologen-dependent enzyme to acetyl CoA or, depending on the electron balance, reduced to ethanol.

P. acetylenicus is the first strict anaerobe known to ferment an unsaturated hydrocarbon in pure culture. Again, the first intermediate of acetylene fermentation is acetaldehyde, which is further dismutated to acetate and ethanol. The enzyme that hydrates acetylene to acetaldehyde could not be demonstrated in a cell-free assay system; perhaps unusual cofactors are also

involved in this reaction. A report on an acetylene-hydratase enzyme in cell-free extracts of an aerobic *Rhodococcus* species (deBont and Peck, 1980) could not be reproduced in our hands either. The function of such an acetylene-hydrating enzyme in an anoxic environment is hard to understand since acetylene is probably not an important substrate in such environments. Perhaps its main role is hydration of other possibly toxic compounds, such as nitriles or cyanides, but there is no experimental evidence so far for such activities.

P. propionicus differs from the other *Pelobacter* species by producing propionate as one of its main fermentation products. Degradation of acetoin, 2,3-butanediol, and ethanol probably follows the same routes via acetaldehyde and acetyl CoA, as outlined above for the other species. The biochemistry of propionate formation from these C-2 compounds has been studied in detail (Schink et al., 1987). The key reaction is catalyzed by pyruvate synthase (pyruvate ferredoxin oxidoreductase), which operates here in the opposite direction to that predicted by its chemical equilibrium. The equilibrium is shifted by the exergonic propionate-forming reaction chain via methylmalonyl CoA; this chain is not coupled to ATP-yielding electron transport phosphorylation in these bacteria. This reaction in *P. propionicus* and other bacteria with similar biochemical capacities (Stams et al., 1984; Samain et al., 1982) is probably responsible for the formation of C-3 compounds from C-2 compounds in significant amounts in anoxic sediments and sludges (Goldberg and Cooney, 1981; Schink et al., 1985).

Applications

All *Pelobacter* strains discussed in this chapter may act as important syntrophic oxidants of primary aliphatic alcohols in sediments and sludges, where they have been found to be predominant (Dubourgier et al., 1986). Of technological interest may be the capacity of *P. venetianus* and similar isolates to degrade polyethylene glycols and to attack nonionic surfactants based on these compounds. These surfactants are of growing interest in the industrial manufacture of detergents, soaps, emulsifiers, etc. Aerobic degradation of polyethylene glycol-containing surfactant wastes creates enormous problems of foam formation in conventional activated-sludge sewage treatment and in natural waters. Anaerobic degradation of most of these compounds to methane can easily be achieved in packed column reactors, in which *P. venetianus*-like anaerobes ferment the polyethylene glycol moieties to substrates for the methanogenic microbial community (Wagener and Schink, 1987).

P. propionicus produces acetate and propionate from the substrates listed in Table 1. Propanol is fermented together with acetate exclusively to propionate. This fermentation may be of interest for biological production of propionate at high purity.

Acknowledgments

The author wishes to thank N. Pfennig and H. G. Trüper for their help on all questions concerning bacterial taxonomy, and in the establishment of this new genus. The assistance of Martin Bomar in compiling the phase contrast photomicrographs shown in Fig. 1 is highly appreciated.

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CHAPTER 187

The Genus *Bdellovibrio*

EDWARD G. RUBY

Fewer than 30 years ago Stolp isolated the first representative of a group of bacteria that form plaques on lawns of other Gram-negative bacteria (Stolp and Petzold, 1962). Subsequently, this group of bacteriolytic microorganisms was described as a new genus, *Bdellovibrio* (Stolp and Starr, 1963). Since that time there has been a rapid increase in our understanding of the ecology, physiology, and biochemistry of the bdellovibrios, as well as the beginning of an understanding of their phylogeny and genetics.

The most significant characteristic that unifies bacteria defined in the genus *Bdellovibrio* is the presence of a distinctive growth phase within the periplasmic space of any of a variety of Gram-negative bacteria. This adaptation to growth in the interior of another bacterium has been accompanied by the evolution of a developmental life cycle that alternates between two (and sometimes three [Burger et al., 1968]) morphologically and physiologically differentiated cell types (Fig. 1). Thus far, all bdellovibrios isolated from nature require the presence of another bacterium to provide an intracellular growth environment, although it is possible to grow at least some bdellovibrios in the presence of extracts of other bacteria (Friedberg, 1978; Gray and Ruby, 1989). Also, as described below, variant strains can be selected that grow on simple nutrient media (Diedrich et al., 1970; Seidler and Starr, 1969b).

The distinctive life style of the bdellovibrios has led to a variety of somewhat confusing terminologies. They have been described both as "parasites" associating with "host" bacteria and as "predators" feeding upon "prey" bacteria.

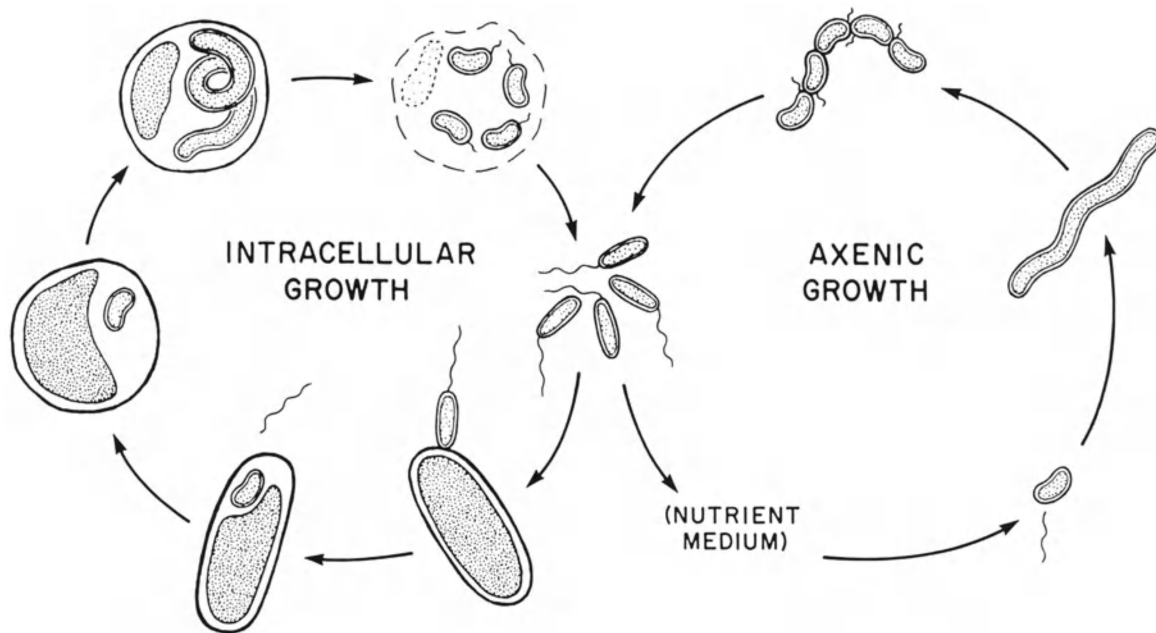

Fig. 1. The two growth patterns of *Bdellovibrio bacteriovorus* 109J. *Left:* wild-type bdellovibrios can grow only within another bacterium, alternating between an extracellular, attack-phase developmental form and an intracellular, growth-phase developmental form. *Right:* variant strains can be isolated that can grow axenically when inoculated into a complex nutrient medium. Many of these variants are facultative, i.e., able to grow either intracellularly or axenically.

This apparent controversy exists because the bdellovibrios express traits that fall within the definitions of both parasites and predators (Starr, 1975). The important point, however, is not what we call them, but that we realize that the success of the bdellovibrios derives from their unique combination of both "predatory" and "parasitic" traits. Our goal is to discover how those traits function together, without being prejudiced by restrictive labels. Accordingly, in this chapter the term "invaded cell" will generally be used, although occasionally "substrate cell" will appear. The latter term is used to emphasize that the invaded cell is used only as a source of organic substrates, and that its viability or metabolism is apparently unimportant to the successful growth and development of the bdellovibrios (Rittenberg, 1983; Varon and Shilo, 1969a).

Other terms that may need clarification, are the "attack-phase" and the "growth-phase," which are differentiated cell stages of the bdellovibrio life cycle. Attack-phase cells are flagellated, single-unit curved rods that do not exhibit chromosome replication or cell proliferation. Growth-phase (or "multiplication-phase" [Shilo, 1984]) cells have no external appendages, can initiate chromosome replication, and increase in size as single, elongating, spiral cells with unseptated cytoplasms. After a bdellovibrio enters a substrate cell it performs a number of degradative and biosynthetic modifications on the outer membrane and peptidoglycan of the invaded bacterium (Ruby, 1989). The resulting spherical structure, consisting of the inactive invaded cell and the developing bdellovibrio, is termed the "bdelloplast." Although the bdellovibrio remains within what was the periplasmic space of the invaded cell, the types and amounts of macromolecular components characteristic of the periplasm change, some being lost to the exterior, and others that are normally found in the cytoplasm becoming available to the bdellovibrio. Thus, the periplasmic compartment appears no longer to be distinctly separated from either the cytoplasm (Cover et al., 1984) or the extracellular environment (Crothers and Robinson, 1971; Ruby and McCabe, 1988). For this reason, the more generic term "intracellular," rather than "intracytoplasmic" or "intraperiplasmic," will be used in this chapter.

Since the publication of the first edition of *The Prokaryotes* (Stolp, 1981), there have been several general reviews of the bdellovibrio life style (Rittenberg, 1979; Thomashow and Rittenberg, 1979; Rittenberg, 1983), as well as more specific reviews dealing with ecology (Varon and Shilo, 1978; Rittenberg, 1979; Shilo, 1984), experimental methods (Rittenberg, 1982), and the cell envelope (Ruby, 1989). This present review treats the subjects of ecophysiology, isolation, identification, and application of the bacteria in the genus *Bdellovibrio,* and in particular reviews the (approximately) 60 papers published on the biology of the bdellovibrios since 1981.

Identification

The genus *Bdellovibrio* is defined in large part on the basis of a life style that includes the use of another Gram-negative cell as the sole source of nutrition (Burnham and Robinson, 1974). There are a number of other bacterial genera whose members prey upon or parasitize other microorganisms for their growth. Isolating bdellovibrios from, or studying them in, the natural environment requires an awareness both of the presence and characterisics of these other microbial bacteriovores, and of how they can be distinguished from the bdellovibrios. Such an awareness is of value not only for the valid identification of bdellovibrios in ecological studies (Enzinger and Cooper, 1976; Varon and Shilo, 1978, Germida and Casida, 1983; Mitchell et al., 1967), but also in providing a sense of the diversity of the organisms that should be considered in a comparative study of intracellular adaptations (Moulder, 1985; Ruby, 1989).

Table 1 lists seven genera of bacteria that have been reported to grow on unicellular organisms. With the exception of *Vampirococcus,* isolates have been maintained in laboratory culture, although often with some difficulty (I. Esteve, personal communication). Major metabolic and cellular characteristics by which they differ from each other and from bdellovibrios are noted (Table 1). While *Micavibrio, Vampirovibrio,* and *Vampirococcus* have been reported to be able to grow upon microorganisms of only a single genus, bdellovibrios as a group can utilize any of a wide variety of Gram-negative bacteria as a substrate cell. Any given strain of bdellovibrio usually has the ability to grow on bacteria of several different genera (Stolp and Starr, 1963; Taylor et al., 1974); for example, *Bdellovibrio bacteriovorus* 109J can be grown on certain strains of *Escherichia, Pseudomonas, Rhizobium, Chromatium, Spirillum,* as well as other genera. The basis for the specificity that is expressed within a strain of bdellovibrio and for the differences between bdellovibrio strains is still unknown, although the working assumption is that this specificity involves specific recognition of surface components of the attacked bacterium (Schelling and Conti, 1986).

Table 1. The characteristics of the major genera of bacteria that feed upon other microorganisms.

Genus	Motility	Metabolism	Association	Dependency[a]	Reference[b]
Myxococcus	Gliding	Obligate aerobe	Unattached	Axenic	1
Ensifer	Flagellar	Obligate aerobe	Attached	Axenic	2
Daptobacter	Flagellar	Facultative anaerobe	Cytoplasmic	Axenic	3
Micavibrio	Flagellar	Aerobe	Attached	Obligate (*Pseudomonas*)	4
Vampirovibrio	Flagellar	Aerobe	Attached	Obligate (*Chlorella*)	5
Bdellovibrio	Flagellar	Obligate aerobe	Periplasmic	Obligate (Gram-negative)	6
Vampirococcus	None	Anaerobe	Attached	Uncultured (*Chromatium*)	7

[a]The kind of host organism to which the parasite is restricted is given in parentheses.
[b]References: 1, Burnham et al., 1981; 2, Casida, 1982; 3, Alguero et al., 1987; 4, Lambina et al., 1982; 5, Mamkaeva et al., 1988; 6, Rittenberg, 1983; 7, Guerrero et al., 1986.

Morphology and Ultrastructure

Bdellovibrios do not have any intrinsically distinctive morphological characteristics themselves and thus are usually recognized during microscopic examination of natural samples only by their association with the bacteria they have invaded (Caiola and Pellegrini, 1984; Wilkinson, 1979). Several excellent electron microscopic studies of the processes of penetration and growth by intracellular bdellovibrios have been reported using laboratory cultures (Abram and Davis, 1970; Abram et al., 1974; Snellen and Starr, 1974; Starr and Baigent, 1966) and have led to a better understanding of the topographical characteristics of this process (Rittenberg, 1983; Rittenberg and Thomashow, 1979).

The bdellovibrios exhibit two (or sometimes three [see below]) distinct cell morphologies that reflect their specific adaptations to either the attack phase or the growth phase of its life cycle. The attack-phase cell is a small (0.25 to 0.40 μm in width, 1 to 2 μm in length) vibrioid to rod-shaped bacterium. It possesses one polar flagellum of 28 nm diameter (Seidler and Starr, 1968) that is ensheathed by a continuous extension of the outer membrane of the cell (Thomashow and Rittenberg, 1985b). However, the flagellar sheath has been shown to be biochemically distinct from the rest of the outer membrane (Thomashow and Rittenberg, 1985a). The entire flagellum is lost from the attacking bdellovibrio after the bacterium attaches to a susceptible cell and before it enters that cell. The outer membrane of the bdellovibrio appears to be a strong structure that can maintain the osmotic integrity of the cell even when its peptidoglycan layer is experimentally removed (Thomashow and Rittenberg, 1978a).

The growth-phase bdellovibrio has the peculiar morphological characteristic of indeterminate growth. The extent of the length of an elongating cell is directly dependent upon the quality of the surrounding environment: intracellular bdellovibrios grow to lengths proportional to the volumes of the invaded cells (Kessel and Shilo, 1976), and the elongation of either axenic or prematurely released bdellovibrios depends upon the concentration of extracellular signals (Eksztejn and Varon, 1977; Gray and Ruby, 1989) that control bdellovibrio differentiation and growth. Cell elongation always occurs with the formation of a spiraling cell of strain specific compactness, and septa and flagella are elaborated only as the growth-phase cell differentiates into a number of attack-phase progeny (Fig. 1).

Bdellocysts

Almost all bdellovibrio strains that have been examined exhibit the simple, dimorphic life cycle illustrated in Fig. 1. However, the bdellovibrios of the *Bdellovibrio* sp. strain W not only are capable of the typical alternation between an attack and growth phase, but also can form a third, resting stage termed the bdellocyst (Burger et al., 1968; Hoeniger et al., 1972). Under unfavorable environmental conditions (Tudor and Conti, 1977a), strain W forms a resistant, multi-layered cyst inside the invaded cell and will eventually differentiate into an attack-phase cell upon the return of favorable conditions (Tudor and Conti, 1977b; 1978). A series of papers dealing with the synthesis and composition of the bdellocyst envelope has appeared (Tudor, 1980; Tudor and Bende, 1986; Tudor and DiGiuseppe, 1988). The significance of this encystment to the environmental biology of bdellovibrios has not been experimentally examined, but it would appear to offer a selective advantage to cells that frequently encounter harsh or changing conditions.

Bacteriophages of Bdellovibrios

There are presently three areas of interest concerning the characteristics of bacteriophages that parasitize bdellovibrios ("bdellophages"): 1) their abundance in the natural environment

may have some, as yet unknown, effect on the density and distribution of bdellovibrios in soil, water, and sewage (Varon, 1974); 2) they have been used in some taxonomic studies to group bdellovibrios with similar bacteriophage specificities (Althauser et al., 1972; Varon, 1974); and 3) their DNA has been used in studies of bdellovibrio transfection (Roberts et al., 1987; Roberts and Ranu, 1987).

Several laboratories have reported the isolation of bdellophages (Althauser et al., 1972; Hashimoto et al., 1970; Sagi and Levisohn, 1976; Varon and Levisohn, 1972), which are most easily obtained by their ability to form plaques on lawns of axenically growing bdellovibrios. Such bdellophages generally are able to attack and propagate within wild-type bdellovibrios provided that the bdellovibrios are grown together with cells that they can invade (so that they may initiate replication), and that the bdellophage attaches to the bdellovibrio just before the bdellovibrio invades another cell (Varon, 1974).

Taxonomy and Phylogeny

The difficulty of developing and applying a traditional taxonomic methodology to a group of bacteria that must be grown in a mixed culture has been responsible for the incomplete state of this area of bdellovibrio research. There are three described species of *Bdellovibrio*—*B. bacteriovorus* (Stolp and Starr, 1963), *B. stolpii,* and *B. starrii* (Seidler et al., 1972), as well as at least two unspeciated groups: the encysting forms represented by *Bdellovibrio* sp. strain W (Hoeniger et al., 1972), and the marine bdellovibrios (Marbach et al., 1976; Taylor et al., 1974). The three described species have been compared by DNA analysis (GC content, genome size, and DNA hybridization), as well as by cytochrome spectra and host range (Seidler et al., 1969; Torrella et al., 1978), and have been separated on the basis of three criteria that are variable and difficult to determine (Stolp, 1981).

The inadequacy of the methodology has resulted in very few attempts to identify large numbers of environmental isolates. Thus, no ecophysiological significance has been assigned to the various species of *Bdellovibrio,* and newly isolated strains are rarely taxonomically described. At present the nomenclatural type culture of *B. bacteriovorus* is strain 100 (ATCC 15356, DSM 5070); the type culture of *B. stolpii* is strain UKi2 (ATCC 27052, DSM 50722); and the type culture of *B. starrii* is strain A3.12 (ATCC 15145, DSM 50712) as described by Stolp (1981).

Further attempts have been made to elucidate the intrageneric arrangement of bdellovibrio strains by emphasizing passive, extracellularly located traits such as phage susceptibility (Varon, 1974), extracellular proteases (Gloor et al., 1974), antigenicity (Kramer and Westergaard, 1977), membrane proteins (Severin et al., 1981), and penicillin-binding proteins (Park and Mahadevan, 1988). Falkner et al. (1989) suggested that the marine and the terrestrial bdellovibrios have different surface antigens, and that this observation, as well as the relatively low GC content characteristic of the marine strains (Torrella et al., 1978), further supports the general consensus that they comprise two widely divergent groups (Stolp, 1981).

While there has been little recent progress in developing the taxonomy of the bdellovibrios, comparisons of sequence similarities of the 16S ribosomal RNAs of representatives of the three described species of *Bdellovibrio* and of the one marine isolate have allowed some speculation upon the phylogenetic relationship of the genus *Bdellovibrio* to other genera of bacteria (Hespell et al., 1984). The analyses of these data support two conclusions: 1) that the bdellovibrios probably form a single, ancient phylogenetic group that has experienced considerable divergence; and 2) that the closest existing relatives of the bdellovibrios are the myxobacteria and the sulfate-reducing bacteria. Further exploration of the molecular phylogeny of the bdellovibrios should provide thought-provoking information about their evolutionary relationships and may produce taxonomically useful probes for ecological studies.

Habitats

Distribution in the Natural Environment

The widespread distribution of bdellovibrios is evidenced by their ready isolation from samples of soil (Keya and Alexander, 1975; Klein and Casida, 1967; Mishustin and Nikitina, 1974; Parker and Grove, 1970), sewage (Dias and Bhat, 1965; Staples and Fry, 1973), freshwater (Fry and Staples, 1974; Guelin and Cabioch, 1970), and sea water (Marbach et al., 1976; Mitchell et al., 1967; Miyamoto and Kuroda, 1975; Taylor et al., 1974). Unfortunately, beyond the documentation of the presence and abundance of bdellovibrios in various natural samples, there have been few ecological investigations specifically designed to identify their normal habitats (Rittenberg, 1979). Rather little information is available to document experimentally what environmental factors may be important in the ecology of this group, except that oxic conditions and an abundance of

Gram-negative bacteria are positive correlating factors in many (Fry and Staples, 1976; Varon and Shilo, 1978), but not all (Williams and Falkner, 1984), studies.

The most complete studies of the ecology of the bdellovibrios are those reported by Williams and his co-workers, who examined the temporal and spatial distribution of these bacteria in estuarine waters. For most of this work, *Vibrio parahaemolyticus* was the prey species used to enumerate bdellovibrios; thus, the results may reflect the ecology of only a subset of all the bdellovibrios present in that environment i.e., those capable of growth on this species. The results of these studies indicated that bdellovibrios are more abundant in estuaries than in open ocean water (Williams et al., 1980). Bdellovibrios lytic to *V. parahaemolyticus* were found to occur at higher concentrations in the warmer, summer months than in the winter (Williams et al., 1980), and to be relatively uniformly distributed within the depth of the water column (Williams and Falkner, 1984). Most recently, a study of the abundance and distribution of bdellovibrios in estuarine sediments has suggested that they maintain their abundance and even appear to proliferate in this environment (Williams, 1988); warm water temperatures, low salinity, and a high concentration of other culturable Gram-negative bacteria are the environmental conditions that correlate positively with bdellovibrio abundance.

Because of the importance of the presence of substrate cells to bdellovibrio survival (Hespell et al., 1974), the biological (and not just the physical and chemical) characteristics of the microbial ecology of the bdellovibrios must be addressed. Several studies have attempted to determine experimentally the minimum concentration of substrate cells that is necessary to support a population of bdellovibrios (Fry and Staples, 1974; Hespell et al., 1974; Keya and Alexander, 1975; Varon, 1981; Varon et al., 1984; Varon and Zeigler, 1978). Of particular importance to this process are the kinetics of successful attachment of the bdellovibrio to a potential substrate cell (Varon and Shilo, 1968), a process in which the surface characteristics of that cell are of demonstrated importance (Varon and Shilo, 1969b). Applications of chemostat methods (Varon, 1979) and theoretical modeling (Dulos and Marchand, 1984; Marchand and Gabignon, 1981) to such questions have also been made.

An additional factor affecting the distribution and success of bdellovibrios in the natural environment is any potential for directed movement that would result in an increased likelihood of encountering a potential substrate cell (Rittenberg, 1979; Shilo, 1984). While bdellovibrios do not seem to express a measurable chemotactic response toward concentrations of suitable substrate cells (Straley and Conti, 1977), they have been reported to accumulate in the vicinity of high concentrations of organic solutes (LaMarre, et al., 1977; Straley and Conti, 1974; Straley et al., 1977). It has yet to be demonstrated whether or not this latter capability would itself direct the bdellovibrios to environments that attract or contain concentrations of actively metabolizing substrate cells.

The Intracellular Environment

A consideration of the natural environment of the bdellovibrios must be concerned both with the habitats and ecological distribution of the attack-phase cell and with the special intracellular environment of the growth-phase bdellovibrio. It has been observed that the intracellular environment may serve not only as a compact and complete nutrient source, but also as a protected environment (Rittenberg and Thomashow, 1979; Shilo, 1984). Thus, the bdelloplast is a relatively safe haven against lethal irradiation (Friedberg, 1977), phages (Varon and Levisohn, 1984), and environmental pollutants (Varon and Shilo, 1981). It seems clear, therefore, that their ability to produce an intracellular growth chamber exerts positive (and perhaps significant) effects on the ecology of the bdellovibrios.

The obligate nature of the intracellular life style of the bdellovibrios has another ecological consequence: it clearly limits the number of environments in which the bdellovibrio can reproduce. The question of why bdellovibrios normally proliferate only when they are within substrate cells has yet to be answered; however, two approaches to this problem are being actively pursued. The first approach is to define the genetic character of the mutation (or mutations) of the variant bdellovibrios that can proliferate in the absence of a substrate cell (Seidler and Starr, 1969b). The introduction of molecular genetics to the study of the bdellovibrios has made this goal feasible (Cotter and Thomashow, 1989). The second approach involves the identification of specific compounds in extracts of substrate cells that can promote axenic growth by these bdellovibrios that otherwise grow only within the intracellular environment of the substrate cell (Crothers et al., 1972; Horowitz et al., 1973; Gray and Ruby, 1989; Reiner and Shilo, 1969).

Isolation

The basic approach to the isolation of bdellovibrios from natural samples bears a resemblance to that used to obtain bacteriophages: dilutions of water, sewage, or soil are mixed with a susceptible bacterial suspension in soft agar and are plated and incubated at an environmentally appropriate temperature; the presence of a growing bdellovibrio clone is indicated by a plaque in the bacterial lawn (Rittenberg, 1982). In principle, two important considerations must be addressed for a successful isolation: 1) an appropriate choice of the lawn-forming prey bacterium must be made; and 2) some physical separation of the bdellovibrios from other plaque-forming microorganisms must be employed to facilitate screening and selection (Varon and Shilo, 1970).

Bdellovibrios, like bacteriophages, express some specificity for the species of their prey (Shilo and Bruff, 1965; Taylor et al., 1974), although the basis for such specificity is not yet well described (Chemeris et al., 1984; Schelling and Conti, 1986). In addition, the choice of a lawn-forming organism must take into account the physiological attributes desired in the isolated bdellovibrios (marine, psychrophilic, etc.). Schoeffield and Williams (1990) suggest that the selection of *Vibrio parahaemolyticus* as the lawn-forming bacterium will reproducibly maximize the efficiency of detection of bdellovibrios in some estuarine water samples. In contrast, the apparent usefulness of *Aquaspirillum (Spirillum) serpens* MW5 as a "universal host" (Torrella et al., 1978) has been recently disputed (S.F. Koval, pers oral communication). While these observations can be of great value in designing experiments, their interpretation in terms of the ecology of bdellovibrios must be approached cautiously (see below).

Because bdellovibrios usually are found in nature in rather low abundance (Klein and Casada, 1967; Fry and Staple, 1974; Williams, 1988) and often are mixed with bacteriophages, myxobacteria, protozoa, and other plaque-forming bacteriovores, a selective filtration step is used to eliminate large bacterial predators and protozoa, thereby enriching the sample in bdellovibrios (Starr and Stolp, 1976; Varon and Shilo, 1978). The relatively slow rate of formation of bdellovibrio plaques differentiates them from plaques formed by bacteriophages.

Enrichment and Direct Isolation of Bdellovibrio (Modified from Stolp, 1981)

An appropriate volume of water sample or a 50-g soil sample suspended in 500 ml of sterile buffer is shaken vigorously for at least 1 h and centrifuged briefly at 2,000 × g to remove large particles or soil. The resulting low-speed supernatant suspension is passed through a series of decreasing pore-sized membrane filters (3.0, 1.2, 0.65, and 0.45 μm). Samples of 100 μl serial dilutions of the final filtrate are combined with 200 μl of a suspension of potential substrate bacteria (10^{10} cells per ml) in 2.5 ml of 0.6% agar at 42°C. The mixture is well agitated and quickly overlaid on a room-temperature, dilute nutrient agar plate, allowed to solidify, and incubated at an appropriate temperature.

Within 24 h, the presence of any viable bacteriophages in the dilutions will be indicated by the appearance of plaques. Because none of these early plaques will be the result of bdellovibrio growth, they can be marked and disregarded. When the plates are viewed again after 2 to 4 days at 30°C, the additional plaques noted are presumptive bdellovibrio clones. Addition of a stain to the medium has been reported to aid in distinguishing bdellovibrio plaques (Williams et al., 1980)

After crushing a small bit of the plaque-containing agar between a cover slip and slide, it should be possible to discern small, rapidly motile bdellovibrio cells among the remains of the lawn bacteria. It should be noted that these numerous manipulations and fractionations of the sample may lead to the loss of an indeterminable portion of the total bdellovibrio population in the sample (Staples and Fry, 1973). Thus, this approach is not appropriate for the quantitative determination of the abundance of bdellovibrios in the sample.

When samples of the natural environment are not being used to estimate the abundance of bdellovibrios (Germida, 1987; Keya and Alexander, 1975; Parker and Grove, 1970; Sullivan and Casida, 1968), but instead to obtain an isolate of bdellovibrios that is lytic towards a particular strain or species of bacterium, the method of "specific enrichment" is most useful. In this approach the sample of water, soil, or sewage is added to a suspension of the desired substrate bacterium in a buffer solution. A period of incubation is chosen during which it is presumed that only those bdellovibrios that can grow upon the enriching bacterium will become enriched in the sample. Because of the lack of added nutrients, the growth of heterotrophic microorganisms in general is discouraged. Bacteriophage development is minimal because the growth of all of the bacteria in the enrichment (except for the bdellovibrios) is minimal.

Specific Enrichment Procedure for Obtaining Bdellovibrio Cultures (Modified from Stolp, 1981)

A culture of the desired substrate bacterium is suspended in DNB medium (0.08% Difco Nutrient Broth, 0.05% Difco Casamino Acid, and 0.01% Difco Yeast Extract in deionized water, adjusted to pH 7.6 with NaOH, supplemented with 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ after sterilization) or HM buffer (10 mM N-2-hydroxyethyl piperizine-N′-2-ethanesulfonic acid adjusted to pH 7.6 with NaOH, and supplemented with 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$) to a density of 10^{10}

cells per ml. To 100 ml of this suspension is added the natural sample that is to be checked for the presence of bdellovibrios (e.g., 100 mg of soil, 0.5 ml of sewage, or 1 ml of river water). The enrichments are incubated with rapid shaking and checked daily by phase-contrast microscopy for the presence of either bdelloplasts or presumptive bdellovibrio cells. When bdellovibrios are apparent, the enrichment is centrifuged at low speed (2,000 × g) for 5 min to remove particles, and filtered through a 0.45 μm pore-sized membrane. The filtrate is diluted and plated onto lawns of the enriching substrate bacterium as described above, and the resulting plaques are checked for the presence of bdellovibrio cells.

There are two special considerations that must be addressed when attempting to isolate bdellovibrios from the marine environment. First, the media and buffers used should contain at least 25% sea water or an appropriate marine salts mixture (Bell and Latham, 1975; Marbach and Shilo, 1978; Taylor et al., 1974) to provide the specific ionic and osmotic requirements of marine bacteria. Second, the sometimes extremely low abundances of bdellovibrio cells in seawater samples require that large volumes of water be processed.

Procedure for Obtaining Marine Bdellovibrio Cultures (Varon and Shilo, 1976)

Either the direct or specific procedures for bdellovibrio isolation can be adapted to marine samples. Both should begin with the concentration of up to 1 l of sea water either by sedimentation in a centrifuge at high speed (10,000 × g for 30 min), or by collection on a 0.1-μm-pore-sized membrane filter. The concentrated material can be used to inoculate soft-agar overlay plates containing cultures of an appropriate prey organism, such as a strain of one of the luminous marine bacteria (Varon and Shilo, 1981).

Preparation of Pure Cultures

The plaques developing from an initial isolation must be purified of contaminating microorganisms by serial transfer. The result of such a process should be a mixed culture containing only the single clone of bdellovibrio, and the strain of bacterium upon which it was isolated and will be maintained. This approach is formally analogous to that applied to the purification of bacteriophage from plaques.

Procedure for Purifying Bdellovibrio Cultures (Modified from Stolp, 1981)

A sample of the bdellovibrios within a developing plaque is removed by inserting a sterile Pasteur pipette tip into the center of the plaque. The agar plug is removed and transfered to 2 ml of HM buffer or a dilute medium solution, and the cells are suspended by vortexing. The liquid suspension is passed through a sterile 0.45-μm-pore-sized membrane filter, and the filtrate collected. Serial dilutions of the filtrate are made in HM buffer and plated for the visualization of individual plaques. Plating is performed by combining 100 μl of bdellovibrio suspension, 200 μl of prey cell suspension (10^{10} cells per ml), and 2.5 ml of 0.6% agar at 42°C, and quickly pouring the mixture onto the top of a DNB agar plate. After incubating the plate for 4 to 7 days, a single plaque is reisolated from this plate, and the process is repeated three times, or until the culture shows no signs of contamination. If difficulties arise from the growth of contaminating heterotrophic bacteria, platings and dilutions can be made on an HM-buffer agar plate to discourage the growth of extraneous bacteria. With this method, substrate cells must be provided at a high turbidity in the soft-agar overlay in order to allow plaque development and visualization in the absence of significant lawn growth.

Manipulation

Cultivation

The success of many experiments using bdellovibrios relies upon the preparation of a healthy, predictably active suspension of attack-phase cells that can be either analyzed directly or combined with a suspension of substrate cells to initiate an intracellular growth cycle. The following procedures have been found to produce attack-phase cells in optimal condition.

Two consequences resulting from the high endogenous respiratory rate (Hespell et al., 1973) characteristic of bdellovibrios, should be remembered: 1) Suspensions of bdellovibrios should be incubated with vigorous agitation, preferably on a rotary shaker, to assure a ready supply of oxygen. Efficient aeration can also be encouraged if the nominal volume of the flask used is at least two to three times that of the cell suspension it contains. 2) Their high respiratory demand causes suspensions of attack-phase cells to lose viability rapidly when maintained in the absence of a source of cells upon which to grow (Hespell et al., 1974; Hespell and Mertens, 1978). Both the addition of a respirable substrate, such as 1 mM glutamate, and the reduction of temperature to 0°C, can ameliorate the effects of the requirements for high endogenous respiratory activity, and prolong both culture viability and synchrony of attack (see below).

The attack efficiency of bdellovibrios is enhanced both by keeping the concentration of organic nutrients (like nutrient broth or yeast extract) in the suspending medium below 0.1% (wt/vol), and by using washed suspensions of both bdellovibrios and substrate cells. Other viability-enhancing conditions for at least some strains include using substrate cell cultures with a low proportion of dividing cells (Rittenberg, 1982), maintaining high concentrations of Ca^{2+}

and Mg^{2+} ions in the suspending medium (Huang and Starr, 1973), the addition of osmoprotectants to cultures at low densities (Varon et al., 1983 and 1984), and the absence of strong irradiation (Friedberg, 1977). The following procedure is recommended for work with bdellovibrios:

Routine Cultivation of *Bdellovibrio Bacteriovorus* 109J (Modified from Thomashow and Rittenberg, 1978b)

Stock Culture. To a 125-ml flask containing 40 ml of HM buffer are added 10 ml of a culture of *Escherichia coli* ML35 (4×10^{10} cells) grown to stationary phase in NB medium (0.8% Difco Nutrient Broth, 0.5% Difco Yeast Extract in deionized water, adjusted to pH 7.6 with NaOH), and 50 to 100 μl of a 7 to 10-day-old stock culture of *Bdellovibrio bacteriovorus* 109J. The flask is shaken overnight at 30°C and, when the culture contains essentially only attack-phase bdellovibrios, it is stored at 4°C.

Large-scale Culture. To a 500-ml flask containing 200 ml of DNB medium is added 40 ml of a culture of *Escherichia coli* ML35 (total addition = 1.6×10^{11} cells) grown to stationary phase in NB medium, and 50 to 100 μl of a fresh *Bdellovibrio bacteriovorus* 109J stock culture. The flask is shaken at 30°C overnight, and the culture observed by phase-contrast microscopy until all of the bdelloplasts have lysed. The culture is cooled on ice, centrifuged at low speed (700 × g for 15 min), and the pellet discarded. The supernatant suspension, containing most of the attack-phase bdellovibrios, is centrifuged at high speed (10,000 × g for 15 min) and the resulting supernatant suspension discarded. The pellet is gently resuspended in HM buffer and harvested by recentrifugation at high speed. Resuspension of the pellet in HM buffer should yield about 6×10^{11} washed bdellovibrio cells.

Enumeration

There are three methods commonly used for enumerating laboratory cultures of bdellovibrios in liquid suspensions. Each method provides a different combination of degrees of accuracy, convenience, speed, and sensitivity. Direct microscopy, either of unstained (Snellen et al., 1978), or fluorescently labeled (Hobbie et al., 1977) cells can be performed on suspensions of bdellovibrios. Microscopy is effective even in the presence of substrate cells because there is usually a significant size difference between the substrate cells and the bdellovibrios. Electronic particle counting has also been used both to count and to size bdellovibrio cells in pure culture (Patinkin, 1975).

To determine the number of viable cells in a suspension, dilutions can either be mixed with a suspension of substrate cells in soft agar and pour plated, or spread on the surface of a nutrient agar medium if the cells are capable of axenic growth (see below). Bdellovibrios will form visible plaques in the lawn after 3 to 4 days and will continue to grow in diameter for a week or more. In the case of axenic strains, small, often yellow, colonies will be apparent within 4 days of incubation at 30°C.

The most convenient and routine method of estimating the concentration of bdellovibrio cells in a relatively dense suspension is by optical density. Both turbidimetric and spectrophotometric approaches have been useful in general laboratory procedures (Varon and Shilo, 1969a). Calibrating such measurements against microscopic counts or viable counts results in a reliable standard curve, especially if the bdellovibrios are cleaned of prey cell debris by washing with HM buffer before their optical density is determined.

Single-Cycle Growth Technique

One of the most intensely studied areas of bdellovibrio biology is concerned with the specific temporal events that make up their developmental cycle (Fig. 1). These events can best be investigated by using a method that produces a large number of bdellovibrios developing in a synchronous manner (Rittenberg, 1982). This is most easily achieved by mixing active, attack-phase bdellovibrios with susceptible substrate cells in a ratio of 2:1, which assures the attack and entry of all the substrate cells within a few minutes. The bdelloplasts formed can then be easily separated from the supernumerary, extracellular bdellovibrios by differential centrifugation, producing a suspension of bdelloplasts within which reside bdellovibrios that have begun, essentially simultaneously, a single, synchronized cycle of growth and development. Analyses of such developing populations at specific times between the initial attack and the final release from the spent bdelloplast can reveal the relative timing of stage-specific developmental events and activities (Matin and Rittenberg, 1972; Rosson and Rittenberg, 1979).

Procedure for Producing Developmentally Synchronized, Intracellularly Growing Bdellovibrios (Thomashow and Rittenberg, 1978b)

An overnight culture of bdellovibrios that have very recently been released from bdelloplasts is cooled to 0°C and harvested by centrifugation at 10,000 × g for 15 min. The resulting pellet of bdellovibrio cells is washed once with cold HM buffer to remove spent bdelloplast debris. A fresh overnight culture of substrate cells (such as *Escherichia coli* ML35) is similarly harvested and washed. Separate suspensions of the bdellovibrios (10^{10} per ml) and the *E. coli* cells (5×10^9 per ml) are maintained on ice until use. A synchronous developmental cycle is begun by mixing equal volumes of the two suspensions and shaking the mixture rapidly at 30°C.

Within 10 to 15 min, all of the *E. coli* cells should be visible as spherical bdelloplasts when viewed under phase-contrast microscopy. At this point the suspension is cooled in an ice bath, and the bdelloplasts are separated from any excess bdellovibrios by centrifugation at 1,000 × g for 5 min. The resulting pellet is resuspended and recentrifuged two more times or until the final pellet contains less than 5% of the excess bdellovibrio cells. The bdelloplast pellet is resuspended in one-half the original culture volume of HM buffer at 0°C. The intracellular bdellovibrios will resume their synchronous development when they are again shaken at 30°C.

Isolation of Axenic Variants

All the strains of wild-type bdellovibrios examined can spontaneously produce variants or mutants that have the ability to grow in the absence of substrate bacteria (Burnham et al., 1970; Stolp, 1981). Such variants arise at a frequency of approximately 10^{-6} to 10^{-8} (Seidler and Starr, 1969b) and can grow on a variety of nutrient media that contain at least a small amount of yeast extract (Ishiguro, 1974). While the frequency of generation suggests that only a single mutational event is required for the loss of substrate cell dependency, the pleiotropic nature of these strains suggests that lesions in any one of several regulatory genes may give rise to this phenotype. It is significant that these variants continue to express the morphological differentiation of the wild-type bdellovibrio, even in the absence of an intracellular life style (Fig. 1). These axenic growth-phase cells have been used to examine characteristics of bdellovibrio growth in the absence of any confounding influences of the invaded cell (Ishigura, 1973; Rosson and Rittenberg, 1981; Rayner et al., 1985; Ruby et al., 1985; Talley et al., 1987).

It has been noted (Diedrich et al., 1970; Varon and Seijffers, 1975) that most axenic variants are facultative and can grow in either of the two modes (axenic or intracellular) shown in Figure 1; however, maintenance of these variants in the absence of substrate cells for numerous transfers often results in the loss of the ability to grow intracellularly. This effect is probably related to the observation that obligately axenic strains have a selective advantage over facultatively axenic strains in chemostat culture (Varon and Seijffers, 1975).

Procedure for Isolating Axenic Bdellovibrio Strains (Modified from Seidler and Starr, 1969b)

The only significant difficulty that must be overcome to isolate axenic variants is that of separating them from the strain of substrate cell on which they have been maintained. Therefore, to obtain a selectable marker, an antibiotic (e.g., streptomycin) is added to a wild-type bdellovibrio culture, which is then propagated on substrate cells for several days until a spontaneously arising streptomycin-resistant bdellovibrio mutant completes lysis of the culture. The antibiotic-resistant bdellovibrios are concentrated by centrifugation and resuspension in HM buffer, and 10^7 to 10^9 cells are spread on NB agar medium containing streptomycin. Under these conditions, the only colonies that form are axenically growing bdellovibrios, which can be further purified by conventional streaking. An improvement in the growth rate of these axenic variants has been reported to occur in the presence of additional osmolytes (Varon and Seijffers, 1977), and the use of freshly poured agar medium and a humidified incubation chamber can enhance colony yields and growth.

Premature Release of Intracellular Bdellovibrios

Because of the unknown genetic nature of the axenic variants and their pleiotropic characteristics (Seidler and Starr, 1969b), these strains are not always appropriate subjects for examining the activities of wild-type, growth-phase bdellovibrios (Ruby et al., 1985). For this reason, a technique has been developed that allows one to obtain viable, wild-type growth-phase cells from the bdelloplast at any time during the intracellular portion of their life cycle (Ruby and Rittenberg, 1983).

Procedure for Premature Releasing Growth-Phase *Bdellovibrio bacteriovorus* 109J (Ruby and Rittenberg, 1983)

Bdelloplasts from a synchronously growing suspension of *Bdellovibrio bacteriovorus* 109J are produced as described above. At the desired stage of development these bdelloplasts are cooled in an ice bath and harvested by centrifugation (5,000 × g for 15 min). The resulting pellet is warmed to room temperature, rapidly resuspended in twice its volume of 10 mM ethylenediaminetetraacetic acid (EDTA) in 120 mM Tris buffer (pH 7.6), and incubated at 30°C for 3 min to permeabilize the bdelloplast's outer membrane. A crude enzyme preparation containing concentrated bdellovibrio lytic enzyme activity (obtained from lysed suspension of bdellovibrios) is added, and, within a few minutes, the growth-phase cells are released from their bdelloplasts. After washing in cold buffer, these cells are metabolically intact and are capable of either differenting into viable attack-phase cells, or responding to isolated substrate-cell signal compounds (Gray and Ruby, 1989). The release process can be enhanced if the bdelloplasts are formed from substrate cells that have a weakened cell envelope (for example, diaminopimelic acid [DAP]-requiring mutants grown for a generation in the absence of added DAP).

Genetic Manipulation

Recent efforts to find methods for transferring DNA into bdellovibrio cells have resulted in two promising approaches: conjugation and transfection. Cotter and Thomashow (1989) have reported a simple method by which anti-

biotic-resistance markers on plasmids in *Escherichia coli* have been mobilized into and expressed by *Bdellovibrio bacteriovorus.* This method is based on a procedure developed by Puhler and his colleagues (Simon et al., 1982) and has been successfully repeated and confirmed by this author (E.G. Ruby, unpublished observations). The advent of the capability to apply molecular cloning techniques to the bdellovibrios, coupled with the limited genetic techniques now in use (Dunn et al., 1974; Lania et al., 1976; Meier and Brownstein, 1976), is certain to be followed by a rapid advance in our understanding of the genetic mechanisms controlling bdellovibrio developmental biology.

The transfection of *Bdellovibrio bacteriovorus* with DNA isolated from the bdellovibrio-attacking bacteriophage MAC-1 (Roberts et al., 1987) has also been reported by one group (Roberts and Ranu, 1987). This procedure may be applicable to certain specialized gene-transfer problems with bdellovibrios, but has not yet been shown to allow transfer of DNA from sources other than MAC-1.

Storage of Cultures

The remarkably high endogenous metabolic rate of bdellovibrios can lead to a relatively rapid loss of culture viability at room temperature (Hespell et al., 1973, 1974). Storage of cultures in the cold (4°C) prolongs viability for weeks to months, and cell survival can be further enhanced by maintaining the bdellovibrios as bdelloplasts. Longer term storage is best achieved at temperatures well below −20°C, and with the addition of a cryoprotectant such as glycerol. It is important to note that although many laboratories maintain bdellovibrio cultures by transfer at weekly intervals, such a procedure may lead to the fixation of unknown variations in the activities or characteristics of the strain over time (Talley et al., 1987). Thus, it is best to prepare a large store of stock cultures in multiple vials, and to open a new vial for subsequent transfer of cultures at least once every few months.

Procedure for Long-Term Storage of Bdellovibrio Cultures (Gherna, 1981)

A freshly lysed culture of bdellovibrio cells is combined with an equal number of substrate cells, and incubated for 30 min at room temperature. One milliliter of the resulting bdelloplast suspension (about 10^9 bdelloplasts) is mixed with either 0.5 ml of sterile 50% glycerol or with 1 ml of a 10% solution of dimethyl sulfoxide in sterile DNB medium. The suspension is placed in a cryo-resistant plastic vial, and precooled by placing it at −20°C for 1 to 2 h. Vials can then be stored either in liquid nitrogen or in a deep freezer (−70°C) for periods of at least 10 years. The frozen specimen can be revived by diluting it at least 50-fold into a suspension of substrate cells and incubating the mixture until the resulting bdelloplasts lyse.

Metabolic Characteristics of Intracellular Growth

An important area of bdellovibrio biology concerns their metabolic adaptions to life in an intracellular environment (Rittenberg, 1983). There are several reasons behind an interest in such adaptions: 1) such adaptions help define the differentiated cell states of the attack-phase and the growth-phase bdellovibrios; 2) they have given us new insight into the range of bacterial metabolic versatility (Rittenberg and Langley, 1975); and, 3) there is evidence for considerable convergence among microorganisms that have chosen to exploit and become dependent upon the intracellular environment (Moulder, 1985; Ruby, 1989). Two classes of characteristic adaptations are among the most striking aspects of bdellovibrio intracellular metabolism: 1) a remarkably high efficiency of growth; and 2) an ability to modify the cellular structures that surround them in the bdelloplast.

All bdellovibrios examined to date are obligate aerobes. There is no obvious metabolic reason why the bdellovibrio life style should require such a restricted metabolism, and many bdellovibrios are able to use both facultative and obligate anaerobes as their substrate cells. Examination of axenically grown *Bdellovibrio stolpii* has revealed the presence of catalase, peroxidase, and an inducible superoxide dismutase activity (von Stein et al., 1982). With the exception of one study (Afinogenova et al., 1979), all indications are that the energy metabolism of most, if not all, bdellovibrios is nonfermentative (Hespell, 1976; Schoeffield and Williams, 1989; Simpson and Robinson, 1968) and is driven by a membrane-associated activity (Gadkari and Stolp, 1975; Friedberg and Friedberg, 1976) that maintains a high energy charge under aerobic conditions (Gadkari and Stolp, 1976). Preferred respiratory substrates include amino acids (Hespell et al., 1973) and the ribose moieties of transported phosphorylated nucleosides (Hespell and Odelson, 1978; Ruby et al., 1985), but not carbohydrates. The presence of glycolytic and tricarboxylic acid (TCA) cycle enzyme activities in bdellovibrio cell extracts (Hespell, 1976) suggests that the inability of these organisms to utilize sugars as a significant respiratory substrate may be due to the lack of an effective sugar transport capability (Ruby, 1989).

The efficiency with which respiration-derived energy is used to convert prey cell material into new bdellovibrio cell material has been calculated to be about twice as high as that typical of all other heterotrophic bacteria tested (Rittenberg and Hespell, 1975). It is clear, however, that the invaded cell rapidly and irreversibly loses its energy-generating and biosynthetic capabilities (Rittenberg and Shilo, 1970), and heat-killed cells are equally effective at supporting bdellovibrio growth and proliferation (Hespell, 1978; Ross et al., 1974). Therefore, the bdellovibrio achieves its high growth efficiency without the energy or biosynthetic parasitism characteristic both of viruses and of other intracellular bacteria (Moulder, 1985). Neither the physiological nor the bioenergetic basis for this phenomenon is known. However, the high growth effeciency may be due in part to the efficient coupling of energy generation and energy utilization (Rittenberg and Hespell, 1975), the unusual ability to transport and incorporate intact phosphorylated nucleosides (including ATP) from the prey (Rittenberg and Langley, 1975; Ruby et al., 1985; Ruby and McCabe, 1986), and the ability to incorporate rather complex portions of the structure of the invaded cell as anabolic precursors (Ruby, 1989).

One of the most striking features of bdellovibrio intracellular metabolism is their ability to modify the components of the prey cell structure. The results of some of these activities are new, functional structures, such as modified cell walls (Thomashow and Rittenberg, 1978d; Ruby and Rittenberg, 1984; Araki and Ruby, 1988). Alternatively, the bdellovibrio can incorporate various substances into its own growing cells: fatty acids (Kuenen and Rittenberg, 1975; Rittenberg and Langley, 1975); lipopolysaccharides (Nelson and Rittenberg, 1981a and 1981b): and possibly even intact and functional outer membrane proteins (Guerrini et al., 1982; Diedrich et al., 1983; Talley et al., 1987) derived from the invaded cell, although this last capability has been disputed (Rayner et al., 1985).

These modifications are believed to be performed by bdellovibrio-produced enzymatic activities (Engelking and Seidler, 1974; Hespell et al., 1975) that function in what was the periplasmic space of the invaded cell, altering the permeability and surface characteristics of the outer membrane (Odelson et al., 1982; Cover and Rittenberg, 1984; Ruby and McCabe, 1988), the peptidoglycan (Thomashow and Rittenberg, 1978b, 1978c, 1978d; Ruby and Rittenberg, 1984), and cytoplasmic membrane (Cover et al., 1984) of the invaded cell. Biochemical characterizations of some of these bdellovibrio activities have been reported recently (Araki and Ruby, 1988; M. Saier, personal communication).

Applications

There are three general classes of applications for which the bdellovibrios have either potential or actual use. The first of these has been described by Varon and Shilo (1981), who use the decrease in the bdellovibrio's ability to function as an attack-phase cell as an indication of the presence of certain pollutants, to which the bdellovibrios show a high sensitivity. This assay procedure is simple because the rate of bdellovibrio attack on a suspension of luminous bacteria is proportional to the loss of light emission by the cell suspension.

A second practical use, for which there appears to be some application for the bdellovibrios, is in the area of water quality. It has been suggested (Daniel, 1969; Fry and Staples, 1976; Guelin and Cabioch, 1970; Lambina et al., 1981) that the bdellovibrios may play a significant natural role in the self-purification of natural water systems. To date there has been no report of any successful efforts to supplement this natural level of activity by artificially increasing the abundance of bdellovibrios in a natural environment.

The area of application that has seen the most actual activity has been the use of members of the genus *Bdellovibrio* for the discovery of novel biochemical and physiological capabilities (e.g. transport, biosynthesis, differentiation control) that are adaptations to life in an intracellular environment (Moulder, 1985; Ruby, 1989). Recent studies, both of the biochemistry of signals involved in the control of bdellovibrio cellular differentiation (Gray, 1989) and of methods of molecular genetics (Cotter and Thomashow, 1989), have firmly established the bdellovibrio life cycle as an important model system for the study of prokaryotic developmental biology.

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CHAPTER 188

The Myxobacteria

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The myxobacteria are Gram-negative, unicellular, gliding bacteria with rod-shaped vegetative cells (Fig. 1). Because of their gliding movement, colonies develop as thin, film-like, spreading swarms, particularly on media low in organic constituents (lean media) (Fig. 2). Under starvation conditions, the myxobacteria undergo an impressive process of cooperative morphogenesis: the vegetative cells aggregate and pile up, and the resulting cell mass differentiates into a fruiting body (Fig. 3). Myxobacterial fruiting bodies show various degrees of complexity, both morphologically and structurally. They typically measure between 50 and

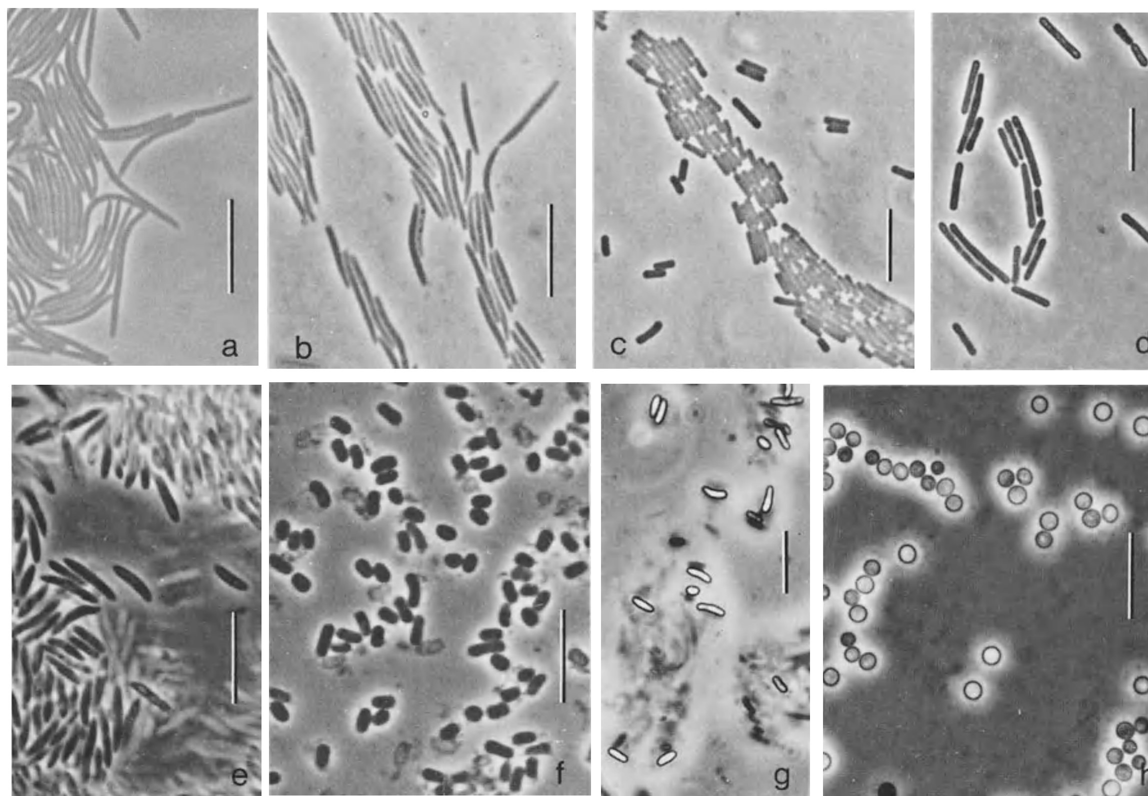


Fig. 1. Various types of myxobacterial cells. (a to d) Vegetative cells. (a and b) The Cystobacterineae type: (a) *Cystobacter ferrugineus,* cells from a liquid culture. (b) *Stigmatella aurantiaca,* cells in situ on agar surface in a chamber culture. (c and d) The Sorangineae type. (c) *Chondromyces crocatus* in a chamber culture. (d) *Sorangium compositum* in a chamber culture. (e to h) Myxospores of members of the Cystobacterineae. (e) Myxospores of *Cystobacter ferrugineus,* from a crushed, degenerated, *Archangium*-like, fruiting body; under oil immersion, the high optical refractility of the myxospores is not apparent. (f) Myxospores of *Cystobacter velatus* from a crushed fruiting body sporangiole; oil immersion. (g) Experimentally induced myxospores of *Stigmatella aurantiaca* on the agar surface in a chamber culture; under the 40× dry objective, the high optical refractility of the myxospores becomes very conspicuous. (h) Myxospores from a *Myxococcus xanthus* fruiting body; the optical refractility of these spherical myxospores is so high that it is recognizable even under oil immersion. All photographs are in phase contrast. Bars = 10 μm.

500 μm, and they can thus be easily seen with the naked eye. Within the maturing fruiting body, a cellular differentiation takes place during which the vegetative cells convert into short, fat, optically refractile myxospores (Figs. 1 and 3). The myxospores are desiccation resistant and allow the organism to survive unfavorable environmental conditions.

The phylogenetic position of the myxobacteria has been elucidated by a comparison of 16S rRNA sequences (Ludwig et al., 1983; Oyaizu and Woese, 1985). According to these data, the myxobacteria belong to the delta branch of the large assembly that has been called the purple bacteria, or the proteobacteria. The delta branch also contains the remotely related genus *Bdellovibrio* and certain sulfate reducers such as the genera *Desulfovibrio* and *Desulfonema* (Stackebrandt et al., 1988). The myxobacteria are currently assigned to twelve different genera (see "Taxonomy of Myxobacteria," this chapter).

Many myxobacterial fruiting bodies are large enough to be seen with the unaided eye and had already been observed by botanists early in the 19th century. However, they continued to be mistaken for fungi for almost a century. The first report in the scientific literature appears to be by Heinrich Friedrich Link (1809), who described a little "gasteromycete," *Polyangium vitellinum,* a taxon that is still valid. The description was accompanied by an exact and beautiful illustration (see Fig. 15) (Link, 1809; Ditmar, 1814). Two more species, *Stigmatella aurantiaca* and *Chondromyces crocatus,* were defined by the British mycologist, M.J. Berkeley (1857), who classified them as hyphomycetes (fungi imperfecti). Again, both taxa are still valid. *Stigmatella aurantiaca* was described two more times as a new fungus under different names and with different systematic positions before it was finally recognized as a myxobacterium by the U.S. botanist Roland Thaxter (1892); for the tortuous history of *Stigmatella,* see Reichenbach and Dworkin (1969). Thaxter was the first to elucidate the astonishing and unexpected life cycle of the myxobacteria, which he published, together with a substantial number of new species, in a series of brilliant articles (Thaxter, 1892, 1893, 1897, 1904). However, the reception by the scientific community was somewhat less than enthusiastic, and it took at least 20 years before Thaxter's work became widely accepted. In the first decade of the 20th century, however, four rather voluminous studies on myxobacteria were published (Baur, 1905; Quehl, 1906; Vahle, 1910; Kofler, 1913). Those articles are full of interesting observations and methods for the isolation and study of myxobacteria, and deal with many problems that later became important topics of research. It may be mentioned in passing that two of those four authors were later to become very influential scientists, one as a geneticist deeply involved in breeding research (Erwin Baur), the other as one of the founders of microchemistry (Ludwig Kofler). In the following decades, research was confined mainly to taxonomic, ecological, and morphological questions (Jahn, 1911, 1924; Krzemieniewska and Krzemieniewski, 1926, 1927a, 1927b, 1930). During that period, new species were discovered and the wide distribution of myxobacteria was recognized. Furthermore, it was recognized that there were two large subgroups that could be distinguished by the shape of their vegetative cells (Krzemieniewska and Krzemieniewski, 1928), and that some myxobacteria degrade cellulose and could be cultivated on a medium containing only cellulose and a few mineral salts (Imshenetski and Solntseva, 1936; Krzemieniewska and Krzemieniewski, 1937a, 1937b). For some time, cellulose decomposition by myxobacteria was a principal focus of interest, mainly in Russia, where it was studied mainly in the laboratory of A.A. Imshenetski (for a review, see Imschenezki, 1959).

Studies utilizing pure cultures of myxobacteria did not become common practice until the middle of the century. Also, until around 1960, it was generally believed that myxobacteria could not grow suspended in liquid media. Because of the difficulty of growing myxobacteria suspended in liquid media, their popularity with microbiologists was not high. Physiological studies were extremely tedious and only moderately conclusive, because the myxobacteria grew only as a film along the glass wall of the container and the surface of the liquid. Still, even under those experimental restrictions, some insights were gained into the nutritional requirements of myxobacteria (Norén, 1955). Loebeck and Klein (1956) and Mason and Powelson (1958) reported that at least some strains of *Myxococcus* grew well as suspension cultures. Subsequently, many more examples of myxobacteria growing in a dispersed manner have been described, so that most strains can now be handled as ordinary bacteria. Ironically, it later turned out (Dworkin, 1984) that dispersed growth of *Myxococcus* (*Mx.**) *xanthus* strains

*In the remainder of this chapter the following abbreviations sometimes are used for the genera of myxobacteria: *An., Angiococcus; Ar., Archangium; Cb., Cystobacter; Cc., Corallococcus; Cm., Chondromyces; Ha., Haploangium; Me., Melittangium; Mx., Myxococcus; Na., Nannocystis; Pl., Polyangium; Sg., Stigmatella; So., Sorangium.*

had been observed as early as 1948, but this information had never been published (Woods, 1948).

Beginning in the 1950s, the emphasis of myxobacterial research shifted more and more to problems in developmental biology, with *Mx. xanthus* as the preferred object. A major breakthrough was the discovery by Dworkin and Gibson (1964) that vegetative cells of *Mx. xanthus* in suspension cultures could be induced to convert into myxospores by the addition of certain chemicals, such as 0.5 M glycerol. This was the first clearly defined experimental system for the investigation of a morphogenetic process in a myxobacterium, and it led to a long series of studies on the morphological, physiological, and biochemical events that take place during induced myxospore formation.

Another major achievement of the past 15 years was the development in the laboratory of Dale Kaiser of an experimental system for the transfer of plasmids, transposons, and genes into and between strains of *Mx. xanthus,* based on the use of coli phage P1 and myxobacterial phages. This set the stage for the development in that organism of methods for the genetic analysis of various processes, including gliding motility and developmental programs, (for reviews, see Kaiser et al., 1979; Kaiser, 1984a, 1986, 1989). The availability of genetic techniques finally made the full potential of *Mx. xanthus* accessible as a model system for the study of morphogenetic processes on the prokaryotic level. While these methods are not easily adaptable to other myxobacteria, a more versatile system is under study that uses conjugation with *Escherichia coli* for the transfer of plasmids and transposons (Breton et al., 1985). This system has already been successfully applied to myxobacteria other than *Mx. xanthus.*

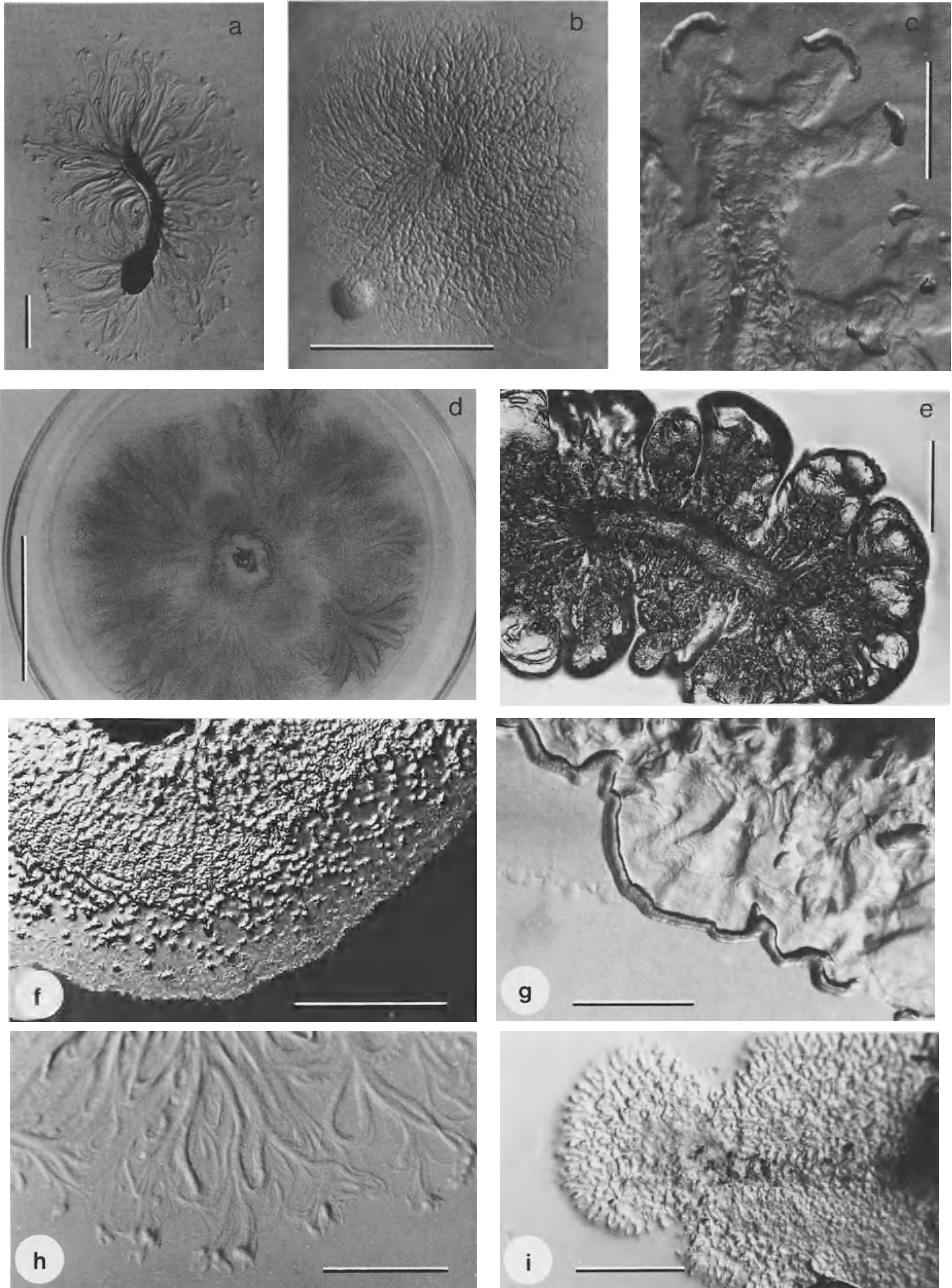
After this solid methodological basis was laid, progress in myxobacterial research gained considerable momentum. Completely unexpected findings have been made in the molecular biology of myxobacteria, e.g., the discovery of a multicopy single-stranded RNA-linked DNA (Dhundale et al., 1987), and the presence of two reverse transcriptases in *Mx. xanthus* (Inouye et al., 1990). Also, the discovery that many myxobacterial strains produce secondary metabolites, most of which are novel compounds, which may have practical applications, will most likely stimulate an even broader interest, in myxobacterial biochemistry, physiology, and taxonomy (for reviews, see Reichenbach et al., 1984, 1988; Reichenbach and Höfle, 1989).

Our knowledge of the myxobacteria has been summarized repeatedly in recent years. Apart from the articles mentioned above, the following reviews should be consulted for further details: 1) for general surveys and taxonomy—Rosenberg, 1984; Reichenbach, 1986; McCurdy, 1989; 2) for molecular biology—Sumiko Inouye et al., 1987; 3) for developmental biology—White, 1975, 1981; Zusman, 1980, 1984, 1990; Kaiser, 1984b; Dworkin, 1985, 1990; Dworkin and Kaiser, 1985; Shimkets, 1987, 1990b; and 4) for biotechnology—Reichenbach, 1988. In addition, several movies are available from the Institut für den wissenschaftlichen Film, Göttingen, Germany, that illustrate cell behavior, colony formation, and developmental processes in various species of myxobacteria (Reichenbach et al., 1965a, 1965b, 1965c, 1965d, 1980; Grimm et al., 1971; Kühlwein et al., 1971a, 1971b).

Habitats

The principal habitats of myxobacteria are soil, dung, decaying plant material, and the bark of living and dead trees. In addition, they are sometimes found in places where they most probably cannot live, but where their resting stages can survive for some time. Thus, several

Fig. 2. Swarm colonies of various myxobacteria. (a, b, d, f, and h). Swarms of members of the Cystobacterineae. (c, e, g, and i). Swarms of members of the Sorangineae. (a) *Stigmatella erecta* on CY agar; dissecting microscope, oblique illumination. Bar = 1 mm. (b) *Myxococcus xanthus,* very young colony on Casitone agar; dissecting microscope, oblique illumination. Bar = 0.5 mm. (c) *Polyangium* sp. on water agar, swarm edge; the spreading colony falls apart into cell clumps, which separate, thereby etching trenches into the agar surface; dissecting microscope, oblique illumination. Bar = 1 mm. (d) *Cystobacter violaceus* (formerly *Archangium violaceum*) on VY/2 agar; from the inoculum in the center, the swarm has spread over the whole agar plate. Bar = 30 mm. (e) *Nannocystis exedens* on Casitone agar; the swarm developed from the ridge in the center, and the agar plate has become deeply corroded; Leitz Aristophot. Bar = 5 mm. (f) *Corallococcus coralloides* on Casitone agar; the central part of the swarm is covered with rudimentary fruiting bodies; Leitz Aristophot; Bar = 5 mm. (g) *Polyangium* sp. on a streak of *E. coli* (which is still recognizable at the left) on water agar; the swarm edge is a compact, ridge-shaped mass of vegetative cells, behind which the agar surface is deeply corroded; dissecting microscope, oblique illumination. Bar = 1 mm. (h) *Stigmatella erecta* on Casitone agar, swarm edge with flamelike protrusions; dissecting microscope, oblique illumination. Bar = 1 mm. (i) *Nannocystis exedens* on a streak of *Micrococcus luteus* on water agar; the entire swarm has sunk into the agar, which is deeply corroded; dissecting microscope, oblique illumination. Bar = 2 mm.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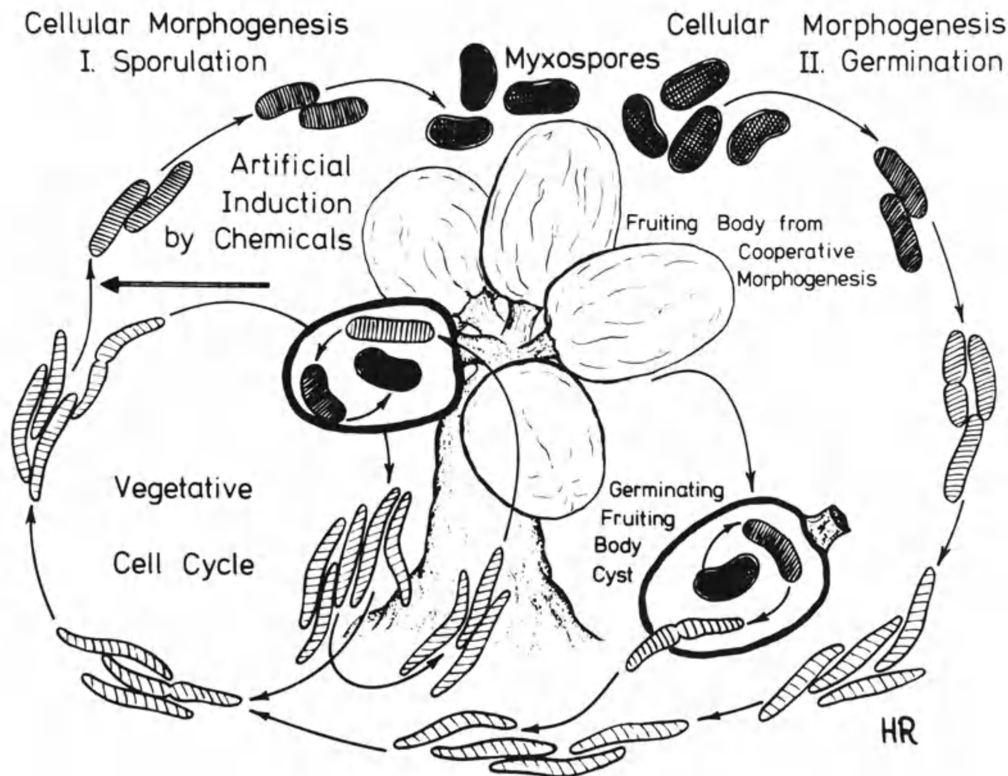


Fig. 3. Cellular morphogenesis in myxobacteria, shown with *Stigmatella aurantiaca* as an example. Fruiting body and cells are not drawn to scale. (From Gerth and Reichenbach, 1978.)

species of myxobacteria have been isolated from seashore sediments (Brockman, 1967; Rückert, 1975b), although no myxobacterium is known that is able to grow at the concentration of salt found in seawater. Typically, in samples with a high salt content, such as salt marshes or certain desert soils, myxobacteria can only be demonstrated when the sample is first desalted before being cultured on a low salt medium (Rückert, 1983). The occurrence of myxobacteria in the peat of sphagnum bogs at a pH of 3.7 and at the low oxygen content of 2.6 mg/liter is also remarkable (Dawid, 1984), because, in cultures, myxobacteria are strict aerobes and without exception do not grow at a pH below 5.5. Of course, in complex habitats the existence of inhomogeneities among the microniches is always conceivable and could explain the presence of myxobacteria under seemingly unacceptable conditions. Another peculiar habitat is the leaf surface, or phyllosphere, of plants (Rückert, 1981). No leaf was sampled higher than 2 m above ground, but all 14 plant species investigated and 52% of the specimens were positive, mostly for *Myxococcus* species. Again, there is no proof that the myxobacteria can really grow on plant leaves, although this may not be totally inconceivable. The presence of myxobacteria deep within caves (Menne and Rückert, 1988) may seem less astonishing because the organisms could easily be washed in and then grow or remain dormant, depending on the local conditions.

A survey of common sources for different myxobacteria is given in Table 1. Most myxobacteria appear to be primarily soil organisms. However, almost all are "micropredators" (Singh, 1947) and are attracted by habitats with rich microbial communities, which they may colonize as secondary substrates. Even if certain myxobacteria can be isolated more easily from sources other than soil, their presence in the soil of the same environment can often be demonstrated. The preferred substrate of a particular species may vary under different climatic and edaphic conditions. Thus, in the central USA, *Chrondromyces* species are common inhabitants of bark and rotting wood, but are rare in soil (Nellis and Garner, 1964; H. Reichenbach, unpublished observations). In India, they are found regularly in soil, in the rhizosphere of plants, on rotting wood, bark, and dung (Agnihothrudu et al., 1959; Singh and Singh, 1971). In Europe, in spite of apparently similar climatic conditions to those found in the USA, they appear to be extremely rare organisms and

Table 1. Convenient and preferential sources for the isolation of different myxobacteria.[a]

Substrate	Myxobacterial species typically found[b]
Soil	*Nannocystis exedens*,[5] *Sorangium cellulosum*,[4] *Archangium serpens*,[4] *Corallococcus coralloides*,[4] *Polyangium* spp.,[3] *Cystobacter* spp.,[3] *Melittangium* spp.,[3] *Myxococcus fulvus*,[2] *Mx. virescens*,[2] *Mx. stipitatus*[2]
Dung of herbivores	*Myxococcus fulvus*,[5] *Corallococcus coralloides*,[5] *Mx. virescens*,[4] *Cystobacter fuscus*,[4] *Cb. ferrugineus*,[4] *Archangium serpens*,[4] *Nannocystis exedens*,[3] *Cb. violaceus*,[3] *Polyangium* spp.,[3] *Stigmatella erecta*,[2] *Mx. xanthus*,[2] *Melittangium* spp.,[2] *Cb. velatus*[1]
Bark and rotting wood	*Stigmatella aurantiaca*,[4] *Chondromyces apiculatus*,[4] *Sorangium cellulosum*,[4] *Corallococcus coralloides*,[4] *Myxococcus fulvus*,[3] *Cm. pediculatus*,[2] *Haploangium* spp.[2]

[a]As pointed out in the section on "Habitats," the frequency of myxobacteria on a specific substrate may vary substantially in different environments. Also, some species may be underrepresented from a certain source because the isolation technique usually applied may not result in the isolation of the particular organism.
[b]Frequency of the encountered species: [5], ubiquitous; [4], very frequent; [3], moderately frequent; [2], relatively rare; [1], rare.

have been found almost exclusively on dung, rotting wood, and bark (Zukal, 1896; Quehl, 1906; Jahn, 1924; Krzemieniewska and Krzemieniewski, 1946). Dawid (1979) tested several thousand samples from the Siebengebirge, a mountain ridge near Bonn, Germany, and obtained only one isolate of *Chondromyces,* a strain of *Cm. apiculatus,* from a piece of rotting wood. Similar results have been obtained by Krezemieniewska and Krzemieniewski (1946) and by one of us (H. Reichenbach) who isolated myxobacteria over decades from all kinds of samples collected at many different sites in Europe.

Myxobacteria are very common in soils of neutral or slightly alkaline pH (Brockman, 1976; Brockman and Boyd, 1963; Dawid, 1978, 1979; Hook, 1977; Kühlwein, 1960; Krzemieniewska and Krzemieniewski, 1927b; McCurdy, 1969a; Michoustine, 1968; Norén, 1950, 1952; Peterson, 1965; Peterson and Norén, 1967; Rückert, 1975a, 1976, 1979; Rückert and Heym, 1977; Sabadoš-Šarić, 1957). They have been isolated from samples collected in tropical rain forests and in the arctic tundra, in steppes, deserts, and in bogs, at sea level as well as at high altitudes. Warm and dry areas, such as the southwestern United States, Mexico, northern India, the Mediterranean countries, and the Canary Islands, have been found to be especially rich in different myxobacteria. The same is true for areas with underground limestone in temperate climates. Still, a myxobacterial species may show an unequal distribution even within the same climatic zone. The case of *Chondromyces* has just been mentioned. Another example is *Stigmatella aurantiaca,* which is common in the middle of the North American continent (Nellis and Garner, 1964; Reichenbach and Dworkin, 1969; McCurdy, 1969a), but very rare in Europe (Krzemieniewska and Krzemieniewski, 1946, isolated myxobacteria in Europe for 15 years before they found their first strain of *Sg. aurantiaca*) The decisive factor that controls the distribution may be the warm, humid American summers which are clearly contrasted in that respect with those in Europe. For unknown reasons, *Sg. aurantiaca* seems to be restricted to rotting wood and bark on both continents. Locations with acid soils and raw humus underground, such as the *Rhododendron* forests of West Virginia (H. Reichenbach, unpublished observations), can be totally devoid of myxobacteria. But under otherwise favorable conditions such as sufficiently high humidity and temperature, myxobacteria have also been isolated from soils with a bulk pH of below 5 (Rückert, 1975a, 1979). In cool, humid environments, such as in Finland, northern Minnesota, and mountain ranges, myxobacteria may be abundant, albeit limited to a few species. Tropical rain forests are not necessarily rich in myxobacteria, perhaps due to the nature of the underground material. Myxobacteria have also been isolated from Antarctic soil samples but at least in one case, the organisms had a temperature optimum around 30°C, which raises some doubts as to whether they were really indigenous (Rückert, 1985). In another instance, however, the isolated bacteria were true psychrophiles that developed, very slowly indeed, at temperatures between 4 and 9°C but did not grow at 18°C (Dawid et al., 1988). While the vegetative stages of the latter organisms closely resembled myxobacteria of the *Polyangium* and *Nannocystis* type, their identify could not be established with certainty since they did not produce fruiting bodies. No mesophilic myxobacteria were found in the Antarctic samples examined by Dawid et al. (1988).

Dung of various animals, especially of herbivores such as rabbit, hare, deer, moose, sheep, and goat, is an excellent substrate for myxobacteria. Rabbit dung in particular was the preferred source of myxobacteria for the early investigators, and in fact appears to serve as a

kind of natural bait. It seems, however, that myxobacteria can also pass unscathed through the digestive tract of animals (Kühlwein, 1950). Also, the observation of myxobacteria on dung pellets collected on the surface of deep snow layers suggests that, occasionally, they are deposited with the dung (Rückert, 1975a; H. Reichenbach, unpublished observations). However, the organisms primarily appear to arise in the surrounding soil after the dung has been dropped. This conclusion is based on the fact that aged dung is a better source of myxobacteria than is fresh dung, that dung collected on soil rich in myxobacteria results in a greater yield of strains than one from poor soil, and that the same species found on dung can also be found in the surrounding soil. A number of myxobacteria are regularly found on dung, such as *Myxococcus fulvus, Mx. virescens, Corallococcus coralloides, Cystobacter fuscus,* or *Stigmatella erecta,* but there is no myxobacterium that depends on dung-derived growth factors, as was thought for some time, and all strains isolated from dung can be cultivated on relatively simple media. Of course, occasional stimulation by substances such as vitamins found in dung extracts is conceivable.

Bark and rotting wood are good sources of certain myxobacteria. This includes the cellulose degraders of the genus *Sorangium,* but several noncellulolytic species are also regularly found in those habitats. Results vary with different species of trees: In Europe, relatively good yields have been reported with bark from living elder (*Sambus racemosa*), beech (*Fagus silvatia*), and black locust (*Robinia pseudacacia*) (Dawid, 1979). In Minnesota and other locations in the USA, bark and rotting wood are reliable sources for *Stigmatella aurantiaca* and *Chondromyces apiculatus.* The latter has also been found regularly on wood samples from southeast Asia, and on dry, rotten "leaves" of *Opuntia* cacti from the Canary Islands (H. Reichenbach, unpublished observations). The difference between the situation in Europe has already been pointed out. Clearly, there are other, less obvious, factors, perhaps climatic ones, that also play a role in the distribution of myxobacteria. However, there are no myxobacteria with an absolute dependence on bark and wood. The cellulose degraders are also common in soil and dung, and because the other species are bacteriolytic, they often grow well on dung, and, in culture, on media that contain peptone or protein. In fact, it is not understood why in nature they grow preferentially on wood and bark. Even when they are found on dung, they may be there mainly because the dung of herbivores is always abundant in plant residues. However, *Haploangium* has only been found on bark and wood so far. It has never been cultivated, so its nutritional requirements are unknown.

Although myxobacteria have repeatedly been isolated from freshwater habitats (Brauss et al., 1967, 1968; Geitler, 1925; Gräf, 1975; Hook, 1977; Jeffers, 1964; Tržilová et al., 1980, 1981), it seems probable that they are merely washed in from the soil. Myxobacteria have been used to classify rivers for the type of pollution present (Gräf, 1975; Tržilová et al., 1980, 1981). While myxobacteria appear not to be typical aquatic organisms, our experience with cultivated strains gives no reason to believe that they cannot survive and thrive in suitable niches in aquatic environments. A clear indication of this is the case of parasitism of a myxobacterium on the green alga *Cladophora,* with fruiting body formation inside the emptied-out algal cells (Geitler, 1925). Another example may be the occurrence of structures resembling myxobacterial fruiting bodies on eggs of the water beetle *Dytiscus marginalis* (Jackson, 1959).

Isolation

Although myxobacteria are common in many types of soil (a teaspoonful of soil is usually sufficient to isolate four or five species) they are rarely mentioned in articles on soil microbiology. The reason is that the usual dilution and plating techniques used for the isolation of soil microorganisms are unlikely to reveal the presence of myxobacteria. Due to the slime matrix they produce, myxobacterial cells do not disperse easily when the soil sample is shaken in water, so that the organisms are highly underrepresented in number when the suspension is plated. Even when other, more suitable methods are used, the estimated numbers probably are only approximate ones; depending on the type of soil, they are in the range of 10^3 to 4.5×10^5/g, and thus under favorable conditions seem to be a rather substantial component (McCurdy, 1969a; Sabadoš-Šarić, 1957; Singh, 1947). Another problem is that, on lean media, the myxobacterial colonies develop as delicate, spreading swarms that are easily overlooked, while on rich media they remain compact and are not recognized as myxobacteria. In addition, they grow relatively slowly so they are often overgrown by other soil microorganisms, particularly fungi. The fruiting bodies, however, are conspicuous and have probably been frequently observed by soil biologists and myxologists.

The isolation of myxobacteria can start from fruiting bodies that have developed on natural substrates, or from swarms growing in agar cul-

tures. These isolations can also be preceded by enrichment and baiting techniques. The methods have frequently been reviewed (Kühlwein and Reichenbach, 1965; McCurdy, 1969a; Peterson, 1969).

Collection and Treatment of Samples

Soil to be used for the isolation of myxobacteria should be taken from the upper few centimeters of the soil profile. Soils rich in higher organisms, and samples collected from between plant roots and near the base of stems, give the best yields. If the material cannot be processed soon after collection, it should be air-dried as quickly as possible because otherwise the sample may become moldy, creating problems later during isolation. A few cubic centimeters of soil are sufficient for most isolation techniques. In general, it is better to have several small samples from different localities than one large batch from a single spot. Dry material can be stored for long periods at room temperature. It appears that, in the natural substrate, myxobacteria are much more stable than, for example, fruiting bodies dried on filter paper. Thus, we have found essentially the same species over a 12-year period of sampling of stored soil samples, and we are regularly able to isolate myxobacteria from samples stored for 10 to 15 years (H. Reichenbach and M. Dworkin, unpublished observations).

With dung, better results are usually obtained if the samples are neither very fresh nor very old, but are completely free of low-molecular weight components. It is very important to dry the samples if they have to be stored for more than one day, and such dry dung will yield myxobacteria even after months and years of storage. Rabbit dung to be used for baiting (see below) should be taken from wild animals; pellets from laboratory or domestic rabbits are usually unsuitable, perhaps because they become soaked with urine in the cage.

Bark and wood for the isolation of myxobacteria must be taken from tree species that are low in resins and tannin compounds, and specimens have to be quickly dried if they are not to be processed immediately. Successful isolations are often obtained with wood that has already been decomposed to a rather soft state, and with bark from the base of the tree and from fallen trees.

Direct Isolation from Natural Substrates

Isolation can often be achieved directly from the natural substrate. Bark of living or dead trees, rotting wood, or dung pellets are kept in large petri dishes lined with two to three layers of filter paper. The samples are soaked in distilled water containing cycloheximide (up to 0.08 mg/ml) for a few hours to suppress the growth of molds. The water is then decanted and appropriate amounts of water are added at intervals during cultivation. If the culture is kept too dry, it may soon be covered with molds; if the substrates are inundated, no myxobacterial fruiting bodies will develop. The air in the incubator should be kept sufficiently humid, so that the crude cultures do not dry out too quickly. The initially saturated system is allowed to dry out gradually over a period of 14 to 21 days; in this way it will eventually pass through the optimum for the development of myxobacterial fruiting bodies. Such crude cultures are likely to contain a variety of fungi, mites, springtails, nematodes, and other organisms that may contaminate other cultures. The danger of a contamination with mites is especially serious and can quickly spoil a culture collection. The cultures should therefore be kept in tightly closed plastic bags or, more conveniently, in a separate incubator, which should frequently be sterilized.

The cultures can be incubated at room temperature (around 20°C) or at 30°C. We have never observed unequivocal differences in the yield of myxobacteria when parallel crude cultures were kept at different temperatures, but we have found that at room temperature, the development of myxobacteria proceeds more slowly and molds tend to spread more vigorously. Thus, we keep such cultures at 30°C (see also Krzemieniewska and Krzemieniewski, 1927b). However, samples from mountains at high altitudes or from arctic environments may contain myxobacteria that are adapted to lower temperatures (see "Habitats," this chapter), and even in forest soils in Central Europe strains are occasionally found that grow reasonably well at 30°C but produce fruiting bodies only at lower temperatures.

Initially, the crude cultures should be inspected for fruiting bodies every day, beginning with the third or fourth day of incubation, because fruiting bodies often spring up and then rapidly collapse and become very inconspicuous. Also, the fruiting bodies may soon become overgrown by other organisms. Most fruiting bodies will appear during the first 10 days, either on the substrate itself or on the filter paper lining; only rarely will anything of interest develop later than 18 to 20 days. Sometimes, fruiting bodies appear very early on the substrate, within hours after moistening; probably, in such cases, desiccated structures have simply been rehydrated and thus have become recognizable, while the fruiting bodies that appear later are produced de novo. In the later phases

of culture development, spherical or ridge-shaped masses of vegetative cells, which are usually bright yellow, orange, or red, can often be observed on the filter paper and can be used for isolation; in this way one may obtain species whose fruiting bodies are too small and inconspicuous to be recognized directly on the substrate. These organisms are almost always members of the Sorangineae, usually *Polyangium* and *Nannocystis* strains, which produce swarms with a massive ridge at the edge. Sometimes, cellulose decomposers may develop on the filter paper lining itself.

Isolation from Soil by Baiting

A baiting technique can be used to isolate myxobacteria from soil (Krzemieniewska and Krzemieniewski, 1926). A large petri dish is filled with soil which is moistened with distilled water up to its water-holding capacity. Autoclaved dung pellets from wild rabbits are partly buried in the soil. The culture is then incubated and examined as described above. Vegetative cells will migrate to the dung pellets, colonize them, and in time develop the characteristic fruiting bodies. Here, as with the technique described above, the discovery of the myxobacterial fruiting bodies is greatly facilitated by the fact that they tend to appear locally in large numbers and in addition often are brightly colored and glistening. With some experience, it is possible to locate them quickly with the unaided eye, despite their relatively small size (50 to 500 μm). For a more careful examination of the culture, a dissecting microscope with incident illumination and a magnification between 10 and 40 × is perfectly adequate (see Fig. 2).

Isolation from Swarms

The bacteriolytic properties of myxobacteria can be used for their enrichment from their natural substrates, particularly from soil and plant debris. Streaks of living food organisms on water agar (WAT agar, see below), either in the form of a cross-streak, three parallel streaks, or a number of circular patches, are inoculated with a small quantity (approximately the size of a lentil) of the material to be analyzed (Singh, 1947). The cross and the patches are inoculated in the center, the parallel streaks at the ends. Care should be taken not to scatter the inoculum over the plate. To facilitate application of the sample, the soil may first be moistened with sterile water and then applied with sterile swabs.

To restrict the development of fungi, the addition of the antibiotic cycloheximide (25–100 μg/ml) to the enrichment medium has been recommended (WCX agar, see below; Brockman and Boyd, 1963). Results are indeed superior when cycloheximide is included, and the cultures can be maintained for a longer period of time. The antibiotic does not control the growth of amoebae, a major nuisance with this isolation technique. Also, it does not result in the selection of particular myxobacteria or in the suppression of any other gliding bacteria, such as *Cytophaga*-like bacteria, flexibacters, *Taxeobacter*, or *Herpetosiphon*, that can also be isolated with this method.

WAT Agar and WCX Agar

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved. After autoclaving, 25 μg cycloheximide may be added per ml from a filter-sterilized stock solution, yielding WCX Agar.

Many bacteria and yeasts can be used as food organisms by the myxobacteria. Food organisms that are readily recognized and can be easily eliminated later are, of course, preferred. Selection for myxobacteria works better if the prey microbes used are alive rather than dead. In the first study, an *Aerobacter* strain was used as the food organism (Singh, 1947). We have found the following bacteria to be useful: 1) *Micrococcus luteus* is easy to distinguish, is nonmotile, and is relatively large, so that it is not readily spread around; while it is not well utilized by soil amebae, neither will all strains of myxobacteria, specifically *Nannocystis* strains, grow on it. 2) *Escherichia coli* is a favorable substrate for myxobacteria, including *Nannocystis*, which can be subcultured indefinitely on it; but soil amebae also use it well, and this sometimes makes *E. coli* inconvenient. 3) Autoclaved yeast, *Saccharomyces cerevisiae*, can be cheaply prepared from commercial bakers' yeast; the pH of the suspension must be adjusted to 7.0, since the yeast cake is usually acidic; unfortunately, the autoclaved yeast is also a good substrate for many contaminants. Generally, living *E. coli* is our preferred food organism.

The cultures are incubated at 30°C and checked under a dissecting microscope for the appearance of myxobacterial swarms and fruiting bodies, at first daily, beginning at the second or third day, and later at longer intervals (See Fig. 2). After about 3 weeks, no additional myxobacteria are likely to appear, and the cultures can be discarded. Most myxobacteria appear within 8 to 14 days. The myxobacteria grow first on the lawn of the food organism, but later often spread over the agar surface between the smears. A fast-spreading organism such as

Cystobacter may reach the end of a streak within 1 to 2 days. If two streaks are made in a crossed pattern, different myxobacteria may develop on each of the four arms. Different organisms also may follow one another on the same streak. As a rule, species of the Cystobacterineae are seen first, while the Sorangineae develop later. Between two and five different myxobacteria can be expected on one plate. Three parallel plates are usually sufficient to secure most of the myxobacterial types that are readily isolated from a sample, with the exception of the cellulose degraders. Fruiting bodies are often produced within the swarms after a couple of days. Fruiting bodies may also appear on the soil crumbs or other particles of the inoculum. To be able to detect the delicate, filmlike, swarms of myxobacteria, one has to use a dissecting microscope with a tiltable mirror so that light can be applied at a shallow angle to the surface of the plate.

The sooner a swarm is recognized, the more easily a myxobacterium can be isolated, as at the beginning, the contaminants may still be confined to the site of the inoculum. Transfers are then made from the swarm edge, which is usually the purest part of the colony.

A disadvantage of isolating myxobacteria from the swarm stage is that most species cannot be identified by their swarm morphology. With some experience, however, at least certain genera or types can be differentiated (see Fig. 2). The ubiquitous *Nannocystis,* for example, produces a network of trails outside the streak of the food organism. These trails are deeply etched into the agar and end with a small cluster of cells. On the streak, there is often a heavy, meandering ridge. *Polyangium* shows a similar pattern, but with coarser structures. Swarms with an edge that consists of a massive, often brightly colored ridge are in general typical for the suborder Sorangineae. *Cystobacter* and *Archangium* swarms are usually tough slime sheets with a pattern of delicate, but conspicuous, branched, radiating veins. The swarms of the Myxococcaceae tend to be smooth, softslimy sheets, often with dense fields of tiny waves or ripples. If there are radial veins, they typically are wavy and meandering. Swarms of *Mx. stipitatus* show a bright yellow fluorescence in ultraviolet (UV) light of 366-nm wavelength (Lampky and Brockman, 1977). The shape of the vegetative cells under the microscope may also provide a clue to the identification of the swarm observed.

Isolation of Cellulose Decomposers

For the isolation of cellulose-degrading myxobacteria of the genus *Sorangium,* a mineral agar with cellulose as the only carbon source is used. While NH_4^+ is an excellent nitrogen source for *Sorangium,* much better results are obtained when NO_3^- is used in the isolation medium, as in ST21 agar. The cellulose is applied in the form of sterile filter paper which is placed on top of the agar surface. Since the cultures have to be incubated for a longer time before the cellulose decomposers can be recognized, it is essential to include cycloheximide in the medium.

ST21 Agar and ST21CX Agar

Solution A:

K_2HPO_4	0.1%
Yeast extract (Difco)	0.002%
Agar	1%

Make up in about two-thirds of the water volume.

Solution B:

KNO_3	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$FeCl_3$	0.02%
$MnSO_4 \cdot 7H_2O$	0.01%

Make up in the remaining water volume.

After autoclaving separately, solutions A and B are combined and 1 ml of trace element solution (see below) is added per liter of medium.

For crude and enrichment cultures, 25 μg of cycloheximide is added per ml from a filter-sterilized stock solution yielding ST21CX agar.

Trace Element Solution (Drews, 1974)

The following formulation is concentrated 10-fold over the original one given in Drews (1974).

$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2$	20 mg
$CuSO_4$	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$ZnCl_2$	20 mg
LiCl	5 mg
$SnCl_2 \cdot 2H_2O$	5 mg
H_3BO_3	10 mg
KBr	20 mg
KI	20 mg
EDTA, Na-Fe^{3+} salt (trihydrate)	8 g

When dissolved in 1 liter of water and filter sterilized, this solution is stable for months at room temperature. The mixture is used at 1 ml/liter medium.

The filter paper is inoculated in the center with a pea-sized amount of soil or plant debris, either in one spot or in the form of a streak. The cultures are incubated at 30°C. Unequivocal cellulose decomposition cannot be expected before 8 to 10 days of incubation, and most *Sorangium* strains are seen only after 10 to 20 days. They are recognized as bright, more or less translucent patches, which are yellow, orange, brown, or even black, often several cm

across, and consisting of macerated cellulose, slime, and usually masses of densely packed fruiting bodies. The latter are responsible for most of the color, but also the vegetative parts of the swarms may be tinged yellow and orange, and at the edges, dense, orange ridges often develop. Besides the sorangia, other cellulolytic bacteria may also appear. The lysed areas always contain complex mixtures of a variety of bacteria, protozoa, and nematodes. In such cultures, particularly at later stages, often noncellulolytic myxobacteria also develop which obviously live off the cellulose degraders (Brockman and Boyd, 1963). They appear in the form of long, heavy, orange ridges, which are the edges of expanding swarms, and are usually strains of *Nannocystis, Polyangium,* or *Chondromyces.* Often fruiting bodies are also produced, not only in the macerated areas, but also on the edges of the intact filter paper, on the inoculum, and on the agar just beyond. Of course, these latter myxobacteria, not being cellulolytic, have to be subcultured on streaks of food bacteria and not again on filter paper. As the fruiting bodies observed in the lysis zones are not necessarily those of the cellulose degraders, in case of doubt, subcultures are best started both from fruiting bodies and from the swarm edge, using both types of substrates.

Isolation from Fresh Water

Sediments and plant and animal debris collected in lakes, ponds, rivers, etc., may be processed in the same way as described above. If free-floating myxobacteria are to be isolated, between 10 and 1000 ml of water is passed through a sterile membrane filter of small pore size (0.15 to 0.45 μm). The filter is then placed right-side up on the surface of rabbit dung agar (another lean medium, e.g., VY/2 agar with a reduced yeast content or CY agar with a lowered peptone concentration would also do). The cultures are incubated at a suitable temperature, e.g., 34°C. After 4 to 5 days, myxobacterial swarms and fruiting bodies may be observed (Gräf, 1975).

Rabbit Dung Agar

20 g of dry rabbit dung (wild rabbits) is boiled in 1 liter of distilled water for 20 min. The filtrate is made up to 1 liter again, and 1.5% agar is added. The pH is adjusted to 7.2, and the medium is autoclaved.

In summary, the various direct isolation techniques all have certain limitations, and each may be more or less appropriate for the isolation of a specific organism. For the cellulose degraders there is little choice. However, with some experience, they may also occasionally be found on dung pellets. Conversely, the cellulose plates are also useful for the isolation of *Chondromyces, Stigmatella, Polyangium,* and *Nannocystis.* While *Myxococcus* is most easily obtained on dung pellets, *Cystobacter fuscus, Stigmatella erecta, Archangium, Corallococcus, Chondromyces,* and *Melittangium* are also often found with this technique. The most generally effective technique is the use of bacterial smears; under these conditions, myxobacteria with small and inconspicuous fruiting bodies as well as strains that do not form fruiting bodies at all under the culture conditions, are reliably isolated. Bark and rotting wood incubated in a moist chamber are in some cases excellent sources for fast and reliable isolation of *Stigmatella aurantiaca* and *Chondromyces* species.

Purification Methods

In the simplest case, pure cultures of myxobacteria can be obtained in one step from fruiting bodies, which usually contain an uncontaminated population of myxospores, at least as long as they are young. By carefully transferring material from fruiting bodies to a suitable agar medium, such as CY or VY/2 agar (see below), pure swarms can be obtained. An excellent tool for such delicate work is a glass rod drawn out into a fine tip. More convenient for a swift operation is the sharp point of a disposable, 1-ml, injection syringe. Direct isolation from fruiting bodies is possible with *Myxococcus* species, because they produce soft-slimy fruiting bodies, and enough myxospores to start a culture can be obtained just by touching the fruiting body at the top. The fruiting bodies of *Chondromyces* and *Stigmatella* consist of sporangioles which are borne on a relatively long stalk that lifts them above the heavily contaminated substrate surface. The sporangioles can be plucked off and used as an inoculum. With all other myxobacteria, the chance of a selective transfer from fruiting bodies are low because they are too intimately interwoven with the substrate and the slime layer of the swarm.

VY/2 Agar

Bakers' yeast	0.5% (by weight of commercial yeast cake)
$CaCl_2 \cdot 2H_2O$	0.1%
Cyanocobalamin	0.5 μg/ml
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved. The yeast may be stored as an autoclaved stock suspension for several weeks. In order to obtain a uniform suspension of the yeast cells in the agar medium, the yeast must be added to the molten medium.

CY Agar

Casitone (Difco)	0.3%
Yeast extract (Difco)	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

ENRICHMENT TECHNIQUES. If direct isolation is not possible, enrichment techniques have to be applied. Fruiting bodies or vegetative cells are transferred to streaks of a food organism on WCX agar. three parallel streaks can be made on one plate, and each streak can be inoculated at both ends. As the swarm is often firmly attached to the substrate or forms a very tough slime sheet, from which tiny bits cannot easily be removed, or because the myxobacteria migrate within the agar, the inoculum often must be cut out with a small piece of agar using the tools mentioned above. An inoculation loop would be much too coarse, and would risk carrying over too many contaminants. The bacterial smears are best inoculated at their edge so that the developing swarm also has a chance to spread over the free agar surface, which sometimes gives a better separation from the contaminants. New transfers can be made from the purest-looking sections of the developing swarms, preferentially from the swarm edge, until swarms are obtained that seem pure enough to justify a transfer to VY/2 or CY agar. A transfer to smears of autoclaved *E. coli* on WCX agar may be necessary as an intermediary step, particularly if the myxobacterium grows only *within* the streaks of the living *E. coli*. Much time can be saved later if the cultures are initially transferred within short intervals (1 to 2 days), before the contaminants have multiplied and spread. While it is advisable to make several transfers from the crude culture—by inoculating all three streaks of the enrichment plate, if possible from different sites—in order to enrich for the organism and to have a choice of contaminants to deal with, transfers can also be made one at a time, so that the three streaks of a plate can be used consecutively.

It may be mentioned that sometimes transferred fruiting bodies do not germinate. This may happen, for example, if fruiting bodies found on a dung pellet have erroneously been identified as a bacteriolytic species (which is indeed the case with almost all dung organisms) and therefore were transferred to a bacterial smear, while in reality the fruiting bodies had been produced by a cellulose degrader. The latter will readily develop if inoculated to filter paper on ST21 agar (see "Isolation of Cellulose Decomposers," this chapter). However, there are also bacteriolytic myxobacteria that do not always germinate on bacterial streaks, e.g., *Cystobacter fuscus*. With these strains it sometimes helps to transfer fruiting bodies directly to VY/2 agar. After germination, the swarms should be inoculated as soon as possible on bacterial streaks, because many contaminants can grow vigorously on VY/2 agar. If a sufficient quantity of fruiting bodies can be obtained from the crude culture, some of them should first be heated in water (see "Purification by Heating," this chapter) before they are inoculated to the growth medium. It should also be kept in mind that there is at least one myxobacterium (*Haploangium*) that will not germinate on any known medium and has not been cultivated so far.

If the myxobacteria are left growing on the streaks for a longer time, they usually will produce fruiting bodies. Fruiting bodies can be very useful as starting material for further, more sophisticated purification steps. Also, fruiting bodies are important for the identification of the isolated strains.

The main contaminants in the enrichment cultures are other bacteria. Occasionally, other gliding organisms, more often small Gram-negative rods, become trapped in the slime of the swarm and be carried around by the moving myxobacteria. These two types of contaminants are often difficult to eliminate. Nematodes, fungi, and especially soil amebae may also become a serious problem.

The first step in purification is to eliminate all animals from the culture because they move very actively and thus quickly spread bacterial contaminants over the plate. The transfer of nematodes can usually be avoided if some caution is observed, because they are large enough to be easily recognized and eliminated. Occasionally, however, a culture is heavily infested with nematodes; or the worms cannot be seen because an opaque substrate is used, e.g., cellulose plates. In these cases, the plate can be frozen by simply storing it in a −80°C deepfreeze for one or two days, which seems to kill all nematodes. After thawing the plate, it is important to make a transfer to a fresh plate immediately after the surface of the plate has softened sufficiently, because the thawed agar will be very runny and soft (Reichenbach, 1983). The amebae, which sometimes multiply extremely fast, can also be killed by exposing the plate to ammonia vapors (M. Aschner, personal communication). We use a 5% ammonia solution in a petri dish and place the culture plate upside down over the ammonia dish for 1 to 2 min. The plate is then left with a closed lid for another 2 to 5 min, and then the myxobacteria are transferred to a fresh plate because the old one is now strongly alkaline. The myxobacteria

usually survive the treatment very well, even when no fruiting bodies are present. A further transfer should be made as soon as possible from the fresh culture because a few amebae, probably encysted stages, sometimes survive. Soil amebae do not seem to be inhibited by cycloheximide, nystatin, or freezing.

Occasionally, cycloheximide-resistant fungi are present, but they can almost always be inhibited by dusting the inoculum with nystatin powder. The myxobacteria grow between the nystatin particles and can then be transferred to another plate.

The remaining contaminants are bacteria. By transferring pieces from the fast-spreading swarm edge, most of the larger and less motile organisms are quickly eliminated, in particular *Bacillus* species and their spores, but the slime matrix of the swarm may still shelter many small, mainly Gram-negative bacteria. Apart from the slime, the relatively slow growth of myxobacteria compared with that of typical contaminants is a major factor that makes purification time-consuming and difficult. Usually it takes 3 to 6 weeks before an isolated strain is pure; only rarely can it be accomplished in one to two days (by direct transfer of fruiting bodies). Fortunately, those isolations that may take one to two years (e.g., a cellulose degrader) are equally rare.

Often spreading growth under selective nutritional conditions is either not sufficient to shake off contaminants, or the procedure becomes too time-consuming. In such cases, a number of more specific purification steps may be tried.

PURIFICATION BY PLATING. Plating of diluted cell suspensions, the technique of choice for the isolation of most soil bacteria, is only partly useful with myxobacteria. The first difficulty is that the slime makes it difficult to suspend the cells homogeneously in water. We have sometimes had success using a small (1 ml) sterilized tissue homogenizer to overcome this problem. If fruiting bodies are available, treatment with ultrasound for 1 to 2 min may result in a suspension of myxospores. (The use of ultrasound to kill contaminants selectively, as suggested by Sutherland, 1976a, has not appeared very promising in our hands.) Cell suspensions can also be produced in a mixer with or without glass beads (McCurdy, 1963). The cell suspensions can be diluted without problems in sterile distilled water, although a special dilution medium (DM) has also been used.

Dilution Medium (DM; McCurdy, 1963)

Soluble starch	0.5%
$MgSO_4 \cdot 7H_2O$	0.05%
K_2HPO_4	0.025%

The second difficulty is that quite a few myxobacteria, particularly members of the suborder Sorangineae, do not readily produce swarms from single cells. Often a long and tedious optimization and adaptation procedure is required before single-cell colonies are obtained, even from pure strains. Nevertheless, with many myxobacteria, a reasonably high proportion of the cell population will grow to form colonies, so that plating may be successful. However, in our experience, plating has a chance only if the myxobacterium is already nearly pure. This may have to do with their relatively low plating efficiency and relatively slow growth rate. CY agar is often an appropriate medium for plating; sometimes the yield is improved if the peptone concentration is increased to 0.8 to 1.2% or if 0.5% glucose is added. ECM agar and SP agar have also been recommended. While VY/2 agar is a good growth medium, its turbidity reduces its utility and, in addition, myxospores sometimes do not germinate on this medium.

ECM Agar (McCurdy, 1963)

Washed cells of *Escherichia coli* (100 mg dry mass per 100 ml) are suspended in a medium containing: $MgSO_2 \cdot 7H_2O$, 0.05%; NaCl, 0.6%; agar 1.5%. The pH is adjusted to 7.2, and the medium is autoclaved. On this medium, myxobacterial colonies are surrounded by lysis zones.

SP Agar (McCurdy, 1963; McDonald and Peterson, 1962)

Raffinose	0.1%
Sucrose	0.1%
Galactose	0.1%
Soluble starch	0.5%
Casitone (Difco)	0.25%
$MgSO_4 \cdot 7H_2O$	0.05%
K_2HPO_4	0.025%
Agar	1.5%
Vitamin solution (see below)	2.5 ml/liter

Vitamin Solution (McDonald and Peterson, 1962)

Thiamine	100 mg
Riboflavin	75 mg
Pyridoxine	75 mg
Ca pantothenate	200 mg
p-Aminobenzoic acid	5 mg
Nicotinamide	75 mg
Choline HCl	200 mg
Folic acid	1 mg
Inositol	1,000 mg
Biotin	0.05 mg
Cyanocobalamin	0.05 mg

These ingredients are dissolved in 1 liter of ethanol. Add 2.5 ml per liter of medium.

It seems not to make much difference whether the diluted cell suspensions are spread on the agar surface or included in pour plates. In the latter case, the agar content of the medium should be reduced to about 1% and temperatures above 40°C of the molten agar should be avoided. Myxobacterial colonies may be recognized by their swarming behavior, which they also tend to show when embedded in agar. On rich media, swarming may be considerably reduced, and the colonies may become compact and look like those of ordinary bacteria. If they are dense enough, however, they may be brightly colored in shades of yellow, orange, red, or purple.

PURIFICATION BY HEATING. This procedure can be used if fruiting bodies with mature myxospores are present. The fruiting bodies are suspended in 1 ml of sterile water and incubated in a water bath. After 10, 20, and 40 min, samples are transferred to CY or VY/2 agar and carefully spread all over the plate. The useful temperature—too high for the contaminant, but still withstood by the myxobacterium—has to be found by trial and error. We usually work at 58°C, a temperature which is survived by most myxospores for at least 10 min. This temperature is often sufficient to kill contaminating bacteria, fungi, and amoebae. Only rarely do myxobacteria tolerate higher temperatures, and some do not withstand 58°C, in which case 56°C or less may still lead to success. It is, however, important to try this method in the later part of the purification process, because then the chances are higher that thermoresistant contaminants, e.g., *Bacillus* spores, will no longer be present. It may take a week or longer at 30°C, before swarms can be seen on the plates, as a substantial part of the myxospore population may also have been killed. However, often swarms are visible within 1 to 2 days.

PURIFICATION WITH ANTIBIOTICS. This procedure may also be useful if mature myxospores are present. Fruiting bodies are suspended in 1 ml of a rich growth medium, e.g., EBS medium (see below). To this is added 0.1 ml of a concentrated mixture of potent antibiotics, and the suspension is shaken overnight at 30°C (Reichenbach, 1983). The myxospores do not germinate in the rich medium, but the contaminants will grow and be killed. If a contaminant is resistant to the inhibitors, the culture will be turbid the next morning, and the procedure must be repeated with a different mixture of antibiotics. (Occasionally the liquid becomes turbid due to suspended dead cells. In this case, the culture fluid can be replaced by fresh medium and the culture incubated a few hours more.) We use three increasingly aggressive combinations of antibiotics, AB-1, -2, and -3 solutions, (see below) which we apply consecutively when necessary. If the supernatant is still clear on the next morning, it is replaced by sterile distilled water, and the fruiting bodies are washed by shaking them for 4 to 6 hr more. They are then transferred to VY/2 or CY agar, or to streaks of autoclaved *E. coli* on WCX agar. Usually swarms develop after 1 to 5 days. Sometimes an individual swarm is still contaminated, although no foreign colonies can be seen on the plate. Therefore, several swarms should always be isolated and tested separately for purity. Again, the procedure is less effective during the early stages of purification, before the majority of the contaminants have been removed. It should be emphasized that the vegetative growth of myxobacteria is, in general, as sensitive to inhibition by antibiotics as is the case with other Gram-negative bacteria. However, the metabolically quiescent myxospores within the fruiting bodies are resistant, and it is their germination that is prevented by the antibiotics.

EBS Medium

Peptone from casein (tryptic digest); Merck, Darmstadt, Germany)	0.5%
Proteose peptone (Difco)	0.5%
Peptone from meat (Merck)	0.1%
Yeast extract (Difco)	0.1%

The pH is adjusted to 7.0. The medium is autoclaved.

AB-1 Solution

Chloramphenicol	20 mg
Streptomycin sulfate	30 mg
Tetracycline HCl	25 mg
Na cephalotin	20 mg

The above ingredients are dissolved in 50 ml water and filter sterilized. The solution remains stable for several weeks at 4°C.

AB-2 Solution

Chloramphenicol	20 mg
Streptomycin sulfate	30 mg
Tetracycline HCl	25 mg
Kanamycin sulfate	35 mg
Erythromycin	25 mg
Polymyxin B sulfate	20 mg

The above are dissolved in 50 ml water and filter sterilized. The solution remains stable for several weeks at 4°C. It is often inhibitory to myxobacteria and should only be used as a last resort.

AB-3 Solution

Chloramphenicol	20 mg
Tetracycline HCl	30 mg
Neomycin sulfate	20 mg
Gentamycin sulfate	30 mg

Collistin methanesulfonate 30 mg

The above are dissolved in 50 ml water and filter sterilized. The solution remains stable for several weeks at 4°C. This mixture is specifically designed to eliminate pseudomonads. It usually serves that purpose but is less well tolerated by the myxobacteria than AB-1 solution.

It has also been suggested that inhibitors, e.g., neomycin and sulfanilamide, be included directly in the isolation medium (McDonald, 1967; McCurdy, 1969a). However, while some myxobacteria can be isolated in this way, we do not recommend this approach. (Cyclohexamide is, of course, an exception, as it does not inhibit prokaryotes. It is remarkable how many soil bacteria can grow in the presence of high doses of powerful antibiotics, and there is always the danger of a counterselection of some myxobacteria. Addition of antibiotics to purification media is a different story, however, as some groups of myxobacteria are naturally resistant to certain potent antibiotics (see later).

PURIFICATION OF CELLULOSE DECOMPOSERS. The purification of *Sorangium* strains is especially tedious and time-consuming because the organisms only grow slowly on the selective substrate, giving the contaminants ample time to multiply. Furthermore, within the water-soaked filter pad, many contaminants spread rapidly over the whole area, complicating the problem of separating the myxobacteria from the other organisms.

The first transfer from the crude culture should be made again to filter paper on ST21CX agar (see "Isolation of Cellulose Decomposers") to retain the selective conditions. For the preparation of subcultures, filter paper is cut into small rectangles, about 1.5 × 3 cm, three or four of which are placed at some distance from each other on the agar surface. The filter pads are inoculated from different parts of the original swarm. In this way, an initial reduction of the contaminants may be achieved. The next step is to remove nematodes and amebae, using the techniques recommended above. To eliminate bacterial contaminants, transfers can be made to streaks of autoclaved *E. coli* on WCX agar to which 250 mg kanamycin sulfate has been added per liter medium (after autoclaving, from a filter-sterilized stock solution: KAN4 agar). All *Sorangium* strains tested so far are completely resistant to kanamycin as well as to neomycin and gentamicin (H. Reichenbach, unpublished observations). Most sorangia grow reasonably well on autoclaved (but not on living) *E. coli*, often forming large, delicate swarms, another advantage of this purification step. Pieces from the swarm edge that appear to be uncontaminated can be transferred to VY/2 agar. The pure strain is reinoculated to filter paper to make sure that the desired organism has been isolated. We have never observed a *Sorangium* strain to have lost its ability to degrade cellulose during isolation. Addition of kanamycin initially to the crude culture would prevent the appearance of many other myxobacteria and at the outset, select for kanamycin-resistant contaminants.

If the method just described fails, heating of the fruiting bodies or treatment with the antibiotic solution may lead to success. If the *Sorangium* isolate is already relatively pure, it may also be transferred to chitin agar (CT7 agar; see Chapter 176) or to cellulose overlay agar (CEL3 agar, see below). Most but not all sorangia also decompose chitin and grow relatively quickly, producing large swarms on chitin agar with chitin as the only carbon, nitrogen, and energy source. Large swarms also arise on cellulose overlay agar, but development is delayed so that the contaminants have a better chance to predominate. On both media, the sorangia penetrate the agar, so that sometimes a pure inoculum can still be obtained from the deeper layers of the plate even though the surface is contaminated.

CEL3 Agar

Cellulose powder	0.5%
KNO_3 (autoclaved separately)	0.1%
Agar	1.0%

Cellulose powder MN 300 from Macherey & Nagel (Düren, Germany) works well. The pH is adjusted to 7.2. The medium is autoclaved and poured as a thin layer on top of ST21 agar plates.

Another strategy often helpful in the purification of sorangia is incubation at 38°C, e.g., on KAN4 agar + autoclaved *E. coli.* Most but not all strains are able to grow at this temperature, sometimes vigorously so, while growth of the contaminants is often prevented.

TESTING FOR PURITY. The final task in pure culture isolation is to check for purity of the culture. Some swarm material may be streaked on nutrient agar, on which myxobacteria, in contrast to most contaminants grow only slowly or not at all. Other media useful for purity control are CY and MYX agar. Although most myxobacteria grow on those media, they are usually easily distinguished from nonmyxobacterial colonies.

MYX Agar

Glutamate Na	0.5%
Yeast extract (Difco)	0.1%

$MgSO_4 \cdot 7H_2O$	0.1%
Glucose (autoclaved separately	0.2%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

In addition, some material may be inoculated into a liquid medium, e.g., nutrient broth or CAS medium and incubated with shaking for one day at 30°C. Under such conditions, myxobacteria usually grow in clumps, flakes, or as a ring around the glass wall, if at all. Dispersed growth at that stage is almost always an indication of contamination.

Myxobacteria can often be differentiated easily from other bacteria by their size, cell shape, and their inability to swim actively. Thus microscopic examination will also often quickly reveal contaminants, although this is not always reliable and requires some experience.

Counting Myxobacteria in Soil

The quantitative determination of myxobacteria in soil is not likely to give accurate results, because, in the swarm and in the fruiting bodies, the cells are always linked together by slime, and in the fruiting bodies cells are often surrounded by the tough walls of the sporangioles. Still, estimates are possible and have been performed in the following way: Small disks (about 2 cm in diameter) impregnated with a food organism are placed on a water agar or a silica gel plate, and each circle is inoculated with a small quantity of soil which has been carefully homogenized and weighed (Singh, 1947). Alternatively, the weighed and homogenized soil is made into a paste with sterile water, and small drops are applied to the ends and centers of cross-streaks of a food organism on water agar, or, for counting cellulose decomposers, to filter paper disks on mineral agar (Brockman, 1976). After incubation, the number of myxobacteria is calculated from the number of swarms and fields of fruiting bodies that have developed. Another approach is to add soil dilutions to ACE agar (McCurdy, 1969a). This method has severe limitations because the medium allows a rapid development of contaminants, and only *Myxococcus* and *Corallococcus* cells can be counted. Nevertheless, the numbers that have been obtained are sometimes quite impressive.

ACE Agar (Antibiotic Cell Extract Agar; McCurdy, 1969a)

SP medium (see "Purification by Plating," this chapter) is supplemented with:

Yeast extract	0.1%
Yeast (or *E. coli*) cells	0.5% (dry weight)
Neomycin	10 μg/ml
Sulfanilamide	10 μg/ml
Cycloheximide	100 μg/ml

The myxobacteria are detected by their swarm morphology and surrounding lysis zones.

Cultivation

Contrary to a belief that still seems widely held, myxobacteria are not particularly fastidious organisms. With the exception of *Haploangium,* all species have been cultivated, and all can be grown in axenic, pure culture. Of course, as with all other organisms, myxobacteria have their peculiarities, but no unusual requirements, and not even particularly complex ones, have been discovered to date. Exotic substrates such as the rabbit dung media that were so popular for a long time are not necessary. Although it is not always easy, fully defined, synthetic media have been developed for some myxobacteria. In connection with possible commercial antibiotic production, quite a few myxobacteria have been cultivated in large-scale fermentors (5 m^3 and greater), often on technical media, such as corn steep powder and soy meal.

Plate Cultures

Myxobacterial cultures are often incubated for extended periods, (1 to 4 weeks), where dehydration may occur, so that plates must be poured thicker than for the usual bacterial work. Also, a high humidity in the incubator is helpful.

All myxobacteria, including the cellulose degraders, seem to grow well on yeast agar (VY/2 agar). The yeast cells are usually decomposed, but not always completely, and sometimes not at all. Vitamin B_{12} is occasionally required (e.g., by many *Polyangium* strains) so cyanocobalamin is included in this standard medium. Typically, on VY/2 agar gliding motility is considerably stimulated, and the swarm colonies tend to become very large. Fruiting bodies are often formed even after several transfers on VY/2 agar, although sooner or later most strains cease fruiting on this medium as well as on most other growth media. Cultures on VY/2 agar are usually rather stable, and most strains need not be transferred more often than every 2 to 3 weeks (30°C). The reason is probably that VY/2 agar is relatively lean, nutrients are only gradually solubilized, and the pH does not change very much. The medium therefore is also well suited for stock cultures.

Another useful medium is CY agar (see "Purification Methods," this chapter). On this medium growth often is more vigorous, but the swarms do not spread so much, may become somewhat slimy, and often the cells die earlier (after 1 to 2 weeks) due to copious ammonia production. The choice of the peptone for media of this type is critical. In general, the most suitable products appear to be pancreatic and tryptic digests of casein, e.g., casitone (Difco), tryptone (Difco), or peptic digests (Marcor, Merck). But other peptones such soybean peptones can also be used. Meat peptones, on the other hand, are usually not useful, perhaps because their phosphate content is too high. In any case, the suitability of a peptone for the myxobacterial strain to be cultivated must first be tested. How critical the quality of the peptone can be is seen from the fact that even the specific batch of a particular peptone brand may make a difference. Thus, for example, not every batch of Difco casitone supported successful induction of myxospore formation with *Stigmatella aurantiaca* (Gerth and Reichenbach, 1978); similar observations have also been made with glycerol induction of myxospores of *Mx. xanthus.* Replacement of peptone by a total protein hydrolysate, e.g., casamino acids (Difco), is often not possible. But casamino acids are a perfect N source for *Cystobacter* and *Sorangium* strains.

Watson and Dworkin (1968) showed that *Mx. xanthus* lacked hexokinase and was unable to take up, metabolize, or grow on sugars. This, however, cannot be generalized for the myxobacteria, despite earlier impressions that this might be the case. Many myxobacteria are known that efficiently metabolize sugars, e.g., *Stigmatella* and *Cystobacter.* In fact, it seems that only the *Myxococcus* species and perhaps some *Corallococcus* strains are unable to use carbohydrates. In some cases, e.g., certain *Corallococcus* strains, polysaccharides such as starch can be utilized, although no mono- and disaccharides can be used. Apparently the starch is degraded to the trisaccharide, which can be metabolized (Irschik and Reichenbach, 1985). Thus, it may sometimes be useful to supplement a peptone medium with starch or glucose. Another beneficial effect of this may be that the pH rises more slowly, so that the culture can be maintained for a longer time. We have found the following formulations useful for plate cultivation:

CYG2 Agar

Casitone (Difco)	0.3%
Yeast extract (Difco)	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. After autoclaving, 0.3% glucose is added from an autoclaved stock solution.

STK2 Agar

Base agar:

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved and poured into petri dishes. After the agar has solidified, the following top agar is added:

Top agar: As base agar, but the agar concentration is increased to 1.5%, and the medium is prepared in about 85% of the final total water volume. In the remaining water is dissolved, as a percentage of the total volume of the top agar:

Soluble starch (see below)	0.2%
K_2HPO_4	0.02%

The starch solution is heated on a water bath under stirring before autoclaving in order to prevent clumping of the starch. The two solutions are combined after autoclaving.

In the form just described, STK2 agar can be used for checking starch degradation. If it is only to be used for cultivation, just the top agar (but with 1.2% agar) is required.

The cellulose decomposers utilize NO_3^- or NH_4^+ as the only N source and can be cultivated on simple synthetic media like CK6 and CA2 agar (see below). They respond favorably to the addition of an organic N source, such as peptone (0.1 to 0.2%), but usually they will not grow in the absence of a suitable carbohydrate, like glucose or starch. *Sorangium* strains thus can also be grown on VY/2, CEL3, and STK21 agar, and many strains will grow as well on CT7 agar, but for the reasons just mentioned, relatively few will grow on CY agar.

CK6 Agar (Modified from Couke and Voets, 1967)

Solution A:

$MgSO_4 \cdot 7H_2O$	0.15%
Fe^{3+} citrate	0.002%
Agar	1.5%

Prepare this solution in 80% of the final water volume.

Solution B:

KNO_3	0.2%
K_2HPO_4	0.025%

Prepare this solution in 10% of the final water volume.

Solution C:

Glucose	0.5%
$CaCl_2 \cdot 2H_2O$	0.15%

Prepare this solution in 10% of the final water volume.

After autoclaving, the three solutions are combined.

CA2 Agar (Mullings and Parish, 1984)

Base agar:

Agar	1.5%

Stock solution A:

KNO_3	7.5 g
K_2HPO_4	7.5 g

These ingredients are dissolved to give 100 ml.

Stock solution B:

$MgSO_4 \cdot 7H_2O$	1.5 g

This is dissolved to give 100 ml.

Stock solution C:

$CaCl_2 \cdot 2H_2O$	0.27 g
$FeCl_2$	0.15 g

These are dissolved to give 100 ml.

After autoclaving, the base agar is supplemented with 1% (v/v) of each of the three stock solutions, 1% (w/v) of glucose (from an autoclaved 20% stock solution) and trace elements.

Since myxobacterial swarms tend to spread quickly, cultures are more conveniently kept in petri dishes than on agar slants in tubes. In the latter, the swarm edge soon reaches the glass surface and the cells dry out and die; also transfer of sticky, tenacious swarms is easier from plates. The plates are inoculated best at one spot at the side to give the swarm as much space as possible. In large swarms, living cells are often found only in a rather narrow band along the edge. Therefore, the inoculum should always be taken from the edge, at least as long as there are no fruiting bodies in the interior zone. Many myxobacteria of the suborder Sorangineae tend to penetrate deeply into the agar, in which case an agar strip has to be cut out and used as an inoculum.

Viable Counting

Accurate viable counts are possible only with strains that grow in a dispersed fashion in liquid media. Plating efficiency has to be tested for every strain. Given below are some media that allow plating efficiencies close to 100%: CT and CTT agar for *Mx. xanthus* FB; PT agar for *Mx. fulvus* Mx f2; and SG agar for *Sg. aurantiaca* Sg a1. CF (clone-fruiting) agar supports the formation of colonies from single cells of *Mx. xanthus,* with the subsequent production of fruiting bodies (Bretscher and Kaiser, 1978). If a plating medium is not satisfactory for a specific strain, usually a modification of the peptone and ion concentrations or the addition of some yeast extract will make it suitable.

CT Agar (Dworkin, 1962)

Casitone (Difco)	2%
$MgSO_4 \cdot 7H_2O$ (autoclaved separately)	8 mM
Potassium phosphate buffer, pH 7.2	0.01 M
Agar	2%

A variation of the pH sometimes improves the results; it has been found that a pH of 7.6 is closer to the optimum than 7.2 (H. Reichenbach, unpublished observations).

CTT Agar (Bretscher and Kaiser, 1978)

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	8 mM
Potassium phosphate buffer, pH 7.6	1 mM
Tris buffer, pH 7.6	10 mM
Agar	1.5%

For semisolid media, agar is at 0.6%. The medium is autoclaved.

PT Agar (for *Mx. fulvus* Mx f2)

Casitone (Difco)	0.4%
Yeast extract (Difco)	0.4%
$MgSO_4 \cdot 7H_2O$	0.2%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

SG Agar (Gerth, 1975)

Casitone (Difco)	1.5%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.2%
Agar	1.5%

The pH is adjusted to 7.0. The medium is autoclaved.

CG Agar (Hagen et al., 1978)

Casitone (Difco)	0.015%
Na pyruvate	0.1%
Na citrate	0.2%
$(NH_4)_2SO_4$	0.02%
$MgSO_4 \cdot 7H_2O$	8 mM
Potassium phosphate buffer, pH 7.6	1 mM
Tris buffer, pH 7.6	10 mM
Agar	1.5%

The medium is autoclaved.

Excellent viable counts, e.g., for survival curves after treatment with mutagens, have been obtained by us with *Mx. fulvus* Mx f2 and *Sg. aurantiaca* Sg a1 by adapting a miniaturized counting technique recommended by Sharpe and Kilsby (1971). One of the plating media mentioned above is prepared with a reduced agar concentration of 0.6%. The bacteria are diluted directly in this soft agar at 40°C, and 0.1 ml drops of appropriate dilutions are put into petri dishes, covered with a drop of sterile agar of the same composition, and incubated at 30°C. It should be noted that vegetative cells of *Mx. xanthus,* and perhaps other myxobacteria as well, are sensitive to temperatures higher than 43°C. Microcolonies are counted under a dissecting microscope. Up to 150 colonies per drop can easily be counted (H. Reichenbach

and M. Dworkin, unpublished observations). The method has several advantages. Without difficulty, 40 drops can be placed into one petri dish, using the bottom and the lid. Besides enormous economy in materials and incubator space, results are obtained earlier (after 3 to 4 days instead of 5 to 8 days) and with superior accuracy (more replicates, e.g., 5 instead of 3; less divergence between replicates; and a better chance to hit the optimum dilution step, because a wide interval of dilution steps can conveniently be plated. Plating efficiency can be 100%. The peripheral drops often show a reduced colony count, probably because they dry more easily. Therefore a ring of sterile agar drops should be placed around the edge of the plate.

Production of Fruiting Bodies in Culture

Usually, fruiting bodies are not produced on media that allow good growth. High nutrient concentrations seem to repress the fruiting process. As already mentioned, on VY/2 agar, fruiting bodies are often produced during several transfers, but a vegetatively propagated strain normally stops fruiting soon after isolation and often cannot be made to fruit again. The reason for this is not really understood; perhaps there is a selection for nonfruiting variants. In several cases, induction conditions for fruiting have been found for certain individual strains. Such strains reliably form fruiting bodies even after many transfers of purely vegetative growth, but other strains of the same species usually do not respond to the same conditions. With freshly isolated strains, fruiting can often be induced by transferring swarm material from a lean growth medium, like VY/2 agar, to plain water agar (WAT agar) or to sterile filter paper pads on water agar. Also, fruiting may continue for some time on streaks of living *E. coli* cells on water agar. The nutrients carried over from the growth medium, and the low nutrient level provided by the lysing *E. coli* cells, respectively, allow reasonable growth for some time, leading to sufficiently high cell densities; the subsequent more-or-less-abrupt depletion of nutrients then somehow triggers development. The need for a high population density for fruiting has been established for several myxobacteria, e.g., *Mx. xanthus* (Wireman and Dworkin, 1975) and *Cm. apiculatus* (H. Reichenbach, unpublished observations).

Using some simple tricks, myxobacterial strains can sometimes be maintained in the fruiting state for a long period. We were able to do so with *Sg. aurantiaca* over a period of years by storing the strain in the form of fruiting bodies on WAT agar at room temperature (20°C). After 2 to 3 months, when the culture began to dry out, the fruiting bodies were inoculated to VY/2 agar and incubated to 30°C. The resulting swarms were immediately reinoculated to Wat agar, incubated for a few days at 30°C during which time the organism began to fruit again, and then stored at room temperature as before. One or two more transfers to VY/2 agar were possible, but after that the strain failed to produce fruiting bodies any longer. This procedure of cycling the culture between rich media that support growth and lean media that induce fruiting body formation will probably work with many different myxobacteria.

With several *Chondromyces* species (*Cm. apiculatus, Cm. crocatus, Cm. pediculatus, Cm. lanuginosus*) we have obtained fruiting bodies when the organism was kept in a mixed culture with cellulose degraders (not myxobacteria) on filter pads placed on ST21 agar plates. The cultures are incubated in the light (on the bench top in the laboratory) at room temperature (20–24°C). Transfers are made every 3 to 4 weeks by inoculating macerated cellulose and *Chondromyces* fruiting bodies together to fresh filter pads on ST21 agar. within 4 to 5 days, new fruiting bodies appear. Using this procedure, several of our strains have continued to produce dense populations of fruiting bodies over a period of years (H. Reichenbach, unpublished observations). The method can also be used with pure cultures of *Chondromyces.* The swarm material is simply inoculated into a developing culture of a cellulose degrader. While *Polyangium* strains often form fruiting bodies with this procedure, myxobacteria of the suborder Cystobacterineae rarely do so. In general, it seems that a slightly reduced temperature (24–28°C) is favorable for fruiting. Also, in two cases, a requirement of light for fruiting body differentiation has been demonstrated—with *Cm. apiculatus* (Reichenbach, 1974a, 1974b) and with *Sg. aurantiaca* (Qualls et al., 1978).

Chamber Cultures

For the study of the social behavior of myxobacteria and of morphogenetic events, chamber cultures that can be observed and followed under the microscope are extremely useful. Fig. 4 illustrates one procedure for making such cultures.

Liquid Cultures

If a freshly isolated myxobacterial strain is cultured for the first time in a liquid medium in a shake flask, the bacteria almost always grow in the form of flakes and nodules and as a film

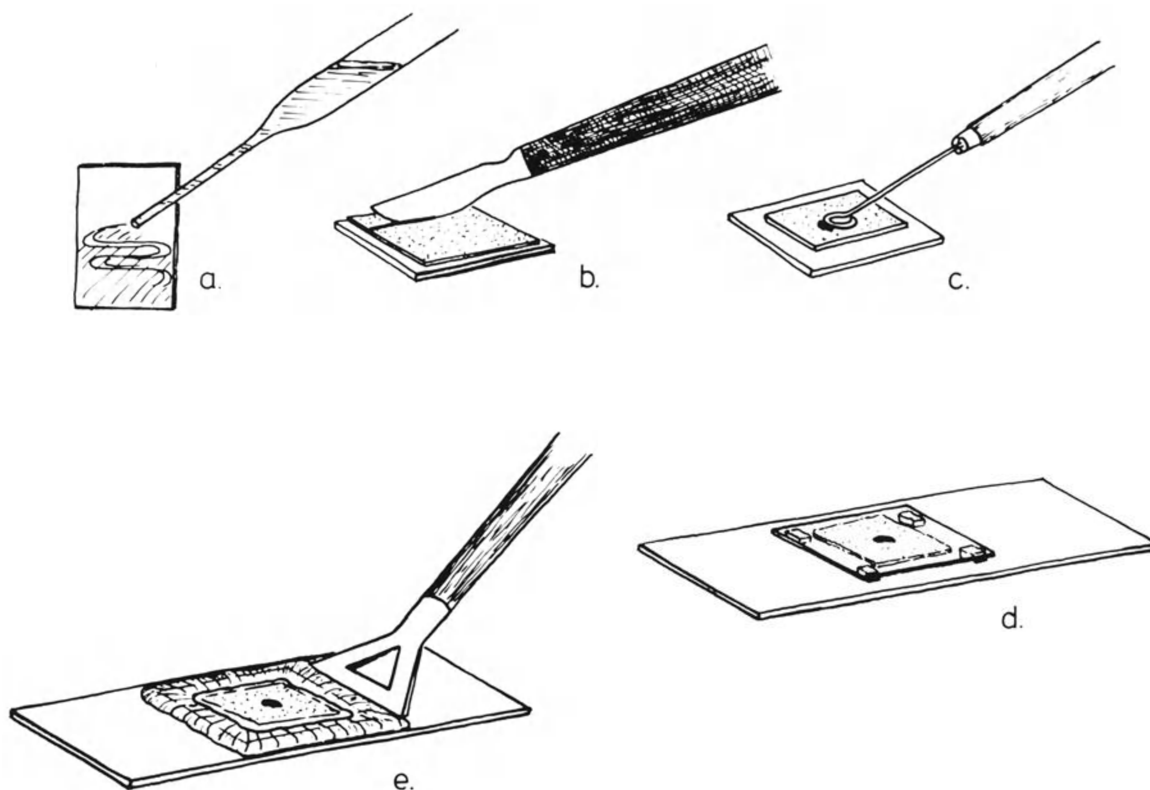


Fig. 4. Method for making a chamber culture: (a) Coat a sterile cover slip (e.g., 40 × 22 mm) with a thin agar layer. The molten agar is applied with a Pasteur pipette; the hotter the agar, the thinner the resulting layer. (b) Using a sterile scalpel, remove the agar layer along the edges of the cover glass. (c) Inoculate the agar film. (d) Mount the cover slip with the agar layer downwards on a sterile slide; tiny pieces of glass from a broken microscopic slide are used as supports. (e) Seal the chamber carefully with a molten mixture of paraffin and vaseline (2:1) and incubate at a suitable temperature. (From Heunert, 1973.)

along the glass wall. In static cultures, they grow as a sheet on the glass in the upper parts of the vessel and as a pellicle along the surface of the liquid. However, if repeated transfers are made from shake cultures, preferentially using the supernatant as an inoculum, strains that grow in a dispersed fashion may be obtained. While with many strains only a few such transfers are required, other strains may resist dispersed growth completely. Thus, for example, while most strains of *Myxococcus* can be grown in dispersed culture, we once transferred a strain of *Mx. fulvus* for months in a variety of liquid media without ever observing the slightest inclination to dispersed growth (H. Reichenbach, unpublished observations). Cells of most members of the suborder Sorangineae show a strong tendency to stick together, and in spite of many attempts, we have never been able to get *Chondromyces, Polyangium,* and *Nannocystis* to grow homogeneously in liquid cultures. The best that could be achieved was growth as tiny flakes with *Nannocystis* and *Polyangium.* With *Sorangium,* however, we have been able to select many completely dispersed strains. The list of myxobacteria that have been grown in a dispersed fashion is very long by now, and, with the exception of the last-mentioned organisms, comprises virtually all taxa. However, even the recalcitrant species which do not grow in a completely dispersed fashion, can at least be cultivated in suspension in liquid medium.

There is still no explanation for the factors which determine dispersed growth. One possibility is that the chemical structure and physicochemical properties of the excreted slime play an essential role. This hypothesis is consistent with several observations: 1) strains growing in a dispersed fashion often show a different swarm structure when returned to agar plates (soft-slimy swarms with rudimentary surface texture and reduced tendency to spread); 2) they usually have lost the ability to form fruiting bodies; and 3) the Sorangineae, which persistently fail to grow in a dispersed state, produce a slime that is chemically different from that of the Cystobacterineae (as shown by the fact that in contrast to the slime of the latter, the slime

of the former does not adsorb Congo red; McCurdy, 1969a).

An alternative possibility is suggested by the work of Kaiser's group. A large number of *Myxococcus* strains representing several different species were all found to possess pili when freshly isolated, and all of them failed to grow in a dispersed fashion in liquid media. This was examined in further detail in *Mx. xanthus* FB, where it was shown that a particular class of motility-deficient mutants had lost the ability to produce pili and now exhibited dispersed growth (Kaiser, 1979; D. Kaiser, personal communication). Of course, both slime and pili may be involved in dispersed growth, the pili reacting with a receptor in the slime at the cell surface. Also, not all myxobacteria may behave in the same way.

Work by Arnold and Shimkets (1988b) and in one of our laboratories (M. Dworkin, unpublished observations) indicates that the fibrils characteristically formed by *Mx. xanthus* (Dobson and McCurdy, 1979) play an important role in the cohesion and swarming behavior of the organism. These fibrils differ from the pili described by Kaiser (1979).

A homogeneously growing strain may start to clump again if growth conditions are changed, e.g., in a medium of different composition. Thus, cells of *Sg. aurantiaca* Sg a1, a strain which grows in a dispersed fashion, form tiny nodules if $CaCl_2$ is added to the medium. Freshly isolated *Cystobacter fuscus* cultures grow homogeneously almost immediately when inoculated into a casamino acids medium, but produces nodules and flakes for many transfers in casitone medium. Cells of *Sorangium cellulosum* strain So ce14 yield a homogeneous suspension when shaken overnight in casitone medium (in which they cannot grow, however); when reinoculated into AMB growth medium, they produce nodules again. It may also be mentioned in this connection that although the myxobacteria are the classical "slime bacteria" (*myxo* is a combining form meaning "slime"), they do not produce copious amounts of slime, either on plates or in liquid media, which rarely become recognizably viscous. Only on certain agar media is a larger quantity of slime sometimes seen, e.g., *So. cellulosum* on CA2 agar.

To facilitate the transition to dispersed growth, the addition of 0.1% agar to the liquid medium has been suggested (Schürmann, 1967). The agar forms tiny flakes that float in the liquid and are colonized by the myxobacteria. However, the selection of truly dispersed strains is not noticeably speeded up by this procedure. Sometimes it is helpful to replace the usual rotary shaker with a reciprocating shaker, since the slime sheets are broken up more efficiently on the latter, and the transition to dispersed growth may be faster, although patience is still required (K. Gerth, personal communication).

Liquid media used for cultivation of myxobacteria are of the same general composition as the agar media mentioned above (see "Direct Isolation" and "Purification," this chapter), but the nutrient requirements stand out more clearly in liquid media, and the organisms respond almost immediately to inadequacies of the medium. The prototype for the bacteriolytic myxobacteria is CT liquid medium, which was designed to provide the most rapid growth (3.5-h doubling time), maximum cell yield (4–5 × 10^9 cells/ml), and uniform cell suspension of *Mx. xanthus* FB (Dworkin, 1962; the medium has the same composition as the CT agar described in "Viable Counts," this chapter, only without the agar). However, most other myxobacteria grow better when the peptone concentration is reduced, as in CAS medium and in MD1 medium (see below). The latter medium was originally developed for *Nannocystis* but it is also often useful for *Archangium, Melittangium,* and *Cystobacter.* Also, in many cases it is superior for starting liquid cultures with newly isolated strains.

CAS Medium

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH is 6.8 and needs no adjustment if Difco casitone is used. The medium is autoclaved.

MD1 Medium (Behrens et al., 1976)

Casitone (Difco)	0.3%
$CaCl_2 \cdot 2H_2O$	0.07%
$MgSO_4 \cdot 7H_2O$	0.2%
Cyanocobalamin	0.5 μg/ml

Trace elements (see "Isolation of Cellulose Decomposers")

The vitamin solution is not needed for most strains. The medium is autoclaved.

What has been said about the choice of peptone in agar media holds also for liquid media. For many myxobacteria the addition of glucose (0.2 to 0.5%), starch, or another useful sugar to the peptone medium may be favorable. In general, the organisms do not grow faster with the carbohydrate, but they grow for a longer time, and they reach a higher cell density; also, the rise of the pH is delayed, so that the culture becomes more stable.

As already mentioned, some myxobacteria, e.g., *Cystobacter* strains, grow well on casamino acids, e.g., in CAC medium.

CAC Medium

Casamino acids, vitamin-free (Difco)	0.2%
Sodium glutamate	0.3%
$MgSO_4 \cdot 7H_2O$	0.1%
Cyanocobalamin	0.5 μg/ml
Trace elements	

After autoclaving, the medium is complemented from autoclaved stock solutions with:

Glucose	0.5%
Phosphate buffer, pH 6.5	0.5 mM

Sg. aurantiaca Sg a15 can be cultivated in the following defined medium:

STG Medium (Kunze et al., 1984)

Casamino acids, vitamin-free (Difco)	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$(NH_4)_2SO_4$	0.1%
$CaCl_2 \cdot 2H_2O$	0.05%
Phosphate buffer, pH 7	1 mM
Trace elements	
Vitamins (see earlier)	

After autoclaving, the medium is complemented with 0.5% glucose from an autoclaved stock solution. Alternatively, fructose, maltose, starch, or dextrin 10 could also be used.

The cost of the medium is of considerable importance when large-scale culture is necessary. Many myxobacteria have been cultivated on various technical media, like corn steep powder, maize gluten, soy meal, skim milk, or yeast cells (Gerth et al., 1983; Kunze et al., 1984, 1985, 1987). Zein medium is an example of a production medium:

Zein Medium (Kunze et al., 1987)

Zein (maize gluten, Maizena, Hamburg, Germany)	1%
Peptone from casein, tryptic digested (Merck, Darmstadt, Germany)	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH is adjusted to 7.3. The medium is autoclaved.

All myxobacteria of the bacteriolytic type rely totally, or to a large extent, on peptides and amino acids for nitrogen, carbon, and energy. As a consequence, relatively large quantities of ammonia are released, which seriously limits growth and productivity via regulatory mechanisms and by making the medium alkaline. Thus, in cultures of *Mx. virescens* Mx v48 concentrations of 35 to 42 mM NH_4^+ and more were measured in the culture supernatant, and 80 to 140 mM within the cells (Gerth and Reichenbach, 1986). On-line extraction of ammonia from a laboratory fermentor via hydrophobic-membrane hollow fibers maintained the NH_4^+ concentration in the medium between 3 and 7.5 mM with dramatic effects: the generation time was lowered from about 4 to 2 hr, the cell density increased by 233%, and the yield of the antibiotic myxovirescin rose from 8 to 115 mg/liter (K. Gerth, personal communication).

For a few strains of bacteriolytic myxobacteria, fully defined, synthetic media have been developed. Examples are given in Table 2. Generation times increase substantially in such media. Thus, with *Mx. xanthus* FB, the doubling times were: 3.5 h in CT medium, 8 h in S medium, and 6.5 in M1 medium; 6.5 h in M1 medium without lysine, 9.5 h in M1 medium without threonine, and 11.5 h in M1 medium without lysine and threonine (Filer et al., 1973).

The cellulose degraders can be cultivated on very simple media, such as CK1 and CK6 medium (see below). The addition of a small quantity of peptone, e.g., 0.05% peptone from a tryptic digest of casein (Merck, Darmstadt, Germany), usually improves growth and keeps the cells in the dispersed state, whereas without peptone they sometimes form cell clusters or tiny clumps (Irschik et al., 1987). Media with peptone as the only N source usually also allow good growth, e.g., AMB medium.

CK1 Medium (Kleinig et al., 1971; Modified from Couke and Voets, 1967)

$MgSO_4 \cdot 7H_2O$	0.3%
$CaCl_2 \cdot 2H_2O$	0.18%
Fe^{3+}-citrate	20 mg/liter
KNO_2	0.2%
K_2HPO_4	0.025%
Glucose	1%

Nitrate and phosphate, and $CaCl_2$ and glucose are each autoclaved together and separate from the other ingredients.

CK6 Medium (Irschik et al., 1987; Modified from Couke and Voets, 1967)

$MgSO_4 \cdot 7H_2O$	0.15%
$CaCl_2 \cdot 2H_2O$	0.15%
$FeCl_2 \cdot 7H_2O$	8 mg/liter
KNO_3	0.2%
K_2HPO_4	0.025%
Glucose	0.5%

Nitrate and phosphate, and $CaCl_2$ and glucose are each autoclaved together and separate from the other ingredients.

AMB Medium (Ringel et al., 1977)

Soluble starch	0.5%
Casitone (Difco)	0.25%
$MgSO_4 \cdot 7H_2O$	0.05%
K_2HPO_4	0.025%

The medium is autoclaved.

MD1 medium (see above) can also be used for cellulose degraders if it is supplemented with

Table 2. Composition (in mg/ml) of five fully defined, synthetic media for bacteriolytic myxobacteria.

Component	*Myxococcus xanthus* FB			*Archangium violaceum* medium[d]	*Cystobacter fuscus*/ *Cb. ferrugineus* Hp16 medium[e]
	S medium[a]	M1 medium[b]	A1 medium		
L-Ala	50	1000	—	50	—
L-Arg	100	100	—	100	—
L-Asn	500	500	100	100	—
L-Asp·K	—	—	5000	—	—
L-Cys	—	100	—	—	—
L-Glu·Na	—	—	—	1000	—
L-Gln·Na	—	—	—	—	1000
L-Gly	50	100	—	50	—
L-His	50	100	—	50	—
L-Ile	500	1000	100	300	40
L-Leu	1000	2000	50	500	—
L-Lys	250	500	—	150	—
L-Met	50	500	10	50	—
L-Phe	—	1000	100	—	40
L-Pro	500	1000	—	250	—
L-Ser	100	200	—	100	—
L-Thr	100	100	—	100	—
L-Trp	50	1000	—	—	—
L-Tyr	600	400	—	500	—
L-Val	100	200	100	100	—
Djenkolic acid	100	—	—	—	—
Glycogen	3000	—	—	—	—
D-Glucose	—	—	—	—	5000
Na pyruvate	—	—	5000	—	—
Spermidine·3HCl	—	—	125	—	—
Cyanocobalamin	—	—	1	—	—
$(NH_4)_2SO_4$	—	—	5000	—	—
$MgSO_4 \cdot 7H_2O$	2000	200	2000	200	1000
$Ca_3(PO_4)_2$	—	—	—	20	—
$CaCl_2$	—	2	1.1	—	—
KCl	—	—	—	20	—
NaCl	—	200	—	—	—
$FeCl_3 \cdot 6H_2O$	—	2	2.7	—	—
KH_2PO_4	—	140	—	—	—
Phosphate buffer	10 mM, pH 7.6	—	1 mM, pH 7.6	—	0.5 mM, pH 6.5
Tris buffer	—	10 mM, pH 7.6	10 mM, pH 7.6	20 mM, pH 7.5	—
Trace elements	—	—	—	—	1 ml/liter

[a]From Dworkin (1962).
[b]From Witkin and Rosenberg (1970).
[c]Minimal medium: from Bretscher and Kaiser (1978).
[d]From Mayer (1967). The organism is in fact *Cystobacter violaceus.*
[e]Reichenbach (1984); H. Reichenbach, unpublished observations. Some strains also require thiamine and/or biotin when growing in this minimal medium.

0.1% glucose or soluble starch (Irschik et al., 1987). There are even some strains that can grow in liquid media, like CB medium containing casitone as the only C, N, and energy source (Sarao et al., 1985), but this must be exceptional, because none of the many *Sorangium* strains we have tested would do so.

CB Medium (Casitone Broth: Sarao et al., 1985)

Casitone (Difco)	0.25%
$MgSO_4 \cdot 7H_2O$	0.05%
K_2HPO_4	0.0025%

For liquid cultures of myxobacteria, ordinary Erlenmeyer flasks are perfectly satisfactory as culture vessels. For agitation, a rotary shaker at about 150 rpm is sufficient. As the oxygen demand of myxobacteria is rather moderate, flasks with baffles give little or no improvement in growth and actually are unfavorable with peptone-containing media because they produce too much foam. Several types of antifoam can be used with myxobacteria, but the type of antifoam acceptable in a certain situation and tolerated by a specific strain must always be tested

first. We have found that with small culture volumes and limited foaming, a few drops of a 0.5% cholesterol solution in acetone works well. In fermentors, as a rule, more efficient antifoams must be applied (Mizrahi et al., 1977; Ringel et al., 1977). We have had good results in many different myxobacterial fermentations with polyoxypropylene-based antifoams at concentrations of 0.02 to 0.03%, for instance, antifoam agents LB625 or M115 (both from Brenntag, Mülheim/Ruhr, Germany; Gerth et al., 1980; Irschik et al., 1983a), or with silicone antifoam agent at 0.005 to 0.02% (Merck, Darmstadt, Germany; these sometimes require further additions later during fermentation; Irschik et al., 1983b; Kunze et al., 1984, 1985). Many, but not all, myxobacteria can be cultivated in the presence of an Amberlite adsorber resin, e.g., with 1% XAD-1180 (Röhm & Haas, Darmstadt, Germany; e.g., Gerth et al., 1983; Kunze et al., 1985). This strategy often is extremely useful for the production and recovery of secondary metabolites that are excreted by the cells.

Mass cultivation of myxobacteria in fermentors has not presented any serious problems. Mechanical stress seems not to be a limiting factor. If no continuous control and regulation of culture parameters is required, cultivation in large flasks (10 to 15 liters) also gives excellent results. The flasks are aerated with three air outlets of sintered glass at the ends of silicone tubings, which are lowered to close to the bottom of the vessel. The aeration produces enough turbulence in the liquid so that additional stirring is unnecessary. The appropriate temperature is achieved by placing the flask in an incubation room or by connecting a length of silicone tubing coiling within the culture broth to a thermostat with a pump. With such an inexpensive system, yields on the order of 8 to 12 g cells/liter (wet weight, corresponds to 2 to 3 g dry weight) can be obtained.

Cultivation in commercial fermentors permits the continuous monitoring of various culture parameters and allows them to be balanced and controlled. Although the optimum conditions depend on the type of fermentor used, the strain, and the medium, some generalizations are possible: In fermentations on the scale of 50 to 5000 liters, typical aeration rates are between 0.01 and 0.2 liter air per liter of medium per min, which, with stirring rates between 150 and 650 rpm, result in oxygen levels between 90 and 100% saturation (Gerth et al., 1980, 1982, 1983; Irschik et al., 1983a, 1983b, 1987; Kunze et al., 1984, 1985, 1987; Mizrahi et al., 1977). In a special study on myxovirescin production with *Mx. virescens* Mx v48 with fed-batch fermentations in a peptone medium, a cell yield of 0.5 g (dry weight) per g oxygen consumed, and of 0.31 g per g peptone was obtained (Nigam et al., 1984). The oxygen consumption was 6.25 g O_2 per liter culture in 55 h. Respiration quotients between 0.3 and 1.3 mol CO_2 per mol O_2 were calculated under different culture conditions, with values of 0.8 to 1.0 during logarithmic growth. The maximum dry weight yield was 3.1 g/liter. The specific growth rate, μ, varied between 0.05 and 0.12/h during exponential growth, depending on the feeding rate.

Other types of special fermentations have also been performed with myxobacteria; for instance, continuous cultures of *Mx. virescens* Mx v48 have been achieved in a chemostat with online extraction of myxovirescin in a vortex chamber (Hecht et al., 1987), and fermentations have been established with alginate-immobilized cells (Vuillemard et al., 1988; Younes et al., 1984, 1987). Cells of *So. cellulosum* So ce12 immobilized in 1-mm alginate beads are extremely stable and could be maintained in fermentors under continuous exchange of the medium for up to 75 days before the cells degenerated (Becker, 1990; K. Becker, personal communication). The system allowed the production conditions for the antibiotic sorangicin to be defined more clearly, and under optimal conditions, volume/time yields five times higher than those measured with suspended cells in batch cultures were obtained.

When fermentations are started with an inoculum of 5 to 10% (v/v), the results are almost always satisfactory. Sometimes the age of the inoculum is a critical factor, and beyond a certain, relatively early stage but still within the logarithmic phase, the cells will start to grow only after a long and erratic lag period or not at all. This has been observed with *Polyangium* and *Sorangium* strains (H. Reichenbach, unpublished observations).

Under optimal growth conditions the doubling times of myxobacteria are between 3.5 and 15 h, with the members of the suborder Sorangineae comprising the most slowly growing myxobacteria. Cell densities of up to 10^{10}/ml can be reached, for example, with *So. cellulosum.* Cell mass yields are in the range of 4 to 12 g wet weight per liter (e.g., Irschik et al., 1983a, 1983b, 1985). The dry mass is 20 to 25% of the wet weight.

The pH range for growth is rather narrow, approximately 6.5 to 8.5; in general, there is no good growth below a pH of 6.6 or above pH 8.0. While some of the cellulose decomposers will grow at a pH down to about 5.0 (Couke and

Voets, 1967; Krzemieniewska and Krzemieniewski, 1937a), below pH 5.8, growth is marginal (H. Reichenbach, unpublished observations). As with other bacteria, the pH in myxobacterial cultures can be stabilized by adding a buffer. HEPES and MOPS buffers (50 mM) are well tolerated by many myxobacteria, and Tris may also be used. (It should be noted that many of the media formulations do not contain phosphate; the low amount of phosphate contained in the peptones—in a 0.2% solution of peptone from casein, 0.5 mM phosphate was present (Becker, 1990)—are usually sufficient to satisfy the phosphate requirement of myxobacteria).

The temperature optimum for growth is usually between 32 and 36°C, and there is a sharp maximum around 38°C. The minimum is less clear. *Mx. xanthus* FB grew exponentially with a generation time of 14 h at 14°C, the lowest temperature tested (Janssen et al., 1977); the lower limit for cellulose decomposition by two *Sorangium* strains was found to be 11°C (Baur, 1905; Krzemieniewska and Krzemieniewski, 1937a; Quehl, 1906). *Mx. fulvus* Mx f2 grows, albeit slowly, at 6°C. However, there also are myxobacteria that obviously have a different temperature range: The psychrophilic myxobacteria from Antarctica have already been mentioned (Dawid et al., 1988), and at the other end of the scale, strains of *Archangium, Polyangium,* and *Sorangium* that are able to grow at 40°C have been known for some time (McCurdy, 1969a). About 70% of the *Sorangium* strains grow at 40°C, and about 80% at 38°C. A variety of other myxobacteria can grow at these somewhat elevated temperatures, e.g., most *Polyangium,* many *Archangium, Cystobacter, Chondromyces,* and some *Mx. virescens* strains, but usually not *Mx. fulvus* and *Corallococcus.*

Most myxobacteria do not require vitamins when growing in the usual complex media, but, as already mentioned, a vitamin dependence, usually for thiamine and/or biotin, may develop when the organism is transferred to a minimal medium. *Sg. aurantiaca* Sg a15 needs both thiamine and B_{12} (Kunze et al., 1984); B_{12} dependence is not unusual and has also been observed with many *Polyangium* (but not with *Sorangium*), some *Nannocystis,* and one strain of *Corallococcus macrosporus.*

The mineral requirements of myxobacteria are similar to those of other bacteria. Myxobacteria seem to need relatively high Mg^{2+} concentrations (the optimum is often around 5 to 10 mM, equivalent to 0.075 to 0.15% $MgSO_4 \cdot 7H_2O$). *Sorangium* strains require Ca^{2+} as well and grow better on media with Ca^{2+} than with Mg^{2+} (McCurdy, 1969a). The addition of Co^{2+} sometimes has a stimulating effect (Gerth et al., 1982; Ringel et al., 1977). *Mx. xanthus* is sensitive to elevated concentrations of monovalent cations (Mason and Powelson, 1958), and the salt tolerance of myxobacteria is generally low. An exception is *Mx. virescens,* which seems to form fruiting bodies better when 0.5% NaCl is added to the medium (Rückert, 1978). This organism can still grow on media containing 1% NaCl, although at a clearly reduced rate, and, in fact, it can be selectively isolated on streaks of *E. coli* on WCX agar at that salt concentration.

Usually, myxobacterial cultures are kept in the dark or, in an incubation room, under continuous illumination. As already mentioned, some myxobacteria produce fruiting bodies only when the cultures are illuminated. Also, a phototactic behavior has been reported for some myxobacteria (Aschner and Chorin-Kirsch, 1970). Illuminated cultures often are much more deeply colored than those grown in the dark, because carotenoid synthesis is induced by light (Burchard and Dworkin, 1966a). As color is sometimes a taxonomically relevant characteristic, cultures used for taxonomic work should at least be intermittently illuminated.

Isolation of Mutants

Mutant strains of myxobacteria can be isolated without difficulty by the standard techniques used with other bacteria, provided strains capable of dispersed growth and procedures for obtaining a high plating efficiency are available. Thus, carotenoid mutants, morphogenetic mutants, motility mutants, and metabolic mutants have been isolated in a number of laboratories. *M*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) is often (but not always) an excellent mutagen for myxobacteria. The useful concentration range is 50 to 150 μg NTG/ml when the cultures are treated for 1 h. Killing curves can conveniently be determined with the miniaturized viable-count technique mentioned previously. Also UV irradiation has successfully been used to obtain mutants of myxobacteria. However, in contrast to NTG, UV was ineffective in generating carotenoid mutants in *Mx. fulvus* Mx f2. Further details on the application of different mutagens and a comparison of their effects on a variety of myxobacteria can be found in the literature (e.g., Burchard and Parish, 1975; Hodgkin and Kaiser, 1977; Grimm, 1978). In recent years, insertional mutations produced with transposons such as Tn5 lac, or with RP4 plasmids, have played an increasingly important role in the genetics of myxobacteria (e.g.,

Downard, 1988; Jaoua et al., 1987; Kaiser et al., 1985; Kroos and Kaiser, 1984; Kroos et al., 1986, 1990; Weinberg and Zusman, 1989).

Preservation Methods

Vegetative cultures of myxobacteria on agar plates kept at room temperature or at 30°C will usually remain viable for about 2 to 3 weeks. Storage of such cultures at 4°C leads to a rapid loss of viability.

If fruiting bodies are produced, the cultures remain viable for a much longer time. One has to be certain, however, that the fruiting bodies do indeed contain myxospores, since under some conditions, the fruiting bodies formed are empty or contain only vegetative cells.

Storage in the Form of Desiccated Fruiting Bodies

Pieces of agar that contain fruiting bodies can be placed on sterile filter paper in an empty petri dish, dried in an evacuated desiccator, and stored as such for years. This method is especially useful with myxobacteria such as *Nannocystis* whose fruiting bodies are mainly located within the agar.

Alternatively, vegetative cells can be transferred from a suitable growth medium, preferably VY/2 agar, to small rectangles of sterile filter paper, about 1.5 × 3 cm in size, on water (WAT) agar, and the cultures are incubated at 28°C. Usually, large numbers of fruiting bodies develop within 2 to 3 days. These fruiting bodies are allowed to mature for about 8 days. The filter pads are then placed into suitable containers, such as screw-cap tubes or small medicine bottles, and dried in an evacuated desiccator over silica gel for a few days. The containers are then tightly closed and stored at room temperature or at 6°C (it is not known which temperature is better). The dried fruiting bodies survive for about 5 to 15 years. This method has the advantage of being easy and cheap, and it conserves the ability of the strain to form fruiting bodies. It works, however, only if the strain has not been transferred too many times since its isolation. The method should therefore be applied as soon as a strain is pure, and, if fruiting is a desired feature, even earlier. Sometimes myxospores are still produced on the filter although fruiting bodies are not formed any more. In that case, the filter can still be dried and a viable preparation produced. The growth medium from which the vegetative cells are taken must not be too rich. Thus, for example, fruiting bodies usually do not appear after transfer from CY agar. Sometimes it is necessary to grow the organism directly on the filter paper in order to obtain fruiting bodies. This may be done on filter pads on water agar after streaking autoclaved yeast or food bacteria onto the filter. In this way, *Chondromyces* strains are sometimes induced to fruit. The cellulose decomposers almost always produce many fruiting bodies when growing on filter paper, which has been placed on ST21 agar. As the fabric of the filter paper is destroyed during growth, the paper can no longer be removed, but the macerated mass may be transferred to a fresh filter pad, which is then treated as described above.

To start a culture from dried fruiting bodies, the filter pad is placed upside down on CY agar, VY/2 agar, or WAT agar with streaks of autoclaved *E. coli*. After 24 h, the filter strip should be shifted to another place on the plate, because reduced oxygen access may prevent germination under the wet paper. When the paper is removed, many of the soaked and softened fruiting bodies will stick to the agar surface.

Alternatively, if a sufficient quantity of fruiting bodies is obtainable, e.g., after growth on VY/2 agar, they may be dried in skim milk after having been transferred to water agar, or, in the case of the cellulose degraders, to filter paper on ST21 agar. The fruiting bodies are suspended in skim milk, and a few drops of the suspension are applied to a freeze-dried milk plug in an ampule. The ampules are kept at 4°C for at least 10 min, and the plug is then dried again for about 5 h (this time at room temperature) in a desiccator connected to an efficient pump. After that, the ampules are filled with nitrogen gas and sealed. Strains thus preserved also retain their ability to form fruiting bodies and may remain viable for a longer time than on filter paper. While drying in skim milk also works well with myxospores, including those that were experimentally induced (Dworkin and Gibson, 1964), results with vegetative cells are highly erratic. Vegetative cells invariably die during drying and only those cultures which contained myxospores survived. Experiments with true lyophilization and with protective substances other than skim milk did not give better results.

An excellent and convenient method for the preservation of myxobacteria is freezing at −80°C. Samples of 1 ml are transferred from exponentially growing liquid cultures to screw-cap tubes and placed directly into the deep freeze. Alternatively, the bacteria may be scraped from the surface of a young agar culture, transferred to 1 ml CAS medium, and frozen. All of the myxobacteria that we have tested so far (26 species) have survived in the deep freeze for 8 to 10 years, the longest period over

which data have been collected. Storage at ultra-low temperature is the best way to preserve strains growing in the dispersed state, nonfruiting, nonsporeforming strains, and mutants. It is also a fast and reliable method for the storage of all kinds of strains of a culture collection. However, if the temperature rises above −30 to −25°C, the cells will die unless they are transferred to a growth medium within 5 to 10 h.

For safe long-term storage, freezing in liquid nitrogen is the method of choice. We use the same cell suspension as described above, but add 5% dimethyl sulfoxide (DMSO) to the suspension medium, which is usually CAS medium. We fill 0.5-ml amounts of the suspension into glass ampules, seal them, and immerse them in liquid nitrogen without further precautions. The DMSO may not be required since all the cells stored without DMSO have survived, thus far for 2 years.

Storage of frozen cell suspensions at −25°C is not recommended, because most myxobacteria will die within several days or, at the most, a few weeks.

To reactivate frozen cultures, the tubes or ampules are immersed in cold water to speed up thawing. The cells must be transferred to fresh medium immediately after they have thawed, because if the cells remain in the original medium after thawing, they will lyse within 15 to 30 min. We generally use VY/2 agar for the reactivation of the culture, usually with good results, but CY agar, CAS medium, and MD1 medium can also be used. If experience is lacking with a certain strain, the thawed suspension should always be inoculated into several different media, including a liquid medium. It sometimes happens, albeit rarely, that recovery will occur only on a specific medium, although the other media normally support growth. For example, we have observed that some strains can be reactivated only in a liquid medium or only on agar plates. If a liquid medium is used, care should be taken not to dilute the organisms too much, e.g., by inoculating a few drops into 100 ml. In some cases, for example with particularly fragile mutants, it is useful to reactivate the cells by embedding them in a growth medium containing 0.6% agar. The soft agar appears to provide a matrix which protects cells that have been slightly damaged by freezing and thawing (D. Zusman, personal communication).

At least some myxobacteria can be stored in a 1% NaCl solution at room temperature. The cell suspension must not be too dense: the liquid should be just visibly turbid, otherwise the cells will lyse overnight. For one strain of *Mx. xanthus,* a suspension of vegetative cells has been kept alive for 5 months in this way. In another case, we suspended vegetative cells and glycerol-induced myxospores of *Mx. xanthus* FB in 1% NaCl solution and kept the suspensions in sealed ampules for 7 years at room temperature. After that time, a culture could be started from the myxospores, but not from the vegetative cells. While the original strain was very sensitive to NaCl, the revived strain grew on CY agar with 1% NaCl. This clearly indicates that in such cases, the bacteria are not completely dormant, and that a selection for certain characteristics may have taken place.

Characterization

Considerable information has accumulated during the past 10 years on the composition, biochemistry, molecular biology, and genetics of myxobacteria.

Morphology and Fine Structure

The vegetative cells of the myxobacteria are rod-shaped, typically, 3 to 6 μm long and 0.7 to 1.0 μm wide. They are found in two morphological types: 1) slender, flexible rods with more-or-less tapering ends, and 2) cylindrical, somewhat rigid rods with bluntly rounded ends (Krzemieniewska and Krzemieniewski, 1928; Fig. 1a–d). These different cell types correlate with a number of other basic characteristics and represent two suborders of the Myxobacterales, the Cystobacterineae and the Sorangineae. The cell shape makes it possible to assign a strain at once to one of the two suborders.

Most electron microscopic studies on myxobacteria are rather old now, and a thorough reinvestigation would be desirable. In addition to *Mx. xanthus*, several other myxobacteria have been studied, including *Mx. fulvus, Mx. virescens, Cystobacter, Stigmatella, Sorangium,* and *Chondromyces* species (Abadie, 1967, 1968, 1971a, 1971b; Galván et al., 1986; Lampky, 1976; MacRae and McCurdy, 1975; McCurdy, 1969b; Reichenbach et al., 1969; Schmidt-Lorenz and Kühlwein, 1968; Voelz, 1965, 1966a, 1966b, 1967; Voelz and Dworkin, 1962). Cytologically, the myxobacteria are typical Gram-negative bacteria. Both the tapered and the cylindrical cell types appear to be identical in fine structure, ultrathin sections revealing a typical triple-layered outer membrane and a thin peptidoglycan sheet. The cells divide by septum formation (Abadie, 1971b; MacRae and McCurdy, 1975). Within the cells, mesosomes and mesosomelike membrane bodies are often seen (Abadie, 1967, 1968, 1971b; Galván et al., 1986; Lampky, 1976; MacRae and McCurdy, 1975;

Voelz, 1965). The visualization of ribosomes was initially a problem, because they were difficult to fix properly (Voelz, 1967), but it later turned out that they are of the usual type. At low phosphate levels, the formation of polysomes seems to be induced in *Mx. xanthus* (Voelz, 1966b). Polysomes were also observed in cells of *Cm. crocatus,* and they were often attached to the cytoplasmic membrane and arranged in a helical fashion (Abadie, 1971b). Unusual disk-shaped, membrane-associated structures have been described from *Cm. crocatus* (MacRae and McCurdy, 1975). In cross-sections they appear as two double tracks, each 7.5–11 nm wide and between 55 and 130 nm long, connected to the interior of the cytoplasmic membrane by 11- to 15-nm-long, fibrillar extensions. Several classes of intracellular granules, or inclusion bodies, have been found. Black granules, apparently produced by all myxobacteria, have been identified as polyphosphate (Voelz et al., 1966). Electron-transparent granules are of at least two types: 1) granules that are pale gray, containing material that does not contrast well; these have been suggested to be polysaccharide (Reichenbach et al., 1969), "slime vacuoles" (Abadie, 1971b; Schmidt-Lorenz and Kühlwein, 1969), or "glycogen" (Voelz et al., 1966). This has been corroborated by light microscopy after performing the periodic-acid Schiff reaction (Schmidt-Lorenz and Kühlwein, 1969). 2) Completely empty, large, circular areas, which are sudanophilic under the light microscope, have been seen in the cells of *So. cellulosum* (Lampky, 1976) and may be lipid material. Also, poly-β-hydroxybutyric acid has been demonstrated chemically in *So. cellulosum* (H. Jansen, personal communication). In cells of *Mx. xanthus* grown under suboptimal conditions, large parts of the cytoplasm sometimes appear to have a crystalloid pattern (Voelz, 1966b, 1967, 1968).

In many myxobacteria, intracellular microtubules and fibrils may be seen (Abadie, 1971b; Burchard et al., 1977; MacRae and McCurdy, 1975; Schmidt-Lorenz and Kühlwein, 1968). The diameter of the microtubules is 10 to 16 nm (with *Cm. crocatus* 15 to 19 nm), that of the fibrils, 4 to 5 nm (with *Cm. crocatus* 7.5 to 10 nm). Once it was thought that microtubules and fibrils were just two different aspects of the same structure depending on the plane of sectioning (Schmidt-Lorenz and Kühlwein, 1968), but this is not the case. The fibrils are usually arranged in bundles which may be 40 nm wide and 3.5 μm long. The bundles usually lie close to the cytoplasmic membrane and often follow the long axis of the cell, sometimes to the cell poles, but they also may cross the cell from one side to the other. A herringbonelike periodicity with a 12-nm spacing has occasionally been seen (Burchard et al., 1977). Also, transverse bands with a very regular, crystal-like pattern may occur (Burchard et al., 1977). Originally, fibrils were found only in cells from swarming agar cultures (Schmidt-Lorenz and Kühlwein, 1968), but they were subsequently found in cells from liquid cultures as well (Burchard et al., 1977; H. Reichenbach, unpublished observations). These systems of fibrils and microtubules have been interpreted as contractile elements responsible for gliding motility by producing contraction waves (Burchard et al., 1977; Schmidt-Lorenz and Kühlwein, 1968), but there is no evidence that those structures can really contract, and they could have some other function or even be artifacts. In *Cm. crocatus,* those structures were assumed to be composed of ribosomes (paracrystalline system: Abadie, 1971b).

An extremely complex structure has been discovered in the surface layers of *Mx. fulvus,* just below the outer membrane, suggested to be the apparatus of gliding motility (Lünsdorf and Reichenbach, 1989). One or several belts with an intricate substructure appear to run helically around the periphery of the cell, beginning at or close to the cell pole. From isolated material, it was deduced that the unit element consists of a long series of tiny rings at a regular, periodic distance, connected to each other by two longitudinal structures, perhaps two fibrils. Several of these unit elements, or strands, form a ribbon, and it could be shown that the rings in those ribbons can change their conformation thereby producing a reduction of the transverse dimension of the ribbon by 40%. The belt is composed of about seven ribbons, and conceivably a coordination of the conformational changes occurring in the ribbons could produce a travelling wave in the belt and concomitantly in the cell surface, which would propel the cell. Nothing is known yet about the biochemistry of the system, and not even the structural details are fully understood. However, the components of the apparatus have been seen also in other myxobacteria, including *Mx. xanthus.*

Gliding Motility

From the very beginning, investigators have been intrigued by the mechanism of gliding motility in the myxobacteria. There are numerous hypotheses, including contractile fibrils (Burchard et al., 1977), a travelling wave along a complex belt below the outer membrane of the cell (Lünsdorf and Reichenbach, 1989), and the polarized excretion of surfactant (Keller et al.,

1983), but none have been proven so far (for reviews, see Burchard, 1980, 1981, 1984). Genetic analysis of the motility system of *Mx. xanthus* has revealed a very complex situation involving at least 33 genes (Hodgkin and Kaiser, 1979a, 1979b; for review, see Shimkets, 1990b). Two almost completely separate motility systems exist. The A system (A for adventurous) controls the movements of individual, independent cells. It comprises 22 loci and may be related to the lipopolysaccharide (LPS; Gill and Dworkin, 1986, 1988). The S system (S for social), comprising 10 loci, controls the gliding movements of groups of cells. If the S system is to operate, the cells must not be farther apart than one cell length. They obviously have to be in contact with one another, and the contact is mediated by pili (Kaiser, 1979) and by strands of LPS (see below). The S system seems to be of central importance in all social activities of the myxobacteria, as in the coherence of the swarm colonies, in the rippling phenomenon, and in fruiting. Usually, a mutant is only nonmotile under all circumstances if it is A^-S^-. A single mutation in one further locus, *mgl*, has the same effect, so that *mgl* appears to be required in the A system as well as the S system. Another set of genes, the "*frizzy*" (*frz*) genes, determine the frequency with which the cells change the direction of their movements (Blackhart and Zusman, 1985). They were discovered because the colonies of Frz mutants differ substantially in size and organization from wild-type swarms, and the cells show an aberrant aggregation behavior. While wild-type cells may reverse their movements every 6 to 7 min, there are some Frz mutants that do so every 2 h or every 2 min. The genes and their gene products have been analyzed in considerable detail. Surprisingly, the Frz CD protein is 40% homologous with the Tar protein, which controls the direction of movement in *Salmonella typhimurium* (McBride et al., 1989). As in the enteric bacteria, *Mx. xanthus,* the direction of movement is controlled by methylation and demethylation reactions (McCleary et al., 1990).

The speed of gliding of myxobacterial cells varies, depending on the substrate and the temperature; typically, it is between 3 and 13 μm/min (*Mx. virescens,* 32°C, on peptone agar, measured in movie frames; Reichenbach, 1966). Gliding cells always deposit slime tracks (Fig. 5). When placed in a liquid such as water, the cells sometimes attach themselves to the glass with one pole and perform relatively fast gyrating movements.

While *Mx. xanthus* cells show directed movements towards latex beads and clumps of prey bacteria (Dworkin, 1983), it has not been possible to demonstrate a chemotactic response to any of a number of chemicals tested (Dworkin and Eide, 1983). Nevertheless, myxobacterial cells clearly have some sensory mechanism that enables them to move toward one another over a distance of at least 10 cell lengths. Gliding cells also respond to delicate structural disturbances in the substrate surface, such as are produced in agar plates by mechanical stress. This results in distorted swarms and unusual fruiting body patterns, a phenomenon which has been named elasticotaxis (Stanier, 1942b). This is interesting insofar as the movements of the cells in this case are not coordinated by intercellular communication but by an external factor, such as the structural pattern of the substrate surface, via a purely physical mechanism. The same mechanism may also play a major role in determining the behavior of cells in the swarm and during fruiting.

Occasionally, branched cells are seen in myxobacterial cultures; they can also translocate, albeit clumsily (Reichenbach, 1965a, 1966).

The nature of the outer surface of the myxobacterial cell is of interest, because it can be assumed that the organization of the surface may be directly involved in gliding motility as well as in certain types of intercellular communication. It seems that the appearance of the cell surface may change substantially with the preparation technique, the culture conditions, and, of course, also with different types of mutants. The underlying rules are only partly understood at the moment. In thin sections, the outer surface appears essentially smooth or, at the most, slightly wavy. The same is normally the case in freeze-etched preparations (Burchard and Brown, 1973). In negative contrast and in shadowed mounts, however, the surface often appears to be covered by short, vesicular blebs and long, irregular, seemingly tubular extensions, approximately 50 nm wide, which are probably protrusions of the lipopolysaccharide (LPS) layer (e.g., Arnold and Shimkets, 1988b; MacRae and McCurdy, 1975, 1976; Schmidt-Lorenz and Kühlwein, 1969). The excretion of this material can be prevented by adding the diazo dye Congo red (5 μg/ml, a concentration which does not interfere with growth) to the culture; as a consequence, *Mx. xanthus* cells are no longer able to agglutinate (Arnold and Shimkets, 1988a, 1988b). This also implies a loss of social (S) gliding and of fruiting. The Congo red-treated cells behave in all these respects like a class of A^+S^- mutants, the Dsp mutants, which indeed do not bind Congo red. It may thus be concluded that the major Congo red receptor is controlled by genes of the S sys-

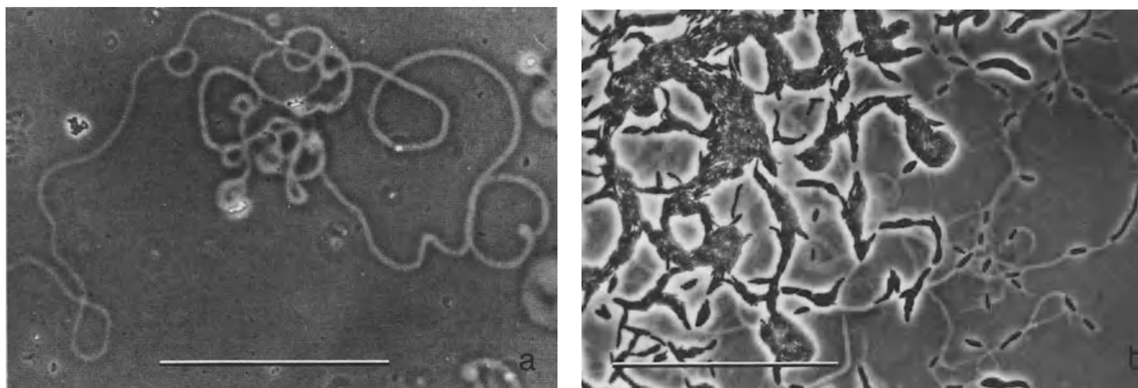


Fig. 5. Slime tracks as seen on thin agar layers in chamber cultures. (a) *Nannocystis exedens.* (b) *Myxococcus fulvus;* the spots seen on the left are cell clusters, not single cells. Bar = 100 μm. Phase contrast.

tem. If the Congo red is removed, wild-type cells begin to produce the tubular material again within 30 min, and their cohesiveness and all associated properties are restored.

Surface Structures

Scanning electron micrographs of mycobacteria often show a system of extracellular strands and fibers which may even form a dense network connecting the cells to each other and to their environment (Arnold and Shimkets, 1988b; Burnham et al., 1981; Lampky, 1976). Some of that material may be identical with the tubular LPS strands discussed above, but some may also be excreted slime. It seems that such networks can also be used by the myxobacteria to entrap prey organisms (Burnham et al., 1981).

Another type of appendage is the thin, polar fimbria, which is 5 to 8 nm wide and may exceed the length of a cell (Arnold and Shimkets, 1988b; Dobson and McCurdy, 1979; MacRae and McCurdy, 1975, 1976; MacRae et al., 1977). Fimbriae have been observed with many different species of both suborders; the only negative result was with *Nannocystis,* but this may have been due to technical problems. The fimbriae may be restricted to one of the two cell poles, and up to six have been seen per cell. In *Mx. xanthus,* two types of fimbriae were distinguishable, a rigid (R) and a flaccid (F) type (Dobson and McCurdy, 1979). The two could be separated and purified and turned out to be very similar in many respects (e.g., in the apparent molecular weight, which was about 7,750 Da; and in their amino acid composition). Only their dissociation behavior differed. The R type disintegrated into 2-nm microfibrils at pH 3.5 to 4.5. Both species of fimbriae agglutinated guinea pig erythrocytes. The F type may be the contracted form of the R type.

Tiny holes in the outer membrane, 6.5 to 8.5 nm wide and surrounded by a collar of 2.6 nm, which sometimes shows a ring of 12 spikes, have been proposed to be the insertion sites of the fimbriae (MacRae et al., 1977). As fimbriae could not be demonstrated on nonmotile mutants (MacRae and McCurdy, 1976), and, in particular, not on mutants lacking S motility (Kaiser, 1979), it has been suggested that the fimbriae may be involved in social motility. In *Mx. xanthus,* four genetic loci (*sglA, sglB, sglG,* and *tgl*) seem to control fimbriation. If *tgl*$^-$ cells come in contact with *tgl*$^+$ cells, their S gliding is restored (Kaiser, 1979). But as pointed out above, S motility is also correlated with the presence of the LPS-containing fibrils. Thus, the cell-cell interactions required for social motility may require the participation of both the fimbriae and the fibrils.

Chemical Composition

Much has been learned in the last decade about the chemical composition of the myxobacterial cell. However, the precise nature of the extracellular polysaccharides characteristically excreted by the myxobacteria remains undetermined, as it is usually accompanied by a variety of other large and small excreted molecules (Sudo and Dworkin, 1972; Hanson and Dworkin, 1974). The excreted slime appears usually to be a heteropolysaccharide containing, among others, *N*-acetyl aminosugars (Sutherland, 1979; Sutherland and Thomson, 1975). Slime from fruiting bodies resembles in its overall composition that produced in vegetative cultures. A large extracellular protein-polysaccharide-lipid complex with proteolytic activity has been isolated from culture supernatants of *Mx. virescens* (Gnosspelius, 1978b). A glycopeptide with a heat-stable blood anticoagulant activity,

named myxaline, has been obtained from the culture broth of *Mx. xanthus* (El Akoum et al., 1987; Masson and Guespin-Michel, 1988). The glycan part contains *N*-acetyl aminosugars, and the peptide is rich in glutamic acid and serine. The excreted slime must be chemically different in the two suborders of myxobacteria, since Congo red (0.01% aqueous solution) stains the slime of the Cystobacterineae, but not that of the Sorangineae (McCurdy, 1969a).

LIPOPOLYSACCHARIDES AND CELL WALLS. There are several studies on the chemistry of the lipopolysaccharides (LPS) of various myxobacteria (Rosenfelder et al., 1974; Sutherland, 1979; Sutherland and Thomson, 1975; Weckesser et al., 1971). Contrary to the statement of Ruiz et al., (1987), myxobacterial LPS, including lipid A, can be readily and completely extracted from the cells by the usual phenol procedure (e.g., Panasenko, 1985; Rosenfelder et al., 1974; Sutherland and Smith, 1973; Sutherland and Thomson, 1975). Typical LPS yields are 0.5 to 1.0% (up to 1.9%) of the cell dry weight. Chemically, the LPS of the myxobacteria resembles that of the enterobacteria. The LPS of *Mx. fulvus* contains mannose, galactose, glucose, rhamnose, arabinose, glucosamine, and 3-O-methyl-D-xylose, a sugar only very rarely seen in bacterial LPS (Rosenfelder et al., 1974; Weckesser et al., 1971). The LPS of other myxobacteria may differ somewhat in its sugar composition, but 2-keto-3-deoxyoctonic acid is always present, and heptose is never present. The 3-O-methyl-D-xylose may or may not be found, and, in some cases, similar nonpolar but thus far unidentified sugars have been observed (Sutherland, 1979; Sutherland and Smith, 1973; Sutherland and Thomson, 1975). While the scant data do not really allow generalizations, a complete lack of rhamnose seems to be characteristic for *Sorangium* and may be another distinguishing characteristic of that suborder. The lipid A moiety contains glucosamine and the usual 3-hydroxy fatty acids, mostly iso-3-OH-C_{15} and iso-3-OH-C_{17} (Rosenfelder et al., 1974). During sporulation of *Mx. xanthus,* the LPS, or at least its polysaccharide part, is completely lost (Sutherland, 1976b), and a capsule composed of an α-1,3-glucan is produced (Sutherland and Mackenzie, 1977). That capsule is ruptured and discarded during germination rather than enzymatically solubilized (Voelz, 1966a). Monoclonal antibodies have also been obtained against O-antigens and against a core antigen of *Mx. xanthus* LPS (Fink and Zissler, 1989a; Gill and Dworkin, 1988). Several O-antigen mutants were defective in the A system but not in the S system of gliding. They were still able to form normal fruiting bodies (Fink and Zissler, 1989b). Endotoxinlike effects (anticomplement test and Shwartzman skin reaction) could be produced with whole cells and myxospores of *Mx. xanthus* (Ruiz et al., 1987).

Mx. xanthus, the only myxobacterium for which the organization of the cell wall has been investigated, has a thin peptidoglycan layer that seems to be organized in patches rather than in a homogeneous sacculus (White et al., 1968). The composition of the peptidoglycan resembles that of *E. coli,* with diaminopimelic acid at the cross-links. It appears that during sporulation, the degree of cross-linking increases. At the same time, galactosamine and glycine accumulate in the wall layer. The pepotidoglycan content of vegetative cells and myxospores is, however, the same (0.6% of the dry weight).

LIPIDS. The fatty acid spectra of the myxobacteria show characteristic differences between the two suborders (Fautz et al., 1981). While all myxobacteria contain iso-branched fatty acids in substantial quantities, mainly saturated $C_{15:0}$ and $C_{17:0}$, the pattern is dominated by them only in the Cystobacterineae (usually between 50 and 70%) (Fautz et al., 1979, 1981; Schröder and Reichenbach, 1970; Ware and Dworkin, 1973; Yamanaka et al., 1988). *Na. exedens* (suborder Sorangineae) is the only myxobacterium so far in which larger amounts (about 30%) of iso-$C_{15:1}$ and iso-$C_{17:1}$ are found.

Even more characteristic is the distribution of hydroxy fatty acids. Although it was overlooked in the earlier studies, a 2-hydroxy (OH) fatty acid iso-2-OH $C_{17:0}$, was first seen as a contaminant from phospholipid in LPS preparations of *Mx. fulvus* and *Cb. ferrugineus* (Rosenfelder et al., 1974). The LPS contains 3-OH fatty acids, exclusively. Later, proof was presented for the chemical structure of the 2- and 3-OH fatty acids of *Mx. fulvus* and *C. ferrugineus,* and it was demonstrated that, in total cell hydrolysates, 2-OH fatty acids, mainly iso-2-OH $C_{17:0}$, constitute a respectable share of the total fatty acids (10 to 12%), while 3-OH fatty acids only play a minor role (2.5 to 4.5%: Fautz et al., 1979; later analyses of Yamanaka et al., 1988, give somewhat lower values of 1 to 10% for 2-OH, and 0.2 to 0.3% for 3-OH). In members of the Sorangineae, no hydroxy fatty acids are seen at all in total cell hydrolysates (Fautz et al., 1981). In members of the Cystobacterineae, two types of phosphatidylethanolamine have been demonstrated, one of which contains exclusively nonhydroxy fatty acids; the other, however, contains 50 to 70% 2-OH fatty acids (Yamanaka et al., 1988). Another major component of the fatty acid pattern of the Cystobacterineae is the

$C_{16:1}$ fatty acid (15 to 39%). This component, too, seems much reduced in the Sorangineae. While all investigators found only iso-branched fatty acids in myxobacteria, one laboratory did report anteiso fatty acids in *Mx. xanthus* (Ruiz et al., 1985). In another study, a clear difference was seen between the fatty acid patterns of *Corallococcus* and those of *Myxococcus* species (Monteoliva-Sanchez et al., 1987): branched and unbranched $C_{17:0}$ fatty acids were present in large quantities in the latter (27 to 38%) but completely absent in the former. While such a clear difference would be useful for taxonomic reasons, it is highly unlikely and is not supported by other investigations (Yamanaka et al., 1988).

The earliest analysis of the phospholipid pattern of a myxobacterium, that of *Mx. fulvus* Mx f2, demonstrated phosphatidylethanolamine as the main component (72%), but phosphatidylglycerol (9%), phosphatidylinositol (7%), and an unknown, ninhydrin-positive phospholipid (8%) were also present (Kleinig, 1972). A small proportion of the phospholipids were alk-I-enyl-acyl (1–15%) and alkyl-acyl compounds; however, they comprised 22% of the phosphatidylinositol compounds. The phospholipid content of the isolated membranes was 25% (dry weight). The occurrence of two species of phosphatidylethanolamine in myxobacteria has already been mentioned (Yamanaka et al., 1988). In another study concerning the membrane composition of *Mx. xanthus,* the inner and outer membranes could be separated (Orndorff and Dworkin, 1980). The outer membrane showed an unusually low buoyant density (1.221 g/ml) which was probably due to an exceptionally high phospholipid content. The main phospholipid in both membranes was phosphatidylethanolamine (60 to 70% of total phospholipid). The other major phospholipids were phosphatidylglycerol and two unknown phospholipids. Phosphatidylserine and cardiolipin were found only in the cytoplasmic membrane. Substantial amounts of lysophospholipids were found in both membranes, although somewhat more in the outer membrane. They obviously indicate lipase activities, which here seem not to be restricted to the outer membrane.

In *Sg. aurantiaca,* too, the main phospholipid was phosphatidylethanolamine (50%), followed by phosphatidylglycerol (12%), and phosphatidylinositol (20%) (Caillon et al., 1983). Very high amounts of lysophosphatidylethanolamine were also found. Each phospholipid had a different fatty acid composition. Alkyl ether linkages were common; phosphatidylinositol occurred only as the dialkyl compound. The structural details of an alk-I-enzyl-acyl-phosphatidylethanolamine, i.e., a plasmalogen, from *Mx. stipitatus* have recently been elucidated (Stein and Budzikiewicz, 1987).

From the amount of phosphorus in the lipid fraction of *Sg., aurantiaca* it has been estimated that only about 40% of the fatty acids are bound in phospholipids (Schröder and Reichenbach, 1970), and, in fact, several nonphospholipids with fatty acids on them have been identified. Ceramides and cerebrosides have been isolated from *Cystobacter fuscus* and chemically characterized (Eckau et al., 1984; Dill et al., 1985). Ceramides are long-chain, 1,3-dihydroxy-2-amino bases (sphinganine) with an amide-bound fatty acid. Cerebrosides are sphingolipids consisting of a ceramide with a C-1-bound sugar. The compounds are common in eukaryotes and, interestingly, they play a role in cell-cell recognition and cell adhesion and also have been found to be fruiting-body inducers in basidiomycetes. Perhaps they play similar roles in myxobacteria. The myxobacterial ceramides all contain 2-hydroxy fatty acids. Also, a novel, long-chain base, 17-methylsphinga-4E,8E-dienine, was found in two of them. The cerebrosides are all galactosides.

The capnoids discovered as a characteristic constituent of the lipids of another group of gliding bacteria, the cytophagas, have not been found in myxobacteria (Godchaux and Leadbetter, 1983). The capnoids are C-1 sulfonic acids of sphinganine bases and their ceramides.

Pigments and Respiratory Components

Carotenoids are the main pigments of most myxobacteria. With the exception of *Na. exedens,* the main compounds are always monocyclic carotenoid glycosides, usually containing glucose, with a fatty acid attached to the sugar via an ester bond (Kleinig et al., 1970, 1971; Reichenbach and Kleinig, 1971). Pigments of this type are rather unusual, but they have been found, for example, in *Nocardia kirovani* (Bacheron et al., 1970). The structure of myxobacton, which is the main pigment of many myxobacteria including *Mx. xanthus,* has been reinvestigated and corroborated (Eckau et al., 1984). It appears that certain pigment types are characteristic for taxonomic subgroups (for a review, see Reichenbach and Kleinig, 1984). *Nannocystis* contains aromatic carotenoids but no glycosides. Members of the suborder Sorangineae produce glucosides with hydroxyl but not with keto functions on the chromophore, and the Cystobacterineae have glucosides with keto but not with hydroxyl functions. In all cases, complex mixtures of carotenoids are found, but only

monocyclic and acyclic compounds occur. The carotenoid content of exponentially growing, late log-phase cultures of *Mx. fulvus* Mx f2 has been determined. Acetone extracts typically represent 0.03% of the dry weight of dark-grown cells and 0.03–0.06% of the dry weight of light-grown cells (Reichenbach and Kleinig, 1971). The carotenoids are located in the (cytoplasmic) membrane, and their concentration there is consequently much higher (0.14% in a membrane preparation of *Mx. fulvus* containing both membranes; Kleinig, 1972). The carotenoid synthesis of myxobacteria is induced by light (Burchard and Dworkin, 1966; Burchard and Hendricks, 1969; Reichenbach and Kleinig, 1971). The genetics of light-induced carotenogenesis has been studied in *Mx. xanthus* and has been found to comprise various control mechanisms. Also, a light-induced promoter has been identified and cloned (e.g., Balsalobre et al., 1987; Martinez-Laborda et al., 1990). The only demonstrated role of carotenoids in myxobacteria is to provide protection against photooxidation (Burchard and Dworkin, 1966a). This has also been demonstrated with carotenoid-free mutants of *Mx. fulvus* Mx f2. The killing effect is seen only if the mutants are illuminated with daylight intensities (about 40,000 lux; H. Reichenbach, unpublished observations). In *Mx. xanthus,* protoporphyrin IX has been found to be a photosensitizer (Burchard and Dworkin, 1966a). Dark-grown cultures, which produce only small amounts of carotenoid, lyse quickly if illuminated. It is not clear, however, that all myxobacteria accumulate protoporphyrin IX as a photosensitizer; it could not be detected, for example, in *Mx. fulvus.* The effect of various inhibitors on the cyclization reaction of carotenoid synthesis has also been studied (Kleinig, 1974, 1975; Reichenbach and Kleinig, 1971). In normally growing laboratory cultures, there is virtually no turnover or degradation of carotenoids. A system for the in vitro synthesis of C_{15} to C_{60} polyprenols from isopentenylpyrophosphate with a crude enzyme preparation from *Mx. fulvus* has been developed (Beyer and Kleinig, 1985).

Pigments other than carotenoids are also produced by myxobacteria. A fluorescent, pale-yellow pigment is characteristic for *Mx. stipitatus* (Lampky and Brockman, 1977) and similar pigments are also found in *Mx. xanthus* (Gerth et al., 1983). On certain media, the colonies of *Cb. violaceus* (= *Ar. violaceum*) develop a deep purple-violet color, probably due to melanoid pigments (Kühlwein and Gallwitz, 1958; Reichenbach, 1965a). Melanoid pigments are also relatively often seen with individual strains of many other species, especially on peptone-containing media, e.g., with *Cystobacter, Archangium, Corallococcus,* and *Stigmatella.* Liquid cultures of *Sg. aurantiaca* in peptone medium turn a deep black color within 1 to 2 h after reaching the stationary phase (Reichenbach and Dworkin, 1969). The slimy swarms of some strains of *So. cellulosum* on CA2 agar become deep violet to black after 2 to 4 weeks. *Mx. virescens* and *Pl. vitellinum* are bright greenish-yellow, especially on protein-containing media. Often the various shades observed with different strains and species are only due to differences in the quantitative proportions of the same pigments. Fruiting bodies, and especially the walls of the sporangioles, are often very intensely colored, but the chemical nature of these pigments is not known.

The respiratory quinones of the myxobacteria are exclusively menaquinones (Kleinig, 1972; Kleinig et al., 1974). A survey of 11 genera and 20 species of both suborders showed that the menaquinone pattern of the myxobacteria is completely uniform and comprises virtually only MK-8 (M.D. Collins, personal commuinication). *Nannocystis* contains substantial quantities of squalene (0.4%) and steroids (0.4% of the dry weight), mainly 3-OH-cholest-8(9)-en-3β-ol (Fig. 6; Kohl et al., 1983). Because these compounds are synthesized de novo, *Nannocystis* is one of the very few prokaryotes that are known to be able to synthesize steroids.

Membranes and Membrane Proteins

A few interesting proteins that have been isolated and characterized from myxobacteria will be briefly mentioned. Most of these are surface proteins that seem somehow to be involved in cell-cell contacts and morphogenetic events.

The membranes of *Mx. fulvus* consist of about 64% protein and 29% lipid, the latter comprising mainly (25%) phospholipid (Kleinig, 1972). About 40 polypeptides could be resolved in membrane extracts of *Mx. xanthus* (Orndorff and Dworkin, 1980). Of those, about 26 were enriched in the cytoplasmic membrane fraction, and 8 to 10 major proteins were found

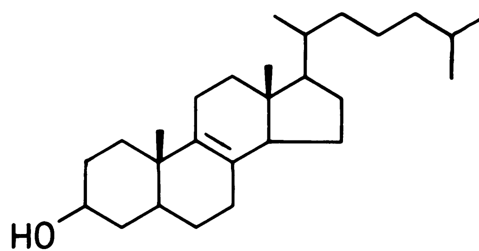


Fig. 6. The sterol 3-OH-cholest-8(9)-en-3β-ol, which is synthesized by *Nannocystis exedens.*

in the outer membrane fraction. None of the latter were, however, identical to the 32-kDa and 44-kDa proteins that are the principal proteins of the outer membrane of *Escherichia coli.* Many membrane and soluble proteins change in amount and rate of synthesis during fruiting. Six of the membrane polypeptides changed substantially during aggregation (Orndorff and Dworkin, 1982): during the early stages of fruiting, protein T disappeared from the soluble fraction of *Mx. xanthus,* when proteins S and U first appeared and then increased dramatically. Protein S (Inouye et al., 1979a) has been particularly well studied. In vegetative cells it amounts to less than 1% of the soluble protein, but during fruiting body formation, its rate of synthesis rises to 15% of total protein synthesis. In the presence of Ca^{2+}, protein S assembles on the surface of the maturing myxospores (Inouye et al., 1979b). Certain domains in the amino acid sequence of protein S show homologies to the Ca^{2+}-binding sites of calmodulin. The functional correspondence of those areas was demonstrated by site-specific mutations, which eliminated the Ca-binding property of protein S together with its ability to accumulate on the myxospores. Protein S also has some homology with the bovine-lens protein, α-crystallin (Inouye et al., 1983a; Teintze et al., 1988; Wistow, 1990). Protein S is coded by two tandem genes which closely follow one another and show an 88% amino acid homology. It appears that the protein species synthesized during fruiting comes exclusively from (downstream) gene 2 (Inouye et al., 1983a, 1983b).

Another development-specific protein is a myxobacterial hemagglutinin, a lectin that is induced during the developmental aggregation of *Mx. xanthus* (Cumsky and Zusman, 1981). It appears to be localized in the periplasmic space or loosely bound to the cell surface and to recognize a galactose-containing, complex receptor. Its distribution over the cell surface is uneven, as it is found in patches mainly at the cell poles (Nelson et al., 1981). The 28-Da protein contains four highly conserved domains of 67 amino acids each, which are reflected in four strong internal homologies in its gene (Romeo et al., 1986).

Several nucleotide-binding proteins have been described. They are of great interest because they could be involved in signal transduction, e.g., during development. In the soluble-protein fraction of sporulating *Mx. xanthus,* a single protein has been discovered that specifically binds cyclic AMP (cAMP). The binding capacity did not change during myxospore development (Orlowski, 1980). Vegetative cells of *Mx. xanthus* contain a cyclic GMP- (cGMP-) and a cAMP-binding protein in the periplasm, and a second cAMP-binding protein in the cytoplasm. The latter appears to be identical with the cAMP-binding protein mentioned above. All three activities varied substantially during aggregation (Devi and McCurdy, 1984a). In the membrane fraction of *Sg. aurantiaca,* a single, GTP- and GDP-binding protein was identified. Proteins of this kind are very common in eukaryotes, but have only rarely been found in prokaryotes. It was suggested that the protein plays a role in the phosphoinositide metabolism of *Stigmatella* (Dérijard et al., 1989).

Using an iodination technique, substantial changes in the pattern of accessible surface proteins of *Mx. xanthus* were shown to take place during development (Maeba, 1983). A glycoprotein, probably a peripheral membrane protein that is present only in vegetative cells, was further characterized (Maeba, 1986). The 74-Da protein contained about 15% carbohydrate, mainly neutral sugars, but also some hexosamines and uronic acids. It made up 1% of the total cell protein and might play a role in cell-cell interactions.

A different approach to the study of the dynamics of the cell surface during development has been the use of monoclonal antibodies against cell-surface antigens (CSAs) of vegetative and developing cells (Gill et al., 1985). In this way, many different CSAs of *Mx. xanthus* have been identified (e.g., Gill and Dworkin, 1986, 1988). While most of the antibodies are directed against cell-surface proteins, some are directed against LPS or, in the case of CSA 1604, against a large, 150-Da complex consisting of at least two protein subunits and LPS (Jarvis and Dworkin, 1989a, 1989b). CSA 1604 is of special interest, because antibodies directed against it block certain stages in the fruiting body development of *Mx. xanthus.* Other monoclonal antibodies are directed against cell-surface proteins that appear uniquely during development (Gill and Dworkin, 1986).

Some proteins have been identified by transfer and expression of myxobacterial genes in *E. coli.* An 18.7-Da, basic protein coded by the *vegA* gene of *Mx. xanthus* turned out to have amino acid homologies with certain histones and may function as a DNA-binding protein. It is essential for vegetative growth (Komano et al., 1987). Also, the *Mx. xanthus* gene *fprA* was shown to code for a protein that binds flavin mononucleotide. This protein, too, is essential for growth. Overexpression of the gene in *E. coli* leads to a spectacular increase in flavin biosynthesis, but the FprA protein seems not be an enzyme in the biosynthetic pathway (Shimkets, 1990a).

The product of the *csgA* gene of *Mx. xanthus,* C factor, which is required for several steps during fruiting body development, was recovered from developing fruiting bodies and characterized (Kim and Kaiser, 1990). The 17-Da protein restores the developmental capacities of *csgA* mutants, acts in a very low and narrow concentration range (1 to 2 $\times$ 10^{-9} M), and appears to represent a signal factor.

Nucleic Acids

The GC content of myxobacterial DNA is between 67 and 72 mol% (as measured by buoyant density and thermal melting, respectively). The GC content appears to be slightly higher (70 to 72 mol%) in the Sorangineae than in the Cystobacterineae (67 to 70 mol%) (Behrens et al., 1976; Mandel and Leadbetter, 1965; McCurdy and Wolf, 1967). The GC content may vary considerably between different DNA segments in the genome; in *Mx. xanthus,* for example, it varies from 36 to more than 80 mol% (Komano et al., 1987).

Repeated efforts have been made to determine the genome size of *Mx. xanthus,* applying widely differing techniques, including cloning of the entire *Mx. xanthus* genome into 141 artificial yeast chromosomes (YACs) (Kuspa et al., 1989; Yee and Inouye, 1981, 1982; Zusman et al., 1978). The most reliable estimate of 9,450 kilobase pairs (kbp) comes from a recent construction of complete physical genome maps of several independent *Mx. xanthus* strains (Chen et al., 1990). The *Myxococcus* genome is thus approximately the same size as the *Streptomyces* genome, and it is twice as large (4,700 kbp) as that of *E. coli.* The genome of *Sg. aurantiaca* appears to be nearly the same size as the *Myxococcus* genome (Yee and Inouye, 1981). It has been reported that exponentially growing cells of *Mx. xanthus* contain just one replicating chromosome with 1.5 genome equivalents (Zusman et al., 1978). The amount of DNA in a freshly divided cell was estimated to be 14 $\times$ 10^{-9} μg, while a logarithmically growing cell contains 20 $\times$ 10^{-9} μg.

The DNA of *Mx. xanthus* strain FB has been found to be methylated at cytosine sites (Yee and Inouye, 1982). In rapidly growing cells, the DNA is undermethylated, and the degree of methylation increases when growth slows. A vegetative promoter and two development-specific promoters have also been characterized (Komano et al., 1987; Inouye, S., 1984).

Mx. xanthus RNA polymerase shows the usual $\alpha\beta\beta'$ subunits composition, but two slightly different sigma factors have been obtained from vegetative cells (Rudd and Zusman, 1982). Gene *rpoD* for a major vegetative sigma factor from *Mx. xanthus* has been characterized (Inouye, S., 1990). It probably corresponds to the previously described protein, sigma I (see above) and consists of 708 amino acids (80,391 Da). The amino acid sequence derived from the base sequence is in excellent agreement with that of *E. coli* sigma-70, but the *Myxococcus* sigma has an additional 100 residues at the amino end. The last 242 amino acids at the carboxy end show 78% and 72% homology with *E. coli* sigma-70 and *Bacillus subtilis* sigma-43, respectively. *Myxococcus* sigma-80 contains an extremely high proportion (40%) of charged amino acids. The polypeptide corresponding to *rpoD* has also been identified. It showed a somewhat higher apparent molecular weight (86 kDa), and there was evidence of a second sigma factor—apparent molecular weight 80 kDa, calculated from the putative gene (73,117 Da). Gene *rpoD* was used as a probe to find other genes for sigma factors in the *Myxococcus* genome, and a developmental sigma factor has thereby been discovered.

A most unusual kind of nucleic acid, msDNA, which was first discovered in *Mx. xanthus,* has raised many intriguing questions (Yee et al., 1984). msDNA has been found in almost all myxobacteria investigated (Dhundale et al., 1985). It is a single-stranded DNA and is present at 500 to 700 copies per genome. The DNA is 162 bases long, and its 5′-end is linked via a 5,′ 2′-phosphodiester bond with a guanidine residue in a branched RNA, msdRNA, which is 77 bases long and comes from a much longer precursor. The RNA has an extremely stable stem-and-loop structure. The coding regions for msDNA and msdRNA are found side by side at one specific site in the *Myxococcus* genome but they are oriented in opposite directions and overlap with 8 bases at their 3′-ends (Dhundale et al., 1987). A very similar msDNA (81% sequence homology) with a corresponding genome structure has been found in *Sg. aurantiaca* (Furuichi et al., 1987a, 1987b). A second species of such DNA, mrDNA, was later discovered in *Mx. xanthus* (Dhundale et al., 1988b). mrDNA is, however, much shorter, and it has a completely different base sequence in the DNA as well as in the RNA, but the RNA still has a similar secondary structure to that of msRNA. Also, there seem to be only 100 copies of mrDNA per genome.

The msDNA is synthesized by a reverse transcriptase with an associated ribonuclease H activity. The enzyme uses a folded RNA precursor as a primer and a template (Lampson et al., 1989). The gene of the reverse transcriptase has been found immediately downstream from the

msdRNA region and codes for 485 amino acids, which show sequence homology with retroviral reverse transcriptases (Inouye, S., et al., 1989). In fact, *Mx. xanthus* actually contains two reverse transcriptases (Inouye, S., et al., 1990). The wide distribution of msDNA in myxobacteria, as well as a comparison of codon usage in the reverse transcriptase gene and in other *Myxococcus* genes, suggests that the system is very old and was not acquired recently (in contrast to a similar system in certain *E. coli* strains). By deletion mutation, msDNA synthesis could be eliminated, but this had no significant effects on growth, motility, and morphogenesis compared to the wild-type strain (Dhundale et al., 1988a).

No evidence has been found for the presence of a poly(dT-dG)·poly(dC-dA) (TG) element in *Mx. xanthus* (Morris et al., 1986).

While the half-life of mRNA is about 3.5 min in vegetative cells of *Mx. xanthus,* 5 to 10 species of mRNA with a much longer half-life (20 to 30 min) could be demonstrated during fruiting body formation (Nelson and Zusman, 1983). One of the long-lived mRNAs is for protein S. The stable mRNA can amount up to 30 to 40% of the total mRNA.

Although no clear evidence has been presented for free, self-replicating plasmids in myxobacteria, several early reports suggest that extrachromosomal DNA may be present. Thus, after induction of chloramphenicol resistance in *Mx. xanthus* RB, a burst of synthesis of circular, covalently linked, extrachromosomal DNA was observed (Brown and Parish, 1976). The resistance was due to chloramphenicol acetylation (Burchard and Parish, 1975). Also, it was possible to transfer resistance to several different antibiotics from R factor-containing strains of *E. coli* to several *Myxococcus* species by conjugation (Parish, 1975). In some cases, extrachromosomal DNA could be demonstrated in the resistant *Myxococcus* strains, and the resistance could be transferred to other *Myxococcus* strains by conjugation.

It is now possible to introduce foreign plasmids into myxobacteria, usually via *E. coli* phage P1 as a suicide vector (Kaiser and Dworkin, 1975; Kuner and Kaiser, 1981; Morris et al., 1978), or by direct conjugation with *E. coli* (Parish, 1975; Breton et al., 1985). The plasmids often survive in the myxobacterial cells but seem always to be integrated in the genome. The genetic information contained in such plasmids is often expressed. A particularly useful approach is the random insertion of Tn*5 lac* segments into the genome of *Mx. xanthus,* which may be turned on by external promoters (Kroos and Kaiser, 1984). In this way, promoters could be identified that became active at certain stages of the developmental program (Kroos et al., 1986). Also, site-specific insertion of external plasmids has been possible, e.g., by incorporating into the plasmid, the *attP* attachment site of lysogenic phage MX-8 (Orndorff et al., 1983), which recognizes only one site, *attB,* in the *Mx. xanthus* genome. Thus, the plasmid is integrated only once and at one specific site (Li and Shimkets, 1988). A useful strategy to increase the integration of plasmids is to insert pieces of the myxobacterial genome into the plasmid (Jaoua et al., 1987). For a survey of the vast literature on myxobacterial genetics, the reader is referred to review articles on this topic (Kaiser et al., 1979; Kaiser, 1984a, 1986, 1989; Shimkets, 1990b).

Phages and Bacteriocines

There are some problems with the isolation of phages for myxobacteria, perhaps because wild strains are usually lysogenic and therefore restrictive. In fact, when the ultracentrifugal pellets of culture supernatants were examined under the electron microscope, all six strains of *Mx. xanthus, Mx. virescens,* and *Mx. fulvus* produced small quantities of phage particles (Brown et al., 1976a). Restriction of phage MX-1 has been shown directly with a strain of *Mx. virescens* (Morris and Parish, 1976). The strain became fully sensitive after curing by UV irradiation. Also, two restriction endonucleases have been demonstrated in the restrictive strain. Such enzymes have been found in many different myxobacteria (Mayer and Reichenbach, 1978). Furthermore, defective phage particles, originally described as rhapidosomes (Reichenbach, 1965b), have been seen in several myxobacteria (Brown et al., 1976a; McCurdy and McRae, 1974; Reichenbach, 1967). Nevertheless, infective phages have been isolated from soil and dung of various animals; these are most likely virulent mutants of temperate phages (Brown et al., 1976a). The first myxobacterial phage to be isolated was MX-1 of *Mx. xanthus* (Burchard and Dworkin, 1966), which has been extensively studied (Brown et al., 1976a, 1976b; Burchard and Voelz, 1972; Tsopanakis and Parish, 1976; Voelz and Burchard, 1971). Like all myxobacterial phages known so far, it is a DNA phage with a genome size of 130 to 150 $\times 10^6$ Da and a GC content of 56 mol% (using buoyant density, a reinvestigation with a chemical method gave 50 to 52 mol%; Brown et al., 1976b). The phage particle has an icosahedral head and a tail with a contractile sheath. MX-1 is a lytic phage with a host range restricted to *Mx. xanthus.* Several more *Mx. xanthus* phages

have been isolated (Brown et al., 1976a; Campos et al., 1978; Geisselsoder et al., 1978; Martin et al., 1978) which often resembled MX-1, but other morphological types with very short tails have also been obtained. A strategy was applied to isolate transducing phages from a collection of *Mx. xanthus* strains using certain sensitive strains as indicators, such as strain FB, which is probably a phage-free and nonrestrictive strain (Morris and Parish, 1976). In this way, several useful transducing phages have been found (Martin et al., 1978), such as MX-8, which has been particularly well characterized (e.g., Orndorff et al., 1983; Stellwag et al., 1985). MX-8 is a generalized transducing phage with a linear, double-stranded DNA (56 kbp). The genome has a terminal redundancy of 8% and is circularly permuted over at least 40% of its length. MX-8 can start a lysogenic cycle in *Mx. xanthus* strains, and its prophage is integrated into the bacterial genome by a site-specific recombination between the *attB* and *attP* sites already mentioned. Another well-studied transducing phage with similar morphology is MX-4 (Campos et al., 1978; Geisselsoder et al., 1978).

During the search for myxobacterial phages, bacteriocinlike activities have also been discovered. In one case, two antibacterial activities have been described, but one was directed against the homologous producer strain, and the other against the totally unrelated genera *Salmonella* and *Cytophaga*; thus, neither conforms to the definition of a bacteriocin (Brown et al., 1976a). In another case, an activity termed xanthacin was obtained from *Mx. xanthus* FB. The enriched preparation inhibited *Cystobacter* and *Melittangium,* but not *Mx. xanthus* or any other *Myxococcus* strains, which again seems not to fit the explicit definition of a bacteriocin (McCurdy and MacRae, 1973). Xanthacin appeared to be a particulate membrane fraction. True bacteriocins were, however, discovered in *Mx. fulvus* strain Mx f16 (Hirsch, 1977). The strain produced three bacteriocins, which could be precipitated from the culture supernatant with $(NH_4)_2SO_4$. They were not inducible by mitomycin. One of them, fulvocin C, was further characterized. As expected for a bacteriocin, it only essentially inhibited *Mx. fulvus* strains (all 16 strains tested), the producer excluded. The chemical structure of fulvocin has been fully elucidated, and it was the first bacteriocin for which this could be achieved (Tsai and Hirsch, 1981). It is a very compact and stable protein (4672 Da, 45 amino acids) with four disulfide bridges. In sensitive strains, fulvocin C leads to a fusion of the outer membranes of adjacent cells. The minimal inhibitory concentration is 0.25 μM.

Myxobacterial Colonies

On media with a relatively low nutrient content, like VY/2 and CY agar, colonies of myxobacteria spread over the agar surface. Such colonies are called swarms. The swarm does not behave like a pseudoplasmodium, as has sometimes been stated in the older literature; that is, the colony does not move as a whole, but simply increases in diameter due to gliding motility and cell division. An exception are the tiny swarmlets that emerge from germinating *Chondromyces* sporangioles, which have already been described in connection with chemotaxis. Also, as discussed earlier, there are obviously mechanisms that keep the cells together.

The appearance of a swarm is a particular function of the species and the medium. On rich media, such as those with a high peptone content (around 1%), myxobacterial colonies may remain small and more or less compact; sometimes they are even circular, convex, and with an entire edge like ordinary bacterial colonies. The typical myxobacterial colony is, however, a swarm sheet consisting of cells and excreted slime; much of the latter is probably composed of interwoven slime trails. The swarms often contain conspicuous veins, rings, and ridges, or even depressions in the agar surface. Such patterns can be very characteristic for specific taxonomic groups (see below). The swarm sheet can sometimes be loosened from the agar surface with water (e.g., for *Cystobacter* and *Stigmatella*); sometimes, however, it sticks tenaciously to the agar surface, occasionally to such an extent that the agar is torn into pieces when the aging swarm sheet contracts (especially for *Sorangium*). In some cases, the swarm sheet becomes so tough and leathery, or rubberlike, that it is very difficult to cut with the inoculating loop (for *Archangium, Cystobacter,* and *Melittangium*); in other cases, it is soft and slimy and can easily be scraped off the plate (for *Myxococcus*). Often the myxobacteria penetrate the agar (even 1.5% agar), and the swarm spreads below the agar surface, sometimes penetrating to the very bottom of the dish (often for *Polyangium*). In such cases, the swarm may completely disappear below the surface, and its former position can be recognized only by a shallow depression. All members of the Sorangineae tend to grow within the agar. However, some members of the Cystobacterineae also do so, although here it is often a consequence of swarm degeneration. Some myxobacteria corrode the agar surface to the extent that they

produce long tunnels or large, cavernlike holes in the plate. This is typical of *Polyangium* and especially of *Nannocystis.*

The swarm edge usually shows characteristic flamelike protrusions, or flares. Sometimes wide, tonguelike depressions are produced (by *Chondromyces* and other members of the Sorangineae), or isolated narrow trenches radiate from the swarm center far into the surrounding agar surface (*Polyangium, Nannocystis*). Occasionally, massive ridges are also seen at the swarm edge (*Chondromyces, Polyangium, Cystobacter*). At such a swarm edge, the swarm may consist of only one cell layer, but as a rule the swarm sheet is composed of many layers on top of one another that quickly pile up behind the margin. When growing on very lean media, most of the swarm may be only one cell thick, although even then the bacteria tend to concentrate themselves in certain areas, such as along radial tracks. In large swarms, most of the cell population may be found in the outer regions, and if no fruiting bodies or myxospores have been produced, the interior may be completely deserted. The speed of the advancing edge is in the range of 1 mm in 9 h (*Cb. violaceus* at 22°C; Reichenbach, 1965) to about 1 mm in 1.6 h (*Cystobacter* and *Stigmatella* strains at 30°C).

Various taxa may be recognized by their swarm morphology. When plate cultures are flooded with an aqueous 0.01% Congo red solution for 5 to 10 min, the swarm sheets of the Cystobacterineae stain a deep violet-red, while those of the Sorangineae remain unstained (McCurdy, 1969a). The swarms of *Myxococcus, Corallococcus,* and *Angiococcus* typically develop meandering radial veins, which may be particularly conspicuous on CY agar; the swarm sheet usually remains soft and slimy. In contrast, long, more or less straight, branching veins are seen in the swarms of *Cystobacter, Archangium, Melittangium,* and *Stigmatella*; the sheet is usually tough. On VY/2 agar, the swarms of most Sorangineae are sunk into the agar and appear as wide shallow depressions at the surface. *Chondromyces* and *Sorangium* often develop long, cablelike veins, on the surface which sometimes curl into a ring at one end. Also, isolated rings are often seen in those swarms. With *Chondromyces* swarms, the swarm edge typically consists of a series of tongue- and shell-like shallow depressions. *Polyangium* swarms on VY/2 agar often penetrate the agar to the bottom and characteristically are surrounded by a more or less perpendicular, curtainlike zone composed of tiny, well-separated cell clumps. *Polyangium* and *Nannocystis* growing on streaks of autoclaved *E. coli* often produce long, radiating, narrow trenches in the agar surface, with small cell clusters (and, later on, fruiting bodies) at the end. The two can often be distinguished because the tracks and cell clumps of *Nannocystis* are more delicate. On mineral salts-glucose media, like CA2 and CK6 agar, *Sorangium* often produces swarms with a beautiful, dense network of branching cablelike veins.

It should be emphasized that the swarm structures described above are usually only seen with strains that have recently been isolated. The swarms of strains that have been transferred many times, or that have been adapted to growth in liquid media, are often very different. Such swarms may be soft-slimy, lacking all morphological differentiation, and, in the case of the Cystobacterineae, sometimes no longer grow on the surface but are sunk into the agar. In addition, a colony dimorphism has been described for *Cb. violaceus* and *Mx. xanthus* and is probably quite common among myxobacteria (Burchard et al., 1977; Grimm and Kühlwein, 1973a, 1973b, 1973c; Reichenbach, 1965a). This dimorphism may be a function of cell motility and consequently the spreading behavior of the colonies (K/G or K/S in *Cb. violaceus*, as well as the amount and consistency of the slime produced, and, probably directly connected to that, the capacity to fruit, and, after transfer to a liquid medium, to grow as a homogeneous cell suspension (*Cb. violaceus*). Also, pigmentation can be affected (deep and pale violet in *Cb. violaceus,* tan/yellow in *Mx. xanthus*). Usually, one colony type is more stable than the other, but segregation in both directions is possible. An explanation at the genetic level is still lacking, but it may involve a recombinase system (Jaoua et al., 1990). Of course, any mutations in genes of the motility system can be expected to have some effect on the appearance of myxobacterial colonies, e.g., the *frizzy* genes of *Mx. xanthus* discussed earlier (see "Gliding Motility") (Blackhart and Zusman, 1985).

In myxobacterial swarms, a very peculiar and striking feature has occasionally been observed: dense fields of regularly arranged, tiny ridges, or ripples. These ridges are usually found in long, parallel rays. They are positioned parallel to one another and perpendicular to the direction of the ray. The distance between the ridges is uniform and is approximately equal to the width of the ridges (Fig. 7). In *Mx. xanthus,* a distance of 45 μm from crest to crest has been measured (Shimkets and Kaiser, 1982). The phenomenon was discovered when movies of developing *Corallococcus* and *Myxococcus* swarms were first made. They showed that the ridges actually move, so that the whole field ap-

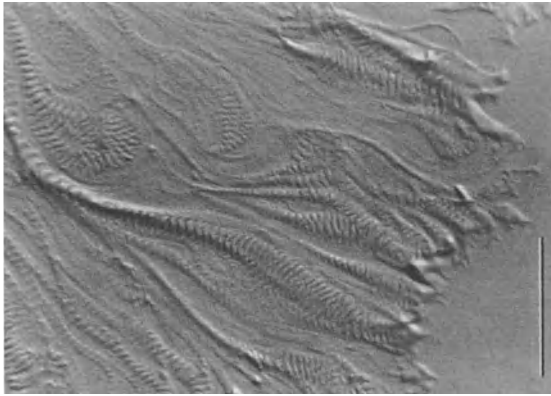


Fig. 7. Oscillating ridges, or ripples. *Stigmatella erecta* on casitone agar; dissecting microscope, oblique illumination. Bar = 200 μm.

pears to oscillate; hence, the ridges were called oscillating waves (Reichenbach, 1965b; Reichenbach et al., 1965a, 1965c). In fact, the movements of the ridges may even speed up and slow down, or pulsate, over a period of several hours. The movement of the ridges is fast enough to be recognized under the dissecting microscope; a speed of about 2 μm/min has been measured in *Mx. xanthus* (Shimkets and Kaiser, 1982). Oscillating waves were later found in many other myxobacteria. They appear to occur with all Cystobacterineae, and occasionally are also observed with members of the Sorangineae, e.g., *Chondromyces.* With *Sg. aurantiaca,* even waves travelling in opposite directions along the same path have been seen (Reichenbach et al., 1980). In *Mx. xanthus,* rippling can be induced by purified peptidoglycan and peptidoglycan components, such as *N*-acetyl-glucosamine, *N*-acetylmuramic acid, diaminopimelic acid, and D-alanine (Shimkets and Kaiser, 1982). The mechanism of cell movement in the oscillating waves and the biological significance of rippling are not understood.

Finally, the shape of myxobacterial swarms may become distorted by stress lines in the agar plate produced, for example, by mechanical pressure and stretching, a phenomenon known as elasticotaxis (Stanier, 1942).

Fruiting Bodies and Myxospores

The outstanding trait of the myxobacteria is their ability to form fruiting bodies and myxospores (Figs. 8, 9, 10, and 11). These morphogenetic processes make the myxobacteria by far the most sophisticated prokaryotes, and the object of considerable scientific interest as model systems for the study of the mechanisms of development. Studies of morphogenesis have provided extensive insights into the biochemistry and genetics of communication, as well as differentiation and morphogenesis on the prokaryotic level, and have also provided clues to the origin of important control mechanisms of eukaryotic development. These are several examples of such clues, including GTP-binding proteins, phosphoinositol cycle, steroid biosynthesis, stress proteins, and retrons. The literature on myxobacterial development is already very voluminous, and many excellent reviews on this topic are available (Dworkin, 1985, 1990; Kaiser, 1984b, 1986, 1989; Kaiser et al., 1979; Shimkets, 1987, 1990b; White, 1975, 1981; Wireman and Dworkin, 1975; Zusman, 1980, 1984, 1990).

The simplest myxobacterial fruiting bodies are mounds of either soft or hardened slime, the latter often having bizarre shapes (*Corallococcus, Archangium*), the former typically having a nob or head with a constriction or a stalk at the base (*Myxococcus*). Most myxobacteria enclose their myxospores in sporangioles, i.e., containers with well-defined walls; the interior of the sporangioles is always hard and tough. The sporangioles may occur singly or in groups; they may rest either directly on or in the substratum, or on simple or branched slime stalks. The fruiting bodies of several species are structures of considerable complexity, and some were mistaken for fungal fruiting bodies for a long time. The following key gives a survey of the different fruiting body types and their relation to the various myxobacterial taxa. It must be kept in mind, however, that fruiting body structure and morphology often degenerate quickly after a few transfers upon isolation. Also, strains may be isolated that produce degenerate fruiting bodies from the very beginning. In such cases, only completely atypical nobs and ridges of slime and myxospores are formed. It may be added here that the fruiting bodies of several myxobacteria have also been studied with the scanning electron microscope, and some very impressive pictures have been obtained in this way (e.g., Brockman and Todd, 1974; Galván et al., 1981; Stephens and White, 1980a; Stephens et al., 1982).

Key to the Fruiting Body Types of Myxobacteria

1. Naked masses of slime and myxospores 2
1′. Myxospores encased in sporangioles 3
2. Fruiting body soft-slimy .... *Myxococcus*
2′. Fruiting body hard, cartilaginous
.... *Corallococcus, Archangium*
3. Sporangioles single 4

3′. Sporangioles in groups 5
4. Single sporangiole located on or in the substrate
.... *Nannocystis, Haploangium*
4′. Single sporangiole on top of a stalk
.... *Melittangium, Sg. erecta*
5. Group of sporangioles on or in the substrate
.... *Angiococcus, Cystobacter, Polyangium, Sorangium, Nannocystis*
5′. Cluster of sporangioles on top of an unbranched stalk
.... *Sg. aurantiaca,* most *Chondromyces* species
5″. Clusters of sporangioles on a branched stalk
.... *Cm. crocatus*

More or less the same types of fruiting bodies are found in both suborders (with the qualification that the Sorangineae produce only fruiting bodies with sporangioles). Still, the course of morphological differentiation may be different with seemingly identical fruiting body types. Thus, *Chondromyces* and *Sg. aurantiaca* both form little treelets, but the stalks and sporangioles are produced in a different way in the two cases. While *Chondromyces* excretes a slime stalk that lifts the undifferentiated cell mass upward, and differentiation of the sporangioles occurs as the last step, *Sg. aurantiaca* piles up a mass of cells with approximately the shape and size of the final fruiting body, then the cells either withdraw from the stalk area or degenerate and lyse in that part, and the sporangioles mature (Fig. 12).

As has already been mentioned, stalks, sporangiole walls, and pigments are produced during fruiting but nothing is known about the chemical composition of these structures and substances. Under the electron microscope, the stalk of *Cm. crocatus* is seen to consist of numerous parallel tubes which are approximately the diameter of a cell and run in direction of the long axis of the stalk (Abadie, 1971b; McCurdy, 1969b). The stalk of *Sg. aurantiaca* was found to be composed of tubules wedged against each other and containing cell debris but only rarely a cell or myxospore; the stalk was surrounded by a thin, dense surface layer resembling the wall that surrounds the sporangioles (Voelz and Reichenbach, 1969). The stalk of *Sg. erecta* also was found to be cell free and to consist of slime, although in this case tubules could not be distinguished (Galván et al., 1987). In contrast, scanning electron micrographs suggested that the stalk of *Sg. aurantiaca* is cellular (Stephens and White, 1980). The contradiction is not yet resolved; perhaps the fruiting bodies examined with the scanning electron microscope had not yet fully matured. In any case, under the light microscope the stalk of *Sg. aurantiaca* looks translucent, unpigmented, and white, and thus completely different from the opaque, dark brown sporangioles.

The base of fruiting body stalks is often clearly disk-shaped. The disks apparently arise early during fruiting body development when the aggregated cells move on a circular path at the site of fruiting.

Little is known about the arrangement of the cells within the developing fruiting body, but it appears that this arrangement, and perhaps rearrangements by migrations of certain cell populations, may play a major role during fruiting body morphogenesis. In *Me. boletus,* a very regular pattern of three or more layers of strictly parallel cells has been observed (Jahn, 1924). The cells are perpendicularly arranged on the outer surface of the fruiting body, and it has been suggested that they are responsible for the secretion of the sporangiole wall. In developing *Cb. fuscus* sporangioles, the cells in the outer layers lie parallel to the surface. There are two hypotheses to explain the formation of sporangioles: Cells either secrete the sporangiole wall (Jahn, 1924) or they fuse together into a wall layer (Vahle, 1910). The sporangioles of the *Cb. fuscus* fruiting body are embedded in a communal, thick, translucent, unpigmented layer, called the matrix or glass envelope (called "Glashülle" by Jahn, 1924). Such additional envelopes are found with most of the genera that produce fruiting bodies in the form of clusters of sporangioles sitting directly on the substrate surface (*Cystobacter, Polyangium,* and *Sorangium*).

The fruiting bodies are produced by the coordinated action of a large number (10^5 to 10^7) of swarm cells that retain their physical individuality throughout this process of cooperative morphogenesis (Fig. 13). Formation of fruiting bodies can schematically be divided into a number of developmental stages: 1) environmental signal(s) to convert the vegetatively growing cells to a developmental path; 2) cell aggregation or accumulation; 3) emergence of molecules on the cell surface that mediate cell adherence; 4) rearrangement or clustering of the cells within the original undifferentiated mass; 5) production of special structural elements (stalks, sporangiole walls); 6) creation of the specific shape of the fruiting body, perhaps the most fascinating event in the whole sequence; and finally, 7) during the maturation phase, conversion of the vegetative cells into myxospores. Under optimal conditions, the bacteria may go through the whole developmental cycle within 12 to 24 h. It has been demonstrated that fruiting body induction as well as the individual steps of differentiation are subject to control by environmental factors such as nutrients, pH, divalent

cations, and temperature. As has already been mentioned, in at least two cases (*Cm. apiculatus* and *Sg. aurantiaca*), normal development takes place only in illuminated cultures (Qualls et al., 1978; Reichenbach, 1974a, 1974b; Stephens and White, 1980). The course of fruiting body formation has been documented for different genera and species in several scientific films (cited earlier), which show the sequence of events during fruiting.

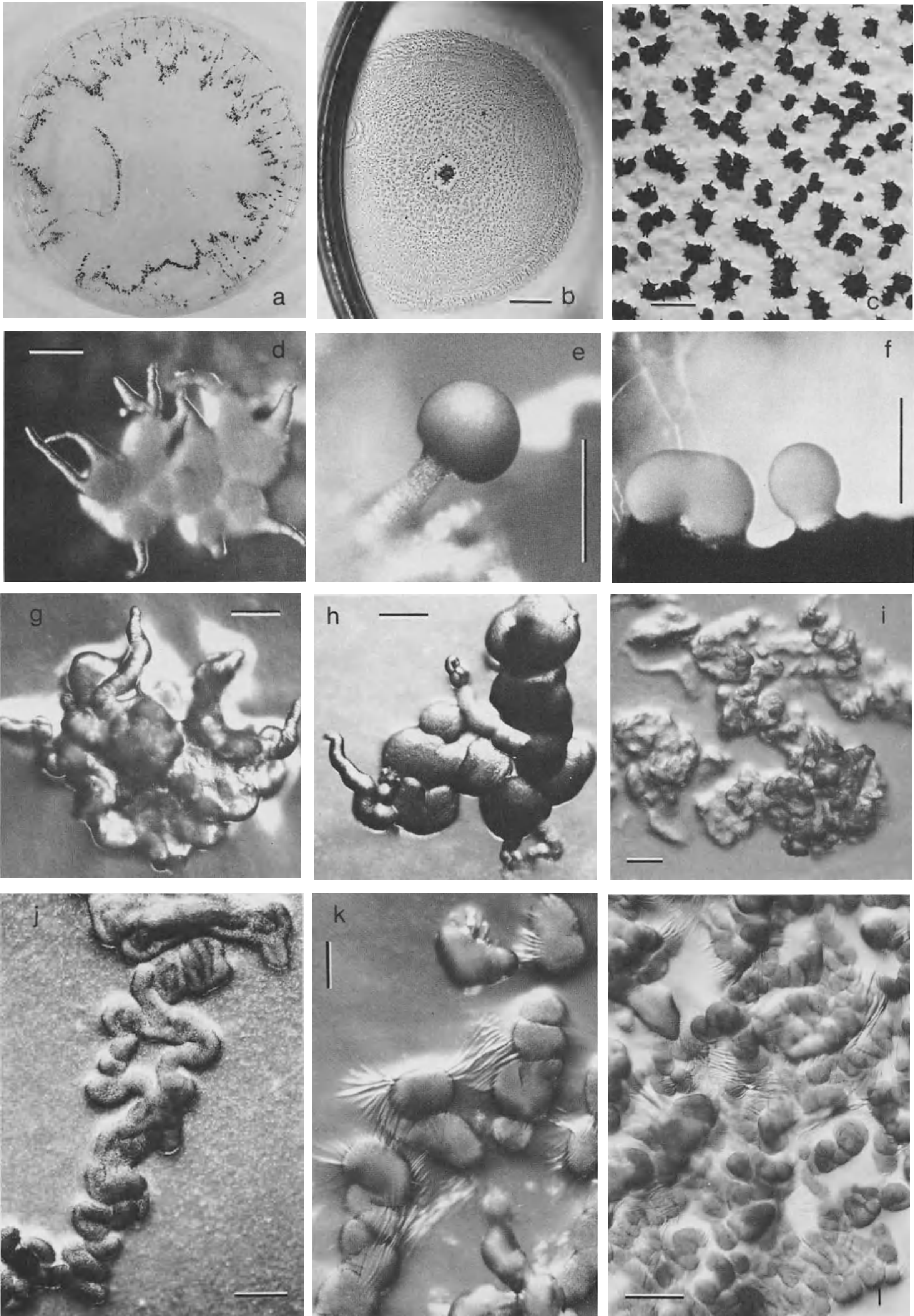
It may be mentioned in passing that, for many myxobacteria, fruiting bodies can also be obtained on submerged substrates; this has been demonstrated with *Polyangium parasiticum* (Geitler, 1925), *Mx. fulvus* (Fluegel, 1963, 1965), and *Mx. xanthus* (Kuner and Kaiser, 1982). *Nannocystis, Polyangium,* and *Sorangium* strains often produce fruiting bodies deep within the agar plate, which is in some sense analogous to submerged conditions. There are, however, other myxobacteria, that seem not to form fruiting bodies under submerged conditions, e.g., *Cm. apiculatus* (H. Reichenbach, unpublished observations).

The distribution of the fruiting bodies on the culture plate is often more or less random, but occasionally striking patterns are observed. Thus, an arrangement in so-called witches rings is often seen in *Myxococcus* and *Corallococcus* cultures. This may perhaps be explained by a periodic depletion of nutrients which then induces fruiting but also a concomitant reduction of the metabolizing cell population. As mentioned, elasticotaxis may also lead to unexpected arrangements.

The terminology used to describe the structural components of myxobacterial fruiting bodies is illustrated in Fig. 14. Although complete consistency with usage of terms in other biological systems is not possible, terms that have already acquired a well-defined meaning in other systems should be avoided if they are inappropriate. The term "fruiting body" has a long tradition of use and may be acceptable in connection with myxobacteria, although in biology, fruiting usually implies sexual processes, and no such events seem to be involved in the case of the myxobacteria. The term "sporangium" has also been applied to these structures, but this term is even more suggestive of sexuality and is best avoided. Most types of fruiting bodies are composed of myxospores and the walled portions containing these myxospores are in fact communal cysts; they have traditionally been referred to as cysts. However, since the term "cyst" in microbiology is applied to quite a different structure, dormant, encapsulated single cells, we propose to use the term "sporangiole" instead, and to accept its obvious shortcomings. The advantage of the term sporangiole over sporangium is that it is a relatively unusual term and has been used only in connection with a restricted group of fungi (Mucorales) where it is not even used with a consistent meaning. Its use here is thus less likely to cause confusion. Incidently, the term sporangiole was applied to these myxobacterial structures by Link (1809) in the very first description of a myxobacterium, *Pl. vitellinum* (Fig. 15).

Inside the maturing fruiting body, the vegetative cells are converted into myxospores. Up to 85 to 90% of the dry weight of a fruiting body may consist of myxospores, the remaining 9 to 15% consisting of polysaccharide (Sutherland, 1979). However, these proportions vary within wide limits even with the same species. In the process of cellular morphogenesis, the slender rod-shaped cells of the Cystobacterineae shorten and fatten substantially and become optically refractile. Also, they surround themselves with an additional layer, a spore coat, or capsule. The capsule is usually so thin that it can be detected only under the electron microscope: it may be only 20- to 30-nm thick in the fruiting body and in glycerol-induced myxospores of *Sg. aurantiaca* (Reichenbach et al., 1969; Voelz and Reichenbach, 1969), and 100-

Fig. 8. Myxobacterial fruiting bodies. (a) *Cystobacter ferrugineus* on VY/2-agar; culture is 4 weeks old; the organism has spread all over the plate and produced numerous dark brown fruiting bodies; the diameter of the plate is 9 cm. (b to d) *Corallococcus coralloides:* (b) swarm on VY/2 agar, with numerous fruiting bodies; the delicate swarm edge and the clear lysis zone in the yeast agar are well recognizable; (c) field of fruiting bodies at higher magnification; (d) a single fruiting body, in situ on the agar surface. (e) *Myxococcus stipitatus,* fruiting body at the edge of a piece of filter paper, in situ. (f) *Myxococcus fulvus,* fruiting bodies on a soil crumb, in situ. (g to j) *Archangium*-like fruiting bodies: (g) *Archangium serpens,* large fruiting body in situ on agar surface; (h) *Cystobacter ferrugineus,* fruiting body with only rudimentary differentiation into sporangioles, in situ on agar surface; (i) *Cystobacter,* probably *Cb. ferrugineus,* with totally degenerated fruiting bodies, in situ on agar surface; (j) *Cystobacter* strain with typical *"Archangium"* fruiting bodies, in situ on agar surface. (k) *Cystobacter velatus,* fruiting bodies in situ on agar surface, covered with a delicately plicated slime sheet. (l) The same organism; at the lower magnification, one can see some of the enormous number of fruiting bodies that may be produced under suitable conditions. Bar = 5 mm in (b), 500 μm in (c), 200 μm in (l), and 100 μm in the remaining micrographs.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nm thick in *Sg. erecta* (Galván et al., 1987). Only the spherical myxospores of *Myxococcus* and *Corallococcus* develop a capsule that is thick enough to be recognized under the light microscope, particularly in negative contrast (Voelz and Dworkin, 1962) or after germination when the capsule remains intact (Reichenbach, 1966). In electron micrographs of thin sections, the laminated capsule of *Mx. xanthus* was found to be up to 350 to 400 nm thick (Voelz and Dworkin, 1962). In the Sorangineae, the morphological changes during myxospore conversion are much less dramatic. The rod cells may shrink a little, and they become optically refractile. The capsule remains very thin (60 nm; Abadie, 1971b) or is not developed at all (McCurdy, 1969b). Physiologically, however, both types of myxospores are equivalent; they are both dormant resting cells.

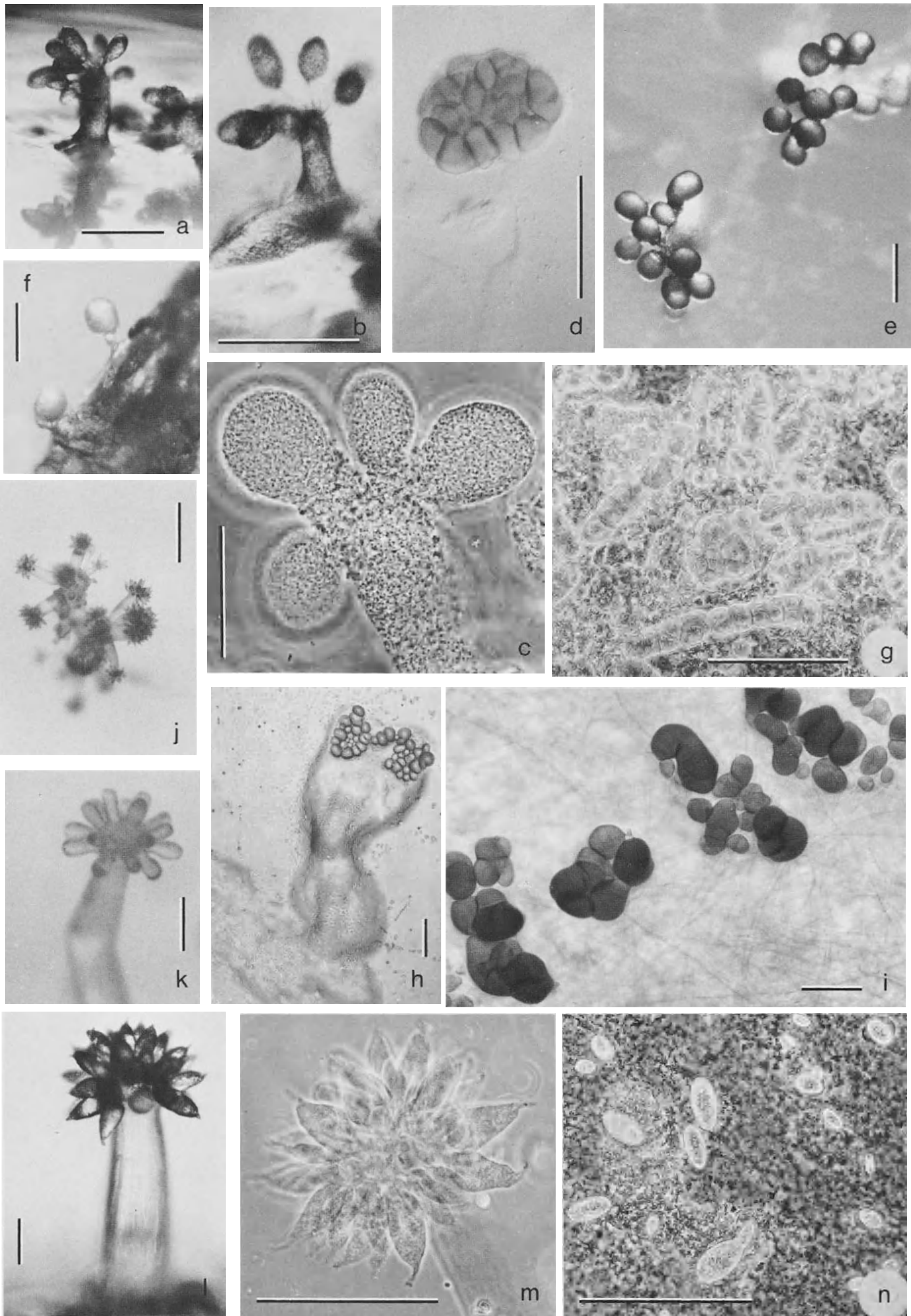
In the past, the resting cells of myxobacteria have also been named spores and microcysts (or pseudo-microcysts in the case of *Cm. crocatus*; Abadie, 1971b). More recently, the term myxospore has been adopted (Dworkin, 1977), because it emphasized the specific nature of the myxobacterial resting cell (in contrast to the term spore, which in bacteria is generally assumed to refer to the endospore) and eliminated the confusion with fruiting body macrocysts and microcysts in the older literature. After what has been said above, there is hardly a need to distinguish the two types of myxobacterial resting cells terminologically, and the term myxospore is now almost universally used for myxobacterial resting cells, regardless of shape and fine structure.

Myxospore formation and fruiting body formation are not always well coordinated. Sometimes only dark myxospores are found in fruiting bodies, which is probably due to strain degeneration. Such myxospores are usually not resistant to desiccation. Also, well-developed fruiting bodies may be found that are, however, completely empty, particularly in older cultures. In such cases, the cells either never converted into myxospores, or they germinated again and left the fruiting body. Especially with *Chondromyces,* fruiting bodies often arise on top of other fruiting bodies, sometimes repeatedly, so that in the end the whole cell mass of the aggregate is used up in the production of stalk material. In these cases, the cells obviously never enter the resting stage.

The myxospores are completely desiccation resistant, in contrast to vegetative cells which are extremely sensitive to desiccation. In addition, they show substantial resistance to ultrasound and UV irradiation. Their temperature resistance is only modest, although clearly above that of vegetative cells; in aqueous suspensions, they can survive at 58 to 62°C for 10 to 60 min (Sudo and Dworkin, 1969).

Cytological changes during myxospore formation have been studied on the ultrastructural level with four species, *Mx. xanthus* (Voelz, 1966a; Voelz and Dworkin, 1962), *Sg. aurantiaca* (Reichenbach et al., 1969; Voelz and Reichenbach, 1969), *Sg. erecta* (Galván et al., 1987; McCurdy and Khouw, 1969), and *Cm. crocatus* (Abadie, 1971b; McCurdy, 1969b). In all four cases, dark granules of polyphosphate and light areas containing some other kind of reserve material can be observed. The latter usually seems to be polysaccharide; in *Sg. aurantiaca,* these polysaccharide granules are coated with a dense layer of ribosomelike particles (Reichenbach et al., 1969). In *Cm. crocatus,* lipid inclusions have been suggested (McCurdy, 1969b). The outer

Fig. 9. Myxobacterial fruiting bodies. (a to c) *Stigmatella aurantiaca:* (a) fruiting body in situ on an agar surface; (b) fruiting body with its sporangioles on exceptionally long peduncles, in situ; (c) early developmental stage of a fruiting body, mounted in "Miracle Mount" (Cunningham, 1972) to make it transparent; the stalk still consists mainly of cells that later migrate into developing sporangioles or degenerate; the young sporangiole in the upper left corner shows the developing sporangiole wall that ends in a collar at the base of the sporangiole; phase contrast. (d) Although this fruiting body strikingly resembles those of certain *Polyangium* (see h) or *Sorangium* species (see g), it still is that of a *Cystobacter, Cb. minor,* as is clearly shown by the shape of the vegetative cells; interference contrast. (e) *Stigmatella erecta,* fruiting bodies in situ on agar surface. (f) *Melittangium lichenicola,* fruiting bodies in situ on a piece of wood. (g) *Sorangium cellulosum* on filter paper, cautiously squeezed slide mount; the shape of the fruiting bodies is often controlled by the orientation of the remnants of the wood fibers and tracheids, along and within which they develop; phase contrast. (h) *Polyangium* sp., fruiting body in crude culture in situ on agar surface; note the deep path that has been etched into the agar surface by the migrating bacterial mass (compare with Fig. 2c) before it encased itself in sporangioles; the tiny dots all over the agar surface are encysted amebae. (i) *Polyangium thaxteri,* fruiting bodies on filter paper, in situ. (j to m) *Chondromyces apiculatus:* (j) cluster of fruiting bodies on surface of filter paper, in situ; dissecting microscope; (k) early (club) stage sporangiole differentiation; dissecting microscope; (l) mature fruiting body; (m) small, mature, fruiting body in "Miracle Mount" (Cunningham, 1972) to make it transparent; the myxospores within the turnip-shaped sporangioles can just be recognized; the slime stalk, in contrast, is cell free; phase contrast. (n) *Nannocystis exedens;* the fruiting bodies are single sporangioles of very variable size and are embedded in the (agar) substrate; slide mount, phase contrast. Bar = 50 μm in (c), 200 μm in (i), 400 μm in (j), and 100 μm in the remaining pictures.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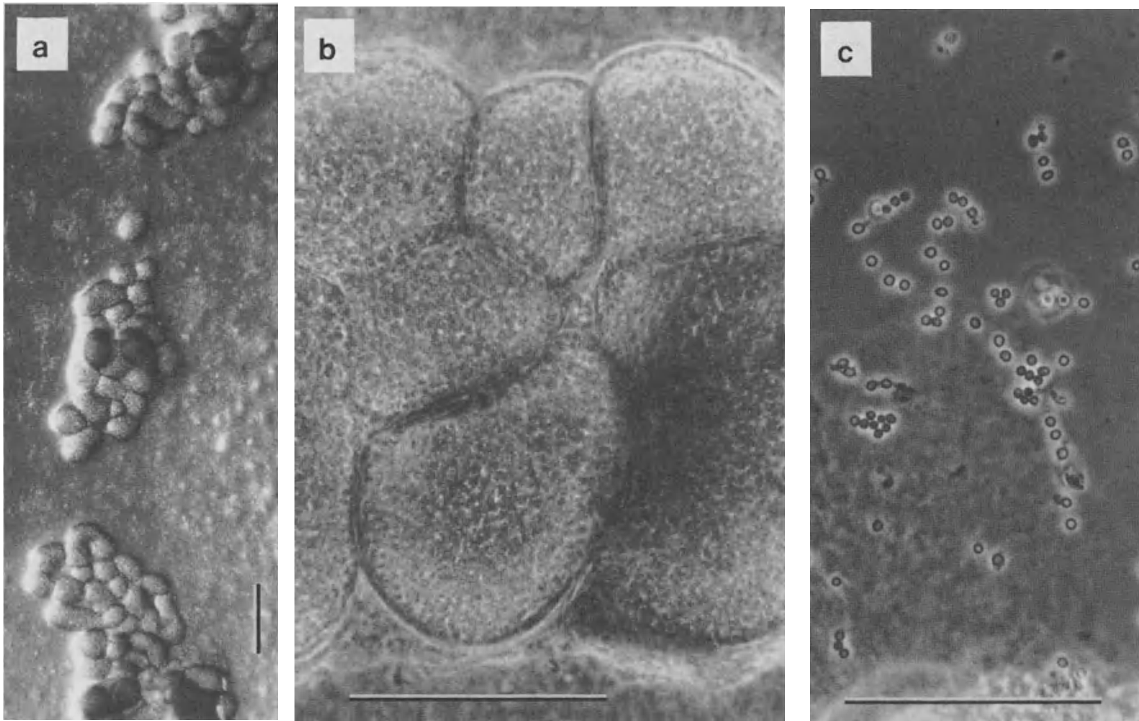
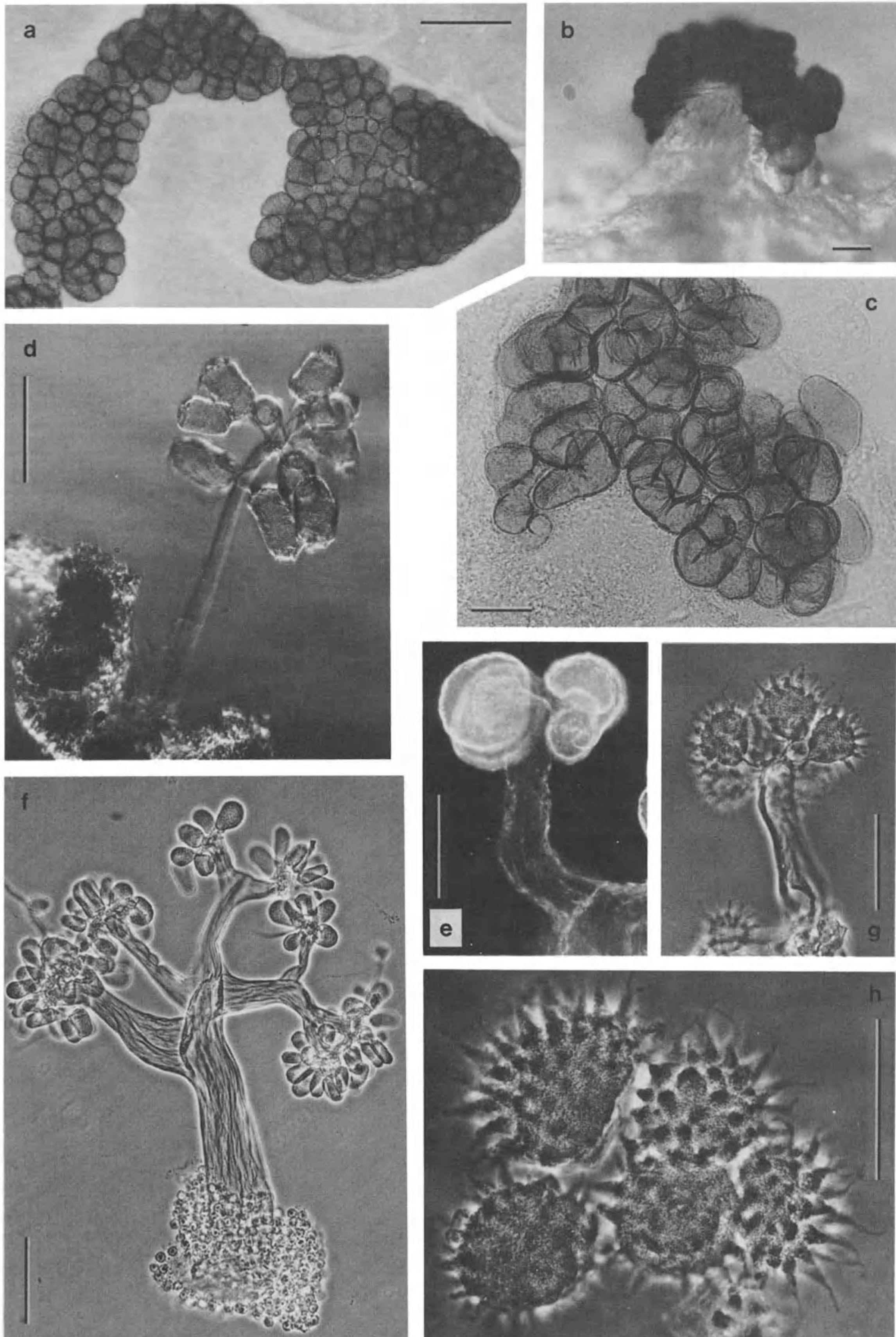


Fig. 10. Myxobacterial fruiting bodies: *Angiococcus disciformis.* (a) In crude cultures and on natural substrates the sporangioles tend to be disk-shaped and loosely stacked but, as shown here, in pure culture, they are more or less spherical and tightly attached to each other. (b) At high magnification, the walls of the sporangioles, although only about 1 μm thick, become clearly recognizable. (c) When the sporangioles are crushed, they release optically refractile, spherical myxospores, shown here at the same magnification as the sporangioles in (b), which look exactly like *Myxococcus* myxospores. (b) and (c) are in phase contrast. Bar = 100 μm in (a), 50 μm in (b) and (c).

membrane, but not the cytoplasmic membrane, is often intensively folded, particularly in glycerol-induced myxospores (Bacon and Eiserling, 1968; Reichenbach et al., 1969; White, 1975); in *Sg. aurantiaca,* tight, budlike membrane bodies sitting in pockets of the cytoplasmic membrane may be seen; in *Cm. crocatus,* the outer membrane may be folded in a concertinalike fashion (Abadie, 1971b). Perhaps the relatively rigid outer layer is not able to adapt itself quickly enough during the fast change in shape of the vegetative cell and responds by folding itself. Otherwise, the cell envelope appears to remain morphologically intact during sporulation and germination.

The chemical composition of myxospores shows some differences from vegetative cells. When myxospores of *Mx. xanthus* and other *Myxococcus* species, or of *Cb. velatus* and *Archangium* species, were extracted with acetic acid, virtually all of the polysaccharide could be removed from the cells. In the extracts, mainly glucose (50 to 70%) and galactosamine (15 to 48%) have been identified. The glucose is part of an α-1-3-glucan. Binding studies with the lectin concanavalin A demonstrated that the glucan is on the outer surface and only present (or accessible) in myxospores. The myxospores of *Cb. velatus* were the only ones that also bound soybean agglutinin (Sutherland and

Fig. 11. Myxobacterial fruiting bodies. (a) *Sorangium cellulosum,* on VY/2 agar in situ (compare with Fig. 9). (b and c) *Polyangium thaxteri;* (b) on agar surface in situ; a mass of tightly packed sporangioles is situated on a stalklike cushion of slime: the whole fruiting body looks like a false morel; (c) when mounted in "Miracle Mount" (Cunningham, 1972), the individual sporangioles can easily be distinguished. (d) *Chondromyces pediculatus,* fruiting body on a soil crumb, embedded in Miracle Mount. (e and f) *Chondromyces crocatus,* both in Miracle Mount: (e) a young, not yet fully differentiated stage; in dark field illumination, the translucent, cell-free stalk and the densely packed cell masses on its top are clearly distinguished; (f) mature fruiting body; at the base of the stalk some encysted soil amebae are seen. (g and h) *Chondromyces lanuginosus,* both in Miracle Mount: (g) survey of the whole fruiting body; (h) view from above showing the end face of the sporangioles covered with little tails. (f to h) in phase contrast. All bars = 100 μm.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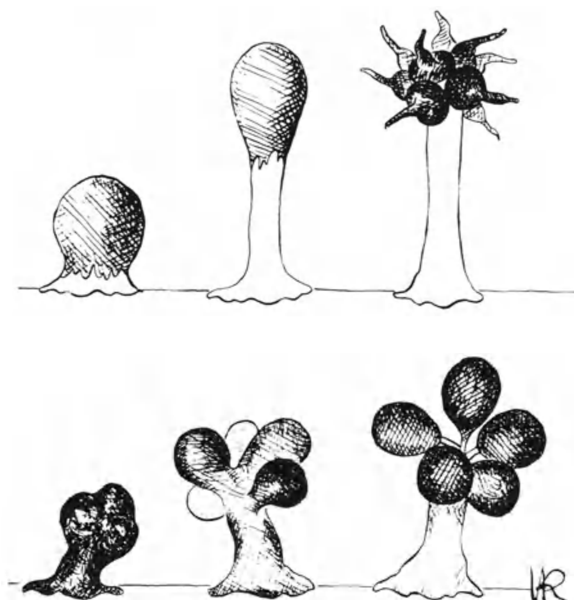

Fig. 12. Similar fruiting body shapes may be reached in different ways, as shown here for *Chondromyces apiculatus* (above) and *Stigmatella aurantiaca* (below), two organisms formerly thought to be closely related. The hatched areas indicate where the cells are located at the various stages.

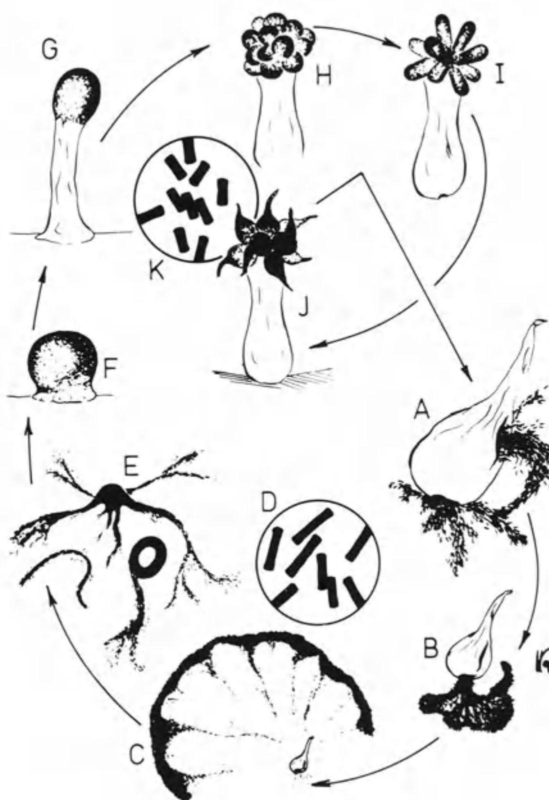

Fig. 13. Cooperative morphogenesis in myxobacteria. The organism shown in the diagram is *Chondromyces apiculatus*. Symbols: A, germinating sporangiole; B, C, development of a swarm colony; D, vegetative cells; E, aggregation of vegetative cells at various places within the swarm. F to J, fruiting body development; F, knob stage; G, excretion of the slime stalk, cells all concentrated in the terminal knob; H, the terminal mass begins to differentiate, forming wartlike protrusions which later elongate into (I) clublike structures and finally mature into (J) turnip-shaped sporangioles; K, myxospores.

Mackenzie, 1977). The coats of glycerol-induced myxospores of *Mx. xanthus,* which, in contrast to fruiting body myxospores, were only 25 to 50 nm thick, could be isolated and chemically analyzed (Kottel et al., 1975). They consisted of 75% polysaccharide (23 to 28% glucose, 50 to 58% galactosamine), 14% protein, 8% glycine, and 0.4% organic phosphorus. While one portion of the coat is already complete 5 to 6 h after induction, another part consisting mainly of glucose is added only after 8 h; perhaps this is the glucan mentioned above. During cell conversion, beginning about 50 min after induction, a rapid accumulation of some galactosamine-containing material on the cell and a concomitant rise in sonication resistance is seen. Quite obviously, this reflects the synthesis of the capsule. The capsule is highly resistant to chemical and enzymatic attack. During germination it is probably broken open mechanically and remains as an empty husk. During the phase of shape change, the degree of cross-linking of the peptidoglycan decreases transiently. It appears to be substantially higher in mature myxospores than in vegetative cells (White et al., 1968). Myxospores of *Mx. xanthus* accumulate large quantities of trehalose: fruiting body myxospores up to 1.1 mg, glycerol-induced myxospores up to 0.3 mg per mg of protein (McBride and Zusman, 1989). The trehalose disappears rapidly during germination and seems to be metabolized in some unknown way. This sugar may be responsible for the high osmotic strength of the myxospores and their elevated resistance to desiccation. In this connection, it is of interest that vegetative cells also store trehalose (up to 0.2 mg per mg of protein) when they are grown in the presence of 0.1 to 0.3 M of certain solutes [sucrose, KCl, NaCl, $(NH_4)_2SO_4$]. *Myxococcus* cultures appear to be unable to utilize added trehalose (H. Reichenbach, unpublished observations). The DNA content of myxospores is higher (27×10^{-9} μg) than that of vegetative cells and corresponds to that of three to four chromosomes (Zusman and Rosenberg, 1968).

Under natural conditions, myxospores are probably produced primarily inside fruiting bodies. In culture, myxospores are sometimes found on the substrate in the immediate neighborhood of fruiting bodies, as if a sporulation

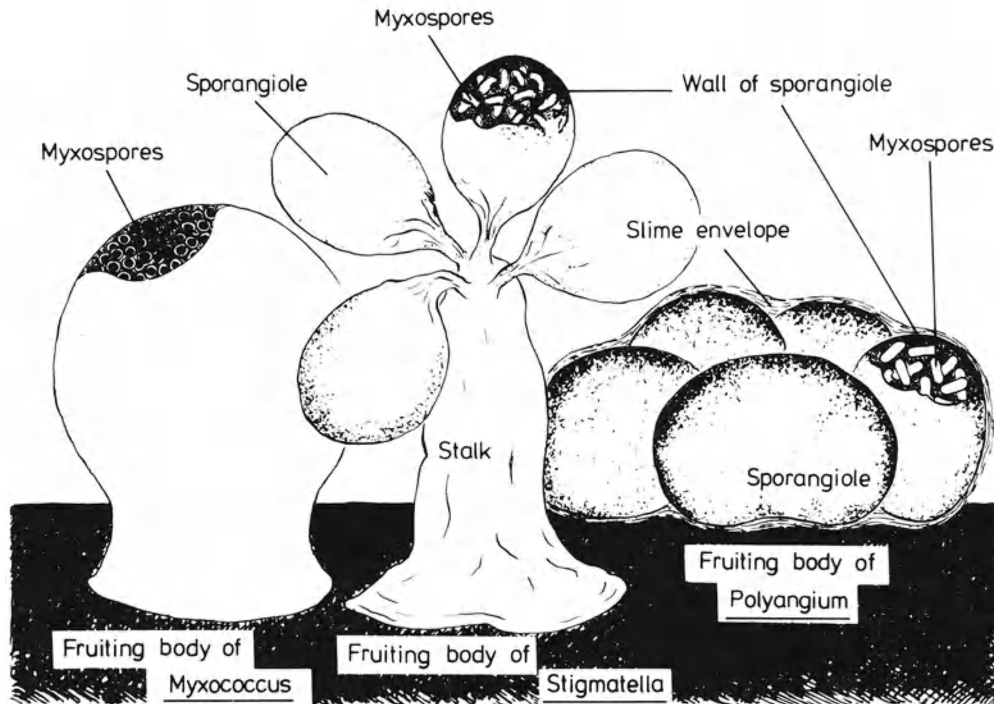

Fig. 14. The terminology for myxobacterial fruiting bodies and their structural components.

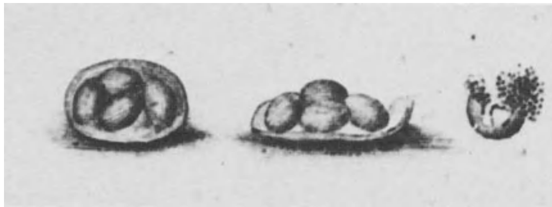

Fig. 15. *Polyangium vitellinum,* probably the first drawing of a myxobacterium to be published (Link, 1809). The original description of this still-valid genus and species reads as follows: "Among the Gasteromycetes in front of *Endogene* is to be inserted: *Polyangium.* Sporangium spherical or distortedly ovoid. Sporangioles very large in relation to the sporangium, ovoid, crammed full with a granular material. The sporangium consists of a thin, translucent, gelatinous, vesicular membrane enclosing four to six ovoid sporangioles which are filled with a colored granular mass. Sporidia could not be distinguished. *P. vitellinum,* with small yolk-colored spots, is to be counted among the smallest (species) of the order. It is found on old tree trunks, either singly or in groups of two or three, and can be recognized by its intense yolk color. Discovered by my friend Ditmar. Is to be inserted among the Gasteromycetes after *Tremella.*" (Translated from the Latin.)

signal had emerged from the maturing fruiting body. This signal seems also to diffuse through cellophane membranes, because, if a swarm is covered with cellophane and the latter is inoculated with the same organism, piles of myxospores are found on the cellophane at exactly the same sites where fruiting bodies have arisen below. Experiments of this kind were an early approach to the study of communication and the mechanisms of fruiting body formation (Fluegel, 1963; McVittie and Zahler, 1962; Reichenbach, 1966). A good experimental system for the study of myxospore formation became available when it was discovered that cellular morphogenesis can be triggered by the addition of glycerol (final concentration 0.5 M) to suspensions of growing cells of *Mx. xanthus* (Dworkin and Gibson, 1964). This became the starting point for biochemical research into cellular morphogenesis (e.g., Dworkin and Sadler, 1966; Sadler and Dworkin, 1966; White, 1975). It was soon found that many other myxobacteria also respond to glycerol induction. *Sg. aurantiaca* is another well-characterized experimental system (Gerth, 1975; Gerth and Reichenbach, 1978; White, 1981). In this organism, almost 40 chemicals have been found that induce sporulation, including monovalent cations (Reichenbach and Dworkin, 1970), various alcohols and amino acids, choline, benzene, indole, and indole derivatives; the latter two are active at particularly low concentrations. The most efficient inducer is 3-methylindole, with an optimal concentration of 0.07 mM. With the aid of mutants that were resistant to one kind of inducer but still responded to another one, the inducers could be divided into

three or four classes. Apparently each class participates in a distinct induction mechanism, each probably acting on a specific receptor. In *Sg. aurantiaca,* sporulation can also be induced by supraoptimal temperatures (40 to 41°C: the temperature has to be controlled very carefully). The change in shape that accompanies the change from vegetative cell to myxospore begins 60 min after the temperature shift, at the same time that it begins after induction with chemicals. Induction by high temperature and by glycerol (130 mM) both have the same effect on the elapsed time to the change in shape; for example, if the temperature is shifted down to 30°C after 20 min at 40.5°C, and glycerol is added at the same time, the shape change begins 40 min later. Heat induction is also possible with other myxobacteria, e.g., *Mx. fulvus* (Gerth and Reichenbach, 1978). The complementation experiment seems to corroborate the idea that the induction event, which may be initiated in different ways, sets in motion a common biochemical pathway, which then leads to cell conversion. The explanation for the effect of elevated temperatures may have to do with the heat shock proteins (HSP) that have been discovered to be induced in *Mx. xanthus* when it is transferred to temperatures above 36°C (Nelson and Killeen, 1986). If cells were heat-shocked at 40°C for 1 h, the time schedule of a following glycerol induction was accelerated, and the myxospore yield improved. It could be shown that the heat shock elicited the expression of genes normally involved in development; thus, 9 out of 18 proteins produced after glycerol induction, including alkaline phosphatase, could also be induced by heat shock (Killeen and Nelson, 1988). In this respect, *Mx. xanthus* appears to be different from *Sg. aurantiaca,* in which the complete developmental program is induced at 40.5°C. Conceivably, myxospore induction by high temperatures reflects an evolutionary root of an ancient stress reaction system which has become connected to the developmental program. In fact, in other organisms, HSP are also induced by stress factors other than heat, like alcohols or oxidants. Also, HSP are expressed during developmental processes in many eukaryotic systems. Therefore, it has been suggested that the HSP stress response system may have been directly involved in the evolution of certain developmental processes (Kurtz et al., 1986). In the case of myxospores, the connection between stress and development is particularly convincing.

The case of the artificial inducers may also be relevant for myxospore formation within fruiting bodies. Natural inducers are perhaps produced and excreted by the isolated, starving cell population crowded together inside the maturing fruiting body. The cellophane experiments discussed above and the presence of myxospores just outside of fruiting bodies may be explained by the occurrence of a diffusible inducer. In this connection, it is of interest that indole derivatives such as formylindole have been isolated from several myxobacteria (W. Trowitzsch, personal communication; Reichenbach et al., 1988), and an increase in the intracellular concentration of glycerol has been correlated with the formation of myxospores in *Mx. xanthus* (Mueller and Dworkin, unpublished observations).

Myxospore germination is induced by the transfer of the dormant cells to a suitable growth medium. Myxospores inside fruiting bodies do not always germinate readily under such conditions, however, especially those with a very thick sporangiole wall like *Cb. fuscus*; perhaps the sporangiole wall delays diffusion of some critical compound. In fact, these walls are so stable and resistant to enzymatic attack that they often remain fully intact as an empty husk long after the germinated cells are gone. As has already been mentioned, high nutrient concentration may also prevent germination (see "Purification," this chapter). Glycerol-induced myxospores of *Mx. xanthus* can be induced to germinate not only by the full growth medium, but also by certain individual amino acids (Gly, Ala, Val, Asp, Glu; at about 10 mM) and inorganic ions (HPO_4^{2-}, Mg^{2+}, Ca^{2+}, NH_4^+; at about 10 mM); heat activation is not required (Ramsey and Dworkin, 1968). It appears that a specific type of early DNA synthesis is essential during germination (Kimchi and Rosenberg, 1976).

Clearly, the function of myxospores is to help the organism survive unfavorable environmental conditions, mainly periods of dry or cold weather and starvation, but, as we have seen in the section on "Habitats," occasionally also other factors such as high salt concentrations, acid pH, and anaerobic conditions. The function of fruiting bodies is more difficult to explain. One hypothesis is that they ensure that a new life cycle is started by a population of cells rather than by single cells (Dworkin, 1973). This property could be essential for organisms that obtain their nutrients by solubilizing extracellular macromolecules with excreted enzymes. A population of cells, in contrast to an individual cell, maximizes the efficiency of extracellular enzymes. Thus, a high cell density will result in higher levels of excreted enzymes, and the utilization of the solubilized substrate will be considerably improved because losses by diffusion into directions not occupied by cells

will be minimized (Dworkin, 1973; Reichenbach, 1974a; Rosenberg et al., 1977).

Nutrition and Energy Metabolism

All myxobacteria are characterized by their ability to decompose biomacromolecules. There are two sharply separated metabolic groups: Group 1 myxobacteria depend on the decomposition of cellulose. They are all classified in one genus, *Sorangium*. The few strains that have been studied in detail are all able to grow on inorganic nitrogen (NH_4^+ or NO_3^-) as the only nitrogen source, although their growth is stimulated if a small quantity (0.05 to 0.1%) of peptone is added. They also grow on organic nitrogen compounds as the only nitrogen source, provided a carbohydrate is also present. A few strains appear even to grow on peptone alone (Sarao et al., 1985). These organisms utilize cellulose and sugars such as glucose equally well, but cellulase production is more or less repressed in the presence of sugar (Couke and Voets, 1967; Krzemieniewska and Krzemieniewski, 1937a, 1937b; Pronina, 1962).

Group 2 myxobacteria, by far the majority of species, depend on an amino acid-containing medium such as peptone for growth. They obtain the required oligopeptides by hydrolyzing proteins. Their dependence on peptides may be such that it becomes difficult, in some cases impossible, to develop defined amino acid media for them. In other cases, the organisms grow on remarkably simple media with glucose, glutamate, and two essential amino acids (see "Cultivation," this chapter). In nature, these organisms seem to subsist mainly on other organisms, which they degrade by means of a variety of hydrolytic exoenzymes. Proteases, nucleases, lipases, glycanases, and cell wall lytic enzymes have been demonstrated. These myxobacteria are particularly efficient in the destruction of other bacteria and of yeast cells, and therefore have been called "micropredators" (Singh, 1947). However, they do not depend on living organisms and could as well be regarded as scavengers. Their association with animal dung is probably due to its rich mixture of living and dead cells of all kinds and to its content of organic matter at every level of decomposition.

All known myxobacteria are strictly aerobic organotrophs. As a prerequisite for aerobic energy generation, *Myxococcus* possesses *a*-, *b*-, and *c*-type cytochromes as well as NADH-oxidase (Dworkin and Niederpruem, 1964; Kleinig, 1972) and a complete tricarboxylic acid cycle (McCurdy and Khouw, 1969; Sarao et al., 1985; Watson and Dworkin, 1968). The genus *Myxococcus* cannot utilize mono- and disaccharides (Watson and Dworkin, 1968), and therefore completely depends on amino acids (or pyruvate) as an energy source (Bretscher and Kaiser, 1978). However, as has already been mentioned, some myxobacteria are able to take up and metabolize oligosaccharides, which they produce from polysaccharides such as starch through the action of special hydrolytic enzymes (Irschik and Reichenbach, 1985). This ability may be more common than presently believed, for virtually all *Myxococcus* and *Corallococcus* strains are able to degrade one or another polysaccharide, e.g., yeast cell wall β-glucan, xylan, and starch.

An overview of the metabolism of myxobacteria has been given by Shimkets (1984). It appears that myxobacterial metabolism is similar to that of other bacteria, and unique biochemical pathways have not been discovered. Of course, myxobacterial metabolism has often been studied mainly in relation to biochemical reorganization during morphogenesis, and many enzymes have been described that are turned on or off at certain stages of development. These data will be found among the reviews cited earlier.

The glycolytic pathway seems to play a major role in the glucose metabolism of *So. cellulosum* (Sarao et al., 1985; D. Hofmann, personal communication). In *Mx. xanthus,* glucose is not metabolized, presumably because hexokinase (ATP-dependent) and pyruvate kinase are absent (Watson and Dworkin, 1968). Nevertheless, an intact phosphofructokinase and a fructose-1,6-diphosphatase are present (Watson and Dworkin, 1968), implying that glycolysis and gluconeogenesis are both possible. The explanation for this apparent contradiction is not obvious. In *Corallococcus coralloides* strain Cc c127, fructose-1,6-diphosphate aldolase activity is very weak, and intracellular hexose seems to be metabolized via the pentose phosphate pathway (Irschik and Reichenbach, 1985). The latter pathway is present in other myxobacteria, e.g., *So. cellulosum* (D. Hofmann, unpublished observations). In fact, this pathway must be present in all myxobacteria, because all are able to synthesize the pentoses required in anabolic reactions. In *Mx. xanthus,* the initial enzyme, phosphoenolpyruvate carboxylase, has been shown to be present (Watson and Dworkin, 1968). The conversion of fructose-6-phosphate into cell wall precursors has also been demonstrated (Filer et al., 1977). In *So. cellulosum,* gluconeogenesis appears to be blocked, which may explain the dependence of this organism on external carbohydrates (D. Hofmann, unpublished observations).

Lysis of Other Bacteria

The capability of myxobacteria to disintegrate whole cells of other microorganisms has attracted much attention (e.g., Beebe, 1941; Imshenetski and Kusjurina, 1951; Norén, 1960a, 1960b; Oxford, 1947), and comparative studies on the kinds of organisms that can be attacked by various myxobacteria have been carried out (Mathew and Dudani, 1955; Nolte, 1957; Oetker, 1953; Singh, 1947). It became apparent from these studies that living as well as dead bacteria could be degraded, and it was suggested that antibiotic substances could be involved in the killing and inactivation of living prey (Finck, 1950; Oxford, 1947; Oxford and Singh, 1946). While bacteriolytic activities of myxobacteria do not depend on the production of growth-inhibiting substances (Norén and Raper, 1962), myxobacteria do indeed synthesize a host of secondary metabolites, and many of them exert strong biological effects (see "Applications," this chapter). The predator-prey relationship between *Myxococcus* strains and the cyanobacterium *Phormidium luridum* in aqueous environments has also been studied in some detail (Burnham et al., 1981, 1984; Daft et al., 1985).

Studies on bacteriolytic activities led to the first studies on myxobacterial exoenzymes (e.g., Bender, 1962; Hart and Zahler, 1966, Haskå, 1969; Hüttermann, 1969; Norén, 1960b; Sudo and Dworkin, 1972). Some of the responsible enzymes have since been purified to varying degrees and have been biochemically characterized. While a complete bacteriolysis obviously requires many different enzymes, all of the studies on bacteriolytic enzymes of myxobacteria have concentrated on peptidoglycan-degrading enzymes. A glucosaminidase, an amidase, and an endopeptidase cleaving D-Ala/diaminopimelic acid and D-Ala/Lys bonds and proteases have been obtained from culture supernatants of *Mx. xanthus* (Sudo and Dworkin, 1972). Three bacteriolytic enzymes have been isolated and highly purified from *Mx. virescens* (Haskå, 1972a, 1972b, 1974); two of them proved to be endo-β-*N*-acetylglucosaminidases. The third enzyme, an endopeptidase, could be purified by adsorption to montmorillonite followed by selective desorption (Haskå, 1974, 1975). The fact that the bacteriolytic enzymes retain some of their activity when they are adsorbed to clay minerals may be of ecological relevance (Haskå, 1981). The addition of C_{11} through C_{15} fatty acids (about 2mM) to suspensions of the target bacteria increased the efficiency of the bacteriolytic enzymes substantially (Haskå et al., 1972). In contrast to the two myxococci, *Cc. coralloides* excreted an enzyme with a muramidase (= lysozyme) activity (Harcke et al., 1972). It may be added here that the "*Sorangium,*" isolate whose enzymes are often discussed in this connection, was in reality a species of *Lysobacter.*

Little is known about the enzymes with which microorganisms other than bacteria are attacked. Living cells of four yeast species could not be degraded by *Mx. xanthus* enzymes, but they were digested after a prior treatment with cysteine or organic solvents, or after heating; in contrast, living cells of the yeastlike fungus *Pullularia (Dematium) pullulans* were partially decomposed (Bender, 1963). Isolated cell walls of *Saccharomyces cerevisiae* also could not be degraded by enzymes from *Mx. fulvus* Mx strain f80 without a brief heat treatment (Borchers, 1982). It appears that the mannoprotein layer on the outside of the yeast cell wall protects the susceptible material. Two inducible enzymes have been demonstrated in the culture supernatant of *Mx. fulvus* Mx f80; 1) a laminarinase with a high specificity for β-1,3-glucosidic bonds cleaving laminaran endolytically with laminaritriose as the main product; and 2) a β-1,3-glucanase that can remove a high-molecular-weight polysaccharide other than β-1,3-glucan from the cell wall. The yeast cell wall is not completely broken down by *Mx. fulvus* Mx f80: The inner, fibrillar, glucan layer and the bud scars remain undigested, as can be seen under the electron microscope.

Most myxobacteria excrete strong, diffusible proteases, as can be readily seen through the clearing action that occurs around colonies growing on skim milk agar. A few of these enzymes have been further characterized: From the culture supernatant of *Mx. virescens,* at least three proteases have been obtained (Gnosspelius, 1978b). One of them has been purified and was found to be an alkaline serine protease (26 kDa) which preferentially cleaved peptide bonds between hydrophobic amino acids. Three different proteases have also been demonstrated in the culture supernatant of *Mx. xanthus* FB (Coletta and Miller, 1986). They have tentatively been classified as alkaline serine proteases. There were qualitative differences among the proteases of different *Mx. xanthus* strains. A coagulase, myxocoagulase (57 kDa), has been isolated from the culture broth of *Mx. fulvus* strain NK35 (Bojary and Dhala, 1989). The enzyme was clearly different from *Staphylococcus* coagulase; it did not contain sugar and was active only on rabbit plasma.

In those cases that have been examined, the bacteriolytic enzymes disappeared from the culture broth during the late exponential phase of

growth while protease activity remained long into stationary phase, either because the proteases were more stable, or because they continued to be produced and/or released (Bender, 1962; Harcke et al., 1971; Haskå and Ståhl, 1971; Nicaud et al., 1984; Sudo and Dworkin, 1972).

In contrast to the extracellular proteases of *Mx. xanthus,* the intracellular proteases appear to be mostly (80%) neutral metalloproteases. During glycerol-induced conversion of vegetative cells into myxospores, a transient increase of two protease activities (at pH 6 and pH 8) was observed (Orlowski and White, 1974).

While it is well established that myxobacteria are able to hydrolyze many other macromolecules by means of diffusible exoenzymes, those enzymes have not been characterized so far. Thus, many myxobacteria decompose starch; quite a few are potent chitin degraders, e.g., all strains of *Cb. fuscus, Cb. ferrugineus, Sg. aurantiaca,* and *Sg. erecta,* and many strains of *Me. lichenicola* and *So. cellulosum.* Also, many myxobacteria appear to attack xylan. Two enzyme activities could be demonstrated in the culture supernatant of the cellulose degrader *So. compositum,* growing in a liquid medium with suspended cellulose powder. The enzymes produced only glucose and cellobiose from cellulose. One was a cellobiase and was destroyed by heating at 55°C for 10 min. After that, an enzyme releasing cellobiose from cellulose was still intact (Couke and Voets, 1968). These two enzymes probably did not constitute the entire cellulase complex of the organism; rather there may exist another endocellulolytic enzyme that was not released from the cells. In fact, in plate cultures of *So. cellulosum* on filter paper or on cellulose powder agar, the lysis zone always exactly coincided with the size of the swarm colony. Thus, there seems to be no diffusible cellulase efficient enough to break down the crystalline cellulose completely.

Antibiotic Sensitivity

There exist relatively few data on the response of myxobacteria to antibiotics. In one study, all myxobacteria tested proved resistant to penicillin and ristocetin, and all were sensitive to erythromycin, chloramphenicol, and tetracycline; most were also sensitive to kanamycin, neomycin, and streptomycin (McCurdy, 1969a). As already mentioned, members of the Sorangineae, and specifically *So. cellulosum,* are resistant to kanamyxin, neomycin, and gentamicin. It was found that almost all myxobacteria are highly sensitive to actinomycin D, which is unusual for Gram-negative bacteria and suggests that they have an unusual, probably more lipophilic, cell surface (Dworkin, 1969). If transposon Tn*5* is introduced into *Mx. xanthus,* it confers on the organism a streptomycin resistance which is not expressed in *E. coli* (Breton, 1984).

Taxonomy and Identification

General Principles

Classification of myxobacteria still rests almost entirely on morphological characteristics, in part for historical reasons, and in part because most of the physiological information is restricted to only a few species of myxobacteria, mostly *Mx. xanthus.* Moreover, the complex morphology of myxobacteria suggests that a morphological classification is more feasible than is the case with most other bacteria. Nevertheless, the present solution does leave quite a few unsolved problems, as will be discussed.

Although some efforts have been made to support and supplement morphological classification by physiological characteristics, the results are relatively scant so far. Comparison of a number of standard physiological tests did not identify useful key characteristics other than the Congo red reaction of slime (McCurdy, 1969a; see also "Characterization," this chapter). This may simply mean, however, that the taxonomically relevant physiological characteristics of the myxobacteria have not yet been recognized, rather than that they do not exist. One taxonomically useful physiological characteristic appears to be cellulose decomposition, and we therefore suggested its use for the definition of the genus *Sorangium.* Investigations with the potentially useful techniques of immunology (Grilione, 1968) and DNA-DNA hybridization (Johnson and Ordal, 1969) have not been pursued beyond a preliminary stage. More recent research has shown that an in-depth investigation of a certain group may indeed reveal chemosystematic and biochemical characteristics of taxonomic weight. Thus, bacteria of the genus *Nannocystis* are unique among the myxobacteria in synthesizing steroids (Kohl et al., 1983) and aromatic carotenoids, and by not producing carotenoid glycosides, as all other myxobacteria do (Reichenbach and Kleinig, 1984). *Mx. stipitatus* appears to be the only myxobacterium that lacks carotenoids, so that its cell mass is colorless; instead, it produces compounds with a strong yellow fluorescence (Lampky and Brockman, 1977). While these compounds may also be produced by other *Myxococcus* species,

in them the fluorescence is masked by the carotenoids unless the cultures are grown strictly in the dark. Furthermore, as has already been discussed in detail, there are significant differences between the fatty acid and carotenoid spectra of the Cystobacterineae and the Sorangineae.

The most serious shortcoming of our present diagnostic scheme of the myxobacteria is that it rests primarily on fruiting body morphology. Although the shape of the vegetative cells and myxospores and the morphology of the swarm colony often permits the assignment of a strain to a suborder, a family, or sometimes even a genus, an exact determination and reliable classification still requires the production of typical fruiting bodies. But many strains form aberrant fruiting bodies, or no fruiting bodies at all, after continued cultivation. If we do not know which species we are dealing with from the crude culture or the early isolation steps, it may be impossible to find out later. The production of atypical fruiting bodies is particularly a problem in connection with the genus *Archangium,* as will be discussed later in more detail.

Another, more basic problem with morphological classification is the apparent convergent evolution which results in more or less identical fruiting body types being produced by taxonomically diverse myxobacteria. A good example is the case of *Chondromyces* and *Sg. aurantiaca* discussed earlier. Similar relationships are found among the genera *Angiococcus, Cystobacter, Polyangium,* and *Sorangium* and have led to considerable confusion in the past. A meaningful taxonomy has to reflect phylogenetic relations, and therefore possible errors in classification due to such convergences have to be carefully tracked down and corrected. However, if other morphological and biochemical characteristics are considered in addition to fruiting body morphology, it should not be too difficult to achieve this.

Another continuing source of confusion in the literature is the inappropriate application of the term myxobacteria to all kinds of unicellular gliding bacteria, such as *Cytophaga* and the *Cytophaga*-like bacteria of the genera *Sporocytophaga* and *Lysobacter.* The lysobacters in particular have often been called myxobacters or classified in the genus *Sorangium* and are consequently often discussed in articles on myxobacteria, e.g., in connection with enzymes and antibiotics.

The phylogenetic position of the myxobacteria is now being put on a solid base by 16S rRNA studies (Ludwig et al., 1983; Oyaizu and Woese, 1985). These studies have shown that the myxobacteria are a phylogenetically coherent group. They further suggest that the nearest, though rather remote, relatives of the myxobacteria are the sulfate-reducing bacteria and the genus *Bdellovibrio,* and that these three groups of organisms cluster in a branch of their own, the delta branch, within the class Proteobacteria (Stackebrandt et al., 1988) (see Chapters 2 and 24). It also has become apparent that there are two deep division lines within the myxobacteria, the major one separating two large groups, which correspond to the two suborders suggested by us earlier, and the minor one allocating a special position to *Nannocystis* within the Sorangineae (Ludwig et al., 1983).

Taxonomy of Myxobacteria

All known myxobacteria are united in the single order Myxococcales. The name has recently been changed from Myxobacterales (McCurdy, 1989) in accordance with Rule 9 of the International Code of Nomenclature of Bacteria (which requires that the name of an order be derived from the name of the type genus). The order may be subdivided into two suborders: Cystobacterineae and Sorangineae (Reichenbach, 1974a). As discussed earlier in this chapter, the members of the suborders differ in cell shape, myxospore development, colony structure, fatty acid, and carotenoid pattern, and in the chemical composition of their slime (Congo red reaction). The suborders are divided into families based on the organization of the fruiting bodies and the shape of the myxospores. The definition of genera and species is based on the same characteristics and on a few complementary physiological facts. There are different possibilities for splitting and rearrangement, and in the absence of more compelling arguments, personal preference must still determine which route to follow. All in all, we can distinguish 12 genera and about 40 different species of myxobacteria, a number that is not likely to increase dramatically in the future. It must be emphasized, however, that myxobacterial taxonomy cannot yet be regarded as settled and we are still confronted with major problems in distinguishing species, e.g., in the genera *Corallococcus, Archangium, Cystobacter,* and *Polyangium.*

A survey of the taxonomy of myxobacteria as favored by us is given in Table 3; it differs in some respects from the taxonomy presented in *Bergey's Manual* (McCurdy, 1989). An annotated and illustrated key to the genera follows below.

Illustrated Key to the Genera of Myxobacteria*

1. Vegetative cells are slender rods with more or less tapering ends, cigar-, boat-, or needle-shaped, about 3.5 to 7 μm long and 0.6 to 0.8 μm wide. Swarms (e.g., on VY/2 agar) tend to remain thin, filmlike, and often show a striking surface structure in form of radial veins and fields of tiny ridges or waves. Swarm edge usually has delicate fringes and flamelike protrusions. Myxospores arise through a striking cellular morphogenesis: the vegetative cells always shorten and fatten substantially. Myxospores always seem to have a capsule, which, however, is often seen only under the electron microscope. Slime and swarm sheets are stained with Congo red. The fatty acid pattern is dominated by branched-chain fatty acids; 2- and 3-hydroxy fatty acids are present in substantial amounts. All species are of the bacteriolytic type.
Suborder: Cystobacterineae 2

1′. Vegetative cells are stout, cylindrical rods, sometimes almost cube-shaped, with broadly rounded ends, about 2.5 to 6 μm long and 0.6 to 1.0 μm wide. Swarms (e.g., on VY/2 agar) tend to sink into and to penetrate the agar, sometimes to the bottom of the plate; they usually form shallow pits and bowllike depressions; their surface structure is often less pronounced, although radial veins, ring-shaped ridges, and fanlike structures may be produced. The agar surface in the swarm area may be more or less corroded. Often, the cells concentrate at the swarm edge as a band or a massive ridge, or they form spherical or kidney-shaped clusters that migrate away from the center and leave deeply etched paths in the agar surface. Myxospores differ only slightly from vegetative cells in shape and seem to possess no capsule or, at the most, a very thin one; they are, however, optically refractile. Slime and colonies do not stain with Congo red. Branched-chain fatty acids are reduced in the fatty acid pattern, and hydroxy fatty acids are completely absent. Several species are cellulose decomposers.
Suborder: Sorangineae 7

2. Myxospores are regular spheres or ovoids with a smooth surface and a heavy capsule, diameter 1.2 to 2.5 μm. Vegetative cells are 3 to 5 μm long, boat- or cigar-shaped. Swarms often consist of a relatively soft slime sheet, although occasionally tough and tenacious sheets are also produced. The surface structure of the swarm is often rudimentary, but characteristic meandering radial veins may develop. Fruiting bodies are simple, spherical, soft-slimy mounds, or cartilaginous columns and ridges that are sometimes branched, both without a distinct outer wall. In the case of *Angiococcus* they consist of spherical or disk-shaped sporangioles.
Family: Myxococcaceae 3

Fig. 16 illustrates the various forms given in the key. The numbers in the figure refer to those in the key.

Comments. The differentiating characteristic of the family is the smooth, spherical myxospore. Myxospores that are nearly spherical are found with three other myxobacteria (*Archangium, Cystobacter, Nannocystis*), which can, however, easily be distinguished by the characters mentioned below.

3. Fruiting bodies are soft-slimy spherical heads or drops, often constricted at the base, in one species with a long slime stalk .... *Myxococcus*
Comments. In agar cultures the shape of the fruiting body often deviates from the type, and only flat, cushionlike mounds or ridges are produced. There are four species: *Mx. fulvus* has white, pink, brick red, or even violet fruiting bodies with small myxospores (1.2 to 1.8 μm). *Mx. xanthus* and *Mx. virescens* produce orange and greenish yellow fruiting bodies, respectively, and have large myxospores (1.8 to 2.5 μm); they may be only one species, although it appears that typical *Mx. virescens* strains produce very large myxospores and often grey, fruiting bodies on VY/2 agar. *Mx. stipitatus* has white-to-brownish fruiting bodies with a long slime stalk; they are often found in groups on a common cushionlike base. Its myxospores are small and they are often, but not always, clearly oval in shape. The swarms show a strong yellow fluorescence with UV light illumination at 366 nm; it is, however, not yet established whether this quality is exclusive for the species.

3′. Fruiting bodies are tough cartilaginous columns or ridges, sometimes staghorn- or coral-branched, very variable in shape, often more or less deeply rooted within the agar .... *Corallococcus*
Comments. This genus has formerly been called *Chondrococcus*. The name is improper because it has been previously used for an alga. In *Bergey's Manual* (McCurdy, 1989), the genus is united with *Myxococcus*, but we prefer to retain it and suggest a new generic name.
Because of its enormous variability, many species have been described in this genus, only two or three of which may be realisitc. But a final decision will require more information. *Cc. coralloides* has small myxospores (1.2 to 1.8 μm) and produces relatively large pink or red fruiting bodies that are often branched or bear finger- or hornlike projections. Strains that also have small myxospores and produce large numbers of tiny, brownish fruiting bodies in dense fields have been described as *Cc. exiguus*. They either represent a species of their own or a variety of the former. *Cc. macrosporus* has large myxospores (1.8 to 2.4 μm) and sturdy, yellow fruiting bodies. In contrast to the other two species, it is relatively rare.

3″. Fruiting bodies consist of tiny, spherical or disk-shaped sporangioles with a definite outer wall
.... *Angiococcus*
Comments. There is only one species, *An. disciformis*. The species as such, has long been absent from descriptions of the myxobacteria although its original description by Thaxter (1904) was very clear. It has since been isolated again, and in fact it is not even particularly rare. The sporangioles appear in dark brown clusters that superficially resemble *Corallo-*

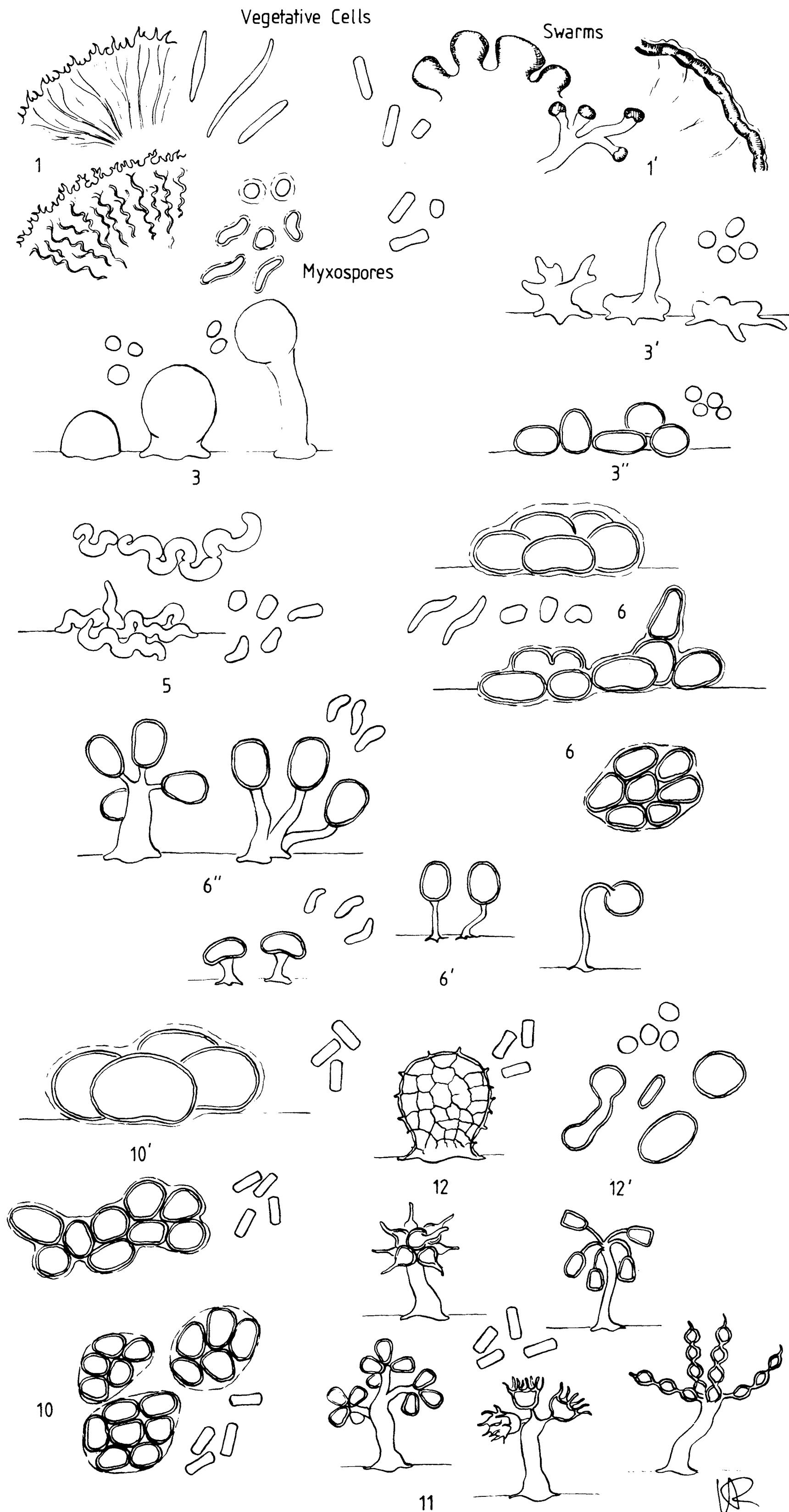

Fig. 16. A diagrammatic illustration of the key to the myxobacterial genera. The numbers in the figure refer to those in the key. The cells shown next to the fruiting bodies are myxospores.

Table 3. Taxonomic survey of the myxobacteria.

Order: Myxococcales	
Suborder:	Cystobacterineae
Families and genera:	Myxococcaceae
	Myxococcus
	Corallococcus (formerly *Chondrococcus*)
	Angiococcus
	Archangiaceae
	Archangium
	Cystobacteraceae
	Cystobacter
	Melittangium
	Stigmatella
Suborder:	Sorangineae
Families and genera:	Sorangiaceae
	Sorangium
	Polyangium
	Haploangium
	Chondromyces
	Nannocystis

coccus fruiting bodies, with which they may often have been confused. It is not yet clear whether this bacterium belongs to the Myxococcaceae, although its swarm resembles that of the other members of the family, and not that of *Cystobacter* to which genus the species has been transferred in the most recent edition of *Bergey's Manual* (McCurdy, 1989). The myxospores are spherical and small (1.2 to 1.8 μm).

4. Fruiting bodies consist of irregular masses of hardened slime, in which the myxospores are embedded, and lack an outer wall. They are either cushions with a bulging brainlike surface, or ridges that consist of meandering rolls, often with rising, fingerlike projections. Myxospores are short, fat rods, often bean-shaped or almost spherical, but clearly less regular than the myxospores of the myxococci. Vegetative cells are long, slender rods with tapering ends. Swarm is thin and spreading (e.g., on VY/2 agar), usually with a pattern of densely crowded, long, radial veins and a tough slime sheet.
Family: Archangiaceae 5
Comments. The family is characterized by the shape and structure of its fruiting bodies, myxospores, and vegetative cells. However, many myxobacteria may form degenerate fruiting bodies that resemble those of the Archangiaceae exactly. This tendency is especially true for the members of the Cystobacteraceae, and if no typical fruiting bodies are observed during isolation and cultivation, there is often no way to discriminate between an *Archangium* and a degenerated *Cystobacter* or *Melittangium.* The situation is further complicated by the fact that the vegetative cells and myxospores of these organisms are of similar shape. It may even be argued that a separate genus *Archangium* does not exist, but this conclusion is probably not correct; because, on natural substrates, one often observes large *Archangium* fruiting bodies side by side with typical *Cystobacter* fruiting bodies, and both organisms retain their original character when isolated. A careful study with modern taxonomical methods would be helpful. Although *Archangium*-type fruiting bodies are also produced by members of the Sorangineae, in this case, the different shape of the vegetative cells allows an easy differentiation from the Archangiaceae.

4′. Fruiting bodies consist of sporangioles with a distinct outer wall, often with stalks. Myxospores are short, fat rods. Vegetative cells are slender rods with tapering ends, either boat- or needle-shaped.
Family: Cystobacteraceae 6
Comments. Aberrant fruiting bodies are common in cultures (see Archangiaceae).

5. The family contains only one genus, *Archangium.* Several species have been described in the genus. However, the variability of *Archangium* fruiting bodies, and the degeneration of the fruiting bodies of other myxobacteria to *Archangium*-like structures, makes it impossible at the moment to distinguish these different species. In fact, it is not even possible to recognize *Archangium* reliably, and it would be more honest to name such strains "*Archangium*-like myxobacteria." *Ar. serpens* is the first species for which *Archangium*-type fruiting bodies have been described, and in the absence of clear, distinguishing characteristics, we prefer to label all relevant strains with this name. *Ar. violaceum* must be regarded as a degenerated *Cystobacter, C. violaceus,* under which name the organism was originally described.

6. Fruiting bodies are clusters of sporangioles that sit directly on the substrate, often embedded in a clearly recognizable, common, transparent slime envelope or covered by a slime sheet .... *Cystobacter*
Comments. There are myxobacteria with very similar fruiting bodies in the suborder Sorangineae, and some of these were originally united with the present *Cystobacter* species in a common genus, *Polyangium,* which, incidently, is the oldest generic name in myxobacterial taxonomy (Link, 1809). The original family name was Polyangiaceae. Subsequently, it was realized that the genus *Polyangium* was heterogeneous. It was therefore split, and the Sorangineae-type species were united with species of the genus *Sorangium.* For reasons of priority, the name of the newly defined genus was *Polyangium,* and for the remaining species the old generic name *Cystobacter* Schroeter 1886 was reactivated. The genus *Sorangium* disappeared completely, and as a consequence the family name Sorangiaceae was also eliminated and replaced by Polyangiaceae, which now, of course, had a completely different meaning than before. We feel this change has led to unnecessary confusion, and therefore shall no longer use the family name Polyangiaceae. Rather we shall redefine the genus *Sorangium* and reintroduce the family name Sorangiaceae.

The species of the genus *Cystobacter* are not well studied, and it is not clear at the moment how many there are. The type species, *Cb. fuscus,* is readily recognizable from its large, glistening, chestnut brown sporangioles. *Cb. ferrugineus* has large, elongated,

dull, dark brown sproangioles; they are often arranged in chains that tend to curve upwards in a fingerlike fashion. Both species have relatively long and slender, often slightly curved myxospores and are strongly chitinolytic. There are strains that produce light to reddish brown and often kidney-shaped sporangioles that are covered by a thin, translucent, and delicately plicated slime sheet; the myxospores are short, fat rods; these strains clearly represent a separate species, *Cb. velatus.* Other strains form somewhat smaller but still large, dull, dark brown, spherical sporangioles that contain myxospores in the shape of short, fat rods; they often produce a deep violet pigment, and appear to constitute the species *Cb. violaceus.* Still other strains form tiny, light to golden brown, spherical sporangioles that are tightly packed together in small clusters and are often embedded in the agar substrate, resembling *Sorangium* or *Polyangium* fruiting bodies in all these respects; but the vegetative cells are long, slender needles, the myxospores short, fat rods, and the swarms of the *Cystobacter* type. These organisms belong to *Cb. minor.* The problem of *An. disciformis* has already been discussed.

6′. Fruiting body consists of a tiny sporangiole sitting on a delicate, white stalk .... *Melittangium*
Comments. Most species were formerly attached to the genus *Podangium.* The type species of *Podangium, Pd. erectum,* has since been transfered to *Stigmatella*; the remaining species have been united in the genus *Melittangium,* so that the genus *Podangium* is now dissolved. There are at least three *Melittangium* species: *Me. boletus* has sporangioles that resemble mushroom caps and are located on very short stalks. *Me. lichenicola* forms spherical, dark brown sporangioles on short and relatively sturdy stalks. *Me. alboraceum* is described as having a spherical sporangiole on a long and bent stalk. There are also strains that produce very tiny and delicate fruiting bodies; they may represent still another species, *Me. gracilipes.* The myxospores are always short, fat rods, the vegetative cells long, slender needles.

6″. Fruiting bodies consist of dull, orange-brown to dark brown, ovoid sporangioles, which are arranged either as several on a common stalk or each on a stalk of its own .... *Stigmatella*
Comments. *Stigmatella* is easily distinguished from *Melittangium*: its fruiting bodies are much coarser, and the vegetative cells are boat-shaped and of moderate length. The myxospores are short, fat rods and often S- or C-shaped. There are two species: *Sg. aurantiaca* with several sporangioles attached to a common stalk, often via tiny peduncles; and *Sg. erecta* with one sporangiole on each stalk. Clear as this may appear, the two species still are sometimes difficult to differentiate, because *Sg. erecta* often produces several fruiting bodies side by side on a common base and sometimes with partially fused stalks, so that the resulting structure may be confused with a *Sg. aurantiaca* fruiting body. These two species occupy different ecological niches: While *Sg. aurantiaca* almost always appears on rotting wood, *Sg. erecta* is found preferentially on dung and sometimes in soil. *Sg. erecta* was formerly classified as *Podangium erectum* (see "Comments" on *Melittangium*), *Sg. aurantiaca* as *Chondromyces aurantiacus.*

7. Characters of the suborder.
Family: Sorangiaceae 8
Comments. See *Cystobacter.*

8. Fruiting bodies consist of several sporangioles clustered together 9

8′. Fruiting bodies consist of solitary sporangioles 12

9. Sporangioles located directly on or within the substrate 10

10. Fruiting bodies consist of tiny sporangioles that are tightly packed together and consequently are often polyhedral rather than spherical. A large number of the small parcels thus formed may lie together in more or less densely packed masses, but also the parcels themselves may become rather large. The fruiting bodies are often produced in enormous quantities, especially on digested filter paper, so that they determine the color of the swarm: bright yellow, orange, all shades of brown, and even pitch black. The vegetative swarm is usually more or less bright orange. Decomposes crystalline cellulose (filter paper) .... *Sorangium*
Comments. We propose to restrict this genus to the cellulose decomposers. Cellulose degradation is such a unique and stable character, and in addition parallels a series of other morphological, physiological, and biochemical characteristics in the respective strains, that a separation of the cellulolytic species seems justified. The organisms have not been sufficiently studied to allow satisfactory classification of species. In addition, it is not always apparent from the original descriptions whether an organism was a cellulose decomposer, because not all strains have been cultivated. Also, the organisms are very variable. There appear to be at least two species: *So. compositum* produces yellow-orange fruiting bodies, *So. cellulosum* brown, gray, or black ones, but this characteristic appears insufficient to differentiate the two species reliably. There are many other morphological, chemosystematical, and physiological differences among strains, but the taxonomic relevance of these observations is presently still under study.

10′. Fruiting bodies consist of more or less spherical sporangioles, that may be yellow, orange, brown, or grey. Several of them may be embedded in a common, translucent slime envelope .... *Polyangium*
Comments. For some time, this genus also contained the species of *Cystobacter* (see there for further comments). *Pl. vitellinum* produces large, golden yellow sporangioles in groups of 1 to 20 in a common envelope. It appears that in culture the color often changes to greenish yellow. *Pl. luteum* has smaller, golden yellow sporangioles embedded in a yellow slime envelope. *Pl. aureum* may be another species of this group of polyangia with orange sporangioles. *Pl. thaxteri* produces large, golden yellow sporangioles that are typically borne as a convoluted mass on a short, stout slime stalk; the whole

structure then resembles the fruiting body of a false morel (*Gyromitra*); often the sporangioles are, however, found directly on the substrate. There are further strains with small sporangioles that are densely clustered together in the same way as with certain *Cystobacter* and *Sorangium* strains. The fruiting bodies are light brown or gray in color and usually are embedded in the agar substrate. Probably, all strains of this type can be classified as *Pl. fumosum* and *Pl. spumosum*. *Pl. parasiticum* seems to parasitize fresh-water algae; it produces red-brown sporangioles within the emptied alga cells. This species has not been reported since its first description in 1925, and its parasitic growth and even its appearance in freshwater may have been an exceptional situation. The polyangia are somewhat difficult to handle and are not well-studied organisms. There are some doubts whether the present genus is really homogeneous.

11. Fruiting bodies consist of a conspicuous, unbranched or branched slime stalk, bearing cluster of bright orange sporangioles .... *Chondromyces*
Comments. The myxobacteria with the most beautiful and complex fruiting bodies are found in this genus. There are five species. One species, *Cm. aurantiacus* had to be transferred to the genus *Stigmatella*. *Cm. crocatus* has a branched stalk with clusters of small, ovoid sporangioles at the ends of the branches. All other species have unbranched stalks. *Cm. apiculatus* produces conspicuous, turnip-shaped sporangioles, and *Cm. pediculatus* produces bell-shaped sporangioles, often drooping on long, delicate peduncles; both species are relatively common, the former especially on rotting wood, the latter often in soil and decaying plant material. *Cm. catenulatus* produces chains of sporangioles, and several chains originate from one stalk. *Cm. lanuginosus* (formerly *Synangium*) has a cluster of a few large sporangioles each of which ends in a ring of long tips; the sporangiole thus resembles the young fruit of a hazelnut.
12. Fruiting bodies are large, solitary sporangioles, golden yellow, with a wrinkled or netlike surface structure when mature, found on bark and decaying wood .... *Haploangium*
Comments. This genus is easily recognized by its peculiar fruiting bodies that so far have only been encountered on the bark of living or dead trees in North America and in Europe. It also has a special position in that these are the only myxobacteria that have resisted all efforts to cultivate them. (The "*Haploangium*" species described and cultivated by Singh and Singh, 1971, were obviously misnamed because their vegetative cells were long, slender rods with tapering ends.) There may be two species that differ in size: *Ha. rugiseptum* with large sporangioles, and *Ha. minus* with small ones.

12′. Fruiting bodies are tiny, ovoid or spherical, solitary sporangioles that are produced in large numbers and are mostly embedded in the substrate. Myxospores are spherical or ovoid. Vegetative cells are often very short, stout, almost cube-shaped. Swarm is etched more or less deeply into the agar. The agar plate may become completely corroded .... *Nannocystis*
Comments. Presently only one species, *Na. exedens*, is known, but probably other species do exist. *Na. exedens* is easily recognized by its typical swarm pattern, which is especially conspicuous on water agar with streaks of living or autoclaved *E. coli*, and by its unique fruiting bodies. The latter may become considerably larger and very irregular in shape when they are produced on the agar surface. On an agar surface, some strains produce composite fruiting bodies that consist of clusters of small sporangioles that are more or less tightly squeezed together. Such strains may represent a different species ("*Na. aggregans*"). *Nannocystis* is perhaps the most common myxobacterium, although it is often overlooked; it is present in virtually every soil sample that contains any myxobacteria. While *Nannocystis* fits, in general, rather well to the other members of the suborder, it shows certain peculiarities that clearly set it apart, such as the nature of its carotenoid pigments and its synthesis of steroids.

Applications

Although myxobacteria have not been used in any industrial process, they do have some potential for biotechnological application. There are also some aspects of their biology which may have economic or environmental impact, even if this has not always been obvious, as described below.

There are no known myxobacterial pathogens for humans, animals, or plants. The fish pathogen *Chondrococcus columnaris* is a *Cytophaga*-like bacterium and has been wrongly classified as a myxobacterium. Because of their many aggressive enzymes, myxobacteria may occasionally contribute to the deterioration of materials such as rawhide or cellulose fabrics (e.g., Heyn, 1957), but it is doubtful that they are an important factor in that respect. However, although not yet demonstrated experimentally, it seems reasonable that they play a substantial role in nature in solubilizing large macromolecules, cell carcasses, and other biological detritus. As myxobacteria may reach rather high population densities in soil, it has been argued that they could become antagonistic to useful soil organisms like *Azotobacter* (Callao et al., 1966). However, although a suppression of *Azotobacter* by *Mx. xanthus* can indeed be demonstrated in the laboratory, it is an open question whether this occurs in nature. Myxobacteria are obviously able to destroy cyanobacteria in mixed cultures in aqueous environments, and it has been suggested that they could perhaps be used to control cyanobacterial water blooms (Burnham et al., 1981, 1984; Daft

et al., 1985). However, that potential has not yet been demonstrated under practical conditions. Still another application of myxobacteria in environmental problems may be their use as pollution indicators (Gräf, 1975; Tržilová et al., 1980, 1981). This scheme is presently being perfected and may eventually become a useful addition to conventional biological water analysis.

Certain chemical compounds produced by myxobacteria may occasionally become troublesome. Many myxobacteria produce a strong earthy smell, which, in the case of *Na. exedens,* has been demonstrated to be due to geosmin (Trowitzsch et al., 1981). While that smell may be pleasant in connection with soil, it is extremely disagreeable in drinking water, from which it has to be removed. The omnipresent myxobacteria could be a major source of that odor.

A good opportunity for application may lie in the field of myxobacterial enzymes, a potential that has not yet been thoroughly investigated. Thus, restriction endonucleases (Mayer and Reichenbach, 1978; Morris and Parish, 1976) and special proteases could become of interest in the future.

The efficient secretion of proteins observed with myxobacteria is unusual for Gram-negative bacteria and points to another conceivable application, as unusual expression hosts for foreign proteins (Breton et al., 1984, 1986; Nicaud et al., 1984). *Mx. xanthus* has been shown to secrete more than 50 different proteins into the medium, amounting to about 4% of the intracellular protein. This is impressive even if the actual number may be somewhat lower because some of the bands may have been due to proteolytic activity (Nicaud et al., 1984). It appears that the outer membrane of the myxobacterial cell is less of a barrier than in most other cells, perhaps due to its more dynamic behavior. It has been demonstrated that two *E. coli* periplasmic enzymes, acid phosphatase and TEM2 β-lactamase, leave the periplasm quickly when they are expressed in *Mx. xanthus* and appear in the extracellular medium (Breton and Guespin-Michel, 1987). The extracellular pectate lyases of *Erwinia* are also expressed in *Mx. xanthus* and secreted into the medium, although in this case the myxobacterial proteases inactivate the lyase (Breton et al., 1986).

Finally, there are some myxobacterial products that could become of interest. The lectin produced by *Mx. xanthus* during aggregation has already been mentioned (Cumsky and Zusman, 1981; Nelson et al., 1981), and there may be more of them. *Mx. xanthus* also secretes a blood anticoagulant, myxaline, which is a heat-stable glycopeptide (El Skoum et al., 1987; Masson and Guespin-Michel, 1988). The same or a similar compound is also produced by other myxobacteria.

The most promising opportunities for biotechnological applications with myxobacteria are at present in the production of secondary metabolites, especially compounds with biological activity such as antibiotics and other inhibitors. Although the ability of the myxobacteria to produce antibiotics has been known for some time (Katô, 1955; Norén, 1953; Norén and Raper, 1962; Oxford, 1947), apart from trivial fatty acids that were found to inhibit the germination of fungal spores (Norén and Odham, 1973), until relatively recently, no chemical structure was proposed for any of these inhibitors. The first myxobacterial antibiotic for which the chemical structure has been elucidated was ambruticin, a potent antifungal compound from *So. cellulosum* (Ringel et al., 1977). In the past decade, about 40 basic structures and 200 structural variants have been discovered in myxobacteria and have been fully characterized chemically. As most of these results have been reviewed (Reichenbach and Höfle, 1989; Reichenbach et al., 1984, 1988), this material is not discussed here in detail. The more significant insights can be summarized as follows: 1) A high proportion of myxobacterial strains produce some kind of compound with biological activity: about 50% among the Cystobacterineae, almost 100% of the *Sorangium* strains. 2) The substances produced are chemically very different, including aromatic, heterocyclic, and polyenic compounds, alkaloids, macrocycles, polyethers, and peptides. A few examples are shown in Fig. 17. 3) Most of these compounds have not been isolated from other organisms and thus appear to be unique to the myxobacteria. The only exceptions are pyrrolnitrin and althiomycin; saframycin has been found with a new variant. 4) The ability to produce a specific compound is typical for a certain strain, and not for all strains of that species. In fact, different strains of the same species may produce totally different antibiotics, but the same antibiotic may also be found in different species and even genera. Interestingly, the border between the two suborders is also a division line for the secondary metabolism, and so far no compound has been found that is produced by representatives of the two different suborders. 5) A strain may produce, at the same time, two or more compounds that are totally different chemically. 6) Often a family of chemical variants of the same basic structure is synthesized by a strain; up to 40 different variants have been isolated from one single strain. 7) The initial yields are usually low, between 0.5 and 20

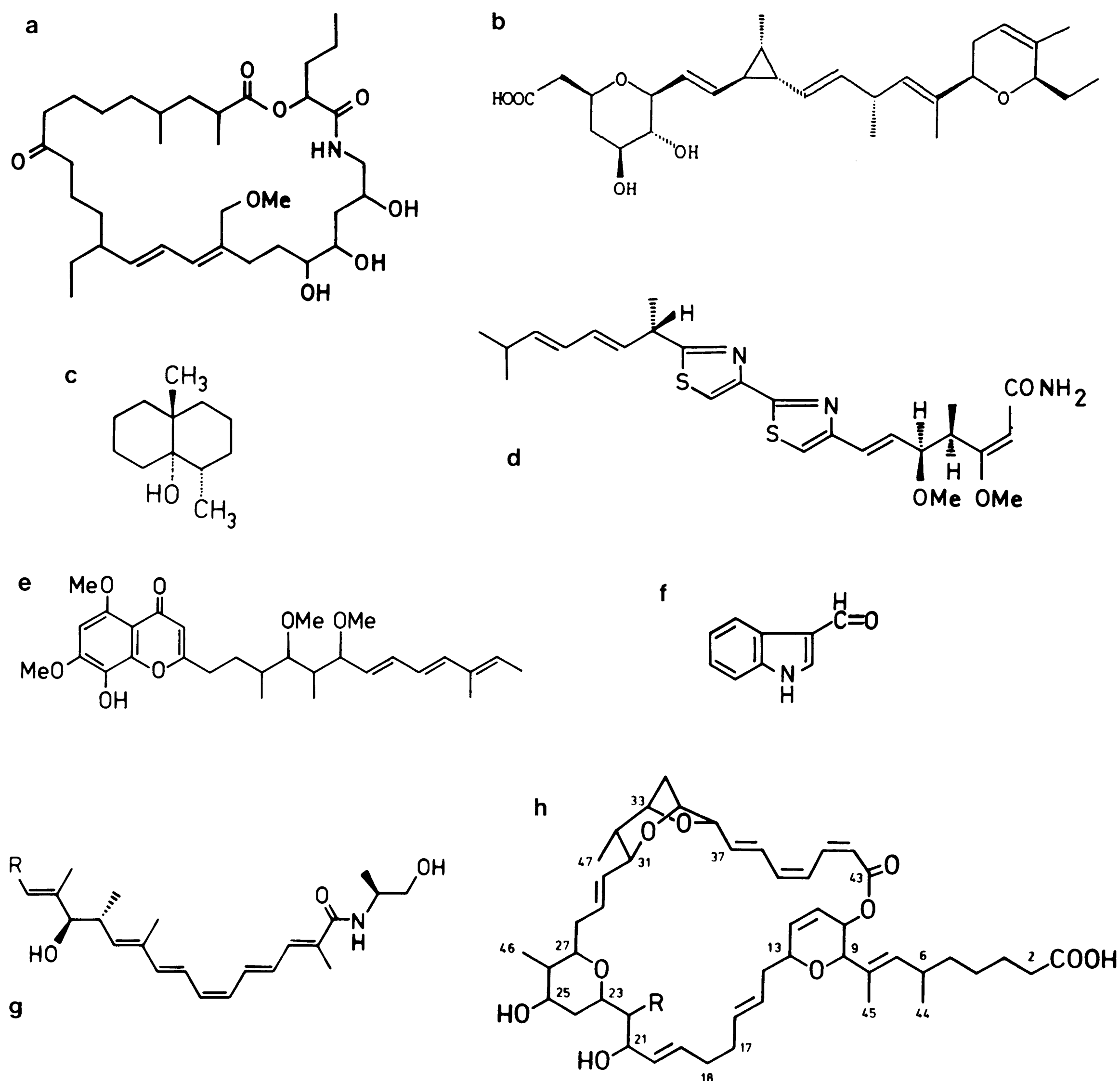

Fig. 17. A few examples of secondary metabolites produced by myxobacteria. (a) Myxovirescin A; the compound, or variants of it, has been described as antibiotic TA and as megovalicins; (b) ambruticin; (c) geosmin; (d) myxothiazol; (e) stigmatellin; (f) 3-formylindole; (g) myxalamid; R is 2-butyl, 2-propyl, ethyl, and methyl for myxalamids A, B, C, and D, respectively; (h) sorangicin; R is OH and H for sorangicin A and B, respectively.

mg/liter, but with conventional methods, yield increases to more than 1 g/liter have already been achieved. 8) Interesting mechanisms of action have been discovered; compounds have been discovered that are specific inhibitors of eubacterial RNA and protein synthesis or are extremely efficient inhibitors of electron transport in respiration and photosynthesis. Two of the latter are used as specific biochemical probes and are commercially available (myxothiazol, stigmatellin). Antitumor and antiviral substances including substances active against the human immunodeficiency virus) have also been found. Among the compounds discovered also are iron transport metabolites of the myxobacteria (myxochelin, nannochelin). At the moment, several of the myxobacterial inhibitors are being intensely studied for potential application and large-scale production.

Antibiotic TA was discovered in *Mx. xanthus* strain TA (Rosenberg et al., 1973), but its structure has not been elucidated so far. Published chemical data, specifically NMR data (Rosenberg et al., 1982) suggests that the compound may be identical to one of the 20 variants of myxovirescin (Gerth et al., 1982; Trowitzsch et al., 1982; Trowitzsch-Kienast et al., 1989). This identification is further supported by the observation that, myxovirescins are produced by many *Mx. xanthus* and *Mx. virescens* strains

(H. Reichenbach, unpublished observations). In fact, the same antibiotic has been described a third time from another *Mx. xanthus* strain (Onishi et al., 1984). Furthermore, the megovalicins (Miyashiro et al., 1988; Takayama et al., 1988) are identical with previously published variants of myxovirescin; a new species, *Mx. flavescens,* has been created to describe the producing organism (Yamanaka et al., 1987), which cannot, however, be distinguished from existing species. Novel compounds with antitumor activity, the glidobactins, have recently been discovered in what seems to be a *Nannocystis* or a *Polyangium* strain (Oka et al., 1988a, 1988b, 1988c). These compounds are acylpeptides with a variable fatty acid moiety and a constant cyclic dipeptide nucleus consisting of two new amino acids. Finally, antibacterial activity has been reported in a strain of *Cc. coralloides,* but chemical data that allow an identification of the compound have not so far been produced (Arias et al., 1979a, 1979b).

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*The films cited may be obtained on loan as 16-mm copies from the Institut für den wissenschaftlichen Film, Nonnenstieg 72, D-3400 Göttingen, Germany. The movies are available free of charge for universities and other educational institutions worldwide, and only the mailing costs must be reimbursed.

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The Genera *Campylobacter* and *Helicobacter*

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Introduction

The genus *Campylobacter* encompasses a diverse group of organisms that are either commensals for pathogens for both humans and animals. Thirteen species are currently included in the genus (Table 1). The new genus *Helicobacter* contains two species previously classified as Campylobacter (Table 1). All of these organisms are Gram-negative, nonsporeforming, oxidase positive, indole negative, asaccharolytic organisms that neither ferment nor oxidize carbohydrates (Smibert 1978, 1984; Penner, 1988). Energy is produced through respiration and the metabolism of amino acids. The GC content for members of the genus falls between 29–39 mol% (Fox et al., 1989; Owen, 1983; Owen and Leaper, 1981; Harvey and Greenwood, 1983b; Ursing et al., 1983). All these organisms are slender, helically curved Gram-negative rods that are 0.2–0.5 μm wide and 0.5–8.0 μm long (Fig. 1). Pairs of cells have an S-shaped or gull-wing morphology while longer chains of cells form spirals. The shorter campylobacter forms move in a rapid, darting manner while spiral forms rotate along the long axis like corkscrews. The wavelength, amplitude, and length of the spirals vary among species. With the exceptions of *Helicobacter pylori* and *H. mustelae,* the organisms are motile by means of a long single, polar flagellum found at one or both ends of the cell (Smibert 1984). *H. pylori* has multiple flagella located at only one end of the cell while *H. mustelae* has both polar and lateral flagella (Fox et al., 1989).

Table 1. Current *Campylobacter* and *Helicobacter* taxonomy.

Species	Subspecies	Biovar
C. fetus	*fetus, venerealis*	
C. jejuni	*jejuni, doylei*	
C. coli		
C. laridis		
"C. upsaliensis"		
C. hyointestinalis		
C. mucosalis		
C. cinaedi		
C. fennelliae		
C. sputorum		*sputorum, bubulus, faecalis*
C. concisus		
C. cryaerophila		
C. nitrofigilis		
H. pylori[a]		
H. mustelae[a]		

[a]The new genus name *Helicobacter* has recently been accepted for these species (Goodwin et al., 1989).

Historical Review of Taxonomy

Campylobacters were originally thought to be members of the genus *Vibrio* and were first cited in the literature by McFadyean and Stockman (1913) as a cause of abortions in ewes. Smith (1918) and Smith and Taylor (1919) associated these curved bacilli, which they named *Vibrio fetus,* with abortion in cattle, thus extending the known host range of this group of organisms. Jones and Little (1931) added winter dysentery in calves to the list of diseases attributable to the new vibrios, calling their isolates *V. jejuni* and Doyle (1944) ascribed swine dysentery to the organism he called *V. coli.* Thus, by 1944, the epithets *V. fetus, V. jejuni,* and *V. coli* had all appeared in the literature and the organisms were well established as veterinary pathogens. It was shortly thereafter that Levy (1946) noted the presence of spiral organisms in the blood of some of 350 people affected with gastrointestinal disease. Unfortunately, the organism seen by microscopy was not recovered by culture from any of these cases. However, in the following year Vinzent et al. (1947) were successful in isolating the organism known as *V. fetus* from the blood of three pregnant women, two of whom went on to have spontaneous abortions. This marked the beginning of the association of these novel vibrios with human disease.

The key observation that initiated the current interest in this group of organisms in human

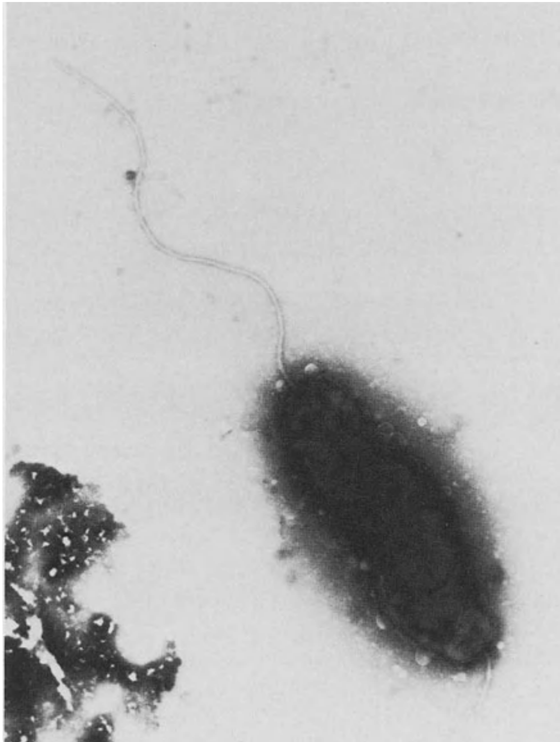

Fig. 1. Electron micrograph of *Campylobacter jejuni* subsp. *jejuni*. Magnification 17,000 ×. Diameter of cells is 0.6 μm.

disease was made by King (1957) who isolated from the blood of a patient an organism that was similar in morphology to the *V. fetus* described by Vinzent et al. (1947) but that differed biochemically and antigenically from *V. fetus*. King named the organism, which grew at 42°C, a "Related Vibrio" and hypothesized that the organism would someday be recovered from the stool of humans with diarrheal illness (King, 1962). This would not be accomplished, however, until 1972 (Dekeyser et al., 1972).

The differentiation of species within the genus *Vibrio* was undertaken by Florent (1959) who described *V. fetus* subsp. *intestinalis*, the pathogen causing septic abortion in sheep, as a spiral organism capable of growing in the presence of 1% glycine and able to produce H_2S, vs. *V. fetus* subsp. *venerealis*, the cause of infectious infertility in cattle, which does not show these characteristics.

Sebald and Véron (1963) demonstrated that the new group of organisms bearing the designations of *V. fetus*, *V. jejuni*, and *V. coli* could be distinguished biochemically and by DNA-base composition from the traditional members of the genus *Vibrio*. They designated a new genus, *Campylobacter*, meaning "curved rod," to encompass the new group. Véron and Chatelain (1973) published a comprehensive taxonomy of the genus *Campylobacter*, designating the organism described as *V. fetus* subsp. *intestinalis* by Florent (1959) as the type species and renaming it *Campylobacter fetus* subsp. *fetus*. Although Smibert took a different view of the taxonomy, designating *V. fetus* subsp. *venerealis* as the type species and renaming it *Campylobacter fetus* subsp. *fetus* (Smibert, 1974), the nomenclature of Véron and Chatelain (1973) has been selected as that which appears in the list of approved bacterial names (Skerman et al., 1980). After a careful review of the literature, Karmali and Skirrow (1984) confirmed that the original description by McFadyean and Stockman (1913) most closely resembles *C. fetus* subsp. *fetus*, which is thus given the position of type species. The name *C. fetus* subsp. *intestinalis* is now defunct and does not appear in Skerman's list of approved bacterial names.

The status of the genus *Campylobacter* continues to be in flux. For example, *C. pylori* and *C. mustelae* have been renamed *Helicobacter pylori* and *H. mustelae* (Goodwin et al., 1989). This change was based on the results of rRNA analysis and other features of the organisms (Lau et al., 1987; Romaniuk et al., 1987; Thompson et al., 1988). Based on 5S and 16S rRNA sequence data, other members of the genus *Campylobacter* may well be moved to other genera in the near future (Thompson et al., 1988), while organisms such as *Wolinella recta*, *W. curva*, *Bacteroides gracilis*, and *B. ureolyticus* may someday be included in the *Campylobacter* genus (Lau et al., 1987; Paster and Dewhirst, 1988). Because these changes are difficult to predict, this chapter will not address those issues but rather will focus on those organisms that currently are accepted in the genus.

Descriptions of Campylobacter Species

C. fetus is the type species of the genus and has two recognized subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. *C. fetus* subsp. *fetus* is a cause of sporadic abortion in cattle and sheep (Bryner et al., 1964, 1971; Garcia et al., 1983). Animals are thought to acquire the organism through contaminated food and water. After intestinal colonization, the organism is apparently spread hematogenously to the uterus where infection causes spontaneous abortion of the fetus. *C. fetus* subsp. *fetus* is an unusual pathogen of humans, although cases of septicemia in immunocompromised hosts have been described (Guerrant, 1978). The organism

also has been recovered from human stool samples (Devlin and McIntyre, 1983; Harvey and Greenwood, 1983a) but the incidence in humans may be underestimated due to the use of selective media and the inhibitory growth temperatures commonly employed for the recovery of *C. jejuni*.

C. fetus subsp. *venerealis*, on the other hand, has a clear tropism for the bovine genital tract where it is a cause of epidemic sterility (Bryner, 1964) and, in some cases, abortion (Garcia et al., 1983). The organism often is carried asymptomatically in the prepuce of infected bulls and is passed to the bovine female genital tract where it causes inflammation and subsequent infertility (Samuelson and Winter, 1966; Hoffer, 1981; Garcia et al., 1983).

C. jejuni has been noted to cause diarrhea (Campbell and Cookingham, 1978; Firehammer and Meyers, 1981) and mastitis in cattle (Lander and Gill, 1985); abortion in sheep (Smibert, 1978); and is a significant cause of enteritis in humans (Butzler and Skirrow, 1979; Blaser and Reller, 1981). *C. jejuni* also has been associated with a wide variety of other human illnesses including septicemia, meningitis, arthritis, urinary tract infection, and Guillain-Barré syndrome (Blaser and Reller, 1981; Blaser et al., 1986a) although these occur less frequently than does enteritis. *C. jejuni* is found in healthy and diarrheic dogs and cats (Fleming, 1983; Fox, 1985; Blaser et al., 1978) and nonhuman primates (Morton et al., 1983) and is part of the normal flora of chickens, turkeys, and many wild birds (Lander and Gill, 1985; Prescott and Bruin-Mosch, 1981; Prescott and Munroe, 1982). It also has been recovered from retail chickens, beef, pork (Harris et al., 1986) and retail mushrooms (Doyle and Schoeni, 1986). In studies where *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* have been distinguished, *C. jejuni* subsp. *doylei* has been isolated from the feces of children (Steele et al., 1985) and from gastric biopsies of adults (Kasper and Dickgiesser, 1985; Owen et al., 1985).

C. coli is isolated routinely from the intestines of healthy pigs, cattle, and sheep (Gebhart et al., 1983; Manser and Dalziel, 1985) and can be recovered from a variety of other domestic animals with and without enteric disease (Prescott and Bruin-Mosch, 1981). It is a significant cause of enteritis in humans (Blaser and Reller, 1981; Steele et al., 1985). However, the incidence of human infection may be underestimated due to the inhibition of some *C. coli* isolates by the selective antimicrobials used to recover *C. jejuni* from clinical samples (Ng et al., 1985).

C. laridis (Benjamin et al., 1983) was originally isolated from seagull feces and is often referred to as the nalidixic-acid-resistant thermophilic campylobacter (NARTC). This organism is an infrequent agent of enteritis and bacteremia in humans (Nachamkin et al., 1984; Tauxe et al., 1985; Simor and Wilcox., 1987) and occasionally is recovered from sheep (Manser and Dalziel, 1985), dogs, monkeys and other avian species (Skirrow and Benjamin, 1980b).

C. hyointestinalis was originally recovered from pigs with proliferative ileitis (Gebhart et al., 1983), although its role in the disease is uncertain. Neither *C. hyointestinalis* nor *C. mucosalis*, a frequent co-isolate of *C. hyointestinalis*, produce the symptoms of the disease when given in pure culture to pigs (Boosinger et al., 1985.) *C. hyointestinalis* is rarely recovered from healthy pigs but has been isolated from the feces of cattle and the intestines of hamsters (Gebhart et al., 1985). This organism also has been recovered from the stool of humans with enteritis or proctitis, although its role in either disease is unclear (Fennell et al., 1986; Edmonds et al., 1987). The use of selective media containing cephalothin or other antimicrobials active against *C. hyointestinalis* and nonoptimal incubation temperatures (i.e., 42°C) limits the recovery of these organisms from human cases.

C. mucosalis (Roop et al., 1985b) was previously classified as *C. sputorum* subsp. *mucosalis* (Lawson et al., 1981) and has been isolated primarily from the intestinal mucosa of pigs (Rowland et al., 1973). This organism may have a role in porcine intestinal adenomatosis or proliferative ileitis although Koch's postulates have not been fulfilled (Lawson and Roland, 1974; Gebhart et al., 1985). It has not been recovered from humans.

"C. upsaliensis," the catalase negative or weak (CNW) campylobacter, was originally isolated from dogs with and without diarrhea (Sandstedt et al., 1983). The name of the organism is in quotation marks since it has yet to be accepted by the International Committee on Systematic Bacteriology and validly published in the International Journal of Systematic Bacteriology. More recently, it has been isolated from the feces of cats and from the feces and blood of humans (Lastovica et al., 1989; Megraud and Bonnet, 1986; Patton et al., 1989; Steele et al., 1985; Taylor et al., 1989; Walmsley and Karmali., 1989).

C. concisus was originally isolated from the oral cavity and gingival crevices of humans with periodontal disease, although no association with oral pathology was established. (Tanner et al., 1981). More recent work has focused on rapid identification and chemotactic responses

of this organism (Tanner 1986; Paster and Gibbons, 1986) as well as its potential role in human enteritis (Vandamme et al., 1989).

C. fennelliae and *C. cinaedi* are associated with enteritis, proctitis, and proctocolitis in homosexual men (Quinn et al., 1984; Totten et al., 1985). Both organisms also have been recovered from the blood of patients with human immunodeficiency virus infection (Pasternak et al., 1984; Cimolai et al., 1987; Ng, V. L. et al., 1987). In addition, *C. cinaedi* is known to be part of the normal flora of hamsters (Gebhart et al., 1989).

C. sputorum is divided into three biovars that can be distinguished phenotypically and by host preference, but not by genetic means (Roop et al., 1985a). *C. sputorum* biovar *sputorum* is a commensal of human gingival crevices (Loesche et al., 1965) and has been recovered from stool samples of humans (Karmali and Skirrow, 1984; Steele et al., 1985). *C. sputorum* biovar *bubulus* appears to be strictly a commensal of cattle and is found in the prepuce of the male and in the female genital tract. It has not been isolated from humans. Finally, *C. sputorum* biovar *faecalis* (Roop et al., 1985a), originally known as *"C. faecalis"* (Firehammer, 1965), has been isolated from sheep feces and bovine genitalia and may be associated with enteritis in cattle (Al-Marshat and Taylor, 1980). There are no human isolates to date.

C. cryaerophila was originally recovered from aborted fetuses of cattle and pigs (Neill et al., 1978; Higgins and Degre, 1979). It also has been recovered from cattle with mastitis, although its role in this disease is uncertain (Logan et al., 1982). Tee et al. (1988) reported the recovery of *C. cryaerophila* from a human fecal sample. In most laboratories isolation temperatures above the optimal growth temperature of this organism (30°C) may prevent the recovery of *C. cryaerophila* (Neill et al., 1980).

Campylobacter nitrofigilis is a nitrogen-fixing organism isolated from the roots of plants growing in salt marshes (McClung and Patriguin, 1980). It has not been recovered from humans or animals. *H. pylori* initially was isolated by Marshall and Warren (1984) and was subsequently named *C. pyloridis* (Marshall et al., 1984). The epithet, however, was grammatically incorrect and was changed to *C. pylori* (Marshall and Goodwin, 1987). It has recently been changed a third time to its current epithet, *H. pylori* (Goodwin et al., 1989). This organism is a cause of type B gastritis in humans and is isolated most frequently from biopsies of the antral region of the stomach (Buck et al., 1986; Marshall, 1990; Rauws and Tytgat, 1989). *H. pylori* also has been recovered from the nonhuman primate *Macaca nemestrina* (Bronsdon and Schoenknecht, 1988). A second species, *H. mustelae,* previously named *C. mustelae* (Fox et al., 1989; Goodwin et al., 1989), has been recovered from the stomachs of ferrets (Fox et al., 1988).

Identification

The individual biochemical, growth, and susceptibility patterns used to distinguish members of the genus *Campylobacter* and the genus *Helicobacter* are shown in Table 2. A discussion of the morphologic and biochemical traits of each species is presented below.

C. fetus

There are two subspecies of *C. fetus,* designated *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis.* These organisms can be distinguished in the laboratory by their cellular morphology and ability to grow in the presence of 1% glycine. The spirals of *C. fetus* subsp. *fetus* have an average wavelength of 1.8 μm and an average amplitude of 0.55 μm, while the spirals of *C. fetus* subsp. *venerealis* are more loosely coiled, with an average wavelength of 2.43 μm and an amplitude of 0.73 μm (Karmali et al., 1981). *C. fetus* subsp. *fetus* can grow in the presence of 1% glycine, although Chang and Ogg (1971) noted that glycine tolerance was bacteriophage-mediated and thus may be a poor test to differentiate these two organisms.

Colonies of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* can be observed after 24–48 h of microaerobic incubation at 25 or 37°C; some strains of *C. fetus* subsp. *fetus* will grow at 42°C (Harvey and Greenwood, 1983b; Edmonds et al., 1985). These organisms will not grow under aerobic conditions but may grow anaerobically if the medium is supplemented with 0.2% fumarate (Veron et al., 1981). Trimethylamine N-oxide (TMAO) will not serve as a terminal electron acceptor for *C. fetus.* Colonies grown on blood agar are 1–2 mm in diameter, smooth, round, convex, entire, shiny, grayish white to tan, and are nonhemolytic. They do not swarm on moist agar and coccoid forms are not observed when these organisms are exposed to air.

Strains of both subspecies are positive for catalase production, nitrate reduction, and growth in 1% bile. They do not reduce nitrite, nor do they hydrolyze urea or hippurate. They are negative for hydrogenase activity by the benzyl viologen assay (Goodman and Hoffman, 1983). H_2S is not produced in triple sugar iron (TSI) agar, and growth is not observed in the presence

Table 2. Biochemical differentiation of *Campylobacter* and *Helicobacter* species.

	Campylobacter								
	jejuni							*fetus*	
	subsp. *jejuni*	subsp. *doylei*	*coli*	*laridis*	*"upsaliensis"*	*cinaedi*	*fenelliae*	subsp. *fetus*	subsp. *venerealis*
Catalase	+	V	+	+	−/w	+	+	+	+
Hydrogen sulfide on TSI	−	−	−	−	−	−	−	−	−
Hippurate hydrolysis	+	+	−	−	−	−	−	−	−
Urease	−	−	−	−	−	−	−	−	−
Nitrate reduction	+	−	+	+	+	+	−	+	+
Tolerance tests									
25°C	−	−	−	−	−	−	−	+	+
42°C	+	−/w	+	+	+	−	−	V	−
1% Glycine	+	+	+	+	V	+	+	+	−
3.5% NaCl	−	−	−	−	−	−	−	−	−
ANA growth in 0.1% TMAO	−	−	−	+	−	NR	NR	−	−
Hydrogen or formate required	−	−	−	−	−	−	−	−	−
Susceptibility									
Nal 30	S	S	S	R	S	S	S	R	R
CF 30	R	S	R	R	S	S	S	S	S

+, most strains positive; −, most strains negative; V, variable reactions; S, susceptible, zone of inhibition >6 mm; R, resistant, no zone of inhibition; NR, not reported; w, weak.
Nal 30 = 30 μg nalidixic acid disc, CF 30 = 30 μg cephalothin disc.

of 3.5% NaCl or 0.04% triphenyltetrazolium chloride (TTC). Both subspecies are resistant to nalidixic acid but are susceptible to cephalothin.

C. jejuni

There are two subspecies of *C. jejuni*, *C. jejuni* subsp. *jejuni*, and *C. jejuni* subsp. *doylei* (Steele and Owen, 1988). The latter subspecies, which is likely the organism first described as *Vibrio coli* by Doyle (1944), is unable to reduce nitrate to nitrite. This is the major difference between the two groups of organisms.

The spiral forms of *C. jejuni* are tightly coiled and have an average wavelength of 1.12 μm and an amplitude of 0.48 μm when measured by phase contrast microscopy (Karmali et al., 1981). This characteristic is shared with *C. coli* and *C. laridis* and distinguishes them from both subspecies of *C. fetus*, whose spirals are less tightly coiled. Cells of *C. jejuni* become coccoid when exposed to atmospheric air.

Colonies of *C. jejuni* subsp. *jejuni* appear after 24–48 h of microaerobic incubation at 37 or 42°C. *C. jejuni* subsp. *doylei* grows well at 37°C but poorly, if at all, at 42°C. No growth of either subspecies is observed at 25°C and anaerobic growth cannot be achieved by the addition of 0.2% fumarate or 0.1% TMAO to the medium (Roop, 1984). Two different colony types have been described (Smibert, 1984). The first measures 1–2 mm in diameter, is raised, round, glistening, and entire, with an opaque center and translucent edge. The second type is flat, with an irregular border and a smooth to ground-glass appearance. These colonies tend to spread along the lines of inoculation producing elongated, coalescing growth. Most strains of *C. jejuni* readily form spreading colonies when grown on moist, freshly prepared media. Both colony types are nonhemolytic on blood agar, are gray to tan in color, but pinkish tan when collected on a loop.

C. jejuni is the only campylobacter capable of hydrolyzing hippurate, making this the key differential test for the species (Harvey, 1980; Hébert et al., 1984). However, hippurate-negative strains of *C. jejuni* have been identified by DNA hybridization (Totten et al., 1987). Most strains of *C. jejuni* subsp. *jejuni* are catalase positive although catalase negative or weakly positive strains of *C. jejuni* subsp. *doylei* occur (Steel and Owen, 1988). *C. jejuni* grows in the presence of 1% glycine and 1% bile but does not produce H_2S on TSI slants. Tolerance to 0.04% TTC is variable. *C. jejuni* is usually susceptible to nalidixic acid, although nalidixic acid resistant mutants have been described (Taylor et al., 1985). *C. jejuni* subsp. *doylei* is susceptible to cephalothin, while *C. jejuni* subsp. *jejuni* is resistant. Strains of *C. jejuni* produce large

Campylobacter									
			sputorum					*Helicobacter*	
			biovar	biovar	biovar				
hyointestinalis	*concisus*	*mucosalis*	*sputorum*	*bubulus*	*faecalis*	*cryaerophila*	*nitrofigilis*	*pylori*	*mustelae*
+	−	−	−	−	+	+	+	+	+
+	+	+	+	+	+	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	V	+	+
+	+	+	+	+	+	+	+	−	+
V	−	+	−	−	−	+	+	−	−
+	+	+	+	+	+	−	−	V	+
+	+	V	+	+	+	−	−	V	V
−	−	−	−	+	+	NR	+	−	−
+	−	−	V	+	+	−	NR	NR	NR
−	+	+	−	−	−	−	−	−	−
R	R	V	V	R	R	S	S	V	S
S	R	S	S	S	S	R	S	S	R

amounts of C19:0 fatty acid (Blaser et al., 1980c).

C. coli

The microscopic and macroscopic appearance of *C. coli* are indistinguishable from those of *C. jejuni.* Biochemically, these organisms also are quite similar with the exception of the hippuricase activity of *C. jejuni.* In addition, Roop et al. (1984) reported that the isolates of *C. coli* they examined produced small bands of H_2S in TSI agar if the medium was fresh and adequate water of syneresis was present at the junction of the slant and the butt. He also found that *C. coli* strains grew in a minimal medium while *C. jejuni* strains did not (Roop et al., 1984).

C. laridis

The microsopic morphology of *C. laridis* is similar to *C. jejuni* in that the cells form tightly coiled spirals of relatively short wavelength and amplitude (Benjamin et al., 1983). Upon exposure to air, the cells undergo a rapid transformation to the coccoid shape.

C. laridis produces colonies after 24–48 h of incubation at either 37 or 42°C. Growth of most strains is enhanced at 42°C. Colonies are 1–1.5 mm in diameter, low convex, smooth, translucent, gray to tan, and entire. They are nonhemolytic on blood agar. Some strains may spread on moist agar.

Isolates of *C. laridis* are catalase positive, reduce nitrate but not nitrite, and grow in the presence of 1% glycine, 1% bile or 1.5% NaCl, but do not hydrolyze hippurate or urea, do not produce H_2S on TSI agar, and are resistant to cephalothin. *C. laridis* can be differentiated from *C. jejuni* and *C. coli* based on its resistance to nalidixic acid, growth in 1.5% NaCl, and growth under anaerobic conditions when 0.1% TMAO is added to the medium. Since nalidixic-acid-resistant mutants of *C. jejuni* and *C. coli* have been described, the use of multiple tests to confirm the identification of these species seems warranted. Urease-positive, nalidixic-acid-susceptible variants of *C. laridis,* confirmed by DNA-DNA hybridization studies, have been reported by Megraud et al. (1988).

"C. upsaliensis"

"C. upsaliensis" strains are slender, curved rods similar to those of *C. jejuni.* They become coccoid after extended incubation.

Colonies appear after 48–72 h of microaerobic incubation at 37°C. Most strains also will grow at 42°C, but not as luxuriantly and there is no growth at 25°C. Colonies are nonhemolytic on blood agar, measure 0.5–1.0 mm in diam-

eter, are translucent, smooth, convex, gray to tan, entire, and are similar in appearance to the colonies of *C. jejuni* and *C. coli*. "*C. upsaliensis*" is either catalase negative or demonstrates a weakly positive reaction. These organisms reduce nitrate but not nitrite and grow in the presence of 1% bile. They do not hydrolyze either hippurate or urea, do not grow in the presence of 1.5% NaCl or 0.04% TTC, and do not produce H_2S in TSI agar. Growth in 1% glycine is variable. These organisms are susceptible to both nalidixic acid and cephalothin (Patton et al., 1989; Walmsley and Karmali, 1989).

C. hyointestinalis

Cells of *C. hyointestinalis* are similar in morphology to those of *C. fetus* subsp. *fetus* (Gebhart et al., 1983). Organisms grow best at 37°C although most strains also grow at 42°C. Some may grow at 25°C. The cells do not become coccoid upon exposure to air.

After 48 h of incubation, colonies measure 1.5–2.0 mm in diameter, are round, convex, shiny, slightly mucoid, and are gray to yellow. The color of colonies gathered on a loop is dirty yellow. These organisms are nonhemolytic on blood agar and do not swarm on moist agar.

C. hyointestinalis strains are potent producers of H_2S as detected in TSI agar. This is a characteristic shared with *C. concisus*, *C. mucosalis*, and *C. sputorum*. Isolates of *C. hyointestinalis* are catalase positive, reduce nitrate but not nitrite, and will grow in the presence of 1% glycine and 1% bile. These organisms do not hydrolyze either hippurate or urea, and will not grow in the presence of 1.5% NaCl or 0.04% TTC. They do not grow aerobically but can grow anaerobically in the presence of 0.1% TMAO (Gebhart et al., 1985). *C. hyointestinalis* strains also produce hydrogenase, which can be detected by the viologen method (Goodman and Hoffman, 1983). They are resistant to nalidixic acid and susceptible to cephalothin.

C. fennelliae and C. cinaedi

Cells of *C. cinaedi* and *C. fennelliae* are slender, curved rods that appear more delicate than those of *C. jejuni*. S-shaped forms can occur but spirals up to 5.0 μm in length predominate. Coccal transformation occurs after prolonged incubation.

C. cinaedi and *C. fennelliae* grow more slowly on primary isolation than other campylobacters; up to 6 days of incubation may be required before colonies are visible. On subculture, after 48 h of incubation, colonies are pinpoint and translucent on blood agar. After additional incubation the colonies appear nonhemolytic, gray to grayish white, translucent, flat, regular, watery, and are less than 1 mm in diameter. Colonies of *C. fennelliae* are whiter and less watery than those of *C. cinaedi* and have a distinctive odor similar to that of bleach. Colonies of both species swarm readily on moist agar, producing a thin translucent film. Growth occurs at 37°C but not at 25 or 42°C. Incubation in a microaerobic environment containing hydrogen enhances growth but growth does not occur either aerobically or anaerobically (Fennell et al., 1984).

In addition to the difference in the odor of the colonies produced on solid media, *C. fennelliae* is nitrate negative. Both *C. fennelliae* and *C. cinaedi* are catalase positive, tolerate 1% glycine and 0.04% TTC, but neither organism tolerates 3.5% NaCl nor produces H_2S in TSI agar. Neither organism hydrolyzes hippurate or urea nor do they grow in the presence of 1% bile. They are susceptible to both nalidixic acid and cephalothin.

C. mucosalis

C. mucosalis is a short, slender rod 0.25 to 0.35 μm in diameter and 1.0 to 2.8 μm long. Coccoid forms and filamentous rods up to 8.0 μm long may be found in older cultures.

Colonies of *C. mucosalis* are 1.5 mm in diameter, round, raised with a flat surface, and are grayish yellow in color. The yellow pigment is observed best when colonies are spread on white paper. The colonies tend to spread on moist agar. *C. mucosalis* will grow between 25 and 42°C.

C. mucosalis are catalase negative, produce H_2S in TSI agar, reduce nitrate, and grow in the presence of both 1% bile and 1% glycine. They do not hydrolyze hippurate or urea, and do not grow in the presence of 1.5% NaCl or 0.04% TTC. While all strains are susceptible to cephalothin some strains have been noted to be resistant to nalidixic acid. Some strains reduce nitrite and tolerate glycine, but these results may vary depending on the basal medium used (Roop et al., 1985b). Growth at 25°C distinguishes this group of organisms from the other catalase-negative campylobacters. The requirement for either hydrogen or formate when the organism is grown under microaerobic conditions is a trait shared only with *C. concisus* and distinguishes them from *C. sputorum*. Anaerobic growth can be achieved in the presence of fumarate and either formate or hydrogen. The cellular fatty acid profile of *C. mucosalis* contains large amounts of lauric acid, a fatty acid which is not found in other campylobacters (Moss et al., 1984).

C. sputorum

Cells of the three biovars of *C. sputorum* are morphologically similar. Cells may be comma-shaped, gull-winged or straight rods, and some filamentous forms up to 8.0 μm in length may be found, primarily in older cultures. These organisms do not exhibit the tightly coiled spiral cells typical of *C. jejuni*. Young cultures may show some darting motility but older cultures are often nonmotile (Roop et al., 1985a).

Colonies of all three *C. sputorum* biovars are 1 to 2.5 mm in diameter, are shiny, round, convex, smooth, gray, and may have an entire or irregular spreading edge. On blood agar some strains exhibit slight alpha hemolysis. These organisms do not swarm on moist agar. The preferred growth temperature is 37°C, although most strains will grow at 42°C and no growth is observed at 25°C. Unlike *C. concisus* and *C. mucosalis,* hydrogen and formate are not required for microaerobic growth although fumarate alone will support anaerobic growth.

C. sputorum biovar *sputorum* is catalase negative, reduces nitrate, produces H_2S in TSI agar, tolerates 1% glycine, and grows in the presence of 1% bile. Most strains grow on 1.5% NaCl but not 3.5% NaCl. They hydrolyze neither hippurate nor urea. Most strains are resistant to nalidixic acid but susceptible to cephalothin.

C. sputorum biovar *bubulus* is catalase negative and *C. sputorum* biovar *faecalis* is catalase positive; this is the only major phenotypic difference between these two biovars. They both reduce nitrate, produce H_2S in TSI agar, grow in 1.5% and 3.5% NaCl, tolerate 1% glycine, and grow anaerobically in the presence of 0.1% TMAO. They do not hydrolyze hippurate or urea and, unlike *C. sputorum* biovar *sputorum,* they do not grow in the presence of 1% bile. Most strains are resistant to nalidixic acid but susceptible to cephalothin.

C. concisus

C. concisus cells are slender, curved, straight, or helical rods 0.5–1.0 μm in diameter and 4.0 μm long.

Colonies of *C. concisus* are 1.0 mm in diameter, convex, translucent, and entire. *C. concisus* will grow at 37 or 42°C but not at 25°C. Either hydrogen or formate is required to sustain growth when incubated microaerobically, while fumarate, in addition to formate or hydrogen, is necessary for growth under anaerobic conditions. No aerobic growth is observed.

C. concisus is catalase negative, produces H_2S in TSI agar, reduces both nitrate and nitrite, and tolerates 1% glycine and 1% bile but not 3.5% NaCl. Neither hippurate nor urea are hydrolyzed and 0.1% TMAO does not support anaerobic growth. Strains are resistant to both cephalothin and nalidixic acid (Tanner et al., 1981; Roop et al., 1985a).

C. cryaerophila

The cells of *C. cryaerophila* are curved rods with loosely coiled spirals that resemble *C. fetus* subsp. *venerealis,* although cells up to 20 μm may be observed.

After 48–72 h of incubation at 30°C, colonies are less than 1 mm in diameter, nonpigmented, smooth, convex, and entire. On subculture, colonies may become flattened and irregular. For primary isolation, organisms require microaerobic conditions and incubation at 30°C. However, on subculture, growth can occur aerobically, microaerobically, or anaerobically. Aerobic growth in this species is unique among campylobacters. Although 30°C is the optimal growth temperature, all strains will grow at 15°C and some may grow in temperatures as low as 5°C or as high as 40°C (Neill et al., 1985).

C. cryaerophila is catalase positive and reduces nitrate. Most strains can reduce nitrite as well, although this is not a universal feature. They tolerate 2.0% NaC1, but not 1.0% glycine or 0.1% TTC. They cannot hydrolyze hippurate or urea nor do they produce H_2S in TSI agar. They do not grow anaerobically in 0.1% TMAO (Neill et al., 1985). These organisms are resistant to cephalothin but susceptible to nalidixic acid.

C. nitrofigilis

C. nitrofigilis, the nitrogen-fixing campylobacter, is a slender, curved rod similar in morphology to *C. jejuni*. In young cultures, the cells are typically short, curved rods, while coccoid forms predominate in older cultures. However, the transition to coccoid forms is slow and apparently is not due to exposure to oxygen, as occurs with *C. jejuni, C. coli,* and *C. laridis* (McClung et al., 1983).

After 72 h of incubation at room temperature, colonies are tan to gray, glistening, low convex, entire, and measure 1 mm in diameter. They are nonhemolytic on blood agar.

The optimum growth temperature for these organisms is 30°C with a range from 10–37°C. *C. nitrofigilis* requires a minimum concentration of 0.5% NaCl for growth in artificial media. The optimum salt concentration is between 1 and 4%, but concentrations as high as 7% can be tolerated. Anaerobic growth of *C. nitrofigilis* can be achieved with the addition of either fumarate or aspartate to the culture medium, but

not with the addition of nitrate. There is no growth under aerobic conditions.

All strains of *C. nitrofigilis* produce catalase, grow in the presence of 3.5% NaCl and reduce nitrate to nitrite. Many isolates can split urea. H_2S formation is not observed in TSI agar. In addition, these organisms are unable to hydrolyze hippurate, do not grow in the presence of 1% glycine or 0.1% TTC, but do, however, have a nitrogenase enzyme and produce pigment from tryptophan (McClung et al., 1983). Isolates of *C. nitrofigilis* are susceptible to both cephalothin and nalidixic acid.

H. pylori and H. mustelae

The cells of *H. pylori* are 0.5–1.0 μm wide and 2.5–5.0 μm in length and are curved rods. On subculture, horseshoe-shapes and gull-wings can be seen. Motility is darting and rapid due to multiple, sheathed flagella with terminal bulbs occurring at only one end of the cell (Fig. 2). The organisms grow best at 37°C under microaerobic conditions. Anaerobic growth does not occur. Rare strains can grow at 42°C, but no growth is observed at 25°C (Fox et al., 1989).

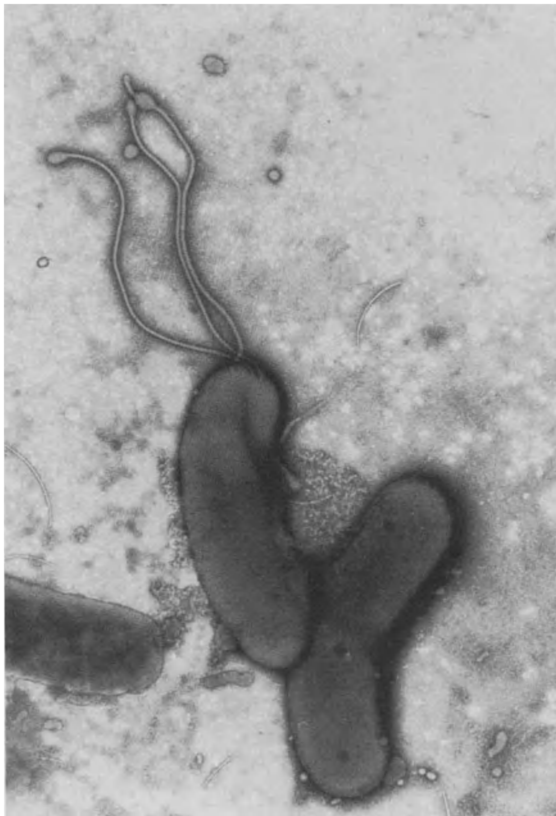

Fig. 2. Electron micrograph of *Helicobacter pylori*. Magnification 17,000 ×. Diameter of cells is approximately 0.5 μm. (Courtesy of Alan Curry and Dennis M. Jones.)

The organism requires a humid atmosphere for growth on agar. Addition of serum to liquid and solid media enhances growth. (Buck et al., 1987).

Colonies appear after 2–7 days of incubation and are 1–2 mm in diameter, opaque, convex, entire, and nonpigmented. Coccoid forms appear after exposure to room air.

H. pylori is catalase positive and strongly urease positive. It does not hydrolyze hippurate nor does it produce H_2S in TSI agar. There is no growth in the presence of 1% glycine or 3% NaCl, nitrate is not reduced, and hippurate is not hydrolyzed. Strains are susceptible to cephalothin but variable in their resistance to nalidixic acid (Dent and McNulty, 1988; Fox et al., 1989). Their strong urease activity differentiates them from members of the genus *Campylobacter* (Hazell et al., 1987). Although some strains of *C. nitrofigilis* demonstrate urease activity, it is not of the same potency as that of *H. pylori*.

H. mustelae is similar in microscopic and colonial appearance. However, several differences in biochemical and susceptibility profiles have been noted. *H. mustelae* is capable of growth at 42°C and does reduce nitrates. It has lateral as well as polar flagella, and is resistant to cephalothin but susceptible to nalidixic acid. It also has strong urease activity (Fox et al., 1989).

Diagnostic Methods

Microscopic Methods of Analysis

The motility of *Campylobacter* species is best observed by suspending the organisms in broth and examining the cells by phase contrast or darkfield microscopy. The wavelength and amplitude of the spiral forms can be measured from photographs of organisms taken under phase contrast microscopy (Karmali et al., 1981). To demonstrate a change from curved rods to coccal forms, organisms are incubated microaerobically for 48 h at 37 or 42°C, then incubated aerobically at room temperature for an additional 48 h. A Gram stain is prepared at this time and examined for a predominance of coccal forms (Karmali et al., 1981). Use of 0.1% aqueous basic fuchsin as the counterstain enhances the detection of campylobacters in Gram stains.

Biochemical Methods

A variety of methods have been used to determine the biochemical and growth characteristics of *Campylobacter* and *Helicobacter* species. Listed here are the methods that we feel are

most reproducible and that can be applied to the majority of these organisms.

Detection of cytochrome oxidase is made by rubbing a colony onto filter paper moistened with a freshly prepared solution of 0.5% tetramethyl-*p*-phenylenediamine dihydrochloride; development of a purple color within 10 s is positive. Catalase production is detected by suspending the organism in a drop of water on a glass slide and adding a drop of 3.0% hydrogen peroxide to the suspension. A more sensitive test for catalase is to add several drops of 3.0% hydrogen peroxide to cells grown on an agar slant containing a medium without blood, such as Brucella agar or TSI agar. In either case, rapid formation of bubbles is considered a positive reaction while delayed appearance of a few bubbles is a weakly positive reaction.

Biochemical tests should be performed on colonies that are no more than 48-h old (72 h for slowly growing organisms). For detection of glucose utilization, H_2S production, urease activity, nitrate reduction, and hippurate hydrolysis, media should be inoculated directly with growth from an agar plate. However, direct inoculation of colonies into tests for tolerance to various substrates may yield falsely positive results. To standardize the inoculum size for these tests, organisms should be suspended in Brucella or Mueller Hinton broth to the density of a 1.0 MacFarland standard. Several drops of this suspension are added to the test medium (Gebhart et al., 1985). Alternately, several drops from the growth at the top of semi-solid Brucella broth can be used (Roop et al., 1984). All tests should be incubated until a positive reaction or significant growth is observed, or up to seven days if the test is negative.

Various basal media have been used for biochemical and tolerance tests. Brucella albimi broth with 0.16% agar (Roop et al., 1984) or fluid thioglycolate medium with 0.1% agar (Gebhart et al., 1983) are reliable and easily prepared. To obtain adequate growth of fastidious campylobacters, such as *C. cinaedi* and *C. fennelliae,* the agar concentration of the semi-solid medium should be increased to 0.3% (Fennell et al., 1984). Use of semi-solid medium permits aerobic incubation and enhances the ability to detect growth by localizing the organisms below the surface of the medium. Other tests may be performed on solid media using bases such as Brucella agar, Mueller Hinton agar, or Yeast Extract Nutrient agar composed of Oxoid BA base No. 2, and 0.1% yeast extract (Benjamin et al., 1983). When performing any tolerance test, a tube or plate containing only basal media must be included as a positive control.

Production of acid from glucose is determined in a semi-solid medium containing 1% glucose and 0.002% phenol red, pH 7.3. Tubes are tightly capped and incubated aerobically. Appearance of a yellow color is a positive result and an indication that the organism is not a campylobacter, since campylobacters neither ferment nor oxidize sugars.

Nitrate reduction is determined in a semi-solid medium supplemented with 0.2% potassium nitrate. When visible growth is present, equal volumes (10 drops) of nitrate reagent A (8 g of sulfanilic acid in 1 liter of 5 N acetic acid) and reagent B (6 ml of *N, N*-dimethyl-l-naphthylamine in 1 liter of 5 N acetic acid) are added. Development of a red color indicates presence of nitrate reductase. If no color develops, zinc dust is added to the medium. Development of a red color after the addition of zinc confirms that nitrate is still present in the medium and that the organism cannot reduce nitrate. Absence of a red color after the addition of zinc indicates that nitrate was reduced to nitrite which was then reduced to nitrogen gas, indicating that the organism has both nitrate and nitrite reductases.

Nitrite reduction is detected in a semi-solid medium containing 0.05% potassium nitrite. When there is visible growth, reagents A and B are added to the medium as for the nitrate test. Absence of a red color is positive for nitrite reduction, while development of a red color is a negative test result.

Production of hydrogen sulfide (H_2S) may be measured by several methods including a TSI agar slant, suspending a strip of lead acetate over a semi-solid medium containing 0.02% cysteine hydrochloride (Vêron and Chatelain 1973), or using a medium containing ferrous sulfate, sodium metabisulfite, and sodium pyruvate as described by Skirrow and Benjamin (1980a). Although the TSI method is not the most sensitive method, we feel that it is the most reproducible of the tests and is the one we recommend. Blackening of the medium is a positive reaction. The medium should be fresh with water of syneresis present at the junction of the butt and slant.

Urease production is detected on Christensen's base urea agar prepared as a slant. Development of a pink color is indicative of a positive test.

To determine glycine tolerance, a semi-solid medium is supplemented with 1.0% glycine. A similar procedure is used for bile tolerance except that the medium is supplemented with oxgall to a final concentration of 1.0%. For salt tolerance, NaCl is added to a semi-solid medium to a final concentration of 3.5%. Tolerance

of 1.5% NaCl can be determined by adding salt to yeast extract nutrient agar plates.

Tolerance of 2,3,5 TTC (Leaper and Owen, 1981; Luechtefeld and Wang, 1982) is determined by incorporation of TTC into Brucella or Mueller Hinton agar to a final concentration of either 0.04%. 0.1%, or 1.0%. If organisms can grow in the presence of TTC, they will reduce the colorless dye to red tetrazolium salts. A standard concentration of TTC has not been established.

The ability of an organism to grow anaerobically in the presence of 0.1% TMAO is determined by a modification of the method described by Benjamin et al. (1983), in which the TMAO is prepared in yeast-extract nutrient broth as listed below.

TMAO Test Broth

Bacto-peptone	1%
Bacto-tryptone	1%
Yeast extract	0.1%
Trimethylamine-*N*-oxide	0.1%
NaCl	0.5%
Agar	0.2%

The medium, made in distilled water, is placed into screw-capped tubes and autoclaved just prior to use. After cooling, the medium is inoculated with a suspension of organisms and the tube is tightly capped. Incubation is at 37°C for 7 days. Growth occurring at the bottom of the tube, which is oxygen free, is indicative of anaerobic growth, a positive test result, while growth only at the surface is microaerobic growth and is a negative test result. (Roop et al., 1984).

Anaerobic growth in the presence of fumarate is assessed by inoculating a suspension of organisms onto Brucella agar supplemented with 0.3% ammonium fumarate. In a similar fashion, 0.2% sodium formate can be added to Brucella agar to determine the ability of this supplement to act as an electron donor. In each case, growth must be compared to that achieved on unsupplemented Brucella agar (Roop et al., 1985a).

Determining the ability of an isolate to grow at 25 and 42°C may be tested in a semi-solid medium or on agar plates. Temperatures must be strictly controlled for this test to be of value. Some investigators recommend 43°C rather than 42°C to identify truly thermophilic organisms. A control test incubated at 37°C, using the same medium and same amount of inoculum, should always be included for comparison.

The rapid method of Hwang and Ederer (1975) is used to detect the ability of strains to hydrolyze hippurate. Cells from an agar plate culture are emulsified in 0.4 ml of 1% aqueous sodium hippurate. After 2 h of incubation at 37°C, 0.2 ml of ninhydrin reagent (3.5% ninhydrin solution in a 1:1 mixture of acetone and butanol) is added and the test is incubated for an additional 10 min. Development of a deep purple color in the ninhydrin layer is a positive reaction. Positive and negative controls must be run simultaneously.

Molecular Methods for Differentiating Campylobacters

Several techniques using either nucleic acid hybridization methods or polyacrylamide gel electrophoresis of proteins have been applied to the identification of *Campylobacter* species isolated in pure culture.

Totten et al. (1985) used whole cell chromosomal DNA extracts from a series of campylobacter isolates in DNA-DNA hybridization reactions to differentiate among strains of *C. cinaedi* and *C. fennelliae* as well as to distinguish hippurate-negative *C. jejuni* isolates from *C. coli* and *C. laridis* (Totten et al., 1987). This spot hybridization method was relatively simple to perform and proved to be very effective for identifying these organisms.

More recently, Romaniuk and Trust (1987) and Moureau et al. (1989) have used restriction-fragment-length-polymorphism analysis of the genes encoding rRNA in campylobacter to differentiate among isolates of various species. Moureau et al. (1989) were able to accurately discriminate among isolates of *C. jejuni, C. coli, C. laridis, C. fetus,* and *"C. upsaliensis"* using a 23-base probe. Similarly, Chevrier et al. (1989), Ezaki et al. (1988), and Ng L.-K. et al. (1987) used several DNA probes to differentiate among species of *Campylobacter,* while Korolik et al. (1988) and Picken et al. (1987) developed probes that were specific for *C. jejuni.*

Hanna et al. (1983) were able to distinguish aerotolerant species of campylobacter from other *Campylobacter* species using polyacrylamide gel electrophoresis patterns. Similarly, Ferguson and Lambe (1984) were able to discriminate between species of campylobacters using soluble protein extracts of campylobacters examined on polyacrylamide gels. Tanner (1986) also used protein profiles to distinguish *C. concisus* from other gingival organisms.

Strain Typing Methods for Campylobacters

Differentiating among strains of campylobacters has been accomplished by several techniques including biotyping (Bolton et al., 1984; Hébert et al., 1982; Lior, 1984; Skirrow and Benjamin, 1980b); serotyping (Lior et al., 1982; Penner and Hennessy, 1980; Hébert et al., 1983); bacteriophage typing (Grajewski et al., 1985); auxotyping (Tenover et al., 1985a; Ten-

over and Patton, 1987); plasmid analysis (Bopp et al., 1985; Bradbury et al., 1983; Tenover et al., 1984); and restriction endonuclease analysis of chromosomal DNA (Bradbury et al., 1984; Collins and Ross, 1984; Kakoyiannis et al., 1984; Owen et. al., 1985).

Antimicrobial Susceptibility Testing in Campylobacter Species

There are several methods available for determining the antimicrobial susceptibility of *Campylobacter* species. The most common methods employed are disk diffusion and agar dilution methods. Of these, the agar dilution test is most accurate and should be considered the method of choice. However, disk diffusion testing often is used in clinical laboratories to determine an organism's susceptibility to cephalothin and nalidixic acid, two markers commonly used in identification schemes. A review of the literature shows that more than 12 combinations of temperature, atmosphere, and length of incubation have been used in an attempt to accurately predict the susceptibility of campylobacters to various agents, yet, the optimum conditions remain unclear. We recommend testing at 37°C for 48 h in a microaerobic environment achieved either through the use of evacuation/replacement, gas-generating envelopes in anaerobe jars, or biobags with microaerobic gas generators. Candle jars are to be avoided since they do not produce optimum microaerobic conditions.

Agar Dilution Method

Mueller-Hinton agar or Brucella agar produce equivalent results when used for susceptibility testing of campylobacters in our laboratory. Other investigators have noted similar results (Wang et al., 1984). The inoculum should be adjusted to deliver 10^6 colony-forming units to the plate. This can be achieved by making a suspension of organisms in saline equivalent to a 1.0 McFarland standard, and diluting the suspension 1:20 in saline before inoculating the agar medium. Plates should be examined at 48 h for all thermophilic campylobacters, although the more fastidious campylobacters may require additional incubation.

Disk Diffusion Method

The inoculum for disk diffusion testing is prepared as for agar dilution. An attempt should be made to read zone sizes after 24 h of incubation. The major disadvantage to this method is the lack of interpretive criteria for zone diameters. Van Hoof et al. (1984) have stated that the National Committee on Clinical Laboratory Standards can be followed for most antibiotics, yet we have found a relatively poor correlation between MICs and zone sizes, particularly for erythromycin.

One suggestion is to use zone/no zone interpretations for susceptibility and resistance. This is particularly helpful for interpreting cephalothin and nalidixic acid zones for the purpose of species identification. For other antimicrobials, particularly those to be used therapeutically, agar dilution testing is recommended if facilities are available.

DNA Probes

DNA probes have been used to detect both tetracycline and kanamycin-resistance genes in *Campylobacter* species (Lambert et al., 1985; Tenover and Elvrum, 1988; Courvalin and Taylor, 1988). Probes, however, only indicate the presence of a resistance determinant or portions of a determinant but do not indicate the phenotype of the organism. Thus, non-functional resistance genes may produce positive hybridization test results that would be interpreted to mean that the organism was resistant to an antimicrobial agent when, in fact, it was susceptible. Probes have primarily been used for epidemiological studies of antimicrobial resistance in campylobacters.

Mechanisms of Antimicrobial Resistance in Campylobacters

A review of the mechanisms of antimicrobial resistance in the thermophilic campylobacters has been presented by Taylor and Courvalin (1988). Of note are the presence in several species of campylobacter of two resistance determinants that probably originated in Gram-positive organisms. The first is the *aphA*-3 kanamycin resistance determinant, first reported in a strain of *C. coli* by Lambert et al. (1985), and sequenced by Trieu-Cuot et al. (1985), and the second is the *tet*O gene, whose DNA sequence was first reported by Sougakoff et al. (1987). Both genes can be carried on transmissible plasmids (Kotarski et al., 1986; Sagara et al., 1987; Taylor et al., 1981; Tenover et al., 1983; Tenover et al., 1985b), as can a new kanamycin resistance gene that may be endemic to *Campylobacter* species, the *aphA*-7 gene (Tenover et al., 1989). Resistance to quinolones, β-lactams, chloramphenicol, macrolide-lincosamide-streptogramin (MLS)-type drugs, streptomycin, spectinomycin, and other aminoglycosides have all been reported in campylobacters (Taylor and Courvalin, 1988).

Isolation

Collection and Transport of Fecal Samples

A fresh fecal sample that is rapidly transported to the laboratory is the optimum specimen for the isolation of enteric campylobacters from animals or humans. Fecal samples that cannot be processed quickly, and all rectal swab specimens, should be transported to the laboratory in alkaline peptone water, pH 8.5, containing 0.05% sodium thioglycolate and 0.025% L-cystine, or, in modified Cary-Blair medium with the agar concentration reduced to 1.6% (Wang et al., 1983). Transport media should be kept cold after inoculation because campylobacters survive longer at 4°C than at ambient temperature (Blaser et al., 1980a).

Collection and Transport of Other Specimens from Animals

Tissue samples or animal carcasses for culture should be kept moist and cool and transported to the laboratory immediately for culture. Air should be excluded as much as possible (Lander and Gill, 1985). For small tissue samples, a transport medium, such as modified Cary-Blair, or cold physiologic saline is recommended.

The lavage and aspiration method of Lander (1983) is recommended for the recovery of campylobacters from the vaginal mucus of cows. *C. fetus* subsp. *venerealis* is best recovered from bulls by collecting material from the prepuce and preputial sac by lavage. Alternately, material can be collected from the mucosal folds and placed in Clark's transport medium (Clark and Dufty, 1978) for transport to the laboratory.

Clark's Transport and Enrichment Medium (Clark and Dufty, 1978)

Fresh bovine serum to which is added

5-Fluorouracil	300 μg/ml
Polymyxin B	100 units/ml
Brilliant green	50 μg/ml
Nalidixic acid	3 μg/ml
Cycloheximide	100 μg/ml

Ten milliliter aliquots of serum containing antibiotics and inhibitors are placed in 30-ml bottles, closed with rubber stoppers, and placed in a boiling water bath for 2 min or until the serum solidifies. The medium is cooled and dispersed with a glass rod. After introducing a microaerobic gas mixture into the bottles (2.5% O_2, 10% CO_2, and 87.5% N_2), they are stored at 4°C for 1 week, after which time they are available for use for up to 4 weeks. Samples containing as few as 100 organisms remain viable in this transport media for up to 48 h. Once in the laboratory, the material can be filtered and applied to either selective or non-selective media, however, the media should not contain cephalothin or polymyxin B (Jones et al., 1985). Incubation of media should be continued for 5 days at 37C and examined at regular intervals.

Filtering Methods

The first clinical samples of campylobacter were recovered using a 0.65-μm filter to remove large particles of stool (Dekeyser et al., 1972). However, the more common application of filters for the recovery of these organisms takes advantage of the highly motile nature of most campylobacters and their slender width which allows them to pass through 0.45–0.65 μm filters leaving other bacterial flora behind. When used in this manner, nonselective media can be substituted for agar plates containing antimicrobic combinations that may prove inhibitory to certain strains of campylobacter (Steele and McDermott, 1984). Steel et al. (1985) isolated a wide diversity of *Campylobacter* species from humans using this method. It should be noted, however, that low concentrations of campylobacters may be missed if the filter technique is used exclusively for stool samples (Goossens et al., 1986).

To perform the method described by Steele and McDermott (1984), fecal samples are diluted 1:10 in sterile saline containing glass beads. 10–12 drops of the suspension are placed on a 47 mm 0.45-μm filter resting on a 6% sheep blood agar plate that had been dried for 30 min. Liquid should not spread over the edge of the filter. The filter is removed and discarded after 10–30 min. and the plate is incubated under microaerobic conditions. The authors note that cellulose triacetate filters worked best in their study.

Alternate filtering techniques using 0.65-μm Sartorius filters or attaching the filters to a Swinney adapter fitted to a syringe, have been used successfully for isolation of campylobacters from animal sources (Gebhart et al., 1983; Lander and Gill, 1985). After passing the material through the filter apparatus, the filtrate is placed on selective or nonselective media. Spreading the filtered inoculum across the medium to obtain isolated colonies will facilitate recognition of multiple species of *Campylobacter*.

Selective Isolation Media

Among the 12 selective media described for the isolation of campylobacters, no single medium is satisfactory for the recovery of all *Campylobacter* species. We will describe five media formulations, each of which offers certain advantages for the isolation of these organisms. The

investigator must decide which species are of interest and then choose the appropriate combination of media. We strongly recommend the use of a selective medium, plus filtration onto a nonselective medium, for maximum recovery of campylobacter isolates from samples.

Several antimicrobial agents have proven troublesome when used for isolation of campylobacters. Cephalothin, which is used in selective media at concentrations of 32 ug/ml, is inhibitory to *C. fetus* subsp. *fetus, C. fetus* subsp. *venerealis, C. jejuni* subsp. *doylei, "C. upsaliensis." C. hyointestinalis, C. cinaedi, C. fennelliae, C. mucosalis,* all subspecies of *C. sputorum, C. nitrofigilis,* and *H. pylori.* Also, strains of *C. coli* that are susceptible to cephalothin have been recovered from cattle (Brooks et al., 1986). Cefoperazone in concentrations of 15–32 μg/ml and cefazolin at concentrations of 15 μg/ml also may inhibit the majority of these organisms. Polymyxin B and colistin are inhibitory to some strains of *C. coli* (Ng et al., 1985), *"C. upsaliensis"* (Steele et al., 1985), and *C. fetus* subsp. *venerealis* (Jones et al., 1985). Rifampin inhibits the growth of *C. cinaedi* and *C. fennelliae* (Flores et al., 1985).

Skirrow's medium was one of the first to be described. It is widely used and permits the recovery of cephalothin-susceptible campylobacters. However, overgrowth by fecal flora is frequently a problem with this medium.

Skirrow's Medium (Skirrow, 1977)

Blood agar base No. 2, to which is added

Lysed horse blood	5%
Vancomycin	10 μg/ml
Trimethoprim	5 μg/ml
Polymyxin B	2.5 IU/ml

The agar is autoclaved, cooled to 50°C, and the blood and antimicrobials added aseptically. The medium is distributed in petri plates.

The medium described by Blaser et al. (1979a) and sold commercially as Campy BAP (BBL, Cockeysville, MD) is similar to that of Skirrow except that defibrinated sheep blood is used and cephalothin is incorporated to more effectively inhibit fecal flora.

Modified Campy BAP (Blaser et al., 1979a)

Brucella agar base, to which is added

Defibrinated whole sheep blood	10%
Cephalothin	32 μg/ml
Polymyxin B	2.5 IU/ml
Trimethoprim	5 μg/ml
Vancomycin	10 μg/ml
Amphotericin B	2 μg/ml

The agar is autoclaved and cooled to 50°C. Blood and antibiotic supplements are added aseptically and the medium is poured into petri plates.

Goossens et al. (1986) described a modification of Butzler's medium Virion (Goossens et al., 1983) that omits colistin and increases the concentration of cefoperazone to 30 μg/ml. This medium provides enhanced inhibition of human fecal flora.

Modified Butzler's Medium Virion (Goossens et al., 1986)

Columbia Blood agar base (Oxoid), to which is added

Defibrinated sheep blood	5–7%
Cefoperazone	30 μg/ml
Rifampicin	10 μg/ml
Amphotericin B	2 μg/ml

The agar is autoclaved and cooled to 50°C. The antibiotic supplements and the sheep blood are added aseptically and the medium poured into petri plates.

An antimicrobial formulation similar to that of modified Butzler's medium Virion that incorporates activated charcoal into the medium in place of blood was described by Karmali et al, (1986). Charcoal was first recommended as an additive to campylobacter selective media by Bolton and Coates (1983). Charcoal effectively enhances the growth of campylobacters when added to enriched media and in some parts of the world, may be less expensive and easier to obtain than sterile animal blood.

Blood-free Medium for Isolating Campylobacters from Feces (Karmali et al., 1986)

Columbia agar base, to which is added

Activated charcoal	4 mg/ml
Hematin	0.032 mg/ml
Sodium pyruvate	0.1 mg/ml
Cefoperazone	32 μg/ml
Vancomycin	20 μg/ml
Cycloheximide	100 μg/ml

The agar and charcoal are autoclaved and cooled to 50°C. The remaining ingredients are added aseptically and the medium is dispensed into petri plates.

Finally, a novel semi-solid medium that takes advantage of the motility of campylobacters to separate them from other organisms present in stool has been described (Goossens et al., 1989). The medium is inexpensive to prepare and the cultures are easy to interpret.

Semi-solid Medium (Goossens et al., 1989)

Mueller Hinton broth, to which is added

Agar	0.4%
Cefoperazone	30 μg/ml
Trimethoprim	50 μg/ml

The broth and agar are combined, autoclaved and cooled to 50°C. The antimicrobials are added aseptically to the medium and poured into 5-cm plates and stored in the dark until used. To inoculate, a loopful of stool is placed at the periphery of the plate. Solid stools are first emulsified in saline. Plates are then incubated in a microaerobic environment. Campylobacters are recognized on the medium by characteristic swarming away from the point of inoculation.

Recovery of *C. hyointestinalis* and *C. mucosalis* from Swine

Gebhart et al. (1983) noted that combination of a filter technique and a selective medium containing trimethoprim-sulfamethoxazole was important for eliminating contamination with *Proteus* species when sampling intestinal tissues of swine with proliferative ileitis. While the 0.65-μm filter resulted in little contamination, it also decreased the number of colonies present on the medium by 40%. On the other hand, trimethoprim-sulfamethoxazole inhibited several of the *C. mucosalis* strains. Thus, a combination of 0.8-μm filters and a selective medium and 0.65-μm filters with a nonselective medium yielded the largest numbers of isolates.

Recovery of *H. pylori* and *H. mustelae*

Biopsies of the antral portion of the stomach or other areas demonstrating gastritis should be sampled via endoscopy and the tissue transported to the laboratory immediately in sterile saline at room temperature. The specimen should be inoculated onto fresh chocolate agar, Skirrow's medium modified by the addition of amphotericin B (5 μg/ml) and substitution of cefsulodin (5 μg/ml) for polymyxin B (Dent et al., 1988), or GC medium base containing either 1.0% soluble starch or 0.2% charcoal (Buck et al., 1987). Since *H. pylori* requires an extremely humid environment for growth on agar, paper towels saturated with sterile water should be placed in the jar with the media. Incubation is at 37°C under microaerobic conditions for up to 7 days.

Recovery of *C. cryaerophila* and *C. nitrofigilis*

Methods for the recovery of these organisms can be found in Neill et al. (1980) and McClung and Patriquin (1980), respectively. Because of the intricacies of the isolation techniques, they are not detailed here.

Enrichment Broths

In general, fecal samples from humans and animals do not require an enrichment broth step if the samples are processed rapidly by the laboratory (Chan et al., 1985; Bolton and Robertson, 1982). However, specimens that have been delayed in transit and specimens that may contain low numbers of organisms, such as those from patients receiving antimicrobial therapy, may benefit from enrichment (Martin et al., 1983). Environmental, milk, and food samples are usually enriched before plating due to the small number of organisms present (Heiseck, 1985). Enrichment is carried out for 24–72 h at 42°C for isolation of thermophilic campylobacters. Two commonly used enrichment broths are described below. Polymyxin B may be omitted from either one for greater recovery of nonthermophilic campylobacters. Most enrichment broths have not been evaluated for recovery of campylobacters other than *C. jejuni* subsp. *jejuni.*

Doyle and Roman Enrichment Broth (Doyle and Roman, 1982)

Brucella broth containing:

Lysed horse blood	7%
Sodium succinate	0.3%
Cysteine hydrochloride	0.01%
Vancomycin	15 μg/ml
Trimethoprim	5 μg/ml
Polymyxin B	20 IU/ml
Cycloheximide	50 μg/ml

Lovetts Enrichment Broth (Lovett et al., 1983)

Brucella broth containing:

Ferrous sulfate	0.025%
Sodium metabisulfate	0.025%
Sodium pyruvate	0.025%
Vancomycin	15 μg/ml
Trimethoprim	7.5 μg/ml
Polymyxin B	5.0 IU/ml

Recovery of *Campylobacter* Species from Milk

To recover campylobacters from milk using the method of Lovett et al. (1983), centrifuge 40 g of raw milk at 34,000 × g for 20 min and discard fat and aqueous layers. Suspend the pellet in 100 ml of Lovett's enrichment broth. Incubate in a microaerobic environment for 24 h at 42°C and filter through 0.65-μm filter. Culture the filter and the filtrate on Skirrow's agar.

Recovery of Campylobacters from Water Samples

For low-turbidity water, Blaser and Cody (1986) recommend filtering 500 cc of water through a 0.45-μm filter and placing the filter face down on a selective medium. For high-turbidity water, the sample should be pre-filtered through

5.0 and 0.6-μm membranes before passing the sample through the 0.45-μm filter. After 6 h of incubation at 42°C, the filter is removed from the plate and a loop is used to streak the inoculum area for isolation of colonies. The plate is then incubated for an additional 24–48 h. It should be noted that as many as 90% of the campylobacters could be retained by the 0.6-μm filter. Thus, low-turbidity waters should not be pre-filtered. An alternate method of processing water samples for recovery of campylobacters has been described by El-Sherbeeny et al. (1985).

Incubation Temperature

The incubation temperature chosen for culture media will have a significant impact on the recovery of campylobacters in the laboratory. With the exception of *C. cryaerophila,* all *Campylobacter* species will grow well on primary isolation at 37°C. Cultures for recovery of *C. cryaerophila* should be incubated at 30°C. However, 42°C is the optimal incubation temperature for the recovery of the thermophilic species *C. jejuni* subsp. *jejuni, C. coli,* and *C. laridis.* If a filter technique is used, the plates should be incubated at 37°C.

Providing Microaerobic Growth Conditions

There are several ways to achieve microaerobic conditions optimal for the propagation of campylobacters. The first, and most expensive, is to use an incubator with an appropriate gas mixture supplied by replaceable tanks. While optimal, this is an option that few clinical laboratories can afford.

For the clinical laboratory, the most convenient option is an anaerobe jar containing a commercially prepared gas-generating envelope. One of two types of gas-generating envelopes can be used. The first is an envelope designed to produce a microaerobic atmosphere of O_2, CO_2, and N_2, and the second is a gas-generating envelope designed to produce an anaerobic atmosphere, which, in the absence of the palladium catalyst causes the hydrogen content of the jar to increase. Increased hydrogen is important for the growth of *C. concisus* and *C. mucosalis* and also enhances the growth of several other *Campylobacter* species. It should be noted, however, that the latter option is discouraged by the manufacturers of the anaerobic gas-generating envelopes since the accumulation of hydrogen within the jar is potentially explosive (Simmons, 1977).

Commercially prepared pouches or plastic bags that contain a small gas generator capable of providing a microaerobic environment for one to two plates are also available and popular in clinical laboratories. These pouches are an effective and convenient means of growing campylobacters, particularly if the laboratory receives relatively few requests to isolate these organisms or has inadequate incubator space to accomodate jars.

Another method of providing a microaerobic atmosphere is evacuation/replacement of the gas in anaerobe jars with a mixture of 10% CO_2, 10% H_2, and 80% N_2. After two cycles of evacuation/replacement, a microaerobic environment, enriched with hydrogen, will be achieved (Gebhart et al., 1983). While this is the most cost-effective method, it is also the most tedious.

A simple and inexpensive method of providing microaerobic conditions uses the Fortner principle in which an agar plate streaked with a rapidly growing facultative anaerobe is included in a sealable pouch with the agar plates used to isolate campylobacters (Karmali and Fleming, 1979). The growth of the facultative organism reduces the oxygen content in the atmosphere surrounding the plates permitting the growth of any campylobacters present.

The final method is use of a candle jar, which provides an atmosphere of approximately 17% O_2 (Luechtefeld et al., 1982). However, we do not recommend the use of the candle jar for primary isolation of campylobacters unless no other system is available since it is less effective than the other methods mentioned for the primary isolation of campylobacters (Luechtefeld et al., 1982). However, it should be noted that the aerotolerance of many campylobacter strains can be increased by adding ferrous sulfate, ferrous metabisulfite, and sodium pyruvate to the isolation medium (George et al., 1978).

Storage and Transport Medium

Sending pure cultures of campylobacter isolates between laboratories often has proven to be a challenge. However, the semi-solid Brucella agar medium described by Wang et al. (1980) has proven effective for transporting a variety of *Campylobacter* species even over periods of up to 3 weeks. The recipe is listed below.

Wang's Transport Medium

Brucella broth, to which is added

Difco agar	0.5%
Defibrinated sheep blood	10.0%

Autoclave the basal medium plus the agar, cool to 50°C, and add the sheep blood. Dispense the medium in 0.5-ml aliquots into small vials. Inoculate the medium with several colonies of a pure culture of campylobacter and incubate the vials with the caps very loose for 24 h at

37°C in a microaerobic atmosphere. After incubation, close the caps and wrap with parafilm before shipping. Campylobacters can be frozen at −70°C for extended periods of time in a 50:50 mixture of trypticase soybroth and heat inactivated fetal calf or horse serum.

Genetic Methods of Detecting Campylobacters

DNA probes consisting of nick-translated whole cell chromosomal DNA specific for *C. jejuni* have been described, but they have not proven useful for detecting this organism directly in clinical samples (Echeverria et al., 1985; Tompkins and Krajden, 1986).

Romaniuk and Trust (1989) have developed oligonucleotide probes directed against rRNA targets that detect either *C. jejuni* and *C. laridis,* or these organisms plus *C. coli.* However, the limits of detection for these probes even when used for culture confirmation was 10^6 colony-forming units. Other DNA probes for the culture confirmation of *Campylobacter* species have been described (Chevrier et al., 1989; Ezaki et al., 1988; Korolik et al., 1988; Picken et al., 1987; Taylor et al., 1989).

Other Diagnostic Methods

Direct microscopic detection of campylobacters using darkfield or phase microscopy (Paisley et al., 1982), as well as immunofluorescent methods (Hodge et al., 1986; Price et al., 1984), have been described in the literature. However, the relatively low sensitivity of these methods have precluded their widespread usage in laboratories.

Serologic tests and immunofluorescent antibody tests are not recommended for the detection of most campylobacter infections in humans or animals. Although serology has been used to monitor herd immunity, tests for *C. fetus* subsp. *venerealis* in individual animals as a diagnostic tool is not recommended (Garcia et al., 1983). The one exception to this rule may be detection of antibodies to *H. pylori* in humans, where serologic tests may well be the current definitive test method. (Perez-Perez et al., 1988).

Finally, a ^{13}C-urea breath test has been described for detection of *H. pylori* in humans. In this test, the patient ingests a solution containing ^{13}C-labeled urea. The release of ^{13}C-labeled CO_2 in the patient's breath, as measured by mass spectrometry, indicates the hydrolysis of the labeled urea by the potent urease of *H. pylori.* While not widely used, the test appears to have merit (Graham et al., 1987).

Applications

Survival

Most studies on the survival of campylobacters in the environment have been undertaken with isolates of *C. jejuni.* These studies have demonstrated that these organisms survive from weeks to months at cold temperatures (4°C) but decrease in numbers relatively quickly at 25°C and even faster at 37°C (Blaser et al., 1980a). *C. jejuni* survived for 3 weeks in feces and milk held at 4°C, 4 weeks in water, and up to 5 weeks in urine. *C. jejuni* also survived in solutions with a pH as low as 3.6. Thus, campylobacters introduced into the environment by humans or animals may serve as a source of infection for weeks to months (Blaser et al., 1980b).

Transmission of campylobacters in water, raw milk, and meat have been reported (Blaser et al., 1979b, 1980b). With regards to control of such transmission cycles, Blaser et al. (1986b) have shown that campylobacters are killed by usual concentrations of chlorine in treated water, while Waterman (1982) has demonstrated that campylobacters are killed by pasteurization methods. In addition, Doyle and Roman have shown that *C. jejuni,* when heated in red meat, has a D value (time for 90% killing) at 60°C of less than 20 s and thus, is readily susceptible to inactivation by heat.

In contrast to its long survival in liquids and feces at low temperatures, *C. jejuni* is susceptible to drying and undergoes significant reduction in numbers when dried. Survival can be enhanced, however, at relative humidities of $<$ 14% at 4°C (Doyle and Roman, 1982). *C. jejuni* has a pH optimum of 6.5 to 7.5, although it will grow over a range of pH 5.5 to 8.0 (Blaser et al., 1980a; Doyle and Roman, 1982). Under stressful conditions, *C. jejuni* assumes a coccoid shape and can remain viable although often nonculturable in aquatic environments (Rollins and Colwell, 1986).

Vaccines

Vaccination remains a key factor in the control of campylobacteriosis in cattle (Bryner et al., 1979, 1988; Clark, 1971; Vasquez et al., 1983). Unvaccinated herds are subject to outbreaks of infertility due to *C. fetus* subsp. *venerealis* causing major economic loss (Bryner et al., 1979, 1988). The importance of booster immunizations has been emphasized for cattle (Berg and Firehammer, 1978). There are at least 10 vaccines currently licensed in the United States for immunizing cattle against *Campylobacter fetus* subsp. *venerealis* (Bryner et al., 1979). Of the

eight that have been evaluated in an animal model, usually the pregnant guinea pig, three resulted in good to very good protection while the remaining five gave minimal to no protection. There are, to date, no vaccines or candidate vaccines suitable for use in humans to protect them against any form of campylobacteriosis.

Methods of Genetic Manipulation of Campylobacters

Campylobacters have not been found to undergo DNA mediated transformation. However, both a shuttle vector (Labigne-Roussel et al., 1987) and a method for introduction of DNA by electroporation (Miller et al., 1988) have been described. Although early studies described the use of transducing bacteriophage for transmitting genetic information between strains of *C. fetus* (Chang and Ogg, 1971), such techniques are not currently used.

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The Genus *Wolinella*

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Introduction

The genus *Wolinella* was proposed (as was the genus *Campylobacter*) for certain species originally classified in the genus *Vibrio* and was placed in the family Bacteroidaceae (Krieg and Holt, 1984). *Wolinella* was named for Dr. Meyer Wolin, an American bacteriologist who first described a microorganism (*Vibrio succinogenes*) with a metabolism that characterizes this genus. Bacteria of the genus *Wolinella* are Gram-negative rods that may be straight, curved, or helical. They are motile by means of a single polar flagellum. They are anaerobic and can utilize hydrogen gas or formate as electron donor with fumarate or nitrate as electron acceptor. Under these conditions, fumarate can also serve as a sole carbon source (Bronder et al., 1982). *Wolinella* species do not oxidize or ferment carbohydrates, but peptides stimulate growth (Gillespie and Holt, 1987). The GC content of their DNA ranges from 42 to 48 mol% whereas the GC content of *Campylobacter* ranges from 28 to 38 mol%.

Except for their GC content, bacteria of the genus *Wolinella* are physiologically, morphologically, and phylogenetically similar to *Campylobacter* species. The phylogeny of *Wolinella* and related genera was examined by analyzing 16S rRNA sequences (Paster and Dewhirst, 1988). These studies demonstrated that the three *Wolinella* species fall into two distinct, but related, subgroups (Fig. 1). One subgroup contained *W. succinogenes, Helicobacter pylori, H. mustelae,* and *Flexispira rappini.* The other subgroup comprised a tight cluster consisting of *Wolinella recta, W. curva,* most species of *Campylobacter,* and two formate-and fumarate-requiring ("misclassified") *Bacteroides, B. gracilis* and *B. ureolyticus* (Fig. 1). *Wolinella recta* and *W. curva* were more closely related to the *Campylobacter* cluster at an average homology of 95% than to *W. succinogenes,* the type species of *Wolinella* (average homology level of 88%). Thus, despite the large differences in GC content of the DNA, it was suggested that *W. recta* and *W. curva* should be considered as members of the genus *Campylobacter* (Paster and Dewhirst, 1988). However, results obtained from other experimental methods will most likely be necessary to resolve the taxonomic status of *Wolinella.*

The overall phylogenetic position of *Wolinella* relative to other Gram-negative bacteria determined from 16S rRNA sequencing (Fig. 2) indicated that the genus *Wolinella* does not belong in the family Bacteroidaceae, to which it is currently assigned. Based on certain unique oligonucleotide signatures in the 16S rRNA sequences, it has been suggested that *Wolinella* is distantly related to members of the proteobacteria group, such as *Escherichia coli* and *Proteus vulgaris* (Stackebrandt et al., 1987; Lau et al., 1987). It is also possible that *Wolinella* and related bacteria are sufficiently different to form a separate phylogenetic grouping or phylum (Romaniuk et al., 1987).

The type strain of *W. succinogenes* is ATCC 29543^T (FDC 602W): *W. recta* ATCC 33238^T (FDC 371): and *W. curva* ATCC 35224^T (VPI 9584). (FDC = Forsyth Dental Center, Boston, MA, USA; VPI = Virginia Polytechnic Institute, Blacksburg, VA, USA.)

Habitats

Bacteria of the genus *Wolinella* do not appear to be free living and have been cultured from the bovine rumen and from human clinical sites.

Wolinella succinogenes

W. succinogenes was originally isolated from bovine rumen fluid (Wolin et al., 1961). One strain (IB4) was isolated from sewage by Yoshinari (Tanner et al., 1984). Nonoral, human clinical isolates that were originally classified as species of *W. succinogenes* are most likely strains of *W. curva,* based upon DNA-DNA ho-

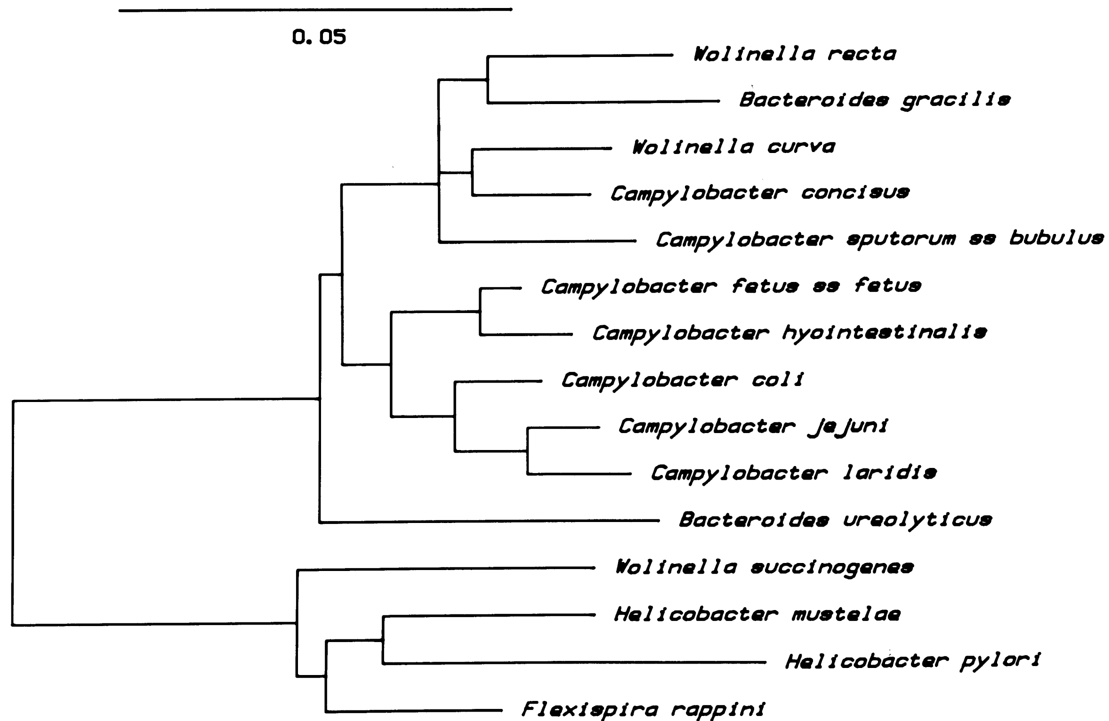


Fig. 1. Phylogenetic tree for *Wolinella* and its closest relatives. Data are based on nearly complete rRNA sequences (B. J. Paster and F. E. Dewhirst, unpublished observations). The scale bar represents the difference in nucleotide sequence determined by taking the sum of all branch lengths in connecting two species. Vertical distance has no meaning.

mologies and serological reactions (Tanner et al., 1984).

Wolinella recta

The primary ecological niche for *W. recta* appears to be subgingival sites (below the gum margin) of the human oral cavity. Isolates described as "vibrio-corroders," dry spreaders, *Campylobacter (Vibrio) sputorum, V. succinogenes, or Bacteroides corrodens* from oral and clinical sites (Smibert and Holdeman, 1976), as well as many of the small motile rods seen on darkfield examination of dental plaque samples, probably are *W. recta. W. recta* has also been isolated from an actinomycotic chest wall mass (Speigel and Telford, 1984).

W. recta may be pathogenic for humans. *W. recta* has been associated (as an anaerobic vibrio) with deep periodontal pockets (Tanner et al., 1979), with gingival (gum) inflammation (Van Palenstein Helderman and Rosman, 1976; Moore et al., 1987), with actively progressing periodontal diseases (Dzink et al., 1988), and with intractable (progressing despite treatment) periodontal lesions (Haffajee et al., 1988). *W. recta* was also isolated from painful human dental root canal (endodontic) lesions (Sundqvist, 1976; Tanner et al., 1984).

Wolinella curva

W. curva has been isolated from various human clinical sites, including subgingival sites, dental root canal (endodontic) lesions, an alveolar abscess, and septicemia (Tanner et al., 1984; Moore et al., 1987). The pathogenicity of this species is unknown as very few strains have been isolated.

Other *Wolinella* species

Additional unnamed strains of *Wolinella* have been isolated from human subgingival sites (Moore et al., 1987). Strains of *Wolinella*-like bacteria were found to colonize periodontal pockets of patients with inflammatory bowel disease. It was suggested that these strains may play a role in the pathogenesis of the disease (Van Dyke et al., 1986). Isolates resembling *Wolinella* but which decomposed hydrogen peroxide were isolated from subgingival sites by Takamori et al. (1982).

Isolation

Isolation of *W. succinogenes*

W. succinogenes was first isolated by enrichment culture from the bovine rumen and clas-

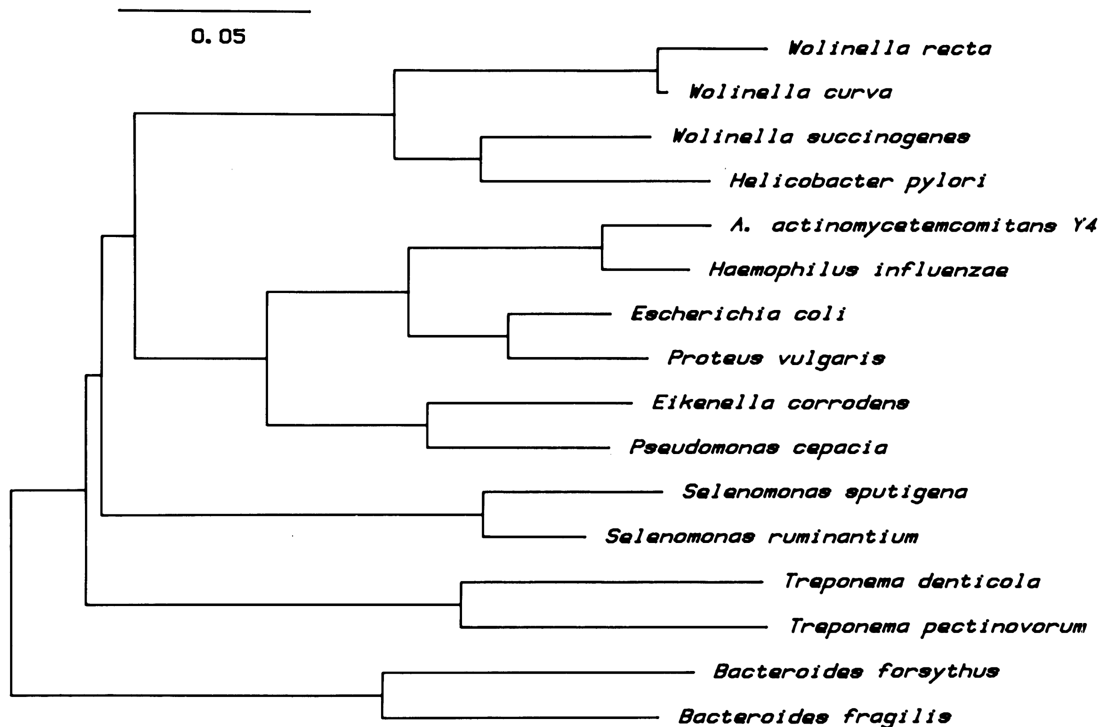


Fig. 2. Phylogenetic tree for species of *Wolinella* demonstrating their overall relationship to other Gram-negative bacteria. The scale bar represents the difference in nucleotide sequence as determined by taking the sum of all branch lengths connecting two species. The genus designation for *A. actinomycetemcomitans* is *Actinobacillus;* Y4 indicates strain FDC Y4 of *A. actinomycetemcomitans* serogroup B.

sified as *Vibrio succinogenes* (Wolin et al., 1961). An inoculum of bovine rumen fluid was serially transferred in an anaerobic methanogenic medium containing formate, sulfide, and inorganic salts. A highly motile vibrio was observed, and subsequently isolated by enrichment in a broth medium containing formate and fumarate. Wolin et al. (1961) recommended using a similar procedure to that first used to isolate additional strains.

Isolation of *W. recta, W. curva* and Other (Unnamed) *Wolinella* species

W. recta and *W. curva* were isolated from human clinical sites when anaerobic techniques were used for sample handling and culture, and specimens were inoculated and grown on enriched media that contained blood, or formate and fumarate (Tanner et al., 1979; Moore et al., 1987; Dzink et al., 1988; Haffajee et al., 1988). An example of isolation procedures used for human subgingival oral sites is as follows. Samples may be removed with a curette or scaler, and placed in a prereduced anaerobically sterilized (PRAS) salt solution. Samples should be dispersed under anaerobic conditions by vortexing, with or without glass beads, or by short periods of sonication at low power levels. Prolonged, powerful sonication techniques disrupt *Wolinella* cells (Olsen and Socransky, 1981).

Dispersed samples should be diluted under anaerobic conditions, and then plated on agar media containing formate and fumarate, with or without sheep blood. Several different basal media including trypticase soy and mycoplasma broth base (BBL) allow isolation of *Wolinella* species. The recipe for one medium (Tanner, 1987) that supports good growth of *Wolinella* species is shown below.

Medium for Isolation and Agar Cultivation of *Wolinella* Species

Brain heart infusion agar	52.0 g
Yeast extract	10.0 g
Sodium formate	2.0 g
Fumaric acid or sodium fumarate	3.0 g
Hemin stock solution (see below)	10.0 ml
Distilled water	950.0 ml
Defibrinated sheep blood	40.0 ml

Hemin Stock Solution:

Hemin	0.05 g
Potassium phosphate dibasic	1.74 g
Distilled water	100.0 ml

Boil the ingredients in a flask to dissolve the hemin. Store at room temperature and protect from light.

Combine the medium components with the exception of the sheep blood. Adjust the pH to 7.0 and sterilize by autoclaving. When the liquid has cooled, add the blood, and pour plates.

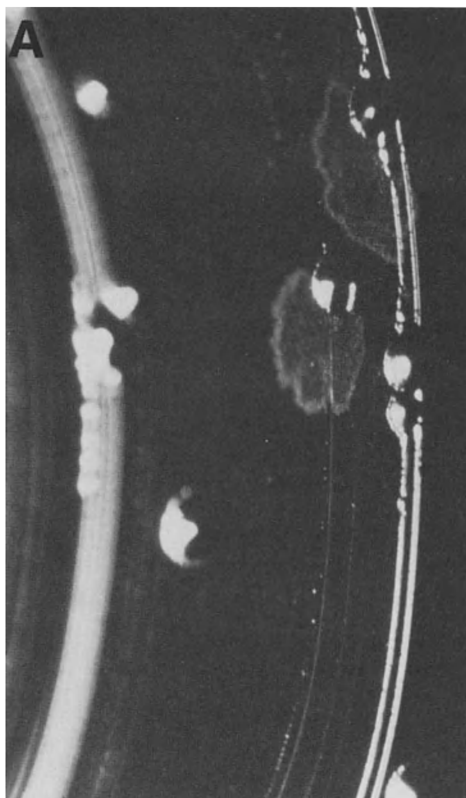
Optimal recovery of oral anaerobic and facultatively anaerobic species is achieved under an anaerobic atmosphere containing hydrogen, for example, 10% H_2, 10% CO_2, 80% N_2.

Wolinella species may be recognized by their colonial morphology (Fig. 3), which varies depending on medium composition. On certain blood agar plates, colonies may spread over the agar surface (dry spreaders), be highly translucent, and have the appearance of droplets of water (Fig. 3A). There may be small convex colonies in addition to the spreading-pitting variant (Fig. 3A). On other media, the same species may pit or corrode the agar surface (Fig. 3B). On media supplemented with formate and fumarate, colonies are larger, convex, and frequently have yellow or pink speckles. No hemolysis has been observed on blood agar.

The following selective medium for *W. recta* and *Bacteroides gracilis* (Hammond and Malonee, 1988) is a modification of the previous medium, replacing blood with sources of sulfate. *W. recta* and *B. gracilis* produce excessive hydrogen sulfide and form small, black colonies on primary isolation. Upon subculture, however, colonies may not turn black on this agar medium.

Selective (Agar) Medium for Isolation of *Wolinella recta* and *Bacteroides gracilis*

Brain heart infusion agar	52.0 g
Sodium formate	2.0 g
Fumaric acid or sodium fumarate	3.0 g
Yeast extract	10.0 g
Sodium thiosulfate	0.3 g
Ferrous sulfate	0.2 g
Hemin stock solution (see below)	10.0 ml
Distilled water	980.0 ml
Menadione stock solution (see below)	10.0 ml
Vancomycin stock solution (see below)	1.0 ml



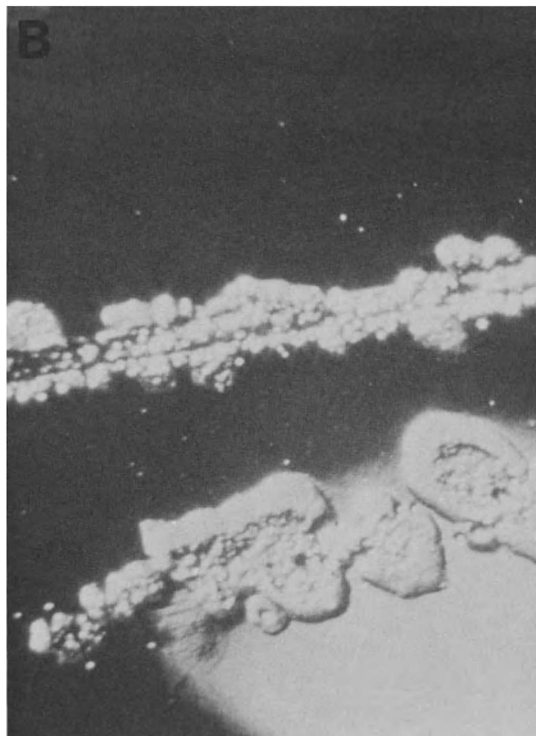


Fig. 3. Colonial morphology of *Wolinella recta*. (A) Agar-spreading (dry-spreading, water drop) colonies and small convex colonial variants of *W. recta*. On blood agar plates unsupplemented with formate and fumarate, the small convex variant is usually 1 mm in diameter, whereas the spreading variant may be up to 5 mm in diameter. (B) Agar-corroding colonies of *W. recta*. This colonial variant is produced from the agar-spreading variant if cells are plated on certain media, including many "home-made" media, as compared to commercially prepared media. Agar-corroding colonies are usually less than 5 mm in diameter.

Hemin stock solution, see recipe for previous agar medium.

Menadione stock solution (100 ml):

Menadione	0.005 g
Ethanol	1.0 g
Distilled water	99.0 ml

Dissolve the menadione in ethanol. Add the water and filter sterilize. Aliquots may be frozen.

Vancomycin stock solution (5 mg/l):

Vancomycin	5.0 mg
Distilled water	4.0 ml

Dissolve the vancomycin in the water in a test tube. Filter sterilize the solution. Aliquots may be frozen.

Combine the ingredients above except the menadione and vancomycin solutions. Adjust the pH to 7.0 and sterilize by autoclaving. When the medium has cooled, add the filter-sterilized menadione and vancomycin solutions, and pour the plates.

Broth Cultivation of *Wolinella* Species

Several broth media have been described for culturing *Wolinella succinogenes*. Wolin et al. (1961) used a medium containing: $(NH_4)_2SO_4$, 0.1%; K_2HPO_4, 0.5%; fumaric acid, 0.3%; sodium formate, 0.3%; yeast extract (Difco), 0.1%; $MgCl_2 \cdot 6H_2O$, 0.02%; and $FeSO_4$, 0.001%; pH 7.0 to 7.2. An improved medium was described by Kafkewitz (1975) consisting of 0.4% yeast extract, 100 mM ammonium formate, 120 mM sodium fumarate, and 0.05% sodium thioglycollate, pH 7.3. When freshly prepared, this broth medium, incubated aerobically, supported growth of several reference strains of *Wolinella,* including representatives of the three named species (Tanner et al., 1984).

In an attempt to improve growth of oral agar-corroding species and species stimulated by formate and fumarate, optical densities of cells of *Wolinella* species was compared following culture in multiple media bases in different combinations with different organic acids and reducing agents (Tanner, 1987). These studies were performed mainly in wells of microtiter plates. Excellent growth of *W. succinogenes* was achieved in yeast extract, 10–15 g/liter; sodium formate, 2 g/liter; sodium fumarate, 3 g/liter; and sodium thioglycollate, 0.5 g/liter. In these studies, the increased concentrations of yeast extract compared to that of the media described by Wolin et al. (1961) and by Kafkewitz (1975) resulted in higher yields of cells. While this medium supported growth of *W. recta* and *W. curva,* cell yields were never as high as with *W. succinogenes.* Maximal growth of *W. recta* and *W. curva* was achieved by the addition of yeast extract at 5 g/liter to complex media including brain heart infusion (BBL), Todd-Hewitt broth (BBL), or mycoplasma broth base (BBL). *W. curva* was stimulated by the inclusion of L-cysteine, 0.5 g/liter, and sodium bicarbonate, 1 g/liter, whereas *W. recta* appeared to prefer sodium thioglycollate, 0.5 g/liter, as a reducing agent.

Gillespie and Holt (1987) studied the growth in broth of the type strain of *W. recta.* Their studies indicated that ammonium formate could substitute for sodium formate and that hemin was not required for growth of *W. recta.* Maximal growth of *W. recta* was achieved in mycoplasma broth base supplemented with 25 mM formate and 75 mM fumarate.

Preservation of Cultures

Wolinella species may be preserved by lyophilization using a dense suspension of cells in broth containing 5% serum and 1% glucose. Cells may also be stored frozen either in liquid nitrogen, or in ultra-low-temperature freezers (−80°C) by suspending young colonies in a broth containing 5% dimethyl sulfoxide prior to slow freezing and storage.

Identification

Wolinella species have been difficult to differentiate from each other and historically were all grouped together as *Vibrio succinogenes* or *Bacteroides corrodens.* However, accurate species identification has become quite feasible using protein profiles from polyacrylamide gel electrophoresis, indirect immunofluoresence, or hybridization with species-specific DNA probes.

Morphology

All species of *Wolinella* are nonsporeforming, Gram-negative rods that are motile by means of a single polar flagellum. Cells are 0.4 μm in diameter and 0.9–4.0 μm long, and each species appears to have a different predominant cell shape with rounded or tapered ends (Table 1; Figs. 4 and 5). *W. succinogenes* cells from a log phase broth culture may appear helical or curved. *W. curva* cells are frequently curved, whereas *W. recta* cells are predominantly straight. Cells of *W. recta* possess a distinctive cell wall surface layer (S-layer) characterized by a hexagonal arrangement of subunits that are in close contact with the outer membrane (Fig. 5; Lai et al., 1981; Kerosuo et al., 1989). S-layers present on archaebacteria and other eubacteria are composed of crystalline protein or glycoprotein (Sleytr and Messner, 1983). Colonial

Table 1. Characteristics that differentiate *Wolinella* species, *Bacteroides gracilis,* and *Campylobacter concisus.*

Characteristic	*Wolinella succinogenes*	*W. curva*	*W. recta*	*Bacteroides gracilis*	*Campylobacter concisus*
Number of strains	2	4	10	7	6
Source of isolation	Bovine rumen	Human clinical	Human clinical	Human clinical	Human clinical
Dominant cell shape	Helical	Curved	Straight[a]	Straight	Curved
SDS-page—double yellow bands (20–25 KDa)	−	−	+	−	−
Growth on agar containing:					
NaF (0.5 g/liter)	+	−	−	+	+
Oxgall (10 g/liter)	+	+	−	V	+
Deoxycholate (1 g/liter)	+	+	−	+	+
Janus green (0.1 g/liter)	+	+	−	+	+
Basic fuchsin (0.032 g/liter)	+	+	−	+	+
Crystal violet (0.005 g/liter)	+	−	−	V	+
Indulin scarlet (0.5 g/liter)	−	+	−	−	−
Penicillin (4–16 μg/ml)	+	+	−	V	V
Polymixin B (2–4 μg/ml)	+	+	−	−	−

+, positive feature; −, negative feature; V, variable feature.
[a] *W. recta* demonstrates an unusual ultrastructure, having hexagonal units in the cell wall.
Data from Tanner et al., 1981, 1984; Tanner, 1986.

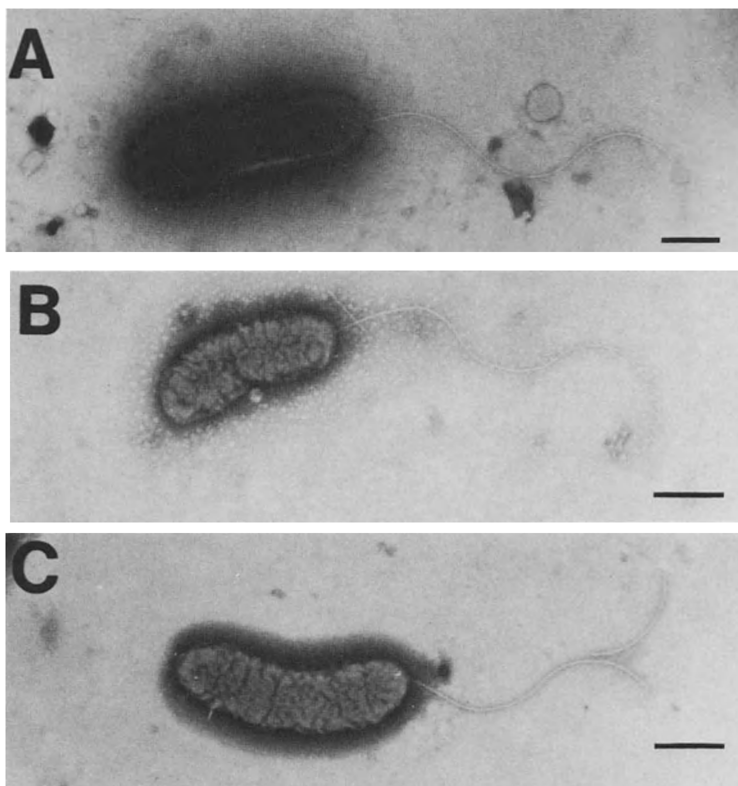


Fig. 4. Electron photomicrographs of negatively stained *Wolinella* cells. (A) *W. recta,* strain FDC 285: a straight cell with a single polar flagellum. (B) *W. curva,* strain VPI 10296: a short curved rod with a typical Gram-negative cell wall surface and a single polar flagellum. Some cells may have bipolar flagella. (C) *W. succinogenes,* strain ATCC 29543 (FDC 602W): a short curved rod with a typical Gram-negative cell wall surface and a single polar flagellum. Bars = 0.5 μm.

morphology (Fig. 3) for *Wolinella* species was described under isolation procedures.

Biochemical Characteristics

Characteristics common to *Wolinella* species are listed in Table 2. *Wolinella* strains grow at 37°C, demonstrate oxidase activity, and produce hydrogen sulfide, but are asaccharolytic and are negative in many routine biochemical tests including enzyme substrate tests (Table 2). Because of their reducing metabolism, *Wolinella* species all reduce benzyl viologen (−421 mV). Growth in broth media of *W. recta* is accompanied by an increase in pH to a maximum of 8.0 (Gillespie and Holt, 1987). While strains

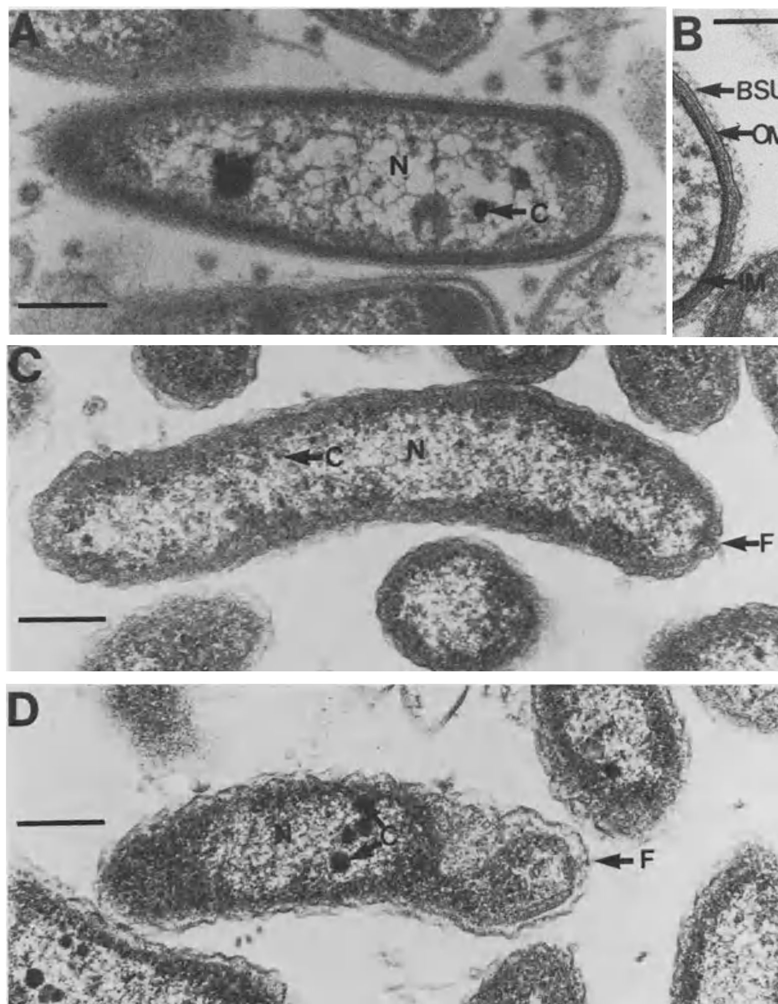


Fig. 5. Transmission electron photomicrographs of *Wolinella* species. (A) *W. recta*. The cell periphery has a well-defined inner membrane, lacks a distinct peptidoglycan layer, and has bullet-shaped subunits covering the outer membrane. Within the cell there is a nuclear region (N) and small electron-dense cytoplasmic inclusions (C). Bar = 0.2 μm. (B) *W. recta*. Section of cell wall illustrating its ultrastructure. The inner membrane (IM), the outer membrane (OM), and the regularly arranged bullet-shaped subunits (BSU) at the cell surface are visible. Bar = 0.1 μm. (C) *W. curva:* a curved-to-helical rod showing the flagellar insertion (F), a nuclear region (N), and small electron-dense cytoplasmic inclusions (C). The ultrastructure of *W. curva* is similar to that of *Campylobacter concisus*. Bar = 0.2 μm. (D) *W. succinogenes:* a curved rod showing the flagellar insertion (F), nuclear region (N), and small electron-dense cytoplasmic inclusions (C). Bar = 0.2 μm.

do not grow on agar surfaces incubated in air, they may grow in an atmosphere containing 5% oxygen (microaerophilic). Characteristics that differ among *Wolinella* species, *Bacteroides gracilis,* and *Campylobacter concisus* are listed in Table 1. *Wolinella recta* is considerably more sensitive to many inhibitory agents, including antibiotics, than is *W. succinogenes, W. curva, B. gracilis,* or *C. concisus.*

Species Differentiation

Determination of *Wolinella* species requires differentiation from other nonfermentative species that either corrode agar or demonstrate a formate- and fumarate-utilizing metabolism (Table 3). *Eikenella corrodens* may be distinguished by its growth on agar surfaces in air with 10% carbon dioxide; growth in the presence of 4 μg/ml clindamycin; lack of active cell motility; lack of growth in media supplemented with formate and fumarate without nitrate; and the presence of lysine and ornithine decarboxylases (Table 3). The other species listed in Table 3 differ physiologically from *E. corrodens.* The formate-and fumarate requiring species proved difficult to differentiate using the characteristics of source of isolation, presence or absence of motility, unreactivity of strains (e.g., of *W. recta* to inhibitors), cell morphology, and urease activity (Tables 1 and 3; Tanner et al., 1987). However, *Wolinella* species can be differentiated using serology, polyacrylamide gel electrophoresis, and DNA probes.

SEROLOGY. All *Wolinella* species are serologically distinct based on agglutination tests (Badger and Tanner, 1981), ELISA (Tanner et al., 1984), and indirect immunofluoresence (Werner-Felmayer et al., 1988; Lai et al., 1989). Different serotypes have been described for *W. recta* using polyclonal antibodies (Badger and Tanner, 1981) and indirect immunofluoresence utilizing different monoclonal antibodies to *W. recta* (Werner-Felmayer et al., 1988). Fluorescently labeled polyclonal antibodies have been

Table 2. Characteristics common to all *Wolinella* species.[a]

Common positive characteristics:	Gram-negative rods; actively motile by a single polar flagellum; 0.4 μm in diameter and 0.9–4.0 μm in length; growth in broth supplemented with formate and fumarate; growth in anaerobic and microaerophilic (5% O_2) atmospheres; reduction of nitrate, nitrite, neutral red, and benzyl viologen; oxidase and benzidine positive; production of hydrogen sulfide, hydrogen, carbon dioxide, and succinate; growth in the presence of Evans blue (0.05 g/liter), malachite green (0.02 mg/liter), brilliant green (0.0125 g/liter), and kanamycin (1, 2, or 4 μg/ml); arginine aminopeptidase positive in API An-Ident tests; GC content, 42–47 mol%.
Common negative characteristics:	No growth in unsupplemented broth media or in media supplemented with only 0.2% nitrate; no growth on agar surfaces in air, or in air containing 10% CO_2; no spores formed; not haemolytic; no reduction in pH when incubated in formate- and fumarate-supplemented media with 1% adonitol, 1% amygdalin, 1% arabinose, 1% dextran, 1% dulcitol, 1% fructose, 1% galactose, 1% glucose, 1% glycerol, 1% glycogen, 1% inositol, 1% inulin, 1% lactose, 1% maltose, 1% mannitol, 1% mannose, 1% melezitose, 1% melibiose, 1% raffinose, 1% rhamnose, 1% ribose, 1% salicin, 1% sorbitol, 1% sucrose, 1% trehalose, 1% xylose, 0.25% esculin, or 0.5% starch; catalase negative; lysine, ornithine, and arginine not decarboxylated; starch, dextran, esculin, casein, DNA, and gelatin not hydrolyzed; hydrogen peroxide, urease, lethicinase, lipase, indole, ammonia, and acetylmethylcarbinol not produced; nitrous oxide, methane, formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, caproate, lactate, and pyruvate not detected; gas not formed under an agar layer over a broth culture; no growth in the presence of NaCl (20 or 40 g/liter) or penicillin (64 μg/ml); negative or weak reactions in API ZYM and API An-Ident tests, except for arginine aminopeptidase.

[a]These characteristics are shared by *B. gracilis, C. concisus,* and *C. sputorum,* except: *B. gracilis* has no flagellum and is not actively motile; *Campylobacter* species have a lower GC content.
Data from Tanner et al., 1981, 1984, 1985.

Table 3. Differentiation of *Wolinella* species from other asaccharolytic agar-corroding, and formate- and fumarate-requiring species.

Characteristic	*Eikenella corrodens*	*Bacteroides ureolyticus*	*B. gracilis*	*Campylobacter sputorum*	*C. concisus*	*Wolinella* species
Growth in Air + 10% CO_2	+	–	–	–	–	–
Lysine and ornithine decarboxylase; growth in 4 μg/ml clindamycin	+	–	–	–	–	–
Cells motile by flagellum	–	–	–	+	+	+
Growth stimulated by nitrate (N), or formate and fumarate (FF)	N	FF	FF	FF	FF	FF
Urease	–	+	–	–	–	–
GC content, mol%	50–58	28	42–47	30–34	34–38	42–47

used to identify *W. recta* from dental plaque samples (Lai et al., 1989).

USE OF GEL ELECTROPHORESIS. Sonicated whole cell preparations of the formate and fumarate-utilizing species were run on SDS-polyacrylamide gels. Each species demonstrated a different protein profile when gels were stained with a conventional silver stain (Fig. 6). *W. recta* was most easily distinguished from these other species by the presence of yellow-stained bands (in contrast to typical black bands) at an approximate molecular weight of 25 kDa Tanner, 1986).

DNA PROBES. *W. recta* can be identified from pure cultures and in dental plaque samples using either whole genomic DNA probes (Gunaratnam et al., 1990) or short oligonucleotide probes (Dix et al., 1990; B. J. Paster and F. E. Dewhirst, unpublished observations). Whole genomic probes (Gunaratnam et al., 1990) were prepared and labeled with a nonradioactive label as described by Smith et al., (1989). Colonies of strains to be identified were "lifted" onto nylon filter membranes, and the DNA was extracted and fixed to the solid support. Positive hybridization reactions were visualized by a nonradioactive color reaction.

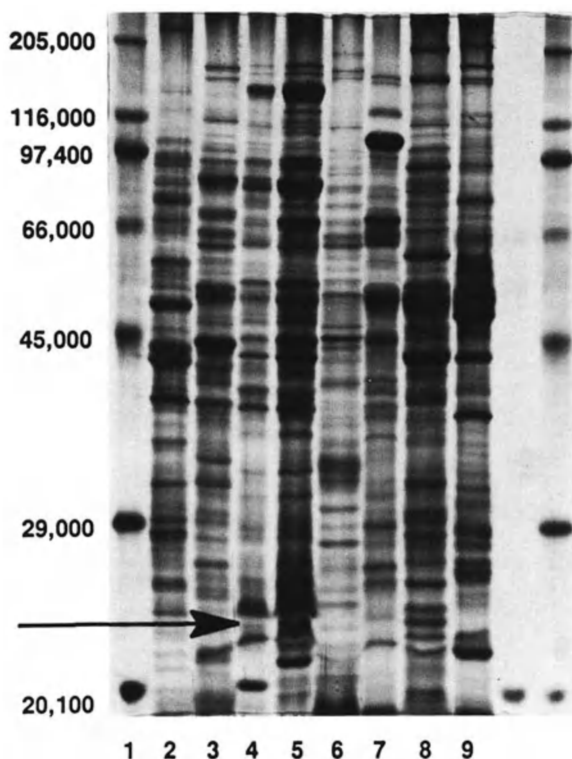


Fig. 6. Silver-stained SDS-polyacrylamide gel of sonicated whole cells of species with a formate and fumarate metabolism. The running and stacking gels contained 14% and 4% acrylamide respectively. The current was run at 15 mA per 0.75 mm thick gel. The arrow indicates the level of yellow-staining bands of *Wolinella recta* isolates in lanes 4 and 5. Lane 1, high-molecular-weight standard mixture; lane 2, *W. succinogenes* ATCC 29543ᵀ, lane 3, *W. curva* ATCC 35244ᵀ; lanes 4 and 5, *W. recta* strains ATCC 33238ᵀ and FDC 267; lane 6, *Campylobacter concisus* ATCC 33237ᵀ; lane 7, *C. sputorum* ATCC 35980ᵀ (VPI S-17); lane 8, *Bacteroides gracilis* ATCC 33236ᵀ; and lane 9, *B. ureolyticus* VPI 7815.

Short DNA oligonucleotide probes (20–24 bases) were designed by comparing 16S rRNA sequences to determine those regions of the molecule that were unique to any given species. Complementary DNA to that region was synthesized, and the probe was labeled with a radioisotope. Probes specific for the RNA of the three *Wolinella* species have been developed, but they have not yet been tested directly on clinical samples (B. Paster and F. Dewhirst, unpublished observations).

Physiological Properties

Wolinella species have generated the interest of several investigators because of their unusual, previously unrecognized, formate- and fumarate-requiring metabolism. Most of the physiological studies have been performed on strains of either *W. succinogenes* or *W. curva.*

Wolinella species can utilize formate or hydrogen gas as an energy source with formate being oxidized to CO_2. *W. succinogenes* can also use hydrogen sulfide as an energy source (Macy et al., 1986). The oxidation of the electron donors can be coupled with any of several electron acceptors, including fumarate and nitrate. Succinate is produced when cells are grown on fumarate, which can also serve as the sole carbon source (Bronder et al., 1982). Fumarate can be replaced by L-malate, L-aspartate, or L-asparagine for *W. succinogenes* and *W. curva* (Wolin et al., 1961; Kafkewitz, 1975).

Oxidation of formate or hydrogen may also be coupled with reduction of nitrate or nitrite, or for *W. succinogenes,* with nitrous oxide or sulfur (Wolin et al., 1961; Yoshinari, 1980; Schröder et al., 1988). Nitrate or nitrite is reduced to ammonia, but not to nitrogen gas (Bokranz et al., 1983). However, nitrous oxide can be reduced to nitrogen via nitrous oxide reductase (Teraguchi and Hollocher, 1989). Sulfur is reduced to hydrogen sulfide via sulfur reductase (Schröder et al., 1988). *W. recta* can grow with oxygen as the sole electron acceptor in continuous culture if the oxygen concentration is maintained below 2–5 μM (Ohta and Gottschal, 1988).

A growth requirement for succinate was described for *W. succinogenes* when nitrate replaced fumarate as the electron acceptor (Niederman and Wolin, 1972), but not nitrous oxide (Yoshinari, 1980). Added succinate was not needed in the presence of substances that could be converted to succinate, including malate, fumarate, and L-asparagine. The succinate requirement could not be replaced by oxaloacetate, pyruvate, bicarbonate, acetate, propionate, butyrate, L-glutamate, D-aspartate, or δ-aminolevulinic acid (Niederman and Wolin, 1972). L-aspartate and L-asparagine could substitute for fumarate, malate, and nitrate as electron acceptors when *W. succinogenes* was grown in the presence of formate.

The electron transport chain is comprised of formate dehydrogenase and fumarate reductase, which are linked by a menaquinone. Formate dehydrogenase was isolated from *W. succinogenes* as a dimer of two identical subunits of molecular weight equal to 110,000, containing a cytochrome *b* that mediated electron transfer to menaquinone (Kröger et al., 1979). Fumarate reductase was isolated in two forms, one of which contained a cytochrome *b* that mediated electron transfer from menaquinone to fumarate reductase (Unden et al., 1980). The reactive sites of formate dehydrogenase appear

to face the outside, while those of fumarate reductase face the inside of the cytoplasmic membrane (Kröger et al., 1980).

Two nitrite reductases (mol. wt: of 63,000) were characterized from *W. succinogenes,* one isolated from the cytoplasm and the other associated with the cytoplasmic membrane (Schröder et al., 1985). In these studies, it was suggested that a hemeC protein with a molecular weight of 22,000 was a form of cytochrome *C.* A cytochrome *C*-containing nitrous oxide reductase (a dimer of mol. wt. 162,000) was also isolated and purified from *W. succinogenes* (Teraguchi and Hollocher, 1989). Sulfur reductase, isolated as sulfur dehydrogenase, is involved in the sulfur respiration of *W. succinogenes* (Schröder et al., 1988). When growth is driven by the reduction of sulfur, a menaquinone is apparently not involved in the phosphorylative electron transport chain.

L-Asparaginase has been isolated from *W. succinogenes* (Kafkewitz and Goodman, 1974) and from strains currently recognized as *W. curva.* (Albanese and Kafkewitz, 1978; Abouchowski et al., 1979; Radcliffe et al., 1979). This enzyme inhibited growth of an in vitro culture of human pancreatic carcinoma cells (Wu et al., 1978) and was thus of interest for its potential antitumor activity.

Genetic information

The G C content of the DNA of *Wolinella* species ranges from 42–46 mol% (Tm). DNA-DNA hybridization studies demonstrated that the currently recognized species were distinct from each other (Tanner et al., 1981, 1984). The phylogeny of *Wolinella* species and related bacteria has been determined by 16S rRNA sequence analysis and was described in the introduction. Two structural genes coding for subunits of the enzyme fumarate reductase from *W. succinogenes* were cloned and expressed in *Escherichia coli* (Lauterbach et al., 1987). No genetic transfer among the species has been observed.

Acknowledgments

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CHAPTER 191

Free-Living Saccharolytic Spirochetes: The Genus *Spirochaeta*

ERCOLE CANALE-PAROLA

The genus *Spirochaeta* includes anaerobic and facultatively anaerobic spirochetes that are indigenous to aquatic environments such as the mud and water of ponds and marshes. These spirochetes occur in nature as free-living forms, that is, their existence does not depend on physical associations with other organisms (Canale-Parola, 1984b).

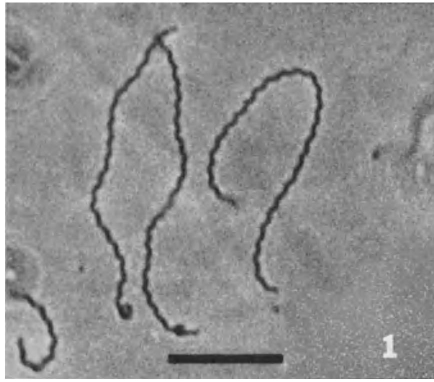
Spirochaeta cells are helically shaped (Figs. 1–12) and possess the typical ultrastructural features of spirochetes (Fig. 13) (Canale-Parola, 1984a). The outermost structure of the cells is an "outer membrane," or "outer sheath," which encloses the coiled cell body ("protoplasmic cylinder") consisting of the cytoplasm, the nuclear region, and the peptidoglycan-cytoplasmic membrane complex (Fig. 13). Organelles ultrastructurally similar to bacterial flagella are located in the area between the outer membrane and the protoplasmic cylinder (Fig. 13). These organelles are essential components of the motility apparatus of spirochetes (Paster and Canale-Parola, 1980) and are usually called "periplasmic flagella." Other names that have been used to designate these motility organelles are "periplasmic fibrils," "axial fibrils," "axial filaments," and "endoflagella." One end of each periplasmic flagellum is inserted near a pole of the protoplasmic cylinder, whereas the other end is not inserted (Fig. 13A). Individual periplasmic flagella extend for most of the length of *Spirochaeta* cells, so that those inserted near opposite ends overlap in the central region of the organism (Fig. 13A). In contrast to flagella of other bacteria, the periplasmic flagella of spirochetes are permanently wound around the cell body and are entirely endocellular (Fig. 13B). Thus, the motility mechanism of spirochetes is different from that of other bacteria, which have flagella that function in direct contact with the external environment and are not wound around the cell body.

With one exception, all the known *Spirochaeta* species (Table 1) have two periplasmic flagella per cell, one flagellum being inserted near each cell pole. The exception is the large spirochete *S. plicatilis* (Table 1), which has as many as 18–20 periplasmic flagella inserted near each end of the protoplasmic cylinder (Blakemore and Canale-Parola, 1973).

Seven species of *Spirochaeta* are presently known (Table 1; Figs. 1–12). One of these, *S. plicatilis,* has not been grown in pure culture, but its ultrastructure and some of its ecological characteristics have been described (Blakemore and Canale-Parola, 1973). Four species—*S. stenostrepta, S. litoralis, S. zuelzerae,* and *S. isovalerica*—are obligate anaerobes. Two other species—*S. aurantia* and *S. halophila*—are facultative anaerobes and characteristically produce carotenoid pigments when growing aerobically (see below).

S. stenostrepta, S. zuelzerae, and *S. aurantia* are freshwater species, whereas *S. litoralis* and *S. isovalerica* are marine species (Table 1) and require Na^+ concentrations ranging from 200 to 300 mM for optimal growth (Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). *S. halophila* was isolated from a high-salinity pond and grows optimally when 750 mM NaCl, 200 mM $MgSO_4$, and 10 mM $CaCl_2$ are present in the medium (Greenberg and Canale-Parola, 1976).

Figs. 1–12. Phase contrast photomicrographs of living *Spirochaeta* cells. Wet mount preparations. Figs. 1 and 2; *S. stenostrepta* strain Z1 (DSM 2028, ATCC 25083). Bars = 10 μm. (From Canale-Parola et al., 1968.) Figs. 3–6; *S. litoralis,* strain R1 (DSM 2029, ATCC 27000). Bar = 5 μm. (From Hespell and Canale-Parola, 1970b.) Figs. 7–9; *S. aurantia* subsp. *aurantia,* strain J1 (DSM 1902, ATCC 25082), irregularly coiled (Figs. 7 and 9) and regularly coiled (Fig. 8) cells. Bars = 10 μm. (From Breznak and Canale-Parola, 1969.) Fig. 10; *S. plicatilis.* Bar = 10 μm. (From Blakemore and Canale-Parola, 1973.) Fig. 11; *S. halophila,* strain RS1 (ATCC 29478). Bar = 5 μm. (From Greenberg and Canale-Parola, 1976.) Fig. 12; *S. zuelzerae,* strain ATCC 19044. Bar = 10 μm. (From Canale-Parola et al., 1968.)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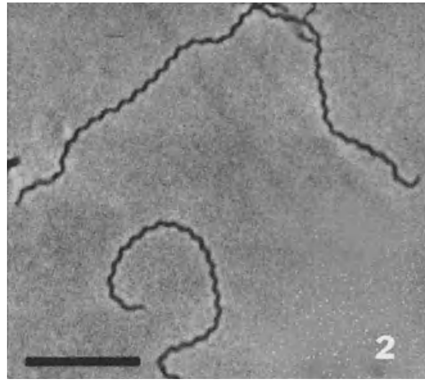
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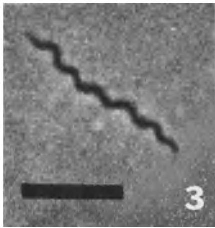
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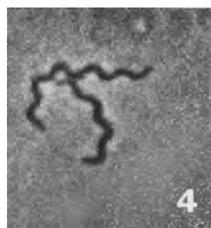
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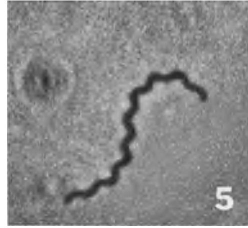
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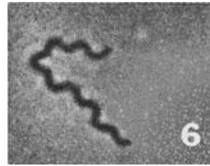
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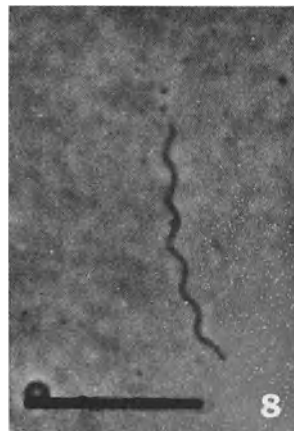
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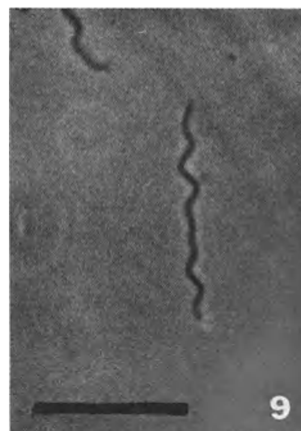
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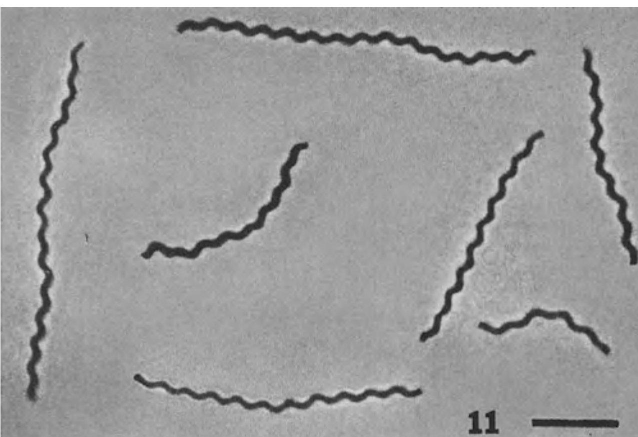
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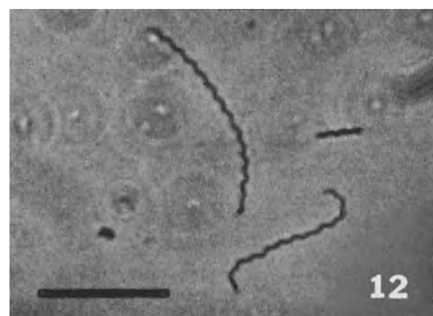
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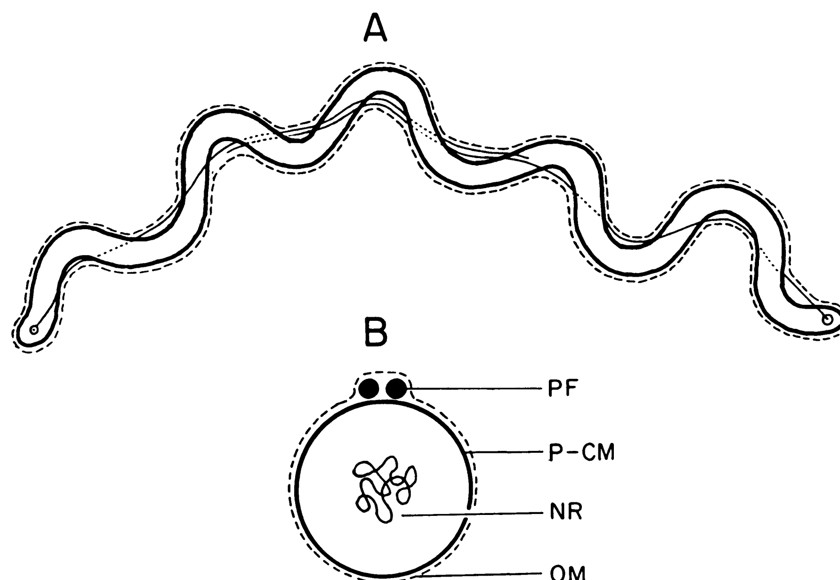

Fig. 13. (A) Schematic representation of a *Spirochaeta* cell. The outermost broken line indicates the outer membrane (outer sheath). The protoplasmic cylinder is represented by the area delimited by the solid line adjacent to the outermost broken line. The cell has two periplasmic flagella indicated by the solid-dotted thin lines wound around the protoplasmic cylinder. The insertion points of the periplasmic flagella are represented by the small circles near the ends of the cell. (B) Schematic representation of a cross section through a *Spirochaeta* cell. PF, periplasmic flagella; P—CM, peptidoglycan-cytoplasmic membrane complex; NR, nuclear region; OM, outer membrane (outer sheath).

Two subspecies of *S. aurantia* are known (Table 1). One of these (subsp. *stricta*) is characterized by significantly narrower coils than the other (subsp. *aurantia*), and its DNA possesses a slightly lower GC content (Breznak and Canale-Parola, 1975; Canale-Parola, 1984b).

Fracek and Stolz (1985) described a strain of anaerobic marine spirochetes isolated from sulfide-rich mud, and concluded that it represented a new species, which they named *Spirochaeta bajacaliforniensis.* The strain is essentially identical to *S. litoralis* with respect to physiological characteristics and GC content of the DNA but differs from the latter spirochete in some minor morphological features. Further work, possibly involving nucleic acid homology studies, is needed to determine whether Fracek and Stolz's isolate is a strain of *S. litoralis* or is, in fact, a new species of *Spirochaeta.*

Ribosomal RNA oligonucleotide cataloging studies have shown that *Spirochaeta* species and most *Treponema* species tested fall within a large cluster which is subdivided into two subclusters (Paster et al., 1984). The smaller of these subclusters includes *S. isovalerica, S. litoralis, S. aurantia,* and *S. halophila.* The larger subcluster consists of *S. stenostrepta, S. zuelzerae,* and all *Treponema* species that were tested except *T. hyodysenteriae.*

Habitats

Species of *Spirochaeta* occur, grow, and persist as free-living organisms in a variety of aquatic environments, such as the water, sediments, and muds of ponds, marshes, lakes, rivers, and oceans. Numerous strains of *Spirochaeta* have been isolated from various freshwater environments (Canale-Parola, 1984b) and from marine muds collected in Pacific and Atlantic coastal regions (Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). *Spirochaeta* cells are present in salt marshes at densities ranging from 10^4 to 10^6 per gram (wet weight) of the top 1 cm of sediment (Weber and Greenberg, 1981). Selective isolation procedures did not yield spirochetes from deep-sea sediments and water from the Sargasso Sea (3,630 m) and the Puerto Rico Trench (8,140 m) (Harwood et al., 1982). These results indicate that although anaerobic and facultatively anaerobic free-living spirochetes are common in marine coastal environments, they are not widely distributed in deep-sea regions. However, Harwood and coworkers (1982) isolated an obligately anaerobic spirochete from a sample collected near a deep-sea (2,550 m) hydrothermal vent at the Galapagos Rift ocean floor spreading center, and they observed bacteria with morphologies typical of spirochetes in surface scrapings from

mussels present near a vent. The occurrence of spirochetes in this deep-sea region may be ascribed to the environmental conditions characteristic of the vent area, which are favorable to the growth of various bacteria.

Halophilic forms (*S. halophila*) with optimum growth temperatures between 35 and 40°C have been isolated from a high-salinity pond located on the Sinai shore of the Gulf of Aqaba (Greenberg and Canale-Parola, 1976). Obligately anaerobic thermophilic spirochetes isolated from New Zealand thermal springs grow optimally at 45 to 50°C (Patel et al., 1985). Other *Spirochaeta* species behave like mesophiles with regard to growth temperatures (Canale-Parola, 1984b).

All species of *Spirochaeta* that have been cultivated are saccharolytic, and they usually lack the ability to utilize compounds other than carbohydrates as oxidizable substrates for growth. Various pentoses, hexoses, and disaccharides, as well as starch, are used as carbon and energy sources (Canale-Parola, 1984b; Greenberg and Canale-Parola, 1976). Even though free-living spirochetes are commonly observed in natural anaerobic environments in which plant material that contains cellulose and pectin is biodegraded (Canale-Parola, 1978; Harwood and Canale-Parola, 1984), none of the *Spirochaeta* species has been reported to ferment these polysaccharides. It may be inferred that in their habitats, which frequently are rich in decaying plant material, *Spirochaeta* species ferment soluble sugars released into the environment by the enzymatic activities of microorganisms that depolymerize plant polysaccharides. Both facultatively and obligately anaerobic species of *Spirochaeta* grow abundantly when the energy source available to them is cellobiose, a major product of cellulose depolymerization, which is produced by the extracellular cellulase systems of some microorganisms (Breznak and Canale-Parola, 1969, 1975; Greenberg and Canale-Parola, 1976; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970a, 1970b). Furthermore, free-living spirochetes, such as *S. aurantia,* exhibit a strong tactic response toward very low cellobiose concentrations (Greenberg and Canale-Parola, 1977a), and possess regulatory systems that enable them to enhance their chemotactic responses when attractants that serve as energy sources are present at very low concentrations in the environment (Terracciano and Canale-Parola, 1984). Inasmuch as cellobiose levels in environments in which cellulose is degraded are likely to be quite low (Smith et al., 1973), a strong tactic response to very low concentrations of cellobiose may confer an important selective advantage on the spirochetes with respect to other cellobiose-utilizing microorganisms.

In addition to their ability to regulate their chemosensory apparatus in response to low substrate concentrations, some species of *Spirochaeta* have developed other strategies to survive in environments lacking or nearly depleted of energy sources. One of these survival strategies has been studied in *S. isovalerica,* a saccharolytic anaerobe that does not utilize amino acids as fermentable substrates for growth, but catabolizes small amounts of L-leucine, L-isoleucine, and L-valine with the formation of isovalerate, 2-methylbutyrate, and isobutyrate, respectively, as end products (Harwood and

Table 1. Characteristics of the seven species of the genus *Spirochaeta*.

Species[a]	Size[b] (μm)	Relationship to O_2[c]	Optimum growth temperatures (°C)	Habitat	GC content of DNA (mol%)[d]
1. *S. stenostrepta*	0.2–0.3 × 15–45	OA	30–37	Freshwater	60.2
2. *S. litoralis*	0.4–0.5 × 5–7	OA	Near 30	Marine	50.5
3. *S. zuelzerae*	0.2–0.4 × 8–16	OA	37–39	Freshwater	56.1
4. *S. isovalerica*	0.4 × 10–15	OA	15–35	Marine	63.6–65.6
5. *S. aurantia*[e]					
subsp. *aurantia*	0.3 × 10–20	FA	25–30	Freshwater	62.2–65.3
subsp. *stricta*	0.3 × 10–20	FA	25–30	Freshwater	61.2
6. *S. halophila*	0.4 × 15–30	FA	35–40	High salinity	62
7. *S. plicatilis*	0.75 × 80–250	Unknown	Unknown	Unknown[f]	Unknown

[a]Pertinent references, which include light and electron micrographs as well as comprehensive descriptions of the species, are the following: species 1, Canale-Parola et al., 1968; species 2, Hespell and Canale-Parola, 1970b, 1973; species 3, Veldkamp, 1960; and Canale-Parola et al., 1968; species 4, Harwood and Canale-Parola, 1983; species 5, Breznak and Canale-Parola, 1969, 1975; species 6, Greenberg and Canale-Parola, 1976; species 7, Blakemore and Canale-Parola, 1973.
[b]Lengths indicated are of the majority of cells. Shorter and longer cells are also present in cultures.
[c]OA, obligate anaerobe; FA, facultative anaerobe.
[d]Buoyant density determinations, except for *S. isovalerica* (based on determination of T_m).
[e]Wavelength of the cell: subsp *aurantia* cells, 2.0–2.8 μm; subsp. *stricta* cells, 1.1–1.5 μm.
[f]Observed in mud from a brackish marsh (Blakemore and Canale-Parola, 1973).

Canale-Parola, 1981a, 1983, 1984). Although these amino acids are not used by *S. isovalerica* as fermentable substrates for growth, their fermentation serves to generate ATP, which is utilized by the spirochetes as a source of maintenance energy. This process allows cells to survive during periods of growth substrate starvation (Harwood and Canale-Parola, 1981a, 1981b, 1982). Other starvation-survival strategies utilized by *Spirochaeta* species may involve ATP generation through metabolism of endogenous RNA (Canale-Parola and Kidder, 1982; Harwood and Canale-Parola, 1984) or metabolism of intracellular polyglucose storage granules (Kropinski et al., 1988).

The large spirochete *S. plicatilis* has been observed within masses of *Beggiatoa* trichomes in samples of black, sulfide-rich marsh mud covered with a layer of marsh water (Blakemore and Canale-Parola, 1973). In this study, the spirochetes were seen to swim freely among the tangled *Beggiatoa* trichomes, and "crept" on or otherwise moved in contact with the surface of the trichomes. The close association with *Beggiatoa* suggested a chemotactic response by the large spirochete toward metabolites produced by the sulfur bacteria. As the level of sulfide generated by biological activities in the mud became low, gradual lysis of the *Beggiatoa* trichomes was observed. This lytic process coincided with a dramatic increase in the number of large spirochetes (Blakemore and Canale-Parola, 1973). Possibly, substances released by the lysing *Beggiatoa* were used as growth substrates for *S. plicatilis.*

Physiology

Motility and Chemotaxis

Three main types of motion are observed in species of *Spirochaeta:* 1) translational motion; 2) rotation of the cell around its longitudinal axis; and 3) flexing motion (Canale-Parola, 1978). In addition to swimming freely in liquid environments, some *Spirochaeta* are able to "creep" or "crawl" on solid surfaces, a movement resembling that of the gliding bacteria (Blakemore and Canale-Parola, 1973). Species of *Spirochaeta* retain their translational motion in environments of relatively high viscosity, e.g., 500 centipoise (Greenberg and Canale-Parola, 1977b). In comparison, flagellated bacteria such as *Escherichia coli, Pseudomonas aeruginosa,* and *Aquaspirillum serpens* are immobilized at viscosities of or above 60 centipoise (Greenberg and Canale-Parola, 1977c).

The motility of a strain of *Spirochaeta aurantia* in liquid environments has been described as follows (Greenberg and Canale-Parola, 1977a; Greenberg et al., 1985): The spirochete usually swims in nearly straight lines (runs) and appears to spin about its longitudinal axis as it progresses through the liquid. From time to time the cell reverses swimming direction, the anterior end of the cell becoming the posterior end. Occasionally, the spirochete stops running, flexes, and then resumes its translational motion. However, upon resuming its translational motion, the spirochete usually alters the direction of its movement, and the previously leading cell end may or may not become the trailing cell end. Thus, *S. aurantia* performs three kinds of behavior (runs, flexes, and reversals), in contrast to *Escherichia coli* which performs two kinds (runs and tumbles). During runs, *S. aurantia* cells have an average linear speed of approximately 16 μm/s (Fosnaugh and Greenberg, 1988). Flexes last from a fraction of a second to several seconds. The average frequency of reversals in cell populations is approximately 0.31 reversals/5 s (Fosnaugh and Greenberg, 1988).

A model that interprets the motile behavior of *S. aurantia* has been proposed (Berg, 1976; Greenberg et al., 1985). According to this model, the two periplasmic flagella of *S. aurantia* rotate, each driven by a motor at the insertion end. Rotation of the flagella in one direction (looking at the cell head on; see Fig. 13B) causes the periplasmic cylinder and the outer membrane to move in directions opposite to each other. Due to its helical shape, the cell rotates about its longitudinal axis and moves along it. Runs occur when the flagellar motor at one cell end rotates clockwise (CW) while the motor at the other cell end rotates counterclockwise (CCW). When both motors switch direction of rotation at the same time, a reversal takes place. A flex is generated during asynchronous switching, i.e., when only one motor switches direction so that both motors are rotating CW or CCW. Asynchronous switching causes the cell ends to twist in opposition to each other, and a flex occurs.

S. aurantia exhibits chemotaxis toward D-glucose, D-xylose, cellobiose, and various other sugars, but not toward amino acids (Greenberg and Canale-Parola, 1977a). Many of the chemoattractants also serve as carbon and energy sources for growth of *S. aurantia.* In the presence of very low concentrations of an attractant that serves as a carbon and energy source, growing cells of *S. aurantia* specifically enhance their chemotactic response toward the attractant and are able to sense concentrations of the attractant much lower than those sensed by cells growing in the presence of excess attractant (Terracciano

and Canale-Parola, 1984). Most likely, the ability to regulate its chemosensory system provides *S. aurantia* with competitive advantages in natural environments deficient in nutrients.

Fosnaugh and Greenberg (1988) carried out an analysis of the motility and chemotaxis behavior of *S. aurantia.* They observed that a population of *S. aurantia* cells spent, on the average, 66% of the time swimming smoothly (runs), 33% of the time flexing, and 1% of the time in reversals. After addition of an attractant (D-xylose, 10 mM final concentration) there was an increase in smooth swimming, a decrease in flexing, and a complete suppression of reversals. From 1.5 to 2 min after addition of the attractant, the population resumed its unmodified behavior. On the basis of their observations and of the above-mentioned motility model for *S. aurantia,* Fosnaugh and Greenberg (1988) postulated that a mechanism for communication between the two flagellar motors is present in this spirochete, and that a motor-switch-synchronizing device is also operating.

Fermentation Products and ATP-Yielding Pathways

Under anaerobic conditions, all species of *Spirochaeta* that have been cultured (Table 1), except *S. zuelzerae,* ferment carbohydrates with formation of acetate, ethanol, CO_2, and H_2 as major end products (Canale-Parola, 1984b; Harwood and Canale-Parola, 1983). *S. zuelzerae* primarily produces acetate, lactate, CO_2, H_2 and a relatively small amount of succinate from carbohydrates (Canale-Parola, 1977, 1984b; Veldkamp, 1960).

S. isovalerica (Table 1), in addition to fermenting carbohydrates, ferments L-leucine, L-isoleucine, and L-valine—forming isovaleric, 2-methylbutyric, and isobutyric acids, respectively, as end products (Harwood and Canale-Parola, 1983). However, *S. isovalerica* requires a fermentable carbohydrate for growth. When *S. isovalerica* is grown in medium containing both glucose and the three above-mentioned amino acids, only a relatively small fraction of the total amount of available amino acids is fermented (Harwood and Canale-Parola, 1981a, 1981b). Under these growth conditions, the ATP derived from amino acid catabolism is estimated to be 4 to 5% of the total ATP formed. Fermentation of the amino acids in the absence of glucose does not support measurable growth of *S. isovalerica,* but serves to generate ATP which is utilized as a source of maintenance energy by the spirochete when fermentable carbohydrates are not available (Harwood and Canale-Parola, 1981b, 1983). In addition to the branched-chain fatty acids, amino acid catabolism by *S. isovalerica* yields small quantities of isobutanol and isoamyl alcohol (Harwood and Canale-Parola, 1981a, 1983).

All *Spirochaeta* species whose metabolic pathways have been studied (species 1, 2, 4, 5, and 6 in Table 1) catabolize glucose to pyruvate via the Embden-Meyerhof pathway (Canale-Parola, 1984b; Harwood and Canale-Parola, 1983). Anaerobic metabolism of pyruvate yields acetyl CoA, CO_2, and H_2 via a clostridial-type clastic reaction (Canale-Parola, 1977; Greenberg and Canale-Parola, 1976). Acetate is formed from acetyl CoA in reactions catalyzed by phosphotransacetylase and acetate kinase. A double reduction involving aldehyde and alcohol dehydrogenases is responsible for ethanol production from acetyl CoA (Canale-Parola, 1977).

When growing aerobically, the two facultatively aerobic species (*S. aurantia* and *S. halophila*) oxidize glucose incompletely, with formation of CO_2 and acetate as major end products. Aerobically, both oxidative phosphorylation and substrate-level phosphorylation are utilized by the two species to generate ATP (Breznak and Canale-Parola, 1972b; Greenberg and Canale-Parola, 1976). The tricarboxylic acid cycle is either absent or plays a minor catabolic role in these two species.

Rubredoxin was detected in cell extracts of the obligate anaerobes *S. stenostrepta* and *S. litoralis,* and of the facultative anaerobe *S. aurantia* (Breznak and Canale-Parola, 1972a; Hespell and Canale-Parola, 1973; Johnson and Canale-Parola, 1973). Rubredoxin was isolated from extracts of both aerobically and anaerobically grown cells of *S. aurantia.* Ferredoxin was present in cell extracts of anaerobically grown *S. aurantia,* but was not found in aerobically grown cells of this bacterium (Johnson and Canale-Parola, 1973).

Isolation

Selective Procedures

Anaerobic and facultatively anaerobic spirochetes are readily isolated from natural environments by means of selective procedures, and usually grow abundantly in ordinary laboratory media. Anaerobic growth yields of the isolates range from 2×10^8 to approximately 10^{10} cells/ml, but commonly are 6×10^8 to 8×10^8 cells/ml (Breznak and Canale-Parola, 1975; Canale-Parola, 1973; Greenberg and Canale-Parola, 1976; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970a,b). Cell popula-

tion doubling times in anaerobic cultures vary from 2.2 to 8.5 h, depending on the species and the growth conditions. Aerobically grown cultures yield from 0.7×10^9 to 1.2×10^9 spirochetes/ml, with doubling times of 2 to 4 h (Breznak and Canale-Parola, 1975; Canale-Parola, 1973; Greenberg and Canale-Parola, 1976).

A procedure in which the antibiotic rifampin (rifampicin) serves as selective agent is quite effective for the isolation of free-living spirochetes (genus *Spirochaeta*) from natural environments (Harwood et al., 1982; Patel et al., 1985; Stanton and Canale-Parola, 1979; Weber and Greenberg, 1981). This procedure, which is described below, is based on the observation that spirochetes in general are naturally resistant to rifampin (Leschine and Canale-Parola, 1986; Stanton and Canale-Parola, 1979). Thus, spirochetes such as *S. stenostrepta* and *S. aurantia* grow in the presence of as much as 100 to 200 μg of rifampin per ml of medium (Leschine and Canale-Parola, 1986), whereas the growth of many other bacteria is inhibited. The resistance of spirochetes to rifampin is probably due to the low affinity of their RNA polymerase for the antibiotic (Allan et al., 1986; Leschine and Canale-Parola, 1986).

Other enrichment procedures used in the isolation of *Spirochaeta* species are based either on one or both of the following selective factors: 1) the ability of spirochetes to pass through filters that retain most other bacteria; and 2) the migratory movement of spirochetes through agar media (Canale-Parola, 1973, 1984b). These selective procedures enrich for species of *Spirochaeta* measuring less than 0.5 μm in diameter (Table 1).

In the enrichment-by-filtration procedure, which is described in detail below, separation of *Spirochaeta* species from most of the microorganisms present in mud or water is achieved by techniques involving filtration through cellulose ester filter discs (e.g., Millipore filters) having an average pore diameter of 0.3 or 0.45 μm. Spirochetes pass through these filter discs because of their relatively small cell diameter (Table 1), and probably also because their motility apparatus enables them to swim freely in liquids as well as to move in contact with solid surfaces.

The enrichment-by-migration procedure uses the ability of spirochetes to move through agar gels or media containing as much as 1 to 2% (wt/vol) agar. This movement or migration occurs primarily within the agar gel, i.e., below the surface of the agar medium. In contrast, flagellated bacteria usually cannot carry out translational movement through gels or media containing agar at the above-mentioned concentrations, although several exceptions have been reported (Greenberg and Canale-Parola, 1977c). Apparently the cell coiling of spirochetes is important for their translational motion through agar gels, inasmuch as this type of movement is impaired in mutant spirochetes lacking the cell-coiling characteristic of the parental strain (Greenberg and Canale-Parola, 1977b).

Migration of spirochetes through agar media results from the unique motility mechanism of these bacteria (Canale-Parola 1977, 1978), as well as from chemotaxis toward the energy and carbon source (Breznak and Canale-Parola, 1975; Greenberg and Canale-Parola, 1977a). The role of chemotaxis in the migration of saccharolytic spirochetes through agar media has been studied (Breznak and Canale-Parola, 1975). When these spirochetes are inoculated in the center of glucose-containing agar medium plates, they grow using this sugar as their energy source. Utilization of the sugar by the spirochetes gives rise to a glucose concentration gradient that moves away from the center of the plate as more of this carbohydrate is metabolized by the spirochetes. Since the spirochetes exhibit chemotaxis toward glucose and are able to move through the agar gel, they migrate into the areas of higher glucose concentration within the gradient. Thus, the spirochetal population follows the outward movement of the gradient and migrates toward the periphery of the plate. This behavior results in the formation of a growth "veil" or "ring" of spirochetes for which glucose serves both as the energy source for growth and as the chemoattractant (Breznak and Canale-Parola, 1975; Canale-Parola, 1973). The veil or ring increases continuously in diameter during incubation and may reach the outer edge of the plate. The migration rate of the spirochetal population is greatest in agar media containing low substrate concentrations (e.g., 0.02% glucose). In these media the substrate becomes rapidly depleted in the region where spirochetes are growing, and the spirochetal population moves toward the outer zone of higher substrate concentrations at a relatively fast rate (Breznak and Canale-Parola, 1975).

In procedures for the isolation of *Spirochaeta* species from natural environments, the chemotactic behavior and the ability of these bacteria to move through agar gels have important selective functions. In a typical isolation procedure, a small, shallow cylindrical hole is made through the surface of an agar medium containing a low concentration of carbohydrate. Rifampin may be included in the medium as an additional selective agent for spirochetes. The medium may be in a petri dish or a small bottle.

A tiny drop of pond water, or of any other material in which spirochetes have been observed, is placed within the hole. The chemotactic, saccharolytic spirochetes in the inoculum multiply and form a growth veil that extends outwardly through the agar medium. Thus, the spirochetes in the veil move away from contaminants, which remain mainly in the vicinity of the inoculation site. Spirochetal cells from the outermost edge of the veil are used to obtain pure cultures by conventional methods, such as streaking on agar medium plates. Isolation procedures involving chemotaxis and movement through agar gels are described below.

Selective isolation techniques have not been developed for the large *Spirochaeta* species, such as *S. plicatilis.*

Enrichment by Migration

When spirochetes are vastly outnumbered by other bacteria in the inoculum, it is advisable to begin the isolation procedure with an enrichment-by-migration step (the principle has been discussed above). A suitable medium (EBM agar medium) for this enrichment has the following composition in g/100 ml distilled water: yeast extract (Difco), 0.1; Trypticase (BBL Microbiology Systems), 0.1; L-cysteine, HC1, 0.05; resazurin, 10^{-4}; agar (Difco), 1. For the enrichment and isolation of marine spirochetes, a mixture of sea water (70 ml) and distilled water (30 ml) is used instead of plain distilled water. The pH of the medium is adjusted to 7.2. After autoclaving, cellobiose is added as a sterile solution to a final concentration of 0.01 g/ 100 ml medium. Rifampin (filter-sterilized solution) may be used as a selective agent (0.5 mg per 100 ml of medium).

The medium is prereduced (Hungate, 1969) and is dispensed into narrow-necked 60 ml glass bottles. During this step and the steps that follow, the medium is maintained in an anaerobic atmosphere by delivering a stream of 0_2-free N_2 into the bottles. The bottles containing the medium are sealed with neoprene rubber stoppers and are placed in a press to hold the stoppers in place during autoclaving. The sterilized medium is allowed to cool to approximately 50°C, and cellobiose and, if desired, rifampin are added. The complete medium is solidified at a 45° angle, so that bottle slants are obtained. Then, a small (5 mm diam., 6 mm depth), cylindrical well is melted halfway down each slant by heating the tip of a thin metal rod and touching the agar with it. Alternatively, the well can be made by aspirating some of the agar medium with a sterile Pasteur pipette connected to a vacuum apparatus. All further manipulations are made either while maintaining an N_2 atmosphere within the bottles (Hungate, 1969) or with the bottles placed within an anaerobic chamber and the rubber stoppers replaced by cotton stoppers.

Before inoculation, any liquid that oozed from the agar medium is removed by suction from the well and the lower part of each slant. The bottle slants are inoculated by carefully placing a small volume of inoculum (e.g., mud) into the well, and are incubated at 30°C. In successful enrichments, the spirochetes form a characteristic, semitransparent growth veil that extends down into the agar medium and diffuses out toward the periphery of the slant, away from contaminating organisms growing in and near the well. The veil usually is visible after 4 to 7 days of incubation. Cells from the edges of the growth veil are used to obtain pure cultures of the spirochetes by means of a procedure involving serial dilutions in a rifampin-containing medium (see below).

Isolation in Rifampin-containing Media

Serial dilutions in melted, rifampin-containing agar medium can be used to isolate spirochetes from materials in which they are present in relatively large numbers (e.g., directly from mud or from the outer edges of growth veils in the bottle slants mentioned above). The spirochete-containing inoculum is serially diluted in tubes of melted (45°C), prereduced RIM medium. This medium is identical to the EBM agar medium described above except that the final concentration of cellobiose is 0.2% (wt/vol), rifampin (0.5 mg/100 ml of medium) is added, and the agar (Bacto, Difco) concentration is 0.8 g/ 100 ml of medium. The medium is dispensed and sealed in anaerobic culture tubes containing an N_2 atmosphere (Hungate, 1969).

Spirochete colonies in the RIM agar medium deeps are recognizable inasmuch as they are spherical and because, as a result of cell migration through the agar medium, they appear as "transparent bubbles," "veil-like growth with a dense center," or "cotton ball-like" (Paster and Canale-Parola, 1982; Stanton and Canale-Parola, 1979; Weber and Greenberg, 1981). To obtain pure cultures, the serial dilution step is repeated at least twice, using cells from spirochete colonies that developed in the RIM agar medium dilution deeps.

Procedures involving serial dilutions in rifampin-containing agar media have been used by Weber and Greenberg (1981) and by Patel et al. (1985) to isolate spirochetes from salt marsh sediments and hot springs, respectively.

Selective Enrichment by Filtration

A filtration technique has been used in the isolation of *Spirochaeta stenostrepta* (Canale-Parola et al., 1967; Canale-Parola et al. 1968), *S. litoralis* (Hespell and Canale-Parola, 1970b), and the facultatively anaerobic *S. halophila* (Greenberg and Canale-Parola, 1976). In all cases, the source of the spirochetes was black mud that had the characteristic smell of H_2S. *S. stenostrepta* was isolated from a mud sample collected from a fresh-water pond, *S. litoralis* from marine mud, and *S. halophila* from the mud of Solar Lake, a high-salinity pond located on the Sinai shore of the Gulf of Aqaba.

Medium for Isolating *Spirochaeta stenostrepta*

Distilled water	875 ml
Glucose	5.0 g
Peptone	2.0 g
Yeast extract	0.3 g
Vitamin B_{12}	10^{-5} g
Phosphate solution	15 ml
Salts solution	100 ml
Sulfide solution	10 ml

The phosphate solution is prepared by dissolving 30 g of KH_2PO_4 and 70 g of K_2HPO_4 in 1,000 ml of distilled water.

To prepare the salts solution, 0.2 g of ethylenediamine tetraacetic acid are added to 800 ml of distilled water and dissolved by heating. The pH of the resulting solution is adjusted to 7.0 with 2.5% KOH. Then the following additions are made: $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.75 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; trace elements solution (below), 5.0 ml. Finally, the volume of the salts solution is adjusted to 1,000 ml with distilled water.

The sulfide solution (2 g $Na_2S \cdot 9H_2O$/100 ml distilled water) is autoclaved separately and added shortly before inoculation to the sterile medium cooled to 30–35°C.

After sterilization by autoclaving, a precipitate is present in the medium, but it disappears as the medium cools. The final pH of the complete medium is 6.9–7.0.

Trace elements solution: A separate solution of each salt listed below, in the amount indicated, is prepared in distilled water. Heating may be required to dissolve some of the salts. The pH of the Na_2MoO_4 and $NaVO_3$ solutions is adjusted to a value below 7. The KI solution is added to the $AlCl_3$ solution and mixed by stirring. Then the other solutions are added, one at a time, to this mixture, with stirring, in the order in which they are listed below.

1. $AlCl_3 \cdot 6H_2O$	0.50 g
2. KI	0.25 g
3. KBr	0.25 g
4. LiCl	0.25 g
5. $MnCl_2 \cdot 4H_2O$	3.50 g
6. H_3BO_3	5.50 g
7. $ZnCl_2$	0.50 g
8. $CuCl_2 \cdot 2H_2O$	0.50 g
9. $NiCl_2 \cdot 6H_2O$	0.50 g
10. $CoCl_2 \cdot 6H_2O$	0.50 g
11. $SnCl_2 \cdot 2H_2O$	0.15 g
12. $BaCl_2 \cdot 2H_2O$	0.15 g
13. $Na_2MoO_4 \cdot 2H_2O$	0.25 g
14. $NaVO_3$	0.05 g

The volume of the final mixture is adjusted to 1,800 ml by adding distilled water, and the pH is adjusted to a value between 3 and 4 with HCl. A yellow precipitate, present at first, is replaced by a fine, white precipitate after a few days. The solution should be mixed thoroughly immediately before it is used and may be stored at room temperature. This trace element solution is a modification of a solution described by Pfennig (1965).

Isolation of *Spirochaeta stenostrepta* (Canale-Parola et al., 1967)

Black mud, from which a strong odor of H_2S could be detected, was suspended in aqueous 0.02% $Na_2S \cdot 9H_2O$. The slurry was filtered through Whatman No. 40 filter paper, and the filtrate was subjected to filtration through sterile cellulose ester filter discs (Millipore, pore diameter 0.45 μm). One ml aliquots of the resulting filtrate were added aseptically to 60-ml glass stoppered bottles, half filled with sterile isolation medium. The bottles were then completely filled with medium, stoppered without trapping air bubbles, and incubated at 30°C (each bottle was covered with a sterile 50-ml beaker). After 5 to 7 days of incubation, the microbial population in many of the bottles consisted predominantly of thin spirochetes. Pure cultures were obtained by use of dilution shake cultures (the medium was covered with sterile paraffin) or by plating serial dilutions and incubating the plates in the absence of O_2 (Bray dishes). The isolation medium solidified with 1.5% agar was used. Spirochetal colonies appeared after 5 to 6 days. After isolation the organisms were also grown in Florence flasks filled with a medium (GYPT) containing (g/100 ml distilled water): glucose, 0.5; yeast extract and peptone, 0.2 each; sodium thioglycolate, 0.05. The pH of this medium was adjusted to 7.0–7.3 before sterilization. The spirochetes were maintained in paraffin-layered stab cultures of medium GYPT containing 1.5% agar, at 5°C and transferred monthly.

Subsurface colonies of *S. stenostrepta* in agar media are white, spherical, and characteristically fluffy in appearance.

S. litoralis and *S. halophila* were isolated by a procedure similar to that used for *S. stenostrepta,* except that different isolation media were used and the incubation temperatures were 22–23°C for *S. litoralis* and 37°C for *S. halophila* (Hespell and Canale-Parola, 1970b; Greenberg and Canale-Parola, 1976). Furthermore, 1 volume of the mud used as the source of *S. litoralis* was suspended in 5 volumes of isolation medium before filtration through filter paper.

Isolation of *Spirochaeta litoralis* (Hespell and Canale-Parola, 1970b)

The isolation medium for the marine spirochete included (g/100 ml distilled water): tryptone (Difco), 0.3; yeast extract (Difco), 0.05; and NaCl, 2.0. To this mix-

ture 2 ml of M potassium phosphate buffer (pH 7.4) and 0.2 ml of a salt solution (see below) were added. The pH of the medium was adjusted to 7.3 with KOH before sterilization. Immediately before inoculation the medium was supplemented with 2 ml of a sterile glucose solution (25 g per 100 ml distilled water) and with 0.5 ml of a sterile $Na_2S \cdot 9H_2O$ solution (10 g per 100 ml distilled water). The final pH of the medium ranged from 7.4 to 7.5.

The salts solution contained (g/75 ml distilled water): tetrasodium ethylenediamine tetraacetate, 1; $CaCl_2 \cdot 2H_2O$, 3.75; $MgCl_2 \cdot 6H_2O$, 12.5; $FeSO_4 \cdot 7H_2O$, 0.5.

To 75 ml of this salt mixture, 25 ml of a trace elements solution (see Medium for Isolating *Spirochaeta stenostrepta*) was added.

After cloning, *S. litoralis* was grown routinely in the isolation medium. Subsurface colonies of *S. litoralis* in agar media are white to cream-colored, and resemble those of *S. stenostrepta* in appearance.

Maintenance procedures: Broth cultures of *S. litoralis* in test tubes remained viable for three days at 30°C. Agar stabs grown at 30°C and then kept either at 5 or 30°C were no longer viable after approximately one week, whether they were layered with paraffin or not. Viable cells were not recovered from lyophilized preparations.

S. litoralis was satisfactorily maintained by using "depression" cultures (Canale-Parola and Wolfe, 1960) prepared by using 1-liter Erlenmeyer flasks each containing 800 ml of isolation medium to which 2 g of agar per 100 ml were added. These cultures, when incubated for 2 days at room temperature and then at 15°C, remained viable for at least three months.

Isolation of *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976)

The isolation medium contained 0.2 g of peptone (Difco) and 0.4 g of yeast extract (BBL) per 97 ml of an inorganic salt solution which had the following composition: $CaCl_2$, 0.04 M; NaCl, 0.85 M; and $MgSO_4$, 0.2 M. In preparing the salt solution, salts were added in the order in which they are listed, to avoid formation of a precipitate. The dihydrate form of $CaCl_2$ and the heptahydrate form of $MgSO_4$ were used. After adjusting the pH to 7.5 with KOH the medium was sterilized by autoclaving. The volume of the medium was brought to 100 ml by adding separately sterilized solutions of glucose and sodium sulfide to final concentrations of 0.5% and 0.05%, respectively.

S. halophila was grown routinely at 37°C in ISM broth (Greenberg and Canale-Parola, 1975), which differed from the isolation medium because maltose replaced glucose, the sodium sulfide was omitted, and the composition of the salt solution was changed (to $CaCl_2$, 0.01 M; NaCl 0.75 M; $MgSO_4$, 0.2 M). Furthermore, BBL yeast extract was replaced by Difco yeast extract which supported higher growth yields of our isolate. *S. halophila* was maintained by storing ISM agar (ISM broth containing 0.75 g agar per 100 ml) plate cultures at 5°C. The cultures were transferred monthly.

Colonies of *S. halophila* are pigmented when growing aerobically but lack pigmentation under anaerobic growth conditions. Colonies grown in air on ISM agar plates were red and appeared circular when viewed from above. A portion of each colony grew above the surface of the medium, and part of the colony extended into the agar. Areas of diffuse growth were present at the periphery of the colonies. Generally, colonies measured 2–6 mm in diameter after 5 days at 35°C but were smaller or larger depending on the number of colonies on the plate and the length of incubation. Cells streaked on ISM agar plates, and grown anaerobically in Bray dishes formed colonies which developed below the surface of the agar medium and were white, spherical, and diffuse.

Cultivation of most of the anaerobic species of *Spirochaeta* described here (Table 1; species 1, 2, 3) and does not require the use of stringent anaerobic procedures (e.g., the Hungate technique). Reducing agents, such as sodium thioglycolate or L-cysteine, are commonly added to the growth media, and other conventional techniques for the growth of anaerobes are used, as discussed elsewhere (Canale-Parola, 1973).

Enrichment by Filtration and Migration

Many strains of *Spirochaeta aurantia* have been isolated by means of a selective technique that combines filtration of the inoculum through a cellulose ester filter disk with migration of the spirochetes in agar media (Breznak and Canale-Parola, 1969; Canale-Parola, 1973; Breznak and Canale-Parola, 1975).

Isolation of *Spirochaeta aurantia* by Filtration and Migration

	Medium HE	Medium PEP
Distilled water	50 ml	100 ml
Peptone	0.1 g	0.5 g
Yeast extract	0.1 g	0.05 g
K_2HPO_4	–	0.01 g
Hay extract	50 ml	–
Agar	1 g	1 g

The pH of medium HE is adjusted to 6.5 before sterilization. To prepare the hay extract, 0.5 g of dried barn hay are boiled in 100 ml of distilled water for 10 min. The boiled mixture is filtered using Whatman No. 40 filter paper. The filtrate is the hay extract.

The inoculum, consisting either of pond or marsh water, or of a water-mud slurry, was prefiltered through Whatman No. 40 filter paper to remove large particles. Then, the enrichment cultures were prepared by depositing one or two drops of the filtrate near the center of each of a number of sterile cellulose ester filter disks (47-mm disk diameter, 0.3- or 0.45-μm pore diameter, Millipore) previously placed on the surface of isolation medium plates. One filter disk had been placed on each plate, approximately in the center. The cultures were incubated at 30°C for 12–24 h to allow spirochetes in the inoculum to move through the filter disk onto the surface of the medium. Then the filter disks were removed aseptically from the plates, and incubation of the plate cultures was continued. Spirochetes that had passed through the filter disks grew and migrated

through the agar medium, forming semitransparent growth veils that diffused toward the edge of the plates. Spirochetal growth veils usually developed in 5–10 days. Generally, 10–20% of the plate enrichments were successful (i.e., yielded growth veils) when spirochetes were present in the inoculum as determined by light microscopy. Analyses showed (Breznak and Canale-Parola, 1975) that the total carbohydrate content of a batch of isolation medium HE was 40 mg/100 ml and the glucose content 5 mg/100 ml. Medium PEP contained 6 mg total carbohydrate, and less than 1 mg glucose per 100 ml. As discussed previously, low carbohydrate concentrations, such as those in isolation media HE and PEP, are used to enhance the rate of spreading of the spirochetal growth veil through the agar medium.

It should be noted that bacteria other than spirochetes may form subsurface, spreading growth veils in the enrichment plates. Most common among these veil-forming bacteria are *Aquaspirillum (Spirillum) gracile* (Canale-Parola et al., 1966) and *Serpens flexibilis* (Hespell, 1977). *Serpens flexibilis* cells are flexible, Gram-negative rods that have bipolar as well as lateral flagella.

Pure cultures of *S. aurantia* are obtained by streaking cells from the outer edge of the growth veil onto isolation medium plates or growth medium plates.

A suitable growth medium for *S. aurantia* contains (g/99 ml distilled water): glucose, 0.2; yeast extract, 0.2; Trypticase (BBL), 0.5. The pH is adjusted to 7.5 before sterilization. When desired, agar (1 g) is added. After autoclaving and allowing the medium to cool, 1 ml of sterile 1 M potassium phosphate buffer (pH 7) is added.

Growth of some *S. aurantia* strains is either partially or totally inhibited in media containing agar (Difco) concentrations higher than 1% (wt/vol). Thus, viable cell counts usually are higher when performed using plates of media containing 0.75 or 1% agar than when the cells are grown in media including agar at higher concentrations. *S. aurantia* cells grown in media containing agar at concentrations higher than 1% frequently are aberrant in morphology. Many usually long, poorly coiled, filamentous cells are present, as well as an abundance of spherical bodies (Breznak and Canale-Parola, 1975).

Surface, aerobic colonies of *S. aurantia* in growth medium (agar, 1%) are light orange to orange, round or nearly round, with slightly irregular edges, and measure 1–4 mm in diameter after 4–7 days at 30°C. The colonies grow mostly within the agar medium, just under the surface, but many have a slightly raised central portion. One strain (Vinzent strain) was found to produce both this type of colony and a "pinpoint" type of colony, measuring approximately 0.5 mm in diameter and growing primarily on the surface of the agar medium (Breznak and Canale-Parola, 1975).

Surface, anaerobic colonies of *S. aurantia* (growth medium, 1% agar) are similar in morphology to the aerobic ones, but are not pigmented. Subsurface anaerobic colonies are white and spherical.

S. aurantia may be maintained on slants of growth medium at 5°C. These stock cultures are transferred monthly.

Maintenance

Maintenance procedures suitable for individual *Spirochaeta* species have been described above. It should be added that cells of all *Spirochaeta* species that have been cultured can be preserved in a viable condition for several years by maintaining them at the temperature of liquid nitrogen. Conventional methods are used to prepare liquid nitrogen stock cultures of spirochetes (Canale-Parola, 1973).

Identification

Free-living, anaerobic and facultatively anaerobic, helical bacteria which possess the ultrastructural features typical of spirochetes (e.g., periplasmic flagella and outer sheath) are classified in the genus *Spirochaeta.*

Differentiation among the obligately anaerobic species (Table 1, species 1–4) may be based in part on determinations of carbohydrate fermentation end products. *S. stenostrepta, S. litoralis,* and *S. isovalerica* form acetate, ethanol, CO_2, and H_2 as major products of glucose fermentation, whereas *S. zuelzerae* produces acetate, lactate, CO_2, H_2, and small amounts of succinate (Canale-Parola, 1984b). In addition, *S. isovalerica* forms small amounts of isovalerate, 2-methylbutyrate, and isobutyrate as fermentation end products when growing in media containing L-leucine, L-isoleucine, and L-valine, as well as a fermentable carbohydrate (see "Physiology," this chapter) (Harwood and Canale-Parola, 1983). *S. stenostrepta* and *S. zuelzerae* are freshwater forms, whereas *S. litoralis* and *S. isovalerica* were isolated from seawater environments and have salt requirements typical of marine bacteria (Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). Further characterization of the obligate anaerobes should take into account interspecific differences in GC content of the DNA and in cell size (Table 1).

Salt requirements constitute an important basis of distinction between the facultative anaerobes *S. aurantia* and *S. halophila* (Table 1, species 5 and 6). The latter species is halophilic and has an absolute requirement for relatively high concentrations of Na^+, Cl^-, Ca^{++}, and Mg^{++} (see above), whereas *S. aurantia* strains have been isolated only from freshwater environments and do not exhibit special salt requirements.

As mentioned previously, *S. halophila* and *S. aurantia* produce pigments when growing aerobically. In both species the pigments are carotenoids. The major pigment of *S. halophila* (strain RS1) is 4-keto-1′,2′-dihydro-1′-hydroxytorulene, whereas *S. aurantia* (strain J1) produces mainly 1′, 2′-dihydro-1′-hydroxytorulene (Greenberg and Canale-Parola, 1975). Nonpigmented mutants of *S. halophila* (Greenberg and Canale-Parola, 1976) and of *S. aurantia* (B. J.

Paster and E. Canale-Parola, unpublished data) have been isolated.

Cells of *S. aurantia* subspecies *stricta* are more tightly coiled than those of *S. aurantia* subspecies *aurantia* (Table 1, footnote[e]).

Criteria used for the identification of *S. plicatilis* (Table 1, species 7) are its large size (Table 1) and its characteristic morphology and motility (Blakemore and Canale-Parola, 1973).

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CHAPTER 192

The Genus *Treponema*

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Introduction and Habitats

The genus *Treponema* is composed of both pathogenic and nonpathogenic species indigenous to humans and animals. They are helical, tightly coiled, motile spirochetes ranging from 5–20μm in length and 0.1–0.4μm in diameter and are best observed by dark-field microscopy. The organisms stain poorly with the usual aniline dyes; however, those that are capable of aniline-dye uptake are Gram-negative. Staining can best be accomplished by the use of silver impregnation or immunofluorescent methods. A general review of the spirochetes is given in Chapter 191.

The truly pathogenic members of the genus are specific for their natural hosts. The four morphologically identical human pathogens and the diseases they cause are: *Treponema pallidum* subsp. *pallidum* (syphilis), *T. pallidum* subsp. *pertenue* (yaws), *T. pallidum* subsp. *endemicum* (nonvenereal endemic syphilis), and *T. carateum* (pinta). The 100% DNA homology between *T. pallidum* subsp. *pallidum* and *pertenue* provides the basis for subspeciation of these two organisms (Miao and Fieldsteel, 1980). *T. pallidum* subsp. *endemicum* was created because it is considered a variant of *T. pallidum* (Smibert, 1984). *T. paraluiscuniculi* (venereal rabbit spirochetosis) and *T. hyodysenteriae* (swine dysentery) are the only treponemes that have been established unequivocally as animal pathogens (Bayon, 1913; Glock and Harris, 1972; Harris et al., 1972a; Hunter and Ross, 1972; Taylor and Alexander, 1971; Todd et al., 1970; Vallejo, 1969). The isolation of *T. hyodysenteriae* from wild rodents suggests a role for these animals as reservoir hosts in the epidemiology of the disease (Joens and Kinyon, 1982).

The only pathogenic treponeme that has been cultured in vitro in or on artificial media is *T. hyodysenteriae* (Harris et al., 1972a, 1972b). Limited multiplication of *T. pallidum* subsp. *pallidum* has been accomplished in a tissue culture monolayer system (Fieldsteel et al., 1981).

Selected differential characteristics of the pathogenic treponemes are shown in Table 1.

Well-defined, cultivable species of treponemes have been found in the oral cavity, intestinal tract, and/or genitalia of chimpanzees, pigs, and dogs, as well as in the rumen of cattle (Bryant, 1952; Cwyk and Canale-Parola, 1979; Hanson, 1970; Harris et al., 1972b; Holdeman et al., 1977; Kinyon and Harris, 1979; Leach et al., 1973; Paster and Canale-Parola, 1982; Paster and Canale-Parola, 1985; Pindak et al., 1965; Smibert and Claterbaugh, 1972; Socransky et al., 1969; Stanton and Canale-Parola, 1979, 1980; Wojciechowicz and Ziolecki, 1979; Ziolecki, 1979; Ziolecki and Wojciechowicz, 1980; Zymet, 1969). In addition, spirochetes resembling treponemes have been observed in the intestinal tract of horses, mice, rats, guinea pigs, opossums, insects, and termites, and in the tissues of brine shrimp (Breznak, 1973; Lee and Philips, 1978; McLeod et al., 1977; Osborn and Bain, 1961; Savage et al., 1971; Turek and Meyer, 1979; Tyson, 1974). Despite the fact that many of these anaerobic and putative microaerophilic host-associated treponemes have been cultured in vitro and subsequently characterized, microscopic studies of animal and human tissues, exudates, and normal flora suggest that these are other species that have not as yet been isolated. Whether these spirochetes, alone or in combination with other organisms, can be implicated as the cause of oral, genital, or gastrointestinal disease remain uncertain; as a result, they should be considered to be nonpathogens.

In humans, as in animals, treponemes are found as part of the normal oral, intestinal, and genital flora (Hanson and Cannefax, 1964; Harland and Lee, 1967; Lee et al., 1971; Parr, 1923; Rosebury, 1962; Ruane et al., 1989; Shera, 1962; Takeuchi et al., 1974). Again, their association with disease remains speculative, and they are considered as nonpathogens. In the oral cavity, they are more prominent in persons with periodontal disease and gingivitis than in people with a healthy gingiva (Addy et al., 1983;

Table 1. Some differential characteristics of pathogenic treponemes.

				Experimental cutaneous lesions in:				
	Natural host	Disease	Cultivable in vitro	Rabbits	Guinea Pigs	Hamsters	Mice	Primates
T. pallidum subsp. *pallidum*	Humans	Syphilis	Limited[a]	Yes	Yes	No	No	Yes[b]
T. pallidum subsp. *pertenue*	Humans	Yaws	No	Yes	No	Yes	No	Yes[c]
T. pallidum subsp. *endemicum*	Humans	Endemic syphilis	No	Yes	Yes	Yes	No	No
T. carateum	Humans	Pinta	No	No	No	No	No	Yes[b]
T. paraluiscuniculi	Rabbits	Venereal spirochetosis	No	Yes	Yes	No	No	No
T. hyodysenteriae	Pigs	Swine dysentery	Yes	No	No	No	No[d]	No

[a]Cultivable only in a tissue culture monolayer system.
[b]In chimpanzees.
[c]In monkeys.
[d]Produces caecitis in CF-1 mice.
Adapted from Smibert (1984).

Armitage et al., 1982; Listgarten and Hellden, 1978; Mikx et al., 1986; Moore et al., 1982, 1983, 1984, 1985, 1987; Offenbacker and Van Dyke, 1985; Simonson et al., 1988). Treponemes and treponeme-like organisms have been isolated from fecal and rectal samples obtained from patients with mild to severe intestinal illness, including homosexual males with and without AIDS (Coene et al., 1989; Jones et al., 1986; Sanna et al., 1982, 1984; Tompkins et al., 1981, 1986; Willen et al., 1985). On the basis of DNA DNA homology studies, some of the treponemes isolated from homosexual men with AIDS appear to be *T. hyodysenteriae* (Coene et al., 1989; Wergifosse and Coene, 1989). The distribution in nature of the known cultivable species and some key unnamed isolates is shown in Table 2.

Additional information on the evolution and biology of treponemes and their distribution in nature can be found in the following reviews (Breznak, 1973; Canale-Parola, 1977, 1978; Harwood, 1984; Holt, 1978; Johnson, 1977; Sell and Norris, 1983; Smibert, 1973, 1976a, 1985).

Morphology and Structure-Function Relationships

Selected General Characteristics

Treponemes, like all spirochetes, (see Chapter 191) are composed of an outer membrane (OM) that delimits the outer cellular surface of the organisms and an inner membrane (IM) that encloses the protoplasmic cylinder (Canale-Parola, 1977; Holt, 1978; Hovind-Hougen, 1976, 1983; Kinyon and Harris, 1979; Johnson et al., 1973; Walker et al., 1989). Unlike the flagella of members of other bacterial genera, spirochetal flagellar organelles lie entirely within the periplasmic space between the IM and OM and are commonly referred to as periplasmic flagella (PF) (Canale-Parole, 1978; Holt, 1978; Hovind-Hougen, 1976; Kinyon and Harris, 1979); older names for PF are endoflagella and axial filaments. The PF attach at either pole of the organism through basal hook structures and wrap around the helical protoplasmic cylinder (Canale-Parola, 1977; Holt, 1978; Hovind-Hougen, 1976). The number of PF and the wavelength of the helix differ among members of this diverse group of organisms. *T. pallidum* and several other treponemes have been shown to possess cytoplasmic fibrils, thin filaments which run the length of the organism just under the cytoplasmic membrane (Hovind-Hougen, 1983). Fig. 1 depicts the ultrastructure of the human pathogen type species, *T. pallidum* subsp. *pallidum.*

Outer Membrane Structure

The outer membrane (OM) of *T. hyodysenteriae* and other cultivable members of the genus *Treponema* appears similar to those of Gram-negative bacteria in structure and chemical makeup (Chatfield et al., 1988; Hovind-Hougen, 1976; E. M. Walker, unpublished observations; Walker et al., 1989; Wannemuehler et al., 1988). However, the lipid-bilayered OM of *T. pallidum* subsp. *pallidum* and *pertenue* differs significantly from these organisms in its strikingly low integral-membrane protein content (Radolf et al., 1989; Walker, unpublished observations; Walker et al., 1989) and its absence of lipopolysaccharide (LPS) (Penn et al., 1985b; Radolf and Norgard, 1988). It is conceivable that the

Table 2. Distribution of cultivable treponemes in nature.

	Oral cavity		Genitals			Intestinal tract		
	Human	Monkey	Human	Monkey	Rumen	Human	Monkey	Pig
T. denticola	+	+						
T. vincentii	+							
T. pectinovorum	+							
T. socranskii	+							
T. skoliodontum	+							
Large oral treponemes	+							
T. phagedenis		+	+	+				
T. refringens		+	+	+				
T. minutum			+					
T. succinofaciens								+
T. bryantii					+			
T. saccharophilum					+			
Pectinolytic rumen treponemes					+			
T. hyodysenteriae								+
T. innocens								+
Large treponemes from homosexual males						+		
Brachyspira aalborgi[a]						+		

Symbols: +, organism present; blank, organism absent.
[a]*B. aalborgi* is included for convenience.

low particle density of the OM of *T. pallidum* subsp. *pallidum* and *pertenue* represents a significant strategy on the part of the organisms for evading the host immune response (Walker, unpublished observations; Walker et al., 1989). The inability to demonstrate LPS in the OM of these *T. pallidum* subspecies does not preclude the possibility that the morphology of the treponemal rare outer membrane protein (TROMP) molecule(s) is determined by lipid-protein interaction (Walker et al., 1989). Studies addressing the integral-membrane protein content and the presence or absence of LPS among the other human treponemal pathogens and the rabbit pathogen *T. paraluiscuniculi* have not been reported.

Subsurface Structures

Treponemal subsurface structures may be defined as those molecules that lie beneath the OM. The most prominent structures are the PF, which mediate the striking translational and axial motility common to all spirochetes (Bromley and Charon, 1979; Goldstein and Charon, 1988; Limberger and Charon, 1986a; Paster and Canale-Parola, 1980). Members of the genus *Treponema* contain two to nine PF inserted into each end of the cell (Smibert, 1984). Intact treponemal PF are composed of multiple polypeptide subunits (Bailey et al., 1987; Blanco et al., 1986, 1988; Cockayne et al., 1987; Hardy et al., 1975; Limberger and Charon, 1986a, 1986b; Norris et al., 1988; Penn et al., 1985a; Radolf et al., 1986; Sand-Petersen et al., 1981). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis has shown that *T. pallidum* subsp. *pallidum* PF is composed of at least four polypeptides with molecular weights of 37, 34.5, 33, and 31 kDa, while the PF of *T. phagedenis* biotypes reiter and kazan 5 are made up of four polypeptides with similar molecular weights (Blanco et al., 1986; Limberger and Charon, 1986a; Norris et al., 1988; Radolf et al., 1986; Sand-Petersen et al., 1981). On the basis of antigenic structure and *N*-terminal amino acid sequence studies, Norris et al. (1988) have divided the flagellar polypeptides of *T. pallidum* and *T. phagedenis* into two classes. The 37-kDa polypeptide of the single sheath or outer surface of these organisms (Blanco et al., 1986; Cockayne et al., 1987; Limberger and Charon, 1986a; Norris et al., 1988; Radolf et al., 1986) has been designated class A; the three proteins related to the core structure of the filament (Cockayne et al., 1987; Penn et al., 1985a), have been designated as class B. The class B proteins of *T. pallidum* subsp. *pallidum* share significant *N*-terminal amino acid sequence homology and antigenic cross-reactivity (Blanco et al., 1986, 1988; Norris et al., 1988) and are encoded by three highly homologous yet distinct structural genes (Champion et al., 1990; Pallesen and Hindersson, 1989). In contrast, the 37-kDa sheath protein of *T. pallidum* subsp. *pallidum* does not possess *N*-terminal amino acid sequence homology with the core proteins (Blanco et al., 1988; Isaacs et al., 1989;

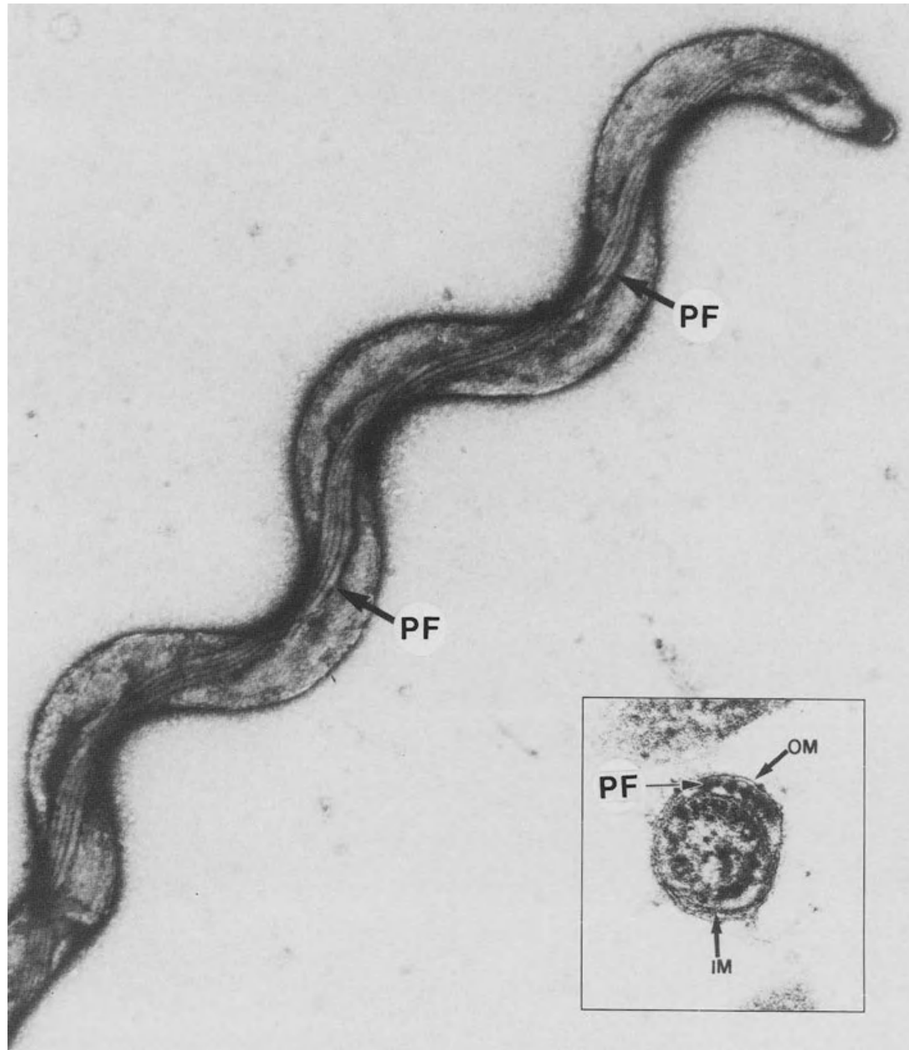

Fig. 1. Transmission electron micrograph of whole-mounted *Treponema pallidum* subspecies *pallidum* negatively stained with uranyl acetate. Inset: transmission electron micrograph of thin-sectioned *Treponema pallidum* subspecies *pallidum*, cross-sectional plane of view. PF, periplasmic flagella; OM, outer membrane; IM, inner membrane. Photograph and permission to use by E. M. Walker.

Norris et al., 1988). The PF of *T. pallidum* subsp. *pallidum* and *T. phagedenis* share considerable antigenic and structural homology (Bharier and Allis, 1974; Blanco et al., 1986, 1988; Hardy et al., 1975; Norris et al., 1988; Penn et al., 1985a; Radolf et al., 1986; Sand-Peterson et al., 1981). Indeed, the PF of *T. pallidum* subsp. *pallidum* may represent a key virulence factor as well as a secondary target for the protective immune response that develops during the course of syphilitic infection. Evidence to support this hypothesis is provided by 1) the presence in normal human serum of complement-dependent treponemicial antibodies to shared PF epitopes (Blanco et al., 1986); 2) the existence of pathogen-unique epitopes on the surface of the *T. pallidum* subsp. *pallidum* Class A and Class B polypeptides (Blanco et al., 1988; Norris et al. 1988); 3) the altered course of disease in response to *T. pallidum* subsp. *pallidum* challenge following vaccination of rabbits with *T. pallidum* subsp. *pallidum* PF (Champion, C. I., Miller, J. N., Lovett, M. A., Blanco, D. R., unpublished observations); and 4) the potential role of motility as a factor that enables the organism to disseminate by invading the tight junctions between endothelial cells (Thomas et al., 1988).

Their unique properties may also be relevant to treponemal vaccine development and to the construction of immunologically specific and sensitive serodiagnostic assays. The cloning and

sequencing of the *T. pallidum* subsp. *pallidum* PF structural genes that encode for the class A and Class B proteins (Champion et al., 1990; Isaacs et al., 1989; Pallesen and Hindersson, 1989) and the ability to express the two genes that encode for the 34.5-and 31-kDa class B polypeptides in *Escherichia coli* (Champion et al., 1990), permit novel approaches to these problems.

The PF of *T. hyodysenteriae* are composed of 37-, 34-, and 32-kDa polypeptides (Miller et al., 1988). Furthermore, it has been shown that the epitopes residing on the 33-kDa PF polypeptide of *T. phagedenis* biotype kazan 5 are found on each of these three proteins. The protection of mice against *T. hyodysenteriae* challenge by vaccination with a crude, cloned PF antigen has been reported (Boyden et al., 1989).

In addition to the proteins that make up the PF subunit structures, several antigenic polypeptides from *T. pallidum* subsp. *pallidum* and *pertenue,* ranging in molecular weights from 12 to 190 kDa, have been identified by immunoprecipitation (Alderete and Baseman, 1981; Baseman and Hayes, 1980; Moskophidis and Muller, 1984), cross-over immunoelectrophoresis (Pedersen et al., 1981; Penn and Rhodes, 1982), and SDS-PAGE-Western blot analysis (Baker-Zander et al., 1985; Fehniger et al., 1984; Hanff et al., 1983, 1982; Lukehart et al., 1982; Thornburg and Baseman, 1983; van Eijk and van Embden, 1982). Characterization of *T. pallidum* subsp. *pallidum* by two-dimensional electrophoresis has revealed at least 78 antigenic polypeptides (Norris and Sell, 1984; Norris et al., 1987). Phase partitioning with the nonionic detergent Triton X-114 has identified seven to eight *T. pallidum* subsp. *pallidum* antigenic polypeptides in the hydrophobic phase with molecular weights of 14 to 57 kDa (Cunningham et al., 1988; Radolf et al., 1988). Recent evidence indicates that the majority of these antigens are, in fact, proteolipids, which may anchor to the cytoplasmic membrane or OM by means of their covalently attached fatty acids (Chamberlain et al., 1989a, 1989b; Swancutt et al., 1990). Three of these proteolipids, the pathogen-specific 47-, 34-, and 15-kDa antigens (Radolf and Norgard, 1988), stimulate immobilizing antibody (47 and 34 kDa), treponemicidal antibody (47 kDa), and lymphocyte proliferative responses (15 kDa) (Baker-Zander et al., 1988; Jones et al., 1984; Robertson et al., 1982). As with the PF of *T. pallidum* subsp. *pallidum,* the role of these proteolipids antigens—as virulence factors, target molecules for the protective immune response in syphilitic infection, as well as their vaccinogenic capabilities—is speculative. However, their pathogen specificity points to the possibility for development of serodiagnostic antigens of high immunological specificity and sensitivity. The cloning, expression, and sequencing of the genes that encode for the 47-, 34-, and 15-kDa antigens (Chamberlain et al., 1988; Hsu et al., 1989; Norgard et al, 1986; Purcell et al., 1989; Swancutt et al., 1986; Swancutt et al., 1989) and other proteins should provide the armamentaria for more detailed and accurate structure-function analysis as well as for the development of more accurate serodiagnostic assays.

Recently, Masuda and Kawata (1989) have isolated and characterized the cytoplasmic fibrils of *T. phagedenis* and other treponemes. The cytoplasmic fibrils are comprised of a single, major, 80-kDa polypeptide; a similar protein is present in *T. pallidum* subspecies and may represent the cytoplasmic fibril subunit in these organisms. The function of the cytoplasmic fibrils is unknown. They may, however, be involved in motility in some way, inasmuch as they run parallel to the endoflagella.

The major 60-kDa polypeptide of *T. pallidum* subsp. *pallidum* has been shown to be the subunit of a multimeric protein homologous to the *groEL* chaperonin protein of *E. coli* (Houston et al., 1990). These chaperonins are found in all prokaryotes and eukaryotes and apparently are involved in protein folding and assembly. Similar proteins exist in the other treponemes.

Isolation, Identification, and Characterization

Human Treponemal Pathogens

METHODS OF ISOLATION AND CULTIVATION. *T. pallidum* subsp. *pallidum* is the only human treponemal pathogen that has been grown in vitro (Fieldsteel et al., 1981). However, continuous in vitro culture has not been achieved, thus precluding the use of this procedure for isolation and identification. Isolation of *T. pallidum* subsp. *pallidum, pertenue,* and *endemicum,* along with the rabbit pathogen *T. paraluiscuniculi,* is conducted for the purpose of experimental investigation; infectious tissue or exudates are inoculated into adult male rabbits by either the intratesticular or intradermal routes. *T. carateum* is isolated by the intradermal inoculation of chimpanzees (see Table 1 for animal susceptibility to cutaneous lesions). Detailed procedures for the preparation of suspensions and methods of inoculation, and accepted criteria for determining infectivity, and identification by dark-field microscopy have been described by Turner and Hollander (1957) and Miller (1971). The development of lesions in these animals characteristic for each

species and subspecies of *Treponema* (Turner and Hollander, 1957; Miller, 1971) as well as the pattern of lesion development in human disease (see Table 1 and Miller, 1987) are useful criteria for identification. Several isolates of *T. pallidum* subspecies continue to be propagated by intratesticular passage in rabbits. The Nichols strain of *T. pallidum* subsp. *pallidum* has been passaged continuously by this route since 1912. It has been studied more extensively than any other isolate and is the strain used in the preparation of treponemal antigens for immunologically specific serodiagnostic assays.

The limited in vitro multiplication of *T. pallidum* subsp. *pallidum* in a tissue culture system was first described by Fieldsteel et al. (1981). Treponemes extracted from infected rabbit tests are inoculated into cultures of Sf1Ep rabbit epithelial cells (American Type Culture Collection, Rockville, MD, USA). The medium used is a modification of Eagle's minimal essential medium with Earl's salts containing 13.9 mM D-glucose, 0.63 mM dithiothreitol, 31.3 mM HEPES (*N*-2-hydroxyethylpiperizine-*N'*2-ethane sulfonic acid) buffer (pH 7.4), 0.126 mg/ml resazurin, and 20% fetal bovine serum or calf serum inactivated at 56°C for 30 minutes. Optimal conditions for growth consist of incubation at 33–35°C in an atmosphere containing 1.5 to 5% O_2, 5% CO_2, and the balance N_2 (Fieldsteel et al., 1982). *T. pallidum* subsp. *pallidum* attaches to the Sf1Ep cells and multiplies on the mammalian cell surface (Fieldsteel et al., 1981; Konishi et al., 1986). Under these conditions, the Nichols strain of *T. pallidum* subsp. *pallidum* can increase in numbers up to 100-fold and maintain viability and infectivity for rabbits for 12 to 15 days. Although several strains of *T. pallidum* subsp. *pallidum* multiply in this system, isolates of *T. pallidum* subsp. *pertenue* and *endemicum* fail to grow (Cox et al., 1984). Attempts to extend multiplication by subculture have thus far been unsuccessful (Norris and Edmondson, 1986a).

Little is known about the growth requirements of *T. pallidum*. Treponemal multiplication does not occur in the absence of mammalian cells; the reason for this dependence has not been determined. The requirement for serum is stringent, and the required components are associated with the protein fraction (Norris and Edmondson, 1986b). Inasmuch as only certain lots of fetal bovine or calf sera are effective in maintaining growth, serum lots must be prescreened for this activity. Human serum inactivated at 56°C for 30 min can also fulfill the serum requirement (Norris and Edmondson, 1986b). Optimal multiplication at 1.5 to 5% O_2 (Fieldsteel et al., 1982) and metabolic and survival studies (reviewed by Cox, 1983; Jenkin and Sandok, 1983; Sell and Norris, 1983) provide evidence that *T. pallidum* subsp. *pallidum* is microaerophilic. The fact that *T. pallidum* subsp. *pallidum* (Nichols) grows best at 33 to 35°C (Fieldsteel et al., 1982) mirrors its in vivo behavior, in which the infection in rabbits is enhanced in cooler regions of the body, such as the testes and exposed areas of the skin (Miller, 1971; Turner and Hollander, 1957).

IDENTIFICATION IN EXUDATES AND TISSUES. The restrictions of in vitro cultivation, together with the impracticability of animal inoculation, preclude the use of these isolation procedures in the diagnosis of treponemal disease. At present, the identification of treponemes in lesion exudates from patients with early acquired treponemal disease or early congenital syphilis is usually attempted by dark-field microscopy, in which identification is based upon the observation of characteristic morphology and motility. Unfortunately, this assay has several limitations that, in light of the similarities in clinical manifestations between treponemal and other diseases and the lack or inconclusive nature of serological reactivity, can and do cause errors in differential diagnosis. The following represent the most important limitations of dark-field microscopy:

1. *Limited criteria for T. pallidum identification:* the organisms observed must exhibit both the characteristic morphology and motility of *T. pallidum* in order to establish identification. The potential presence of host indigenous, nonpathogenic treponemes with similar characteristics in the oral cavity, genitalia, and gastrointestinal tract preclude an unequivocal identification from these sites. Indeed, dark-field examination of lesions within the oral cavity and the anal region is most often contraindicated due to the potential presence of nonpathogenic spirochetes.
2. *Necessity for rapid examination of prepared slides:* the relatively rapid loss of motility in the presence of atmospheric O_2 necessitates that slide specimens be examined within a short period of time after preparation (15–30 minutes) to ensure the observation of characteristic movement. This restriction not only reduces the chances for accurately identifying organisms in the exudate but also reduces the probability of observing the treponemes that might be present.
3. *Relatively poor sensitivity:* dark-field examination of lesion exudates from patients with early treponemal disease may be negative, despite the presence of *T. pallidum.* This lack of sensitivity is not surprising. Dark-field examination of *T. pallidum* suspensions con-

taining 10^6 organisms per ml was the equivalent of 1 organism per high dry field (HDF) when utilizing a calibrated microscope (J. Miller, unpublished observation). By extrapolation, 10^3 treponemes per ml of lesion exudate, a significant number of organisms, would present as 1 organism per 1,000 HDF and would not be detectable.

4. *Technical difficulty:* The identification of *T. pallidum* by dark-field microscopy requires the availability of a specially equipped microscope as well as considerable training, experience, and expertise. This combination is generally not available at most health care facilities. As a result, dark-field examination may not be done or may be performed incorrectly. Again, because antibody may be lacking or its presence is inconclusive, this creates a serious problem for both physicians and health care officers responsible for controlling spread of the disease.

These limitations associated with dark-field examination have been recognized since the procedure was first described, and, as might be expected, attempts have been made to develop a more efficient method. A proposed assay utilizes the principle of direct immunofluorescence and is referred to as the Direct Fluorescent Antibody Test for *T. pallidum* (DFA-TP) (Kellogg, 1970). With this procedure, lesion material or tissue sections are fixed to a microscopic slide and combined with Flourscein isothiocyanate labeled rabbit anti-*T. pallidum* immunoglobulin conjugate previously absorbed with cross-reacting nonpathogenic treponemes to render it specific for *T. pallidum.* Following a 30-min incubation period and washing, the slide is examined for the presence or absence of treponemes by fluorescence microscopy. This immunological procedure provides the opportunity to examine accurately not only smears from oral and anal lesions, but also tissue sections for the presence of treponemes. In addition, the organisms need not be motile, thus eliminating the need for immediate examination. The use of adequate controls with this assay is essential, because conjugates of poor specificity due to incomplete absorbance of cross-reacting antibodies may give misleading results.

More detailed information on the collection, submission, preparation, and examination of specimens for dark field examination and DFA-TP is contained in the excellent review by Larsen et al. (1984).

Silver impregnation methods have proved valuable in the identification of treponemes in tissues. However, their technical difficulty, poor reproducibility, and presence of artifacts resembling treponemes often make identification difficult. The modified Krajian procedure is one of many available silver impregnation methods and is described in detail by Miller (1971), along with techniques for tissue fixation, embedding, and sectioning.

A discussion of nontreponemal and treponemal tests in the serological diagnosis of treponemal infection is beyond the scope of this chapter. However, an excellent detailed presentation of the principles, procedures, interpretations of results, precautions, and potential problems associated with these serological assays is contained in the review by Larsen et al. (1984).

Treponemes Cultivable in or on Artificial Media

NUTRITIONAL AND ATMOSPHERIC REQUIREMENTS FOR GROWTH. A summary of some key nutritional and atmospheric requirements of the cultivable treponemes is given in Table 3.

Treponemes cultured in or on artificial media are strict anaerobes or microaerophiles. Growth and propagation require patience and an appreciation of their nutritional and metabolic requirements. These requirements differ for each species. Some treponemes need serum in the culture medium while others need short-chain fatty acids (Smibert, 1973, 1976a). Usually, any animal serum can be used in final concentrations ranging from 5 to 10%. All sera used in a medium must be inactivated at 60°C for 30 min. The serum provides the required long-chain fatty acids. There is very little free fatty acid in serum, and the long-chain acids are part of the neutral fats and phospholipids present. Most treponemes have a lipase that acts to liberate fatty acids. The only phospholipid in serum found to be used by five species was lysophosphatidylcholine (Trevathan et al., 1982). Two different pathways of phospholipid metabolism were proposed for two groups of the species studied. The long-chain fatty acids required by treponemes are not the same for all species studied. Van Horn and Smibert (1982) reported that *T. denticola* and *T. vincentii* require oleic acid (*cis*-18:1[9]). Elaidic acid (*trans*-18:1[9]) also supports growth but to a lesser extent than the *cis* form. No other short-or long-chain fatty acids support growth of these two species. The main component of serum containing the fatty acids that support growth is the alpha-globulin fraction. Serum albumin is also needed to "detoxify" or bind the fatty acids in order to keep their concentration at a nontoxic level (van Horn and Smibert, 1982). Johnson and Eggebraten (1971) reported that *T. phagedenis* requires a pair of fatty acids, one being saturated

Table 3. Some nutritional and atmospheric requirements of cultivable treponemes.

	Serum[a]	Volatile fatty acids[b]	Thiamine pyrophosphate[c]	Requires carbohydrate[d]	Aerated medium[e]
T. denticola	+	−	+	−	−
T. vincentii	+	−	+	−	−
T. skoliodontum	+	−	+	−	−
T. phagedenis	+	−	−	+	−
T. refringens	+	−	−	−	−
T. minutum	+	−	−	−	−
T. hyodysenteriae	+	−	−	+	+
T. innocens	+	−	−	+	+
Large treponemes from homosexual males	+	−	−	+	+
Brachyspira aalborgi[f]	+	−	−	−	−
T. socranskii	−	+	−	+	−
T. pectinovorum	−	+	−	+	−
T. bryantii	−	+	−	+	−
T. succinofaciens	−	+	−	+	−
T. sacchrophilum	−	+	−	+	−

[a]Inactivated animal serum.
[b]Rumen fluid or volatile fatty acid mixture.
[c]Addition of filter-sterilized TPP to medium.
[d]Needs a fermentable carbohydrate as an energy source for growth.
[e]Addition of some air (oxygen) to prereduced medium.
[f]*Brachyspira aalborgi* is included for convenience.

with at least 14 carbons and the other acid unsaturated with at least 15 carbons with one, two, or three double bonds. Elaidic acid (*trans*-18:1(9)) can be used in place of the pair of acids. Lemcke and Burrows (1980) reported that *T. hyodysenteriae* requires cholesterol for growth and that serum can be replaced by cholesterol and serum albumin. Sitosterol and cholestanol are the only sterols that can replace cholesterol. Stanton (1985) reported that the organism needs cholesterol and the phospholipid phosphatidylcholine, (Stanton, 1987; Stanton and Cornell, 1987). The sterol can be obtained from serum or red blood cell membranes. Cholesterol is converted to cholestanol, which is incorporated into the treponemal cell membrane. Thus, this organism can grow with only cholesterol and phosphatidylcholine added to the culture medium. The requirement for short-chain fatty acids can only be met by the addition of either rumen fluid or a mixture of short-chain fatty acids (Smibert, 1973, 1976a).

Socransky et al. (1969) reported the isolation of an oral treponeme that needs isobutyric acid. Smibert and Claterbaugh (1972) reported isolates from pig rectal and intestinal samples that require glucose, heme, isobutyric acid, and *n*-valeric acid. Hardy and Munro (1966) described an oral isolate that needs isobutyric acid, and Wegner and Foster (1966) reported on a rumen isolate that needs both isobutyric acid and *n*-valeric acid. Rumen fluid from cattle will supply acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, and *n*-valeric acid as well as heme. A medium with rumen fluid can be autoclaved and is used in the medium at final concentration of 20 to 30%. The short-chain fatty acid requirement can also be met by an artificial rumen fluid (volatile fatty acid and heme mixture) described by Caldwell and Bryant (1966). (See also Holdeman et al., 1977, for the formula of medium 10, which contains the artificial rumen mix; and Smibert et al., 1984, for the mixture used in oral treponeme cultures.)

Artificial Rumen Fluid Mixture (Caldwell and Bryant, 1966)

Acetic acid	17 ml
Propionic acid	6 ml
n-Butyric acid	4 ml
n-Valeric acid	1 ml
Isovaleric acid	1 ml
Isobutyric acid	1 ml
DL-Methylbutyric acid	1 ml

Adjust the pH to 7.0.
Add 0.31 ml of the above to 100 ml of medium.

Heme Solution

Heme solution is prepared by combining 50 mg of heme or hemin, 1 ml of 1 N NaOH, and 100 ml distilled water. Add 1 ml to 100 ml of medium.

While rumen fluid, the above artificial rumen fluid mixture, and the heme solution can be

autoclaved, serum must be filter sterilized using a 0.2-μm-pore-size membrane filter. Both serum and rumen fluid (or fatty acid mixture and heme,) can be used together in the same medium in order to have a universal medium that can support growth of both kinds of treponemes.

Unfortunately, there are also other nutritional considerations to be considered. The serum-requiring oral treponemes require thiamine pyrophosphate for growth (Austin and Smibert, 1982), while the short-chain fatty acid-requiring oral, intestinal, and rumen treponemes need a fermentable energy source in the medium. Glucose will serve as an energy source for many strains while other carbohydrates *may* be the best energy source for others. Some organisms have a need for a unique energy source; for example, *T. pectinovorum,* an oral species, requires pectin (or glucuronic or galacturonic acid) for growth (Smibert and Burmeister, 1983); no growth occurs with other carbohydrates. Even some serum-requiring treponemes may need a fermentable energy source for growth; *T. phagedenis* needs glucose for growth in a serum-containing medium (George and Smibert, 1982a, 1982b).

Atmospheric conditions are also important factors to be considered for the growth of treponemes. Most organisms require a highly reduced medium for optimum growth, while others need either a less reduced medium or a small concentration of air in a reduced medium. As a rule of thumb, the short-chain fatty acid-requiring organisms need a highly reduced medium, while most of the serum-requiring treponemes have a lesser need for a well-reduced medium. *T. hyodysenteriae* and *T. innocens* will not grow in a highly reduced medium. For growth in roll tubes, 1 ml of air is injected into each tube. Although, *T. hyodysenteriae* will grow in a system containing 1 to 5% O_2, maximum cell yields occur when the culture is stirred in a 1% atmosphere. No growth occurs in a medium with less than 1% O_2 or with more than 5% O_2 (Stanton and Lebo, 1988). When growing these organisms on blood agar medium, it is best to use ordinary aerobic plates (not prereduced) and to incubate them in anaerobic jars using conventional anaerobic methods. The treponemes isolated from the intestinal tract of homosexual males with diarrhea (Jones et al., 1986) also require 1% O_2 for growth. Two procedures for growing most treponemes in order to attain high yields are the roll-tube and the anaerobic chamber methods (Rosebury and Reynolds, 1964); the roll tube method is the most convenient.

METHODS OF ISOLATION AND MICROSCOPIC OBSERVATION. Isolation of treponemes can be accomplished with an anaerobic chamber, anaerobic jars, or roll tubes. When either a chamber or anaerobic jar system is used for isolation, solid media in agar plates are used. When a roll-tube system is used, the isolation medium is usually liquid or semisolid. Anaerobic jars should be used properly to ensure good anaerobic conditions when isolating treponemes with strict anaerobic requirements. The cold catalyst should be changed after one or two uses and replaced with fresh catalyst. A used catalyst can be reactivated by placing the pellets in an oven at 110–130°C for several hours. The gas mixture used in anaerobic jars can be either a hydrogen-generating system, such as the GasPak (BBL) envelope, or a tank gas mixture containing H_2 and CO_2, such as 95% H_2–5% CO_2. Samples for isolation of treponemes can be used directly or diluted in anaerobic broth. They can be either fecal, rumen, genital, intestinal, intestinal mucosal scrapings, or oral plaque. All samples to be cultured for treponemes should be examined by dark-field microscopy. The number and size of the treponemes should be noted, and the data matched with the results of isolation. Subsequent observations for growth and multiplication should be conducted by dark-field microscopy.

GENERAL METHODS. The following general methods have been used to isolate treponemes from samples that contain other faster growing bacteria:

SELECTIVE MEDIA. These are media that contain antibiotics to suppress other bacteria (Leschine and Canale-Parola, 1980; Smibert et al., 1984; Songer et al., 1976; Stanton and Canale-Parola, 1979). Samples are either streaked onto agar plates or inoculated into a broth medium containing antibiotics. This method will suppress most of the oral, intestinal, or rumen flora and allow growth of the slower growing treponemes in the selective medium (Fiehn and Frandsen, 1984; Lee and Philips, 1978; Leschine and Canale-Parola, 1980; Smibert et al., 1984; Songer et al., 1976).

Selective Method of Smibert for Isolation of Oral, Genital, and Intestinal Treponemes (Smibert and Burmeister, 1983; Smibert et al., 1984)

The samples are placed in a tube with a prereduced buffer containing small glass beads, and the cells dispersed on a Vortex mixer for 5–10 seconds. Serial 10-fold dilutions are made and each dilution inoculated into tubes of prereduced Oral Treponeme Isolation (OTI) broth (see below) containing rifampin (2 μg/ml)

and polymyxin (800 U/ml). The cultures are incubated at 37°C for 1 to 2 weeks and the presence of treponemes determined weekly by dark-field microscopy.

OTI Medium of Smibert

Polypeptone (BBL)	5.0
Heart infusion broth (BBL)	5.0 g
Yeast extract	5.0 g
Pectin	0.8 g
Glucose	0.8 g
Soluble starch (Difco)	0.8 g
Sucrose	0.8 g
Maltose	0.8 g
Xylose	0.8 g
Ribose	0.8 g
Fructose	0.8 g
Sodium pyruvate	0.8 g
K_2HPO_4	2.0 g
NaCl	5.0 g
$MgSO_4$	0.1 g
L-Cysteine-HCl	0.68 g
Clarified rumen fluid	500 ml
Distilled water	500 ml

Adjust the pH to 7.0–7.2.

OTI agar medium contains 0.8% purified agar (Oxoid), while OTI broth medium contains 0.16% agar. OTI base medium is boiled, and the broth base is put into tubes containing an atmosphere of nitrogen. The tubes are rubber stoppered and autoclaved in a rack, which is then placed into a press in order to keep the stoppers in place. OTI agar base medium is autoclaved in a flask, cooled to 45°C under a stream of N_2. (See Holdeman et al., 1977, for details of preparing prereduced media.) The agar medium is poured into petri dishes, and then placed in anaerobe jars when the agar solidifies. The jars are evacuated and filled with a gas mixture of N_2, H_2, and CO_2; GasPak (BBL) envelopes may be used. The plates are held prereduced in the jars until used. Heat-inactivated serum and thiamine pyrophosphate are added to OTI medium to a final concentration of 5–10% and 0.75–1.5 mg/100ml respectively (100 ml of serum and 1 ml of an aqueous solution of thiamine pyrophosphate, 500 mg/ml). Occasionally, organisms require a filter-sterilized yeast autolysate, which is added to the medium at a final concentration of 5%. The yeast autolysate is prepared by adding 56 g of dry Bakers yeast to 200 ml of distilled water and incubating at 56°C for 4 days. The yeast cells are centrifuged and removed and the clear supernatant fluid is filter sterilized.

Selective Method of Stanton and Canale-Parola for Isolation of Rumen Treponemes (Paster and Canale-Parola, 1985; Stanton and Canale-Parola, 1979)

Rumen samples are serially diluted into agar medium tubes containing 1 μg/ml of rifampin. The gas phase of the prereduced medium is CO_2. After incubation at 39°C, typical treponemal colonies are picked with a Pasteur pipette and subcultured in broth medium. Colonies in agar tubes are described as round "cotton balls," a "veil-like" growth with denser center, and like a "transparent bubble."

Selective Rumen Fluid Cellobiose (RFC) Agar

Clarified rumen fluid	30 ml
Salt solution A	20 ml
Salt solution B	20 ml
Resazurin (0.1% solution)	0.1 ml
Distilled water	19 ml
L-Cysteine-HCl	0.1g
Noble agar (Difco)	0.7 g

Adjust the pH to 6.7–7.0.

Salt solution A contains $CaCl_2$, 0.45 g; $MgSO_4$, 0.45 g; and 1 liter distilled water. Salt solution B contains KH_2PO_4, 2.25 g; K_2HPO_4, 2.25g; NaCl, 4.5 g; $(NH_4)_2So_4$, 4.5 g; and 1 liter distilled water. The base RFC medium is boiled to reduce resazurin and dispensed into culture tubes using a gas phase of CO_2 after which the tubes are rubber stoppered and autoclaved. $NaHCO_2$ (5%) and cellobiose (10%) stock solutions are filter sterilized and pipetted into each tube. Rifampin (10 μg/ml) is added to the $NaHCO_2$ stock solution. One ml of the $NaHCO_2$ solution and 0.1 ml of the cellobiose solution is added to 8.9 ml of base medium. Final concentration of rifampin is 1 μg/ml. RFC broth is made without agar.

GM-1 and NOS Selective Method for Isolation of Oral Treponemes (Blackmore and Canale-Parola, 1976; Leschine and Canale-Parola, 1980; Weber and Canale-Parola, 1984).

Gingival samples are taken with sterile toothpicks, and the toothpicks placed into tubes containing either prereduced GM-1 or NOS medium in a N_2 atmosphere. The tubes are shaken and serially diluted into tubes of melted GM-1 or NOS agar containing 2 μg/ml of rifampin. After incubation at 37°C, colonies appearing like "cotton balls" and "transparent bubbles" are removed with a Pasteur pipette and subcultured.

GM-1 Medium

Trypticase (BBL)	0.5 g
Yeast extract	0.25g
NaCl	0.25 g
Sodium thioglycolate	0.05 g
L-Cysteine-HCl	0.1 g
Distilled water	100 ml

The medium is adjusted to a pH of 7.4, autoclaved, and then cooled rapidly to 35°C. The sample is inoculated into the medium after adding the following supplements (per 100 ml of medium): 0.2% thiamine pyrophosphate, 0.3 ml; volatile fatty acid solution, 0.5 ml; 10% sodium bicarbonate, 0.5 ml; and heat-inactivated rabbit serum, 0.3 ml. The volatile fatty acid solution consists of 0.5 ml each of isobutyric, DL-2-methylbutyric, isovaleric, and valeric acids dissolved in 100 ml of 0.1 N KOH. Rifampin is added to the medium at a final concentration of 2 ug/ml.

NOS Medium

Heart infusion broth (Difco)	1.24 g
Trypticase (BBL)	1.0 g
Yeast extract	0.25 g
Sodium thioglycolate	0.05 g
L-Cysteine-HCL	0.1 g
L-Asparagine	0.025 g

Glucose	0.2 g
Distilled water	100 ml
Noble agar (Difco)	0.7 g

The base medium is supplemented with the following: 0.2% thiamine pyrophosphate, 0.3 ml; volatile fatty acid mixture prepared as in GM-1 medium, 0.2 ml; 10% sodium bicarbonate, 2.0 ml; and heat-inactivated rabbit serum, 2.0 ml. Each of the supplements are filter sterilized and added to the prereduced base medium. Other carbohydrates such as pectin can be used in place of glucose in the NOS medium to isolate treponemes with different carbohydrate requirements. Fiehn and Frandsen (1984) reported that rifampin 2 μg/ml; polymyxin, 800 U/ml; and nalidixic acid, 75 μg/ml in GM-1 blood agar give the best recovery of oral treponemes and suppress most other kinds of oral bacteria.

Selective Method for Isolation of *T. hyodysenteriae* from Pigs with Swine Dysentery (Songer et al., 1976)

The mucosa of the large intestine of pigs is washed to remove intestinal contents. Colonic mucosal scrapings of lesions from pigs with acute swine dysentery are streaked onto selective isolation agar medium that has not been prereduced. The inoculated plates are incubated in anaerobic jars containing 80% H_2 and 20% CO_2; a GasPak envelope (BBL) can also be used. The plates are examined after 2, 4, and 6 days of incubation for areas of beta hemolysis with little or no surface growth in the hemolytic zone. The hemolytic zone is scraped with a loop, streaked onto Trypticase soy agar plates supplemented with citrated bovine blood, and incubated in an anaerobic jar at 37°C for 3–5 days.

TSA-5400 Selective Method for *T. hyodysenteriae*

Trypticase soy agar (BBL)	100 ml
Citrated bovine blood	10%
Spectinomycin (Upjohn)	400 μg/ml

The agar plates are poured under aerobic conditions and can be stored like ordinary aerobic media. Other antimicrobial agents can be used in the medium for isolation of this organism. The medium can be supplemented with polymyxin B, 200 U/ml, or spectinomycin, 200 μg/ml; vancomycin, 25 μg/ml; and colistin sulfate, 25 μg/ml (Jenkinson and Wingar, 1981; Kitai et al., 1987).

Selective Method for Isolation of Large Treponemes from Human Feces (Hovind-Hougen et al., 1982; Jones et al., 1986; Sanna et al., 1982; Tomkins et al., 1986)

Stool specimens from homosexual males with diarrhea are examined by dark-field microscopy for the presence of treponemes. Positive samples are streaked onto selective medium.

Selective Method of Jones et al. (1986)

Trypticase soy agar (BBL)	100 ml
Citrated human blood or defibrinated horse blood	5%
Spectinomycin	400 μ/ml
Polymyxin B	5 μg/ml

Plates are incubated in anaerobic jars for 5–7 days at 37°C. A haze of growth on the surface of the agar is examined for treponemes and subcultured onto fresh blood-agar selective plates; slight beta hemolysis is sometimes seen. Tomkins et al. (1986) used a similar medium containing 400 μg/ml of spectinomycin, while Sanna et al. (1982) employed Trypticase soy-7% horse blood agar supplemented with 400 μg/ml of spectinomycin and 15 μg/ml of rifampin. Houvind-Hougen et al. (1982) used Trypticase soy-10% calf blood supplemented with 40 μg/ml of spectinomycin and 5 μg/ml of Polymyxin B for the isolation of *Brachyspira aalborgi.*

Selective Method of Lee and Philips (1978) for Isolation of Putative Treponemes from the Intestines of Mice and Rats

Blood agar base no. 2 (Oxoid)	100 ml
Lysed horse blood	7%
Polymyxin B	80 μg/ml

The cecum of mice and rats is removed and a piece of tissue washed in physiological saline. The mucosal surface is scraped and inoculated onto the selective medium. Plates are incubated for 2–3 days in anaerobic jars.

MEMBRANE-FILTER METHODS. Membrane-filter methods have been described by Hardy et al. (1964); Hunter and Ross (1972); Loeche and Socransky (1962); Smibert and Burmeister (1983); Smibert et al. (1984); Smibert and Claterbaugh (1972); Socransky et al. (1969); and Taylor and Alexander (1971). They utilize the ability of treponemes to move through the pores of a membrane filter that will hold back most other bacteria. These methods are limited, therefore, to the isolation of treponemes smaller than most other bacteria. Samples are inoculated onto membrane filters that are placed on top of agar media, which may also contain antimicrobial agents that make the media selective. Motile treponemes migrate through the pores of the filter and grow in the agar medium. The unavailability of Ionagar No. 2 from Oxoid has necessitated the substitution of Purified agar from Oxoid (R. Smibert, unpublished observations).

Membrane-Filter Method of Smibert for Isolation of Intestinal and Oral Treponemes (Smibert and Burmeister, 1983; Smibert and Claterbaugh, 1972; Smibert et al., 1984)

Oral, intestinal, or rumen samples diluted 10-fold are mixed with a Vortex mixer for 1 min. Several drops of each dilution are placed onto the surface of a 45-mm diameter (0.22-μ pore size) membrane filter which is on the surface of any of the agar medium mentioned in this Chapter. A sterile O-ring, 1 inch in diameter, is first placed on the filter, and a seal of sterile 2% agar is placed around the O-ring. The O-ring prevents other bacteria from swarming or being washed off the filter and growing on the agar medium. The plates are incubated in an

anaerobic jar in a gas mixture containing 95% H_2 and 5% CO_2 at 37°C for 1–2 weeks. GasPak envelopes (BBL) or anaerobic chambers can also be used. The membrane filter is removed and a white haze of growth is seen in the depth but not on the surface of the agar. A plug of growth near the leading edge of the haze is cut out with a loop or Pasteur pipette and examined by dark-field microscopy for treponemes. A larger plug of agar is removed and placed in a tube of prereduced broth medium. The medium used for isolation of treponemes by this method contains serum, rumen fluid, or volatile fatty acid-heme solution. The rumen fluid and/or volatile fatty acid solution can be omitted from the medium if culturing only for serum-requiring organisms. Short-chain fatty acid-requiring treponemes do not require serum in the medium.

Agar plates with filter and O-ring should be prereduced by incubation in anaerobic jars prior to use. Uninoculated and inoculated plates should be kept in separate anaerobic jars under a stream of CO_2. This procedure prevents the medium from becoming oxidized and limits the plates' exposure to air.

Antibiotics such as rifampin (2μ/ml) and polymyxin B (800 U/ml) can be included in the media; other antibiotics such as nalidixic acid (75μ/ml) can also be used (Fiehn and Frandsen, 1984).

Smibert and Claterbaugh (1972) Isolation Medium

Polypeptone (BBL)	0.5 g
Yeast extract	0.5 g
Glucose	1.4 g
Soluble starch	0.5 g
Ammonium sulfate	0.5 g
L-Cysteine-HCl	0.68 g
Resazurin	1.6 mg
Bovine rumen fluid	280 ml
Distilled water	220 ml
Salt solution	500 ml
Purified agar (Oxoid)	8 g

Adjust to pH 6.5 (yields a pH of 7.2–7.5 after autoclaving).

The salt solution contains K_2HPO_4, 1 g; KH_2PO_4, 1 g; $NaHCO_3$, 10 g; NaCl, 2 g; $CoCl_2$, 0.0034 g; $NaMoO_4$, 0.0034 g; $MnSO_4$, 0.0034 g; $CaCl_2$, 0.2 g; $MgSO_4$, 0.2 g; and 1 liter distilled water.

Membrane-Filter Method of Taylor and Alexander (1971) for Isolation of Pig Intestinal Treponemes

Colon samples are diluted in prereduced peptone-yeast extract broth and centrifuged at 700× for 10 min. The supernatant fluid is filtered through a 0.65μ Millipore filter, and a few drops of the filtrate spread onto the surface of prereduced 10% horse blood agar plates. The plates are incubated in an anaerobic jar for 48 hours. Areas of complete hemolysis with no apparent surface growth are examined for treponemes by dark-field microscopy and subcultured.

Membrane-Filter Method of Hunter and Ross (1972) for Isolation of Pig Intestinal Treponemes

Pieces of large intestinal mucosa and mucosal scrapings from pigs with swine dysentery are suspended in a small volume of nutrient broth and incubated for 4 hours at 37°C. A sterile 0.22-μm or 0.65-μm membrane filter is placed on the surface of isolation agar medium. A ring of sterile petrolatum is placed around the filter and 2 drops of the broth sample are placed on the center of the filter. Plates are incubated for 5 days at 37°C and examined for the typical haze of treponeme growth in the agar.

Hunter and Ross (1972) Isolation Medium

Brain heart infusion broth	50% vol/vol
Spirolate broth (BBL)	50% vol/vol
Purified agar (Oxoid)	0.8%
Inactivated rabbit serum	10%
Mucin (1%; filter-sterilized)	2 ml/100 ml

Adjust the pH to 7.8.

Membrane-Filter Method of Hardy et al. (1964) for Isolation of Oral Treponemes

Gingival scrapings are spread over 0.45-μm or 0.3-μm membrane filters on isolation agar medium and the plates incubated for 7 days at 37°C. A hazy growth under the filter is plugged and fresh plates streaked and incubated for 14 days at 37°C.

Hardy et al. (1964) Isolation Medium

Spirolate broth (BBL)	3 parts
Brain heart infusion broth (BBL)	1 part
Sodium thioglycolate	0.1%
Inactivated rabbit serum	10%
Purified agar (Oxoid)	0.8%

Adjust the pH to 7.0–7.2.

Membrane-Filter Method of Blake (1968) for Oral Treponemes

Oral samples are inoculated onto membrane filters that are placed on the surface of agar medium. Membrane filters are cemented to Lucite plastic rings in order to prevent other bacteria from being washed off the filter and contaminating the isolation medium agar. The plates are incubated for 5–7 days at 37°C and the agar under the filter examined for treponemal haze.

Blake (1968) Isolation Medium

Tryptone (Difco)	3%
Yeast extract	0.5%
NaCl	0.25%
L-Cysteine-HCl	0.075%
Glucose	0.5%
Purified agar (Oxoid)	0.8%
Inactivated horse serum	10%

Adjust the pH to 7.4.

Membrane-Filter Method of Socransky et al. (1969) for Oral Treponemes

Samples are inoculated onto the surface of 0.22-μm pore size Millipore filters placed on the surface of the agar and the plates incubated for 2–7 days at 37°C.

Isolation Medium of Socransky et al. (1969) for *T. macrodentium*

PPLO agar (BBL)	100 ml
Glucose	1 mg/ml
Nicotinamide	400 μg/ml

Spermine tetrahydrochloride	150 μg/ml
Sodium isobutyrate	20 μg/ml
Thiamine pyrophosphate	5 μg/ml

Adjust the pH to 7.0.

Isolation Medium of Socransky et al. (1969) for *T. denticola*

Spirolate broth (BBL)	45% vol/vol
Brain heart infusion broth	45% vol/vol
Sodium thioglycolate	0.025%
Asparagine	0.025%
Tryptone (Difco)	0.025%
Purified agar (Oxoid)	0.7%
Inactivated rabbit serum	10%

Adjust the pH to 7.0.

WELL-PLATE METHODS. Many early investigators used well-plate methods to isolate treponemes from oral, genital, and fecal samples. These methods are based upon the ability of treponemes to migrate through agar and thus move away from other bacteria and colonize. The methods can be used with any of the media already described. Furthermore, antimicrobial agents can be added to make the medium selective and to reduce bacterial contamination (Hampp, 1957; Hanson, 1970; Hanson and Cannefax, 1964; Rosebury, 1962; Rosebury and Foley, 1942; Rosebury et al., 1951).

Well-Plate Method of Hanson for Oral, Fecal, and Genital Treponemes (Hanson, 1970; Hanson and Cannefax, 1964)

This method may be used to isolate treponemes or other spirochetes from the oral cavity, anus, and genitalia of the chimpanzee, monkey, dog, cat, rabbit, rat, mouse, hamster, guinea pig, gerbil, fox, raccoon, skunk, and opossum. Samples are collected with sterile swabs and held until used at 1–5°C in 3 ml of broth containing 20 U of polymyxin B. The swab is then pressed against the side of the tube to remove excess broth and debris. Isolation agar medium in 14 × 100-mm petri dishes containing a well 10 mm in diameter and 12–14 mm deep is inoculated with 0.2 ml of the holding broth. The plates are incubated at 37°C in an anaerobic jar. After 5–7 days incubation, a white haze of growth is seen 1–3 cm from the edge of the well. Agar plugs are cut from the hazy area with a Pasteur pipette and subcultured. The authors used a medium with 0.001% crystal violet for initial subculture and after several transfers omitted the dye from the medium.

Hansen (1970) Holding and Isolation Medium

Spirolate broth (BBL)	45%
Brain heart infusion broth	45%
Sodium thioglycolate	0.025%
Asparagine	0.025%
Tryptone (Difco)	0.025%
Inactivated rabbit serum	10%
Agar	0.7%–0.8%

The broth medium is the same but without the agar.

Well-Plate Method of Hampp (1957) for Oral Treponemes

Twenty ml of agar medium is placed in a sterile 30-ml beaker. A well 5 mm in diameter and 15 mm deep is cut into the agar with a sterile pipette. The beaker is covered with a sterile cup or aluminum foil. At frequent intervals, the water of syneresis is removed from the well. The sample is placed in the well being careful not to touch the lip of the well. The beakers are incubated in anaerobic jars and examined after 4–7 days at 37°C for a hazy growth in the agar several cm from the well.

Well-Plate Method of Rosebury for Oral Treponemes (Rosebury, 1962; Rosebury and Foley, 1942; Rosebury et al., 1951)

Ten ml of medium is placed into 50 × 14-mm dishes; the depth of the medium should be at least 7 mm. Prior to use, a well approximately 4 mm deep is cut in the center of the agar with a Pasteur pipette having a tip approximately 2 mm in diameter. The plate is inoculated by stabbing one side of the well obliquely about 2 mm deep. Inoculated plates are inverted and incubated in anaerobic jars at 37°C.

MISCELLANEOUS ISOLATION METHODS. Two other methods have been used for isolating treponemes. The first is used in the laboratory of one of the authors (R. Smibert) either for primary isolation or, mostly, to purify cultures of contaminating bacteria either not inhibited by antimicrobial agents in selective media or capable of moving through membrane filters because of their small size. A parabiotic chamber (Bellco Glass, Inc., Vineland, NJ, catalogue no. 1945) with rubber stoppers on each tube is fitted with a 25-mm-diameter membrane filter. The pore size of the filters may be 0.15, 0.22, 0.30, or 0.45 μm. The sterile chamber is assembled and gassed with a stream of nitrogen while both tubes of the chamber are partially filled with sterile, prereduced, antibiotic-containing broth or semisolid medium. One tube is inoculated with the sample or contaminated culture, and the chamber incubated for 2–3 days at 37°C. The treponemes migrate through the filter separating the two tubes of the chamber and grow in the broth in the other tube. The broth is examined by dark-field microscopy for treponemes. Any broth medium can be used with this method.

The second method has been used by Stanton and Canale-Parola (1979) and Paster and Canale-Parola (1985) for the isolation of rumen treponemes. Dilutions of a sample are inoculated into agar tubes composed of an agar medium containing rifampin, 1μg/ml. The fluffy, hazy colonies of treponemes are picked by stabbing through the agar into the colony with a Pasteur pipette.

Subculture Procedures

The isolation procedures described above separate treponemes from other kinds of bacteria, but not from different species of treponemes. Mixed treponemal cultures must be streaked for isolated colonies, identified to species, and, if not previously recognized, must be fully characterized prior to the conduct of further studies. Inasmuch as the greatest number of motile and viable treponemes are found at the outer edge of the hazy colony, small plugs of agar near the leading edge of the haze are transferred to broth or semisolid medium. The best methods for subculture of most treponemes are the roll-tube method using prereduced media and the anaerobic chamber method (Holdeman et al., 1977). In the roll-tube system, a gas mixture of 90% nitrogen and 10% carbon dioxide is used. Broth culture medium is usually the same as the isolation medium. Thus, any medium described in the "Isolation" section can be used for subculture. The agar is omitted or replaced with 0.16% agar if a semisolid medium is desired. When transferring cultures, it is best to use large inocula. For most treponemes, well-reduced culture media are necessary for good growth.

Treponema hyodysenteriae, T. innocens, and the large treponemes isolated from the feces of homosexual male patients with diarrhea (Jones et al., 1986) will not grow in highly reduced media such as prereduced roll-tube media. They can be subcultured by streaking ordinary aerobic (i.e., not prereduced) blood agar plates, such as Trypticase soy agar (BBL) with 5–7% blood. The plates are incubated in an anaerobic jar under anaerobic conditions for 4–5 days at 37°C. The plates have areas of complete or almost complete hemolysis. Faint growth of small clear colonies can be seen in the hemolytic area by examining the plates under oblique lighting. Cultures must be transferred once a week. Growth in broth requires the addition of 10% inactivated serum in place of blood. Kinyon and Harris (1974) described growth of *T. hyodysenteriae* in Trypticase soy broth (BBL) with fetal calf serum. Aerobic Trypticase soy broth is pipetted into culture tubes gassed with CO_2. The tubes are rubber stoppered, the rack placed in a press, and autoclaved. After cooling, 10% fetal calf serum is added to each tube. Stanton and Lebo (1988) found that *T. hyodysenteriae* does not grow under strict anaerobic or normal aerobic conditions but requires between 1 and 5% O_2 with 1% being necessary for optimal multiplication. The air space above prereduced media, such as Tryticase soy broth-serum or prereduced brain heart infusion broth with 10% inactivated bovine serum in culture tubes, is injected with 1 ml of air, after which the medium is inoculated with the culture, and the tubes are rubber stoppered and incubated. When using a VPI anaerobic inoculator, the gas flow is shut off and the tubes inoculated and restoppered. This allows sufficient air into the headspace of the culture tube to allow good growth of the organism. For mass culture in a flask of prereduced medium, air (30–40 ml/1-liter flask containing 500 ml of medium) is inoculated into the headspace of the flask with a 50- or 100-ml syringe fitted with a sterile membrance-filter holder so that approximately 1% O_2 is in the headspace. In order to obtain maximum cell yield, a stirring bar is placed into each tube or flask and the cultures stirred on a magnetic stirrer; a shaker may also be used. It is not known if the organisms are respiring or if they are able to grow only in a medium with a certain O/R potential. One of us (R. Smibert, unpublished observations) has found that *T. innocens* and the large treponemes from homosexual male patients with diarrhea also need air injected into prereduced media for growth and stirring for maximum cell yields.

Colonization

After isolation and subculture, single colonies of treponemes must be picked for further study. Obtaining single-colony, pure-culture isolates is most difficult, time consuming, and frustrating. Any medium mentioned in the isolation section of this chapter can be used to colonize treponemes. In selecting a medium, there are several considerations. First, a medium with serum will only isolate treponemes that require long-chain fatty acids. Secondly, many oral treponemes require thiamine pyrophosphate. Lastly, many oral, rumen, and intestinal treponemes require both short-chain fatty acids found in rumen fluid and a fermentable energy source. Treponemes grow into the agar medium and spread into the agar. Surface colonies are not usually seen. Thus, the agar should be softer than the usual 1.5% in order to allow the treponemes to migrate. Agar medium for colonization should contain 1.25–1.3% Bacto-agar or Noble agar. If Purified agar (Oxoid) is employed, 0.7–0.8% should be used in the medium. When prereduced agar plates are required, they should be prereduced in an anaerobic jar for several days prior to use. Two anaerobic jars are used, one containing the uninoculated prereduced media and the other to receive the plates as they are inoculated. Both anaerobic jars are gassed with a stream of O_2-free CO_2 while they are open. CO_2 is heavier than air and prevents atmos-

Table 4. Key characteristics of *Treponema* species.

	Size	Need rumen serum	Glucose	Fructose	Lactose	Mannitol	Sucrose	Arabinose	Rhamnose	Maltose	Raffinose	Xylose	Pectin	Indole	Hydrolyzes esculin
T. phagedenis bt. reiter	M	S	A	A	A	A	–	–	–	–	–	–	–	+	–
T. phagedenis bt. kazan	M	S	A	A	A	A	–	–	–	–	–	–	–	+	+
T. socranskii subsp. *socranskii*[a]	S	R	A	A	–	–	A	A	A	A	V	A	V	–	–
T. socranskii subsp. *buccalis*[a]	S	R	A	A	–	–	A	A	A	V	V	A	V	–	–
T. socranskii subsp. *paredis*	S	R	A	A	–	–	A	–	–	A	V	V	V	–	–
T. pectinovorum	M	R	–	–	–	–	–	–	–	–	–	–	A	–	–
T. succinifaciens	M	R	A	–	A		–	A	–	A	–	A			
T. bryantii	M	R	A	–	A		A	A	–	–	–	A	–		
T. saccharophilum	L	R	A	A	A	–	A	A	–	A	A	–	A		
T. hyodysenteriae[b]	L	S	A	A	A	–	A	–	–	A	A	–	A	+	+
T. innocens[b]	L	S	A	A	A	–	A	A	–	A	A	A	A	–	+
T. denticola bt. denticola	M	S	–	–	–	–	–	–	–	–	–	–	–	+	+
T. denticola bt. comandonii	M	S	–	–	–	–	–	–	–	–	–	–	–	–	+
T. vincentii	M	S	–	–	–	–	–	–	–	–	–	–	–	+	V
T. refringens bt. refringens	M	S	–	–	–	–	–	–	–	–	–	–	–	+	+
T. refringens bt. calligyrum	M	S	–	–	–	–	–	–	–	–	–	–	–	+	+
T. minutum	S	S	–	–	–	–	–	–	–	–	–	–	–	+	+
T. skoliodontum	S	S	–	–	–	–	–	–	–	–	–	–	–	–	–

Abbreviations: Size: S = small treponeme (0.15–0.24 μm in diameter), M = medium treponeme (0.25–0.32 μm), L = large treponeme (>0.35 μm). Need rumen serum: S = serum, R = rumen fluid or volatile fatty acid mixture. A = acid production with a pH of 6.0 and below in 95% of strains; – = no acid production or negative test result; + = positive test result; V = variable result or test result negative in less than 90% of strains.

[a]*T. socranskii* subsp. *socranskii* and *T. socranskii* subsp. *buccalis* cannot be differentiated by a biochemical test. They can be differentiated by DNA homology and by a serologic (slide-agglutination) test.

[b]*T. hyodysenteriae* produces a strong beta hemolysis on blood agar plates, while *T. innocens* produces a weak hemolysis on blood agar plates. Data in this table on *T. hyodysenteriae* and *T. innocens* are from tests done in aerated medium with the tubes shaken.

Table 5. Some key characteristics of nonfermenting *Treponema* species.

Species	Phosphatase	H_2S	Indole	Growth in 1% glycine	Produce formate	Produce acetate and butyrate	Convert fumarate to succinate
T. denticola bt. denticola	+	+	+	–	–	–	–
T. denticola bt. comandonii	+	+	–	V	–	–	–
T. vincentii	–	+	+	–	–	+	–
T. refringens bt. refringens	–	+	+	–	–	–	+
T. refringens bt. calligyrum	–	+	+	+	–	–	+
T. minutum	–	+	+	+	+	–	–

Symbols: +, positive; –, negative; V, variable.

Table 6. Major fermentation products of *Treponema* species.

	Ethanol	Propanol	Butanol	Acetic acid	Formic acid	Propionic acid	*n*-Butyric acid	Lactic acid	Succinic acid	H_2 gas
T. phagedenis	+	+	+	+	+	tr	+	−	−	−
T. socranskii	−	−	−	+	−	−	−	+	+	−
T. pectinovorum	−	−	−	+	+	−	−	tr	−	−
T. succinifaciens	−	−	−	+	+	−	−	+	+	−
T. bryantii	−	−	−	+	+	−	−	−	+	−
T. saccharophilum	+	−	−	+	+	−	−	−	−	−
T. hyodysenteriae	−	−	−	+	−	−	+	−	−	+
T. innocens	−	−	−	+	−	−	+	−	−	+
T. denticola	−	−	−	+	−	tr	tr	−	−	−
T. vincentii	−	−	−	+	−	−	+	−	−	−
T. refringens	−	−	−	+	−	−	−	−	−	−
T. minutum	−	−	−	+	+	−	−	−	−	−
T. skoliodontum	−	−	−	+	−	−	−	−	−	−

Symbols: +, a major product; −, not present; tr, trace or small amount present or a trace amount only occasionally found by gas liquid or high-pressure liquid chromatography.

Table 7. GC content of the DNA of *Treponema* species.

Species	GC content (mol%)[a]
T. pallidum	52–54 (1)
T. phagedenis bt reiter	39–39 (1,2)
T. phagedenis bt. kazan	38–39 (1,2)
T. refringens	39–43 (1,2)
T. denticola	37–38 (2)
T. vincentii	44 (3)
T. succinifaciens	36 (4)
T. pectinovorum	39 (5)
T. hyödysenteriae	25–26 (6)
T. innocens	25–26 (6,7)
T. socranskii subsp. *socranskii*	51–52 (8)
T. socranskii subsp. *buccalis*	50–51 (8)
T. socranskii subsp. *paredis*	50–51 (8)
T. bryantii	36 (9)
T. saccharophilum	54 (10)
T. minutum	37 (3)

[a]The numbers in parentheses correspond to the following citations: 1, Smibert (1984); 2, Miao and Fieldsteel (1978); 3, Smibert (1985); 4, Cwyk and Canale-Parola (1979); 5, Smibert and Burmeister (1983); 6, Miao et al. (1978); 7, Kinyon and Harris (1979); 8, Smibert et al. (1984); 9, Stanton and Canale-Parola (1980); and 10, Paster and Canale-Parola (1985).

pheric O_2 from seeping into the open jars. The streaked plates are incubated upright for 1 to 2 weeks. Anaerobic chambers can also be used for colonizing treponemes. Another method used by one of the authors (R. Smibert) is prereduced agar medium in rubber stoppered 3-, 4-, or 6-ounce prescription bottles. The bottle-plates are gassed with a stream of O_2-free N_2 or a mixture of 95% N_2 and 5% CO_2, then streaked, restoppered, and incubated for 1 to 2 weeks. Pour bottle-plates can also be used. Colonies of treponemes are subsurface and become larger in diameter as the cultures grow. They may be several millimeters in diameter and appear as a white, translucent haze in the medium. Some have an irregular edge and appear like bits of cotton in the medium, while others have an entire edge that may or may not appear denser than the center of the colony. Colonies are picked with a loop or a plug is taken with a Pasteur pipette and subcultured. All cultures must be examined by dark-field microscopy to determine purity.

One of us (R. Smibert) employs the OTI medium (see "Selective Media") as a general purpose medium for isolation, maintenance of stock cultures, and as an agar medium with 0.7% Purified agar (Oxoid) for colonization of these organisms.

Christiansen (1964) colonized *T. phagedenis* and *T. refringens* in the following medium:

Medium of Christiansen

Fluid thioglycolate	100 ml
Purified agar (Oxoid)	0.7%
Inactivated rabbit serum	10%

Hardy et al. (1963) colonized *T. denticola, T. refringens, T. phagedenis,* and *T. vincentii,* using three different media. The plates are streaked and quickly put into anaerobic jars for 6–14 days.

Hardy Medium for *T. phagedenis* and Oral Treponemes

USP alternate thioglycolate broth	100 ml
Inactivated normal calf serum	10%
Purified agar (Oxoid)	0.7%

Hardy Medium for *T. refringens* and *T. phagedenis*

Trypticase (BBL)	3%
Yeast extract	0.5%
Glucose	0.5%
NaCl	0.25%
L-Cysteine -HCl	0.2%
Inactivated rabbit serum	10%
Purified agar (Oxoid)	0.7%
Adjust the pH to 7.2.	

Hardy Medium for *T. denticola* and *T. vincentii*

Thioglycolate broth	3 parts
Brain heart infusion broth	1 part
Inactivated rabbit serum	10%
Purified agar (Oxoid)	0.7%

Hanson and Cannefax (1965) colonized 48 strains representing *T. denticola, T. refringens, T. phagedenis* and *T. vincentii.* Colonies appeared in 7–8 days.

Medium of Hanson and Cannefax (1965)

Spirolate broth	45%
Brain heart infusion broth	45%
Sodium thioglycolate	0.05%
Inactivated rabbit serum	10%
Agar	2.5%

Identification and Characterization

During the last few years, several new treponemal species have been created and their identification accomplished using conventional bacteriologic methods. Many other species of treponemes are yet to be isolated, characterized, and named. The base medium for identifying and characterizing treponemes can be any medium described in this chapter. One of us (R. Smibert) uses a prereduced peptone-yeast extract-basal (PY) medium with a gas phase of either N_2 or 95% N_2 and 5% CO_2. This medium and its preparation are described in the *Anaerobe Laboratory Manual* (Holdeman et al., 1977). Carbohydrates and other compounds

used in the study of treponemes are added to the PY medium. It is supplemented with 10% inactivated serum, thiamine pyrophosphate, and either 30% rumen fluid or the artificial rumen fluid mixture.

Basal PY Medium for Treponemes (Holdeman et al., 1977):

Peptone	0.5 g
Trypticase	0.5 g
Yeast extract	1.0 g
Resazurin	0.16 mg
L-Cysteine-HCl	0.5 g
Salt solution (see below)	4.0 ml
Distilled water	100.0 ml

Salt solution:

$CaCl_2$, anhydrous	0.2 g
$MgSO_4$, anhydrous	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaCl	2.0 g
Distilled water	1 liter

All media with carbohydrates are checked for acid production after 3 to 5 days incubation. A pH of 6.0 or below is considered positive for acid formation. A carbohydrate-containing culture (preferably glucose) is kept for determining end products of fermentation by gas chromatography or high-pressure liquid chromatography. A culture must be examined for end products even if the pH is above 6.0 because some organisms metabolize amino acids and neutralize an acid pH.

Antibiotic susceptibility of these organisms can be determined by the addition of various concentrations of antibiotics to inoculated broth medium (Abramson and Smibert, 1971a, 1971b) or by the addition to the broth medium of one or more high-concentration susceptibility disks (Abramson and Smibert, 1972); after a suitable incubation period, the tubes are assayed for growth inhibition.

Key characteristics for the identification of cultivable treponemes are described in Tables 4–7. Additional information on identification of treponemes can be found in: Cwyk and Canale-Parola (1979); Holdeman et al. (1977); Kinyon and Harris (1979); Paster and Canale-Parola (1985); Smibert (1973, 1976a, 1976b, 1984, 1985); Smibert and Burmeister (1983); Smibert et al. (1984); and Stanton and Canale-Parola (1980).

Preservation of Cultures

Most cultivable treponemes are transferred to a suitable medium weekly for experimental use. However, *T. pallidum* subsp. *pallidum, pertenue,* and *endemicum* and *T. paraluiscuniculi* require propagation in the rabbit testis as a source of treponemes for the conduct of experiments. Cultures and rabbit testicular extracts of treponemes can be stored in the frozen state in liquid N_2 or in a mechanical freezer at $-70°$ to $-80°C$. For preservation over several years, sterile cryoprotective agents such as 10–15% glycerol or 3% dimethylsulfoxide (DMSO) are added to the cultures and extracts in sterile plastic cryotubes. Prior to freezing, the cryotubes containing cultivable treponemes are gassed with N_2 until the top is secured. In order to revive the cultures and extracts, they are removed from storage and thawed at room temperature. Cultivable treponemes are transferred to fresh medium and incubated at 37°C for 4–6 days. The human and rabbit pathogens are inoculated into rabbit testes for growth and multiplication.

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CHAPTER 193

The Genus *Borrelia*

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The genus *Borrelia* is one of the four genera of the family Spirochaetaceae and is comprised of pathogenic spirochetes that are transmitted by arthropod vectors. With the exception of louse-borne relapsing fever, the borrelioses are zoonoses. In the past, due to the inability to culture the spirochetes and conduct the usual morphological and physiological studies for their classification, the borreliae have been classified into species solely on the basis of their arthropod vector. In addition, the antigenic variability associated with some of the borreliae has prevented the use of serological methods for their identification. With the formulation of a culture medium by Kelly (1971), and with subsequent improvements by Stoenner (1974) and Barbour (1984), many of the borreliae can now be cultivated in vitro and taxonomic methods based on molecular biology can be applied to their classification. Nineteen species of Borreliae have been recognized.

Borreliae are slender (0.2–0.5 μm × 8–30 μm) helically shaped bacteria (Fig. 1). They are considered to be Gram-negative, but are visualized best with Giemsa's stain. *Borrelia* shares the same basic ultrastructural features as other members of the Spirochaetaceae (see also Chapter 191). These features include an outer membrane that encloses the protoplasmic cylinder and periplasmic flagella inserted subterminally and bipolarly in the protoplasmic cylinder. An amorphous slime layer has been observed on the surface of several borreliae (Hovind-Hougen, 1976). Muramic acid (Ginger, 1963) and ornithine (Klaviter and Johnson, 1979) have been identified as components of the borrelial cell wall. The periplasmic flagella are unsheathed and overlap in the central region of the cell (Holt, 1978). The number of periplasmic flagella per cell end varies from 7 to 20 (Hovind-Hougen, 1976). Because of the internal location of the periplasmic flagella, cells maintain their motility in environments of relatively high viscosity.

Borrelia species are relatively slow-growing microaerophilic spirochetes. The cells contain an iron superoxide dismutase, but not catalase or peroxidase (Austin et al., 1981), and are resistant to metronidazole (Johnson et al., 1984a). They grow well in the temperature range of 30–35°C with generation times of 12–24 h at the latter temperature. Their nutritional requirements have not been fully defined but appear to be complex. *Borrelia* require N-acetylglucosamine (Kelly, 1971) and long-chain fatty acids, which are incorporated unaltered into cellular lipids (Livermore et al., 1978; Pickett and Kelly, 1974). They utilize glucose as a major energy source, with lactic acid as the predominant metabolic end product (Kelly, 1976).

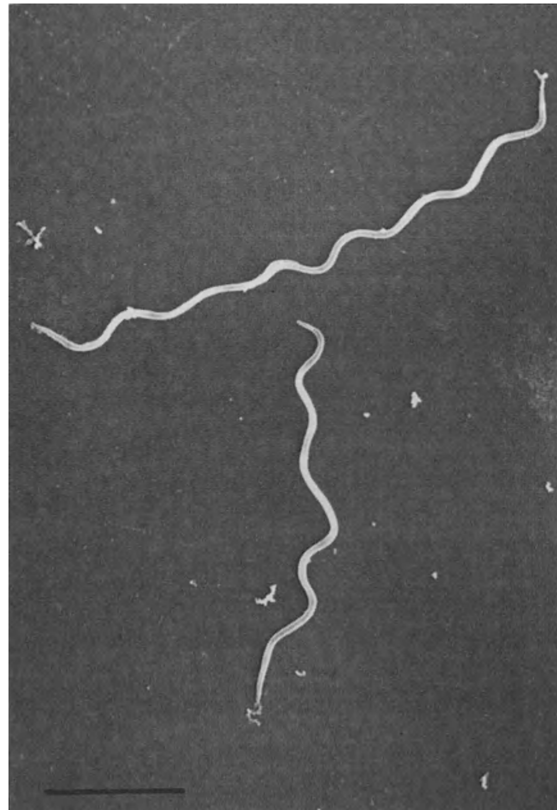

Fig. 1. *Borrelia burgdorferi* strain 297, human cerebral spinal fluid isolate, as seen by scanning electron microscopy. Bar = 5 μm.

Borrelia species do not produce any known toxins and may elicit an acute inflammatory response in the host. They are considered to be extracellular parasites, as they are predominately confined to the plasma space of their mammalian hosts. The remittent fevers characteristic of relapsing fever borreliae are due to antigenic variations among the infecting populations of spirochetes.

Habitats

All known borreliae are arthropod-borne pathogens and are transmitted by the human body louse or various species of hard and soft ticks. The principle species of *Borrelia* and their primary vectors are listed in Table 1. The validity of the assignment of some of the species of *Borrelia* is questionable. For example, on the basis of DNA homology studies, a high degree of relatedness (76% to 100%) was observed among *B. hermsii, B. turicatae,* and *B. parkeri,* indicating that these borreliae probably comprise a single species (Hyde and Johnson, 1984). Conversely, some *B. burgdorferi* isolates from the United States and Europe appear to be genetically dissimilar (LeFebvre et al., 1989).

Presence in Lice

B. recurrentis is the etiological agent of louse-borne (epidemic) relapsing fever and is the only

Table 1. Characteristics of *Borrelia* species of medical and veterinary significance.

Species	Disease	Vector	Geographic distribution
B. recurrentis	Louse-borne relapsing fever	*Pediculus humanus* subsp. *humanus*	Worldwide
B. hermsii	New World tick-borne relapsing fever	*Ornithodoros hermsi*	Canada, western USA
B. turicatae	New World tick-borne relapsing fever	*O. turicata*	Southwestern USA
B. parkeri	New World tick-borne relapsing fever	*O. parkeri*	Western USA
B. mazzottii	New World tick-borne relapsing fever	*O. talaje*	Southwestern USA, Mexico, Central and South America
B. venezuelensis	New World tick-borne relapsing fever	*O. rudis*	Central and South America
B. duttonii	Old World tick-borne relapsing fever	*O. moubata*	Central, eastern, and southern Africa
B. hispanica	Old World tick-borne relapsing fever	*O. marocanus*	Spain, Portugal, Morocco, Algeria, Tunisia
B. crocidurae, B. merionesi, B. microti, B. dipodilli	Old World tick-borne relapsing fever	*O. erraticus*	Northern and eastern Africa, Near and Middle East, southeastern Europe
B. persica	Old World tick-borne relapsing fever	*O. tholozani*	Middle East, Greece, Central Asia
B. caucasica	Old World tick-borne relapsing fever	*O. verrucosus*	Iraq, southwestern USSR
B. latyschewii	Old World tick-borne relapsing fever	*O. tartakowskyi*	Iraq, Iran, Afghanistan, southcentral and southwestern USSR
B. burgdorferi	Lyme (borreliosis) disease	*Ixodes dammini*	Midwestern and eastern USA
		I. pacificus	Western USA
		I. ricinus	Europe
		I. persulcatus	Asiatic USSR, China, Japan
B. anserina	Avian borreliosis	*Argas persicus* and other *Argas* spp.	Worldwide
B. theileri	Bovine borreliosis	*Rhipicephalus evertsi, Boophilus microplus, B. annulatus, B. decoloratus*	South Africa, Australia, Brazil, Mexico
B. coriaceae (?)	Epizootic bovine abortion	*Ornithodoros coriaceus*	California

species of borreliae that is louse-transmitted. The human is the only known reservoir of this spirochete. The human body louse, *Pediculus humanus* subsp. *humanus,* becomes infected after feeding on a spirochetemic individual and remains infected during its life span of approximately 10 to 61 days. The ingested borreliae enter the louse midgut where they penetrate the gut epithelium to gain entrance to the hemolymph, in which they multiply. They do not infect the salivary glands or ovaries of the lice. Accordingly, the infection is not passed to offspring of infected lice via transovarial transmission, nor do humans become infected via infectious saliva while the lice are feeding. Rather, infection is thought to occur when spirochetes are liberated from the lice and enter the host through the bite wound when the lice are crushed on the skin by scratching. Since body lice prefer the normal body temperature of 37°C, to higher temperatures they are likely to leave a febrile person to seek an afebrile person, which results in the rapid transmission of infection during epidemics.

Presence in Ticks

The borreliae are also parasites of both soft and hard ticks. Two human diseases caused by infected ticks are the tick-borne (endemic) relapsing fevers and Lyme (borreliosis) disease. Numerous species of *Borrelia* are responsible for the tick-borne relapsing fevers and are distributed worldwide in both tropical and temperature climates (Table 1). Argasid (soft) ticks of the genus *Ornithodoros* serve as the vectors of these spirochetes, with wild rodents being the major reservoir for the borreliae. Occassionally, lizards, toads, turtles, and owls can harbor these spirochetes. The infection is maintained in reservoir animals by tick bites. Humans are accidental hosts, with the possible exception of infections by *B. duttonii.* In East Africa, this spirochete is carried by the domestic tick, *O. moubata,* and the reservoir for the borreliae appear to be humans.

The course of development of relapsing fever borreliae in ticks varies considerably from that in lice. Ticks become infected while obtaining a blood meal from spirochetemic animals. A generalized infection ensues, and spirochetes can be found in a variety of tissues, including the salivary glands, the coxal glands on the legs, and the ovaries. Humans and animals become infected while the tick is feeding, either through infectious saliva and/or coxal fluid entering via the bite wound or skin. Ticks are durable vectors of borreliae, as they are able to survive months to years without a blood meal. In addition, the spirochetes are able to survive in the ticks for similar periods of time. In contrast to lice, female ticks can transmit borreliae transovarially to their progeny, and the spirochetes can be maintained during transition from one developmental form to another (e.g., larvae to nymph to adult).

The Ixodidae (hard) ticks are the primary vectors of *B. burgdorferi,* the etiological agent of Lyme disease (Table 1). The association between *B. burgdorferi* and *Ixodes dammini,* the vector of this disease in northeastern and north-central USA, has been studied extensively (Benach et al., 1987; Burgdorfer et al., 1982; Burgdorfer et al., 1989; Ribeiro et al., 1987). This relationship is similar to that occurring in other ticks of the *I. ricinus* complex. The larval and nymphal forms of *I. dammini* commonly become infected with *B. burgdorferi* when obtaining a blood meal from infected rodents. The white-footed mouse, *Peromyscus leucopus,* is a primary reservoir for the spirochete. In contrast to the tick- and louse-borne relapsing fever borreliae that egress from the midgut a few days after ingestion and invade the hemolymph, *B. burgdorferi* remains in the midgut until the next blood meal (Burgdorfer, 1989; Ribeiro et al., 1987). At that time, it penetrates the basal membrane of the midgut, invades the hemolymph and various tissues, including the salivary glands, and is transmitted to the host via infectious saliva. Transovarial transmission occurs less frequently in the *Ixodes* ticks as compared to the *Ornithodoros* ticks.

Several species of *Borrelia* are of veterinary importance. However, little information is available on the development of these borreliae in their tick vectors.

Medical Importance of Borreliae

Relapsing Fevers

Subsequent to exposure to an infected louse or tick, the relapsing fever borreliae gain access to the blood and the lymph. After an incubation period of 4 to 18 days and after the spirochetes have multiplied to levels of 10^6 to 10^8 cells per ml of blood, the illness begins abruptly with shaking chills, fever, headache, and fatigue, which persist for 3 to 7 days. Following an afebrile period of 5 to 10 days, a second symptomatic episode may occur. Additional relapses of decreasing time and intensity are characteristic of the tick-borne disease but are uncommon in the louse-borne disease. The relapses

are the result of antigenic variation in the borreliae. The borreliae are able to undergo a number of cyclic antigenic variations. When antibodies to the prevailing antigenic type appear, the organisms "disappear" from the peripheral blood and are replaced by a different antigenic variant in a few days. If the host is untreated, this process may be repeated several times, depending upon the infecting strain of *Borrelia.*

During the acute phases of the illness, borreliae may be seen in blood smears stained with Giemsa or Wright stain and counterstained with crystal violet (Felsenfeld, 1971). The mortality rate in louse-borne relapsing fever ranges as high as 40% in untreated cases, but can be less than 5% following antibiotic treatment. Oral tetracycline (30 mg/kg of body weight), given in four divided doses at 6-h intervals for 10 days, is considered the drug of choice (Foster, 1977). Shortly following the initiation of antibiotic therapy, a Jarisch-Herxheimer reaction occurs, manifested by chills, fever, headache, and joint and muscle pains. This reaction is quite pronounced in louse-borne relapsing fever. Clinical descriptions of relapsing fever in humans have been detailed by Bryceson et al. (1970).

Louse-borne relapsing fever has been reported primarily from African countries, particularly Ethiopia and Sudan. Some outbreaks have occurred in the South American Andes. Tick-borne relapsing fevers are more sporadic in occurrence. In North America, most of the cases occur within the distributional area of *Ornithodoros hermsi* or *O. turicata* (Table 1). In the United States, tick-borne relapsing fever is underreported because it is seldom diagnosed. Unless a patient history of wilderness exposure or camping in rodent-infected areas is given and the patient is aware of a tick bite, the illness is usually not suspected during the initial period of fever (Fihn and Larson, 1980).

Lyme (Borreliosis) Disease

Lyme disease is a multisystem illness that primarily affects the skin, nervous system, heart, and joints (Steere, 1989). The disease was brought to the attention of the medical community as the result of an outbreak of arthritis in adults and children in Lyme, CT, USA, and the adjacent communities of Old Lyme and East Haddam. The resulting epidemiological and clinical investigations led to the identification of a new disease entity, Lyme disease (Steere et al., 1977).

The hallmark skin lesion of Lyme disease, erythema chronicum migrans, was first described in Europe in 1908 (Afzelius, 1921) and in the USA in 1969 (Scrimenti, 1970). According to the CDC, Lyme disease is the most common vector-borne infection in the USA. Cases have been reported in 43 states, with three areas of concentration: from Massachusetts to Maryland in the northeast; Wisconsin and Minnesota in the midwest; and California and Oregon in the west. Most European countries have also reported cases, as have republics in the USSR. In addition, the disease has been reported in China, Japan, Africa, and Australia.

Lyme disease can be divided into early and late infection. Approximately 7 to 9 days following the initial tick bite, the early (localized) infection is marked by the pathognomonic skin lesion, erythema migrans (in 60 to 80% of patients), and accompanied by fever, regional lymphadenopathy, or minor constitutional symptoms (Steere, 1989). The erythema migrans lesions usually fade within 3 to 4 weeks, even when untreated, but they may recur. Weeks to months later (disseminated disease), patients may develop meningoencephalitis, Bell's palsy, myocarditis, migrating musculoskeletal pain and intermittent attacks of arthritis, especially in the large joints such as the knee. As the infection progresses (late or persistent infection), the episodes of arthritis last for months rather than weeks. Also, syndromes of both the central and peripheral nervous system may occur more than a year after the initial infection. Persistent infections in Lyme disease may also develop as acrodermatitis chronicum atrophicans (ACA), the late skin manifestation of the disease, which has primarily been observed in Europe (Asbrink and Hovmark, 1988; Kaufman et al., 1989). The skin lesion typically begins as a bluish-red discoloration of swollen skin on an extremity. This lesion may occur at an earlier site of erythema migrans. The inflammatory stage of ACA may persist for years or decades and may lead to atrophy of the skin. Transplacental transmission of *B. burgdorferi* has been reported; however, congenital infection with adverse outcome, such as perinatal death, appears to be unusual (Schlesinger et al., 1985; Weber et al., 1988).

After the first several weeks of infection, most patients have a positive antibody response to the spirochete. The specific IgM response is detectable after the third week and peaks after 6 to 8 weeks (Steere et al., 1983). The *B. burgdorferi*-specific IgG response peaks during the second to third month of infection and may persist for years. Since the direct visualization and culture of *B. burgdorferi* from patient specimens is difficult, serologic determinations are currently the most practical mode of laboratory

diagnosis. False negative results occur early in the disease, whereas false positives may occur in patients with a variety of other diseases, including syphilis, Rocky Mountain spotted fever, autoimmune disease, and neurologic disorders. Differential diagnosis is difficult.

Early Lyme disease usually responds well to antimicrobial therapy. However, cases with persistent joint or central nervous system complications tend to be more resistant to treatment. Adults with the early manifestations generally respond to doxycycline (100 mg orally, 2× daily, 10–30 days) or amoxicillin (500 mg orally, 4× daily, 10–30 days). Children younger than 8 years of age are generally treated with amoxicillin (250 mg orally, 3× daily, 10–30 days). Neurologic manifestations and other abnormalities are treated with ceftriaxone (Rocephin) (2 g, intravenously, 1× daily, 14 days). Treatment failures have occurred with all of these regimens, and retreatment may be necessary. Since patients who have already developed a mature anti-borrelia IgG response often remain seropositive after apparently successful antibiotic therapy, the presence or absence of circulating antibodies to *Borrelia* following treatment is not a reliable indicator of cure (Dattwyler et al., 1989). Descriptions of clinical presentations and the medical progress of Lyme disease have been reviewed by Steere (1989).

Veterinary Diseases

Borrelia anserina, the type species of the genus, is the etiological agent of avian borreliosis, a highly fatal disease of geese, ducks, turkeys, and chickens. Mammals are resistant to this infection. The disease is widespread, occurring in many countries including Europe, Asia, Australia, and South, Central, and North America. The vectors of *B. anserina* are species of *Argas* ticks. The disease begins with a high fever; then the birds become cyanotic and develop diarrhea. Spirochetes are present in the blood early during the disease and relapses do not occur.

Borrelia coriaceae is the putative etiological agent of epizootic bovine abortion, a disease of major economic importance in California (Lane et al., 1985). This recently recognized species of *Borrelia* (Johnson et al., 1987) is carried by the soft tick *Ornithodoros coriaceus* (Lane et al., 1985).

Borrelia theileri is responsible for a mild disease of cattle, horses and sheep. The illness is characterized by one to two episodes of fever, weight loss, weakness, and anemia. The vectors of this spirochete are species of the hard ticks *Rhipicephalus* and *Boophilus.* The disease has been reported in South Africa and Australia.

Isolation

The study of *Borrelia* was greatly facilitated by the development of a culture medium by Kelly (1971) that would support the growth of the spirochetes. Stoenner enriched the basic formulation of Kelly with the addition of yeast extract and tissue culture medium (Stoenner et al., 1982). Subsequent modifications by Barbour (1984) resulted in BSK (Barbour-Stoenner-Kelly) medium, which has allowed the isolation of borreliae from a variety of tissues. The success of in vitro culture of *Borrelia* is usually dependent on the quality of the animal serum or albumin used in the media preparation (Callister et al., 1990).

Liquid BSK II Medium for Growth of *Borrelia* (Barbour, 1984)

1. To 900 ml of glass-distilled water, add the following:

N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, Sigma)	6.0 g
Neopeptone (Difco)	5.0 g
Sodium citrate	0.7 g
Glucose	5.0 g
Sodium bicarbonate	2.2 g
TC Yeastolate (Difco)	2.5 g
Sodium pyruvate	0.8 g
N-acetyl glucosamine (Sigma)	0.4 g
Bovine serum albumin, fraction V	50.0 g

2. Adjust pH of medium at 20–25°C to 7.6 with 1 N NaOH.
3. Sterilize by filtration with air pressure (0.2-μm membrane filter).
4. Add 100 ml of sterile 10X concentrate CMRL 1066 without glutamine (Gibco).
5. Add 200 ml of sterile 7% gelatin (Difco), which has been dissolved in boiling water.
6. Add 64 ml of sterile, heat-inactivated rabbit serum (Pel-Freeze Biologicals, Inc., Rogers, AR).
7. Dispense to glass or polystyrene tubes or bottles. Fill to 90% capacity and cap tightly.
8. Store complete medium at 4°C.

Semisolid medium is prepared by the addition of 100 ml of sterile 1.89% Seakem LE agarose (Gibco), which has been dissolved in boiling water. The agarose solution is added just prior to dispensing the medium.

Borrelia can be isolated from *Ornithodoros* and *Ixodes* ticks using semisolid BSK medium. The hemolymph and most tissues of *Ornithodoros* ticks can be used as a source of the relapsing fever borreliae and *B. coriaceae.* In contrast, *B. burgdorferi* is usually restricted to the midgut of *Ixodes* ticks. Susceptible animals (young mice, rats, or hamsters) can be used for the isolation of borreliae by inoculation of tick tissue suspensions or by allowing infected ticks to feed on the animals. The borreliae may be isolated from the blood during the first 7 days

of infection and from the kidney, spleen, and bladder after 14 days using the procedures described below.

Borrelia strains are isolated from blood by inoculating one to two drops per 10 ml of semisolid BSK medium. Tissue samples are homogenized in liquid BSK medium (10% wt/vol) with a Stomacher Lab Blender (Tekmar, Cincinnati, OH). Larger tissue debris is allowed to settle and duplicates of 1:10-dilutions of the supernatant are made in the semisolid BSK medium. Cultures are incubated at 30—35°C and examined by darkfield microscopy for spirochetes at weekly intervals.

B. burgdorferi has been isolated and subcultured from blood, CSF, synovial fluid, and skin specimens from Lyme disease patients. In contrast to relapsing fever, very few borreliae are present in the acute stage of Lyme disease. They cannot be detected visually in the blood, and most isolation attempts have been unsuccessful (Rawlings et al., 1987; Steere et al., 1983). Only one successful isolation and subculture has been reported for the synovial fluid (Schmidli et al., 1988). Spirochetes are more readily isolated from the CSF when central nervous system involvement occurs and from biopsy specimens of the skin lesion of erythema migrans and acrodermatitis chronicum atrophicans (Asbrink and Hovmark, 1988; Berger et al., 1985; Rawlings et al., 1987). *B. burgdorferi* has not been isolated from the urine of patients with Lyme disease. Despite successes in isolating *B. burgdorferi* from patient specimens, no standard method for processing samples can be recommended at this time. Although borreliae usually can be detected in culture media within 1 to 3 weeks of incubation, some isolates may not be visible for several months.

Borreliae are best preserved by the addition of glycerin (final concentration, 10%) to blood or plasma from infected animals, or to culture media, and stored at −70°C or in liquid nitrogen.

Identification and Characterization

Members of the genus *Borrelia* have been identified by ecological and biochemical characteristics. All species in this genus are transmitted to vertebrates by hematophagous arthropods. In most cases, borreliae differentiation was based on identification of the specific vector that transmits the spirochete, on the host (humans, animals, or birds), and on the variable infectivity of isolated borreliae for different species of laboratory animals (Burgdorfer, 1976; Davis, 1956; Felsenfeld, 1971). More recently, genetic techniques have been used to characterize these spirochetes.

Borrelia DNA has a GC content of 27 to 32 mol%, which is lower than that reported for *Treponema* and *Leptospira* (Hyde and Johnson, 1984). DNA homology studies have shown that the three North American relapsing fever borreliae, originally speciated on the basis of vector specificity, constitute a single species of *Borrelia* (Hyde and Johnson, 1984). In addition, the Lyme disease spirochete and the *O. coriaceus* spirochete were determined to be new species of *Borrelia* (Johnson et al., 1984b; Johnson et al., 1987). Through the use of restriction endonuclease analyses and hybridization studies, it has been possible to detect similarities and differences among strains of *B. burgdorferi* (LeFebvre et al., 1989). The detection and identification of *Borrelia* has also been accomplished through the use of DNA probes (Schwan et al., 1989).

Molecular Biology

The DNA organization of the *Borrelia* appears to be unique in the Spirochaetaceae and perhaps among the prokaryotes as well in having linear DNA plasmids. In contrast to other spirochetes, the *Borrelia* species were found to contain plasmids (Hyde and Johnson, 1984). Plasmids were detected in all of the *Borrelia* examined, which include *B. hermsii, B. parkeri, B. turicatae, B. crocidurae, B. burgdorferi, B. coriaceae,* and *B. anserina,* the type species of this genus (Hyde and Johnson, 1986). A small 9.2-kb supercoiled plasmid present in a fresh tick isolate of *B. burgdorferi* was found to exist as a stable dimer and to contain methylated DNA (Hyde and Johnson, 1988). In addition to circular plasmids, *Borrelia* species contain linear plasmids, a form of DNA that was previously thought to be unique to eukaryotes (Barbour and Garon, 1987; Plasterk et al., 1985). Also, the linear plasmids of *B. burgdorferi* were shown to have covalently closed termini, another form of DNA thought to be restricted to eukaryotes and viruses (Barbour and Garon, 1987).

Linear plasmids of *B. hermsii* are believed to carry silent copies of serotype-specific variable major proteins (VMP) of the organism (Meier et al., 1985). The expression locus and the active copies of the VMP genes are located on different linear plasmids of the cell (Plasterk et al., 1985). Antigenic variation (switching) occurs due to the transposition of the structural genes to the expression locus. In contrast, the DNA of *B. duttonii,* another agent of relapsing fever, was reported to consist of a number of distinct segments (Hayes et al., 1988). DNA sequences that

coded for the VMPs of *B. duttonii* appeared to be present on high-copy-number segments. However, recent experiments using in situ lysis procedures provide evidence that *B. duttonii* contains a circular chromosome, in addition to at least two linear plasmids and one supercoiled plasmid (Perng et al., 1990). *B. burgdorferi* is similar to *B. hermsii* in that they both have the genes for surface proteins located on a linear plasmid. Genes encoding outer-surface protein A and outer-surface protein B are present on a 49-kb linear plasmid of this spirochete (Barbour and Garon, 1987). The organization and expression of these genes have been studied extensively (Bergstrom et al., 1989; Bundoc and Barbour, 1989; Howe et al., 1986). *B. burgdorferi* will lose plasmids, as a result of in vitro cultivation (Barbour, 1988), as well as infectivity for hamsters (Johnson et al., 1984c). Schwan et al. (1988) reported that a fresh *B. burgdorferi* tick isolate carried nine plasmids, including seven linear plasmids ranging in size from 49 to 16 kb and two circular plasmids of 27 and 7.6 kb. Continuous cultivation of this strain in BSK medium resulted in the loss of both a 7.6-kb circular plasmid and a 22-kb linear plasmid, in addition to a corresponding loss of infectivity in mice. Other genes of *B. burgdorferi* that have been isolated and characterized include those encoding a 60-kb common antigen (Hansen et al., 1988) and a major flagellin protein (Gassman et al., 1989).

Ferdows and Barbour (1989) examined the genome of *B. burgdorferi* cells using pulsed-field gel electrophoresis. The results of their studies indicated that *Borrelia* spirochetes have linear chromosomes. However, similar studies conducted at another laboratory concluded that members of the genus *Borrelia* contain the circular chromosome typical of prokaryotic organisms (Perng et al., 1990).

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The Genus *Leptospira*

SOLLY FAINE

Bacteria of the genus *Leptospira* are ubiquitous, associated with most mammals, and with marsupials and certain amphibia, or found free-living in soil and surface waters. Some strains of leptospires can cause an acute generalized infection, characterized by fever and hemorrhages, called leptospirosis. Leptospires may remain and grow in the kidneys of animals that recover from the acute infection but remain renal excretors or carriers, shedding leptospires in their urine. Humans may be infected but do not remain carriers and are thus not natural hosts.

The disease leptospirosis is economically important because it causes loss of productivity in livestock. It also causes loss through illness and death in humans in contact with either domestic livestock or wildlife carriers. It is thus a significant occupational hazard for people in rural or agricultural communities and in selected occupations in direct or indirect contact with animals (see "Economic Importance of Leptospirosis").

The leptospires are unusually thin, coiled, aerobic or microaerophilic bacteria, which cannot be stained easily for light microscopy, do not grow as surface colonies on conventional media, and are usually observed in wet preparations by darkfield microscopy. They are thus studied almost exclusively in fluid cultures, leading to special difficulties in characterization, identification, and nomenclature, many of which have now been overcome with the development of techniques that are independent of culture on solid media.

As a result of hesitation to define the genus and its species in inappropriate and nondefinitive terms, a system of classification and nomenclature based on serology was adopted, linked with primary division into animal pathogens and nonpathogens. This system has been satisfactory, if not ideal, for diagnosis, prognosis, epidemiology, and treatment of leptospirosis in animals and humans. The pathogenic leptospires were grouped into an "interrogans" complex, later the *interrogans* species, comprising numerous serovars with individual names, arranged by serological relationships into serogroups. The nonpathogens were part of a "biflexa" complex, later the *biflexa* species, similarly grouped by serovar (Faine and Stallman, 1982). The official description is thus of two species, *Leptospira interrogans,* and *L. biflexa.* The names of the component serovars of each species are conventionally written in italics. Serologically related serovars are grouped for convenience into serogroups, which have no formal taxonomic status, and whose names are written in capitalized Roman letters. Yasuda et al. (1987) classified leptospires into genospecies, based on the GC content of the DNA and on DNA homology groups. Some of the genospecies were given new names while existing names were used for others. This classification is still incomplete because the GC content and consequent genospecies status of many serovars has not yet been published. Among those strains designated by Yasuda et al. (1987), there are relationships which do not correspond entirely with their serological classification (see "Genetic Composition, and Classification," below, and Table 2).

A third species, believed to be nonpathogenic, formerly described as *Leptospira illini* (Hanson et al., 1974), was reclassified into a new genus, *Leptonema,* according to its morphology, genetics, and growth characteristics (Hovind-Hougen, 1979). Another species, *L. parva,* was defined by Hovind-Hougen et al. (1981b) on the basis of morphology, growth, and DNA composition. In addition, there are halophilic water leptospires, classified as members of *L. biflexa,* which differ from other biflexa type leptospires mainly in their salt tolerance (see Table 1). Table 1 lists characteristics of a number of species of *Leptospira.*

Most studies assume that all leptospires are similar, that findings on any strain can be compared with any other of the same or different serovars, and that any differences related to serovar truly reflect serovar classification. The hypothesis that differences may be related to geno-

Table 1. Characteristics of species of the genera *Leptospira* and *Leptonema*.

Species	GC content (mol%)[a]	Pathogenic[b]	Growth[c] 11°	37°	$CuSO_4$[d]	8AZ[e]	DAP[f]	Lipase[g]
Leptospira interrogans:								
L. interrogans[h]	34.9 ± 0.9 to 40.7 ± 0.6	+	−	+	−	−	−	V
L. interrogans[i]	34.9 ± 0.9	+	−	+	−	−	−	V
L. borgpetersenii[i]	39.8 ± 0.3	+	−	+	−	−	+	−
L. inadai[i]	42.6 ± 0.9	+	−	+	+	+	−	+
L. noguchii[i]	36.5 ± 1.2	+	−	+	−	−	−	−
L. santarosai[i]	40.7 ± 0.6	+	−	+	−	−	+	−
L. weilii[i]	40.5 ± 0.7	+	−	−	−	−	−	−
Leptospira biflexa:								
L. biflexa[h]	33.5 ± 0.2 to 37.6 ± 0.7	−	+	V	+	+	+	+
L. biflexa[i]	36.0 + 0.3	−	+	V	+	+	+	+
L. meyeri[i]	33.5 ± 0.2	V	−	+	+	+	+	+
L. wolbachii[i]	37.2 ± 0.5	−	−	−	−	+	+	+
L. parva[h]	48.7 ± 0.4	−	−	+	+	+	−	+
Leptonema illini[h,b]	54.2 ± 0.2	−	+	−	−	+	+	+

Symbol: +, has the characteristic; −, does not have the characteristic; V, variable, or literature conflicts.
[a]From Yasuda et al. (1987).
[b]+, some or all serovars are known pathogens; −, none are pathogenic.
[c]Growth in Tween-albumin medium.
[d]100 mg/l added to growth medium[a].
[e]225 mg/ml 8-azaguanine added to growth medium[a].
[f]10 mg/l 2,6-diaminopurine added to growth medium[a].
[g]Trioleinase activity.
[h]A currently designated species (Kmety and Dikken, 1988).
[i]Characteristics and nomenclature for new genospecies[a] derived by genetic grouping of leptospires included in the currently designated species.

species rather than serovar remains to be explored.

Ecophysiology

Cell Structure

Members of the genus *Leptospira* (Faine and Stallman, 1982; Johnson and Faine, 1984) are thin helicoidal bacteria with a coil amplitude of approximately 0.1 to 0.15 μm and a wavelength of about 0.5 μm. Frequently one or both ends are hooked in fluid cultures, but straight variants occur (Faine and van der Hoeden, 1964). Unspecified nuclear material, ribosomes, and mesosomes are enclosed in a peptidoglycan complex wound in a helix and surrounded by an outer envelope (OE). An outer membrane, (variously described as three or five layers depending on the method of fixation), totally enclosing each leptospire, is composed of protein, lipid, and lipopolysaccharide (LPS). Extrusions, presumably of LPS, and blebs can be seen to originate from the OE in some preparations. Heat (Faine et al., 1964), ethanol, sodium deoxycholate (Yanagawa and Faine, 1966), lysozyme, detergents (Auran et al., 1972), and NaCl all can remove OE to produce spherical sacs approximately 1.5–2 μm in diameter, sometimes seen as granules in aging cultures. The OE is necessary for leptospiral integrity; in intact organisms antibodies react only with surface antigens of the OE, which prevents access to subsurface antigens. OE preparations are immunogenic and have been used as vaccines. Nothing is known of the details of membrane structure.

Leptospiral flagella (previously known as "axial filaments"), presumed to mediate motility (see Chapter 191), are inserted into the cell wall in a manner typical of a Gram-negative cell, with insertion of a knob, two pairs of discs connected by a rod, and a hook area. However, *Leptonema* has a Gram-positive type of flagellar insertion with a single pair of discs (Hovind-Hougen, 1979). Each leptospire or leptoneme has two flagella within the helix, apparently in a groove in the cell wall, each originating near one end, closer to the tip in straight forms of leptospires compared with hooked forms. Flagella removed mechanically (Nauman et al.,

Table 2. Classification of selected serovars of *Leptospira*.

Serogroup	Serovars
Interrogans group	
Australis	*australis*[a], *bratislava, fugis, jalna*[a], *lora, muenchen*
Autumnalis	*autumnalis*[a], *bim, bulgarica, butembo, erinaceiauriti, fortbragg*[c], *mooris, rachmati*
Ballum	*arboreae, ballum*[b], *castellonis*
Bataviae	*bataviae*[a], *brasiliensis, claytoni, paidjan*
Canicola	*benjamini, broomi, canicola*[a], *portlandvere, schueffneri*[a]
Celledoni	*celledoni*[d], *whitcombi*
Cynopteri	*cynopteri, tingomaria*
Djasiman	*djasiman*[a], *sentot*
Grippotyphosa	*canalzonae, grippotyphosa*[a], *valbuzzi*
Hebdomadis	*borincana*[e], *hebdomadis*[a], *kremastos*
Icterohaemorrhagiae	*birkini, copenhageni*[a], *icterohaemorrhagiae*[a], *lai, naam, smithi*[a]
Javanica	*javanica*[b], *poi, sofia, sorexjalna*
Louisiana	*louisiana*[c]
Manhao	*lincang, manhao*
Mini	*beye, georgia, mini*[b], *perameles, szwajizak*
Panama	*cristobali, panama*[c]
Pomona	*kunming, pomona*[a], *proechimys*
Pyrogenes	*mycastoris, pyrogenes*[a], *robinsoni, zanoni*[a]
Ranarum	*evansi, ranarum*[f]
Sarmin	*sarmin*[d], *waskurin, weaveri*
Sejroe	*balcanica, caribe, dikkeni, hardjobovis*[b], *hardjoprajitno*[a], *istrica, medanensis, saxkoebing*[a], *sejroe*[b], *wolffi*[a]
Shermani	*shermani*[e]
Tarassovi	*atlantae*[e], *bakeri*[e], *navet*[e], *rama, tarassovi*[b], *tunis*
Biflexa group	
Andamana	*andamana*[g]
Codice	*cdc* (or *codice*)[h]
Holland	*holland, zoo*
Semaranga	*semaranga*[f], *patoc*[g]

[a]*L. interrogans* (Yasuda et al., 1987).
[b]*L. borgpeterseni* (Yasuda et al., 1987).
[c]*L. noguchii* (Yasuda et al., 1987).
[d]*L. weilii* (Yasuda et al., 1987).
[e]*L. santarosai* (Yasuda et al., 1987).
[f]*L. meyeri* (Yasuda et al., 1987).
[g]*L. biflexa* (Yasuda et al., 1987).
[h]*L. wolbachii* (Yasuda et al., 1987).
Data from literature sources including Yasuda et al. (1987) and Kmety and Dikken (1988).

1969; Chang and Faine, 1974) and purified by differential centrifugation (Kelson et al., 1988) appear as coiled structures, approximately 15–18 nm in cross-sectional diameter and of variable length, some of which are sheathed. The core contains presumably protein globular structures arranged regularly, either linearly or helically, for the length of the flagellum. Flagellar antigens, assumed to be peptides, cross-react widely in contrast to the high specificity of LPS agglutinating antigens (Kelson et al., 1988).

A cell wall (Yanagawa and Faine, 1966) surrounded by a cytoplasmic membrane (Hovind-Hougen, 1979) contains the cytoplasmic contents and determines the helical leptospiral shape. Microfibrils originating in the cell wall (Yanagawa and Faine, 1966) were isolated from *L. biflexa* and characterized by Yanagihara and Mifuchi (1968) as a 72-kDa homogeneously migrating protein, which also contained carbohydrates in a protein:CHO ratio of 1:0.8. Cytoplasmic microtubules are characteristic of *Leptonema* (Hovind Hougen, 1979). Inclusions of NaCl were seen in a halophilic strain (Muggia) of *L. biflexa* (Hovind-Hougen et al., 1981a).

Chemical Composition

The chemical composition of leptospires, which includes a relatively high proportion of lipids (Johnson, 1976) and long-chained fatty acids (including the unusual positional isomer *cis*-11-hexadecanoic acid) (Johnson et al., 1970), is influenced by strain, growth conditions, and culture medium. Various pentoses, hexoses, heptoses, and hexosamines are found; fatty acids include hydroxylauric, palmitic, and oleic acids as major constituents in *L. interrogans* serovar *copenhageni* (Johnson, 1976; Vinh et al., 1986a). The cell wall contains diaminopimelic acid and 4-*o*-methyl mannose (Yanagihara et al., 1983). Some of the constituent lipids are responsible for the endotoxin-like properties of a specific cytotoxic glycolipoprotein (GLP) obtained from *copenhageni* leptospires with lysozyme and acetic acid precipitation (Vinh et al., 1986b).

LPS and surface protein antigens (Nunes-Edwards et al., 1985) are found in OE, along with variety of OE proteins, with significant differences among the strains of the serovars *copenhageni, pomona,* and *hardjo.* A group of non-agglutinating monoclonal antibodies to *copenhageni* reacted solely with a 37-kDa protein localized in the OE (Jost et al., 1988).

Leptospiral LPS prepared by hot phenol-water methods and extracted from either water or phenol phases or both, differs from the LPS of many other Gram-negative bacteria because it is not toxic and was reported not to contain authentic 2-keto-3-deoxyoctonic acid (KDO) (Shimizu et al., 1987; Vinh et al., 1986a), although electron microscopically it had a similar

ribbon form and globular ultrastructure. Significant variation in LPS occurs among serovars; the LPS of *hardjo bovis* contains KDO, which needs prolonged acid hydrolysis to release it in at least some serovars or genospecies, possibly because of the presence of substituted sugars (Vinh et al., 1989). Equivalents to "rough" or "smooth" type bacterial morphology are not recognized in leptospires, but their LPS bands in electrophoretic gel patterns are analogous to those of either rough or smooth organisms (Vinh et al., 1986a). The lipid A fraction analog has not been studied in detail, but preparations from the serovars *copenhageni* and *hardjo* lack hydroxymyristic acid.

Alkali treatment of LPS produces soluble erythrocyte-sensitizing antigenic and protective polysaccharides (Hindle and White, 1934; Faine et al., 1974), whereas antigenic and immunogenic polysaccharides, including oligosaccharides comprising four or more residues, were derived by acid hydrolysis. Among them, mannosamine has been characterized as an epitope reacting with monoclonal antibodies to serovar *hardjo* (Vinh et al., 1989; Jost et al., 1989).

Metabolic Activities

Various metabolic enzymes, not in use as differentiating criteria, have been identified in leptospires, including catalase, originating intracellularly; oxidase, which can be used to identify colonies in agar; fatty-acid desaturating enzymes; β-oxidative enzymes; lipases and phospholipases; aminopeptidases; tricarboxylic acid cycle enzymes; glyoxylate cycle and isoleucine deaminase enzymes; glycosidases; esterases; and various arylamidases. Four strains of halophilic leptospires were all oxidase-positive, but otherwise variable in enzyme activities (Cinco et al., 1975).

Phospholipases specific for erythrocyte membrane phospholipids (sphingomyelin, lecithin, and phosphatidyl choline) have been identified both by enzyme and hemolytic activities in *L. interrogans* serovars *bataviae, canicola, grippotyphosa, pomona,* and other pathogens, as well as in *L. biflexa* serovars; (Kasarov, 1970). Saprophytic leptospires contain phospholipases A and C, while the pathogens have only phospholipase A. There are two phospholipase groups among pathogenic leptospires: those containing a calcium-dependent sphingomyelinase and lecithinase, and those with neither; saprophytes produced a calcium-independent lecithinase alone (Kasarov and Addamiano, 1970). A sphingomyelinase C hemolysin from serovar *hardjo* (del Real et al., 1989) and an uncharacterized hemolysin from serovar *pomona* (Dain et al, 1988) have been cloned recently. Lipase activity groups have also been recognized (sec Table 1).

Lipases may allow leptospires attached to surfaces to "graze" on nutrient fatty acids at a solid-liquid interface (Kefford et al., 1983), to which they are attracted by a chemotactic gradient.

Genetic Composition and Variation

Leptospiral DNA may be used in genetic studies like other bacterial DNA. Cloned DNA or nucleotide sequences complement enzyme defects or deletions (Zuerner and Charon, 1988; Yelton and Cohen, 1986), or are expressed as antigenic leptospiral proteins (Doherty et al., 1989); either DNA fragments or specific sequences (Zuerner and Bolin, 1988; Yelton and Peng, 1989) have been used as probes for identification or diagnosis (see below), and leptospiral protein antigens have been cloned and expressed in recombinant *Escherichia coli* (Doherty et al., 1989).

Neither bacteriophages nor plasmids have been identified in *Leptospira* or *Leptonema,* but transformation between leptospires in fluid culture has been reported. The absence of an understood means of genetic transfer between leptospires handicaps research and knowledge of leptospiral genetics.

The GC content of leptospiral DNA falls in the range of about 33.5–40.7% (Yasuda et al., 1987). On the basis of genetic and other characterisitics (Table 1), six new species were defined among leptospires classified serologically in the species *L. interrogans.* These were named *L. borgpeterseni, L. inadai, L. interrogans, L. noguchii, L santarosai,* and *L. weilii.* Three new genospecies, *L. biflexa, L. meyeri, L. wolbachii,* were also identified by DNA-DNA homology and similarity in GC content of their DNA among leptospires classified serologically as *L. biflexa* (see "Classification and Nomenclature," this chapter) (Table 2). *Leptonema illini* and *Leptospira parva* each fell into separate new species (Table 1).

Closely related or apparently identical serovars may have different restriction endonuclease patterns (Hathaway et al., 1985), especially when a variety of restriction endonucleases is used, and conversely, antigenic variants selected by monoclonal antibodies may have similar endonuclease profiles (Yamaguchi et al., 1988) compared with their parent strains. Nevertheless, grouping of leptospires by their restriction

endonuclease patterns broadly follows the conventional serological groups.

Classification and Nomenclature

The genus name *Leptospira,* given by Noguchi in 1917, means a "very thin spiral (= helical)" form (Faine and Stallman, 1982). As new types of *Leptospira,* obviously different pathogenically and ecologically but indistinguishable morphologically or culturally with the methods then available, were recognized in the 1920s and 1930s, the only criteria for identification and classification of these slowly growing, metabolically sluggish, hard-to-see bacteria, was serology. The serovar was adopted as the basic taxon, and an arbitrary criterion—10% residual titer of standardized hyperimmune rabbit sera after carefully controlled cross-absorption—was adopted for definition of a new serovar. There is currently sufficient partial cross-reaction to be able to group different but similar serovars together in 23 serogroups which have ecological, diagnostic, pathogenic, and epidemiological significance, but no formal taxonomic status. (Dikken and Kmety, 1978; Faine, 1982; Faine and Stallman, 1982; Johnson and Faine, 1984; Kmety and Dikken, 1988). In general, pathogenic leptospires are in separate groups on the basis of their genotype, serotype, lipase, and other characteristics from saprophytes (Table 1). The pathogenic leptospires are all placed in a single provisional species, *L. interrogans,* and all non-pathogenic types in a provisional species, *L. biflexa.* Within these are over 200 individually-named serovars, resulting in a cumbersome if unavoidable trinomial nomenclature (Table 2). Some serovars identical by serotyping are not homogeneous when compared by DNA-DNA homology, GC content, and DNA restriction endonuclease patterns, and sometimes also by lipopolysaccharide composition and structure (Le Febvre and Thiermann, 1986; Vinh et al., 1989). This causes as yet unresolved problems in identification, nomenclature, and classification, particularly because of the practical relevance of serological classification. Taxonomic applications of monoclonal antibodies (Terpstra et al., 1985; Ono et al., 1987) reveal one or more serogroup-specific epitopes in some serogroups (Farrelly et al., 1987; Vinh et al., 1989), while in others there appears to be a mosaic of epitopes whose relative quantitative distribution corresponds with and probably mediates serovar specificity (Adler et al., 1989). Common serogroup antigens, identified qualitatively by the techniques of ELISA or immunoblotting, are useful for broad classification.

Physiology

Growth

Leptospires grow by elongation of the cell body followed by a constriction and pinching off at a flexible joint in the middle of the cell. Eventually, the two halves separate. A new flagellum develops at the new free end and grows in toward the middle of each daughter cell. Pairs are common. Under some poorly-understood culture conditions, separation fails and chains of leptospires are formed. Very long forms also occur, sometimes in chains, particularly where amounts or types of fatty acid in the medium are inadequate. Thus, there are different requirements for cell division and for separation.

Leptospires well adapted to their environment grow with a doubling time of 6–8 h in fluid or solid laboratory media at 28–30°C, or in vivo. Freshly isolated pathogens or environmental strains may take days or weeks to adapt to laboratory conditions, with doubling times as long as 14–18 h. The lag phase may last for days, especially for new isolates, but is short or imperceptible with laboratory cultures grown from relatively large inocula (approximately 5–10% of culture volume) subcultured from vigorously growing cultures. The stationary phase is reached at culture densities of approximately 10^8 cells/ml in serum media and 10^8–10^9 cells/ml or more in cultures in Tween-albumin media. The culture density is especially enhanced if the culture is well aerated, and it can persist at these levels for days or weeks. The growth rate in solid or semisolid medium is not known. Cultures stored in the dark at room temperature may be viable for years.

L. biflexa and related saprophytic strains tend to grow faster. *Leptonema illini* grows to maximum culture densities of about 10^9 cells/ml in about 24–48 h at 30°C in trypticase soy broth or leptospiral media.

Nutritional Requirements

The basic nutritional requirements for the growth of leptospires include nitrogen in the form of ammonia, derived from its salts or from the deamination of asparagine by asparaginase in serum in the medium; carbon in the form of long-chain (C_{15-18}) fatty acids; thiamine; cyanocobalamin; and oxygen. Pyruvate enhances growth from small inocula in some strains. In general, sugars are not metabolized, and short-chain fatty acids cannot be utilized unless long-chain fatty acids are also available. Although these are general requirements throughout the genus, there are differences between the sapro-

phytic and the parasitic members, and some variability within the latter.

Some of the parasitic leptospires, identified so far by serovar rather than species, require long-chain unsaturated fatty acids, while all the others and the saprophytic group utilize saturated fatty acids. Members of the latter group also produce lipases, while only some of the parasitic ones do. Long-chain fatty acids are required for growth, but are broken down by β-oxidation; leptospires contain relatively high concentrations of long-chain saturated, and some unusual unsaturated, fatty acids, mainly as constituents of their lipopolysaccharides.

Energy requirements of leptospires are derived from oxidation of fatty acids. End products of metabolism are undefined. The metabolic enzymes described are assumed to act as mediators of conventional metabolic pathways, but there is little direct evidence, virtually none of it recent. In addition to a need for thiamine (5–25 mg/1) and cyanocobalamin (20 μg/1), optimum iron concentrations (10^{-5}M) and traces of copper and manganese are required. Leptospires synthesize their own pyrimidine bases, allowing 5-fluorouracil (100 mg/1) to be used as a selective agent in culture media (Johnson, 1981).

Metabolism

Leptospires have an aerobic or a microaerophilic metabolism. Addition of approximately 1:100,000 concentration of a redox indicator, such as 1-naphthol-2-sodium sulfonate indo-2,6-dichlorophenol (Eh −0. 180 mV at pH 7.2) or 2,6-dichlorophenol-indophenol (Eh −0.200 mv at pH 7.2) (Lawrence, 1951), to semisolid or solid media results in discoloration of the medium below the disk, or at and for a few mm around the subsurface colony sites before and when colonial growth appears. The optimum pH for growth of leptospires and leptonemes is in the region 7.2–7.4. Acid, even at pH 6.8–6.9, is rapidly lethal, whereas alkaline conditions up to pH 7.8–7.9 are tolerated. Growth in laboratory media at those extremes is poor. An acid urinary pH, as in some carnivores or omnivores, will prevent the urinary excretion of live leptospires, but not necessarily their continued growth in renal tubules. Soil composition and pH have important ecological effects on the survival of excreted pathogenic leptospires and their spread to new hosts via surface waters (Elder et al., 1986).

Leptospires and leptonemes appear to be essentially aquatic organisms. Dry conditions are lethal, and they cannot be recovered from cultures, body fluids or tissues, or surface waters after drying under normal conditions. They will survive rapid freezing as in lyophilization, at −20 to −70°C in blocks of tissue, or for a few days in chilled meat (Peet et al., 1983). They do not survive for long under anaerobic conditions, nor do they appear to survive intracellularly following ingestion by phagocytes. Nevertheless, they appear to flourish in nature in reducing environments where oxygen levels are low, as in swamp water, soil, the renal tubules of mammalian hosts, and intercellularly or interstitially in the tissues of a host animal during the acute stages of infection.

Leptospires are very sensitive to the action of detergents, including sodium deoxycholate and soaps. Since long-chain fatty acids essential for growth and metabolism are also toxic, they must be made available to the leptospires at nontoxic concentrations. They are usually absorbed to a detoxifying absorbent such as serum albumin, charcoal, or, in nature, particles or surfaces from which they are released to the leptospire. Leptospires may be attracted to such surfaces by a nutrient gradient. In laboratory media serum albumin used as an additive in powder form or in animal serum serves as the absorbent.

Saprophytic leptospires and leptonemes will grow at temperatures as low as 11–13°C, a property useful for identification (Table 1). Their optimum temperature for growth is in the range 25–28°C. Pathogenic serovars will not grow at 13°C, and have an optimum around 28–30°C after adaptation in the laboratory. Recent mammalian isolates can be maintained at 37°C for many subcultures, provided they are transferred frequently while growing vigorously. Non-pathogens and avirulent pathogens do not survive 0.85% NaCl in Korthof medium at 37°C but can grow at 30°C. Halophilic leptospires require sodium concentrations from 0.22–0.44 M and can tolerate up to 0.65–0.75 M (Cinco et al., 1975). Under these conditions, their oxidases and tributyrinases are active, and they are otherwise similar to other *biflexa* serovars.

None of the leptospires survive in the laboratory at temperatures above 41–42°C. Leptospires have been preserved in liquid nitrogen for at least 3 years (Alexander et al., 1972). All the non pathogens, including the halophiles, can grow in the presence of 10 mg/1 of $CuSO_4$. The salt concentration of optimum culture media for leptospires contains only about one-10th of the salt concentration of physiological saline, which is lethal to many strains. Very low salt concentrations are well tolerated; leptospires survive long periods in stream or tap water, but washing in water or dilute buffer accompanied by strong centrifugation for laboratory studies

removes the outer envelope. All the nonpathogens will grow in the presence of 225 mg/l of 8-azaguanine or 5–10 mg/l of 2-6–diaminopurine; some of the pathogens will also grow in the latter.

Most leptospires are susceptible in vitro to a wide variety of antibiotics at low, therapeutically useful, levels (Faine and Kaipainen, 1955; Broughton and Flack, 1986; Oie et al., 1983; Alexander and Rule, 1986). Resistance to neomycin allows its use as a selective agent. Resistance to penicillin or streptomycin develops readily in culture but is not considered to be a problem clinically. Mutual antagonistic activity between leptospires in solid media in petri dishes has been described but not characterized.

Sera from animals apparently never infected with pathogenic leptospires contain IgM molecules capable of reacting with surface receptors in the OE of nonpathogens (biflexa type) (Johnson and Muschel, 1966) and avirulent pathogens (Faine and Carter, 1968). This reaction may determine survival after entry to an animal host. Immunoglobulins appear in the animal urine in the acute stage of infection and in early convalescence, as a result of overflows of serum antibodies through damaged kidneys. Urinary antibodies may also occur in animals which are renal carriers, even though they are excreting leptospires, in the absence of detectable structural or functional renal abnormality. Leptospires excreted in urine may be coated with host immunoglobulin and be refractory to agglutination by serum antibodies.

Pathogenicity in Relation to Habitat

The reason why some leptospires are pathogenic and some are not is still unclear. It has not been proven that all the biflexa type leptospires isolated from animal urine were pathogenic. All the recognized pathogens and nonpathogens fall into distinct genetic groups. (Table 1). Among the pathogenic varieties there are unanswered questions of why some animals are resistant, or conversely, why they are susceptible to infection with certain serovars. Ecological considerations will determine the chances of leptospires surviving in the environment after excretion from their hosts, and thus the chances of infection of a fresh susceptible animal.

After entry and growth in the host animal, pathogenic leptospires must have mechanisms to evade host defenses and to produce the sometimes severe damage to host tissues. Both virulent and avirulent leptospires attach to cell surfaces (Vinh et al., 1984), and virulent leptospires may attach preferentially by their ends to fibronectin, collagen, and laminin in the extracellular matrix of cells (Ito and Yanagawa, 1987a and 1987b). Details of leptospiral attachment to the surfaces of renal tubular epithelial cells and the relationship of the mechanisms to those described by Kefford et al. (1983) for attachment of *L. biflexa* to noncellular surfaces are obscure (Ballard et al., 1986), but the issue remains central to the understanding of the pathogenesis, epidemiology, and prevention of leptospirosis.

Among pathogens, virulence for any or all animals may be lost on culture passage, because avirulent organisms better able to grow in culture conditions are selected. Virulence may be regained by animal passage, or by selecting organisms better adapted to survive and grow in vivo (Faine, 1957). Susceptibility to serum-innate IgM antibody may be a mechanism determining virulence. The effector on the leptospire may be a carbohydrate found in virulent organisms.

Although leptospiral LPS is not significantly pyrogenic and lacks some other typical endotoxic properties, a glycolipoprotein from *L. interrogans* serovar *copenhageni* was toxic for mice and cytotoxic for cultured cells, acting by means of constituents that were uniquely leptospiral long-chain fatty acids, possibly by competitively inhibiting the incorporation of naturally occurring lipids in the cell membrane. The toxicity in vitro was obscured in the presence of serum albumin, which absorbed the toxic lipids (Vinh et al., 1986b). A similar action of this or another toxin on platelet membranes could account for platelet aggregation and consequent coagulation defects, which are features of severe leptospirosis. Phospholipase activity is related to selective hemolytic activity for various animal species, consistent with the host range of pathogenic leptospires being determined by the specificities of their phospholipases for the phospholipids of the cell membrane of the host species. While hemolytic activity in vivo has not been proven directly, the hemolysins are specifically neutralized by post-infection immune serum. Changes resembling punched-out holes were found by electron microscope in erythrocytes of calves with leptospirosis and are consistent with either cytotoxin or hemolysin activity (Thompson and Manktelow, 1986), and autoimmune hemolysis may occur from the absorption of complement and antibody binding to leptospiral antigens on erythrocytes (van der Ingh and Hartmann, 1986).

Specific immunity in leptospiroses is mediated by humoral (antibody) means alone. Very low levels of specific immunoglobulins are

protective if they are surface-active (agglutinating) on leptospires and are opsonic. Increased resistance with age in animals parallels maturation of the B-cell immune response. The rate at which antibodies are formed in relation to the rate of growth of the leptospires in the host is a major determinant of the outcome of infection (Faine, 1957, 1962).

The only known determinants of immunity in leptospirosis are opsonization and phagocytosis. In both cases, the only leptospiral components recognized as reacting are LPS and its constituents; the reacting molecules, identified using opsonic and protective monoclonals, are polysaccharides of the side chains of the LPS and are located in the outer envelope (Jost et al., 1988, 1989). Serovar specificity depends on the nature of the LPS antigen polysaccharides.

Isolation

Special techniques are used for the isolation of any type of leptospire from environmental or clinical sources (Johnson, 1981; Faine, 1982, 1988) because leptospires grow comparatively slowly and are relatively susceptible to acids and other metabolic products of accompanying and contaminating bacteria or fungi. Fluid media are usually used, with filtration or selective agents to eliminate contaminants. Growth is less reliable in solid media which can be used in some circumstances but are likely to dry out during prolonged incubation.

Leptospires (including pathogenic varieties) and leptonemes have been isolated from surface waters (rivers, streams, lakes, soil water, mud). In these sites they pose a hazard to anglers, farmers, military personnel, and boaters. There are sometimes suprisingly high concentrations even in fast-flowing rivers. Sensitive culture methods and freedom from contamination are essential for isolation from these sources. Suitable specimens are water, soil, or mud samples collected into dry containers (to avoid contamination with leptospires in water in the containers). Although direct inoculation into selective culture medium, with or without prior serial dilution, may be used, it is usually desirable to concentrate the specimen and necessary to remove or reduce contaminating microorganisms. Cultures need to be checked in case more than one type of leptospire is present.

In general, leptospires, but not other bacteria, will pass through a membrane filter of 0.22 μm pore size; the filtrate is cultured. Preliminary passage through a prefilter or a 0.45 μm membrane will facilitate filtration. An older, effective, but less acceptable, method is to use rapid biological filtration of leptospires into the bloodstream from the peritoneal cavity of mice, hamsters, or guinea pigs (Faine, 1982). The same methods of filtration, growth on solid media, or animal inoculation may be used to purify contaminated cultures.

Leptospires may be isolated from the kidneys of renal carriers or from alkaline urine, as well as from blood and all other tissues and body fluids of acutely infected animals or humans. Autolysed or contaminated specimens or acid urine are unsuitable for examination. Specimens collected aseptically are desirable. The urine is centrifuged, and the sediment inoculated into fluid standard or selective medium.

Blood taken aseptically from human or animal sources in the acute stages of infection may be cultured by adding 0.1–0.5 ml to 10–50 ml of EMJH medium and incubating at 30°C. Blood specimens for culture may be transported after aseptic addition of EDTA or other anticoagulants (see below). Tissues removed aseptically at autopsy can be ground and extracted with buffered saline or culture medium, and the supernatant, after settling, can be used for inoculation of culture media or animals (whose blood can then be cultured). Details of methods can be found in the references cited above. (Alexander, 1985; Faine 1982, 1988).

Culture Media—Principles and Preparation

Traditional leptospiral media were dilutions of 8–10% v/v serum, usually from rabbits, in a diluted nutrient broth or peptone solution. The development of bovine albumin detoxicant and polysorbate 80 (Tween 80) as a nutrient source (Johnson, 1981) allowed the isolation of more exacting strains and the maintenance of cultures under reproducible conditions. Water used to dissolve nonautoclavable filter-sterilizable additives must be previously heat sterilized, and containers used for filtration should be dry and preferably sterilized by heat to avoid contamination from filter-passing leptospires.

Details of the composition, recipes, and methods of preparation of commonly used culture media may be found in recent references (Johnson, 1981; Faine, 1982; Alexander, 1985; Faine, 1988). Serum media, e.g., Korthof medium, Stuart medium, or Schüffner-Verwoort medium, commonly contain approximately 0.8–1.0 g/l peptone and 8–10% pooled slightly hemolysed rabbit serum, phosphate-buffered at approximately 0.01M to a final pH in the range 7.2–7.6. The serum is added to the autoclaved base medium and the final mixture sterilized by passing through a 0.22 μm filter membrane, or sterile serum may be added aseptically to the

autoclaved base. Protein media without serum use bovine serum albumin (BSA) as a detoxicant and are based on the oleic-acid-albumin media of Ellinghausen and MacCulloch, modified by Johnson and Harris (EMJH medium) (Johnson, 1981). The quality of the water and the albumin (fatty acid content, dryness) are critical. Albumin should be from batches tested for suitability, from known manufacturers. In an extreme, the albumin and the Tween may be purified before use by extraction with solvents (Adler et al., 1986).

Growth is indicated by a birefringent swirl in the medium on shaking; some strains never achieve greater density, while others reach visible turbidity, seldom opacity, indicating growth to approximately 10^9 cells/ml or greater. Cotton plugs should not be used because toxic fatty acids may be leached from the cotton into the medium.

Many laboratory strains will grow in protein-free medium (Bey and Johnson, 1978; Adler et al., 1986) with careful attention to water quality, if the Tweens are purified by extraction of free fatty acids, their further oxidation minimized, and the proportions of different Tweens (Tween 20, Tween 40, Tween 80) are balanced for the specific strain. Frequent subculture is necessary during growth on protein-free medium. In cultures without protein detoxicants, some strains produce lipases whose end products are especially toxic to leptospires, including themselves. Strains need to be subcultured in gradually reducing protein concentrations to adapt to protein-free media, which are generally not suitable for attempts at fresh isolations from clinical or environmental sources. A limited range of adapted strains are maintained under protein-free conditions and used for vaccine production. *Leptonema* can grow in trypticase soy broth to reach concentrations approximating 10^9 cells/ml in 24–48 h at 30°C, although there are no studies of growth rates or nutritional requirements. Sodium-dependent leptospires were grown in a medium containing 0.25 g NH_4Cl, 0.25 g Tween-85, 10.0 g BSA, 10 mg thiamine, and 1 mg cyanocobalamin in 1 liter of artificial sea water (Cinco et al., 1975).

SOLID MEDIA AND SEMISOLID MEDIA. Most leptospires will grow in or on media solidified with agar (Faine, 1982), provided the agar is not toxic and fungal contamination and dehydration during storage and incubation are avoided. Following surface or stab inoculation of semisolid or solid media (0.2–0.8% agar), leptospires grow in disks (Dinger disks) below the surface, although colonies may appear at first in the depths. In solid medium (0.8–1% agar), poorly visualized colonies appear in the depths of the medium in 2–20 days, depending on the leptospire and the quality of the medium, and the colonies may spread centripetally as gray transparent spheres up to 2 mm in diameter with defined edges. In shallow media, as in a petri dish, a colony may form a white-edged ring covering several centimeters, representing a spreading, inverted hemispherical colony. The advancing edge of the spreading growth, which can be stained with oxidase reagent, is dense with leptospires, and can be picked as a plug with a sterile pasteur pipet for transfer or for microscopic examination. These colonies, which are the analogs of the Dinger disks, were first described by Larson and Cox (1957). Growth on or in solid media is brownish-grey by transmitted light, but whitish by reflected light and very hard to detect in uncolored media. Incorporation of a 1:100,000 concentration of a redox indicator (see "Physiology," this chapter) enhances visibility. The potential of growth in colonies on the surface of solid (2%) agar (Wood et al., 1981) has not been exploited.

L. biflexa strains will grow at a lower concentration of serum or BSA, more quickly (visible or even maximum growth in 24 h at 30°C), and more densely, than most *L. interrogans* strains. The reasons for these differences have not been elucidated.

SELECTIVE AND DIFFERENTIAL MEDIA. Knowledge of special growth requirements has allowed the development of selective culture media, containing 2-6-diaminopurine, 8-azaguanine, or neomycin, for growing leptospires in the presence of contaminants (Faine, 1982, 1988). An enriched medium for better isolation of leptospires from animals was described by Ellis et al. (1982).

Tissue cultures may be contaminated with filter-passing *L. biflexa* in solutions made with unheated tapwater used for culture media or with pathogenic leptospires introduced with primary cultures of animal kidneys, inadvertently derived from carrier animals.

Techniques of Culture and Subculture

Recent accounts and full details of special techniques demanded by the special characteristics of leptospires and their media are given in Johnson (1981); Faine (1982, 1988). Culture purity and density may be checked under darkfield microscopy (see below).

Identification

Methods and Techniques

Leptospires are poorly visualized after staining with strong carbol fuchsin or other dyes. Silver

may be deposited on the surface but the techniques are exacting and details are easily obscured. Stains are not used for light microscopy, except silver, although immunofluorescence or immunoperoxidase can be used in sections of human or animal tissues. The usual routine method is darkfield microscopy. Electron microscopy is useful for identification and classification, and immunoelectron microscopy, especially with immunogold, has been used for experimental identification of structures whose antigens have been recognized serologically.

Low-power darkfield microscopy using a dry condenser is the standard method for checking growth in cultures, and for reading agglutination tests (Faine, 1982). To observe fine details of morphology at the limits of optical microscopy, it is necessary to use a high-resolution oil immersion objective (N.A. 1.25–1.3) equipped with a diaphragm or funnel stop to match the corresponding oil immersion condenser (N.A. 1.2–1.4). A thin slide, a very thin cover glass, both extremely clean, and a thin preparation sealed against movement resulting from drying currents are necessary for technically satisfactory conditions. Phase contrast, which can reveal some detail in leptospires and the spherical "granules" (Faine et al., 1964) derived from them, is unsuitable for routine observation of cultures. Immunostains are useful for both cultures and tissue or environmental specimens if the serovar is known or if a broadly cross-reacting antiserum is used, and also as a tool for research on the specificity of antigens, on monoclonal antibodies, and for localization of antigenic sites. Electron microscopy is not usually used for the routine identification of leptospires or related genera (except for *Leptonema*) but combined with negative staining and immunogold-labeled polyclonal or monoclonal antibodies, it has demonstrated the location of antigens and epitopes corresponding to flagella (Kelson et al., 1988), lipopolysaccharides, and the outer envelope (Jost et al., 1989).

Characteristics and Criteria

There are no reliable criteria to differentiate various types of *Leptospira* from one another or from *Leptonema* by darkfield microscopy (Faine and Stallman, 1982; Johnson and Faine, 1984). Leptospires are typically thin, bright, beaded, motile, rodlike structures, about 0.2 μm in diameter, ranging from approximately 8–25 μm in length; when viewed with very high magnification they are seen to be helical. The rod appears rigid except at the ends distal to the flagellar insertions where it is flaccid. There is rapid, but sometimes irregular and erratic, translational movement in either direction. Rapid rotation on the long axis causes the ends to bend against the direction of translational movement to give a hooked appearance at one or both ends. Straight (not hooked) variants have a much shorter flaccid portion and flagellar inserts much closer to the end. Flagella, the outer envelope, and other structures cannot be seen by light microscopy, but can sometimes be visualized with difficulty following specific immunostaining with appropriate selective antibodies. Internal contents in granular forms can be seen by darkfield and by phase contrast microscopy; electron microscopy shows that these are rolled-up, protoplasmic cylinders within a membrane.

In semisolid media, leptospires move with a characteristic screw-like motility, flexing and winding around obstacles. The rigid form is resumed immediately in fluid media. Motility is enhanced proportionately with the viscosity of the medium.

Leptospira can be differentiated from *Leptonema* by electron microscopy and by growth in trypticase soy broth (TSB) medium, and *Leptospira interrogans* serovars from those of *L. biflexa* by growth at 13°C and in 8-azaguanine (Table 1). All *Leptospira* require long-chain fatty acids, usually detoxified with serum albumin in culture, but *Leptonema illini* grows in TSB medium. A monoclonal antibody reacting exclusively with nonagglutinating antigens in members of the *Leptospira interrogans* group in ELISA tests (Jost et al., 1988) is useful for rapid identification. Other genera of spirochetes (see Chapter 191) are readily differentiated from *Leptospira* and *Leptonema* by their morphology, motility, and number of flagella, and by serology (Johnson and Faine, 1984; Baker-Zander and Lukehart, 1984; Magnarelli et al., 1987).

"Bacterial restriction endonuclease analysis" (BRENDA) (Robinson et al., 1982) is useful for relatively rapid presumptive identification and classification of isolates and for subclassification and revision of taxonomic categories, as is the determination of the GC content and DNA homology grouping.

The officially recognized classification is serological (Faine and Stallman, 1982; Johnson and Faine, 1984; Kmety and Dikken, 1988) measured by agglutination of live cultures, with antibodies against heat-labile antigens (Dikken and Kmety, 1978). Details of the serological methods are in the references above and in Faine (1982). For the microscopic agglutination test (MAT), serial dilutions of sera are mixed with an equal volume of culture, incubated at 30–37°C for 1 h or longer, and a loopful of each

dilution examined by darkfield microscopy for agglutination, taking 50% agglutination as the endpoint. Cross-reactions between serovars are common. The strain reacting to the highest titer is not necessarily the homologous serovar for the antiserum being used. Serovar classification cannot be done without cross absorption. LPS patterns after gel electrophoresis and immunoblotting may assist in identification of serologically apparently identical strains. (Vinh et al., 1989).

Mouse monoclonal antibodies produced against most of the main serogroups of *Leptospira* following immunization with whole leptospires, sonicated suspensions, or chemical fractions include all classes of murine immunoglobulins, with cross-reacting serovar specificities often different from rabbit, human, bovine, or other animal immunoglobulins. Generally, these reactions confirm taxonomic relationships that have been revealed by agglutination absorption studies (Ono et al., 1982; Farrelly et al., 1987; Terpstra et al., 1987; Jost et al., 1988). Species-reactive antigens *(L. interrogans)* and serogroup- as well as serovar-specific and other epitopes, some of them not involved in agglutination, have been identified by ELISA, thus solving some classification problems. Judiciously chosen monoclonals can expedite the tedious serological identification of isolates.

Applications

Diagnosis of Leptospirosis

The same diagnostic principles apply for human or animal leptospirosis. Ideally, the infecting agent should be recognized, cultivated, and identified, but this is infrequently achieved because of inadequate or late clinical suspicion, slow growth, and the need for specialized techniques. Indirect, usually serological, evidence of infection is commonly used, but tests of molecular diagnosis by DNA or RNA probes are currently being explored. Additional, nonetiological tests such as histological, hematological, and biochemical changes may assist diagnosis.

The diagnosis of illness requires different techniques from screening for epidemiological surveillance. Rapid, generic diagnosis is often urgently required in humans or animals to decide whether or not an illness is leptospirosis, and, if so, which type (for management, therapeutic, prognostic, and epidemiological reasons) at a time when therapeutic intervention and management can affect the progress of the disease. Screening tests provide evidence of an underlying level of subclinical infection in a population, in addition to those diagnosed as clinical cases, but speed is seldom vital in these cases. Low titer reactors are significant because they indicate previous infection.

The techniques of specimen selection, microscopy, cultivation, agglutination, immunofluorescence, ELISA, and identification are described above and in Faine (1982) and Faine (1988).

CULTURE OF SPECIMENS. Usually blood, urine, and cerebrospinal fluid (CSF), but sometimes necropsy and biopsy tissues are cultured. A significant dilution of at least 1:50 of blood in medium is recommended. Urine should not be acid. Growth is seen as a birefringent swirl, confirmed by darkfield microscopy, with care to ensure that similar looking fibrin threads are not mistaken for leptospires.

Tissues such as kidney or liver should be cut or ground aseptically into very small fragments, and no more than approximately 5% (w/v) inoculated, to avoid anaerobiosis in the medium. Fragments or suspensions of tissues may also be inoculated directly onto the surface of solid medium in plates. Growth is recognized by the appearance of small gray-white subsurface colonies or by a ring of growth around the inoculum site.

MICROSCOPY. Darkfield microscopy is generally used for direct examination of specimens; electron microscopy is seldom valuable. Properly controlled and interpreted histological examination of tissues stained for leptospires by silver, immunofluorescence, or immunoperoxidase assay offers a presumptive, though frequently accurate, diagnosis. Attempting direct identification of leptospires in body fluids or tissues by darkfield microscopy is not recommended, especially for novices, although certain identification of leptospires by microscopy can speed diagnosis and management.

SEROLOGICAL DIAGNOSIS. Most leptospirosis is diagnosed by serology, using MAT. ELISA tests for IgM or IgG or both, sometimes used in parallel, or immunoblots or immunobeads have not yet gained acceptance as substitutes for agglutination because their diagnostic interpretation is still unclear. MAT antibodies react with LPS polysaccharides and are relatively specific for the infecting serovar and serogroup, although the highest titer does not necessarily indicate the infecting serovar because there are cross-reactions between serovars and individual differences in immunoglobulin response to the antigens. Only isolation and identification of the

infecting leptospire can prove its identity. Unless the infecting serovar is known, it is necessary to test sera against a battery of serovars representative of the serogroups known to exist locally, preferably using locally isolated strains.

A titer of 1:400 or higher in a single serum specimen or a rising 4-fold titer between consecutive specimens is considered significant in an acute infection, although there are extreme differences in titers in acute leptospirosis caused by different serovars. Very high titers of 10,000 or more with the serovars *icterohaemorrhagiae* or *copenhageni* and low responses of 800–1600 with *hardjo* are typical both in humans and animals. Antibodies in humans last for months to years. There is little reliable data on persistence in animals. Effects on persistence of antibody titers of antibiotic treatment, initial height of titer, infecting serovar, immunoglobulin class, or possible continuous or repeated exposure to the same or other leptospires have not been adequately assessed, although this information is vital for understanding immunity and for evaluating immunization.

ELISA tests have been more valuable in screening than in diagnosis, possibly because of the current lack of standardization and experience in interpretation. Serum IgM antibodies in carrier pigs correlate with urinary shedding (Ballard et al., 1984). The immunoblot test using culture or LPS on nitrocellulose is a sensitive method now being developed for rapid diagnosis (Watt et al., 1988), and DNA restriction endonuclease patterns expedite preliminary classification of isolates. Direct diagnosis by using gene probes, including polymerase chain reaction, for recognition of leptospires in tissues or body fluids, or for identification of isolates has been explored recently (Millar et al., 1987; van Eys et al., 1988, 1989; Zuerner and Bolin, 1988).

PROSPECTS FOR IMPROVED SERODIAGNOSIS. Serological diagnosis always depends on the presence of detectable antibody. No tests, however sensitive, can detect antibody in the first day or two of infection in a novice patient before it has been produced, yet when diagnosis is needed most urgently. Monoclonal antibodies with either genus or serovar specificity can aid identification of isolates. Their main potential use in diagnosis is primarily in research for characterizing specific or generic antigens for faster, more sensitive, or more appropriate tests. Genotypic classification of leptospires by DNA homology is discussed above.

Vaccines: Principles of Immunity and Immunization

All evidence shows that immunity to initial infection or to reinfection is humorally derived and is specific for the serovar (sometimes serogroup). Such immunity is mediated by any class of immunoglobulin with the appropriate epitope specificity (Jost et al., 1989). Immunity follows both infection and vaccination; its duration is uncertain, but experimentally it coincides with detectable agglutinating antibody, even at very low levels. Killed vaccines of whole leptospires are widely used for animals (dogs, pigs, cattle), and, in Asia, for humans, conventionally in two initial injections about a month apart, followed by an annual booster. Vaccines of outer envelope preparations and leptospires grown in protein-free media are effective and believed to cause fewer side effects, but still probably owe their efficacy to their LPS content. Experimentally, both leptospiral LPS alone (Jost et al., 1989; Masuzawa et al., 1989) and a derived polysaccharide containing the protective antigen (Jost et al., 1989) are effective vaccines. At present there is no evidence of any protein or peptide protective antigen; none of the proteins cloned into recombinants have been protective. Conversely, none of the genes for the known protective LPS or polysaccharide antigens have been cloned. The biological disparity between the leptospires and the expression vectors tested makes expression of cloned genes especially difficult.

Economic Importance of Leptospirosis

The main economic importance of *Leptospira* lies in their ability to produce infection in animals and humans. The economic factors in leptospirosis (Faine, 1982, 1990) include direct or indirect costs of

- abortion, failure to thrive, loss of milk production, and associated veterinary costs in domestic and commercial livestock and dogs, with potential for malnutrition and impoverishment in individuals and communities dependent upon animal sources of protein, especially in subsistence economies;
- vaccination and surveillance (vaccines, veterinary attention, laboratory tests, evaluation and administration), including testing and certification of serum samples from large numbers of livestock before international transport;
- human illness, suffering, medical care, and loss of productive working time and capacity;
- production and evaluation of vaccines and vaccination programs for animals and humans;
- rodent control in livestock management, pastures (including sugar cane plantations and ricefields), and food processing;
- occupational disease surveillance and control, protective clothing, and insurance for work-

ers in industries where there is a significant risk (e.g., milkers of cows, slaughterers, assistants, inspectors, and laborers at abbatoirs, military personnel).

Biotechnological applications are confined to the production and development of vaccines and diagnostic reagents at present.

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CHAPTER 195

The Family Chlorobiaceae

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The species and genera included in the physiological and ecological group of the green sulfur bacteria (*Chlorobium* and related genera; the Chlorobiaceae) share a number of particular characteristics which suggest that these genera may be genetically related. However, until now only a few strains have been studied with respect to the similarities of the oligonucleotide sequences of their 16S rRNA (Woese, 1987) and their 5S rRNA (van den Eynde et al., 1990). Thus, the Chlorobiaceae cannot yet be considered as a family of genetically related genera comparable to the Chromatiaceae (Fowler et al., 1984) (see also Chapter 170). The closest relatives of the Chlorobiaceae analyzed so far form a cluster containing *Bacteroides fragilis* and other *Bacteroides* species; with respect to all other groups of anoxygenic phototrophic bacteria, the Chlorobiaceae appear to be a well-isolated group (S_{AB} value < 0.25).

Habitats

Freshwater Habitats

In contrast to bacteria of the Chromatiaceae, visible, mass accumulations of green sulfur bacteria are rare in freshwater habitats. The first reports are from Szafer (1910) and Strzeszewski (1913) who described layers of green bacteria in the effluents of sulfur springs in Poland. Chlorobiaceae occurred at the highest sulfide concentrations, while purple sulfur bacteria appeared downstream at lower sulfide concentrations. Nonetheless, *Chlorobium* species are widely distributed in the anoxic mud and sediments of all freshwater habitats where their presence can be established, using an appropriate enrichment culture method.

The largest blooms of green sulfur bacteria in freshwater enrichments occur at the chemocline of stratified holomictic or meromictic lakes. Czeczuga (1968) reported that the green layer at the chemocline in Wadolek Lake, Poland, was dominated by *Chlorobium limicola*. In the Bolschoje-Kichier Lake, USSR, the green layer was formed by *Ancalochloris perfilievii* and *Pelodictyon luteolum* (Gorlenko and Lebedeva, 1971). A very dense layer of a green *Pelodictyon* species at a depth of 2m in Knaak Lake, Wisconsin, was studied by Parkin and Brock (1981). In many lakes, green and purple sulfur bacteria occur together in the layer, for example, in the Pluss-See (Northern Germany; Anagnostidis and Overbeck, 1966) and in the Schleinsee and Buchensee (Lake Constance region, Vetter, 1937; Overmann and Tilzer, 1989). In these lakes, the Chlorobiaceae are represented by species of *Ancalochloris* and *Pelodictyon,* and by the Pelochromatium and/or Chlorochromatium consortium. It is not uncommon for the green sulfur bacteria to proliferate just below the Chromatiaceae (Caldwell and Tiedje, 1975; Gorlenko et al., 1983). This can be understood if it is considered that the Chlorobiaceae, with their highly effective light-harvesting chlorosomes, are unique with respect to their modest radiation requirements. In order to grow at comparable growth rates, Chlorobiaceae require only about one-fourth of the light intensity of the purple bacteria (Biebl and Pfennig, 1978).

Lakes with Chromatiaceae and brown Chlorobiaceae species have also been studied by Bergstein et al. (1979) and Veldhuis and van Gemerden (1986).

Symbiotic Consortia of Green Sulfur Bacteria in Freshwater Habitats. One of the outstanding features of the green sulfur bacteria is their participation in the only known symbioses between bacteria. In these so-called consortia, a central chemoorganotrophic, usually motile, bacterium is covered by synchronously dividing, green sulfur bacteria. Although these consortia were originally given binominal names, such names are illegitimate as long as they are applied to symbiotic associations (Pfennig and Trüper, 1974; Trüper and Pfennig, 1971). At present the following consortia are known: Chlorochromatium aggregatum, Chlorochromatium glebulum, Cylindrogloea

bacterifera, Pelochromatium roseum, and Pelochromatium roseoviride (Gorlenko and Kuznetsov, 1971).

The Chlorochromatium consortium (Lauterborn, 1915; Chloronium mirabile, Buder, 1913) consists of a single, rod-shaped, polarly flagellated, motile, colorless, central bacterium which is covered by 6–12 regularly attached, rod-shaped, nonmotile, green cells of the *Chlorobium limicola* type. A high degree of metabolic interdependence is indicated by the synchronous growth and division of all cells of the consortium. Furthermore, the nonmotile phototrophic *Chlorobium* symbionts produce a positive phototactic response in the motile central bacterium: the consortia are capable of accumulating at the illuminated side of the culture vessel. Thus, because of the symbiosis, the nonmotile *Chlorobium* cells attain the ecologically significant capacity to respond to different light intensities, as do the motile Chromatiaceae (Pfennig, 1980).

In addition to the green Chlorochromatium consortium, there are two other brown ones. Lauterborn (1915) and Utermöhl (1925) first described the brown Pelochromatium roseum consortium. Here, the symbiotic phototrophic bacterium is a strain of *Chlorobium phaeobacteroides*. For the Pelochromatium roseoviride consortium (Gorlenko and Kuznetsov, 1971), the motile central bacterium is surrounded by an inner layer of 6–12 cells of *Chlorobium phaeobacteroides* and a second outer layer consisting of *Pelodictyon luteolum*-like cells.

Until now, the symbiotic consortia have only been studied in enrichment cultures. While the phototrophic components can readily be isolated in pure culture, all attempts to grow or identify the motile chemotrophic central bacteria have been unsuccessful (Pfennig, 1980).

The symbiotic consortia thrive in all kinds of freshwater habitats containing anoxic, sulfide-bearing mud and water: ditches, pools, ponds, and stratified lakes. Usually, they occur together with motile and/or gas-vacuole-containing, purple sulfur bacteria (Anagnostidis and Overbeck, 1966; Caldwell and Tiedje, 1975; Dubinina and Kuznetsov, 1976; Gorlenko et al., 1983).

MEROMICTIC LAKES. A special environment between holomictic freshwater lakes and shallow marine habitats are the deeper meromictic coastal lakes. Since the interface between the stagnant anoxic and sulfide-containing seawater and the overlying fresh or brackish water is very stable, the blooms of phototrophic sulfur bacteria are almost permanently present throughout the year. In many of these lakes, the brown Chlorobiaceae, in particular, *Chlorobium phaeobacteroides* and *C. phaeovibrioides*, are the dominant species. Examples are Lake Faro, Messina, Sicily (Trüper and Genovese, 1968); Lake Mogilnoye, Kildin Island, USSR (Gorlenko et al., 1978); Spanish Mediterranean coastal lakes (Guerrero et al., 1987); and Bietri Bay of Ebrie Lagoon, Ivory Coast (Caumette, 1984). Other meromictic lakes with reddish-brown layers are Fayetteville Green Lake (USA; Culver and Brunskill, 1969) with *Chlorobium phaeobacteroides*, and Repnoe Lake (USSR; Gorlenko et al., 1973) with the brown *Pelodictyon phaeum*.

Montesinos et al. (1983) determined the selective conditions under which the brown Chlorobiaceae compete successfully with the green species for the formation of blooms in lakes, studying the ratio between the green *Chlorobium limicola* and the brown *Chlorobium phaeobacteroides* in eight Spanish lakes. The results were compiled, together with data from 22 other lakes from around the world; the presence of the green or the brown species was compared with the depth at which the phototrophic bacterial plate was located. It is obvious from their list that green Chlorobiaceae were dominant in lakes with blooms near the surface (2–4 m) whereas, in blooms between 5- to 9-m depth, green and/or brown species developed. If the blooms occurred between 9- and 25-m depth, they were exclusively formed by the brown species.

Water selectively removes certain wavelengths of light and Montesinos et al. (1983) confirmed the distributions seen in their field data by laboratory experiments with pure and combined cultures of green and brown *Chlorobium* species in filtered light. The brown Chlorobiaceae have a selective advantage over the green species in the narrow band of blue-green radiation (450–550 nm) that penetrates deepest in the water column. Only the light-harvesting carotenoids of the brown species, isorenieratene and β-isorenieratene have a broad absorption band between 450 and 550 nm, allowing these species to effectively absorb the available blue-green radiation (Pfennig, 1989). The carotenoids of the purple sulfur bacteria also absorb in this region. However, these bacteria require higher radiation intensities for comparable growth rates (Biebl and Pfennig, 1978) and, therefore, cannot compete with the brown Chlorobiaceae at limiting light intensities.

Marine Habitats

An impressive example of the capacity of brown *Chlorobium* species to survive on incredibly low

amounts of blue-green radiation is the recent discovery of bacteriochlorophyll (bchl) *e* at an 80-m depth in the Black Sea. On the RV *Knorr* cruise 134, leg 9 in May 1988 (US-Turkish Oceanographic Expedition to the Black Sea), hydrogen sulfide was detected at depths of 80 m in the western basin (42°50'N, 32°00'E). In the acetone extracts of filtered samples from the sulfide interface between 68- and 92-m depth, four homologs of bchl *e* and the carotenoids isorenieratene and β-isorenieratene were identified (Repeta et al., 1989). These pigments indicated the presence of brown *Chlorobium* species. From cultures prepared from parallel membrane-filter samples of water from the 80-m depth, bacteria resembling *Chlorobium phaeobacteroides* have been obtained by N. Pfennig and J. Overmann (unpublished observations).

In shallow marine lagoons, stagnant seawater pools, and salt marsh environments, mass developments of green sulfur bacteria may occasionally occur. Even if not bloom-forming, the typical marine green sulfur bacterium, *Prosthecochloris aestuarii* (Gorlenko, 1970), can almost always be isolated from anoxic water and mud. Two other typically marine species have been described: *Chlorobium vibrioforme,* (Trüper, 1970; Caumette, 1984, 1986) and *Chloroherpeton thalassium* (Gibson et al., 1984).

Isolation

Preparation of Culture Media

Suitable culture media for the Chlorobiaceae are very similar to those for the Chromatiaceae. The medium composition and preparation procedures given in Chapter 170 for the Chromatiaceae can be used. Instead of trace element solution SL 12, however, the following EDTA-free solution SL 10 has also been successfully used (Overmann and Pfennig, 1989).

Trace element stock solution SL 10

Distilled water	1 liter
HCl (37%)	8.5 ml
$FeCl_2 \cdot 4H_2O$	1.5 g
H_3BO_3	6 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$ZnCl_2$	70 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg

The salts are dissolved in the order given, and the solution is sterilized by autoclaving. This solution is used at 1 ml per 1 liter of medium.

Selective Enrichment of Green Sulfur Bacteria

Water, mud, or sediment samples from anoxic sulfide-containing natural habitats (freshwater or marine samples) usually contain both green and purple sulfur bacteria, even if the color of the sample may suggest the presence of only one group. Chlorobiaceae are selectively enriched in medium 1 or 2 (see Chapter 170) at pH values between 6.6 and 6.9 (not above pH 7.0). The bottles are continuously illuminated with a daylight-fluorescent lamp at light intensities between 5 and 300 lux and an incubation temperature of 20 to 30°C. At high light intensities (500–2,000 lux) and temperatures between 30 and 35°C, green *Chlorobium* and *Prosthecochloris* species (marine; Trüper, 1970; Matheron and Baulaigne, 1972) will be selectively enriched over purple sulfur bacteria. At very low light intensities (5–50 lux), the Chlorobiaceae are selectively enriched (even with tungsten lamps) due to their unique capacity to grow at light intensities that are too low to support the growth of other phototrophic sulfur bacteria (Biebl and Pfennig, 1978).

Low-light intensities, together with diurnal light and dark changes and temperatures around 20°C, may be used to enrich for certain species of Chlorobiaceae that are outgrown by *Chlorobium* or *Prosthecochloris* at high-light intensities and high temperatures. The enrichment of the gas-vacuole-containing green sulfur bacteria of the genus *Pelodictyon* (Pfennig and Cohen-Bazire, 1967; Overmann and Pfennig, 1989) and of the Chlorochromatium and Pelochromatium consortia can be expected at low sulfide concentrations (< 2 mM) in medium 1 between 20 and 100 lux, and at 15 to 20°C.

With the amount of sulfide initially present in the medium, only very limited growth can be expected. After all the sulfide and sulfur are photooxidized, the bacteria stop growing and will be damaged by further illumination. In order to keep the enrichments growing and to obtain high cell yields, it is necessary to feed the cultures several times with a sterile, neutralized sulfide solution, as described in Chapter 170.

If the green or brown Chlorobiaceae to be isolated were first detected by microscopic observation in a water or mud sample from nature, liquid enrichment cultures should not be used. Such enrichment cultures may select for species that are not the dominant form in the sample. In order to isolate the various bacteria in the same proportions as they are present in the original sample, the latter is used directly as an inoculum for deep agar dilution series. Medium 1 (Chapter 170) is used with the addition of 3

mM acetate, which serves as an additional carbon source and results in the development of much larger colonies. The agar tubes are incubated at room temperature and at light intensities (fluorescent light) not higher than 50–200 lux to provide the least selective conditions.

Identification

The properties of the species of the Chlorobiaceae are listed in Table 1. Members of the Chlorobiaceae lack assimilatory sulfate reduction and require a reduced sulfur compound as a sulfur source. They can assimilate some simple organic carbon compounds in the presence of sulfide and carbon dioxide. Many strains need vitamin B_{12}. Cell multiplication occurs by binary fission; only *Pelodictyon clathratiforme* and *P. phaeoclathratiforme* show ternary fission leading to the formation of three-dimensional nets. *Chloroherpeton thalassium* is the only motile species of the Chlorobiaceae; it consists of unicellular gliding filaments. Fig. 1 shows the morphology of some typical species of the Chlorobiaceae.

The most conspicuous difference between the Chlorobiaceae and the purple sulfur bacteria (Chromatiaceae and Ectothiorhodospiraceae) is the bacteriochlorophyll pigments. Thus, despite their trivial names of green and purple bacteria, it is not the color of colonies or cell suspensions, because there exist green and brown species in the Chlorobiaceae as well as brown, orange, purple, red, and green species in the purple sulfur bacteria. The easiest differentiation between the Chlorobiaceae and the purple sulfur bacteria is by recording the absorption spectra of living cell suspensions, as described in Chapter 170. The wavelengths typical for the three presently known bulk (antenna) bacteriochlorophylls in living cells of Chlorobiaceae (and some of the Chloroflexaceae) are: bchl *c*, 745–755 nm; bchl *d*, 715–745 nm; and bchl *e*, 710–725 nm (Gloe et al., 1975; Pfennig and Trüper, 1977). Fig. 2 shows absorption spectra of living cell suspensions of two *Chlorobium* species. The in vivo absorption characteristics of bchls *c* and *e*, as well as of the most common carotenoids, are represented. In such spectra the typical maxima of the reaction center bchl *a* of the Chlorobiaceae does not show up due to its minute amount as compared with the amount of antenna bacteriochlorophylls.

When physiology, morphology, and pigments of a newly isolated strain have been determined, identification with a known species is possible in most cases; if not, further properties of the new strain must be studied. We recommend the determination of the GC content of the DNA and a study of ultrathin sections under the electron microscope, in order to verify the presence of chlorosomes (see later for discussion of these unique structures of the green bacteria).

The description of a new species of the Chlorobiaceae should be based on pure cultures and, if possible, on more than one strain. Besides nomenclatural items, a new description should contain detailed information about cell morphology (color, size, shape, motility, mode of division, presence of gas vacuoles, slime capsules, sheaths); occurrence of cell aggregates; fine structure; photosynthetic pigments (color of cell suspensions, types of bchl and carotenoids); and GC content. The following physiological data should be given: oxygen tolerance; sulfide tolerance; salinity requirements: optimum growth temperature and pH; sulfur, nitrogen, and carbon sources utilized; vitamins or other growth factors required; and capacity for photo-versus chemotrophy, organo- versus lithotrophy, and hetero-versus autotrophy. An indication of natural habitats is also necessary.

Finally, a type strain must be designated and deposited in one of the recognized national type culture collections, preferably ATCC, DSM, NCIB, or NTHC, as required by the International Code of Nomenclature of Bacteria (Lapage et al., 1975).

Physiology

During photosynthetic metabolism in the Chlorobiaceae, light is harvested in special organelles, the chlorosomes. These are lens- to cigar-shaped bodies that are attached to the inner side of the cytoplasmic membrane in direct association with the plasma membrane. The chlorosomes contain the antenna bacteriochlorophylls and transfer energy to the reaction center bacteriochlorophyll in the plasma membrane. The reaction center bacteriochlorophyll is always bchl *a* in the Chlorobiaceae.

Although cyclic photophosphorylation in the Chlorobiaceae works like that in the Chromatiaceae (Drews, 1989), the mechanism for NAD^+ reduction is quite different. In the Chlorobiaceae, the primary photosynthetic electron acceptor is not a bacteriopheophytin like in the purple bacteria, but an iron-sulfur protein with a reduction potential of -540 mV. This allows the green sulfur bacteria to directly reduce NAD^+ via reduced ferredoxin. The electron removed by noncyclic electron flow is replaced by external electrons entering the system at a less electronegative site via cytochrome c_{555}, which again donates them to the reaction center bac-

Table 1. Properties of the species of the Chlorobiaceae.

Species	Shape, size (μm)	Aggregate pattern	Gas vacuoles	Color of cell suspension	Predominant bchl	Predominant carotenoid[a]	GC content (mol%)	Type or neotype strain	Special requirement
Ancalochloris perfilievii	Sphere, 0.5–1.0 with prosthecae up to 2.0	Microcolonies	+	Green	ND	ND	ND	—	Vit. B_{12}
Chlorobium chlorovibrioides	Vibrio, 0.3–0.4 × 0.7–1.4	Spirals	−	Green	*c* or *d*	chl	54	DSM 1370	2–3% NaCl
C. limicola	Rod, 0.7–1.1 × 0.9–1.5	Chains	−	Green	*c* or *d*	chl	51.0–58.1	DSM 245	—
C. phaeobacteroides	Rod, 0.6–0.8 × 1.3–2.7	Chains	−	Brown	*e*	irt	49.0–50.0	DSM 266	Vit. B_{12}
C. phaeovibrioides	Vibrio, 0.3–0.4 × 0.7–1.4	Spirals	−	Brown	*e*	irt	52.0–53.0	DSM 269	2% NaCl, vit. B_{12}
C. vibrioforme	Vibrio, 0.5–0.7 × 1.0–1.2	Spirals	−	Green	*d* or *c*	chl	52.0–57.1	DSM 260	2% NaCl
Chloroherpeton thalassium	Rod, 1.0 × 8–30	Unicellular gliding filaments	− and[b] +	Green	*c*	γ-car	45–48.2	ATCC 35110	1–3% NaCl, vit. B_{12}
Pelodictyon clathratiforme	Rod, 0.7–1.2 × 1.5–2.5	Nets	+	Green	*c* or *d*	chl	48.5	—	Vit. B_{12}
P. luteolum	Ovoid, 0.6–0.9 × 1.2–2.0	Clumps, Spheres	+	Green	*c* or *d*	chl	53.5–58.1	DSM 273	0–3% NaCl
P. phaeoclathratiforme	Rod, 0.75–1.1 × 1.5–3	Nets	+	Brown	*e*	irt	47.9	DSM 5477	—
P. phaeum	Vibrio, 0.6–0.9 × 1.0–2.0	Spirals	+	Brown	*e*	irt	ND	DSM 728	3% NaCl, vit. B_{12}
Prosthecochloris aestuarii	Sphere, 0.5–0.7 × 1.0–1.2 with prosthecae	Chains	−	Green	*c*	chl	52.0–56.0	DSM 271	1–18% NaCl, vit. B_{12}
P. phaeoasteroidea	Sphere, 0.3–0.6 × 0.5–0.8 with prosthecae	Chains	−	Brown	*e*	irt	52.2	DSM 1378	0.5–2% NaCl

Abbreviations: +, present; −, absent; ND, not determined.
[a]chl, chlorobactene; irt, isorenieratene; car, carotene.
[b]Depending on phase of growth.
Adapted from: Gibson et al., 1984; Gloe et al., 1975; Gorlenko, 1972; Gorlenko and Lebedeva, 1971; Gorlenko, 1974; Mandel et al., 1971; Overmann and Pfennig, 1989; Pfennig and Trüper, 1971; Puchkova and Gorlenko, 1976; Trüper and Pfennig, 1971.

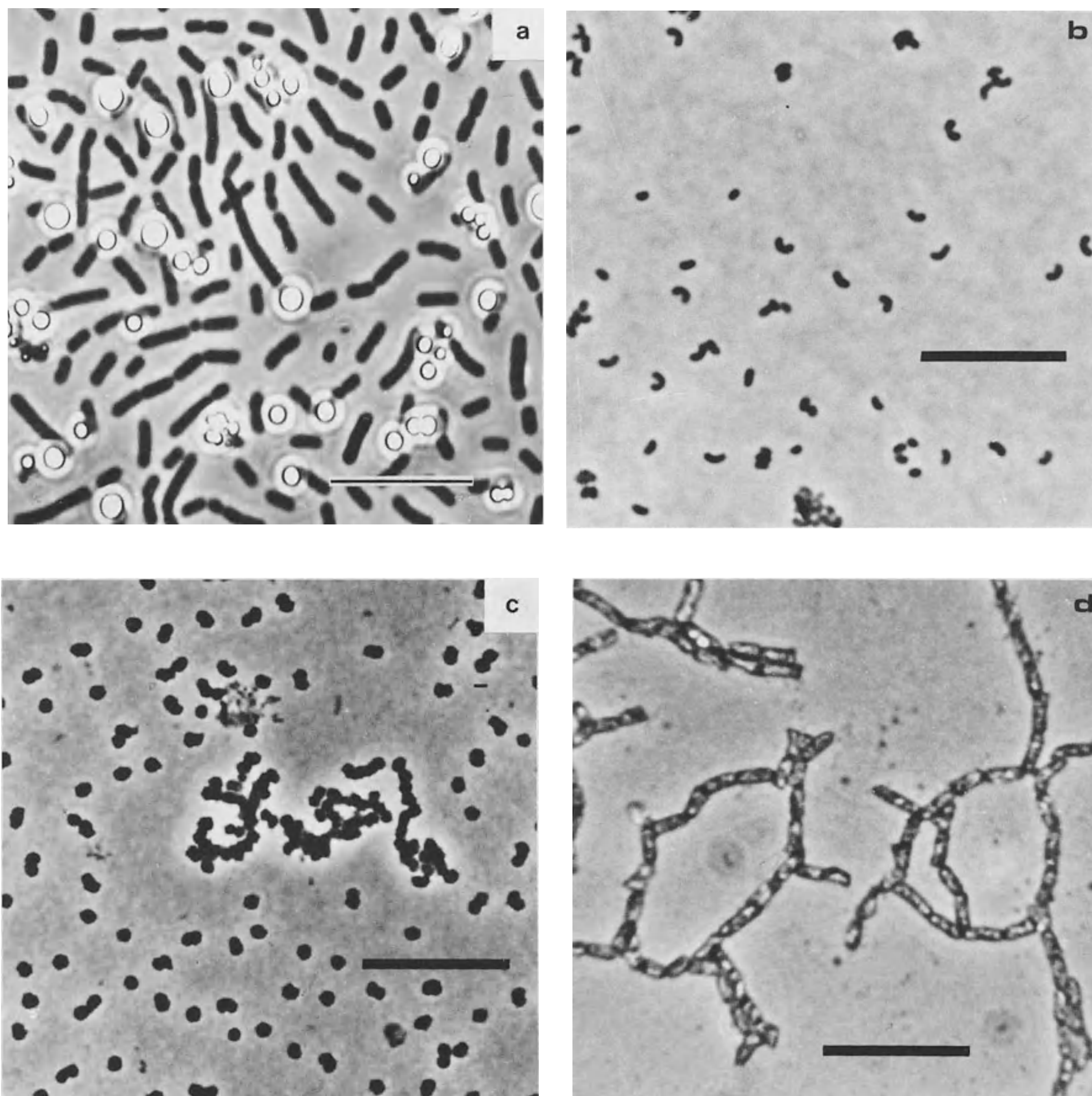

Fig. 1. Morphology of the Chlorobiaceae (phase contrast micrographs). Bars = 10 μm. (a) *Chlorobium limicola.* (b) *Chlorobium phaeovibrioides.* (c) *Prosthecochloris aestuarii.* (d) *Pelodictyon clathratiforme.*

teriochlorophyll. Cytochrome c_{555} behaves in analogy to cytochrome c_2 in purple bacteria, whereas menaquinones replace ubiquinones as mobile intramembranous hydrogen carriers. For further details, see Drews (1989).

Lippert and Pfennig (1969) demonstrated that in the Chlorobiaceae, molecular hydrogen utilization occurs in the majority of species and strains. Autotrophic growth in the green sulfur bacteria is, however, dependent on the presence of a reduced source of sulfur, as these organisms are not capable of assimilatory sulfate reduction.

The uptake hydrogenases of *Chlorobium* species are not yet fully understood (Gogotov, 1986). In general, the enzymes appear to be membrane-bound and probably contain nickel and iron-sulfur clusters. The natural electron acceptors of hydrogenases in *Chlorobium* are rubredoxin or cytochromes (Gogotov, 1986).

All species of the Chlorobiaceae utilize sulfide and elemental sulfur as photosynthetic electron donors (Trüper, 1981a, 1989). The stoichiometry of sulfide oxidation with respect to CO_2 fixation is more variable than that of the Chromatiaceae due to incomplete formation of sulfate (Van Niel, 1931).

During oxidation of sulfide, hydrophilic "elemental sulfur" appears in the form of globules outside the cells.

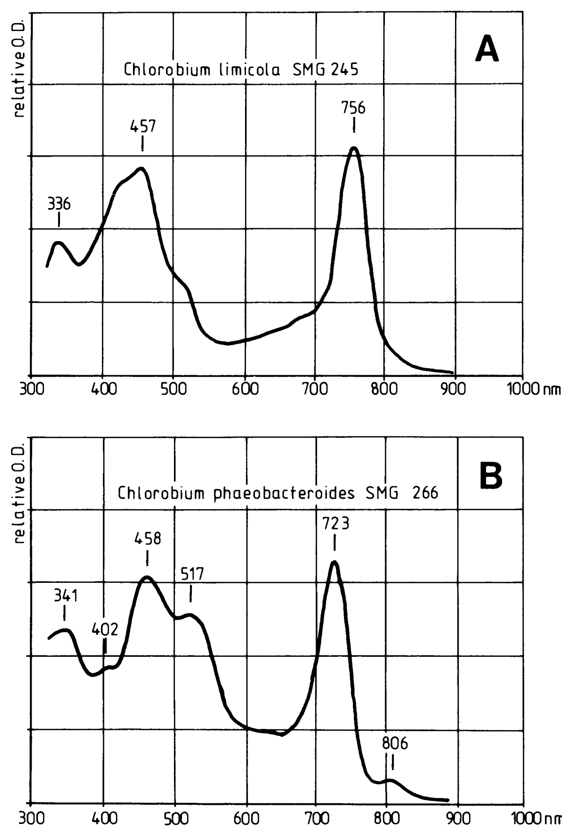

Fig. 2. Absorption spectra of living cell suspensions of green and brown *Chlorobium* species. (A) *Chlorobium limicola,* with bchl *c* (756 nm) and the carotenoid chlorobactene (457 nm). (B) *Chlorobium phaeobacteroides,* with bchl *e* (723 nm) and the carotenoids chlorobactene (458 nm) and isorenieratene (517 nm).

Utilization of thiosulfate as a photosynthetic electron donor by Chlorobiaceae is restricted to the *"thiosulfatophilum"* biovars of the two species *Chlorobium limicola* and *C. vibrioforme.* Of these, only *C. vibrioforme* biovar *thiosulfatophilum* forms extracellular sulfur globules from thiosulfate (Steinmetz and Fischer, 1982). The thiosulfate-utilizing biovars form thiosulfate as free intermediate during phototrophic sulfide oxidation, and they are able to disproportionate elemental sulfur in the light in the absence of carbon dioxide (Trüper et al., 1988).

The enzymology of sulfur metabolism in the Chlorobiaceae is not yet fully resolved. It appears that the oxidation of sulfide to "elemental sulfur" is mediated by *c*-type cytochromes. The formation of thiosulfate from sulfide is catalyzed by flavocytochrome *c* (Fischer, 1984). Adenylylsulfate reductase was shown to occur in *Chlorobium* species (Trüper and Peck, 1970) and was purified by Kirchhoff and Trüper (1974). Splitting of adenylylsulfate and liberation of sulfate is performed by ADP sulfurylase in *C. vibrioforme* (Trüper and Fischer, 1982; Khanna and Nicholas, 1983; Bias and Trüper, 1987) and exclusively by ATP sulfurylase in *C. limicola* (Bias and Trüper, 1987).

In the Chlorobiaceae, autotrophic CO_2 fixation occurs via a reverse tricarboxylic acid cycle, as was first proposed by Evans et al. (1966). Key enzymes of this cycle are the ferredoxin-dependent α-ketoglutarate and pyruvate synthases, and an ATP-dependent citrate lyase (Sintsov et al., 1980; Ivanovsky et al., 1980). The careful ^{14}C-labeling studies by Fuchs et al. (1980a, 1980b) and respective ^{13}C-NMR studies by Paalme et al. (1982a, 1982b), together with the characterization of the citrate lyase (Antranikian et al., 1982), have provided unequivocal final evidence for this distinctive mechanism of autotrophic CO_2 fixation.

With respect to the utilization of organic carbon, the Chlorobiaceae show little versatility (Trüper, 1981b). They are obligately photolithoautotrophic even when utilizing acetate or pyruvate (so far the only organic carbon sources known to be used) showing a strict dependence upon a simultaneous supply of bicarbonate and sulfide or thiosulfate.

Although the best nitrogen source for growth is the ammonium ion, the majority of strains of the Chlorobiaceae are able to fix dinitrogen (for reviews, see Vignais et al., 1985; Hallenbeck, 1987).

Further aspects of ultrastructure, energy transfer, metabolism, phylogeny, physiology, and ecology are dealt with in a book on green photosynthetic bacteria edited by Olson et al. (1988).

Applications

Chlorobiaceae have been used for the removal of the obnoxious (and poisonous) smells of hydrogen sulfide during anaerobic waste treatment (Kobayashi et al., 1983). Cork and Cusanovich (1979) presented a process for the continuous disposal of sulfate by a mutual bacterial system consisting of *Desulfovibrio desulfuricans* and *Chlorobium limicola.* Cork (1982; Cork and Ma, 1982; Cork et al., 1983, 1985) developed a system using *C. limicola* as the biocatalyst in the production of elemental sulfur and organic carbon (biomass) from acid gas (containing H_2S and CO_2), an alternative to the Claus desulfurization process used in cleaning sour natural gases.

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CHAPTER 196

The Genus *Bacteroides* and Related Taxa

HAROUN N. SHAH

History

Bacteroides fragilis, type species of the genus *Bacteroides* was first described by Veillon and Zuber (1898) as *Bacillus fragilis* but was later transferred to the genus *Bacteroides* by Castellani and Chalmers (1919). *B. fragilis* has been referred to as *Fusiformis fragilis* (Topley and Wilson, 1929), Ristella fragilis (Prévot, 1938) and *Bacteroides fragilis* subspecies *fragilis* (Holdeman and Moore, 1974). Studies on more than 300 morphologically and physiologically similar isolates which were saccharolytic and showed growth stimulation with bile revealed phenotypically discernible groups within a "continum of variants" (Holdeman and Moore, 1970). As a result, *Bacteroides fragilis* was regarded as a single species which comprised the following; *B. fragilis* subspecies *fragilis, B. fragilis* subspecies *distasonis, B. fragilis* subspecies *eggerthii, B. fragilis* subspecies *ovatus, B. fragilis* subspecies *thetaiotaomicron, B. fragilis* subspecies *uniformis,* and *B. fragilis* subspecies *vulgatus* (Holdeman and Moore, 1974).

All subspecies were later shown to be genetically distinct by DNA-DNA hybridization (Johnson, 1973) and were reinstated to species rank by Cato and Johnson (1976). Similar studies by Johnson et al. (1986) led to the recognition of three additional related species, *B. caccae, B. merdae,* and *B. stercoris.* Biochemical, chemical, and molecular biological data indicate that this group of 10 species form a taxonomically coherent group of species that differs so markedly from other taxa within the currently defined genus (Holdeman et al., 1984) that they require separate generic status. Accordingly, the description of the genus *Bacteroides* was amended recently to restrict it only to the *"B. fragilis* group of species" (Shah and Collins, 1989) and includes all the above taxa.

The moderately saccharolytic, pigmented/nonpigmented organisms comprise a large group of 15 species which share many common phenotypic properties. The earliest reported member of this group, *Bacteroides melaninogenicus* was described by Oliver and Wherry (1921). Previously regarded as a single species (see Chapter 197) with one asaccharolytic subspecies (*B. melaninogenicus* subspecies *asaccharolyticus*) and two moderately saccharolytic subspecies (*B. melaninogenicus* subspecies *melaninogenicus* and *B. melaninognicus* subspecies *intermedius*), all subspecies or serogroups (Lambe, 1974) have been elevated to species rank (Holdeman and Moore, 1974). The species *B. oralis (B. oralis* var. *oralis* and *B. oralis* var. *elongatus)* was separated from *B. melaninogenicus* almost exclusively on the basis of pigment production by the latter (Loesche et al., 1964). This heavy reliance on pigmentation, together with poor circumscription of the species *B. oralis,* led to the species becoming a repository for a large and varied collection of strains which could not be accommodated elsewhere. Furthermore, the only three available reference strains of *B. oralis* (ATCC 15930 [VPI9085], 7CM, and J1) were all assigned to new species (Fig. 1). Strain ATCC 15930 was assigned to *B. melaninogenicus* because of the formation of black pigment (Holbrook and Duerden, 1974; Holbrook et al., 1977) and later to *B. loescheii* (Holdeman and Johnson, 1982). J1 was described as *"B. ruminicola-like"* due to its capacity to ferment xylose and arabinose (a specific property which *B. oralis* does not possess) while strain 7CM was transferred to *B. vulgatus* on the basis of DNA-DNA hybridization data and its growth stimulation of bile (Shah and Collins 1981). Two strains, HS4 and VPI 8906D, which conformed to the description of *B. oralis* had high interstrain DNA-DNA homology but low genetic relatedness with a newly proposed neotype strain of *B. oralis* (ATCC 33269). Strains HS4 (NCDO 2354) and VPI 8906D were shown to comprise the nucleus of a new species, *B. buccalis* (Shah and Collins, 1981), while *B. veroralis* (Watabe et al., 1983) and *B. oulorum* (Shah et al., 1985) were proposed for phenotypically similar but genetically distinct species. Biochemical and chemical studies showed that strain J1 and two related stains NP333 (NCDO 2352) and WPH61 were genetically similar but differed sufficiently from

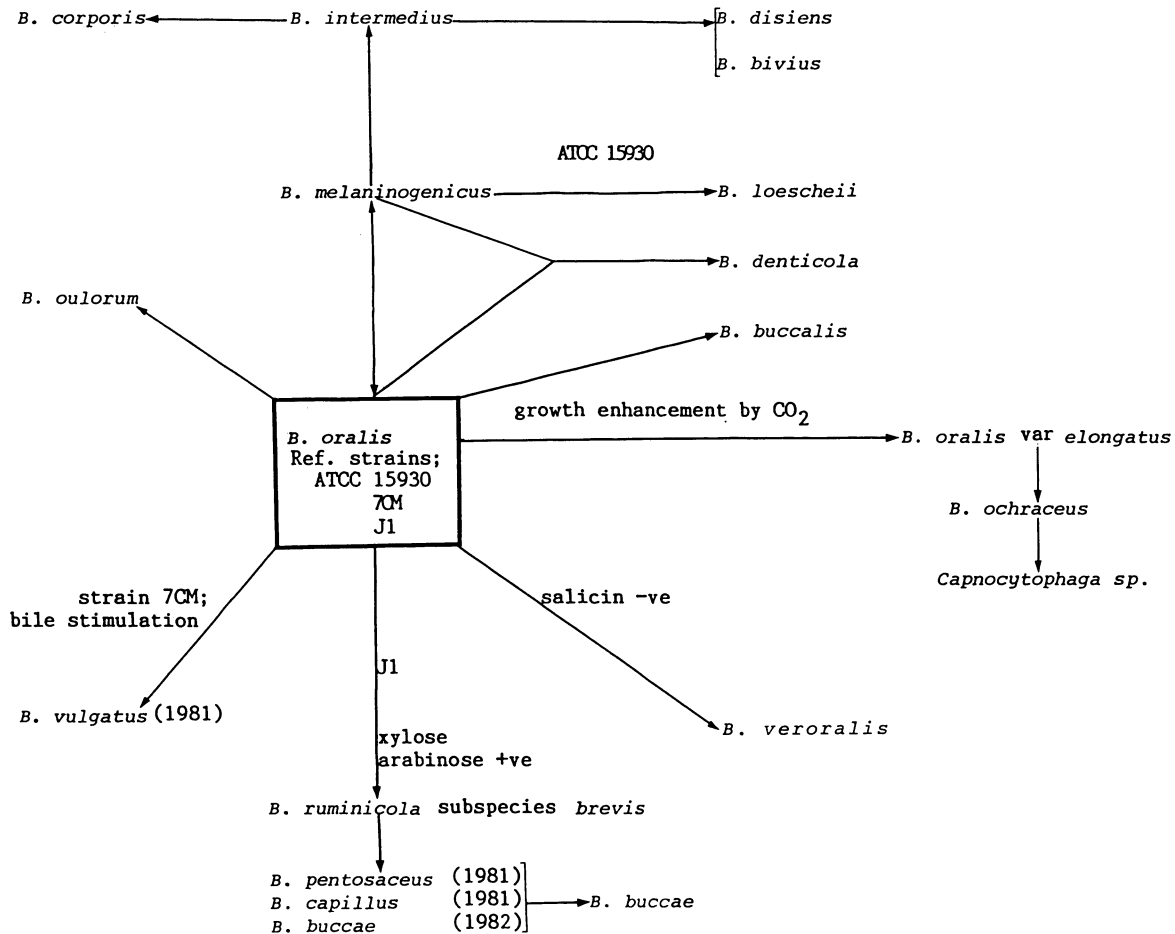

Fig. 1. Current status of isolates that previously belonged to *Bacteroides oralis.*

other species to warrant their placement in a new species, *B. pentosaceus* (Shah and Collins, 1981). Phenotypically and genetically similar strains, for which the species *B. capillus* was proposed, were isolated from localized periodontitis sites (Kornman and Holt, 1981). *B. pentosaceus, B. capillus,* and the newly proposed *B. buccae* (Holdeman et al., 1982) were reported to be genetically similar and the specific epithet *B. buccae* took precedence (Johnson and Holdeman et al., 1985). *B. oris* (Holdeman et al., 1982) and *B. heparinolyticus* (Okuda et al., 1985) share many common properties with these species but are generally less fermentative. The description of *B. denticola* (Shah and Collins, 1981) was emended to include both pigmented and nonpigmented strains (Holdeman and Johnson, 1982). The less saccharolytic pigmented species *B. intermedius* was shown to comprise another genetically distinct taxon for which the name *B. corporis* was proposed (Johnson and Holdeman, 1983). *B. bivius* and *B. disiens* are phenotypically similar to *B. intermedius* but do not form pigment on blood agar (Holdeman and Johnson, 1977). This large group of species, generally referred to as the *"B. melaninogenicus-B. oralis* group," form a biochemically related group of species which differs from *B. fragilis* and other recently proposed genera and should be assigned to a new genus (Shah and Collins, 1989). Most species of the *"B. melaninogenicus-B. oralis"* group are frequently isolated from the oral cavity and are moderately fermentative.*

In contrast to the above species, most of the remaining taxa do not colonize the oral cavity and are generally nonfermentative (Holdeman et al., 1984). They represent a diverse collection of species quite unrelated to the type species *B. fragilis* or to each other. Consequently, several new genera have been proposed, many of which are monospecific (see Shah and Collins, 1989). Examples of some species which have long been established and since reclassified, include *Tissierella praeacuta* (Collins and Shah, 1986b) for *B. praeacutus* (1908); *Anaerorhabdus furcosus*

*Recently reclassified in a new genus *Prevotella.*

(Shah and Collins, 1986) for *B. furcosus* (1898); *Sebaldella termitidis* (Collins and Shah, 1986a for *B. termididis* (1962); *Fibrobacter succinogenes* (Montgomery et al., 1988) for *B. succinogenes* (1950); *Megamonas hypermegas* (Shah and Collins, 1982a for *B. hypermegas* (1963); *Mitsuokella multiacidus* (Shah and Collins, 1982b for *B. multiacidus* (1974); *Rikenella microfusus* (Collins et al., 1985) for *B. microfusus* (1978), and *Ruminobacter amylophilus* (Stackebrandt and Hippe, 1986) for *B. amylophilus* (1956). Although several other species such as *B. capillosus, B. coagulans,* and *B. ureolyticus* have not been reclassified, biochemical and chemical properties so far examined indicate that they warrant separate generic status (see reviews by Shah and Collins, 1983, and Collins and Shah, 1987).

Recently, many new species have been proposed as members of the genus *Bacteroides.* Many of these species bear little resemblance to *classical Bacteroides* and their current taxonomic positions remain uncertain. Table 1 (from Shah and Collins, 1989) gives an overview, and where possible the taxonomic position of these species, while Fig. 2. gives the distribution of mol% GC values among these organisms.

General Characteristics

More than 50 species of *Bacteroides* are currently listed in *Bergey's Manual of Systematic Bacteriology* (Holdeman et al., 1984) and the Approved Lists of Bacterial Names (Moore et al., 1985) In the past, any Gram-negative, obligately anaerobic, nonsporeforming rod which could not be assigned to the genus *Fusobacterium* or *Leptotrichia* has been placed in the genus *Bacteroides.* Poor definition of the genus, including its wide range in DNA-base composition (about 28–61 mol% GC) (Holdeman et al., 1984) has allowed isolates which share only a few common properties to be deposited in the genus *Bacteroides.* Previously we have suggested (Shah and Collins, 1983; Collins and Shah, 1987) that the generic description should be emended to include only the type species *B fragilis* and related taxa. Thus, the genus *Bacteroides* should be restricted to those species which fulfill the following criteria:

1. Obligately anaerobic, Gram-negative, nonsporeforming rods.
2. Saccharolytic, and produce major levels of acetate and succinate as metabolic end products.
3. Contain enzymes of the hexose monophosphate shunt-pentose phosphate pathway such as glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), in addition to malate dehydrogenase (MDH) and glutamate dehydrogenase (GDH).
4. Have a DNA-base composition within the range 40–48 mol% GC.
5. Possess sphingolipids.
6. Contain a mixture of long-chain fatty acids with predominantly straight-chain saturated, anteiso-methyl branched and iso-methyl branched acids.
7. Possess menaquinones with MK-10 and MK-11 as major components.
8. Contain *meso*-diaminopimelic acid as the diamino acid of its peptidoglycan.

On the basis of the above criteria the following species are currently considered to constitute the revised genus *Bacteroides: B. fragilis* (type species), *B. caccae, B. distasonis, B. eggerthii, B. merdae, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis,* and *B. vulgatus.* This more restricted definition of the genus *Bacteroides* (Shah and Collins, 1989) contains only saccharolytic, nonpigmented species. The growth of all species is stimulated by bile and, with the exception of *B. splanchnicus,* are the only Gram-negative, anaerobic, nonsporeforming rods which possess high levels of both G6PDH and 6PGDH (Shah and Collins, 1989). Although *B. splanchnicus* superifically resembles members of the *"B. fragilis"* group, biochemical, chemical, and genetic data indicate that they are taxonomically unrelated. *B. splanchnicus* differs from the *"B. fragilis"* group in producing propionic acid as a major metabolic end product, possessing menaquinones with nine isoprene units, and containing mainly 13-methyl-tetradecanoic acid (see review, Shah and Collins, 1983). Subsequent rRNA-homology studies by Johnson and Harich (1986) revealed less than 20% relatedness between *B. splanchnicus* and the *"B. fragilis"* group, which further supports the exclusion of *B. splanchincus* from the genus *Bacteroides* sensu stricto.

The group of species referred to above as the *"B.melaninogenicus-B.oralis"* group comprises many of the species commonly isolated from the oral cavity, and which have DNA-base compositions between 40–50 mol% GC (Shah et al., 1990; Reddy and Bryant, 1977). Using the revised definition of the genus *Bacteroides,* it is evident that this group constitutes a new genus, quite unrelated to *B. fragilis,* for which the name *Prevotella* has been proposed (Shah and Collins, 1990). These species are moderately saccharolytic, produce mainly acetic and succinic acids in a glucose medium, and do not grow in media containing 20% bile. The pigmented species such

Table 1. Outline of the current taxonomic positions of *Bacteroides* species and related taxa.

Species	Taxonomic status or comment	Reference(s)
Species currently designated *Bacteroides*		
B. caccae, B. distasonis, B. eggerthii, B. fragilis, B. merdae, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis, B. vulgatus	*Bacteroides* (emended definition)	Shah and Collins, 1989
B. bivius, B. buccae, B. buccalis, B. corporis, B. denticola, B. disiens, B. intermedius, B. heparinolyticus, B. loescheii, B. melaninogenicus, B. oralis, B. oris, B. oulorum, B. ruminicola, B. veroralis, B. zoogleoformans	*B. melaninogenicus-B. oralis* group; (now reclassified as *Prevotella* gen. nov.)	Collins and Shah, 1987; Shah and Collins, 1983; Shah and Collins, 1990
B. levii, B. macacae, B. salivosus	Not *Bacteroides;* possibly related to *Porphyromonas*	Collins and Shah, 1987; Love et al., 1987; Shah and Collins, 1983
B. ureolyticus, B. gracilis	Not *Bacteroides;* possibly related to *Campylobacter* or *Wolinella*	Paster and Dewhirst, 1988
B. capillosus	Not *Bacteroides;* generic position uncertain	Collins and Shah, 1987; Shah and Collins, 1983
B. cellulosolvens	Not *Bacteroides;* generic position uncertain	Murray et al., 1984
B. coagulans	Not *Bacteroides;* generic position uncertain	Collins and Shah, 1987; Shah and Collins, 1983; Elleman, 1988
B. forsythus	Not *Bacteroides;* generic position uncertain	Tanner et al., 1986
B. galacturonicus	Not *Bacteroides;* generic position uncertain	Jensen and Canale-Parola, 1986
B. helcogenes	Not *Bacteroides;* generic position uncertain	Benno et al., 1983
B. nodosus	Not *Bacteroides;* generic position uncertain	Collins and Shah, 1987; Shah and Collins, 1983
B. pectinophilus	Not *Bacteroides;* generic position uncertain	Jensen and Canale-Parola, 1986
B. pneumosintes	Not *Bacteroides;* generic position uncertain	Collins and Shah, 1987; Shah and Collins, 1983
B. polypragmatus	Not *Bacteroides;* generic position uncertain	Moore et al., 1985
B. putredinis	Not *Bacteroides;* generic position uncertain	Collins and Shah, 1987; Hammann and Werner, 1983; Shah and Collins, 1983
B. pyogenes	Not *Bacteroides;* generic position uncertain	Benno et al., 1983
B. splanchnicus	Not *Bacteroides;* generic position uncertain	Hammann and Werner, 1983; Shah and Collins, 1983; Collins and Shah, 1987
B. suis	Not *Bacteroides;* generic position uncertain	Benno et al., 1983
B. tectum	Not *Bacteroides;* generic position uncertain	Love et al., 1986
B. xlanolyticus	Not *Bacteroides;* generic position uncertain	Scholten-Koerselman et al., 1986
New genera for reclassified species		
Anaerorhabdus	Formerly *B. furcosus*	Shah and Collins, 1986
Fibrobacter	Formerly *B. succinogenes*	Montgomery et al., 1988
Megamonas	Formerly *B. hypermegas*	Shah and Collins, 1982a
Mitsuokella	Formerly *B. multiacidus*	Shah and Collins, 1982b
Porphyromonas	Formerly *B. asaccharolyticus, B. endodontalis,* and *B. gingivalis*	Collins and Shah, 1987; Shah and Collins, 1983, 1988
Rikenella	Formerly *B. microfusus*	Collins et al., 1985
Ruminobacter	Formerly *B. amylophilus*	Stackebrandt and Hippe, 1986
Sebaldella	Formerly *B. termiditis*	Collins and Shah, 1986a
Tissierella	Formerly *B. praeacutus*	Collins and Shah, 1986b

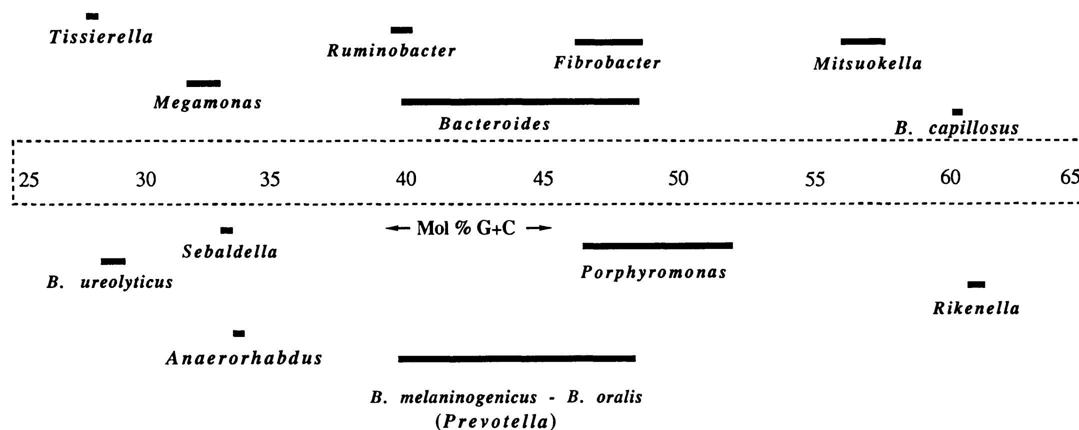

Fig. 2. Distribution of GC content of the DNA of *Bacteroides* and related genera.

as *B. melaninogenicus, B. loescheii, B. corporis, B. intermedius,* and some strains of *B. denticola* produce both protoheme and protoporphyrin pigments. *B. melaninogenicus, B. loescheii* and pigmented strains of *B. denticola* fluoresce brilliantly under ultraviolet (UV) radiation (365 nm) due to the production of mainly protoporphyrin (Shah et al., 1979). These species have the ability to demetallate the iron-containing protoheme molecule leaving a highly conjugated structure which fluoresces under UV radiation (Shah et al., 1979). The darker pigmented colonies of *B. intermedius* and *B. corporis* accumulate an excess of protoheme over protoporphyrin. Before these colonies become entirely black the nonpigmented cells at the center of the colonies also fluoresce. This property is, therefore, useful for recognizing at least some species of the *"B. melaninogenicus-B. oralis"* group and has been of major consequence in elucidating the taxonomic interrelationships of this group of species. Other members of this large cluster include *B. bivius, B. buccae, B. buccalis, B. disiens, B. heparinolyticus, B. oralis, B. oulorum, B. oris, B. ruminicola, B. veroralis,* and *B. zoogleoformans.* These species differ from the *"B. fragilis* group" in that they are sensitive to bile and lack G6PDH and 6PGDH (Shah and Williams, 1982).

B. levii and *B. macacae* resemble *Porphyromonas* species in producing mainly protoheme and possessing a similar dehydrogenases pattern (Shah and Collins, 1988). The similarity in metabolic end products (butyric and acetic acids) and a compatible GC content of 45-48 mol further reinforces the similarity between *B. levii* and *Porphyromonas* (Shah and Collins, 1988). However, like *B. macacae, B. levii* is weakly saccharolytic. *B. macacae* further differs from the rest of this group in producing mainly propionic and succinic acids as major metabolic end products and possessing a lower GC content of 42–44 mol. Two broad groups of species are further discernible by DNA-base composition: a high GC group with base compositions within the range 56–61 mol% and a low GC group of 28–37 mol%. The high GC group, which comprises *Mitsuokella multiacidus* (Shah and Collins, 1982b), *Mitsuokella dentalis* (56–58 mol%) (Haapasalo et al., 1986), *Rikenella microfusus* (60–61 mol%) (Collins et al., 1985), and *Bacteroides capillosus* (60 mol%) share few common properties with each other or *Bacteroides* sensu stricto. *Mitsuokella multiacidus* is a highly saccharolytic organism that produces major levels of lactic acid in addition to acetic and succinic acids (Holdeman et al., 1984). *B. capillosus* is asaccharolytic, producing only low levels of acetic and succinic acids, while *R. microfusus* is very weakly saccharolytic but produces major levels of similar acids (Kaneuchi and Mitsuoka, 1978).

Microorganisms with DNA-base compositions within the range 28–37 mol% GC are phenotypically and genetically so different from each other and from *B. fragilis* as to warrant their placement in several new genera. With the exception of *Megamonas hypermegas* and *Sebaldella termitidis,* all species are nonfermentative. The metabolic end products of these species/genera vary considerably, in accord with their chemotaxonomic heterogeneity (see review, Collins and Shah, 1987).

Habitat

Members of the genus *Bacteroides* and related taxa normally colonize the upper respiratory, intestinal, and female genital tracts. Normal feces for example, contains 10^{11} "*Bacteroides fragilis* group" species per gram compared to

facultative anaerobes which approximate 10^8 per gram (Drasar and Hill, 1974). Other species such as the "*B. melaninogenicus-B. oralis* group" are associated with dental plaque. Although these organisms are normally considered as part of the normal commensal flora of the mucous membranes of humans and animals, they are the most important anaerobic bacteria associated with human infections (Finegold et al., 1985). The infections caused by these organisms are often related to their primary site of isolation. Thus, species of the "*B. fragilis* group" account for between 15–54% of the anaerobic flora in intra-abdominal infections, between 32–68% in perirectal abscesses, and between 26–50% in decubitus ulcers (Finegold et al., 1985). Conversely, their numbers reach only 3% of the anaerobic flora in infections of the head and neck, do not occur in dental diseases, and are less than 4% of the anaerobic flora of transtrachael aspirates and pleural fluid (Finegold et al., 1985). In contrast, members of the "*B. melaninogenicus—B. oralis* group" can account for up to 72% of the anaerobic flora of head and neck infections and can reach up to 50% in pleural fluid (Finegold et al., 1985). However, they account for less than 10% of the anaerobic flora of infections below the waist. This pattern of infection suggests that the source of these organisms in soft tissue infections are probably derived from their natural habitat. The normal site of isolation of these species and others are given in Table 2.

Isolation

Methods available for specimen collection, transport, and anaerobic culture have been dealt with in detail by Dowell and Hawkins (1974), Holdeman et al. (1977), and Sutter et al. (1985). The following media have been selected from various recommended media (Sutter et al., 1985) for primary isolation of anaerobic bacteria, with particular reference to *Bacteroides* and Gram-negative, anaerobic bacteria of human origin.

Total Counts

This can be carried out on either Brucella blood agar supplemented with vitamin K, and hemin

Table 2. Normal site of isolation of *Bacteroides* and other Gram-negative, nonsporeforming anaerobes.

Species/genus	Normal site of isolation[a]
"*Bacteroides fragilis* group"	Colon
"*B. melaninogenicus-B. oralis* group"	Oral cavity
B. bivius, B. disiens	Female genital tract
Other species labelled "*Bacteroides*"	
B. capillosus	Mouth and colon of humans and animals
B. cellulosolvens	Sewage sludge
B. coagulans	Intestinal and urogenital tract
B. forsythus	Dental plaque
B. gracilis	Oral cavity
B. helcogenes	Swine intestinal tract
B. levii	Bovine rumen
B. macacae	Oral cavity of macque monkey
B. nodosus	Infected hoofs of sheep and goats
B. pneumosintes	Naso-pharyngeal, gingival crevice
B. polypragmatus	Sewage sludge
B. putredinis	Colon of humans and animals
B. pyogenes	Swine intestinal tract
B. ruminicola species	Rumen of sheep, cattle, and other ruminants
B. suis	Swine intestinal tract
B. tectum	Oral flora of dogs and cats
B. ureolyticus	Respiratory, intestinal, and genital tracts
"*Bacteroides*" spp. that have been reclassified	
Anaerorhabdus furcosus	Colon of humans and animals
Fibrobacter succinogenes	Rumen of sheep, cattle, and other ruminants
Megamonas hypermegas	Intestinal tract of poultry
Mitsuokella multiacidus	Colon of humans and pigs
Rikenella microfusus	Ceca of poultry
Ruminobacter amylophilus	Rumen contents of cattle
Sebaldella termitidis	Intestinal contents of termites
Tissierella praeacuta	Intestinal tract

[a]Except where stated, sites refer to humans.

Table 3. Presumptive identification of Gram-negative, anaerobic, nonsporeforming rods.

	Kanamycin (1 mg) susceptibility	Colistin (10 μg) susceptibility	Growth in 20% bile	Nitrate reduction	Urease production	Pigment/ fluorescence
B. fragilis group	R	R	+	−	−	−
Pigmented species	R	V	−	−	−	+(−)
Nonpigmented species	R	V	−	−	−	−
B. ureolyticus-like group	S	S	−	+	V	−
Fusobacterium-Leptotrichia	S	S	−(+)	−(+)	−	−

R, resistant; S, susceptible; V, variable; +, all strains positive; −, all strains negative; +(−), most strains positive, few negative; −(+), most strains negative, few positive.
Adapted from Sutter et al. (1985).

solution or Wilkins-Chalgren agar. The composition of these media is as follows:

Brucella Blood Agar (BBL or Difco)

Pancreatic digest of casein	1.0 g
Peptic digest of animal tissue	1.0 g
Glucose	0.1 g
Yeast autolysate	0.2 g
NaCl	0.5 g
Sodium bisulfite	0.01 g
Agar	1.5 g
Distilled water	100.0 ml

Adjust to pH 7.0, boil agar to dissolve, then sterilize at 121°C for 15 min. The medium is then cooled to 50°C and defibrinated sheep blood (5% [v/v] added aseptically and dispensed.

Wilkins—Chalgren Agar

Trypticase	1.0 g
Gelysate	1.0 g
Yeast extract	0.5 g
Dextrose	0.1 g
NaCl	0.5 g
L-arginine-free base	0.1 g
Sodium pyruvate	0.1 g
Vitamin K	0.05 mg
Hemin	0.05 mg
Agar	1.5 g
Distilled water	100.0 ml

Boil to dissolve agar and autoclave at 121°C for 15 min.

The addition of kanamycin and vancomycin to laked blood Brucella agar medium (KVLB) inhibits most facultative anaerobic bacteria and permits the selection of mainly *Bacteroides* species. Its composition is as follows:

Kanamycin-Vancomycin Laked Blood Agar (KVLB)

Brucella agar	4.3 g
Hemin solution (5 mg/ml)	0.1 ml
Vitamin K, solution (10 mg/ml)	0.1 ml
Kanamycin solution (100 mg/ml)	0.075 ml
Distilled water	100.0 ml

The medium is autoclaved at 121°C for 15 min, then cooled to 50°C, and 1 ml of a vancomycin solution (7.5 μg/ml) and laked sheep blood (blood frozen overnight and then thawed) added.

Bile-containing media are generally used to selectively isolate members of the "*B. fragilis* group" of species. The growth of microorganisms such as those of the "*B. melaninogenicus-B. oralis* group" of species, which colonize the oral cavity, is inhibited on these media. Bacteroides Bile Esculin Agar is one such medium which is successfully used (Sutter et al., 1985) to isolate *B. fragilis* and related species. Its composition is as follows:

Bacteroides Bile Esculin Agar

Trypticase soy agar	40.0 g
Oxgal	20.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Hemin solution	2.0 ml
Gentamicin solution (40 mg/ml)	2.5 ml
Distilled water	1 liter

The pH is adjusted to 7.0, and the medium autoclaved at 121°C for 15 min and dispensed.

When successfully isolated, organisms can be maintained by weekly subculture on blood agar plates. Cultures for long-term storage are best kept at −70°C in liquid nitrogen, or lypholized.

Growth in Liquid Media

Most of the species described above use both carbohydrates and nitrogenous substrates as energy sources. Consequently, all media designed for the bulk growth of these microorganisms generally contain glucose and either proteose peptone or some other commercial protein hydrolysate. NaCl (0.5%) is generally required

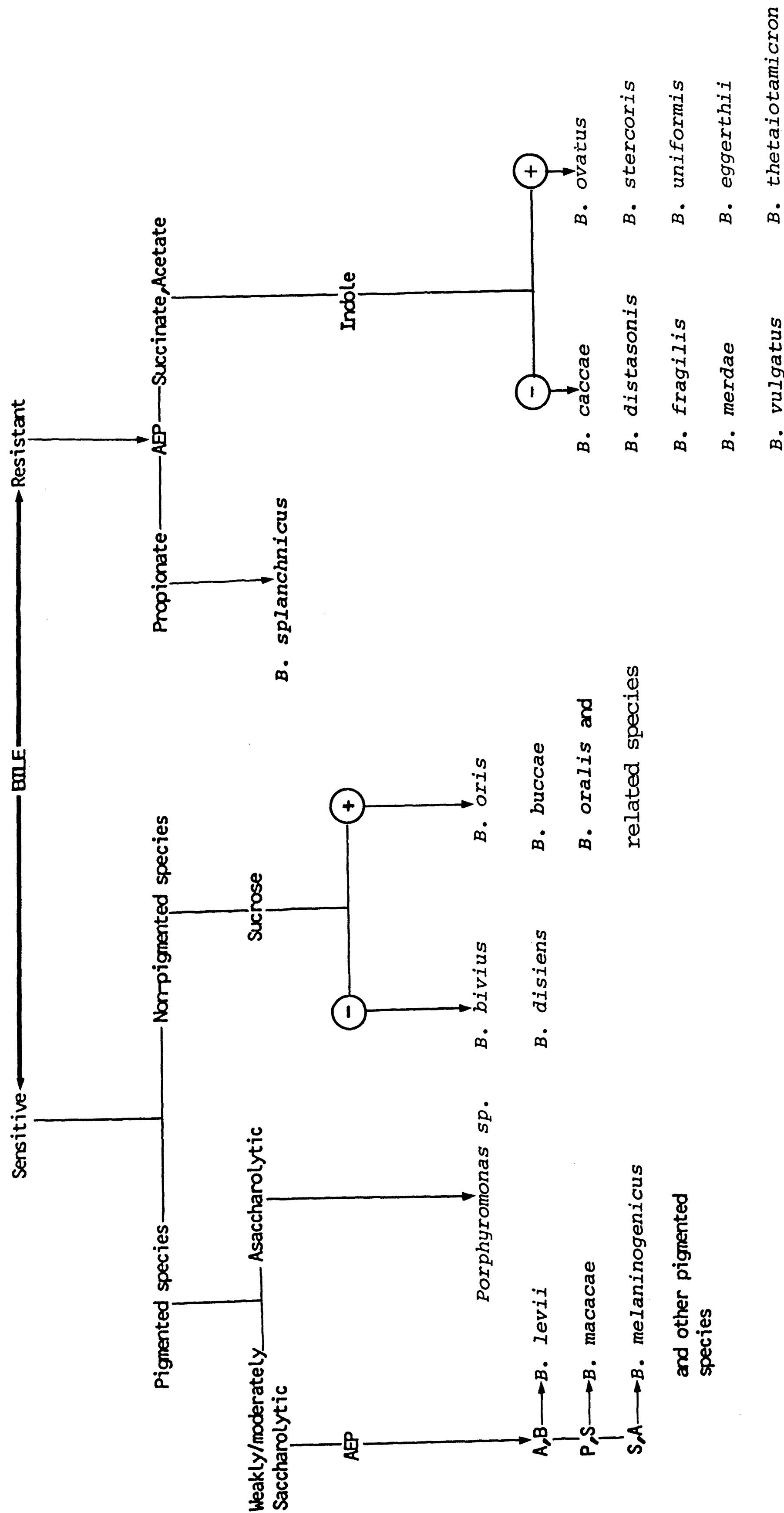

Fig. 3. Further identification of *Bacteroides* and phenotypically related taxa (see Table 7 for abbreviations).

Table 4. Differentiation of *B. fragilis* and related species by sugar fermentation and other diagnostic tests.

	Fermentation					Indole	Catalase
	Sucrose	Rhamnose	Arabinose	Trehalose	Salicin	production	formation
B. fragilis	+	−	−	−	−	−	+
B. distasonis	+	−	+	−	−	−	+
B. vulgatus	+	+	+	−	−	−	−
B. ovatus	+	+	+	+	+	+	−
B. thetaiotaomicron	+	+	+	+	−	+	+
B. uniformis	+	−	+	+	+	+	−
B. caccae	+	+	+	+	−	−	−
B. merdae	+	+	−	+	+	−	−
B. stercoris	+	+	−	−	−	+	−
B. eggerthii	−	+	+	−	−	+	−
B. splanchnicus	−	−	−	−	−	+	−

+, property present; −, property absent.

while cysteine is used both as a reducing agent and a growth requirement. Heme (for cytochrome production) replaces the need for whole blood while vitamin K is used as a precursor for menaquinone biosynthesis (Shah and Collins, 1980). Species that colonize the rumen often require either volatile fatty acids or rumen fluid. Details of culture media such as PYG or medium 10 (Caldwell and Bryant, 1966) are given by Holdeman et al. (1977). Medium BM (Shah et al., 1976), which is simple in composition and has been used very successfully for the culture of nearly all such species isolated from humans is given below:

BM

Trypticase	1.0 g
Proteose peptone	1.0 g
Yeast extract	0.5 g
NaCl	0.5 g
Glucose	0.5 g
Cysteine hydrochloride	0.075 g
Distilled water	100.0 ml

Sterilize, cool, then add 1% (v/v) of filter sterilized hemin-menadione solution (see below).

Hemin-Menadione Solution

Hemin solution is first prepared by adding 1 ml of 1 N NaOH to 50 mg of crystalline heme, followed by 98 ml of water. To this is added 1 ml of menadione solution (5 mg/ml in 95% ethanol) and the mixture filter sterilized and stored at −20°C. The hemin and menadione solution is added aseptically at 1% (v/v) to sterilized media.

Identification of Bacteroides and Other Gram-negative, Anaerobic Rods

The system of Sutter et al. (1985) involves the use of Gram stain, cell and colonial morphology, susceptibility to special antibiotic disks (placed on the first quadrant of a streaked plate), and tests for nitrate reduction, growth in bile, urease production, and observation of pigment production/UV fluorescence, This system enables a preliminary grouping of isolates (Table 3) into the following major categories:

1. *B. fragilis* group,
2. Pigmented group,
3. *B. ureolyticus*-like group,
4. Nonpigmented group,
5. *Fusobacterium-Leptotrichia* group.

Commercially available multitest systems such as API 20A and RAP-ID ANA can be used for most of the test listed above (see, for example, Hofstad, 1980). However, for many of these systems, the database for identification is still inadequate and cannot be relied upon entirely. Gas-liquid chromatography (GLC) of the acidic end products (AEP) of isolates is necessary for accurate identification to the species level and is employed in all current identification schemes.

Leptotrichia buccalis, which produces major levels of lactic acid, and *Fusobacterium* species, which produce predominantly butyric acid, could be readily separated from the remaining species, which produce mainly acetic and succinic acids and occasionally lower levels of other volatile short-chain acids. Growth in the presence of bile and pigment production on blood agar are major characteristics for delineating these taxa to recognizable groups (see Fig. 3).

Some species possess unique properties which make them readily recognizable. Thus, the growth of *B. gracilis* is stimulated by formate or hydrogen and fumarate or nitrate (Tanner et al., 1981). *B. zoogleoformans* grows as a very viscous, ropy-like mat in liquid medium (Cato et al., 1982). Both *B. ureolyticus* and *B.*

Table 5. Biochemical and chemical characteristics useful in the differentiation of Gram-negtive, nonsporeforming, rods.

Taxon	DNA-base composition (mol% GC)	Dibasic[a] amino acid of the peptidoglycan	Dehydrogenase[b] enzymes present	Predominant[c] cell fatty acid	Major menaquinone	References
B. fragilis group	40–48	DAP	G6PDH, 6PGDH, MDH, GDH	$aiC_{15:0}$	MK-10	Miyagawa et al., 1979
B. fragilis					MK-11	Shah and Collins, 1980, 1983
B. splanchnicus	40	DAP	G6PDH, 6PGDH, MDH, GDH	$iC_{15:0}$	MK-9	Miyagawa et al., 1979 Shah and Collins, 1980, 1983
B. melaninogenicus-B. oralis group	40–52	DAP	MDH, GDH	$aiC_{15:0}$ $iC_{15:0}$ $C_{15:0}$	MK-10, MK-11, MK-12, MK-13	Miyagawa and Suto, 1980 Shah and Collins, 1980, 1983 Collins and Shah, 1987
Nonpigmented nonfermentative						
B. coagulans	37	–	GDH	$C_{16:0}$ $C_{18:1}$	–	Collins and Shah, 1987
B. capillosus	60	–	GDH	$C_{14:0}$ $C_{16:0}$	–	Collins and Shah, 1987
B. putredinis	–	–	MDH, GDH	$iC_{15:0}$	–	Collins and Shah, 1986b
B. ureolyticus	28–30	DAP	–	$C_{18:1}$	–	Jackson et al., 1978 Paster and Dewhirst, 1988 Prefontaine and Jackson, 1972
Anaerorhabdus furcosus	34	– (Not DAP)	G6PLH	$C_{18:1}$ $C_{18:0}$	–	Shah and Collins, 1986
Tissierella praeacuta	28	mDAP	6PGDH	$iC_{15:0}$	–	Collins and Shah, 1986b
Weakly fermentative						
Rikenella microfusus	60–61	DAP	MDH	$iC_{15:0}$ $C_{15:0}$	MK-8	Collins et al., 1985
Fibrobacter succinogenes	45–51	DAP	–	$C_{16:0}$ $C_{15:0}$	–	Montgomery et al., 1988 Miyagawa et al., 1979
Fermentative						
Megamonas hypermegas	32–35	DAP	G6PDH, 6PGDH, MDH	$C_{15:0}$	–	Shah et al., 1983 Shah and Collins, 1982a
Ruminobacter amylophilus	40–42	DAP	–	$C_{16:0}$ $C_{14:0}$		Miyagawa et al., 1979, 1981 Stackebrandt and Hippe, 1986
Sebaldella termitidis	30–32	–	–	$C_{16:0}$ $C_{18:1}$	–	Shah and Collins, 1986a
Mitsuokella multiacidus	56–58	mDAP	MDH	$C_{16:1}$	–	Shah and Collins, 1982b Shah et al., 1985

[a]DAP, diaminopimelic acid; mDAP, *meso*-DAP.
[b]G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase.
[c]$C_{14:0}$, tetradecanoic acid; $C_{16:0}$, hexadecanoic acid; $C_{16:1}$, hexadecenoic acid; $C_{18:1}$, octadecenoic acid; $iC_{15:0}$, 13-methyltetradecanoic acid; $aiC_{15:0}$, 12-methyltetradecanoic acid; MK-n, menaquinone with n (n = 8 to 13) isoprene units; –, not tested or not known.

Table 6. Distinguishing characteristics of species of the *B. melaninogenicus-B. oralis* group.

	B. melaninogenicus	*B. bivius*	*B. buccae*	*B. buccalis*	*B. corporis*	*B. denticola*	*B. disiens*	*B. heparinolyticus*	*B. intermedius*	*B. loescheii*	*B. oralis*	*B. oris*	*B. oulorum*	*B. ruminicola*	*B. veroralis*	*B. zoogleoformans*
Pigment[a]	+	−	−	−	+	V	−	−	+	+	−	−	−	−	−	−
Xylose	−	−	+	−	−	−	−	+	−	−	−	+	−	V	−	V
Arabinose	−	−	+	−	−	−	−	+	−	−	−	+	−	+/W	−	V
Cellobiose	−	−	+	+	−	−	−	+	−	+	+	+	−	+/W	+	+
Rhamnose	−	−	$+^-$	−	−	−	−		−	−	+	$-^+$	−	−	−	−
Salicin	−	−	+	−	−	−	−	+	−	−	+	+	−	+/W	−	V
Sucrose	+	−	+	+	−	+	−	+	+	+	+	+	−	+	+	+
Lactose	+	+	+	+	−	+	−	+	−	+	+	+	+	+/W	+	+
Indole	−	−	−	−	−	−	−	+	+	$-^+$	−	−	−	−	−	$-^+$
Esculin hydrol.	−	−	+	+	−	+	−	+	−	$+^-$	+	+	−	+	+	+
NAG[b]	+	+	−	+	+	+	V		−	+	+	+	+	+	+	+
α-Fuc[c]	$+^-$	+	−	+	−	+	−	+	$+^-$	+	+	+	+	+	+	
β-Xyl[d]		−	+	−				+			−	+	−			
β-Glu[e]	−	−	+	+	−	−	−	+	−	$+^-$	+	+	+		V	
Gly[f]	−	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−

W, weak reaction; +, positive reaction; −, negative; V, positive or negative reaction; $+^-$, most reactions positive; $-^+$, most reactions negative.
[a]Pigment, pigment production on blood agar.
[b]NAG, *N*-acetylglucosamineidase.
[c]α-Fuc, α-Fucosidase.
[d]β-Xyl, β-Xylosidase.
[e]β-Glu, β-Glucosidase.
[f]Gly, glycine aminopeptidase.

forsythus often form pits on the surface of blood agar plates (Tanner et al., 1986); the former also possesses urease activity (Jackson and Goodman, 1978). Other species which bear the label *"Bacteroides,"* such as *B. cellulosolvens,* ferment only cellulose and cellobiose (Murray et al., 1984). *B. tectum,* an oral isolate from cats and dogs, atypically grows in the presence of bile (Love et al., 1986), whereas the swine intestinal species *B. helcogenes* grows only poorly (Benno et al., 1983). The growth of other swine colonic species *B. pyogenes* and *B. suis,* is, however, inhibited by bile (Benno et al., 1983). Some major characteristics of these taxa are given below.

Differentiation of Species

A combination of biochemical/chemical tests can be used to obtain unequivocal identification of species within the "*B. fragilis* group," the "*B. melaninogenicus-B. oralis* group," and other Gram-negative nonsporeforming, anaerobic rods. For convenience, the biochemical and chemical tests are listed separately here, but in practice they may be carried out in parallel.

The production of indole and catalase and the fermentation of sucrose, salicin, rhamnose, and arabinose can be used to differentiate members of the "*B. fragilis* group" (Table 4). Species of this group possess DNA-base compositions within the range 40–48 mol% GC, have *meso*-diaminopimelic acid in their peptidoglycan, contain enzymes of the hexose monophosphate shunt-pentose phosphate pathway, possess major levels of 12-methyl-tetradeconic acid (anteiso-C_{15}) and contain predominantly menaquinones with 10 and 11 isoprene units (Table 5). *B. splanchnicus* differs from this group in producing butyric acid as a metabolic end product, contains predominantly 13-methyl-tetradecanoic acid (iso-C_{15}), and possesses menaquinones with nine isoprene units (MK-9) (Table 5).

Members of the "*B. melaninogenicus-B. oralis* group" can be identified to the species level by a combination of fermentation reactions and the production of specific constitutive enzymes (Table 6). These species have DNA-base compositions within the range 40–52 mol% GC (Ta-

ble 5) but differ significantly from those of the "*B. fragilis* group" in the absence of the pentose phosphate pathway enzymes (G6PDH and 6PGDH). The menaquinone composition of some species are highly distinctive. Thus, the principal respiratory quinone of *B. melaninogenicus, B. intermedius* and *B. oulorum* is MK-10, whereas for *B. denticola* and *B. veroralis* it is MK-11 (Shah et al., 1985). *B. pentosaceus* possesses almost equal levels of MK-12 and MK-13, while *B. buccalis* contains predominantly MK-12 (Shah and Collins, 1981) and *B. oralis* MK-13 (Shah et al., 1985).

Members of the remaining species/genera have not been studied in such detail as the above species, but they can be readily identified from available data. Table 7 highlights the major biochemical reactions for differentiating these species. Many of these microorganisms are nonfermentative, thus the metabolic end products produced are probably derived from the fermentation of nitrogenous substrates in the medium. The DNA-base compositions, dehydrogenase enzymes pattern, cellular fatty acid composition and major menaquinones of various taxa are given where known (Table 5).

Outlook for Biotechnology

Industrially important compounds synthesized by a range of Gram-negative, anaerobic bacteria have not been exploited commercially due to difficulties encountered in genetic manipulation of these organisms, and poor expression of their genes in distantly related cloning systems such as phage lambda in *E. coli* (see Salyers et al., 1987; Shoemaker et al., 1986). *B. fragilis* is clinically the most important member of this large group of microorganisms (Finegold et al., 1985), consequently the physiology and pathogenicity of this species has been widely studied. *B. fragilis* grows on a simple defined medium, has a fast generation time (1–2 h) and several potential cloning vectors have been described. These include bacteriophages (Tartera and Jofre, 1987; Tartera et al., 1989), antibiotic resistant conjugative plasmids (Callihan et al., 1983; Shoemaker et al., 1986), and cosmids which are genetically engineered to promote the expression and transfer of genes to and from members of the Bacteroidaceae (Shoemaker et al., 1986). Thus, the shuttle vector pE5-2 has been used to transfer genes to *P. gingivalis* and *B. intermedius* (Progulske-Fox et al., 1989). It should,

Table 7. Salient features of species currently or formerly referred to as *Bacteroides*.

Species	Features
B. capillosus	NF; AEP trace, A, S; 60 mol% GC
B. cellulosolvens	NF, except for cellobiose and cellulose; 43 mol% GC
B. coagulans	NF; produces indole, gelatin digested; AEP, low levels of A
B. forsythus	NF; α-fucosidase positive, trypsin and β-galactosidase by API ZYME; 44–48 mol% GC
B. galacturonicus	WF; pectin and polygalacturonate fermented to produce F, A, and L; 36 mol% GC
B. gracilis	NF; nitrate reduced to nitrite; AEP, S
B. helcogenes	F; β-hemolysis; AEP, A, S
B. nodosus	NF; highly proteolytic; AEP, low levels of A, P, and S; 45 mol% GC
B. pectinophilus	WF; pectin and polygalacturonate fermented to F, A, and L; D-fructose positive; 45 mol% GC
B. pneumosintes	NF; AEP, low level of A
B. polypragmatus	F; motile; indole produced; trehalose positive; AEP, A
B. putredinis	NF; indole produced, gelatin digested; AEP, iV, S; Phe; 50–52 mol% GC
B. pyogenes	F; growth inhibited on bile; xylan negative; AEP, A, S
B. suis	F; growth inhibited in bile; xylan positive; AEP, A, S
B. tectum	WF; growth on bile; cellobiose positive; APE, A
B. xylanolyticus	WF; pectin and polygalacturonate fermented to F, A, and L; D-fructose positive; 45 mol% GC
Reclassified Species:	
Anaerorhabdus furcosus	NF; some strains, ferments glucose weakly cells may be Y-shaped; AEP, L
Fibrobacter succinogenes	WF; glucose, cellobiose, and lactose weakly fermented; AEP, A, S
Megamonas hypermegas	F; growth in bile, large cells 2 to 3 by 5 to 11 μm; AEP, A, P, L
Mitsuokella multiacidus	F; nitrate reduced to nitrite; AEP, A, L, S
M. dentalis	NF; AEP, A, S
Rikenella microfusus	WF; spindle-shaped cells; growth in bile; AEP, A, S
Ruminobacter amylophilus	F; ferments carbohydrate polymers such as starch (few monomers); AEP, F, A, S
Sebaldella termiditis	F; ferments xylose but not arabinose; AEP, F, A, L
Tissierella praeacuta	NF; cells motile, peritrichous flagella; gelatin weakly digested; AEP, A, B, iV
B. ureolyticus	NF; nitrate reduced; urease produced; AEP, A, S

F, fermentative; WF, weakly fermentative; NF, nonfermentative; AEP, acid end product; A, acetic; B, butyric; F, formic; iV, isovaleric; L, lactic; Phe, phenylacetic acid; S, succinic acid.

therefore, be possible to clone such genes as the elastase of *B. coagulans* or *B. nodosus* or the fibrinolysin of *B. putredinis* for clinical use, utilizing *B. fragilis* to obtain high expression of these enzymes.

Unlike the above species, genetic transfer and expression has been achieved between *B. nodosus* and the phylogenetically unrelated species *Psuedomonas aeruginosa* for the development of a pili-based *B. nodosus* vaccine (Stewart and Elleman, 1987). This approach offers a means of large-scale production of pili thus avoiding problems associated with culturing the fastidious anaerobic parent organism, *B. nodosus.*

Many of the species isolated from the rumen of sheep have important industrial properties (Bryant and Robinson, 1962; Hentges and Smith, 1985). Thus, *Fibrobacter succinogenes* or *B. cellulosolvens* may find application for the anaerobic digestion of cellulose. Recently, the cellodextrinase gene from *F. succinogenes* has been cloned in *E. coli* (Gong et al., 1989). *B. ruminicola* subspecies *ruminicola* and *B. xylanolyticus* may find useful application in the degradation of xylan while other plant macromolecules such as pectin could be fermented by *B. pectinophilus* and *B. ruminicola* subspecies *ruminicola.* The pigmented species, particularly *B. melaninogenicus, B. loescheii,* and *B. denticola* produce significant levels of protoporphyrin (see Chapter 197) from the demetallation of protoheme (Shah et al., 1979). Porphyrins are both difficult and expensive to synthesize but have important uses in biomedicine. These organisms could, therefore, be used directly to produce such compounds, or their genes could be inserted either into *B. fragilis* and then into *E. coli* to circumvent the difficulties encountered in large-scale production with anaerobic bacteria.

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CHAPTER 197

The Genus *Porphyromonas*

HAROUN N. SHAH

History

Black-pigmented bacteria resembling bacteroides were first reported by Oliver and Wherry (1921). The distinctive pigment produced on blood agar was considered to be melanin and consequently the organism was called *Bacteroides melaninogenicum*. Because of its requirement for X and V growth factors, the species was described as *Haemophilus melaninogenicus* in the third edition of *Bergey's Manual of Determinative Bacteriology* (Bergey et al., 1930). However, in the fifth edition of *Bergey's Manual* (Roy and Kelly, 1939), the generic name *Bacteroides* was adopted, as suggested by Castellani and Chalmers (1919), and *H. melaninogenicus* was reclassified as *Bacteroides melaninogenicus* (Roy and Kelly, 1939).

Prevot (1938), however, considered the genus name invalid and used the generic name *Ristella;* hence *B. melaninogenicus* is often referred to in earlier literature as *Ristella melaninogenica.* Schwabacher et al. (1947) reported that the black pigment produced by this species was incorrectly identified as melanin and suggested that the specific epithet be altered to *B. nigrescens.* Wilson and Miles (1945) considered these organisms as belonging to the group *Fusiformis* and adopted the name *Fusiformis nigrescens* for the pigmented species. Subsequently, the generic name *Bacteroides* took precedence in the seventh edition of *Bergey's Manual* (Kelly, 1957).

Despite its biochemical heterogeneity (see, for example, Courant and Gibbons, 1967; Sawyer et al., 1962), it was suggested that *B. melaninogenicus* be retained as a single species with both nonfermentative and saccharolytic strains. In the eighth edition of *Bergey's Manual* (Holdeman and Moore, 1974), *B. melaninogenicus* was therefore regarded as a single species, with three subspecies based on saccharolytic potential and types of metabolic end products. Thus, saccharolytic strains were assigned to *B. melaninogenicus* subspecies *melaninogenicus,* moderately saccharolytic strains to *B. melaninogenicus* subspecies *intermedius,* and nonfermentative strains to *B. melaninogenicus* subspecies *asaccharolyticus.*

Heterogeneity within *B. melaninogenicus* subspecies *asaccharolyticus* was first demonstrated by electrophoretic mobility patterns of malate dehydrogenase (MDH) and differences in DNA base compositions (Shah et al., 1976). Strains with the slower MDH mobility and lower GC content were of oral origin whereas strains with the faster MDH mobility and higher GC content were isolated from nonoral sites. Studies of the same strains by polypeptide patterns by SDS gel electrophoresis revealed two distinct profiles (Swindlehurst et al., 1977), one with major bands in the low molecular weight region (the slow MDH, low GC group) while isolates of the other group possessed a more evenly dispersed polypeptide pattern (the fast MDH, high GC group). As a result, strains of the latter group of *B. melaninogenicus* subspecies *asaccharolyticus* were then elevated to species rank by the ICSB Taxonomic Subcommittee as *B. asaccharolyticus* (Finegold and Barnes, 1977). Genetic heterogeneity between this group of isolates was confirmed (Shah and Hardie, 1979) and oral strains were reclassified in a new species, *B. gingivalis* (Coykendall et al., 1980). Studies of their cellular fatty acids (Shah and Collins, 1980; Mayberry et al., 1982), menaquinone composition (Shah and Collins, 1980), and DNA-DNA hybridization (Shah and Collins, 1983; van Steenbergen et al., 1982) further supported the placement of these organisms into separate species.

Studies of the asaccharolytic, pigmented oral isolates from endodontal infections and infected root canals with periapical destruction (Sundqvist, 1976) led to the recognition of another genetically distinct species, *B. endodontalis* (van Steenbergen et al., 1984), and the tentative proposal of *B. dentalis* (Haapasalo et al., 1984). Both taxa appeared to be synonymous and the validly published *B. endodontalis* took precedence. The asaccharolytic, pigmented species *B. asaccharolyticus, B. gingivalis.* and *B.*

endodontalis form a biochemically and/or chemically coherent group of species quite distinct from other pigmented bacteroides or the type species of the genus, *B. fragilis.* Ribosomal RNA homology (Johnson and Harich, 1986) and 16S rRNA cataloging (Paster and Dewhirst, 1988) data have confirmed the distinctiveness of these asaccharolytic taxa, for which the genus *Porphyromonas* has now been proposed (Shah and Collins, 1988). Major contributions that led to the establishment of this genus are shown in Table 1.

General Characteristics

Members of the genus *Porphyromonas* are characterized by the production of porphyrin pigments (Shah et al., 1979). The dark brown/black pigment, originally thought to be melanin, has been identified as protoheme and the light brown, UV-fluorescent compound as protoporphyrin (Shah et al., 1979). These pigments are produced on blood agar plates from 3–10 days after subculture. Extracted as their dimethyl esters, the electronic spectrum of the brown/black protoheme dimethyl ester shows a major band (Soret) at 385 nm with additional maxima at 512, 541, and 643 nm (Fig. 1). By contrast, the UV-fluorescent, protoporphyrin dimethyl ester has its Soret band at 407 nm and additional maxima at 505, 541, 575, and 629 nm (Shah et al., 1979; Fig. 1).

Growth in a liquid medium is confluent with cells being typically coccobacilli to rods (0.4–0.8 by 1.0–3.5 μm. All members of the genus produce indole or catalase and do not hydrolyze esculin or starch (Holdeman et al., 1984). The three species are nonfermentatitive and utilize nitrogenous substrates such as trypticase or proteose peptone as energy sources (Shah and Williams, 1987a). Members of the genus produce a complex mixture of acidic end products, with butyric acid being particularly distinctive (Holdeman et al., 1984). Unlike other pigmented species or members of the genus *Bacteroides,* which contain predominantly 12-methyl-tetradecanoic acid (*anteiso*-$C_{15:0}$) as their long-chain fatty acid, *Porphyromonas* species possess mainly 13-methyl-tetradecanoic acid (iso-$C_{15:0}$) (Shah and Collins, 1980). The latter taxa also contain MDH and glutamate dehydrogenase (GDH) but differ from *Bacteroides* sensu stricto in lacking enzymes of the hexose monophosphate shunt/pentose phosphate pathway (i.e., glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase) (Shah and Williams, 1982). The peptidoglycan of all strains so far examined lacked diaminopimelic acid. The electron transport system comprises mainly cytochrome b while menaquinones are the sole respiratory quinones present. Members of the genus *Porphyromonas* possess a GC content of DNA within the range 46–54 mol%.

Habitats

Although studies of the habitat of *Porphyromonas* species are still inconclusive, current evidence indicates that these species colonize different sites in the body. The type species *P. asaccharolytica* can be isolated from a wide range of nonoral sites such as the cervix, ear, intestine, genitalia, and from a variety of infections (Dowell and Lombard, 1981; Finegold et al., 1985). Strains have been isolated from blood, amniotic fluid, umbilical cord, empyema, peritoneal and pelvic abscesses, endometritis, and wound infections (Duerden, 1980a, 1980b; Goldstein et al., 1984; Lambe, 1974; Slots and Genco, 1979). There are only very limited reports of *P. asaccharolytica* strains from the oral cavity (Mayrand et al., 1984; Shah et al., 1976). *P. gingivalis* appears to colonize solely subgingival sites of the oral cavity but has also been recovered from the tongue, tonsils, saliva, and rarely from supragingival dental plaque (Shah et al., 1976; van Winklehoff et al., 1988). *P. gingivalis* was shown to constitute less than 5% of the cultivable subgingival flora of individuals with gingivitis (Slots, 1982; Slots et al., 1978; White and Mayrand, 1981; Zambon et al., 1981) but its numbers can increase dramatically in advanced periodontitis (Duerden, 1980b; Newman, 1979; Speigel et al., 1979; Tanner et al., 1979; White and Mayrand, 1981). It is therefore widely accepted that *P. gingivalis* is present below detectable levels in periodontally healthy sites but that their numbers reach measurable levels with disease development. *P. endodontalis* appears to colonize an even more restricted niche than the two other species. Although it has been isolated from mixed oral infections, it is mainly recovered from infections of endodontal origin (Duerden, 1980b; van Winklehoff et al., 1988) and has been reported widely from root canal infections (Haapasalo, 1986). Since both *P. gingivalis* and *P. endodontalis* are recovered almost exclusively from diseased sites, its primary source of infection remains unknown. These organisms are not present in the healthy oral cavity before development of permanent teeth (Slots, 1982) but may become important with the onset of periodontitis. Phenotypically related species have been reported from a wide range of animals,

Table 1. Major contributions that led to the proposal of the separation of the genus *Porphyromonas* from "*Bacteroides*" *melaninogenicus* subspecies "*asaccharolyticus*."

References	Results	Comments
Shah et al. (1976)	MDH mobility; strains with either slow and fast migrating enzymes; GC content; 46–48 and 52–54 mol%	First evidence of heterogeneity, two centers of variation evident
Swindlehurst et al. (1977)	Differences in SDS polypeptide pattern; two group discernible	Further evidence of heterogeneity, the pattern of profiles corroborated earlier data (Shah et al., 1976)
ICSB Taxonomic Subcommittee report, Finegold and Barnes (1977)	High MDH mobility cluster and high GC group regarded as a distinct entity	*Bacteroides melaninogenicus* subsp. *asaccharolyticus* elevated to species rank as *B. asaccharolytyicus*
Shah and Hardie (1979)	DNA-DNA hybridization, less than 10% difference between both groups of strains	Evidence for separate species now unequivocal. Strains now assigned to group 1 (low GC, slow MDH). Group 2 (high GC, fast MDH), *B. asaccharolyticus*)
Mayrand (1979)	Phenylacetic acid reported as a new metabolic end product	Strains which correspond to group 1 produce phenylacetic acid
Shah and Collins (1980)	Differences in cellular fatty acids and menaquinone composition	Group 1 strains shown to have high levels of iso-$C_{15:0}$ and MK-9. Group 2, *B. asaccharolyticus* had low levels of iso-$C_{15:0}$ and MK-10
Coykendall et al. (1980)	DNA-DNA hybridization data	Low GC (group 1 strains) proposed as *B. gingivalis*
van Steenbergen et al. (1984)	GC and DNA-DNA hybridization data	*B. endodontalis* sp. nov. from dental root canals proposed
Haapasalo et al. (1984)	GC, electrophoretic mobilities, and ultrastructure	New pigmented, asaccharolytic variant from dental root canals, "*B. dentalis*" tentatively proposed
Johnson and Harich (1986)	Ribosomal RNA homology studies among the *Bacteroides* species	*B. asaccharolytics* and *B. gingivalis* recovered in a distinct cluster. *B. endodontalis* was not tested
Shah and Collins (1988)	Biochemical and chemical heterogeneity	Data show the three species form a coherent group that differed significantly from *B. fragilis* (type species). The new genus *Porphyromonas* proposed for *B. asaccharolyticus, B. gingivalis* and *B. endodontalis.* The type species is *P. asaccharolytica*

MDH, malate dehydrogenase; iso-$C_{15:0}$, 13-methyl-tetradecanoic acid; MK-9 and MK-10, menaquinones with nine (MK-9) or 10 (MK-10) isoprene units.

such as cats, monkeys, jaguars, horses, pigs, guinea pigs, and herbivors, but the genetic relatedness of these microorganisms is still unknown. Among these animal isolates, several genotypes and phenotypes have been reported (Laliberté and Mayrand, 1983; Love et al., 1984). Further studies are therefore necessary to ascertain whether at least the "oral" human species are indigenous or exogenous to the oral cavity.

Isolation

Growth on Solid Media

Porphyromonas species can be isolated and maintained on blood agar base with 5% (wt/vol) horse or defibrinated sheep's blood. Neither of its normal cofactors, hemin or menadione, are required if the blood agar plates are freshly made or stored at 4°C for less than 1 week. Other media such as modified Brucella agar or Wilkens-Chalgren agar are commonly used. Prior to 1976, vancomycin-kanamycin blood agar plates (7.5 μg/ml) were routinely used for primary isolation of these organisms from dental plaque. Studies in our laboratory first showed that vancomycin inhibited the growth of the saccharolytic strains whereas colistin (10 μg/ml) inhibited the growth of the saccharolytic taxa such as *B. melaninogenicus* and *B. intermedius* (Shah et al., 1976). In subsequent studies, vancomycin was therefore excluded and colistin (10 μg/ml) was added to

blood agar plates. Other workers have confirmed the inhibitory effects of vancomycin on the growth of these organisms (van Winklehoff and de Graaff, 1983) and a selective medium containing colistin, bacitracin, and nalidixic acid has been reported for *P. gingivalis* (Hunt et al, 1986). The composition of some of these media are as follows:

Modified Brucella Agar

Brucella agar (BBL/Difco)	4.3 gm
Hemin solution (5 mg/ml)	0.1 ml
Vitamin K solution (10 mg/ml)	0.1 ml
Distilled water	100 ml

The medium is autoclaved at 121°C for 15 min and 5 ml of sterile defibrinated sheep's blood is added aseptically.

Wilkins-Chalgren Agar

Trypticase	1.0 g
Gelatin peptone	1.0 g
Yeast extract	0.5 g
Dextrose	0.1 g
NaCl	0.5 g
L-Arginine-free base	0.1 g
Sodium pyruvate	0.1 g
Vitamin K	0.05 mg
Hemin	0.5 mg
Agar	1.5 g
Distilled water	100.0 ml

The medium is autoclaved at 121°C for 15 min.

Selective Medium for *P. gingivalis* (Hunt et al., 1986)

Columbia agar base	42.5 g
Agar (BBL)	6.5 g
Colistin sulfate	10.0 mg
Demineralized water	1,000 ml
Heat to dissolve agar, then add	1.0 ml
Hemin-nalidixic acid (prepared by dissolving 5.0-mg hemin and 15.0 mg of nalidixic acid in 5 ml 1 N NaOH)	
Vitamin K, (1% (wt/vol) in alcohol)	10.0 mg

The pH is adjusted to 7.3, and the medium is autoclaved at 121°C for 15 min. The medium is cooled to 50°C, and 50 ml of defibrinated sheep blood and filter-sterlized bacitracin solution (1 μg/ml final concentration) is added aseptically and dispensed as required.

Growth in Liquid Media

Nutritional studies have shown that *Porphyromonas* species require nitrogenous substrates as a source of energy (Shah and Williams, 1987a). Peptides are the preferred energy source but limited growth is possible on amino-acid-containing media, such as casamino acids or a defined medium (Shah and Williams, 1987a, 1987b; Seddon et al., 1988). Unlike some *Bacteroides* species that colonize the rumen, *Porphyromonas* species cannot replace its nitrogen requirement with ammonia (H. N. Shah, unpublished observations). Thus, all media reported to date contain a commercial source of peptides such as trypticase, proteose peptone, yeast extract, or tryptone at approximately 1% (wt/vol). Glucose is not catabolized, but up to 50% of it can be utilized by some species as a source of carbon. Cysteine is used both as a reducing agent and as a growth requirement. Heme (for cytochrome production) replaces the need for whole blood while vitamin K is added as a precursor for menaquinone biosynthesis (Shah and Collins, 1980). Some of the commonly employed growth media include BM (Shah et al., 1976), tryptone-yeast extract broth (TYB), and proteose peptone-yeast extract glucose broth (PYG) (Holdeman and Moore, 1970). The composition of some of these media are given below.

BM Medium (Shah et al., 1976)

Trypticase	1.0 g
Proteose peptone	1.0 g
Yeast extract	0.5 g
NaCl	0.5 g
Glucose	0.5 g
Cysteine hydrochloride	0.075 g
Distilled water	100.0 ml

The medium is cooled and a solution of 1% (vol/vol) filter sterilized hemin-menadione solution is added.

Hemin-menadione solution: Hemin solution is first prepared by adding 1 ml of 1 N NaOH to 50 mg of crystalline heme, followed by 98 ml of water. To this add 1 ml of menadione solution (5 mg/ml in 95% ethanol) and filter-sterilized and store the mixture at −20°C. The hemin—menadione solution is added aseptically at 1% (vol/vol) to sterilized media.

Tryptone-Yeast Extract Broth

Tryptone	1.0 g
Yeast extract	0.5 g
Cysteine hydrochloride	0.05 g
Hemin	0.5 mg
Vitamin K	0.05 mg
Distilled water	100.0 ml

The medium is adjusted to pH 7.2 and autoclaved at 121°C for 15 min. Bovine serum (2.0 ml) is added aseptically after the medium has cooled.

A chemically defined medium for *P. gingivalis* has been described (Seddon et al., 1988). This medium yields poor growth but may be used as a basal medium for physiological studies. Minimal media can be formulated from it

by the addition of protein hydrolysates (Seddon et al., 1988). The composition is as follows.

Synthetic Medium

Aspartic acid	100 mg
Glutamic acid	100 mg/l
Phenylalanine	100 mg/l
Alanine	100 mg/l
$MgSO_4 \cdot 7H_2O_4$	700 mg/l
KH_2PO_4	200 mg/l
KH_2PO_4	1000 mg/l
$CaCl_2$	100 mg/l
NaCl	100 mg/l
L-Cysteine hydrochloride	75 mg/l
Hemin	5 mg/l
Menadione	0.5 mg/l

The pH of the medium is adjusted to 7.4 and is autoclaved at 121°C for 15 min.

Identification

The genus *Porphyromonas* belongs to the family Bacteroidaceae; a large family of Gram-negative, obligately anaerobic, nonsporing, nonmotile rods. In the current edition of *Bergey's Manual of Systematic Bacteriology* (Holdeman et al., 1984), three genera, *Bacteroides, Fusobacterium*, and *Leptotrichia*, are described on the basis of their characteristic metabolic end-product profile. Thus, *Fusobacterium* produces mainly butyric and acetic acids, *Leptotrichia* produces lactic acid, while strains that possess neither profile are assigned to the genus *Bacteroides*. Work throughout the 1980s has shown that the genus *Bacteroides* (Shah and Collins, 1983; Collins and Shah, 1987) contains an extremely heterogenous collection of species that differ significantly in cellular morphologies and are biohemically and physiologically very diverse. The host specificity of these species and their pathogenic potential vary considerably and this diversity is reflected in the wide range in GC content of the DNA approximately 28–61 mol%). The type species of *Bacteroides, B. fragilis* (42 mol% GC), differs from *Porphyromonas* species (46–54 mol% GC) in DNA base composition and in major metabolic properties (Table 2).

Porphyromonas species can be readily distinguished from other members of the family Bacteroidaceae by the distinctive black, pigmented colonies on blood agar plates after 2–5 days growth. In contrast to the pigmented saccharolytic species such as *Bacteroides intermedius, B. denticola*, or *B. melaninogenicus, Porphyromonas* species form smooth, slightly mucoid, wet, shiny, convex colonies, 1–3 mm in diameter, that darken progressively from the edge of the colony towards the center after 6–10 days incubation. Eventually the whole colony becomes black due to protoheme production. Growth in liquid media is not significantly affected by the addition of carbohydrates and the terminal pH remains near neutral irrespective of its presence. Protein hydrolysates such as proteose peptone, trypticase, or yeast extract markedly enhance growth. The optimum temperature for growth is 37°C. The major metabolic end products from BM or PYG medium are acetic and butyric acids, together with lower levels of propionic, isobutyric, and isovaleric acids (Table 2).

Malate dehydrogenase and glutamate dehydrogenase are present whereas glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are absent (Table 2). Proteinases are produced but the range of substrates hydrolyzed varies. Amino acids, such as aspartate and asparagine, are fermented but these species generally possess only a limited ability to ferment amino acids. Indole is produced but nitrate is not reduced to nitrite nor is starch or esculin hydrolyzed.

Studies by Caroff et al. (1987), Johne et al. (1988), Kumada et al. (1988), and Brondz et al. (1989) have demonstrated the presence of 3-deoxy-D-manno-2-octulosonic acid (KDO) (previously believed to be absent) in the lipopolysaccharide of *Porphyromonas*. The peptidoglycan does not contain diaminopimelic acid as the diamino acid. The principal respiratory quinones are unsaturated menaquinones with 9 or 10 isoprene units. Both nonhydroxylated and 3-hydroxylated long-chain fatty acids are present. The nonhydroxylated fatty acids are composed of predominantly isomethyl branched types (iso-$C_{15:0}$) and lower levels of straight-chain saturated acids. The 3-hydroxylated fatty acids are the straight-chain saturated types. The GC content of the DNA ranges from 46–54 mol%. Characteristics that may be useful in distinguishing *Porphyromonas* from other commonly occuring genera of the Bacteroidaceae are shown in Table 2.

Differentiation of *Porphyromonas* Species

Though DNA-DNA hybridization clearly separates the three species of *Porphyromonas*, physiological tests for distinguishing these species are still limited. Phenotypically related taxa such as *Bacteroides levii* and *B. macacae* can be separated from these species by their ability to weakly ferment glucose, lactose, and/or mannose (Holdeman et al., 1984). *B. salivosus*, a

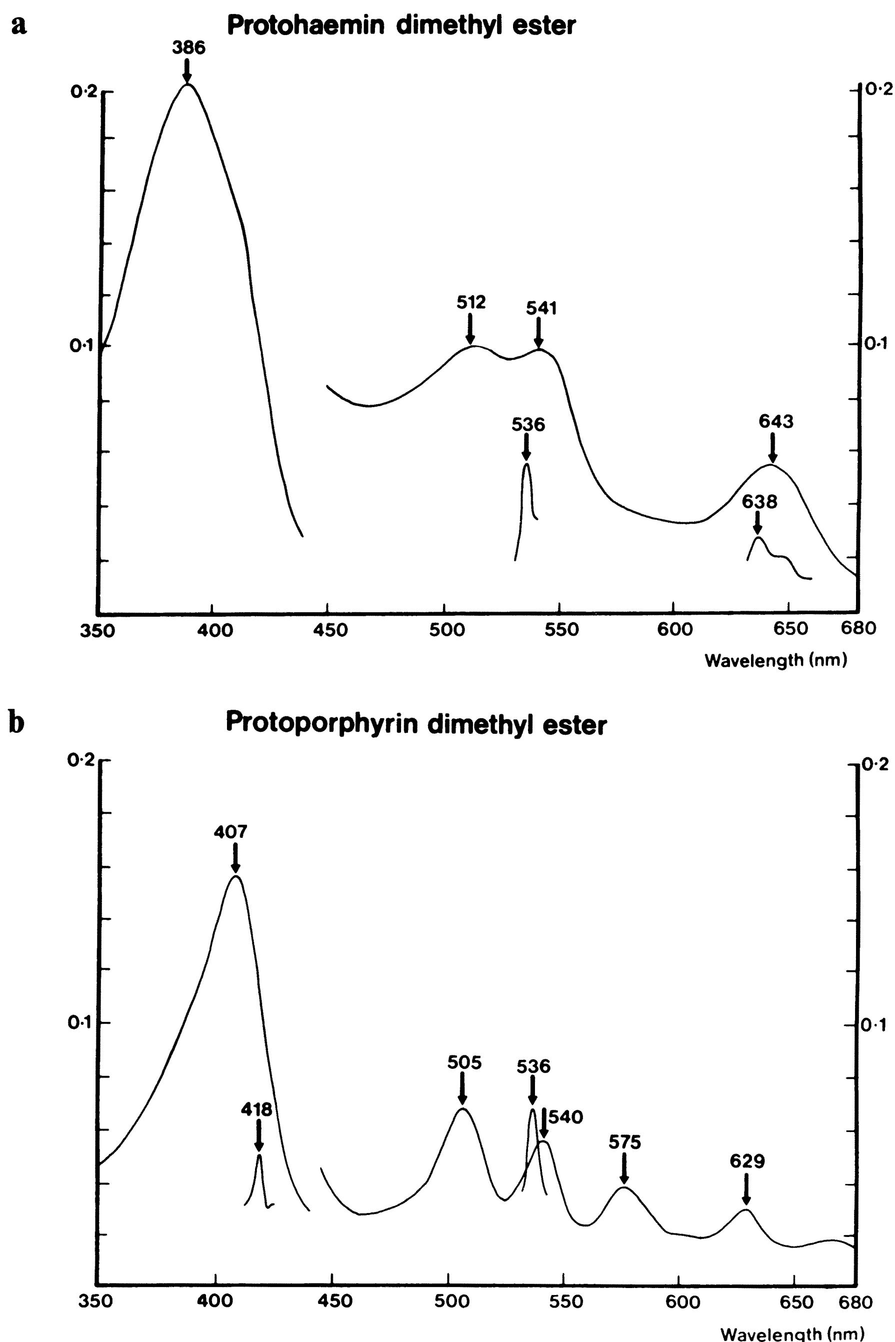

Fig. 1. Absorption spectra of *Porphyromonas* pigments (a) protohaemin dimethyl ester and (b) protoporphyrin dimethyl ester. Calibrations at 418, 536 and 638 nm were done with holmium glass.

phenotypically similar species isolated from cats (Love et al., 1987) resembles *P. gingivalis* in producing phenylacetic acid and has hydrolytic activity to α-*N*-benzoyl-L-arginine-4-nitroanalide (L-BAPNA) but differs by its low GC content (42–44 mol%) and its inability to agglutinate sheep erthrocytes. Most strains of *P. asacharolytica* so far tested possess α-fucosidase activity, thus distinguishing them from *P. gingivalis* and *P. endodontalis*. *P. gingivalis* strains are characterized by the presence of menaquinones with nine isoprene units whereas *P. asaccharolytica* possesses menaquinones with 10 isoprene units. The polyprenyl side chain of *P. endodontalis* is not yet known. The electrophoretic mobility of MDH and differences in DNA base compositions (Table 3) further differentiates the three species of *Porphyromonas*.

Table 2. Differentiation of *Porphyromonas* from *Bacteroides* and other related taxa.

Genus	Metabolism[a]	Indole production	Major metabolic end products from glucose broth[b]	Major long-chain fatty acids	Dehydrogenases present[c]				GC content (mol%)
					MDH	GDH	G6PDH	6PGDH	
Porphyromonas	NF	+	A, B, PA	iso-$C_{15:0}$	+	+	−	−	46–54
Bacteroides sensu stricto (*B. fragilis* and related sp.)	F	V	A, S	anteiso-$C_{15:0}$	+	+	+	+	40–48
B. melaninogenicus–*B. oralis* group of species	MF	V	A, S	anteiso-$C_{15:0}$	+	+	−	−	40–48
"*Bacteroides*" sp. with a high GC: e.g., *B. capillosus, Mitsuokella* (formerly *B. multiacidus*), Rikenella (formerly *B. microfusus*)	V	−	A, S, or L	$C_{14:0}$ $C_{16:0}$ $C_{16:1}$ iso-$C_{15:0}$	V	V	−	−	56–61
"*Bacteroides*" sp. with a low GC: e.g., *Tissierella* spp. (formerly *B. praeacutus*), *B. coagulans, B. putredinis*	NF	V	A, B, iV, P L, or S	$C_{15:0}$, $C_{16:0}$ $C_{18:0}$, $C_{18:1}$ iso-$C_{15:0}$	V	V	V	V	28–37

+, positive growth; −, negative growth; V, variable.

[a]F, Fermentative; NF, nonfermentative.

[b]Abbreviations; A, acetic acid; B, butyric acid; L, lactic acid; P, propionic acid; iV, isovaleric acid; S, succinic acid; PA, phenylacetic acid.

[c]Abbreviations; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.

Table 3. Major differential characteristics of species of the genus *Porphyromonas*.

Characteristics	*P. asaccharolytica*	*P. gingivalis*	*P. endodontalis*
Phenylacetic acid produced as a metabolic end product in glucose broth	−	+	−
Haemagglutination	−	+	−
Cysteine proteinase	−	+	−
α-Fucosidase	±[a]	−	−
N-acetyl glucosaminidase	−	+	−
Menaquinone composition	MK-10	MK-9	+[b]
Malate dehydrogenase electrophoretic mobility	Moderate	Slow	Fast
GC content (mol%)	52–54	46–48	49–51

P. asaccharolytica is the type species.
[a]Most strains positive.
[b]Menaquinone present but number of isoprene units not determined.

Clinical Importance and Outlook for Biotechnology

Members of the genus *Porphyromonas* have been isolated from a wide range of infectious sites (see reviews, Moore, 1987; van Winklehoff et al., 1988). In general, animal model experiments have shown that *P. gingivalis* produces spreading lesions that nearly always result in death of the animal whereas *P. asaccharolytica* and *P. endodontalis* lesions are localized and do not cause death (see review, Mayrand and Holt, 1988). Most studies relating to virulence and pathogenicity of these species have therefore focused on *P. gingivalis* partly for these reasons and partly because of its clearer association with disease. However, despite extensive investigations throughout the 1980s, the pathogenic mechanisms involved in the initiation and development of periodontal disease, for example, are still not fully understood. Current evidence indicates that virulence is multifactorial and a broad approach to the study of this subject is necessary to elucidate the underlying mechanisms involved in periodontal disease development. Contributions have therefore come from a wide range of disciplines and a number of interesting functions have been identified (reviewed by Mayrand and Holt, 1988).

Putative virulence factors so far proposed include surface components such as fimbriae, surface receptors (e.g., hemagglutinins), capsular material, membrane vesicles/blebs, and lipopolysaccharide, in addition to a wide spectrum of both extracellular and intracellular tissue-degrading enzymes such as collagenases, proteinases, glycosaminoglycan-depolymerizing enzymes, heparinases, and nucleases (see Mayrand and Holt, 1988). Capsular material and culture supernatants of *P. gingivalis* have been shown to decrease the chemotactic response of polymorphonuclear leukocytes. Resistance to phagocytosis is strongly associated with the presence of a capsule (Okuda and Takazoe, 1973, 1988; Okuda et al., 1987) and encapsulated strains were shown to remain viable during abscess formation (Takazoe et al., 1971). Mutants that lack a capsule are rapidly phagocytosed but such mutants are pleiotrophic, being deficient in several other properties (Shah et al., 1989). Differences, however, occur in chemical composition and antigenicity of *P. gingivalis* capsules and further studies are required to demonstrate a clearer association (Okuda and Takazoe, 1988). About 50% of *P. gingivalis* strains possess fimbriae but differences in antigenic specificity occurs (Suzuki et al., 1988). Unlike many bacterial species, these structures are not associated with hemagglutinating activity but appear to function in attachment to both host and bacterial cells (Grenier and Mayrand, 1987). Fimbriae were recently shown to bind specifically to gingival fibroblasts resulting in subsequent stimulation of thymocyte activating factor, which in turn stimulated mytogen-induced thymocyte proliferation (Hanazawa et al., 1988).

Unlike that of many Gram-negative bacteria, the lipopolysaccharide of *P. gingivalis* has been shown to possess relatively low toxicity (Koga et al., 1985). Initially this was considered to be due to the absence of KDO and other core sugars but is now attributed to the lack of phosphorylation in the lipid A backbone of the molecule (Johne et al., 1988). Both *P. gingivalis* and *P. endodontalis* possess vesicles (Shah et al., 1976; Haapasalo et al., 1984; Grenier and Mayrand, 1987). Their small size (approximately 50–150 nm) allows them to cross epithelial barriers, which cannot be penetrated by whole cells. They promote bacterial attachment between homologous cells and can mediate attachment between species that do not normally aggregate (Grenier and Mayrand, 1987). Vesicles possess

many of the properties of the parent cell, such as proteinase and collagenase activities or hemagglutination, and it is now widely believed that they deliver potent hydrolytic enzymes into subgingival sites, with the potential to cause tissue damage. Furthermore, vesicle-bound enzymes may function to release peptides (from host tissue and secretions) to support the nutritional requirements of these species in vivo.

It is therefore evident that due to the multifactorial nature of virulence of this species, molecular genetic techniques must be used to investigate the role of each putitive virulence factor separately to enable assessment of their relative contribution. Such studies are now being undertaken in several laboratories.

The biotechnological potential of *Porphyromonas* species is probably limited to *P. gingivalis* because of its wide spectrum of hydrolytic enzymes. However, all species utilize protoheme and under certain conditions are able to demetallate it to produce protoporphyrin (Shah et al., 1979, Fig. 2). Porphyrin compounds have

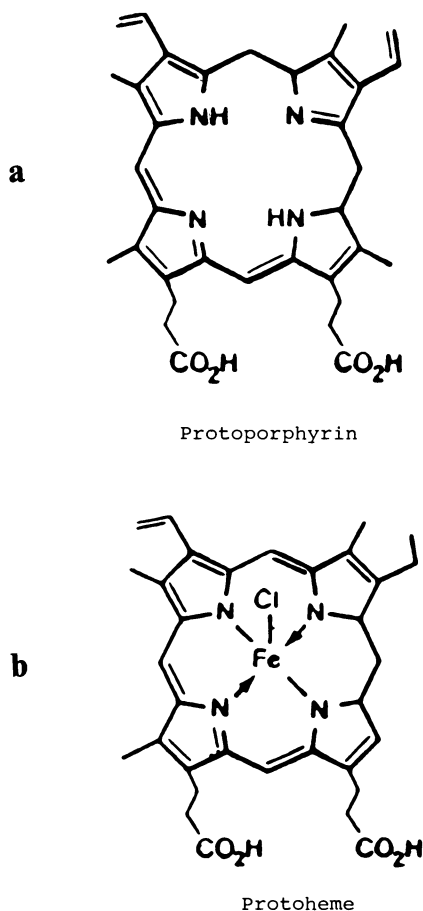

Fig. 2. Major porphyrins produced by *Porphyromonas* species (a) protoporphyrin and (b) protoheme.

wide application in biomedicine but are expensive to synthesize chemically. The potential therefore exists to utilize these organisms for the production of porphyrins to which various residues could be chemically attached.

Although the hydrolytic enzyme activity of *P. gingivalis* is now well documented, the useful biotechnological features of these enzymes are not yet fully realized. The proteinases comprise several types, of which the cysteine proteinase has now been characterized (Shah et al., 1990). Others such as the serine proteinase, the glycylprolyl peptidase, glycosaminoglycan-depolymerizing enzymes, and nucleases, have been purified but await detailed characterization before they can be commercially exploited. For example, it may be possible to treat septic thrombophlebitis (in which heparin, a naturally occurring acidic mucopolysaccharide inhibits the clotting of blood) with the heparinase of *P. gingivalis.* Several such examples exist where the hydrolytic enzymes of *P. gingivalis* may find biomedical applications. However, before this can be achieved, an understanding of the regulation and expression both in the host and in genetic vectors need investigation. Such studies on the thiol proteinase of *P. gingivalis* are in progress (Roberts et al., 1990). Recently the hemagglutinin of *P. gingivalis* has been successfully cloned into *Escherichia coli* (Progulske-Fox et al., 1989a, 1989b). This study represents the first successful transfer of *P. gingivalis* genes into a vector, and thus opens the field to further genetic manipulation of specific macromolecules.

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CHAPTER 198

The Genera *Flavobacterium, Sphingobacterium,* and *Weeksella*

BARRY HOLMES

The name *Flavobacterium* was proposed in 1923 for a genus of the family Bacteriaceae, encompassing the rod-shaped, nonendosporeforming, chemoorganotrophic bacteria (Bergey et al., 1923). Most of the pigmented bacteria of the family were segregated in the tribe Chromobactereae, which contained four genera of aerobic bacteria separated from each other by differences in color. These genera were *Chromobacterium, Flavobacterium, Pseudomonas,* and *Serratia,* for the purple, yellow, green fluorescent, and red strains, respectively. This emphasis on pigmentation (a character shared by genetically diverse bacteria [Weeks, 1969]) for taxonomic assignment to *Flavobacterium* has given the genus a dubious reputation in the past (McMeekin et al., 1972; Weeks, 1969), and as a consequence the genus has served too frequently as a repository for pigmented bacteria that possess the general attributes of *Flavobacterium* but had not been subjected to detailed classification studies. Taxonomic heterogeneity and general uncertainty have characterized *Flavobacterium* from its inception, and its history is a record of proposals to achieve credibility for the genus.

Stanier (1947) recognized that the cytophagas have more than a casual phenetic resemblance to pigmented, Gram-negative eubacteria such as *Flavobacterium* and the relationship to the cytophagas has dominated the taxonomic consideration of *Flavobacterium.* Differentiation of flavobacteria from cytophagas has depended primarily upon demonstration of the gliding movement and colonial translocation characteristic of the latter bacteria, but absence of these features has not deterred assignment of flavobacterial species to *Cytophaga* (Mitchell et al., 1969). Freshly isolated cytophagas have the unusual ability to use a great variety of complex natural polymers, e.g., agar, alginates, cell walls, cellulose, chitin, DNA, keratins, lipids, pectin, porphyrins, proteins, RNA, and starch, as nutrients. This ability is not a general property of flavobacterial species, although strains of some species may hydrolyze casein, chitin, gelatin, or starch.

The problem of differentiating *Flavobacterium* from *Cytophaga* has been discussed extensively (Christensen, 1977a; McMeekin et al., 1972; Mitchell et al., 1969; Weeks, 1969). An outcome would be more nearly possible if two issues were resolved. These are the heterogeneity of *Flavobacterium* and the differentiation of nonmotile flavobacteria from cytophagas. A primary requirement for the resolution of both issues is an acceptable definition of *Flavobacterium.* The concept of *Flavobacterium* was hardly altered in successive editions of *Bergey's Manual of Determinative Bacteriology* until the fifth (Bergey et al., 1939), which eliminated from the genus the least well-described species and the polarly flagellated strains. Those known to be Gram-positive were excluded in the seventh edition (Weeks and Breed, 1957). In the eight edition (Weeks, 1974) the genus remained heterogeneous, as evidenced by the two disparate reported ranges of GC content of the DNA: 30–42 and 63–70 mol%. Only in *Bergey's Manual of Systematic Bacteriology* (Holmes et al., 1984a) did the genus at last become reasonably homogeneous by including only nonmotile low GC strains. Interspecies DNA-DNA hybridization studies of the latter organisms showed a background level of hybridization of about 20%, which may represent a common DNA complement (Callies and Mannheim, 1980; Owen and Holmes, 1981).

Despite sharing several features, the low GC flavobacteria nevertheless show some heterogeneity and fall into four natural groups (A–D, Table 1), as previously suggested by Holmes and Owen (1981). The strains of group D, so far found only on mammalian mucus membranes, are susceptible to penicillin and are nonpigmented; they now comprise the genus *Weeksella.* Strains of the remaining groups are free-living, show resistance to a wide range of antimicrobial agents and are yellow-pigmented (Table 1). These organisms constitute the genus *Flavobacterium* and they can be divided into

Table 1. Differentiation of the flavobacteria.

Character	Habitat	Resistant to antimicrobial agents	Yellow pigment	Indole production	Oxidation of carbohydrates	Proteolytic activity
Group A: *Flavobacterium balustinum, F. breve, Flavobacterium* species group IIb *(F. gleum, F. indologenes), F. indoltheticum, F. meningosepticum*	Free-living	+	+	+	+	+
Group B: *Flavobacterium odoratum*	Free-living	+	+	−	−	+
Group C:[a] *Flavobacterium mizutaii, F. multivorum, F. spiritivorum, F. thalpophilum, F. yabuuchiae*	Free-living	+	+	−	+	−
Group D: *Weeksella virosa, W. zoohelcum*	Strict saprophyte	−	−	+	−	+

+, character present; −, character absent.

[a]Yabuuchi et al. (1983) have proposed that *F. mizutaii, F. multivorum,* and *F. spiritivorum* should be placed in a separate genus *Sphingobacterium.*

groups A–C on the basis of differences in indole production, oxidation of carbohydrates, and proteolytic activity (Table 1). A detailed taxonomic study of these organisms by techniques such as DNA-rRNA hybridization is necessary to determine their phylogenetic relationships and therefore whether they all belong in the same genus. Pending such a study, it seems undesirable to divide the genus further or to admit to it taxa of uncertain affiliation, otherwise the genus is in danger once more of becoming heterogeneous. The limited data available from DNA-rRNA hybridizations (Bauwens and De Ley, 1981) indicate that *F. breve, F. meningosepticum,* and *F. odoratum* may not be closely related, and that *F. aquatile,* the type species of the genus (and itself represented by a single strain), is not closely related to the above three species. For other reasons, *F. aquatile* had been considered unrepresentative of the genus. Consequently, Holmes and Owen (1979) requested that *F. breve* be made the type species instead. Their request was subsequently denied, and thus if the genus is further subdivided in the future the name *Flavobacterium* will remain with *F. aquatile* and the majority of organisms currently recognized as *Flavobacterium* will be transferred to one or more new genera. For the above reasons, *F. aquatile* is not considered in the tables in this paper (its characters are given by Holmes et al., 1984a). Perhaps prematurely, the genus *Sphingobacterium* has been proposed by Yabuuchi et al. (1983) for organisms in group C (Table 1) based on the fact that most of these organisms have been shown to possess novel sphingolipids in their cell walls. As this character is not easily determined routinely, and pending further study and possible revision of the genus as a whole, the group C strains are here retained in *Flavobacterium.* (Although all strains in group C have valid combinations of their specific epithets with *Flavobacterium,* there has as yet been no proposal to transfer *F. thalpophilum* or *F. yabuuchiae* to *Sphingobacterium.)*

With *Flavobacterium* better defined, it is now possible to reassess the differentiation of this genus from *Cytophaga.* It has been suggested that organisms of both these genera, which share a distinctive cellular fatty acid composition, a characteristic menaquinone system, and a low GC content, should all be included in *Cytophaga* (Oyaizu and Komagata, 1981). However, this view does not take into account the heterogeneity of the *Flavobacterium-Cytophaga* group as revealed by the preliminary DNA-rRNA hybridization results of Bauwens and De Ley (1981). More recent DNA-DNA hybridization studies (Bernardet, 1989) reveal no appreciable homology between *Cytophaga johnsonae* and *Flavobacterium* species of groups A, B, and C. Oligonucleotide cataloging (Paster et al., 1985) places *C. johnsonae* and *F. aquatile* in the same group while finding that *F. breve* is only peripherally related to the same group. Cataloging also places *Cytophaga* and

Flavobacterium in the same eubacterial "phylum" as *Bacteroides.*

In summary, while *Flavobacterium* is now more homogeneous than it has ever been, further work is necessary to group the species into additional genera so as to reflect their phylogenetic relationships. Among the flavobacteria of clinical origin at least, these generic groupings may well be reflected by the groups A–C as defined in Table 1. While these organisms would appear to be distinct from *Cytophaga,* other flavobacteria, including *F. aquatile,* may be more closely related to *Cytophaga.* The latest arrangement of including the families Bacteroidaceae and Cytophagaceae plus a newly proposed family "Flavobacteriaceae" in the order Cytophagales (Reichenbach, 1989) is a good reflection of our present knowledge of the phylogenetic relationships of these organisms.

Habitats

Yellow-pigmented, nonfermentative, Gram-negative, nonmotile, rod-shaped bacteria, which have been placed in the genus *Flavobacterium* or have been termed flavobacteria, have been isolated from fresh and marine waters, soil and ocean sediments, foods and food-processing plants, and clinical materials. These bacteria are widely distributed in nature and are especially common in water, which would explain their seeming omnipresence.

Nonclinical Sources

Flavobacterial strains have been isolated most commonly from freshwater and marine environments, and many general bacteriological surveys of such habitats have reported their presence (for example, ZoBell and Upham, 1944). The numerical taxonomic studies of Hayes (1963) and of Floodgate and Hayes (1963) used marine strains isolated from fish surfaces, sea water, and marine mud; the samples were taken from the north Atlantic region, western North American coast, and Florida. Comprehensive investigations of dairy and meat-processing industries have shown numerous flavobacteria (McMeekin et al., 1971, 1972; Jooste et al., 1985), including *Weeksella*-like organisms (Botha et al., 1989). The bacteria have also been found in chlorinated cooling water of vegetable canning plants and were the cause of characteristic spoilage following post-sterilization contamination (Bean and Everton, 1969). *F. multivorum* has been isolated from soil (Hayward and Sly, 1984; Pichinoty et al., 1985).

Clinical Sources

Flavobacteria have consistently been found among the nonfermentative, Gram-negative bacteria isolated from clinical specimens such as blood, urine, infected wounds, and feces (Pickett and Manclark, 1970; Pickett and Pedersen, 1970a, 1970b; Tatum et al., 1974). Their frequency of occurrence is usually low, 1% or less, and their pathogenicity is low-grade or doubtful. They are, however, extremely resistant to many antimicrobial agents (and so may well colonize the patient on intensive chemotherapy for an infection caused by some other organism). Such resistant strains are found in each of groups A–C: *F. breve* (Holmes et al., 1978); *F. meningosepticum* (King, 1959); Group IIb of King (Tatum et al., 1974; this taxon includes *F. gleum* [Holmes et al., 1984b] and *F. indologenes* [Yabuuchi et al., 1983]); *F. odoratum* (Holmes et al., 1977, 1979); *F. multivorum* (Holmes et al., 1981); *F. spiritivorum* (Holmes et al., 1982) and *F. thalpophilum* (Holmes et al., 1983).

F. meningosepticum, the best-known pathogen in the genus, is associated with a sometimes fatal meningitis of infants and has been isolated from their throats, spinal fluid, and blood as well as from throats of normal adults (King, 1959; Holmes, 1987). *F. odoratum* has also been a cause of ventriculitis (Macfarlane et al., 1985). Both *F. multivorum* and *F. odoratum* have been reported as a cause of bacteremia (Freney et al., 1987; Potvliege et al., 1984; Prieur et al., 1988), while both *F. odoratum* and *F. thalpophilum* have been described in wound case reports (Davis et al., 1979; Hansen et al., 1988). *Weeksella virosa* appears to be almost exclusively associated with the female genital tract (Holmes et al., 1986a; Reina et al., 1989), and it seems more prevalent in sexually promiscuous women (Mardy and Holmes, 1988). *W. zoohelcum* is commonly found in the canine oral cavity and has been isolated from human dog-bite wounds (Holmes et al., 1986b). However, one strain has been reported as a cause of meningitis in a child following a dog-bite (Bracis et al., 1979) and another as a cause of septicemia (Noell et al., 1989).

Isolation

Nonclinical *Flavobacterium*

Flavobacteria are chemoorganotrophic and not difficult to isolate, although maintenance of the cultures sometimes presents a problem.

General studies do not require enrichment procedures and usually nutrient agar-type media are used. Studies of marine, pigmented bac-

teria, for example, have used seawater-agar media, such as the following:

Marine *Flavobacterium* Medium (Hayes, 1963)

Beef extract (Lab Lemco)	10 g
Peptone (Evans)	10 g
Agar (Difco)	15 g
Aged seawater	750 ml
Distilled water	250 ml

Adjust pH to 7.2–7.3.

Isolation of yellow-pigmented bacteria from food and food-processing equipment used a similar medium:

Food *Flavobacterium* Medium (McMeekin et al., 1971)

Beef extract (Oxoid L20)	10 g
Peptone (Oxoid L37)	10 g
NaCl	5 g
Agar (Oxoid No. 3)	12 g
Distilled water	1 liter

Incubation temperatures were similar to that of the environment, i.e., 20–25°C., and incubation times were about 4 days.

Weeks (1955) used medium M1, which contains lesser amounts of nutrients, for both isolation and maintenance of flavobacterial cultures.

Medium M1 (Weeks, 1955)

Proteose peptone (Difco)	5 g
Yeast extract (Difco)	1 g
Beef extract (Difco)	2 g
NaCl	3 g
Agar	12 g
Distilled water	1 liter

pH 7.2–7.4.

It is not unusual to find that media containing relatively large amounts of the individual nutrients, such as those used by Hayes (1963) and McMeekin et al. (1971), are not as well suited to maintenance of flavobacterial cultures as are media with lower concentrations. A study on maintenance by Christensen and Cook (1972) dealt primarily with the isolation of cytophagas but flavobacteria were included. In general, media such as PMYA II containing small amounts of nutrients were superior.

PMYA II (Christensen and Cook, 1972)

Peptonized milk	1 g
Yeast extract	0.2 g
Sodium acetate	0.02 g
Agar	15 g
Distilled water	1 liter

This medium was excellent for detection of cytophagal gliding and colonial swarming, which is useful in differentiating between flavobacteria and cytophagas.

Flint (1985), when developing a selective agar medium for the enumeration of *Flavobacterium* species in water, found that on nutrient agar alone less than 10% of colonies recovered were yellow-pigmented; however, when a nutrient agar medium containing kanamycin at 50μg/ml was used, this figure increased to 70%.

Clinical *Flavobacterium*

Tatum et al. (1974) have described in detail the procedures for isolation of flavobacteria from clinical sources, such as those of *F. meningosepticum* and Group IIb. Primary plating of a clinical specimen is usually onto blood, chocolate, eosin-methylene blue, or MacConkey agar, but other media may be used. Incubation temperature is 35–37°C, but the bacteria will grow at room temperatures (20–25°C). Other workers have found ordinary nutrient agar quite satisfactory (for example, Holmes et al., 1977, 1978).

Preservation of cultures

Pickett and his colleagues have used cystine-trypticase agar (BBL) for maintenance of cultures of the clinical flavobacteria. Long-term preservation was carried out using brucella broth (BBL) containing 10% glycerol and the cultures were stored at −50°C (Pickett and Pedersen, 1970a). Isolates can also be stored freeze-dried.

Identification

Assignment of a culture to *Flavobacterium* now rests primarily upon the attributes ascribed to the genus (Holmes et al., 1984a). Strains are strictly aerobic, Gram-negative, nonmotile, yellow-pigmented, free-living nonfermenters with low GC content and do not show gliding motility; clinical strains at least are highly resistant to a wide range of antimicrobial agents. Since these features are quite general, the personal judgment of the investigator is a major contribution, especially for the nonclinical strains which have not been studied as thoroughly as clinical strains.

Clinical isolates can usually be identified by assigning them initially to one of the groups A–D on the basis of the characters given in Table 1 and then using the appropriate table for spe-

cies determination. Methods for determining some characters useful in the differentiation of flavobacteria (indole formation, hydrolysis of DNA, starch, and urea, and pigment production) have been compared by Pickett (1989). Just as the flavobacteria may eventually comprise several genera, so new species are set to emerge from the existing recognized species. It is apparent from Tables 2–4 that many species are heterogeneous in phenotypic characters and such is the genomic diversity among the flavobacteria that many recognized species comprise very few known strains. Indeed, several are represented by a single strain.

Among the taxa in group A, *F. balustinum* (from heart blood of fish) and *F. indoltheticum* (from the marine environment) are represented by single strains and extensive DNA-DNA hybridization studies of phenotypically similar strains have ascribed no additional strains to the former and only three to the latter (A. G. Steigerwalt and D. J. Brenner, personal communication). Similar DNA relatedness studies reveal that in *F. breve* there is a "core" of closely related strains with other strains showing lower levels of relatedness to the "core" strains (Owen and Holmes, 1980). Owen and Snell (1976) showed that of the strains representing the then six serovars of *F. meningosepticum,* the type strain of the species, which represented serovar A, showed much lower levels of relatedness to strains of the other serovars (B–F) than did these strains to each other. Work on this species was extended by Ursing and Bruun (1987), who found only three other strains that showed appreciable homology to the type strain; the remaining 48 strains could be divided into four subgroups. Despite the genomic differentiation of these 52 strains into two major groups, no correlation of these groups could be found with

Table 2. Characteristics for practical identification and differentiation of the *Flavobacterium* taxa of group A.

Test	*F. balustinum* (1 strain)	*F. breve* (7 strains)	*F. indoltheticum* (1 strain)	*F. meningosepticum* (49 strains)	*Flavobacterium* species group IIb (55 strains)
Acid from ammonium salt-sugar medium containing:					
Glucose	+	6/7[a]	+	42/49	+
Arabinose	−	−	−	1/49	13/55
Cellobiose	−	−	−	4/49	3/55
Ethanol	+	−	−	28/49	9/55
Glycerol	−	−	−	38/49	35/55
Lactose	−	−	−	27/49	−
Maltose	−	6/7	+	46/49	+
Mannitol	−	−	−	31/49	3/55
Sucrose	−	−	−	−	12/55
Trehalose	−	−	−	42/49	48/55
Xylose	−	−	−	3/49	9/55
Esculin hydrolysis	+	−	+	47/49	52/55
Growth at 42°C	−	−	−	6/49	15/55
Indole production (Ehrlich reagent)	+	+	+	24/49	53/55
Nitrate reduction	+	−	−	2/49	16/55
Nitrite reduction	−	−	−	18/49	14/55
Starch hydrolysis	−	−	−	−	36/55
Urease production	−	−	−	16/49	11/55
β-D-Galactosidase production	−	−	−	48/49	15/55
GC content (mol%)	33.1	32.4 ± 0.6[b]	33.8	37.0 ± 0.5	35 to 38.5
Number of strains tested for GC content	1	10	1	8	13

+, all strains tested positive; −, all strains tested negative.

[a]Number of strains showing characteristic/number of strains tested.

[b]Mean ± standard deviation.

The phenotypic results were derived from previous work at the National Collection of Type Cultures, as follows: *F. balustinum, F. indoltheticum, Flavobacterium* species Group IIb (Holmes, 1983), *F. breve* (Holmes et al., 1978), and *F. meningosepticum* (Holmes, 1987). The GC values were derived as follows: *F. balustinum* and *F. breve* (Owen and Holmes, 1980), *F. meningosepticum* (Owen and Lapage, 1974; Owen and Snell, 1976), *F. indoltheticum* and *Flavobacterium* species group IIb (B. Holmes and R. J. Owen, unpublished observations).

Table 3. Characteristics for practical identification and differentiation of the group C flavobacteria.[a]

Test	*F. mizutaii* (3 strains)	*F. multivorum* (28 strains)	*F. spiritivorum* (11 strains)	*F. thalpophilum* (7 strains)	*F. yabuuchiae* (2 strains)
Acid from ammonium salt-sugar medium containing:					
Adonitol	2/3[b]	−	−	+	−
Arabinose	1/3	+	3/11	+	−
Ethanol	−	−	+	−	+
Glycerol	−	27/28	+	+	+
Mannitol	−	−	+	−	+
Rhamnose	+	13/28	1/11	+	−
Gelatinase production (plate method)	1/3	4/28	+	6/7	+
Growth at 42°C	−	−	−	+	−
Hydrolysis of 2-napththyl phosphate at pH 5.4[c]	NT	+	+	+	−
Nitrate reduction	−	−	−	+	−
Nitrite reduction	+	−	−	−	−
Urease production	1/3	27/28	+	+	+
Extracellular deoxyribonuclease production	−	17/28	+	6/7	+
GC content (mol%)	41.0 ± 0.8[d]	39.6 ± 0.5	41.4 ± 0.4	45.0 ± 0.8	41.4 ± 0.5
Number of strains tested for GC content	3	11	6	7	2

+, all strains positive; −, all strains negative; NT, not tested.
[a]These organisms are also known as the sphingobacteria.
[b]Number of strains showing characteristic/number of strains tested.
[c]Tested in standard API ZYM gallery.
[d]Mean ± standard deviation.
The phenotypic results for *F. mizutaii* were taken from National Collection of Type Cultures records (B. Holmes, unpublished observations), and the GC contents for these strains were obtained from Yabuuchi et al. (1983). The results for *F. multivorum* were derived from Holmes et al. (1981), those for *F. thalpophilum* from Holmes et al. (1983), and those for *F. spiritivorum* and *F. yabuuchiae* from Holmes et al. (1988).

Table 4. Characteristics for practical identification and differentiation of *Weeksella* species.

Test	*W. virosa* (29 strains)	*W. zoohelcum* (30 strains)
Urease production	−	+
Growth at 42°C	+	1/30[a]
Growth on MacConkey agar	+	−
Growth on *β*-hydroxybutyrate	+	−
GC content (mol%)	37.3 ± 0.5[b]	35.7 ± 0.8
Number of strains tested for GC content	4	4

+, all strains tested positive; −, all strains tested negative.
[a]Number of strains showing characteristic/number of strains tested.
[b]Mean ± standard deviation.
The phenotypic results and GC values for *W. virosa* were derived from Holmes et al. (1986a) and those for *W. zoohelcum* from Holmes et al. (1986b).

antimicrobial susceptibility (Bruun, 1987), crossed immunoelectrophoresis (Bruun and Høiby, 1987), or phenotypic characters (Bruun and Ursing, 1987). The name *F. indologenes* was proposed for all strains of group IIb (Yabuuchi et al., 1983), despite the fact that it is genomically heterogeneous (Owen and Snell, 1976). The name *F. gleum* was proposed for several group IIb strains that show a high level of DNA-DNA relatedness (Holmes et al., 1984b). More recent DNA-DNA hybridization studies of group IIb strains (A. G. Steigerwalt and D. J. Brenner, personal communication) show that, while several strains display high levels of relatedness to the respective type strains of either *F. gleum* or *F. indologenes,* as many strains again remain to be assigned to one or more genomic groups. Until this problem is further investigated, it would seem prudent to continue referring to the taxon by its original designation of *Flavobacterium* species group IIb.

Group B comprises only *F. odoratum,* easily recognized by its fruity odor (Holmes et al., 1977). Although only ten strains have been studied by DNA-DNA hybridization, there is again genomic heterogeneity in the taxon with the strains falling into three genomic groups (Owen and Holmes, 1978).

With the exception of *F. multivorum,* all the other species in groups C and D have been ex-

amined for DNA-DNA relatedness (*F. mizutaii, F. spiritivorum,* and *F. yabuuchiae* [Holmes et al., 1988]; *F. thalpophilum* [Holmes et al., 1983]; *Weeksella virosa* [Holmes et al., 1986a]; *W. zoohelcum* [Holmes et al., 1986b]). These studies showed that the species were genomically homogeneous except for *F. spiritivorum,* which contained two strains showing high levels of relatedness to each other but lower levels to other strains of *F. spiritivorum;* these two strains now constitute *F. yabuuchiae* (Holmes et al., 1988).

Although there is clear evidence for additional genospecies in *Flavobacterium* (perhaps in *Weeksella* also [Botha et al., 1989]), some of these may not be recognized formally as new species unless phenotypic characters can be found to differentiate them. In addition, there are phenotypically discernible taxa which remain unnamed pending a full taxonomic study. These organisms are groups IIc, IIe, IIh, and IIi; they are all saccharolytic and the last three, at least, are indole producers (Rubin et al., 1985). They may therefore well be additional members of *Flavobacterium* group A, especially as strains of Groups IIe and IIh are known to contain menaquinone 6 and lack sphingolipids (Dees et al., 1986), which would also exclude them from group C (the sphingobacteria). Some differentiation of the flavobacteria can be achieved by gas liquid chromatographic analysis of volatile fatty acids produced in culture (Rasoamananjara et al., 1988). Serology has only been applied to *F. meningosepticum,* where types A–O are recognized; however, strains representing some of these serotypes are biochemically atypical of the species and may be strains of *F. breve* (Holmes et al., 1984a).

Identification of the nonclinical flavobacteria seems to be essentially a matter of deciding whether nonmotile flavobacteria are cytophagas. In this connection, it is a requirement to know whether or not an isolate demonstrates gliding movement and colonial translocation as defined by Henrichsen (1972). It is also necessary to know the DNA base composition and whether the culture can utilize a variety of complex macromolecules in its nutrition. Cultures that display properties of typical cytophagas do not present a problem since these would not be classified as flavobacteria; cultures not having such properties could be considered flavobacteria. Isolates that do not display cytophagal gliding but do demonstrate colonial translocation would not be cytophagas according to Henrichsen (1972). Cultures that are believed to display gliding motion but which do not show colonial translocation probably should not be assigned to the Cytophagaceae. Christensen (1977b) believed that colonial spreading would occur in such cultures if the correct experimental conditions were used.

It has already been stated that *F. aquatile* (from water) is not included in the identification scheme given in this chapter. Also not included are the more recently described species *F. thermophilum* (Loginova and Egorova, 1978) and *F. branchiophila* (Wakabayashi et al., 1989), the latter of which causes bacterial gill disease in freshwater fishes. The former is a thermophile, probably a *Thermus* species (see Chapter 205) and the latter, like *F. aquatile,* neither grows at 37°C nor grows well on nutrient agar. Two further new species, both halophiles from an Antarctic Lake, *"F. gondwanense"* and *"F. salegens,"* may be proposed (T. A. McMeekin, personal communication). Whether these more recently described organisms truly belong in the genus remains to be determined. DNA-DNA hybridization studies (Bernardet, 1989) reveal that *F. branchiophila* has higher levels of relatedness to *Cytophaga* species than to *Flavobacterium* species. The other validly published species in the genus that are not mentioned here are of uncertain taxonomic position and do not belong in *Flavobacterium* for the reasons given by Holmes et al. (1984a).

Physiological properties

Within the group A flavobacteria, the main cellular fatty acid components in *F. breve* are iso-$C_{15:0}$ and 3-OH iso-$C_{17:0}$ acids (as they are also present in *F. aquatile*); *F. meningosepticum* contains in addition, significant amounts of 2-OH iso-$C_{15:0}$ and is similar to *Flavobacterium* species group IIb (Moss and Dees, 1978; Oyaizu and Komagata, 1981). *F. odoratum* (group B) differs from members of group A in containing iso-$C_{15:0}$, iso-$C_{17:1}$, 3-OH iso-$C_{15:0}$ and 3-OH iso-$C_{17:0}$ acids (Oyaizu and Komagata, 1981). Among the group C flavobacteria (or sphingobacteria), *F. multivorum* at least contains iso-$C_{15:0}$ and 2-OH iso-$C_{15:0}$ as major fatty acid components while the main cellular lipids are sphingophospholipids (Yabuuchi et al., 1981), as indeed they also are in *F. mizutaii, F. spiritivorum,* and *F. thalpophilum* (Dees et al., 1985; Yabuuchi et al., 1983). The group C organisms also contain major quantities of menaquinone 7 but no menaquinone 6, in contrast to the flavobacteria of groups A and B which contain major amounts of menaquinone 6 but no menaquinone 7 (Dees et al., 1985). These characters further support the case for classifying the group C organisms in the separate genus *Sphingobacterium* but they rely on characters not easily determined routinely.

Cells of all *Flavobacterium* species are nonmotile in both hanging drop preparations and in soft agar; it is generally accepted that they lack flagella. However, Weeks (1955) examined the type strain of *F. aquatile* by electron microscopy and observed structures that he thought were like "pseudoflagella." By light microscopy, Webster and Hugh (1979) observed what they thought to be nonfunctional flagella on both the type strain of *F. aquatile* and that of *F. meningosepticum,* but this was not confirmed by Thomson et al. (1981) using electron microscopy (although extracellular appendages of a nonflagellar nature, like those on gliding bacteria, were seen in *F. aquatile*).

The yellow pigments produced by these organisms are nonfluorescent and insoluble in growth media; in *F. aquatile* the pigment is carotenoid (principally zeaxanthin; O. B. Weeks, unpublished observations) whereas in *F. breve* and *F. odoratum* they are not carotenoid but are probably of the flexirubin type.

Clinical strains are generally resistant to many antimicrobial agents, including amikacin, ampicillin, carbenicillin, gentamicin, kanamycin, polymyxin B, streptomycin, and tobramycin. Although an R plasmid conferring resistance to ampicillin, carbenicillin, and erythromycin has been reported in a strain of *F. odoratum* (Kono et al., 1980), detectable plasmids have been rarely reported (Owen and Holmes, 1981) and resistance in these organisms is therefore possibly associated with chromosomal genes.

Applications

No applications for strains of the genera *Flavobacterium, Sphingobacterium,* or *Weeksella* are yet known. However, suitable strains of *"Flavobacterium keratolyticus"* (isolated from soil in Japan), an organism not only without standing in nomenclature but possibly also not even a member of the genus *Flavobacterium,* naturally produce an endo-β-galactosidase. This enzyme exposes the human red blood cell cryptantigen Tk and is therefore potentially useful in blood transfusion laboratories for determining the type of end-link sugar on the red cell membrane that carries the blood group antigen (Liew et al., 1982). Growth measurements with a supposed *Flavobacterium* strain have proved useful in determining the concentration of maltose- and starch-like compounds in drinking water, where these compounds may contribute to undesirable bacterial growth in distribution systems (Van Der Kooij and Hijnen, 1985).

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CHAPTER 199

The Order Cytophagales

HANS REICHENBACH

In the past decade much has been learnt about the order Cytophagales and about some of the organisms belonging to it, although the majority of its members are as unfamiliar as ever. On the basis of 16S rRNA studies, we can now delimit the group with some confidence and have a well-founded idea of its phylogenetic position (Paster et al., 1985; Woese et al., 1985). Accordingly, the Cytophagales appear to be distantly related to the *Bacteroides* group, and these two together comprise one of the main branches, perhaps a phylum, in the bacterial phylogenetic system. The substructure of the *Cytophaga* branch of the phylum is more difficult to evaluate. There is a main line on which we find unicellular gliders—*Cytophaga* (*Cy.**) *johnsonae*, *Cy. lytica*, *Cy. aquatilis* = *Flavobacterium* (*Fv.*) *aquatile*, and *Sporocytophaga* (*Sp.*) *myxococcoides*—but at a lower level unicellular nonmotile bacteria (*Fv. breve*, i.e., low GC, true flavobacteria) are also found. At a still lower level, a cluster branches off which comprises the unicellular gliders—*Flexibacter* (*Fx.*) *filiformis* = *Fx. elegans* Fx el, *Cy. heparina*, and *Taxeobacter* = Myx 2105), unicellular nongliding flavobacteria (*Fv. ferugineum*), but also filamentous, multicellular, gliding (*Saprospira*) and nonmotile bacteria (*Haliscomenobacter*). It is obvious from these data that our present definition of genera does not reflect the phylogenetic situation, and also that the grouping in families and perhaps orders needs to be reconsidered. Before that is done, however, 16S rRNA sequences of further species should be determined.

From what has been said above it is clear that only a preliminary characterization of the order is possible at present. In this chapter, the order Cytophagales is restricted to unicellular gliding bacteria; the relevant genera are listed in Table 1. Filamentous, gliding bacteria of the genus *Saprospira* may or may not belong to the order, and they are sufficiently different to justify a separate treatment (see Chapter 200); contrary to earlier suggestions, these organisms are not apochlorotic cyanobacteria (Reichenbach et al., 1986). Unicellular, gliding bacteria of the genus *Lysobacter* have been found to be closely linked to the xanthomonads and thus belong to the gamma branch of the Proteobacteria (Woese et al., 1985); consequently they are discussed separately (see Chapter 176) It should be mentioned that, in the past, lysobacters have often erroneously been classified as cytophagas; this is discussed in Chapter 176. On the other hand, the cytophagas have been regarded as myxobacteria for some time and accordingly named; *Cy. columnaris* has even been classified in the myxobacterial genus *Chondrococcus* (now *Corallococcus*). However, as pointed out above, the two groups are not phylogenetically related. The nonmobile bacteria that cluster with the Cytophagales are discussed in Chapter 198 (*Flavobacteria*) and Chapter 201 (*Haliscomenobacter*).

The Cytophagales as outlined above are all unicellular, gliding, Gram-negative bacteria. All

Table 1. Survey of the taxonomy of the order Cytophagales of the *Bacteroides-Flavobacterium-Cytophaga* branch.

Order: Cytophagales
Family: Cytophagaceae
Genera: *Cytophaga*
- *Sporocytophaga*
- Unnamed *Cytophaga*-like bacteria (several genera)
- *Flexibacter*
- *Microscilla*
- *Flexithrix*
- *Capnocytophaga*
- *Taxeobacter*
- (*Saprospira*)[a]

[a]*Saprospira* may or may not belong to the order. It is discussed separately in Chapter 200.

*In this chapter, the following abbreviations sometimes are used for the genera of the order Cytophagales: CLB, *Cytophaga*-like bacteria; *Cp.*, *Capnocytophaga*; *Cy.*, *Cytophaga*; *Ft.*, *Flexithrix*; *Fv.*, *Flavobacterium*; *Fx.*, *Flexibacter*; *Mc.*, *Microscilla*; *Sa.*, *Saprospira*; *Sp.*, *Sporocytophaga*; *Tx.*, *Taxeobacter*.

have rod-shaped cells, which may differ substantially in shape. They may be short or long, delicate or stout, with tapering or rounded ends (Fig. 1). Two genera exhibit a controlled and cyclic shape change: *Sporocytophaga* produces resting cells in the form of spherical microcysts, and some *Flexibacter* species alternate between very long and extremely agile thread cells and very short, almost spherical and nonmotile rod cells (Fig. 2). Many of the other species are more or less pleomorphic, with cell populations that consist of short and very long rods and chains of cells, particularly in older cultures.

The typical colonies are spreading swarms (Fig. 3). Sometimes they are filmlike and may cover the whole culture plate within a few days. In other cases, they only expand slowly or remain more or less compact. In a few instances there is also rhizoid growth. Many Cytophagales produce brightly colored colonies in shades of yellow, orange, or brick red. The yellow and orange colonies often change immediately into red, purple, or brown if covered with a 10% KOH solution. This color change is due to flexirubin-type pigments (Fig. 4), which have so far only been found in organisms of this group (including flavobacteria). The organisms belonging to the Cytophagales may be aerobic, microaerophilic, capnophilic (CO_2-requiring), or facultatively anaerobic. They are all organotrophs, many of them able to degrade biomacromolecules like proteins, chitin, pectin, agar, starch, or cellulose. They are ubiquitous, abundant, and probably play a major role in the turnover of matter in nature (e.g., Ruschke, 1968; Ruschke and Rath, 1966). Some may also be of practical interest.

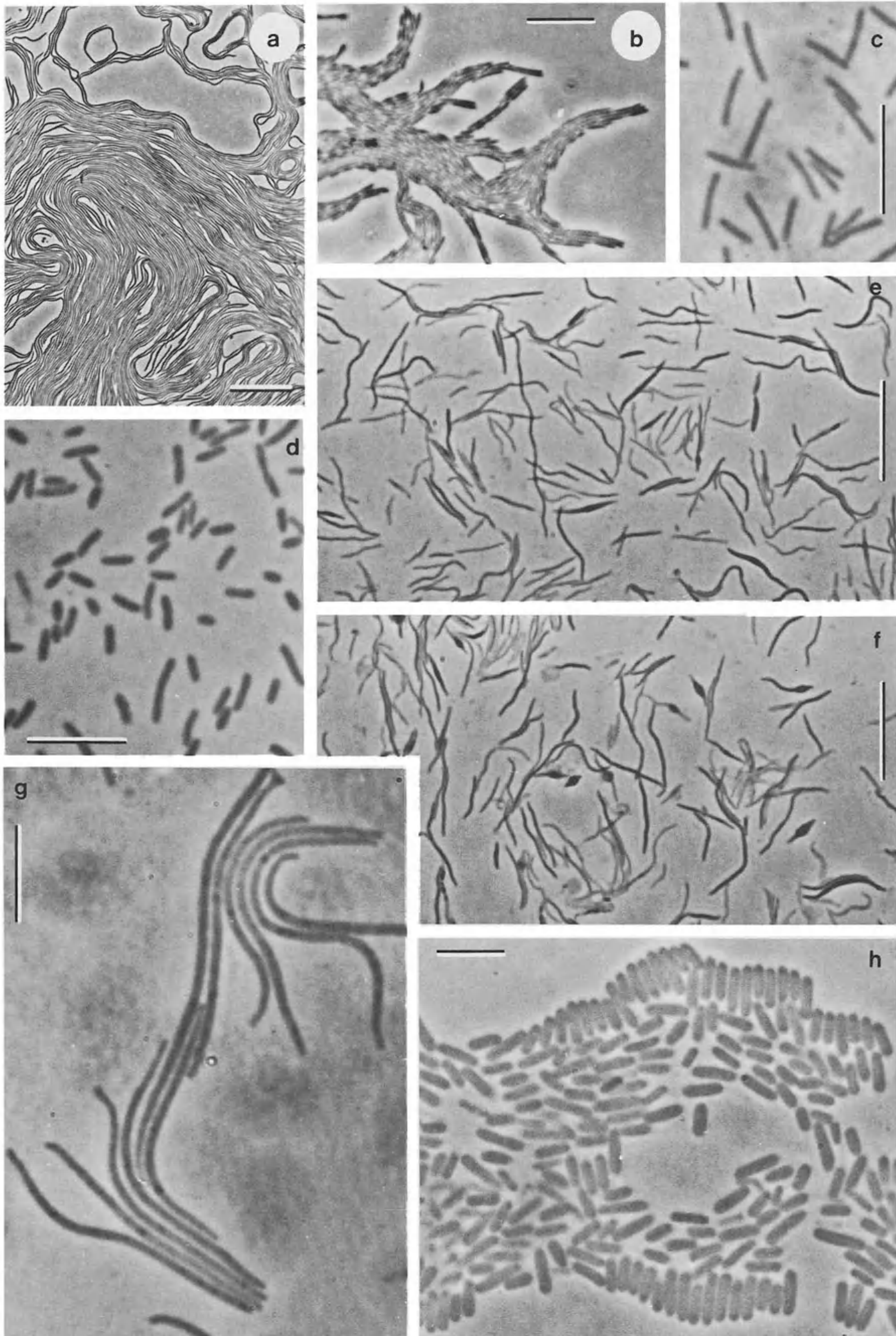
A few reviews on the Cytophagales and their subgroups have been published since the first edition of *The Prokaryotes* and can be recommended here for further information (several chapters in *Bergey's Manual of Systematic Bacteriology*, vol. 3, 1989, for detailed discussions of the taxonomic problems; also, Reichenbach and Weeks, 1981; Shewan and McMeekin, 1983; and on practical aspects: Reichenbach, 1988; see also: Bernardet, 1989; Jooste, 1985; Kath, 1990).

Habitats

The Cytophagales are by far the most common of all gliding bacteria. They are found in enormous numbers in certain habitats, mainly those rich in organic material. They can adapt, however, to rather low nutrient levels (Höfle, 1982, 1983) and probably usually subsist on biomacromolecules, which they are specialized to degrade. The main habitats of the Cytophagales, and thus convenient sources for their isolation, are soils at or close to neutral pH, decaying plant material, and dung of animals, particularly that of herbivores. In freshwater environments, they are found on river banks and lake shores, in estuaries, aerobic bottom sediments, and algal mats. Certain *Cytophaga*-like bacteria (CLB) have also been found in large numbers freely floating in water, especially during the cold season, e.g., in Lake Constance, the Mindelsee, and the Feldsee in southwestern Germany (Gräf, 1962a; Reichardt, 1974; Ruschke and Rath, 1966). The principal cellulose decomposers in southern Canadian lakes appear to be cytophagas (Hoeniger, 1985). CLB are common in sewage plants where, in the aerobic regions, CLB may constitute 5 to 10% of the total cultivatable bacterial population (Güde, 1980). Here, too, a seasonal variation in the number of CLB can be observed, with a maximum in the winter months. Typically, the CLB are particularly enriched in those stages at the end of the purification process, where only recalcitrant organic molecules remain.

In marine environments, the Cytophagales are abundant near the shores: on living and dead seaweeds, in aerobic and anaerobic bottom sediments, and on decaying sea animals, such as crustaceans with their chitin exoskeleton. Marine cellulose degraders have been found on fishing nets in Japan where they once were a serious problem in the deterioration of the fab-

Fig. 1. Cell shape of the Cytophagales in phase contrast. (a) A brick red, marine, agar decomposer, probably a *Microscilla* species, in chamber culture; the long, flexible, thread cells have tapered ends. Bar = 25 μm. (b) CLB from soil, in chamber culture, showing the typical arrangement of cells at the swarm edge. Bar = 10 μm. (c) *Cytophaga lytica,* a yellow, marine, agar-digesting CLB, from SP2 liquid medium. Bar = 5 μm. (d) *Cytophaga succinicans,* a facultatively anaerobic freshwater CLB, from AO agar. Bar = 5 μm. (e and f) *Cytophaga aurantiaca* type strain, a cellulose-degrading true *Cytophaga,* from glucose-glutamate agar. The population in (e) consists of delicate, flexible rods, which are pleomorphic with very long and with slightly swollen cells; in (f), cells from older parts of the colony begin to produce dark, lemon-shaped inflations, which later degenerate to spheroplasts. Bar = 10 μm in both pictures. (g) *Flexibacter flexilis* type strain, from starch agar; one of the thread cells (top edge of the photograph) shows the beginning of branching at one end. Such branching is not uncommon among the Cytophagales. Bar = 5 μm. (h) *Taxeobacter* species, cells in situ on water agar with a streak of living *E. coli,* showing a characteristic palisade pattern. Bar = 5 μm.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ric (Kadota, 1956). Cellulose degraders seem, however, not to be common in marine habitats; at least we have failed so far to isolate any from European coasts. Little is known about the distribution of CLB in the open sea, and a careful investigation of this point would be desirable. As CLB are not likely to form spreading colonies on rich media, the "flavobacteria" that are often observed in ecological studies of various aquatic environments perhaps may frequently really be CLB instead.

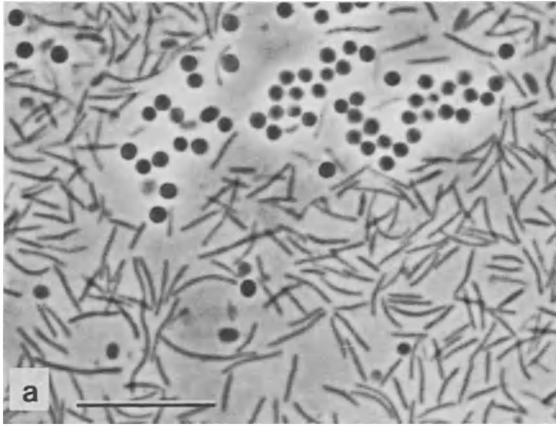
In estuarine habitats in the upper Chesapeake Bay, CLB were found to constitute a substantial proportion of the chitin-degrading bacterial flora (Reichardt et al., 1983), among them many facultatively anaerobic, flexirubin-positive strains resembling *Cy. johnsonae* and *Cy. aquatilis*. These organisms tolerated various levels of salt, but did not depend on elevated salt concentrations.

However, the boundaries between land and sea appear in general to be rather sharp for CLB. This may be concluded from the observation that virtually all strains of CLB and *Flexibacter* isolated by us from terrestrial and freshwater habitats contain, in addition to carotenoids, flexirubin-type pigments, while strains isolated from the sea coast usually produce carotenoids exclusively and only exceptionally produce flexirubin-type pigments (Reichenbach et al., 1981). Since enormous numbers of terrestrial CLB must constantly be washed or blown into the sea, this can only mean that terrestrial strains cannot easily adapt to and become established in the marine environment. An interesting exception may be *Cy. flevensis*. This is an agar-decomposing organism that does not produce flexirubin-type pigments, both characteristics which would speak for a marine origin. It has, however, been isolated from fresh water of the Isselmeer in the Netherlands (van der Meulen et al., 1974). Since the Isselmeer is a part of the North Sea that has been separated from the open sea for decades and has gradually become constituted with fresh water, marine bacteria would have had ample time to adapt to gradually changing conditions and thus may have had a chance to survive. These observations also suggest that marine and terrestrial CLB may be less closely related than was thought before; this is indeed supported by 16S rRNA analyses (Paster et al., 1985).

In recent years, it has been discovered that CLB may also occur in milk and dairy products and perhaps occasionally contribute to deterioration (Jooste, 1985; Jooste et al., 1985). Even more serious are sporadic reports of CLB as human pathogens. CLB in the air humidifier system of a textile plant was found to be responsible for a hypersensitivity pneumonitis of workers; it was later described as a new species, *Cy. allerginae* (Flaherty et al., 1984; Liebert et al., 1984). The *Capnocytophaga* species live in the tooth pockets of humans (Leadbetter et al., 1979). Although a role for them in periodontic disorders has been discussed, it seems that the contribution of capnocytophagas to that disease is only a minor one at the most. But life-threatening septicemias and other kinds of general infections have been observed repeatedly, and not only in immunocompromised patients as has originally been thought (e.g., Forlenza et al., 1980; Paerregaard and Gutschik, 1987; Shlaes et al., 1982). Fortunately, the organisms respond well to antibiotics (Forlenza et al., 1981; Gräf and Morhard, 1966).

An economically serious problem are the fish-pathogenic CLB. These organisms are gaining even more importance as fish cultivation is intensified. First discovered in aquarium fishes in the USA (Davis, 1922), infections with CLB became a major concern in the 1940s in the western USA, where they caused severe losses among salmonid fishes in rivers and lakes and in fish hatcheries. The infections were then intensively studied, and it was discovered that more than one species of CLB was involved, causing several different diseases with diverse symptoms. The two most important diseases were columnaris disease, brought about by *Cy. columnaris* (= *Fx. columnaris* = *Chondrococcus columnaris*), and cold water disease by *Cy. psychrophila*. One factor that may have contributed to these outbreaks may have been the warming of the water by electric power plants, mainly along the Columbia River. It appears that, as a rule, unfavorable living conditions for the fish, like confinement, crowding, and sub-

Fig. 2. Cytophagales with a cyclic change in cell shape, shown in phase contrast. (a and b) *Sporocytophaga myxococcoides* from a membrane of regenerated cellulose on ST6 agar; in (a), most cells are still vegetative rods, but young microcysts and intermediary stages of cell conversion can already be seen; in (b), mature, optically refractile microcysts are present. (c to e) *Flexibacter filiformis* (formerly called *Fx. elegans* Fx el) from VY/2 agar; (c) a slide mount from the very edge of the swarm colony shows very long, delicate, flexible, and extremely agile thread cells without, or with only very few, cross walls; (d) at some distance from the edge, the thread cells have become much shorter by fragmentation; (e) finally, the cell population consists mainly of very short rods which are also clearly fatter and darker and are no longer motile. Bars = 10 μm.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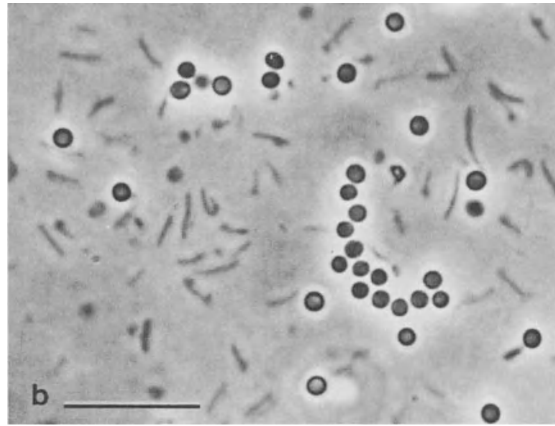
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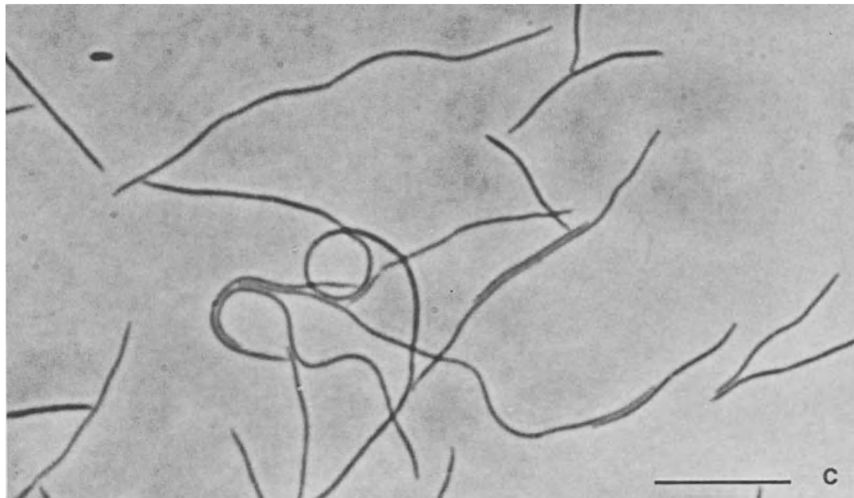
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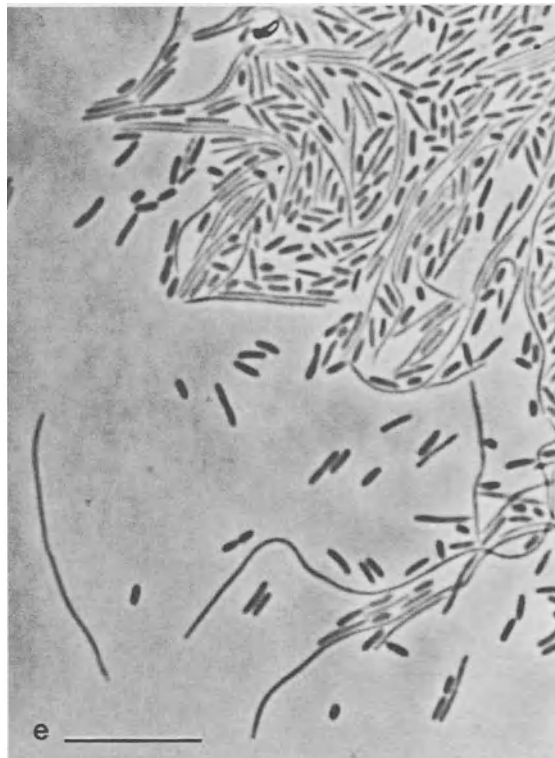
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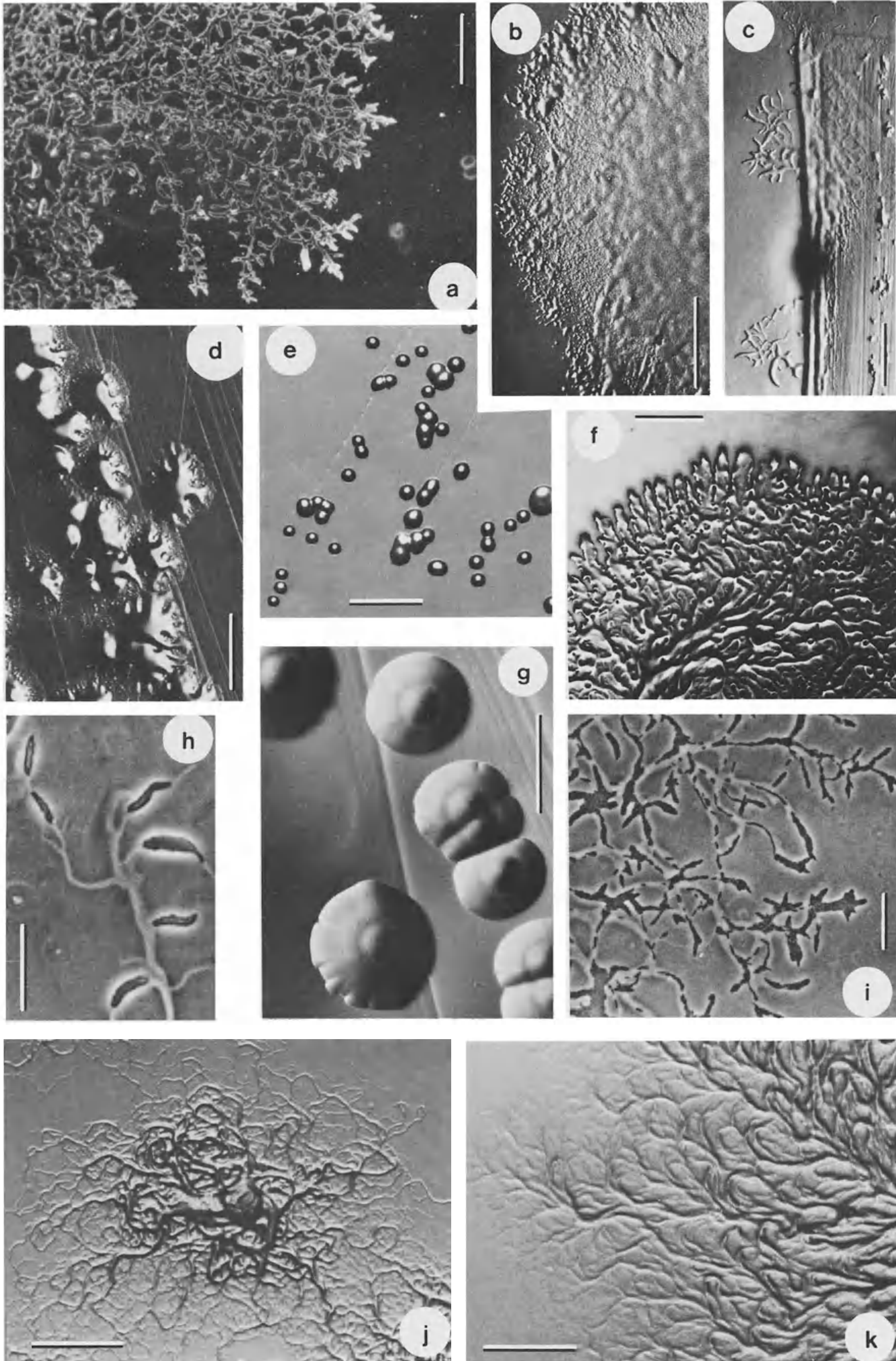
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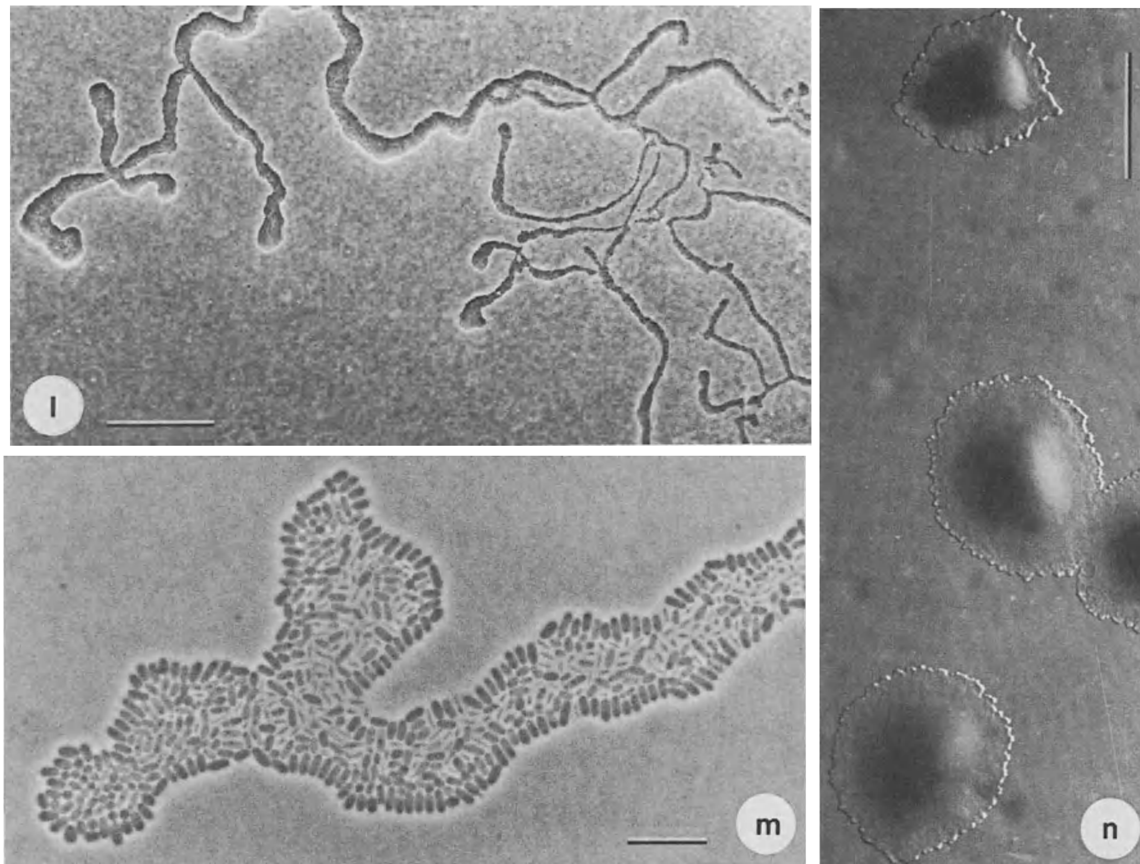

Fig. 3. Colonies of various diverse Cytophagales. (a) Spreading colony of a CLB from soil, growing in chamber culture, dark field. Bar = 100 μm. (b) *Flexibacter filiformis* Fx el, swarm colony on agar plate. Bar = 3 mm. (c) CLB from soil; typical swarm colonies emerge at the side of a streak on an agar surface. Bar = 1 mm. (d and e) CLB from soil growing on two different agar media. (d) On the poorer substrate, the colonies are relatively large and show some spreading; (e) they remain small and compact on the rich medium. Bar = 1 mm in both. (f) CLB from soil, swarm colony on CY agar showing a distinct surface pattern. Bar = 1 mm. (g) *Cytophaga flevensis,* an agar-digesting CLB from freshwater; as a relatively rich agar substrate was used, the colonies remained rather compact. Bar = 2 mm. (h) *Flexibacter filiformis* Fx el, chamber culture; dense clusters of cells are sitting in conspicuous slime tracks; phase contrast. Bar = 30 μm. (i) CLB from soil, chamber culture, edge of a swarm colony with a network of slim tracks; phase contrast. Bar = 30 μm. (j and k) *Cytophaga columnaris,* a fish-pathogenic CLB from freshwater; swarm colonies on MYX agar (j) and AO agar (k), showing the typical growth pattern of the organism. Bar = 1 mm in both. (l to n) *Taxeobacter ocellatus* Tx ol (= Myx 2105): (l and m) on water agar with a streak of living *E. coli,* the organism typically spreads out, producing long, tendril-like strips; (m) at a higher magnification, the unusual palisadelike arrangement of the cells becomes apparent; both phase contrast; (n) after plating on CY agar, relatively compact, brick red colonies begin to spread out. Bar = 100 μm in (l), 10 μm in (m), and 2 mm in (n).

optimal water temperature, favor infections by CLB (e.g., Anacker and Ordal, 1959; Becker and Fujihara, 1978; Bullock, 1972; Colgrove and Wood, 1966; Collins, 1970; Davis, 1949; Fujihara and Hungate, 1972; Fujihara et al., 1971; Garnjobst, 1945; Ordal and Rucker, 1944; Pacha, 1968; Pacha and Ordal, 1970; Pacha and Porter, 1968; Rucker et al., 1953; Snieszko and Bullock, 1976). Fish diseases caused by CLB are now known worldwide, and they often are a serious threat to fish culturists (e.g., Ajmal and Hobbs, 1967; Bootsma and Clerx, 1976; Deufel, 1974; Ghittion, 1972; Lehmann, 1978; Song et al., 1988; Wakabayashi and Egusa, 1974; Wakabayashi et al., 1970a, 1970b). In Europe, CLB diseases have been observed only since about 1965, but it is not clear whether the pathogens were there before but had previously been overlooked, or whether infections have finally become established on a larger scale because environmental conditions have deteriorated. It appears that in the past few years *Cy. psychrophila* has begun to spread through Europe; again, the reasons are not known (Bernardet, 1989; Bernardet and Kerouault, 1989). Very recently, flavobacteria have also been found to be

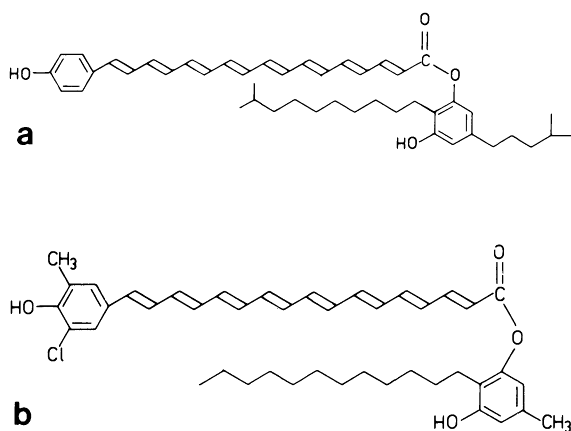

Fig. 4. Flexirubin-type pigments characteristic for members of the Cytophagales. (a) *Cytophaga*-flexirubin from CLB. (b) Chloroflexirubin from a *Flexibacter* species.

involved in fish diseases (*Fv. branchiophila*), and it will be interesting to see if they are related to fish pathogenic CLB. The data on DNA-DNA hybridization with *Cy. columnaris* were insignificant (Wakabayashi et al., 1989). Further, CLB diseases have been encountered in marine environments (e.g., Anderson and Conroy, 1969; Bullock, 1972; Campbell and Buswell, 1982; Hikida et al., 1979), apparently due to infections by *Fx. maritimus* (Bernardet et al., 1990; Wakabayashi et al., 1986). CLB and flavobacteria obviously belong to the bacterial flora that colonize the eggs of cod and halibut, but in this case detrimental effects have not been demonstrated (Hansen and Olafsen, 1989). However, CLB may have a role in disorders of other marine animals; they have been linked to the destruction of the hinge ligaments of cultivated oysters (Dungan et al., 1989). Based on what is known so far, it may be concluded that the infection mechanism of the CLB pathogenic for fish is connected to their very strong protease activity, and in some, also DNase activity.

Isolation

There are no selective methods that are universally applicable for the enrichment and isolation of the diverse members of the Cytophagales. The general strategy for their isolation is to provide conditions that promote spreading growth on surfaces, and then to pick organisms that produce spreading colonies or swarms (Fig. 3) for subcultivation. The nutrient level is usually kept low in the medium used for crude and enrichment cultures in order to stimulate gliding motility and delay development of contaminants. By combining this approach with the use of special substrates in the isolation medium, nutritional specialists among the Cytophagales may be enriched more specifically, e.g., cellulose and chitin degraders. The isolation procedure thus always requires the use of plate cultures. Liquid media have only been used successfully for baiting cellulose decomposers (see below).

With some experience, it will often be possible to recognize members of the Cytophagales microscopically, because many have a characteristic cell morphology (Fig. 1 and 2). If that is not the case, the appearance of the colonies may provide a clue. The property of swarming presents some difficulties since some organisms cannot be or, only with difficulty, can be induced to form spreading colonies, even when gliding motility can be recognized unequivocally by microscopic examination of wet mounts or by the occurrence of slime trails in chamber cultures. This is why quite a few CLB were originally classified as *Flavobacterium* species. On the other hand, spreading growth is, of course, shared by other gliding bacteria. But there are only two other groups of organotrophic, unicellular gliders: the myxobacteria, which usually show a totally different swarm morphology and typically produce fruiting bodies in crude cultures (see Chapter 188); and the lysobacters, which have a peculiar cell morphology, which is, however, shared by some CLB (see Chapter 176). In many cases, pigmentation may be a useful criterion. As already mentioned, many, although not all, members of the Cytophagales are bright yellow, orange, or red. These pigments are always cell-bound (in contrast to some or all of the *Lysobacter* pigments, which, in addition, are usually much paler). If growth is heavy, pigmentation can already be easily recognized in the colonies; alternatively it may be observed after the cells have been concentrated by scraping them together. A large group of soil and freshwater strains of *Cytophaga*, *Sporocytophaga*, CLB, and *Flexibacter* produce flexirubin-type pigments that, upon addition of alkali, turn from orange to purple (Achenbach et al., 1978; Reichenbach et al., 1974) This test can easily be applied to culture plates. In this case, the yellow or orange colonies will turn a much deeper color, either red, purple-violet, or brown, when spotted with 10% KOH solution. The color change differs from that of the pure pigments because of the presence of other pigments, usually carotenoids, which alter the flexirubin absorption spectrum. The color change reaction is reversible upon addition of HCl. If a gliding bacterium gives a positive result with this color test, one can be certain that it belongs to one of the above-mentioned groups of organisms.

The only nongliding bacteria known to contain flexirubin-type pigments are the phylogenetically related, low GC (true) flavobacteria (Reichenbach et al., 1980; Weeks, 1981). It should be understood, however, that the described color change is a phenolate reaction that can also be observed with other pigment types. In fact, a similar color reaction has been found in *Brevibacterium linens*, which contains the phenolic carotenoid 3,3′-dihydroxy-isorenieratene (Kohl et al., 1983). Nonmotile, Gram-positive *Brevibacterium*, of course, cannot easily be confused with the organisms discussed here.

Sources for the isolation of Cytophagales will be found in the section on "Habitats." If a broad representation of those organisms is desired, the samples should be processed soon after collection, especially if the samples are from aquatic or humid environments, because in such samples, oxygen depletion and desiccation in particular can quickly lead to the elimination of sensitive species. Thus, in my experience, the cellulose-degrading *Cytophaga* species are easily lost in this way. On the other hand, many species of Cytophagales are completely desiccation resistant; I have been able to isolate strains of *Sporocytophaga*, *Flexibacter*, CLB, and *Taxeobacter* from samples that had been air-dried and stored for 5 to 10 years at room temperature.

Methods for the Isolation of Cytophagales

Small samples of promising natural material are inoculated either as individual spots or as one to three well-separated streaks onto the surface of dry water agar (WAT agar) plates. For marine samples, seawater agar (MS1 or SW2 agar) is used. Alternatively, media with a very low nutrient content may be used (CYT, MS4, SP2, SP6 agar). Finely divided samples such as soil may also be sprinkled over the plate, as a way of multi-spot inoculation. This can be advantageous because the organisms tend to stick to tiny particles and thus may be distributed in the sample very unevenly. To reduce the growth of fungi, cycloheximide (2.5 mg/100 ml) should be added to the isolation medium (not necessary with marine samples).

WAT Agar and WCX Agar

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved. After autoclaving, 25 μg cycloheximide may be added per ml from a filter-sterilized stock solution for WCX agar.

MS1 Agar

Agar	1.5%

In natural seawater.

The pH is not adjusted. The medium is autoclaved.

SW2 Agar

Na acetate	0.002%
NH_4Cl	0.1%
Agar	1.5%

In artificial seawater (see below).

The pH is adjusted to 7.2. The medium is autoclaved.

Artificial Seawater (Dawson et al., 1969)

NaCl	24.7g
KCl	0.7g
$MgSO_4 \cdot 7H_2O$	6.3g
$MgCl_2 \cdot 6H_2O$	4.6g
$CaCl_2$ anhydrous	1.0g
$NaHCO_3$	0.2g

Dissolve in 1 liter water. To prevent precipitation, $CaCl_2$ and $NaHCO_3$ are autoclaved as separate stock solutions.

CYT Agar

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$CaCl_2 \cdot 2H_2O$	0.05%
$MgCl_2 \cdot 7H_2O$	0.05%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

MS4 Agar

$(NH_4)_2SO_4$	0.1% (autoclaved separately)
Glucose	0.2% (autoclaved separately)
Agar	1.5%

In natural seawater.

The pH is not adjusted. The medium is autoclaved.

SP2 Agar

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.02%
Na acetate	0.002%
Agar	1.5%

In artificial seawater.

The pH is adjusted to 7.2. The medium is autoclaved.

SP6 Agar

Casitone (Difco)	0.03%
Yeast extract (Difco)	0.01%
Agar	1.5%

In artificial seawater.

The pH is adjusted to 7.2. The medium is autoclaved.

The plates are incubated at 30°C, at room temperature, or at 12°C, depending on the origin of the samples. Cultures started from aquatic sources are usually incubated at the lower temperature range because many aquatic CLB will not grow above 25 to 27°C.

The cultures are checked at regular intervals of 1 to 2 days for spreading colonies. It takes between 24 h and several weeks for spreading organisms to appear, depending on the organ-

ism, the incubation temperature, and the nutrient concentration in the culture, which may have been substantially augmented by the introduced sample material. As the spreading swarms are often filmlike and extremely delicate, the plates must be examined with a dissecting microscope equipped with a tiltable mirror so that side illumination can be used from below. Some experience is required before gliding organisms can be reliably distinguished from flagellated bacteria, which occasionally also spread out (Fig. 3b and 3d). A good distinguishing characteristic is usually the pattern of the swarm edge which, for gliding bacteria, is usually dentate with fine flamelike projections, while for flagellated organisms, it tends to be entire or coarsely lobed. However, certain *Bacillus* strains that form long filaments may also spread out with flamelike tongues at the edge of their colonies. Amebae or, in marine samples, gliding diatoms like *Nitzschia putida* may mislead the investigator. Usually a look through the microscope will quickly dispel all doubts; thus, e.g., no gliding bacterium is known that is able to swim actively (that is move without being in contact with a surface).

The baiting technique used for the isolation of myxobacteria (see Chapter 188) also often yields members of the Cytophagales. Living cells of *Escherichia coli* are streaked cross-wise or in three parallel lines onto water agar and inoculated with a suitable sample. The organisms develop along the *E. coli* streaks, often without lysing them, and spread as an extremely thin film, usually a monolayer, far out over the agar surface between the streaks. The technique is in essence nothing else than an enrichment in a low nutrient medium.

If the Cytophagales are present in sufficiently high numbers, the samples can be suspended in sterile water, diluted, and plated. Media with low nutrient concentrations, like CYT and SP6 agar, are well suited. Media with a somewhat higher nutrient concentration, like CY, SP2, and SAP2 agar may also be used. They result in higher colony counts, but spreading of the gliding bacteria may be reduced or suppressed completely (Fig. 3i and 3j). Here, the color of the colonies may provide a useful lead. It should be kept in mind, however, that dilution plating is often not very successful, because the organisms tend to stick to particles and may be lost during the first few dilution steps.

CY Agar, CYG2 Agar, and CYCX Agar

Casitone (Difco)	0.3%
Yeast extract (Difco)	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved. The medium may be supplemented with 0.3% glucose, which is separately autoclaved, for CYG2 agar. If the medium is used for plating of dilution steps, 25 μg cycloheximide may be added per ml after autoclaving from a filter-sterilized stock solution, for CYCX agar.

SAP2 Agar

Tryptone (Difco)	0.1%
Yeast extract (Difco)	0.1%
Agar	1.5%

In artificial seawater.

The pH is adjusted to 7.2. The medium is autoclaved.

Water samples may be plated directly or upon dilution. Often, however, it will be necessary to concentrate the bacteria by passing the sample through a membrane filter. The filter is then incubated right-side up on a suitable culture plate, either as a whole or after having been cut into several sectors; the latter approach increases the chance of isolating several different organisms. If media with somewhat higher nutrients are used, like CY or SAP2 agar, the incubation time is shortened and, thus, the danger of overgrowth reduced. But spreading of the organisms may become more or less suppressed, so that members of the Cytophagales can only be recognized by their color and cell shape. The strategy that is best applied depends on the load of contaminants expected. Some of the smallest gliding bacteria can move through the filter pores (at 0.3- to 0.5-μm pore size) and form colonies on the agar surface below. This can be used as a selective technique. In this case, the filter should be removed from the agar surface after about one day to prevent contaminants from also moving through the filter.

Isolation of Nutritionally Specialized Organisms

AGAR DECOMPOSERS. Agar decomposers are very common in marine environments and can be obtained by the methods outlined above. They are easily recognized, because their colonies rest in shallow craters or in deep pits, and are surrounded by large, uncolored, "gelase" zones if the plate is flooded with iodine solution (Stanier, 1941; Veldkamp, 1961). Some organisms liquify the agar very quickly so that they can only be isolated if widely separated colonies are obtained after plating. Here, the filter migration technique described above is sometimes useful. A few agar-decomposing CLB have also been found in freshwater habitats near the sea coast, viz. *Cy. flevensis* (van der Meulen et al., 1974) and *Cy. saccharophila* (Agbo and Moss, 1979).

Chitin Decomposers. Chitinolytic bacteria are very common among the Cytophagales. Samples are streaked out on chitin agar, e.g., CT6 or CT7 agar (for marine strains, on CT8 or CT9 agar). The chitin is only added in a thin top layer in order to conserve chitin, which is relatively laborious to prepare, and to prevent the chitin from settling on the bottom of the dish out of reach of the organisms; in addition, superior results are obtained in this way because the cleared zones in the opaque chitin layer appear sooner and become more pronounced (Stanier, 1947; Veldkamp, 1955, 1965). Good sources for chitin decomposers are soil, material from sewage plants, and the shells of dead crustaceans. All known chitin-degrading Cytophagales can also grow very well without chitin, e.g., on CY agar, and they can be isolated by the general techniques described above. Thus, they can be purified by plating on, e.g., CY or SAP2 agar.

CT6 Agar

Top layer:

$MgSO_4 \cdot 7H_2O$	0.1%
K_2HPO_4	0.02%
Agar	1.5%

The pH is adjusted to 7.5. After autoclaving, enough chitin is added to give an easily recognizable turbidity, which should be achieved with about 0.5% chitin. The chitin is taken from an autoclaved stock suspension of reprecipitated material; the suspension must be dense enough so that the volume to be added to the medium does not exceed 30% The preparation of the chitin is described in Chapter 176. The medium is poured as a thin layer on top of the following:

Base agar:

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved.

CT7 Agar

Top layer as for CT6 agar. The base agar is WAT agar. On this medium only organisms that can use chitin as the only carbon and nitrogen source are able to grow.

CT8 Agar

Top layer:

K_2HPO_4 (autoclaved separately)	0.02%
Agar	1.5%

In artificial seawater.

The pH is adjusted to 7.5. After autoclaving, chitin is added, as for CT6 agar. The medium is poured on SW2 agar plates.

CT9 Agar

Top layer as for CT8 agar; base agar is SP2 agar.

Cellulose Decomposers. Decaying plant material or soil is sprinkled on filter paper that has been placed on mineral salts agar, e.g., ST6 agar (for marine organisms, SW5 agar may be used).

ST6 Agar and ST6CX Agar

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$MnSO_4 \cdot 7H_2O$	0.01%
$FeCl_3 \cdot 6H_2O$	0.02%
Yeast extract (Difco)	0.002%
Agar	1%
K_2HPO_4	0.1% (autoclaved separately)

After autoclaving, a filter-sterilized trace element solution is added, and, for crude cultures, 25 μg cycloheximide per ml from a filter-sterilized stock solution, for ST6CX agar. A recipe for a trace element solution is found in Chapter 188.

SW5 Agar

$(NH_4)_2SO_4$	0.1%
Yeast extract (Difco)	0.02%
Agar	1%
K_2HPO_4 (autoclaved separately)	0.1%

In artificial seawater.

The medium is autoclaved.

It appears that the type of filter paper used does not make much difference; we get good results with thin, moderately dense, high-quality filter paper (68 g/m², ash 0.1 to 0.2%, more than 95% α-cellulose). Growth of cytophagas on mineral salts agar with cellulose is usually considerably stimulated by Ca^{2+}, Mn^{2+}, and $Fe^{2+/3+}$ (Bortels, 1956). While media with NO_3^- as the nitrogen source are often recommended in the literature, in my experience, media with NH_4^+ are far superior for the isolation of cytophagas and sporocytophagas, at least on agar media.

As an alternative, enrichment cultures may be started with strips of filter paper immersed in a mineral salts solution, e.g., Dubos medium or ST5 medium, in test tubes, so that part of the filter paper remains above the surface of the liquid. The tubes are then inoculated. After incubation for 1 to 3 weeks, glassy, translucent yellow to orange or red spots on the paper at the air-liquid interface indicate growth of cellulose-decomposing cytophagas and sporocytophagas. The yellow and orange spots usually give the flexirubin reaction when a drop of 10% KOH solution is added, but some (terrestrial) *Cytophaga* strains are flexirubin-negative. It appears that, in general, enrichment on plates gives better results because it allows the use of a heavier inoculum. However, an enrichment in liquid medium may be useful when habitats are investigated that are poor in cellulose degraders,

such as marine environments. For the latter, good results have been reported with KM7 mineral medium and incubation at 25°C (Kadota, 1956). As already mentioned, fresh samples should be used for isolating *Cytophaga* strains.

Dubos Mineral Medium (Dubos, 1928)

$NaNO_3$	0.05%
K_2HPO_4 (autoclaved separately)	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
KCl	0.05%
$FeSO_4 \cdot 7H_2O$	0.001%

The pH is adjusted to 7.2. The medium is autoclaved.

ST5 Mineral Medium (Stanier, 1942)

$(NH_4)_2SO_4$	0.1%
K_2HPO_4 (autoclaved separately)	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
$CaCl_2 \cdot 7H_2O$	0.01%
$FeCl_3$	0.002%

The pH is adjusted to 7.0 to 7.5. The medium is autoclaved. For enrichment cultures, 25 μg cycloheximide is added per ml from a filter-sterilized stock solution.

KM7 Mineral Medium (Kadota, 1956)

$NaNO_3$	0.05%
K_2HPO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
$FeSO_4 \cdot 7H_2O$	0.01%

In natural seawater.

The pH is adjusted to 7.2. The medium is autoclaved. This medium was originally developed for a different purpose, which explains the inclusion of relatively small amounts of $MgSO_4$ in a seawater medium.

The purification of cellulose decomposers may be a tedious procedure that requires skill and considerable patience, and it almost always takes several weeks or months. The first subcultures are best started on filter paper placed on mineral salts agar. Now, however, small (about 10 × 15 mm) rectangular filter strips are usually used and inoculated independently from different sites of the crude culture. The filter paper pieces, three or four per plate, are kept well separated to prevent the passage of contaminants from one to the other. Of course, organisms such as flagellated bacteria, amebae, ciliates, and nematodes will spread quickly within the water-soaked filter pad. Before further purification is attempted, they should first be eliminated. To get rid of amebae, which are almost always abundant in cultures started from soil, the plate is exposed to the vapors of a 5% ammonia solution for 2 min (M. Aschner, personal communication). After another one to two minutes of incubation, the cytophagas are transferred to a fresh plate. If there are very many amebae, the procedure may have to be repeated after the cellulose decomposers have grown up. If nematodes are still there, the plate is frozen at −80°C for 2 days. The cellulose decomposers are transferred to a fresh plate as soon as possible after thawing. Next, contaminating bacteria have to be removed, which is the most difficult step. After the load of contaminants has been reduced by repeated transfers to filter paper on mineral salts agar, the cellulose decomposer may be transferred to cellulose overlay agar; we have often had good results with CEL1 agar.

CEL1 Agar

Top layer:

$(NH_4)_2SO_4$	0.1%
Cellulose powder	0.5%
Agar	1.0%

Cellulose powder MN300 from Macherey and Nagel (Düren, Germany) works well.

The pH is adjusted to 7.2. The medium is autoclaved and then poured as a thin layer on top of ST6 agar plates.

The cellulose decomposers penetrate the agar medium and spread within the cellulose layer, concommitantly producing a clear lysis zone. Most or all of the contaminants stay close to the inoculation site, so that after 1 to 3 weeks, a pure, or at least improved, inoculum can be obtained from the edge of the lysis zone.

Favorable results are sometimes obtained by inoculating a small rectangle of filter paper that has been placed on top of cellulose overlay agar, preferably close to one side of the plate. In this case, the cellulose decomposers grow profusely on the filter paper, and from there they spread within the cellulose agar, while the contaminants tend to remain in the macerating filter paper where they find higher nutrient concentrations.

We also find carboxymethyl cellulose media, like ST10 agar, useful for purification of cellulose decomposers. On this medium, too, they spread within the substrate; this can be recognized from a slowly increasing, shallow depression in the surface of the plate. Growth is usually very thin and can barely be seen, if at all; but bacterial cells are found under the microscope if slide mounts are made from the depressed area. Transfers are made from the edge of the depression zone after some time.

ST10 Agar

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$FeCl_3$	0.02%
K_2HPO_4 (autoclaved separately)	0.1%
Casitone (Difco)	0.2%

Carboxymethyl cellulose	1.5%
Agar	0.6%

Carboxymethyl cellulose Sigma no. C-5013, sodium salt, high viscosity, works well.

After autoclaving, standard trace elements (see Chapter 188) and, if used for contaminated strains, 25 μg cycloheximide per ml are added from filter-sterilized stock solutions.

Another technique for purification is to transfer the cellulose decomposers to a membrane filter (cellulose nitrate, 0.3 to 0.4 μm pore size) on cellulose overlay agar or, better, on a membrane of regenerated cellulose (e.g., no. 11604/50/N, pore size 0.8 μm, from Sartorius, Göttingen, Germany) on ST6 agar. The cytophagas will migrate through the membrane filter and produce colonies on the substrate below. If regenerated cellulose is used, these colonies appear particularly early and show a sharp margin. It may, however, still take from 1 to 3 weeks before colonies develop. They are often bright yellow and spread very slowly. With this technique, the time of removal of the top membrane is crucial, because other bacteria may soon grow through the membrane and contaminate the cellulose degraders again. Therefore, it is best to start several parallel cultures and to remove the top membrane at various different times, e.g., after incubation at 30°C overnight, for 1 day, and for 2 days.

With sporocytophagas, final purification is often possible by cautious heating of microcysts suspended in water. The heat resistance seems to vary substantially from strain to strain, and probably also depends on the maturation stage. For example, the thermal death point was reached after 10 min at 68°C with one strain of *Sp. myxococcoides*, while another strain survived 10 min at 70°C; a strain of *Sp. ellipsospora* was killed after 10 min at 58°C (Imshenetsky and Solntseva, 1936; Sijpesteijn and Fåhraeus, 1949). We found that occasionally a strain may even survive boiling for 10 min. Results are usually satisfactory in our laboratory if we incubate microcysts at 58°C and take samples after 10, 20, and 40 min. The heated samples may be streaked on CEL1 agar, on filter paper on CY agar, or on CA2, CA13, or HP18 agar.

CA2 Agar (Mullings and Parish, 1984)

Base agar:

Agar	1.5%

Stock solution A:

KNO_3	7.5 g
K_2HPO_4	7.5 g

Dissolved to give 100 ml.

Stock solution B:

$MgSO_4 \cdot 7H_2O$	1.5 g

Dissolved to give 100 ml.

Stock solution C:

$CaCl_2 \cdot 2H_2O$	0.27 g
$FeCl_3$	0.15 g

Dissolved to give 100 ml.

After autoclaving, the base agar is supplemented with: (a) 1% (v/v) of each of the three stock solutions: (b) 1% (w/v) of glucose (from an autoclaved 20% stock solution); and (c) standard trace elements (see, e.g., Chapter 188).

CA13 Agar

As for CA2 agar, but the KNO_3 in stock solution A is replaced by 0.5 g of $(NH_4)_2SO_4$.

HP18 Agar

Na glutamate	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. After autoclaving, 0.1% glucose (from an autoclaved stock solution) and standard trace elements are added.

Of course, plating of diluted cell suspensions would seem to be the method of choice for the purification of cellulose-degrading cytophagas. However, this can only be done if the organisms are already enriched to near purity since colonies composed of single cells are apparently not produced by these organisms, not even on glucose-containing media, such as those just mentioned, that allow massive growth of pure cultures. Whenever isolated colonies are obtained upon plating, these colonies probably always start from cell clusters, and these are normally contaminated when heavily contaminated cultures are used to prepare the suspension. Further, the cellulose degraders produce a lot of slime which makes it difficult to suspend the cells uniformly in a liquid. After plating on cellulose-overlay agar, colonies which are surrounded by a lysis zone appear only after weeks of incubation, and this gives contaminants ample time to develop and overgrow the cellulose degraders. Gliding and spreading bacteria become a particular nuisance under such conditions. It may be mentioned here that, when glucose is used in a medium, care has to be taken to sterilize the glucose separately, either by filtration or by autoclaving since cellulose-degrading cytophagas are extremely sensitive to toxic products arising when glucose is autoclaved together with other medium compounds (Stanier, 1942). Cellulose-degrading sporocytophagas and cytophagas have been known for many years (Hutchinson and Clayton, 1919; Winogradsky, 1929), but relatively few pure strains are available. They are very common in

soil and decaying plant material (for their occurrence, isolation, and cultivation, see Fåhraeus, 1947; Imschenezki, 1959; Imshenetski and Solntseva, 1936; Kadota, 1956; Krzemieniewska, 1930, 1933; Stanier, 1942; Stapp and Bortels, 1934; Veldkamp, 1965).

PECTIN DECOMPOSERS. Pectin-decomposing CLB can be obtained by plating samples, e.g., lake water, on pectin overlay agar like PEK1 agar or on PEK7 agar. Pectolytic organisms cause shallow depressions in the pectin gel or liquefaction; on PEK7 agar, the colonies are surrounded by clearing zones in a background of finely divided, dispersed pectin flakes or granules, which may be clearly seen, however, only under a dissecting microscope. If such plates are flooded with iodine solution, colonies of pectolytic organisms are surrounded by light yellow halos in a brown plate. The preparation of pectin media is somewhat tricky, and the results depend decisively on the type of pectin used. We found pectin from apple, 38% esterified (from Roth, Karlsruhe, Germany), useful.

PEK1 Agar (Güde, 1973)

Base agar:

Part A:

NH_4Cl	1 g
$CaCl_2 \cdot 2H_2O$	3 g
In 100 ml water.	

Part B:

K_2HPO_4	0.5 g
In 50 ml water.	

Part C:

$MgSO_4 \cdot 7H_2O$	0.2 g
Agar	15 g
Tris HCl buffer, 1 M, pH 8.0	100 ml
In 750 ml of water.	

After autoclaving the solutions are combined, standard trace elements (see Chapter 188) are added, and plates are poured.

Top layer:
12 g pectin is slowly dissolved in 300 ml water of 70°C, the pH is adjusted to 7.2, and the medium is autoclaved for exactly 10 min at 110°C. The solution is poured as a thin layer on base agar plates and will form a gel within 5 h. During autoclaving, the medium may become dark, but this does not interfere with its usefulness.

PEK7 Agar

Top agar:

Casitone (Difco)	0.2%
Pectin	0.5%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved and poured on CY agar plates.

Pectolytic CLB are very common in freshwater (Güde, 1973). They have also been isolated from soil samples after enrichment by percolation of a 1% pectin solution through the samples (Dorey, 1959; his *Fv. pectinovorum* is actually a CLB: Christensen, 1977b). The pectolytic CLB do not depend on pectin but may be stimulated by the presence of a carbohydrate like glucose. They can easily be purified by plating a diluted cell suspension on, e.g., CY agar + 0.3% glucose (= CYG2 agar) or on MYX agar.

MYX Agar

Na glutamate	0.5%
Yeast extract (Difco)	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
Glucose (autoclaved separately)	0.2%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

FISH PATHOGENS. CLB pathogenic for fish can usually be isolated and cultivated on relatively simple media. For freshwater strains, AO agar has been used with good results (e.g., Anacker and Ordal, 1955, 1959; Bullock, 1972; Pacha and Ordal, 1967; Snieszko and Bullock, 1976).

AO Agar (Anacker and Ordal, 1955)

Tryptone (Difco)	0.05%
Yeast extract (Difco)	0.05%
Beef extract	0.02%
Na acetate	0.02%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved. The agar concentration may be varied depending on the purpose for which the medium is used; a reduction to 0.9% is recommended for the first isolation from fish (for AOW agar), and to 0.4% for stock cultures (for AOS agar).

Infested material from gills, skin lesions, or from internal organs, such as kidney or spleen, is streaked on agar plates and incubated at 12 to 18°C or at 30°C depending on the origin of the fish. Initially, media containing diluted fish infusion were used for isolation (Ordal and Rucker, 1944), but this seems not to be required, at least not in all cases, because similar results have been obtained with beef extract (Bernardet, 1989). However, sometimes, the fish pathogens grow very slowly at first, and need an adaptation period before they can conveniently be handled (Ordal and Rucker, 194). This is not totally unusual, for it has been observed that a CLB that had been growing for a long time in a chemostat lost its ability to grow on plates, so that the plate count dropped to 20% of the viable cell count (Höfle, 1983). The

medium used for isolation is, of course, not exclusive for pathogenic CLB, or CLB at all, and many kinds of saprophytic bacteria will grow on it as well. This may become a problem especially if the fish has already been dead for some time, because it is quickly colonized by putrefying organisms. A study of the drug resistance of a collection of *Cy. columnaris* strains revealed that the organism is resistant to a number of antibiotics (Fijan and Voorhees, 1969), and by the addition of neomycin (5 μg/ml) + polymyxin B (10 units/ml) to AO agar, the isolation yields could be improved (Fijan, 1969). The pathogenic CLB can be recognized on the plate by their yellow to orange colonies, which give a positive flexirubin reaction, and, in the case of *Cy. columnaris*, by a very typical, rhizoid-colony morphology. Spreading of the colonies is not always well pronounced, especially with *Cy. psychrophila*; but this organism can easily be recognized, because it does not grow at 30°C and, in contrast to environmental CLB, does not utilize sugars.

For isolating CLB pathogenic for marine fish AOW agar prepared with artificial seawater (= MAOW agar) or, for estuarine sources, with one-third-strength artificial seawater (= BAOW agar) are useful. Also, a richer medium, Marine Agar (Difco), which is a peptone (0.5%)-yeast extract (0.1%) agar (1.5%) in artificial seawater, has been used with good results (Hikida et al., 1979; Campbell and Buswell, 1982). In another case, the pathogen proved more fastidious and could only be isolated and cultivated on an agar medium containing 5% of an enzymatic digest of fish muscle in addition to peptone (0.1%) and yeast extract (0.1%) in seawater (Anderson and Conroy, 1969). In this case it was also shown that the organism depended on a high salt concentration, and that a NaCl solution could not replace seawater. In fact, the fish—rainbow trout with severe lesions on the jaws and snout ("eroded mouth disease")—could be healed simply by transfer to fresh water. *Cy. columnaris*, on the other hand, is completely inhibited by 1% NaCl (Fijan and Voorhees, 1969; Pacha and Ordal, 1970).

THE GENUS *CAPNOCYTOPHAGA*. The capnocytophagas are normal inhabitants of the human oral cavity where they can be found in tooth pockets and in the dental plaque. They may also occur in large numbers in periodontal disorders and infected lesions in the mouth. The genus was established by Leadbetter et al. (1979), but the organisms are very probably identical with the fast-gliding, anaerobic, slender rods discovered 20 years earlier during a study of fusospirilloses of the human oral cavity, which were described under the name *Sphaerocytophaga* (Gräf, 1961). In fact, they were known even before that time, but as *Bacteroides* species. Although the capnocytophagas have a strictly fermentative metabolism, they are not particularly sensitive to oxygen and grow both in the presence and the absence of oxygen, provided an elevated level of CO_2 is supplied. The capnocytophagas are not fastidious at all and can easily be isolated and cultivated (Forlenza and Newman, 1983; Gräf, 1961; Leadbetter et al., 1979). Originally they were isolated on 10% sheep's blood agar. The samples were streaked onto the agar surface, and the cultures were incubated for 7 to 8 days at 37°C in Fortner plates, i.e., a co-culture with a facultative anaerobe. Spreading colonies were obtained, which showed a reddish hue and consisted of cells that were more or less yellow when scraped together (Gräf, 1961).

Leadbetter et al. (1979) recommended the following procedure: The sample material is quickly transferred to a screw-cap tube containing a modified Ringer's solution (NaCl, 0.9%; KCl, 0.042%; $CaCl_2 \cdot 2H_2$, 0.025%). Plaque material may be homogenized by a short burst of ultrasound (5 to 10 sec). Within 30 min after sampling, streaks are made on trypticase soy broth agar (TSB agar), which may be supplemented with 5% sheep's blood; the blood is, however, not essential. The cultures are incubated at 37°C in an atmosphere of $H_2 + CO_2 + N_2$ (GasPak, Baltimore Biological Laboratories = BBL). After 2 to 3 days, spreading colonies may be observed, but higher yields are obtained after 5 days (100% rather than 45%: Forlenza and Newman, 1983). There are three types of colonies—grey, pink, and yellow—but the cell masses of all three are yellow when scraped together. The shapes of the colonies depend critically on the ingredients used for preparing the medium: With stocks from other suppliers than BBL, nonspreading, small, compact colonies were obtained on the same type of medium (variations of this kind could, however, also be a result of using different batches of a medium component from the same supplier). The strains are purified by repeatedly streaking out material from swarm edges.

TSB Agar (Leadbetter et al., 1979)

Trypticase soy broth (BBL)	3%
Agar	3%

The pH is 7.3 (unadjusted). The medium is autoclaved.

Trypticase soy broth consists of:

Trypticase peptone	1.7%
Phytone peptone	0.3%
NaCl	0.5%
K_2HPO_4	0.25%

Glucose	0.25%

TSB agar may be supplemented with 5% sheep's blood (for TS-blood agar).

The capnocytophagas may also be first enriched by suspending sample material in trypticase soy broth without glucose in screw-cap tubes. After tightening the lids, the tubes are incubated at 37°C for 3 days. After that, large numbers of slender fusiform rods may be found in the liquid. Isolates are obtained by streaking the cell suspension on the media mentioned above.

It may be mentioned here that free-living CLB are also known that require CO_2 for fermentative growth on glucose—for example, the facultative anaerobe *Cy. succinicans* (Anderson and Ordal, 1961a, 1961b).

In addition to capnocytophagas, CLB that are not obligatory fermentative and that do not require CO_2 have also been found in dental plaque (London et al., 1982). The organisms could be isolated on blood agar media similar to those mentioned above, e.g., Columbia blood agar (BBL) or modified Schaedler agar with 5% defibrinated horse blood. The plates were incubated at 37°C in a GasPak atmosphere, but the bacteria can grow equally well in a normal atmosphere. The organisms were recognized by their spreading swarm colonies.

Purification

Purification of most members of the Cytophagales is relatively uncomplicated. It is usually sufficient to suspend, dilute, and plate samples from enrichment cultures by conventional methods. In some instances, e.g., with *Cy. columnaris*, the preparation of homogeneous suspensions may be difficult because the cells are embedded in a tenacious, sticky slime, but use of a small tissue homogenizer normally solves this problem. The plating medium should contain somewhat higher nutrient concentrations than the enrichment medium because high plating efficiency is desirable, and spreading growth is not required and should in fact be repressed. Some suitable media are CY agar, sometimes better with glucose (e.g., CYG2 agar); MYX agar; and, for marine strains, SAP2 agar. Often, pure strains are quickly obtained by repeatedly transferring material from the edges of spreading swarm colonies. These transfers should be made carefully, preferably by cutting out a little block of agar with the swarm edge using the sharp needle of a 1-ml disposable syringe. For this technique, media that favor gliding motility are, of course, preferable. The specific problems encountered with certain groups of the Cytophagales have already been discussed.

Cultivation

Most bacteria of the order Cytophagales can be cultivated on relatively simple media. VY/2 agar and CY agar have proved useful for all terrestrial and freshwater organisms except the cellulose decomposers. Cultures usually remain viable longer on VY/2 agar because growth is lighter and the pH is not changed as much as on CY agar. VY/2 agar is also useful for deciding whether an organism is a glider because it stimulates spreading growth. If heavier growth is desirable, CY agar may be enriched with glucose as, e.g., in CYG2 agar. Growth of some CLB is much stimulated by the presence of a carbohydrate. The cellulose decomposers grow very well on filter paper placed on CY agar. Other media suitable for their cultivation are listed above. If these gliding bacteria are cultivated on mineral salts-glucose media (CA2 and CA13 agar) they often grow only if a heavy inoculum is applied.

VY/2 Agar

Bakers' yeast (by fresh weight of commercial yeast cake)	0.5%
$CaCl_2 \cdot 2H_2O$	0.1%
Cyanocobalamin	0.5 μg/ml
Agar	1.5%

The cyanocobalamin is usually not required.

The pH is adjusted to 7.2. The medium is autoclaved. The yeast may be stored as an autoclaved stock suspension for several weeks. A uniform distribution of the yeast is obtained if the medium is melted before the yeast is added.

Many bacteria of the *Cytophaga* group produce heavy growth on HP6 agar, MYX agar, and similar media. Many will also grow well on mineral salts-glucose media like CA2 and CA13 agar. Special media that allow the detection of the ability of a strain to decompose certain macromolecules have already been mentioned. A medium useful for the demonstration of starch decomposition is STK2 agar.

HP6 Agar

Na glutamate	1%
Yeast extract (Difco)	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
Cyanocobalamin	0.5 μg/ml
Glucose (autoclaved separately)	0.5%
Agar	1.5%

The cyanocobalamin is usually not required.

The pH is adjusted to 7.2. The medium is autoclaved.

STK2 Agar

Base agar:

Casitone (Difco)	0.1%

Yeast extract (Difco)	0.05%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2 The medium is autoclaved.

Top agar:
As base agar, but agar concentration increased to 1.5%. The medium is prepared with only about 85% of the total water volume. In the remaining water are dissolved, referring to the total volume of the top agar, the following:

Soluble starch	0.2%
K_2HPO_4	0.02%

The starch solution should be heated on a water bath before autoclaving in order to prevent clumping of the starch. The two solutions are combined after autoclaving, and the mixture is poured as a thin layer on plates of the base agar. Starch degradation can be discovered if, after a suitable incubation time, the culture plates are flooded with an iodine solution, e.g., Lugol's solution diluted 1:5.

The fish pathogens can be cultivated on AO agar but usually also grow well on CY, MYX, and HP6 agar. Most strains do not utilize carbohydrates at all. The capnocytophagas grow on TSB agar with or without sheep's blood.

The same types of media can be used for the marine Cytophagales, only prepared with natural or artificial seawater instead of distilled water. No case is known in which the latter would not do, but a simple NaCl solution is often not suitable (e.g., Anderson and Conroy, 1969). The tolerated salinity range differs depending on the organism and often depends on the origin of the strain (e.g., Lewin and Lounsbery, 1969; Reichardt et al., 1983). Marine cellulose decomposers may be cultivated on filter paper placed, e.g., on SW5 agar. Alternatively, the mineral salts-glucose media as described for the terrestrial organisms may be used, when prepared with seawater.

The type of peptone added to a medium is not as crucial in the case of the Cytophagales as it is for myxobacteria; nevertheless, very different results may be obtained with different preparations and even with different batches. An example has already been given for the capnocytophagas. In our experience, enzymatic digests of casein, e.g., casitone (Difco) or peptone from casein (Marcor, Merck, and Oxoid) are the most universally useful peptones. Soy peptones also often give excellent results. Addition of yeast extract may stimulate growth appreciably. Again, not all preparations are equally satisfactory; good results are obtained, e.g., with Difco yeast extract. Further, it should be kept in mind that quite a few bacteria of this group grow on inorganic nitrogen sources, and it is always worthwhile to test an isolate for that possibility

As a rule, members of the Cytophagales immediately produce homogeneous cell suspensions when inoculated into liquid media and shaken. Typically, the liquid shows a silky shimmer when rotated. Rarely, the bacteria grow in tiny granules or flakes, and then probably always are surrounded by thick capsules or slime layers. The medium sometimes becomes more or less viscous, especially when sugars are included, and harvesting the cells by centrifugation may then make difficulties. In one such case, the addition of 10% $(NH_4)_2SO_4$ (w/v) resulted in satisfactory separation (Verma and Martin, 1967).

Most Cytophagales grow well in liquid media that contain peptone, e.g., CAS liquid medium or FXA liquid medium, and for marine organisms, SP5 liquid medium. Addition of a sugar, such as in FXAG liquid medium, may stimulate growth considerably and may even be a prerequisite for cultivation. Glucose, galactose, mannose, saccharose, and maltose are often readily utilized. The sugar has two effects: it is a convenient carbon and energy source, and it is partly transformed into acids that delay the rise of pH of the medium that results from ammonia production. Shifts of pH may cause problems in shake cultures. Concentrations of buffers high enough to stabilize the pH reliably are often inhibitory to growth. With *Fx. filiformis* (former *Fx. elegans* Fx el), appreciable inhibition was observed at the following buffer concentrations (at a pH of about 7.0): citrate above 15 mM; phosphate, Tris HCl, and Tris-maleic acid-KOH above 20 mM; 3-morpholino-propane sulfonic acid (= MOPS) above 50 mM (E. Fautz, personal communication).

CAS Liquid Medium

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH is 6.8 and needs no adjustment if Difco casitone is used. The medium is autoclaved.

FXA Liquid Medium

Casitone (Difco)	1%
Yeast extract (Difco)	0.2%
$MgSO_4 \cdot 7H_2O$	0.1%

Other similar, enzymatically digested, casein peptones may also be used, e.g., Peptone from casein, tryptically digested (Merck, Darmstadt, Germany).
The pH is adjusted to 7.0. After autoclaving, 0.2% of separately autoclaved glucose may be added, for FXAG liquid medium.

SP5 Liquid Medium

Casitone (Difco)	0.9%
Yeast extract (Difco)	0.1%

In artificial seawater.

The pH is adjusted to 7.2. The medium is autoclaved.

The fish pathogens can be cultivated in the same media mentioned for their isolation, e.g., AO broth (AO agar without agar) (e.g., Bernardet and Kerouault, 1989; Bullock, 1972; Pacha, 1968). A supplement to AO broth of 5% fish peptone, prepared from brook trout, has been recommended and gave superior results with some isolates, although it was not strictly required; it may sometimes be useful with fresh isolates (Kincheloe, 1962). For marine fish pathogens, AO broth in seawater or TCY liquid medium may be used (e.g., Hikida et al., 1979).

TCY Liquid Medium (Hikida et al., 1979)

Tryptone (Difco)	0.1%
Casamino acids, technical (Difco)	0.1%
Yeast extract (Difco)	0.02%
NaCl	3.13%
KCl	0.07%
$MgCl_2 \cdot 2H_2O$	1.08%
$CaCl_2 \cdot 2H_2O$	0.1%

In distilled water.

The pH is adjusted to 7.0 to 7.2. The medium is autoclaved.

One study showed that growth of *Cy. columnaris* can be substantially improved by a fine-tuning of the medium formulation (Song et al., 1988). The optimal medium was SO broth, which was a modification of a medium used by Shieh (1980), from which glucose, pyruvate, and citrate have been omitted, because these compounds had no effect on growth. The medium was much superior to tryptone-yeast extract and tryptone-yeast infusion broth. The generation time was reduced to 150 min compared to 210 min in the next-best medium.

SO Liquid Medium (Song et al., 1988)

Peptone	0.5%
Yeast extract	0.05%
Na acetate	0.001%
$BaCl_2 \cdot H_2O$	0.001%
K_2HPO_4	0.01%
KH_2PO_4	0.005%
$MgSO_4 \cdot 7H_2O$	0.03%
$NaHCO_3$	0.005%
$CaCl_2 \cdot 2H_2O$	6.7 mg/liter
$FeSO_4 \cdot 7H_2O$	1.0 mg/liter

The capnocytophagas are obligatory fermenters and grow luxuriantly in trypticase soy broth (TSB agar without agar) supplemented with 1% glucose; the cultures are incubated under the exclusion of oxygen (Forlenza et al., 1981).

Many Cytophagales can also be cultivated in simple synthetic media, e.g., SY liquid medium. Stabilization of the pH is especially difficult in this case and is effectively achieved only in bioreactors.

SY Liquid Medium

KH_2PO_4	0.07%
$Na_2HPO_4 \cdot 2H_2O$	0.14%
$(NH_4)_2SO_4$	0.2%
$MgSO_4 \cdot 7H_2O$	0.02%
$FeSO_4$	5 mg/l
$MnSO_4$	5 mg/l

After autoclaving, 1% glucose is added from a separately autoclaved stock solution.

Cellulose decomposers grow well in liquid media with powdered cellulose, e.g., in CEL1 liquid medium or in Dubos liquid medium with 0.5% cellulose. We use, with good success, a peptone-mineral salts medium with cellulose powder (= M9 liquid medium). Also, CAS liquid medium with cellulose powder (0.5%) often allows good growth. Further, many strains of cellulose degraders may be cultivated in glucose-containing liquid media such as GLU liquid medium (Kath, 1990; Verma and Martin, 1967).

CEL1 Liquid Medium

ST5 liquid medium is supplemented with 0.1% powdered cellulose (e.g., MN300 for chromatographic purposes from Macherey and Nagel, Düren, Germany), autoclaved separately as a stock suspension.

M9 Liquid Medium

Base medium:	
NH_4Cl	0.1%
K_2HPO_4	0.075%
Casitone (Difco)	0.3%
Yeast extract (Difco)	0.09%
Supplement A:	
$MgSO_4 \cdot 7H_2O$	4.5%
Supplement B:	
$CaCl_2 \cdot 2H_2O$	0.2%
$FeCl_3 \cdot 6H_2O$	0.15%
$MnSO_4 \cdot H_2O$	0.15%
Supplement C:	
Cellulose powder	10%

After autoclaving all solutions, 1% (v/v) of supplements A and B, 5% (v/v) of supplement C, and filter-sterilized standard trace elements (see, e.g., Chapter 188) are added to the base medium.

GLU Liquid Medium (Kath, 1990)

The percentages indicated for each ingredient are with respect to the final volume of the medium.

Solution A:	
NH_4Cl	0.1%
K_2HPO_4	0.075%
Casamino acids (Difco)	0.3%
$Na\text{-}Fe^{III}\text{-}EDTA$	0.002%

HEPES 1.19%
In 90% of the total volume.
The pH is adjusted to 7.2.

Solution B:
$MgSO_4 \cdot 7H_2O$ 0.045%
$CaCl_2 \cdot 2H_2O$ 0.01%
Glucose 0.5%
In 10% of the total volume.

After autoclaving, solutions A and B are combined, and standard trace elements are added from a filter-sterilized stock solution (see, e.g., Chapter 188).

Most members of the Cytophagales can be grown without difficulties on a large scale in bioreactors. In ordinary batch cultures, typical yields obtained are between 10 and 15 g wet weight, or 2 to 4 g dry weight per liter on peptone media with sugar; the ratio of dry to wet weight is 0.20 to 0.25. Generation times are in the range of one to several hours. Thus, e.g., *Fx. filiformis* Fx el grows in peptone media at 30°C with a doubling time of 2 h and 15 min; in mineral salts-glucose medium (SY liquid medium) it grows with a doubling time of 6 h and 20 min (H. Fink, personal communication). *Tx. ocellatus* grows at 30°C with generation times of 1 h and 50 min in peptone liquid medium, 2 h in dilute peptone medium, and 3 h and 30 min in casamino acids-glucose medium.

Little is known about specific mineral and vitamin requirements by members of the Cytophagales. At least, they appear not to be particularly fastidious in that respect. In a study on trace element effects on cellulose-degrading *Sporocytophaga* growing in a mineral salts-cellulose medium, a stimulation of cellulose decomposition by Ca^{2+}, Fe^{2+}, Mn^{2+}, and sometimes Cu^{2+} was demonstrated (Bortels, 1956). Certain Zn salts (ZnO, ZnS, 0.1%) were found to encourage growth of a series of cytophagas and CLB; $ZnSO_4$ (0.1%) suppressed swarming and slime production on plates, and its use in isolation media was suggested (Mehra et al, 1967). In a comparative study of a large number of different marine Cytophagales, a dependence on thiamine and cyanocobalamin was found in a few instances (Lewin and Lounsbery, 1969).

Except for the capnocytophagas, the Cytophagales are strict aerobes. Those that are known to grow anaerobically are usually facultative anaerobes. Some are able to respire on nitrate. Not one of the tested strains could use fumarate as an electron acceptor (Callies and Mannheim, 1978). Several Cytophagales require carbon dioxide for fermentative metabolism, so that care should be taken that enough CO_2 is present in the atmosphere when strains are being tested for anaerobic growth (GasPak, candle jar, Fortner plates, carbonate in the medium). The only strictly fermentative Cytophagales known so far are the capnocytophagas, which are, however, not oxygen sensitive but do require an increased level of CO_2; if that is provided, they can grow even under aerobic conditions (Leadbetter et al., 1979). They need CO_2 because they depend on the activity of phosphoenolpyruvate carboxykinase for growth (Kapke et al., 1980). There seem also to be *Capnocytophaga* strains that grow at CO_2 levels found in air. The existence of strictly anaerobic Cytophagales is not yet excluded.

All known Cytophagales are restricted to pH values between 6 and 8. Their temperature range is wide, from about 0°C to more than 40°C. On plates, most strains will grow more or less at 30°C. In contrast, liquid cultures of many strains, particularly those isolated from aquatic environments, grow only, or grow much better, at lower temperatures (usually between 18 and 26°C).

Preservation

Agar cultures of many Cytophagales stay viable for months when stored at low temperatures (2 to 8°C). At room temperature or higher, plate cultures can usually be kept for 2 to 6 weeks. In general, organisms from aquatic environments are more sensitive than those from soil. Media that are rich in peptone or contain higher concentrations of a sugar are always less suitable for stock cultures, because they produce heavier growth and lead to unfavorable pH shifts. Good media for stock cultures are often VY/2, CYT, and SAP2 agar. For the fish pathogens, a reduction of the agar concentration in AO medium to 0.4% is advantageous. Cellulose decomposers are kept best on filter paper on top of CY or ST6 agar.

For long-term storage, essentially all standard methods for the preservation of bacteria can be applied to the Cytophagales. In our experience, the following methods give excellent results.

Drying in Skim Milk

From a young plate culture, a heavy cell suspension is prepared in about 0.7 ml of sterile skim milk. Three to five drops of this suspension are placed on a plug of skim milk predried by lyophilization in an ampule. The plug must not become solubilized but only wetted by the amount of added cell suspension. The loaded ampules are stored at 4 to 6°C until the whole set has been finished (not longer than one h), and are then dried in a desiccator over silica gel at a good vacuum for about 4 h at room tem-

perature. The desiccator is kept overnight under vacuum, then pumped again for 15 min, and filled with nitrogen gas (preferably by attaching it to a nitrogen-filled balloon). Then the ampules are sealed and stored at room temperature or at 4 to 6°C (we do not yet know whether there is a difference in survival times). So far, organisms have been revived by us after up to 10 years of storage at room temperature. Successful lyophilization is also reported for *Cy. columnaris* (Anacker and Ordal, 1959). Lyophilized cultures of *Cy. columnaris* and *Cy. psychrophila* which had been kindly supplied to us by Dr. R.E. Pacha (Ellensburg, Washington) could be reactivated without problems after 26 and 20 years, respectively.

Skim Milk Preparation (for Preservation)

40 g of powdered skim milk (e.g., Oxoid L31; must be free of antibiotics) are dissolved in 200 ml water and filtered through gauze. The solution is distributed into tubes (5 ml per tube) or ampules (1 ml per 5-ml ampule), and autoclaved at exactly 115°C for 15 min. May be stored in the cold for several weeks.

Storage at Ultra-Low Temperatures

From a dense liquid culture, e.g., in FXA or CAS liquid medium, 2-ml amounts are pipetted into screw-cap tubes and placed directly into a deep freeze at −70 to −80°C. Samples of marine organisms, e.g., in SP5 liquid medium, should be kept in an inclined position during freezing to prevent cracking of the tubes. Cellulose decomposers have been successfully frozen in CEL1 liquid medium.

Alternatively, a heavy loopful of cells from a plate culture is deposited in 1 ml of a peptone-containing liquid medium, like the ones mentioned above, and frozen in the same way. The peptone medium can also be used for cellulose decomposers. Fish pathogens can be frozen at −70°C as agar cultures in AO agar with 0.9% agar; they were found to remain viable for at least one year (Fijan and Voorhees, 1969). Of course, all cultures for preservation should be young, well-growing cultures in an optimal state.

Alternatively, cell suspensions in peptone liquid media may be stored frozen in liquid nitrogen. A special freezing program is not required. We routinely add 5% dimethyl sulfoxide (DMSO) to the suspension medium, although our experience suggests that this is not necessary. In a systematic study on cryopreservation of Cytophagales, mainly marine ones, good survival in liquid nitrogen was demonstrated when the cells were suspended in a medium containing 10% glycerol, while 10% DMSO proved less suitable. Also without additives, at least one cycle of freezing and thawing was usually well tolerated. Many strains remained viable even for some weeks at −22°C without additives, but eventually, after varying storage times, most strains died (Sanfilippo and Lewin, 1970).

For revival, the frozen tube or ampule is quickly thawed, best by placing it into cold water. Immediately after the medium has become liquid, the bacteria are transferred to a suitable growth medium. For reviving a culture, it is advisable to use several different agar and liquid media in parallel, because one or the other may be more appropriate depending on the organism. Also, we found that facultative anaerobes are occasionally better reactivated under anaerobic conditions; they sometimes refuse to start to grow in an aerobic atmosphere, even when they thrive ordinarily very well under such conditions. Thus far we have tested survival at −80°C for up to 15 years, and in liquid nitrogen for up to 10 years and have not yet observed any losses.

Storage in 50% Glycerol at −25°C

Using a young plate culture, a heavy cell suspension is prepared in 50 mM phosphate buffer, pH 7.2. Of this suspension, 1 ml is transferred to a screw-cap tube that contains 2 ml of 87% glycerol precooled to −25°C. The suspension is thoroughly mixed and stored at −25°C in an ordinary household deep freeze. Marine organisms may be suspended in SP5 liquid medium; 1 ml of the suspension is then transferred to 2 ml of precooled 87% glycerol. Alternatively, a heavy loopful of cells is suspended directly in precooled SP5 liquid medium + 87% glycerol (1:2, vol/vol).

To start an active culture, a loopful of the suspension is streaked on a suitable agar medium. The remaining suspension may be returned to the deep freeze, provided the temperature was kept low during handling. CLB preserved by this method may survive for at least several years (I. Hirsch, personal communication), but we have in some cases observed losses, particularly with marine strains, so this technique is advisable for long-term storage, especially when better alternatives are available.

Storage in 0.9% Saline or in Distilled Water

Starting from young plate cultures, cell suspensions are prepared in 0.9% saline or in distilled water. The cell density should not be very high; we usually prepare a suspension of about 10^7 to 10^8 cells/ml (a suspension just faintly turbid), and this stock suspension is then diluted fivefold. Then, 0.5-ml amounts of both suspensions

are transferred in parallel to 1-ml ampules, which are sealed under air and stored at room temperature (18 to 22°C) in the dark. Marine organisms may be suspended in artificial seawater. In our laboratory, a number of *Flexibacter* and CLB strains stored in saline for 7 years could be reactivated with ease and produced heavy growth within 2 days; it is likely that they would have survived for even longer periods. However, the organisms probably are not completely at rest in such suspensions and continue to grow, even if extremely slowly, so that over a long period variant strains may arise.

Characterization

Cell Morphology and Cytology

The cells of all Cytophagales without exception are rod-shaped (Fig. 1 and 2). The proportions and dimensions of the rods vary, however, over a wide range. The CLB have very short to moderately long rod cells; the short ones are often relatively fat, and the longer ones may be rather slender; they typically measure 0.5 to 1.0 × 2 to 8 μm; the cell ends are usually slightly tapered. The cells of *Capnocytophaga* resemble those of CLB with moderately long rods. *Taxeobacter* has relatively plump cylindrical rods with rounded ends. The rod cells of the cellulose decomposers (*Sporocytophaga, Cytophaga*) tend to be moderately long and very delicate, measuring 0.3 to 0.7 × 3 to 8 μm; *Cytophaga* cells are often spindle-shaped. *Flexibacter* and (*Microscilla*) have long thread cells which contain either very few or no cross-walls, and typically measure 0.4 to 0.8 × 10 to 50 μm. Many species readily form spheroplasts. Especially in cultures of cellulose-decomposing true cytophagas, large numbers of lemon-shaped intermediary forms can often be seen (Fig. 1f). This was a source of much confusion for the early investigators (who had no phase contrast microscopes), and fancy developmental cycles have been constructed on such observations (for a review, see Imschenezki, 1959). Inflated, spindle-shaped rods may still be motile and viable and may recover, but it is doubtful whether such stages are of any advantage for the organism, e.g., for a rearrangement of the genetic apparatus, as has been suggested. Lemon-shaped degeneration forms are also typical for *Taxeobacter*. Under starvation, a *Cy. johnsonae* strain was observed to produce coccoid cells which looked like spheroplasts, did not show a peptidoglycan layer, often had lost most of their cytoplasm, and were osmotically stable (Reichardt and Morita 1982). Nevertheless, they were found to be viable, and at least some of them could grow out again into rods. It was suggested that they represent survival forms. Spheroplastlike cells have also been interpreted as resting stages in several other Cytophagales (e.g., Gräf, 1961, 1962; Bauer, 1962), and generic names have even been proposed based on such observations ("*Sphaerocytophaga*," "*Sphaeromyxa*"). But the evidence for a function as resting forms is not convincing in any of those cases.

Several Cytophagales change their cell morphology in response to environmental conditions. Marine *Flexithrix dorotheae* is described to grow quasi-mycelially with long, sheathed filaments that release short, gliding fragments, which may again grow into filaments (Lewin, 1970). Unfortunately, the type strain, which is the only known strain of that organism, does not show this behavior any more; at least I have not been able to verify it in spite of many efforts.

The vegetative cells of *Sporocytophaga* may convert by shortening and fattening into small (diameter 1.2 to 1.4 μm), spherical, optically refractile microcysts. The microcysts are desiccation resistant; we have isolated strains from dry soil samples that had been stored for up to 9 years at room temperature. Their high heat tolerance, which allows them to survive at 60°C, in some cases even at 100°C for a short time, has already been mentioned.

Fx. filiformis (Fx el) and related species go through a spectacular shape change in the course of culture development (Fig. 2c to 2e). When inoculated into a liquid medium, e.g., CAS or MD1 liquid medium, the organism grows into long (15 to 50 μm and more), slender, flexible cells. These cells are not the usual filaments or trichomes, for they contain no cross-walls or septa, or only very few widely separated ones (10 to 30 μm); I therefore call them thread cells. The thread cells are extremely agile and can glide, bend, twist, and wriggle like little worms. When the culture ages, the cells become shorter and shorter and may finally end up a very short coccobacilli (this stage is not always reached). The shorter rods also become slightly fatter and darker, which clearly indicates that the shortening is not simply a fragmentation of the thread cells. Under certain conditions the organism may grow exclusively in the long or in the short form. One factor that determines the length of the cells is obviously population-density connected. The same cycle can also be observed in plate cultures, e.g., on VY/2 agar, on which fast-spreading swarms are produced. Here, agile thread cells are found at the edge, while shorter and shorter rods are found moving towards the center of the colony. Below a certain

length, the rods become nonmotile. The same developmental cycle has been described for *Chitinophaga*, only there the coccobacilli were interpreted as microcysts (Sangkhobol and Skerman, 1981); this appears, however, not to be correct. A study on the length control of *Flexibacter* strain FS-1 revealed that the long thread cells grew exponentially with a generation time of 90 min at 30°C. When the culture was shifted to 35°C they divided into three or four cells within the following 120 min. The culture continued to grow exponentially with about the same growth rate, but with shorter cells. When the culture was shifted back to 30°C, the shorter cells stopped dividing immediately but continued to grow, and the thread cells reappeared. When the thread cells divided, the constriction always occurred in the middle of the cell. The biochemical and genetic mechanisms that control all these fascinating morphogenetic processes are not at all understood and have not been thoroughly studied. In particular, *Fx. filiformis* should provide a beautiful model system for such investigations (Humphrey and Marshall, 1980; Poos et al., 1972; Reichenbach et al., 1974; Simon and White, 1971).

The fine structure of the cells of the Cytophagales is essentially that of typical Gram-negative bacteria, with a few remarkable peculiarities. The outer surface of the cells is usually corrugated or undulated and densely covered with cushionlike knobs and folds (e.g., Bovallius, 1979; Follett and Webley, 1965; Humphrey et al., 1979; Oyaizu et al., 1982; Strohl, 1979; van der Meulen et al., 1974). Often, vesicular and long, uneven, tubular extensions of the outer membrane and LPS can be seen (e.g., Follett and Webley, 1965; Holt et al., 1979a; Kuhrt and Pate, 1973; London et al., 1982; Martin et al., 1968; Pate and Ordal, 1967a; Pate et al., 1967; Simon and White, 1971). These strands may become as long as 0.5 μm and have been shown to contain LPS components, like 2-keto-3-deoxyoctonate (Humphrey et al., 1979). All Cytophagales excrete slime, sometimes in substantial quantities, and this slime can usually be distinguished as a dense network of fine fibril surrounding the cells (e.g., Bovallius, 1979; Humphrey et al., 1969; Martin et al., 1969; Pate and Ordal, 1967b; Ridgway and Lewin, 1973; Ridgway et al, 1975; Strohl and Tait, 1978; Verma and Martin, 1967). The microcysts of *Sp. myxococcoides* are encased in a massive, fibrillar capsule, about 180 nm thick (Holt and Leadbetter, 1967; the diameter of the microcysts as determined in this study was an astonishing 2 to 2.5 μm, which is much greater than is usually measured in the light microscope [1.2 to 1.4 μm]).

The cell envelope of the Cytophagales consists of a thin peptidoglycan layer and the usual outer membrane. In marine, filamentous *Fx. polymorphus* (clearly different from *Flexibacter* as defined here, and of uncertain taxonomic position), an unusual S-layer was discovered on top of the outer membrane (Ridgway, 1977; Ridgway and Lewin, 1973, 1983; Ridgway et al., 1975) The layer consists of densely packed, goblet-shaped subunits, which have a complicated structure and are perhaps rooted in the cytoplasmic membrane. Another very interesting structural detail has been detected in the cell envelope of certain CLB (Pate and Chang, 1979). Here, tiny rings were seen and could be isolated from cell lysates. The rings were about 20 nm wide and 10 nm thick, and were arranged in long chains that seem to form a large, regular, netlike superstructure. It was hypothesized that these rings might be homologous with the flagellar rotors, and that their rotation would somehow propel the cell. It may be added here that the mechanism of gliding motility has been studied for many years with several Cytophagales, but so far without a clear answer (e.g., Burchard, 1984; Chang et al., 1984; Duxbury et al., 1980; Glaser and Pate, 1973; Godwin et al., 1989; Lapidus and Berg, 1982; Pate, 1985; Speyer, 1953; Wolkin and Pate, 1984). What can be said is that there is some motility apparatus in the cell surface, because latex beads move along the surface of immobilized cells (Lapidus and Berg, 1982); that the excreted slime is essential for gliding because it acts as a Stefan adhesive allowing easy translocation but preventing efficient detachment of the cells from the substrate (Humphrey et al., 1979); that the cells rotate around their long axis during gliding (Godwin et al., 1989); and that the energy for gliding may not be provided by ATP, at least not exclusively (Pate and Chang, 1979; Ridgway, 1977). The speed of gliding may be on the order of 100 to 150 μm/min, with large deviations in both directions, depending both on the organism and on environmental conditions (e.g., Duxbury et al., 1980; Garnjobst, 1945; Lapidus and Berg, 1982; Speyer, 1953; Stanier, 1942).

Fine longitudinal fibrils, which were arranged in wide bands just below the outer membrane, have been discovered in *Cy. columnaris* (Pate and Ordal, 1967b). They were suggested to represent the machinery of gliding motility. The fibrils could indeed not be seen in nonmotile mutants (Glaser and Pate, 1973). In freeze-etched preparations, tiny beads of about the same diameter as the fibrils can be seen on the inner surface of the outer membrane (Burchard and Brown, 1973). When the cells are fixed with

glutaraldehyde before being fractured, those beads arrange themselves in long lines which resemble fibrils. Thus it seems possible that the fibrils seen in ultrathin sections are merely fixation artifacts.

Lysing cells of *Cy. columnaris* release unusual tubular structures that appear to derive by invagination from the cytoplasmic and mesosomal membranes (Pate et al., 1967). They are composed of lipid and protein, although the number of protein bands is much reduced in comparison with the cytoplasmic membrane (Kuhrt and Pate, 1973). Similar tubules are also known from other Gram-negative bacteria including *Sporocytophaga* (e.g., Holt and Leadbetter, 1967; Martin et al., 1968; Yamamoto, 1967); they are not identical with rhapidosomes as has occasionally been suggested. The latter were originally observed in *Saprospira* (see Chapter 200 for a detailed discussion); they were later found also in other bacteria, e.g., *Sp. myxococcoides* (Pate et al., 1967) and may be phage tails.

The cells of the Cytophagales divide in the usual way by formation of a septum, which is normally connected with a mesosome (e.g., Holt and Leadbetter, 1967; Poos et al., 1972; Ridgway et al., 1975).

Appendages like fimbriae and pili seem never to have been observed with Cytophagales. Pili were, however, isolated from *Fv. branchiophilum*, where they supposedly are important for the attachment of this fish pathogen to gill filaments (Heo et al., 1990).

In several *Capnocytophaga* strains, acid and alkaline phosphatases could be localized by cytochemical and immunochemical reactions in the periplasmic space; in fact the enzymes were associated with the outer membrane, probably the LPS, and about 12% of the activity was exposed on the outer cell surface. Most of the enzyme molecules were released from the cells when the cultures became older, a good part of them in a membrane-bound form (Poirer and Holt, 1983b, 1983c). While the bacteria normally cannot penetrate the periodontal tissue it seems conceivable that enzymes like the phosphatases may do so and thus are responsible for damage inflicted to the host by *Capnocytophaga.*

Chemical Composition

The peptidoglycan appears as a clearly distinguishable layer in ultrathin sections of all Cytophagales studied in this respect; it is between 2 and 5 nm thick, but increases to 9 nm in *Sporocytophaga* microcysts (e.g., Pate and Ordal, 1967a; Verma and Martin, 1967a). The murein sacculus has been isolated from *Cy. hutchinsonii* and *Sp. myxococcoides* and chemically analyzed (Verma, 1970; Verma and Martin, 1967a, 1967b). It had the composition of a typical Gram-negative peptidoglycan with L-Ala, D-Glu, *m*-DAP, D-Ala, and 70% direct cross-linking between *m*-DAP and D-Ala. Diaminopimelic acid (0.2 to 0.3% of the dry weight) was also observed in an early study on two flexibacters; the diamino acid came most probably from the cell wall (Holm-Hansen et al., 1965). The same organisms appear also to contain bound ornithine (0.08 to 0.7% of the dry weight); the origin of that amino acid is obscure (Holm-Hansen and Lewin, 1965; it could come from an ornithine-containing lipid, see below). Also, the lipopolysaccharide (LPS) of the Cytophagales seems to closely resemble that of the enterobacteria. The LPS of *Fx. filiformis* Fx el (= Fx ½) was found to contain mannose, galactose, glucose, rhamnose, ribose, glucosamine, galactosamine, and small quantities of xylose, arabinose, 2-keto-3-deoxyoctonate (KDO), and heptose (Rosenfelder et al., 1974). From 17 strains of CLB and one strain each of *Cy. aurantiaca* and *Sp. myxococcoides*, the LPS was extracted with phenol, and its sugar composition was subsequently analyzed (Sutherland and Smith, 1973). LPS yields were between 0.4 and 3.4% of the dry weight (in addition, substantial amounts of polysaccharide could be harvested from the culture supernatant by ultracentrifugation). The LPS of all strains contained a high proportion of ribose (in the absence of an appreciable ultraviolet (UV) absorption at 260 nm), mannose, and glucosamine; further, most LPS preparations yielded glucose, galactose, and galactosamine, and 7 out of 19 also rhamnose. The hexosamines remained after removal of lipid A by mild hydrolysis. Because of analytical problems, KDO and heptose could not be determined reliably; but KDO always appeared to be present, whereas heptose remained questionable and was there in very small amounts, if at all. The LPS of *Flexibacter* BH3 contained galactose, glucose, arabinose, rhamnose, glucosamine, deoxysugar, and KDO, but heptose could not be detected (Humphrey et al., 1979). An endotoxin, which was highly active immunologically as well as in the *Limulus* test, was isolated from a CLB; its main carbohydrate constituents (above 0.5% of the total) were rhamnose, fucose, mannose, galactose, glucose, glucosamine, galactosamine, and an astonishing 34% of (tentatively identified) heptose; KDO is not mentioned (Flaherty et al., 1984). The inner and outer membrane of *Cy. johnsonae* were separated and showed densities of 1.18 and 1.14 g/

cm^3, respectively; the outer membrane contained the sulfonolipids that will be discussed later, but little or no KDO (Godchaux and Leadbetter, 1988). Occasionally, members of the Cytophagales produce copious amounts of extracellular slime. In the case of *Cy. hutchinsonii* and *Sp. myxococcoides*, that slime was found to be an acidic heteropolysaccharide consisting of xylose, arabinose, mannose, glucose, and glucuronic acid; the slime of *Sporocytophaga* also contains galactose (Martin et al., 1968; Verma and Martin, 1967b). This slime seems to be broken down enzymatically in the later stages of the culture. *Flexibacter* BH3 produces an extracellular, water-soluble, glycoprotein slime with glucose, fucose, galactose, and some uronic acid in the carbohydrate part (Humphrey et al., 1979). The extracellular slime of *Cy. columnaris* was determined to consist of a homopolymer of α-(1-4)-linked D-galactosamine (Johnson and Chilton, 1966).

The fatty acid patterns of the Cytophagales consistently show two peculiarities (Collins et al., 1982; Fautz et al., 1979, 1981; Godchaux and Leadbetter, 1984; Kath, 1990; Liebert et al., 1984; Oyaizu and Komagata, 1981; Walker, 1969):

1. The dominant species are branched fatty acids, mainly 15:0 (15 to 45% of the total; in *Capnocytophaga* up to 77%: Collins et al., 1982). Branching is mostly of the iso type, but small amounts of anteiso fatty acids are usually also present. Only *Cy. hutchinsonii* contains larger quantities of anteiso fatty acids, almost exclusively 17:0 (26% of the total), but as the identification was tentative, this should be reinvestigated (Walker, 1969). It was also stated that *Capnocytophaga* and *Sporocytophaga* exclusively contain anteiso fatty acids (Holt et al., 1979a), but at least for *Capnocytophaga* this could not be corroborated (Collins et al., 1982). In one study, 1 to 3% of the total fatty acids from CLB have been identified with (delta 17)-cyclopropane fatty acids (Oyaizu and Komagata, 1981). Other major fatty acids are straight chain 16:1, 16:0, and 15:0, usually in that order. The double bond has been found to be in the *cis* configuration and in the C-11 to C-12 position (Kath, 1990; Oyaizu et al., 1982; Walker, 1969).
2. There are always substantial quantities (15 to 55% of the total fatty acids) of 2- and 3-hydroxy fatty acids (Collins et al., 1982; Fautz et al., 1979, 1981; Kath, 1990; Liebert et al., 1984; Oyaizu and Komagata, 1981; Oyaizu et al., 1982). In *Capnocytophaga*, only 3-hydroxy fatty acids could be identified (Collins et al., 1982). Only a small part of these hydroxy fatty acids seem to rise from LPS where, in addition, exclusively 3-hydroxy fatty acids are found (Rosenfelder et al., 1974).

Most of them rather appear to have their origin in novel sulfonolipids, the capnoids, which were originally discovered in *Capnocytophaga* but have since been isolated from many other Cytophagales and may indeed by a chemosystematic marker for that group (Godchaux and Leadbetter, 1980, 1983, 1984). The capnoids are essentially sulfonated sphingolipids, in which the hydroxyl on C-1 of the sphinganine base, capnine, is replaced by a sulfonic acid group. Free capnine was found only in *Capnocytophaga*; normally the amino group on C-2 bears an amide-bonded, long-chain, fatty acid as in true ceramides. The sulfonilipids may comprise as much as 20% of the cell's total lipids and are localized in the outer membrane (Godchaux and Leadbetter, 1988). It appears that there is a connection between sulfonolipids and gliding motility, for capnoid-negative mutants of *Cy. johnsonae* are no longer able to glide, but gliding may recover when sulfonolipid synthesis is restored (Abbanat et al., 1986). The biosynthesis of the capnines is presumably due to the reaction of cysteine or, more likely, of cysteic acid with a matching fatty acyl-CoA compound (Abbanat et al., 1985; White, 1984).

The fatty acid pattern of marine *Fx. polymorphus* is unique among the Cytophagales in that it consists of relatively low amounts of (mainly iso-) branched species and large quantities of polyunsaturated fatty acids, viz. 18% 20:5 (Johns and Perry, 1977). This, together with the ultrastructural details discussed above, suggests that this organism may not be correctly classified among the Cytophagales.

It is interesting to note that very similar lipid patterns to those just discussed have been demonstrated in nonmotile but phylogenetically related flavobacteria, i.e., a high proportion of branched and of 2- and 3-hydroxy fatty acids (e.g., Dees et al., 1979; Fautz et al., 1981; Moss and Dees, 1978; Oyaizu and Komagata, 1981; Yabuuchi et al., 1983; Yano et al., 1976) and the occurrence of capnoids (Godchaux and Leadbetter, 1983) and typical sphingophospholipids and ceramides (e.g., Asselineau and Pichinoty, 1983; Yabuuchi et al., 1983; Yano et al., 1983).

The complex lipids of the Cytophagales are still incompletely known. In all organisms that have been investigated (*Capnocytophaga*, *Cy. hutchinsonii*, *Sp. myxococcoides*) phosphatidylethanolamine was by far the major phos-

pholipid (Collins et al., 1982; Holt et al., 1979c; Iizuka et al., 1987; Walker, 1969). The phospholipid content of the capnocytophagas was between 30 and 36% of the total cellular lipids when estimated as acetone-soluble lipid. The organisms also contained an ornithine lipid, some phosphatidylserine, substantial amounts of a polyprenol, and 5% squalene; two laboratories report the absence of phosphatidylglycerol (Collins et al., 1982; Holt et al. 1979a) while in a third laboratory (Iizuka et al., 1987), this compound was detected along with phosphatidylinositol and cardiolipin. In *Cy. hutchinsonii*, 45% of the dominating 16:1 fatty acid was recovered from phosphatidylethanolamine; no other phospholipid could be demonstrated in this organism, but it contained substantial quantities of two unidentified glycolipids (Walker, 1969).

The respiratory quinones of the Cytophagales are exclusively menaquinones (Callies and Mannheim, 1978; Collins et al., 1982; Kleinig et al., 1974; Oyaizu and Komagata, 1981). Different species contain either MK-6 or MK-7, and the distribution of the two types may be of taxonomic significance. Thus, all cellulose decomposers contain MK-7 and differ in that respect neatly from most terrestrial CLB, which are presently classified with them in the same genus but contain MK-6 (M.D. Collins, personal communication). The respiratory quinone of *Capnocytophaga* is MK-6 with traces of MK-5 (Collins et al., 1982), that of *Flexibacter* is MK-7 (Kleinig et al., 1974). Again, the true flavobacteria are also menaquinone organisms (Callies and Mannheim, 1978; Oyaizu and Komagata, 1981).

Most Cytophagales synthesize pigments. Apart from an occasional strain that produces a dark, diffusing, probably melaninlike substance (e.g., "*Cy. krzemieniewskae*": Stanier, 1941; *Fv.* (*Cy.*) *uliginosum*: ZoBell and Upham, 1944), the pigments are always cell-bound. Chemically they are of two kinds, either carotenoids or flexirubin-type pigments. The two pigment types may occur in the same organism but then are typically localized in different places: the carotenoids in the cytoplasmic, and the flexirubins in the outer membrane (Irschik and Reichenbach, 1978).

It had been suggested for many years that the yellow and orange pigments that can be extracted from many Cytophagales might be carotenoids (e.g., Anderson and Ordal, 1961a; Fox and Lewin, 1963; Lewin and Lounsbery, 1969; Verma and Martin, 1967a). Unequivocal proof of zeaxanthin (4,4′-dihydroxy-β,β-carotene) could be provided for the yellow pigment of marine *Ft. dorotheae* (Aasen and Liaaen-Jensen, 1966c); the same pigment was demonstrated by chromatographic comparison in marine *Cy. lytica* (Lewin and Lounsbery, 1969), and it was later chemically identified also in a terrestrial CLB (*Cy. johnsonae*: Achenbach et al., 1978b). Two monocyclic ketocarotenoids, flexixanthin and deoxyflexixanthin, have been isolated from a flexibacter (probably *Fx. roseolus*: Lewin and Lounsbery, 1969); their chemical structures have been elucidated (Aasen and Liaaen-Jensen, 1966b). *Cy. diffluens* appears to contain the monocyclic dihydroxy carotenoid saproxanthin (Lewin and Lounsbery, 1969), which was originally isolated from *Saprospira grandis* and identified by chemical methods (Aasen and Liaaen Jensen, 1966a).

A novel pigment type, the flexirubins, which later turned out to be of considerable chemosystematic relevance, has been discovered in *Fx. filiformis* (formerly *Fx. elegans* Fx el: Achenbach et al., 1974, 1976; Reichenbach et al., 1974). The chromophore is an omega-phenyloctaenic acid which is connected via an ester bond to a resorcinol bearing two hydrocarbon chains (Fig. 4). This basic chemical structure may be modified by variation of the length and branching of the hydrocarbon chains on the resorcinol, and by the introduction of additional substituents on the omega-phenyl ring, specifically methyl and chlorine; actually, for all pigment species, chlorinated counterparts are found in every flexirubin-producing organism (for reviews of their chemistry and biosynthesis, see: Achenbach, 1987; Achenbach et al., 1978). In this way, a large variety of different flexirubin-type pigments arise, and one single strain may synthesize more than 25 different compounds (Achenbach et al., 1979) Still, certain structural types seem to be characteristic for certain taxa, e.g., a methyl in the *meta* position on the omega-phenyl ring seems to occur only in *Flexibacter*. Biosynthetically, the omega-phenyl ring with the first three carbon atoms of the chain derive from tyrosine, the rest of the chain from acetate, and the resorcinol ring with its hydrocarbon substituents from acetate and various starter molecules, like propionate or isovalerate, via an orsellinic acid homologue; only the methyl on the omega-phenyl comes from methionine; the linking of the two halves of the molecule is the last biosynthetic step (Achenbach et al., 1972, 1982, 1983). A total synthesis of flexirubin has also been performed (Achenbach and Witzke, 1977). Flexirubin-type pigments have been found so far only in the Cytophagales and in the true flavobacteria (Achenbach et al., 1981; Reichenbach et al., 1981; Weeks, 1981). However, not all Cytophagales contain flexirubin-type pigments, and even

closely related species, and perhaps even strains of the same species, may differ in that respect. Still, the presence of these pigments in a strain appears to be a reliable criterion that the isolate belongs to the Cytophagales and flavobacteria. The existence of flexirubin-type pigments in a strain can be demonstrated in three ways: 1) As already mentioned, the colonies show a reversible color shift when covered with a 20% KOH solution (see color plate I in *The Prokaryotes*, 1st edition). 2) This preliminary test can be confirmed by a simple thin-layer chromatography of an acetone extract of the bacteria, with an extract of a known flexirubin-producer as a reference; this test can be made even more reliable by recording the absorption spectra of the eluted spots and by performing the alkali reaction in the cuvette (Reichenbach et al., 1974). 3) Finally, as the only other bacterial pigments that give a similar color reaction appear to be aromatic carotenoids (Kohl et al., 1983), the pigments may be specifically labeled by feeding radioactive tyrosine (flexirubins) or mevalonic acid (carotenoids); the result is analyzed by autoradiography of the chromatograms (Fautz and Reichenbach, 1980). Incidentally, the color reaction of cellulose-decomposing cytophagas or sporocytophagas with alkali was already observed long ago, although its meaning was not understood at the time (Fåhraeus, 1947; Walker and Warren, 1938).

Occasionally, strains of CLB with a thin, filmlike growth show a striking greenish-reddish iridescence (see color plate I in *The Prokaryotes*, 1st edition). This is probably a physical color due to interference effects. Also, it has been suggested that the greenish color that is sometimes observed in cultures of Cytophagales may arise from the combination of a yellow cell mass and dark melaninlike pigments (Lewin and Lounsbery, 1969).

Pigment production by Cytophagales may be influenced by environmental conditions. Thus, *Cy. succinicans* is colorless when grown anaerobically, but yellow-orange under aerobic conditions (Anderson and Ordal, 1961a). Illumination may stimulate carotenoid synthesis; but the synthesis of the flexirubins is light-independent. In the latter case, however, the specific pigment content of the cells varied substantially with some other culture parameters; in particular, a high phosphate content and a low pH appear to reduce pigment synthesis (Reichenbach et al., 1974).

Little else is known about the chemical composition of the Cytophagales. The amino acid composition of the protein of a cellulose-decomposing cytophaga was analyzed and found to be rich in nutritionally valuable, essential amino acids (Chang and Thayer, 1975). The GC content of the DNA of the Cytophagales varies over a wide range from 28 to 65 mol% (e.g., Behrens, 1978; Kath, 1990; Mandel and Leadbetter, 1965; Mandel and Lewin, 1969; Oyaizu and Komagata, 1981). The base sequences of large chunks of the 16S rRNA of a series of *Cytophaga*, *Sporocytophaga*, and CLB strains have been determined (Kath, 1990). An unusual multicopy, single-stranded DNA-RNA species (msDNA) discovered in myxobacteria (see Chapter 188) was also found in *Fx. filiformis* (= *Fx. elegans* Fx el), but it was ruled out for *Cy. johnsonae* and *Tx. ocellatus* (Dhundale et al., 1985); it should be interesting to reinvestigate the isolated occurrence of msDNA in *Flexibacter* with the inclusion of other strains.

Phages and Bacteriocins

Phages have been reported for several CLB and appear not to be difficult to obtain (Anacker and Ordal, 1955; Kingsbury and Ordal, 1966; Pate et al., 1979; Richter and Pate, 1988; Stürzenhofecker, 1966; Valentine and Chapman, 1966). There is considerable morphological variability. Most of the phages bear a tail, which usually seems to be contractile. However, two temperate phages have been isolated from *Cy. johnsonae* strains that appear to have no tails; these phages also were sensitive to chloroform, and their plaque formation was inhibited by agar (Richter and Pate, 1988) In all cases that have been investigated, the phages contained double-stranded DNA. A phage of *Cy. columnaris* could not be propagated in the presence of streptomycin (70 μg/ml), probably because the antibiotic prevented the injection of the phage DNA into the host, which for its part was resistant to streptomycin (Kingsbury and Ordal, 1966). Much higher phage yields were often observed when $CaCl_2$ (1 to 4 mM) was present during infection. Interestingly, nonmotile mutants of *Cy. johnsonae* were completely resistant to all phages tested on them (Pate et al., 1988; Wolkin and Pate, 1986).

A bacteriocinlike activity was discovered in a strain of *Sp. myxococcoides* (Tchan and Giuntini, 1950). The strain inhibited other *Sp. myxococcoides* strains, but not *Cy. aurantiaca*; the activity could not be propagated, and no phage particles could be demonstrated under the electron microscope. Bacteriocin activity was found in many strains of the fish pathogen *Cy. columnaris* (Anacker and Ordal, 1959b). The target specificity of the bacteriocins produced by different *Cy. columnaris* strains varied, so that with the aid of seven selected bacteriocin preparations, 134 strains could be grouped into nine

sensitivity classes. No connection could be seen between a certain bacteriocin class and its distribution in nature. The same results were observed for serological classes of *Cy. columnaris* (Anacker and Ordal, 1959a). Bacteriocin activities were recorded in 20 out of 30 tested strains of *Cy. johnsonae* (Richter and Pate, 1988). The activities were destroyed by protease treatment and acted bactericidally on sensitive strains. With respect to their specificity, two types could be distinguished: 1) bacteriocins that acted on motile and nonmotile strains, and 2) ones that, like the phages discussed above, attacked only motile strains. With the latter, nonmotile mutants could be selected.

The occurrence in various members of the Cytophagales of rhapidosomes, which appear to be tails of defective phages, has already been mentioned.

Colony Morphology

The colonies of the Cytophagales typically are spreading swarms (Fig. 3). Spreading growth depends, however, on several environmental factors, so that organisms that are well able to move by gliding still may not produce swarms (e.g., Chang et al., 1984; Perry, 1973; Wolkin and Pate, 1984). Thus, e.g., 70% of yellow bacteria isolated from sewage plants showed gliding motility under the microscope, but only 25% also formed spreading colonies (Güde, 1980). It has been found in many cases that low nutrient concentrations favor swarming (e.g., Agbo and Moss, 1979; Bauer, 1962; Garnjobst, 1945; Wolkin and Pate, 1984). Therefore, if spreading is to be observed, agar media with a low peptone content (0.1% or less) should be used; also, VY/2 agar stimulates swarming in many cases. Other factors that may influence the spreading behavior are the kind of nutrients supplied, temperature, and humidity. No general rules can be given; however, often a relatively low temperature and a high humidity favor spreading.

The morphology of the swarm colonies varies very much, as is to be expected with such a large assembly of widely differing organisms. Often the swarms are almost unstructured, thin sheets; sometimes their surface is covered with flat mounds, tiny warts, or with veins; or it appears fibrous and feltlike; or the swarms form a network which may become confluent towards the center of the colony. While the swarm sheet is always somewhat slimy, the production of copious amounts of slime is relatively rare; it is observed, e.g., when the cellulose decomposers are grown on a glucose-containing agar (see also Strohl and Tait, 1978). The slime is usually soft, so that the cells can easily be scraped off the plate; but sometimes it becomes very tough, and the cell mass can hardly be removed from the agar surface, e.g., with *Cy. columnaris* (Garnjobst, 1945) and *Cy. uliginosa*. The organisms also often penetrate the agar, and some species even grow and spread preferentially within the plate, e.g., microaerophilic and agar-attacking organisms (e.g., Veldkamp, 1961). Colony variations have been reported for several species (Anderson and Ordal, 1961a; Bachmann, 1955; Oyaizu et al., 1982; Pacha, 1968; Stanier, 1942, 1947; Veldkamp, 1961). In one case, migrating microcolonies were observed (Strohl and Tail, 1978). The colonies of the cellulose decomposers on filter paper are slowly expanding, glassy, unstructured, slimy patches that are usually bright yellow to orange. Grey areas in the colonies of *Sporocytophaga* usually signal generous microcyst formation.

A sickening odor has been reported for *Cy. columnaris* on nutrient agar (Garnjobst, 1945). On peptone-containing media, and particularly with liquid cultures, a strong, cheesy smell is produced by many CLB. The cellulose degraders produce a pleasant sour-fruity odor when growing on glucose agar.

Physiology and Enzymology

With a few exceptions, the physiology and enzymology of the various members of the Cytophagales has not been thoroughly investigated, and virtually no information is available about most basic biochemical pathways. Further, many of the studies were published a long time ago, and a reevaluation of the data in the light of modern biochemical insights would be desirable. On the other hand, nothing discovered so far suggests that the metabolism of these bacteria would differ in some basic manner from the metabolism of other comparable organisms.

Most Cytophagales are strict aerobes, but there also are microaerophilic species, facultative anaerobes, and organisms with a strictly fermentative metabolism (Anderson and Ordal, 1961a, 1961b; Bachmann, 1955; Hirsch, 1980; Leadbetter et al., 1979; Stanier, 1947; Veldkamp, 1961). It appears that all Cytophagales that are capable of fermentative growth require elevated CO_2 levels (optimum 0.3 to 1% $NaHCO_3$: *Cy. fermentans*, *Cy. succinicans*, *Cy. salmonicolor*, *Capnocytophaga*). For strictly fermentative *Capnocytophaga* it has been shown that CO_2 is necessary because the organism depends on phosphoenolpyruvate carboxykinase (PEPCK) in order to synthesize ATP and the terminal electron acceptor, oxalacetate; the lat-

ter is reduced under regeneration of NAD to the level of succinate, which is then excreted as the major fermentation product. Obviously PEPCK replaces in that organism the more usual pyruvate kinase. The enzyme is regulated by the energy charge of the cell: low ATP levels stimulate, high ones inhibit PEPCK activity (Kapke et al., 1980). A condensation of CO_2 with phosphoenolpyruvate was also suggested for fermentatively growing *Cy. succinicans* (Anderson and Ordal, 1961b). The reaction was stimulated by GDP. A CO_2-independent fermentation of fructose-1,6-diphosphate could be performed in cell extracts supplemented with lactate dehydrogenase. The data could be well reconciled with the mechanism outlined for *Capnocytophaga*. As *Cy. succinicans* is a facultative anaerobe, it is likely to have alternative pathways starting from PEP. The fermenting organisms may utilize various sugars, mannitol, and polysaccharides such as agar or starch; however, as a rule, organic acids are not suitable fermentation substrates; one exception is *Cy. succinicans*, which grows anaerobically on pyruvate. Fermentation products are mainly succinate and acetate, usually also propionate and formate, and sometimes small quantities of lactate and ethanol. *Cy. salmonicolor* produces CO_2 and H_2 during fermentative growth (Veldkamp, 1961).

Nitrate respiration allows vigorous growth of *Cy. johnsonae* var. *denitrificans* (Stanier, 1947). More recently, a CLB was isolated that grew anaerobically with NO_3^-, NO_2^-, and N_2O as terminal electron acceptors (Adkins and Knowles, 1984, 1986) If small quantities of sulfide (0.4 μM) were available, this organism was able to reduce N_2O even in the presence of acetylene in concentrations (4 kPa) that normally inhibit N_2O reduction. This is also of interest because acetylene-inhibited N_2O reduction is generally accepted as a measure of denitrification in soils. However, other denitrifying CLB (*Cy. johnsonae* strains) did not show acetylene-insensitive N_2O reduction (Adkins and Knowles, 1986). The strains differed in their ability to reduce various nitrogen compounds. In one case, N_2O reduction turned out to be inducible by nitrite, but not by nitrate. Anaerobic growth under reduction of nitrite or N_2O has also been found among flavobacteria (Pichinoty et al., 1976). Growth with fumarate as the terminal electron acceptor was not possible with any of the Cytophagales and flavobacteria tested, but several of the organisms, e.g., *Cy. hutchinsonii* and *Cy. johnsonae*, could use fumarate for limited NAD regeneration under anaerobic conditions (Callies and Mannheim, 1978). As already mentioned, no strict anaerobes are known among the Cytophagales.

It appears that all Cytophagales, including the cellulose decomposers, grow on organic nitrogen compounds, e.g., peptones, as the sole nitrogen source. Many also use the same compounds for carbon and energy and grow vigorously on peptone media, even if, as a rule, carbohydrates are the preferred carbon and energy sources. Some organisms, however, require the addition of a carbohydrate, e.g., apparently all cellulose decomposers. On the other hand, there are also organisms that cannot utilize carbohydrates at all, e.g., *Cy. psychrophila* (Pacha, 1968) and many *Cy. columnaris* strains (e.g., Song et al., 1988a).

Many Cytophagales can be cultivated on inorganic nitrogen compounds. NH_4^+ is usually preferred to NO_3^-. The latter can sometimes not be utilized at all, thus by *Cy. fermentans* (Bachmann, 1955). The spectrum of utilizable carbon compounds is wide and varies with the individual organisms. Glucose is almost always accepted. One exception is the *Cy. diffluens* group: these bacteria require some other sugar like galactose or sucrose (Lewin and Lounsbery, 1969). Acid is often produced from carbohydrates even under aerobic conditions (e.g., Oyaizu and Komagata, 1981; van der Meulen et al., 1974). *Sp. myxococcoides* metabolizes glucose via the Embden-Meyerhof-Parnas pathway (Hanstveit and Goksøyr, 1974), as does *Cy. johnsonae* (Reichardt and Morita, 1982a).

Many of the Cytophagales are able to degrade biomacromolecules, particularly all kinds of polysaccharides, like agar, starch, cellulose, yeast cell-wall β-glucan, succinoglycan (Oyaizu et al., 1982), pectin, alginate, and heparin. As many of the responsible exoenzymes potentially are of some practical interest, these activities will be discussed under "Practical Aspects," this chapter.

DNA and RNA are efficiently degraded by many Cytophagales (e.g., Greaves et al., 1970; Mitchell et al., 1967). A particularly strong DNAse activity is observed with *Cy. columnaris*. It seems, however, that no organism is able to grow solely on nucleic acids Phosphatases are very common among the Cytophagales (e.g., Christensen, 1977a; Hirsch, 1980; Reichardt et al., 1983) An alkaline and an acid phosphatase of *Cp. ochracea* have been isolated and studied in detail (Poirier and Holt, 1983b). The enzymes are able to remove phosphate from phosphoseryl residues of phosvitin and may play a role in the dephosphorylation of phosphoproteins. CLB are able to grow on washing-powder polyphosphates as phosphate sources; the polyphosphates are well tolerated

up to 0.0025% and are rapidly hydrolyzed (Ruschke and Köhn, 1970). Lipolytic activities are also known from Cytophagales, e.g., cleavage of tributyrin and of Tweens (e.g., Christensen, 1977a; Hirsch, 1980; Jooste et al., 1985; Oyaizu et al., 1982; Reichardt et al., 1983), but the responsible enzymes seem never to have been characterized.

The patterns of antibiotic sensitivities have been determined for many members of the Cytophagales, although the data often are not really comparable because of differences in methods (e.g., Agbo and Moss, 1979; Arlet et al., 1987; Christensen, 1977a; Forlenza et al., 1981; Gräf and Morhard, 1966; Reichardt et al., 1983; van der Meulen et al., 1974; Warke and Dhala, 1968). It appears that many organisms are relatively resistant to penicillins, polymyxin B, aminoglycosides, and chloramphenicol. Kanamycin resistance has been used in the isolation of cellulose-decomposing *Cytophaga* and *Sporocytophaga* strains (2.5 to 10 mg kanamycin sulfate per liter); but it is not known whether these organisms are always resistant to that antibiotic (Rivière, 1961a). Capnocytophagas respond to several conventional antibiotics and are thus easy to control.

A genetics of the Cytophagales is still nonexistent.

Classification and Identification

As mentioned in the introduction, 16S rRNA studies have shown that the Cytophagales are a main branch, or phylum, of their own in the bacterial phylogenetic system (Paster et al., 1985). They are found there together with some other, phenotypically rather different bacteria; although our current knowledge suggests that some common characteristics may indicate connections between those groups, on this basis, probably, nobody would have dared earlier to propose the existence of a natural relationship among all those bacteria. Thus, e.g., *Capnocytophaga* resembles *Bacteroides* morphologically and metabolically to such a degree that it was formerly classified in this genus. Also, sphingolipids, in general rare in bacteria, are found in *Bacteroides* and in certain flavobacteria (but also in nonrelated myxobacteria). Further, the 16S rRNA tree of descent demonstrates unequivocally that the present genera of the Cytophagales are heterogeneous, and that we may even need new definitions for families and perhaps orders.

The order Cytophagales is defined here using conventional phenotypic characteristics so that new isolates can be classified relatively easily and reliably. As discussed in the introduction, only part of the group of phylogenetically related bacteria is included in the phenotypically defined order. As defined here, the order Cytophagales contains unicellular, Gram-negative, rod-shaped, gliding bacteria, that often exhibit a pronounced cellular shape change, or at least pleomorphism. Most species produce more or less brightly colored, yellow, orange, pink, or red colonies. The yellow and orange ones often give a positive flexirubin reaction. The respiratory quinones are exclusively menaquinones.

This definition presents us with two problems: First, a practical one—how to distinguish cytophagas from *Lysobacter*, which shares many of the above-mentioned characteristics. Differences in cell shape and pigmentation, as mentioned in the introduction, would quickly differentiate most, but unfortunately not all, Cytophagales from *Lysobacter*. In case of doubt, more subtle characteristics have to be examined. It seems that all lysobacters degrade chitin, while many Cytophagales do not. The GC content of *Lysobacter* DNA is high, between 65 to 70 mol%, values that are only reached by red *Taxeobacter* among the Cytophagales. The respiratory quinones of *Lysobacter* are ubiquinones (Q-8), while all Cytophagales appear to contain menaquinones exclusively (M.D. Collins, personal communication). Finally, no marine and no parasitic lysobacters have been found so far.

The second problem is how to integrate the flavobacteria into the order Cytophagales. The old genus *Flavobacterium* was plainly heterogeneous, but after exclusion of the high-GC-content species, the remaining, low-GC-content (30–45 mol%) species appear to be naturally related among themselves and with the Cytophagales. Those species of flavobacteria for which 16S rRNA catalogs exist are connected to the phylogenetic tree of the Cytophagales at various levels and are often found close to the gliding species. This suggests that the genus *Flavobacterium* is still heterogeneous, and that a gliding species may be more closely related to a nonglider than to another glider. The situation became even more confusing after several organisms that were originally described as nonmotile and consequently classified as *Flavobacterium* species were later discovered to glide; they would therefore have to be classified among the cytophagas. This is the case for the type species of *Flavobacterium*, *Fv. aquatile*, and for *Fv. pectinovorum*, *Fv. uliginosum*, and *Fv. heparinum*. The (nonmotile) flavobacteria also have, in common with the Cytophagales, menaquinones as the only respiratory quinones, and often flexirubin-type pigments. Therefore, most proba-

bly, gliding motility will lose its determining character in this taxonomic group, and eventually there may be order(s), families, and perhaps even genera that comprise motile and nonmotile species, just as there are in groups of flagellated bacteria. But before the new boundaries can be defined, more data, especially molecular and chemosystematic ones, are needed.

As set out above, the Cytophagales are restricted here to gliding organisms which can be differentiated into genera according to the following key:

Key to the Genera of the Cytophagales

1. Cellulose decomposers that grow on filter paper as the only carbon source; may utilize inorganic or organic N sources; cannot grow on peptone alone but require in addition a carbohydrate, like cellulose (filter paper) or a suitable sugar (e.g., glucose) 2

1′. Do not attack filter paper cellulose; usually grow well on peptones, but some are much stimulated by carbohydrate or depend on it for fermentative growth 3

2. Produce microcysts .... *Sporocytophaga*

2′. Do not produce microcysts, terrestrial .... *Cytophaga*

2″. Do not produce microcysts, marine .... *Cytophaga* (?)

3. Strictly fermentative; require CO_2 and carbohydrate for growth; inhabitants (mainly) of the oral cavity of man and mammals .... *Capnocytophaga*

3′. Strict aerobes or facultative anaerobes 4

4. Vegetative cells in young cultures are long (20 to 100 μm) thread cells 5

4′. Vegetative cells in young cultures are short to moderately long (2 to 15 μm) rods 6

5. Soil and freshwater bacteria; several species show a very conspicuous (cyclic) cellular shape change: in young cultures, long (20–30 μm), extremely agile thread cells; in older cultures, short immotile rods or coccobacilli; many strains contain flexirubins; GC content, 40 to 50 mol% .... *Flexibacter*

5′. Marine organisms; cellular shape change not observed .... *Microscilla*

5″. Marine organisms; very long (sometimes multicellular and nonmotile) filaments which may or may not have a sheath and release shorter, but often still rather long, gliding thread cells; colonies yellow due to zeaxanthin, no flexirubins; GC content, 37 mol% .... *Flexithrix dorotheae*

6. Short, stout rod cells with rounded ends, often arranged at the edges of the colonies in a palisadelike fashion; colonies more or less brick red; GC content, 55 to 65 mol% .... *Taxeobacter*

6′. Cell shape very variable: short to very short, but sometimes also moderately long (5 to 15 μm) rods, delicate or plump, with rounded or tapering ends; colonies often fast spreading, filmlike swarms, yellow to orange, marine organisms sometimes pink; many terrestrial isolates contain flexirubin-type pigments, marine isolates usually do not; in soil, freshwater, and marine habitats; some are fish pathogens; GC content, 30 to 40 mol% .... *Cytophaga*-like bacteria (CLB)

(6″.) Cells are relatively short, cylindrical rods with rounded ends, but often long thread cells or cell chains are also found in cultures; colonies (slowly) spreading, often rather slimy, pale grey to greenish yellow, brick red, or brown due to diffusing pigments; respiratory quinone is Q-8; GC content, 65 to 70 mol%: *these organisms do not belong to the Cytophagales* .... (*Lysobacter*)

The cellulose decomposers were historically the first cytophagas described, and thus taxonomically they represent the type of the whole group. For many years it has been suggested that these cellulose decomposers are rather specialized organisms that are not closely enough related to the other organisms of the group to be united with them in one genus. Experimental evidence for this hypothesis has been provided recently: Surveys of the respiratory quinones of gliding bacteria showed that many CLB contain MK-6: but all cellulose decomposers contain MK-7 (Oyaizu and Komagata, 1981; M.D. Collins, personal communication). Quantitative fatty acid analyses, DNA-DNA hybridization data, and especially comparisons of 16S rRNA base sequences suggest that the two type strains of the named species of the cellulose-decomposing cytophagas and of several new isolates are relatively closely related among themselves. Further, it could be shown that *Sporocytophaga* strains are equally closely related among themselves and, finally, that the latter are relatively closely related to the cellulose-decomposing cytophagas, but that other CLB, like *Cy. johnsonae* and *Cy. heparina*, are much farther off from both (Kath, 1990). The conclusion is that the genus *Cytophaga* should indeed be restricted to the cellulose decomposers. New genera will have to be defined for the other organisms hitherto classified in the genus *Cytophaga*, but this should not be done until after the boundaries between the various groups have been clearly established by molecular taxonomy. In the present situation it may be best to follow the practice used in the present review and to group these other organisms under the label "*Cytophaga*-like bacteria (CLB). *Sporocytophaga* appears to be a separate and equally natural genus, at least as far as cellulose decomposers are concerned. Sporocytophaga isolates can easily be recognized by the presence of microcysts; these are usually produced in enormous numbers in enrichment cultures, and their formation can also be reliably induced with pure strains that no longer produce them in ordinary plate cultures when the strain is grown in M9 liquid medium (Kath, 1990). *Sp. cauliformis* appears to be a misnomer (Gräf, 1962a): it seems neither to decompose cellulose nor to form microcysts and probably belongs to the large assembly of CLB.

Another source of confusion is the distinction between *Flexibacter* and *Cytophaga*. The definition of the genus *Flexibacter* given here is based on the original description by Soriano (1945, 1947), but it is in disagreement with that used in *Bergey's Manual*, eighth edition (Leadbetter, 1974) The definition of *Flexibacter* has recently been changed again in *Bergey's Manual of Systematic Bacteriology* (Reichenbach, 1990), where it is now placed in accordance with the ideas presented here. (A detailed explanation of the rather complex taxonomic situation may be found in the latter reference, but it will not be repeated here.) The organisms united in the present genus *Flexibacter* have long, highly flexible cells. The organisms also have in common a somewhat higher GC content of 40 to 50 mol% (Behrens, 1978; Mandel and Lewin, 1969) and they all seem to contain MK-7 as respiratory quinone (M.D. Collins, personal communication). The type species is *Fx. flexilis*, which shows rather sluggish movements and is flexirubin negative. Much more fascinating is *Fx. elegans* Soriano (1945), which for many years was regarded as the typical *Flexibacter*, and about which many details have been published, especially about strain Fx el (e.g., Achenbach et al., 1978; Behrens, 1978; Fautz et al., 1979, 1981; Hirsch, 1979; Paster et al., 1985; Poos et al., 1972; Rosenfelder et al., 1974; Reichenbach et al., 1974, 1981; Simon and White, 1971). In young cultures and at the edges of spreading swarms, the organism has long and extremely agile thread cells. These cells change their shape in the later stages of the culture, becoming shorter and shorter and finally becoming nonmotile (Fig. 2; Poos et al., 1972; Simon and White, 1971). Under certain conditions, the organism may grow exclusively in the short form, but the cells readily grow out into thread cells again if conditions are changed. Soriano (1945) did not mention any shape change, perhaps because he did not observe it, perhaps because he thought his cultures were contaminated, or perhaps because he regarded his organisms to consist of multicellular filaments and therefore judged fragmentation to be commonplace. But his description of *Fx. elegans*, and especially of the movements of the thread cells, leave no doubt that his and our organisms are at least closely related. It was later discovered that there existed in the literature an even earlier description that exactly fitted *Fx. elegans* as outlined above, shape change, color, and physiology included—that of *Myxococcus filiformis* by Solntseva (1940), whose only mistake was to misinterpret the short cells as myxospores. Unfortunately, the situation was further confounded by the description under the name of *Fx. elegans* of an organism that quite obviously was not identical with Soriano's species (Lewin, 1969; Lewin and Lounsbery, 1969) As the name *Fx. elegans* has been conserved with Lewin's stain as the type species, it has been proposed to change the name of the organisms discussed above into *Fx. filiformis* (Reichenbach, 1990). To complicate things even more, an organism has been described more recently under the name of *Chitinophaga*, without any reference to the publications cited above, which is clearly closely related or identical with *Fx. filiformis* (Sangkhobol and Skerman, 1981). In this case, microcysts have again been postulated, but the published pictures are not at all convincing, and when studying the type strain the present author has never observed anything even remotely resembling a microcyst (H. Reichenbach, unpublished observations). In the 16S rRNA phylogenetic tree, *Fx. filiformis* Fx el is found in a side branch far away from *Sporocytophaga* and the CLB (Paster et al., 1985). It remains to be established whether the genus *Flexibacter* in its present state is really homogeneous. The genus is restricted so far to soil and freshwater organisms; many, but not all, contain flexirubin pigments, which have in fact been discovered in strain Fx el. The marine counterpart of *Flexibacter* may be *Microscilla*; no cellular shape change is known for this genus (Lewin, 1969; Lewin and Lounsbery, 1969; Pringsheim, 1951) It has not been well studied, and the status of the genus and its relation to the other Cytophagales is unknown.

The CLB are unquestionably a very large and heterogeneous assembly of terrestrial and marine bacteria. They will probably have to be distributed over several new genera, as discussed above. In the absence of a sound taxonomy, it is not even remotely possible at the moment to estimate the number of existing species. From DNA-DNA hybridization data and other studies it may be deduced that there are many more genera than are named so far. Some efforts have been made to establish a reliable taxonomy for the fish pathogens, which would obviously be of great practical importance. DNA-DNA hybridization data showed that the fish pathogens *Cy. columnaris*, *Cy. psychrophila*, and *Cy. maritima* are all well-separated, homogeneous, genomic species, which show little relatedness among themselves or to the many environmental species included in the studies (Baxa et al., 1987; Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989; Bernardet et al., 1990). There are, however, strong indications that these are not the only fish pathogens among the CLB, and that there are still other, hitherto undescribed species (Kent et al., 1988; Pyle and

Shotts, 1981). Further, the data seem to suggest that the fish pathogens are restricted to fish and cannot be isolated as free-living, environmental strains, although the latter possibility is difficult to rule out definitely. Information about an environmental reservoir of the fish pathogens would, of course, be of central importance for epidemiological considerations and control measures. Serological data speak also against a close relationship between fish-pathogenic and environmental CLB (Pacha and Ordal, 1970; Pacha and Porter, 1968). Serological tests have therefore been very useful for a quick diagnosis of the fish pathogens, which is important for practical reasons. Early studies showed that species-specific antigens exist in *Cy. columnaris* and *Cy. psychrophila* (Anacker and Ordal, 1959; Bullock, 1972; Pacha and Porter, 1968). *Cy. columnaris* has in addition at least seven type-specific antigens, which can be used to subdivide a collection of 325 strains into four serological groups; no link could be seen between those serological groups and levels of virulence (Anacker and Ordal, 1959; Pacha and Ordal, 1970). Fluorescence-labeled specific antibodies against *Cy. maritima* could be used to demonstrate the presence of the pathogen in various tissues (Baxa et al., 1988). Incidentally, the fish themselves develop antibodies against *Cy. columnaris*, and these seem to limit natural infection and can be applied to protection by vaccination (e.g., Becker and Fujihara, 1978; Liewes et al., 1982).

In another study, the isoenzyme patterns of 106 strains of spreading and nonspreading, yellow-pigmented bacteria from fish and other habitats were used to classify CLB. While one group coincided neatly with isolates that had been identified with *Cy. columnaris*, the other three groups were clearly heterogeneous, one of them even containing other *Cy. columnaris* isolates (Starliper et al., 1988). Thus, the results were inconclusive, and isoenzyme studies do not appear promising for taxonomic purposes.

The recently discovered genus *Taxeobacter* (H. Reichenbach, unpublished observations) has a special position among the soil Cytophagales. It is distinguished from the CLB by its cell morphology, the arrangement of the cells in spreading colonies (Figs. 1 and 3), and the brick red color of the latter. It is a common organism that can be cultivated without difficulties and has probably only been overlooked because it is not easily isolated by conventional techniques. By 16S rRNA cataloging, *Taxeobacter* (= Myx 2105), has been located in the *Fx. filiformis* side branch close to *Cy. heparina* (Paster et al., 1985).

As already mentioned, *Capnocytophaga* has been known for many years under different names. It is a fermenter requiring an atmosphere with 5% CO_2, at least for isolation, and produces mainly acetate and succinate from glucose. Initially only known from the human oral cavity, related species have since been found also in animals, e.g., in dogs (Brenner et al., 1989) and in the rice rat (Shklair and Ralls, 1988). The genetic homogeneity of the three human species, and the identity of one of them with *Bacteroides ochraceus* (type strain) has been established by DNA-DNA hybridization (Williams and Hammond, 1979; Williams et al., 1979).

Practical Aspects

The Cytophagales are of considerable practical interest. Their importance becomes even more impressive, if we take also into consideration the many isolates classified as *Flavobacterium* in the literature, which would be justified because those strains may often have been CLB with unrecognized gliding motility; and even if they were true flavobacteria, they would be closely related organisms. A general review of the practical aspects of gliding bacteria including the Cytophagales has been published (Reichenbach, 1988).

Bacteria belonging to this large complex of species are found virtually everywhere in nature, at least in aerobic and microaerobic environments. They definitely play a major role in the turnover of matter. Their activities are, of course, not always beneficial for humans. Some undesired effects with respect to the dairy industry (see also Cousin, 1982; Guamis et al., 1987) and the rotting of cellulose fabrics have already been mentioned. Other examples of this less pleasant side are: the participation of pectolytic CLB in the spoilage of vegetables (Liao and Wells, 1986; Lund, 1969) and of proteolytic CLB in the putrefaction of fish, although in the latter case, gliding bacteria may be associated with freshly caught fish and are gradually replaced by other bacteria during deterioration (Cho et al., 1984; Gennari and Tomaselli, 1988; Liston, 1960; Shewan, 1971). Also in nature the actions of CLB are not always beneficial. Thus, e.g., CLB have been found tunneling in the cell walls of seagrass leaves, apparently killing the leaves in the process (Porter et al., 1989). A case of symbiosis was discovered in calcareous sponges of the genus *Clathrina*; the bacterium may be sponge-specific and has been associated with the sponge for a long time (Burlando et al., 1988). There may yet be many more examples

of similar symbioses to be discovered. One such case may be the yellowish-brown bacterium living in the mycetomes of the granary weevil *Sitophilus granarius* (Bhatnager and Musgrave, 1970).

In a different context, the destruction of organic molecules may become a positive asset. The occurrence of large populations of CLB in sewage plants has already been discussed (see also Bauer, 1962). Cellulolytic *Cytophaga* and *Sporocytophaga* seem to play a role during composting of cattle manure (Godden and Penninckx, 1984). Many articles describe the decomposition by flavobacteria of recalcitrant chemicals such as pesticides, and, as just explained, some of these could really have been CLB. Thus, flavobacteria have been found to attack chlorobenzoic acid (Baggik 1985), pentachlorophenol (Brown et al., 1986; Crawford and Mohn, 1985), parathion (Mulbry et al., 1986), nylon oligomers (Negoro and Okada, 1982), biphenyl and phenanthrene (Stucki and Alexander, 1987), and aliphatic diols (Willetts, 1983), to give just a few examples. The digestion of hardwood mesquite (*Prosopis* sp.) with a cellulose-decomposing *Cytophaga* strain has been studied with the aim of improving the feed quality of the plant material for cattle (Chang and Thayer, 1975). It was shown that the *Cytophaga* protein is particularly rich in essential amino acids, but feeding experiments with mice gave unsatisfactory results.

The production by members of the Cytophagales of special enzymes for technical application could be another field of practical interest. Not too much is known about such enzymes, but a number of potentially useful hydrolase and lyases has indeed been discovered and more or less characterized. The following examples may illustrate this. The *Cytophaga* and *Sporocytophaga* species are very active cellulose decomposers and have been studied since the beginning of the century (e.g., Berg et al., 1972; Fåhraeus, 1947; Hutchinson and Clayton, 1919; Imshenetski and Solntseva, 1936; Krzemieniewska, 1930, 1933; Rivière, 1961; Sijpesteijn and Fåhraeus, 1949; Stanier, 1942; Winogradsky, 1929). Initially it was believed that enzymatic attack required close contact between cell and fiber, but later it was found that in the culture supernatant of *Sp. myxococcoides* there were also enzymes that could solubilize cellulose, at lest to a certain degree (Kauri and Kushner, 1985; Osmundsvåg and Goksøyr, 1975; Vance et al., 1980). With other organisms, however, cell-free cellulases could not be demonstrated (Chang and Thayer, 1977). Also, transglycosylations to β-1-3 and β-1-6 oligosaccharides (Charpentier, 1965), and a conversion of β-D-glucose into α-D-glucose by the activity of an exoglucanase (Charpentier and Robic, 1974) have been demonstrated. Relatively little is known about enzymes themselves, and a reinvestigation using modern methods would be desirable.

Another group of enzyme producers known for some time are the pectolytic CLB (e.g., Donderski, 1982; Dorey, 1959; Güde, 1973). The responsible enzyme is a randomly cleaving polygalacturonate lyase, which catalyzes a trans-elimination reaction; a pectin methyl esterase could not be found (Kurowski and Dunleavy, 1976; Sundarraj and Bhat, 1971). Only one single enzyme was found in *Cy. johnsonae*, and this enzyme was the smallest one (35 kDa) of all pectate lyases characterized (Liao, 1989). The pectolytic activity of *Cy. johnsonae* made water-stored spruce logs more permeable for preservatives and was therefore considered to be of practical relevance (Kurowski and Dunleavy, 1976; Ward and Fogarty, 1974).

Although chitin degradation is often observed among CLB and flexibacters, relatively little is known about the enzymes (Donderski, 1984; Reichardt et al., 1983; Stanier, 1947; Sundarraj and Bhat, 1972) The enzymes of *Cy. johnsonae*, a chitinase and a chitobiase, may be excreted or cell-bound. Deacetylation seems to be the first degradation step. The chitinolytic strain of *Cy. johnsonae* studied by Veldkamp (1955) was either a *Lysobacter*, judging from the strain deposited at NCIB (no. 8501), or a *Flexibacter*, as may be concluded from the published description of the organism.

Several enzymes from CLB that degrade polysaccharides derived from marine algae have been characterized, mainly agarases (Duckworth and Turvey, 1969a, 1969b, 1969c; van der Meulen and Harder, 1975, 1976), but also a porphyranase (Turvey and Christison, 167), and a large (100 kDa) extracellular carrageenase (Sarwar et al. 1987).

Many Cytophagales efficiently hydrolyze starch (e.g., Christensen, 1977a), but the enzymes never seem to have been studied. In two cases, special enzymes have been found in CLB because they had been screened for them for an application in structural studies. A strain was found and identified with *Cy. johnsonae* that produced endo-β-glucanases, which could then be used to study the composition of yeast cell walls (Bacon et al., 1970; Webley et al., 1967). The discovery of a strain that attacked heparin (Payza and Korn, 1956a, 1956b) proved a decisive contribution to the elucidation of the chemical structure of this complex heteropolysaccharide (e.g., Dietrich, 1969). The organism was first classified as *Flavobacterium he-*

parinum but later recognized to be a CLB (Christensen, 1980). Enzyme production has to be induced by the addition of heparin, and yields are not particularly good. Later, another (tentatively classified) *Flavobacterium* strain was isolated that produced heparinase constitutively and at a 10-fold higher level (Joubert and Pitout, 1985). The enzyme may be of considerable practical interest for the deheparinization of blood.

Many Cytophagales excrete strong proteinases. A few of these enzymes have been further characterized because of their unusual specificities. In a screening, strains were discovered that solubilized autoclaved feathers and wool and could grow on these substrates as the only C and N sources; they did not attack the native material (Christison and Martin, 1971; Martin and So, 1969). The organisms resembled *Cy. johnsonae* in their characteristics but were never clearly classified. The keratinolytic enzyme appears to be associated with acidic polysaccharides on the surface of the cells. *Capnocytophagas* show strong aminopeptidase activities (e.g., Nakamura and Slots, 1982). In a screening for arylaminopeptidases among oral bacteria, several different activities at high levels were discovered in capnocytophagas, including monopeptide-, dipeptide-, and tripeptide-cleaving enzymes, some of which were only seen in capnocytophagas, e.g., enzymes attacking ala-, leu-, his-, lys-, and met-β-naphthylamines (Suido et al., 1986). Such proteolytic enzymes could contribute to the occasional pathogenicity of the organisms. Also the fish-pathogenic CLB show very strong proteolytic activities, which may be responsible for at least part of their infectivity. In an effort to prepare compounds with immunoadjuvant activity from cell walls of *Staphylococcus epidermis*, an interesting endopeptidase was isolated from the culture supernatant of the gliding bacterium *Cytophaga* B-30 (Kawata et al., 1984). The identity of the organism is not unequivocally established, and the description does not rule out the possibility that it was really a lysobacter. The enzyme released long-chain polysaccharide peptides from *Staphylococcus*, but little or nothing from *Streptococcus* and *Micrococcus* peptidoglycan. In cells and in the culture supernatant of *Cytophaga* NCMB 1314, an agent was discovered, presumably an enzyme, that solubilized active cholinesterase from fish muscle (plaice, *Pleuronectes platessa*). Again, the identity of the organism is not completely clear. Release of this "S-factor" into the medium was stimulated by cultivation under magnesium limitation (Bovallius, 1978, 1979; Lundin, 1968; Lundin and Bovallius, 1966).

Substantial phospholipase A_2 activity has been seen in *Capnocytophaga* (*Cp.*) *ochracea* strains; it has been suggested that this enzyme is a factor contributing to the pathogenic potential of the organism (Sandholm et al., 1988).

Interesting immunological effects may be produced with certain cell components of the Cytophagales. The allergenic activity of *Cytophaga allerginae* endotoxin, leading to humidifier fever, a lung disorder, has already been mentioned; the chemical composition of the endotoxin has been determined (Flaherty et al., 1984; Liebert et al., 1984). The purified endotoxin proved to be a novel mitogen that induced peripheral blood lymphocytes in vitro to synthesize IgG and IgA; as the immunoglobin induction is not inhibited by cyclosporin A, it seems to be T-cell independent (Alevy and Compas, 1987). Another potent mitogen called gliding bacteria adjuvant (GBA) was recovered in relatively large quantities (140 to 400 mg/l) from the culture supernatant of the *Cytophaga* GB-2. The psychrotrophic bacterium was first isolated from contaminated fetal calf serum. GBA was free of endotoxin and protein and perhaps consisted of a complex slime polysaccharide. It stimulated mouse B lymphocyte proliferation in vitro, the production of immunoglobulins, and the release of colony-stimulating factor and interleukin 1 by macrophages (Shiigi et al., 1977; Usinger et al., 1985). Purified exopolysaccharide from *Capnocytophaga* cultures activated the human complement system in vitro (maximum effect at 28 μg/ml plasma); this activation may be a factor leading to tissue destruction in periodontal lesions (Bolton and Dyer, 1986). A large (155-kDa), lectinlike protein was isolated from the outer membrane of *Cp. gingivalis* (Kagermeier and London, 1986). The lectin appears to be specific for neuraminic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine, and it is probably responsible for coaggregation with other bacteria, like *Actinomyces israelii*, in the formation of dental plaque. Similar adhesins with specificities for rhamnose and fucose were found on *Cp. ochracea* (Weiss et al., 1987). A lactose-specific, lectinlike receptor was demonstrated on *Cp. sputigena* and *Cp. gingivalis* (Saito et al., 1988).

Some members of the Cytophagales have been found to produce very interesting secondary metabolites. In a screening for antibiotic activities, about 20% of the 270 tested strains showed inhibitory effects (Reichenbach et al., 1984). Several β-lactams were isolated from *Flexibacter* strains, including new monobactams with oligopeptide side chains (Cooper et al., 1983; Sing et al., 1983) and monobactams with formylamino- and glucuronic acid substi-

tuents, the formacidins (Hida et al., 1985; Katayama et al., 1985). Monobactams with a dehydroasparagine residue were obtained from *Cy. johnsonae* (Kato et al., 1987a, 1987b), and deacetoxycephalosporin C and 7-formylamidocephalosporins from a *Flavobacterium* (Shoji et al., 1984; Singh et al., 1982, 1984). *Cy. uliginosa* (formerly *Fv. uliginosum*) contributed marinactan, a heteropolysaccharide with marked antitumor activity in mice (Umezwawa et al., 1983).

Finally, there are some important medical and veterinary aspects of the Cytophagales: Flexibacteria are able to pass through membrane filters of 0.1-μm pore size used for sterilizing seawater; the contaminants could be eliminated only by pasteurization (Little et al., 1987). Immunological effects of cell components and products of diverse Cytophagales and resulting health problems have already been reviewed. A further example are the mitogenic and immunomodulatory effects exerted by cell wall fractions from a *Capnocytophaga* strain which had been isolated from a patient with juvenile periodontitis; the effects were also given by fractions that were free of LPS and peptidoglycan (Murayama et al., 1982). It is not known to what extent or whether *Capnocytophaga* contributes to periodontal disorders. Cytopathogenic effects were observed for "*Sphaerocytophaga*" (Gräf, 1962b, 1962c). These bacteria were present in large numbers in acute and chronic inflammations in the human oral cavity. In cell cultures (calf kidney cells), they caused cell lysis, and they destroyed human leukocytes in vitro. When inoculated subcutaneously into guinea pigs, they produced inflammations and ulcerations, but were not pathogenic for mice and rabbits when applied intraperitoneally and intravenously, respectively. It was found that neutrophils from patients with large *Capnocytophaga* populations in dental infections showed abnormal behavior, and that such behavior could be induced in normal neutrophils when they were exposed to sonic extracts of *Capnocytophaga* (Shurin et al., 1979). Sonic extracts of *Cp. sputigena* showed moderate toxicity for human gingival fibrablasts as indicated by inhibition of cell proliferation and thymidine incorporation (Stevens and Hammond, 1988). Heat-killed *Cp. gingivalis* cells elicited strong endotoxin reactions in the *Limulus* and the Shwartzman test (e.g., Fumarola et al., 1981). As capnocytophagas are usually found in large numbers in cases of severe destructive periodontitis, especially in juvenile periodontitis, and as they produce similar symptoms when inoculated into healthy gnotobiotic rats, they may indeed not be completely harmless, even if they are always present in the normal buccal flora (Forlenza and Newman, 1983). Perhaps they also play a certain role in the "piggyback" transport of nonmotile bacteria into periodontal pockets. While the situation appears complex and somewhat controversial with periodontal pathogenicity, the case is clear when general infections occur (see also Hawkey et al., 1984). Newly described *Cp. canimorsus* and *Cp. cynodegmi* have been considered to be responsible for septicemia and localized wound infections in humans after dog bites; the organisms seem occasionally also to be transmitted by cats (Brenner et al., 1989). Capnocytophagas respond well to antibiotics, like various β-lactams, quinolones, chloramphenicol, and tetracycline (see also Arlet et al., 1987).

As already discussed in the section on "Habitats," the fish-pathogenic CLB are showing a steadily increasing economic impact on fish cultivation and, to a lesser extent, also on wildlife, in fresh water as well as in seawater. *Cy. columnaris*, *Cy. psychrophila*, *Cy. maritima*, and *Fv. branchiophilum* appear to be the major pathogens and have been reported worldwide, but other species are probably also involved (see also Amin et al., 1988; Farkas and Oláh, 1984; Kuo et al., 1980, 1981; Kusuda and Kimura, 1982; Ostland et al., 1989; Spangenberg, 1975). The outbreaks of disease may follow a seasonal pattern (e.g., Kuo et al., 1981). This is easily understandable, for the fish, as well as the pathogen, usually have well-defined temperature optima; thus, for example, *Cy. columnaris* is a typical warm-water, *Cy. psychrophila* a cold-water pathogen (e.g., Snieszko, 1974). Losses due to infections by CLB can be severe and sometimes approach 100% within a few days. Individual strains of the same species may differ substantially in their virulence. It seems that the pathogens become a problem mainly when the fish are kept under suboptimal conditions. The bacteria are sensitive to certain antibiotics, e.g., chloramphenicol, tetracyclines, and erythromycin, but better suited for practical application are chemotherapeutics like nifurpirinol or Mefarol. As pointed out already, control of the disease may also be effected by vaccination or transfer of the fish to water with a higher or lower temperature or salinity, depending on the specific case. Numerous studies on the various aspects of fish diseases caused by CLB have been published in the last 40 years, and some references to specific topics are: *preconditioning by environmental factors*: Chen et al., 1982; Chowdhury and Wakabayashi, 1989a, 1989b; Hanson and Grizzle, 1985; Snieszko, 1974; *experimental infection*: Baxa et al., 1987b, Chow-

dury and Wakabayashi, 1989a; Kuo et al., 1987b; *histopathology*: Pacha and Ordal, 1967; and *control measures*: Amin et al., 1988; Deufel, 1974; Liewes et al., 1982; Ostland et al., 1989; Snieszko, 1953, summarizes the older literature.

Finally, it seems safe to predict that, beyond the examples given earlier, more diseases and disorders of aquatic animals other than fish will be discovered that are connected to CLB.

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CHAPTER 200

The Genus *Saprospira*

HANS REICHENBACH

Gram-negative gliding bacteria that form helical, multicellular filaments are grouped in the genus *Saprospira* (Fig. 1). All known species live in aquatic environments. They are moderately common but relatively difficult to isolate, and therefore have not been studied very well, although some of them can be cultivated without problems. Relatively little has been added to our knowledge of *Saprospira* since the first edition of *The Prokaryotes* (Reichenbach and Dworkin, 1981).

The genus *Saprospira* was defined by Gross (1911). He described two marine species, *S. grandis* and *S. nana,* of which only the former has since been isolated by other investigators. The two organisms differed considerably in their dimensions, as shown in Table 1, which lists some characteristics of the known strains of the genus.

It seems, however, that at least three other investigators had observed saprospiras before Gross. Kolkwitz (1909) described a freshwater saprospira under the name of *Spirulina albida.* He obviously believed his organism to be an apochlorotic blue-green "alga" (cyanobacterium).

In 1875, van Tiegham (1880) observed a delicate white scum that resembled *Beggiatoa* covering the mud in the water course of an old mill. Under the microscope, he discovered that the organism consisted of long, fine, helical filaments that were so tightly wound up that the coils touched. The helices moved by rotation around their long axis. The long filaments were also actively bending. He called this organism *Spirulina alba,* thereby deliberately emphasizing its close affinity to the blue-green algae of the same genus. Although his description is rather scant, it seems almost certain that he was dealing with a *Saprospira* species.

In the same year, Warming (1875) observed a very large helical organism in marine debris from the Danish coast and named it *Spirochaeta gigantea.* From his careful description, one can deduce that this organism, too, may have been a *Saprospira* species.

Not much was published in the five decades following the definition of the genus. In addition, until the middle of the 20th century, *Saprospira* was regularly regarded as belonging to the spirochetes, which caused considerable confusion. Gross (1912) himself stressed the similarity between *Cristispira,* which was also first defined by him, and *Saprospira.* To him and most other investigators at the time, the latter was essentially a spirochete without a crista or an axial filament. Unfortunately, in stained preparations, both organisms also showed a multichambered aspect, which was in fact a staining artifact but obscured the difference in organization of the two bacteria. Dobell (1912) described a new freshwater species, *Saprospira flexuosa.* From the careful description given by Dobell, one may deduce that his organism really was a *Saprospira,* perhaps *S. albida* as suggested by Lewin (1962), although this point cannot be decided because we do not know how many different freshwater saprospiras exist. During a study on helical bacteria in the digestive tract of oysters, Dimitroff (1926) observed several types of organisms which he regarded as saprospiras. One he identified with *S. grandis,* the others he described as new species, *S. lepta* and *S. puncta.* His account of the movements of his isolate of *S. grandis* leaves considerable doubt whether the bacterium was really *Saprospira.* On the other hand, he observed true cristispiras under the microscope and distinguished their very fast movements quite clearly from those of *Saprospira.* If one takes into account the fact that before the days of the phase contrast microscope, it was not so easy to observe living bacteria, it is easier to understand this uncertainty. It might be worthwhile to investigate again for the presence of saprospiras in shellfish. The two new species could never be observed by Dimitroff in the living state so their motility behavior is not known. They both had long, tapering ends with a sharp point, which is not known for any *Saprospira* species. The giant, gliding "spirochete" observed by Soriano in an enrichment culture and very briefly mentioned by him in the legend to a rather suggestive draw-

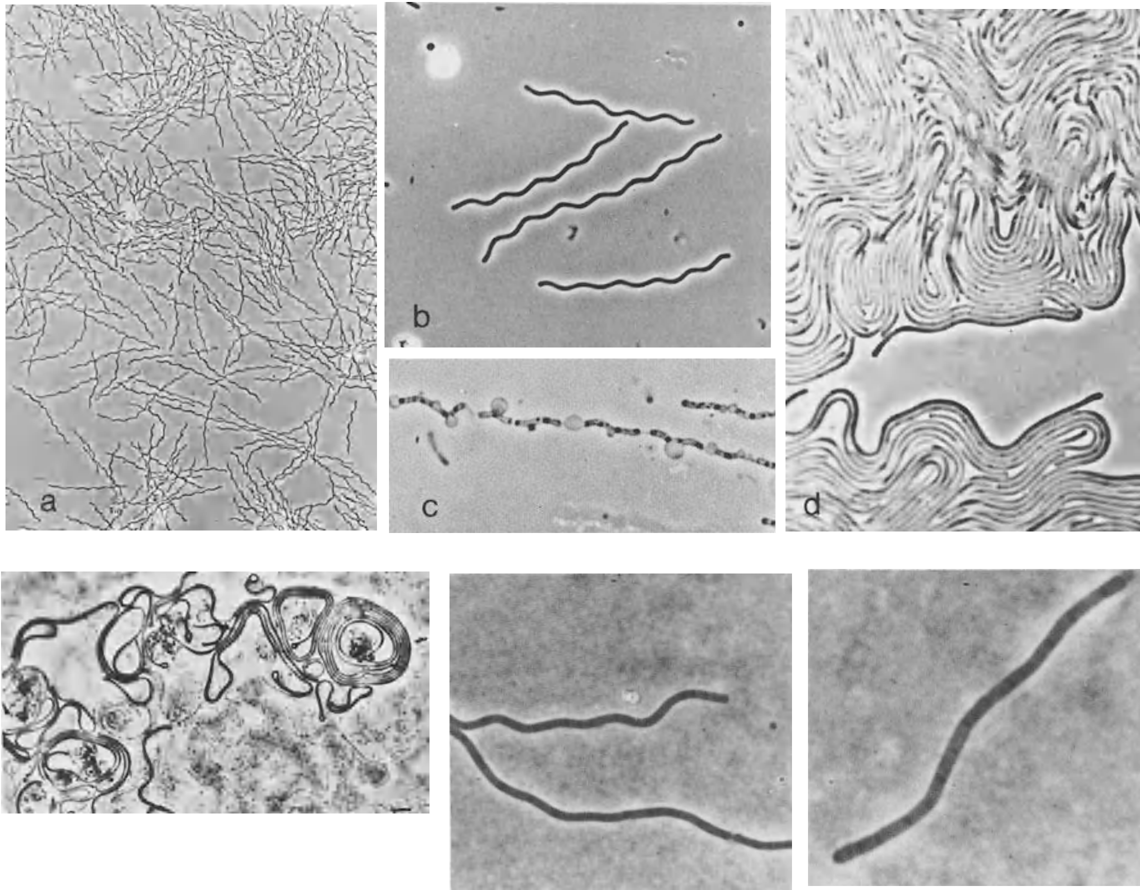

Fig. 1. Phase contrast photomicrographs of *Saprospira grandis.* (a) Helical filaments from a 1-day-old liquid culture, survey picture; × 180. (b) Filaments at higher magnification; × 500. (c) Decaying filament showing the individual cells; × 900. (d) Growing in the narrow space between agar and cover glass in a chamber culture, the filaments have lost most of their helical shape and concomitantly their ability to glide; × 920. (Lower left) Nonhelical filaments in a chamber culture with large, optically refractile, terminal bulbs; × 470. (Lower center and lower right) At very high magnification (× 1000 and × 1200; Zeiss Axiomat) the cross-walls in the filaments are visible. The lower filament shows a constriction near one end, perhaps one possible way of filament fragmentation.

ing (Soriano, 1945), was probably a (freshwater) saprospira. Dyar (1947) described the isolation and cultivation of a freshwater "spirochete." This organism resembled *Saprospira* in many respects, and indeed was later identified with *S. albida* by Pringsheim (1963). Skuja (1948) described the new species, *Achroonema spiroideum,* from Swedish lakes. The organism was later equated with *S. albida* (Lewin, 1962), but the very lax coils typical for *Achroonema spiroideum* leave some doubts whether this assignment is correct.

Many details about the morphology and physiology of the saprospiras were elucidated between 1960 and 1970 through the research of Lewin and his collaborators, who also defined several new species and emended the taxonomic position of the genus (Lewin, 1962, 1965a, 1965b, 1969, 1972; Lewin and Lounsbery, 1969; Lewin and Mandel, 1970). The phylogenetic relationship of *Saprospira grandis* was recently established by 16S rRNA oligonucleotide cataloging: The organism belongs to the *Bacteroides-Flavobacterium-Cytophaga* phylum (Paster et al., 1985). A relationship with the cyanobacterium *Spirulina* has specifically been ruled out (Reichenbach et al., 1986).

A short review of the genus *Saprospira* has recently been published (Reichenbach, 1989). A movie showing the movements and the development of the swarm colonies of *Saprospira* is also available (Reichenbach et al., 1975/1976; Reichenbach, 1980).

Habitats

The saprospiras appear to live exclusively in aquatic environments. *S. grandis* and other marine species have been isolated from sand and

Table 1. Characteristics of the *Saprospira* strains.

Strain	Morphological characteristic (μm)[a]					GC content (mol%)	Habitat
	Length of filament	Diameter of filament	Width of helix	Pitch of helix	Length of cell		
S. grandis, Gross 1911	6–100	0.8		6–6.5	1.5–2.2		Marine
S. grandis, Lewin 1962	10–500	0.8–1.2	1.5–2	4–10	1–2.5	46–48	Marine
S. grandis, Dimitroff 1926[b]	50–90	1.2–1.4[c]		20–28			Oyster, marine
S. grandis, Reichenbach 1980	15–450	0.8–0.9	1.4–1.8	5–6.5	2.7–5.5	47	Marine
S. nana, Gross 1911	36	0.5		2.3–3	1.5–3		Marine
S. gigantea, Warming 1875	About 400	1.5–3	5–9	25–40			Marine
S. toviformis, Lewin and Mandel 1970	10–500	0.8	1.5	4–9	1–2.5	38	Marine
S. lepta, Dimitroff 1926[b,d]	54–92	0.5	1.6–4.8	5–13			Oyster, marine
S. puncta, Dimitroff 1926[b,d]	60–100	0.9–1.2		4–8			Oyster, marine
S. albida, Kolkwitz 1909		1	4–5				Fresh water
S. albida, Dyar 1947	About 400		2	3–6.5			Fresh water
S. albida, Lewin 1965b	10–500	0.8–1.2	1.5–2	3–7	2–3	40–43	Fresh water
S. albida, Ashton and Roberts 1987	10–450	0.8–0.9	1.4–1.6	2.9–4.9	2.1–2.7		Fresh water
S. flexuosa, Dobell 1912[e]	3–50	0.8	2	3			Fresh water
S. spiroidea, Skuja 1948[f]	10–250	0.3–0.5	2.8–5.7	9–25	3–15		Fresh water
S. flammula, Lewin 1965b	10–500	1.0	1.5	3–4	2–3	48	Fresh water
S. thermalis, Lewin 1965a	10–500	1.0	1.5–2.5	7–17	2–5	35–37	Fresh water

[a]In the older literature, measurements were often taken from fixed and stained specimens which may have been considerably distorted.
[b]From the description by Dimitroff it cannot be decided with confidence whether his organisms were true saprospiras.
[c]It is not clear whether Dimitroff was talking about the width of the filament or the width of the helix.
[d]With tapering ends.
[e]Probably actually *S. albida.*
[f]*Achroonema spiroideum* was equated with *S. albida,* but it forms a very lax helix, and, in this respect, it resembles *S. thermalis* more closely than *S. albida.*

mud, and collected at the sea coast at many different locations all over the world (Gross, 1911; Lewin and Lounsbery, 1969; Lewin and Mandel, 1970). One strain of *S. grandis* has been obtained from a rotting crab carapace (Reichenbach, 1980). As discussed above, several species may possibly be found within oysters (Dimitroff, 1926). Saprospiras are common also in fresh water (Lewin, 1965b). They seem to prefer eutrophic conditions and are often associated with algae. Saprospiras have been obtained from the mud in rivers and lakes (Kolwitz, 1909; Pringsheim, 1963), from planktonic algae and cyanobacteria, as well as from the upper layers of surface waters, in which the filaments may be free-floating (Dobell, 1912; Skuja, 1948; Brunel, 1949; Ashton and Robarts, 1987), from decaying organic matter in a sulfur spring (Dyar, 1947), from a bog (Jarosch, 1967), and from sewage plants, including activated sludge (Kolkwitz, 1909; Cyrus and Sladká, 1970; Sladká and Ottová, 1973). *S. thermalis* was first isolated from a hot spring in Iceland, but the organism is not thermophilic and in fact not even thermotolerant, for its maximum temperature is 35–37°C (Lewin, 1965a). Only one strain of *S. albida* was ever isolated from soil. The sample came from Canada, but unfortunately nothing was said about the water conditions of that soil (Lewin, 1965b).

Interesting details about the interaction of a saprospira species with the cyanobacterium *Microcystis aeruginosa* in a South African reservoir have been published (Ashton and Robarts, 1987). Usually *Saprospira* is found in the surface layers of the lake at a very low density of less than 10 filaments per liter, but during *Microcystis* blooms, its density increases to more than 10,000 filaments per liter, and the saprospiras colonize and lyse the floating colonies of the cyanobacterium. Curiously, the *Saprospira* seems selectively to attack a toxic variant of *M. aeruginosa,* which is dominant at the site for most of the year. It has been suggested that saprospiras could be useful to control this undesired organism.

Isolation

No specific enrichment methods are known for saprospiras. Gross (1911) described a collection technique that makes use of the tendency of *Saprospira* to attach itself to a substrate surface. He filled a large petri dish with a sample of marine sand, covered it with sea water, placed coverslips on the water surface, and left them floating overnight. Most of the saprospiras stuck to the glass surface and could be removed from the crude culture.

To isolate saprospiras, samples are taken from appropriate habitats (see above) and placed as well-separated streaks or spots onto the surface of dry agar plates. To prevent excessive growth of contaminants, the media should contain nutrients in low concentrations only. For marine samples, MS1, SW2, or SP6 agar can be used. It appears that for saprospiras, natural and artificial seawater are equivalent, so that the following media could be prepared with either one.

MS1 Agar

> Agar 1.5%
> Natural seawater
>
> The pH is not adjusted. The medium is autoclaved.

SW2 Agar

Agar	1.5%
NH_4Cl	0.1%
Na acetate	0.002%
Artificial seawater (see below)	

The pH is adjusted to 7.2. The medium is autoclaved.

Artificial Seawater (Dawson et al., 1972)

> The following recipe has been used by us with good results: NaCl, 24.7 g; KCl, 0.7 g; $MgSO_4{\cdot}7H_2O$, 6.3 g; $MgCl_2{\cdot}6H_2O$, 4.6 g; $CaCl_2$ (anhydrous), 1.0 g; $NaHCO_3$, 0.2 g. Bring to 1 liter with water. To avoid precipitation, $CaCl_2$ and $NaHCO_3$ are autoclaved as separate stock solutions.

SP6 Agar

Casitone (Difco)	0.03%
Yeast extract (Difco)	0.01%
Agar	1.5%
Artificial seawater (see above)	

The pH is adjusted to 7.2. The medium is autoclaved.

The freshwater saprospiras are often more fastidious, and most investigators were only able to maintain their isolates for longer periods in mixed cultures, e.g., in fouling soil suspensions such as soil enriched with proteinaceous material in water (Pringsheim, 1963). Repeatedly, it has been found that small quantities of H_2S are stimulatory, although the organisms do not really depend on it (Dobell, 1912; Dyar, 1947). Perhaps the good results with Pringsheim's technique were also due to the production of H_2S from the added protein. Dyar (1947) seems to have been the first to succeed in isolating and cultivating a strain of *S. albida.* She used a leaf-infusion agar with a few drops of blood for isolation.

Several freshwater saprospiras were isolated by Lewin using LEW1 agar (Lewin 1965a, 1965b).

LEW1 Agar (Lewin, 1965b)

$Ca(NO_3)_2{\cdot}4H_2O$	0.05%
$MgSO_4{\cdot}7H_2O$	0.01%
K_2HPO_4	0.01%
Tryptone (Difco)	0.01%
Agar	1%

Yeast extract (Difco) can also be used in place of tryptone.

The crude cultures are incubated at 30°C, room temperature, or in the refrigerator, depending on the starting material. For freshwater strains, 23–27°C is recommended (Lewin, 1965a). Although marine *S. grandis* has a temperature optimum around 30°C, it is useful to keep crude and enrichment cultures at 6°C, since the organism still grows and spreads reasonably well at that temperature, while the growth of contaminants is considerably reduced (H. Reichenbach, unpublished observations). The crude cultures are examined from time to time under a dissecting microscope. Oblique illumination should be applied to make delicate, spreading colonies also visible. Already after 3 to 4 days, or, at lower temperatures, after a longer interval of up to 2 to 3 weeks, spreading colonies with long, rootlike tongues (Fig. 2) or with irregular, flamelike edges may be found. This is the typical appearance of colonies of

gliding bacteria. *Saprospira* colonies often also show a very regular striped pattern (Dyar, 1947; Reichenbach et al., 1975/76; Reichenbach, 1980), which is seen with other screw-shaped bacteria, e.g., *Methanospirillum hungatii* (Ferry et al., 1974). A microscopic control should show the typical *Saprospira* filaments (Fig. 1) and their characteristic movements (Reichenbach et al., 1975/76). Further purification is achieved by making transfers from the spreading edges of the swarm colonies; it is best to cut out a piece of the colony using the sharp point of a 1-ml disposable syringe. The material is streaked on the same medium as before, or, as soon as the colonies appear sufficiently pure, also on a richer medium e.g., SAP2 agar for marine, and LEW2 and LEW3 agar for freshwater strains.

SAP2 Agar

Tryptone (Difco)	0.1%
Yeast extract (Difco)	0.1%
Agar	1.5%
Artificial seawater (see above)	

The pH is adjusted to 7.2. The medium is autoclaved.

LEW2 and LEW3 Agar (Lewin, 1965a).

The recipe is the same as for LEW1 agar, but the tryptone concentration is raised to 0.03% and 0.3%, respectively.

Finally, diluted filament suspensions may be plated on the richer versions of the above-mentioned media. The most troublesome contaminants are other gliding bacteria and, in marine samples, agar decomposers. Both groups should be eliminated at the very beginning of the enrichment process.

Cultivation

All *Saprospira* strains that have been studied so far are strictly aerobic organotrophs and seem to prefer amino acids as carbon, nitrogen, and energy sources, often without an alternative. Many marine strains grow well on relatively simple media, such as SAP1 or SAP2 agar.

SAP1 Agar (Lewin, 1962)

Tryptone (Difco)	0.5%
Yeast extract (Difco)	0.5%
Agar	1.5%
Artificial seawater (see above)	

The pH is adjusted to 7.2. The medium is autoclaved.

The marine saprospiras can be cultivated without difficulties and with good yields in liquid media, such as SP5 liquid medium or SAP1 liquid medium (as for SAP1 agar, but without agar). They usually form homogeneous suspensions in shake cultures.

SP5 Liquid Medium

Casitone (Difco)	0.9%
Yeast extract (Difco)	0.1%
Artificial seawater (see above)	

The pH is adjusted to 7.2. The medium is autoclaved.

An almost fully defined medium is reported for *S. grandis* (Lewin, 1972). It is composed of nine essential amino acids, mineral salts, and an unknown growth factor present in yeast nucleic acid hydrolysate. The marine saprospiras almost always require at least half-strength sea water, but *S. toviformis* and a few *S. grandis* strains also grow at freshwater salt concentrations (Lewin and Lounsbery, 1969).

Among the freshwater saprospiras, *S. thermalis* is the least fastidious one and can be grown on relatively simple media, e.g., LEW3 agar (see above) and in LEW4 liquid medium. A synthetic medium also has been developed (Lewin, 1965a).

LEW4 Liquid Medium (Lewin, 1965a)

Casamino acids (Difco)	0.1%
Sodium glutamate	0.1%
Glucose (sterilized separately)	0.1%
Tris (hydroxymethyl) aminomethane	0.1%
$MgSO_4 \cdot 7H_2O$	0.01%
KNO_3	0.01%
KNO_3	0.01%
$CaCl_2 \cdot 2H_2O$	0.01%
Sodium glycerophosphate	0.01%
Thiamine	1 mg/liter
Cobalamine	1 μg/liter
Fe	0.5 mg/liter
Zn	0.3 mg/liter
B, Co, Cu, Mn, Mo	0.1 mg/liter each

The pH is adjusted to 7.5. The medium is autoclaved.

Good growth was also obtained in a medium containing the mineral salts (and glycerophosphate) of LEW4 liquid medium plus tryptone (Difco) 0.02% and glucose 0.02%. If the tryptone is replaced by casamino acids, the addition of the vitamins became necessary (as in LEW4 liquid medium). The casamino acids in turn can be replaced by 0.2% sodium glutamate and three essential amino acids, i.e., leucine (50 mg/liter), isoleucine (50 mg/l), and valine (100 mg/liter). Growth was substantially faster when tyrosine (100 mg/liter) or phenylalanine (250 mg/liter) were also added.

The freshwater saprospiras of the *S. albida* type are more difficult to cultivate. Dyar (1947)

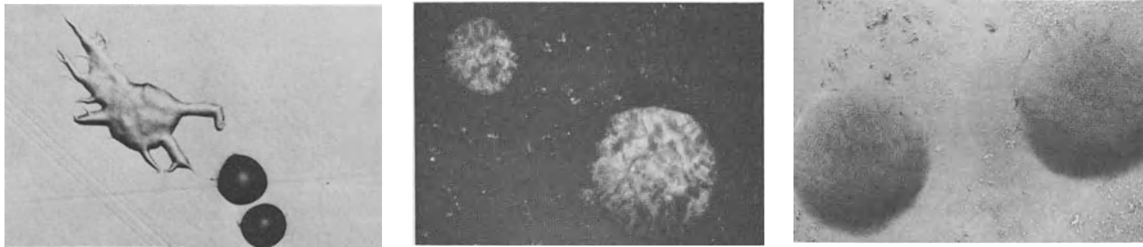

Fig. 2. Colonial morphology of *Saprospira grandis*. (Left) Colony on peptone-seawater agar together with two colonies of a nongliding organism. The *Saprospira* colony is rather tough and remains relatively compact; it spreads mainly along the grooves scratched into the agar surface by the inoculation loop. Leitz Aristophot; × 7.2. (Middle) Compact colonies on SP2 agar, in dark field; × 33. (Right) Colonies under oblique illumination; × 33. In both cases, the appearance of the colonies suggests some orderly arrangement of the filaments; in bright field, a pattern of fine, parallel, concentric stripes can be seen; Zeiss Axiomat.

could grow pure cultures on blood agar containing 5–10% erythrocytes and 1–1.5% agar. The organism did not grow if blood serum or heat-sterilized blood was used, which seems to indicate that it required either a heat-labile growth factor or enzyme in erythrocytes. Lewin (1965b) recommended a semisolid medium, LEW5 agar, for those strains, but even then they grow rather slowly.

LEW5 Agar (Lewin, 1965b)

Tryptone (Difco)	0.1%
Yeast extract (Difco)	0.1%
Gelatin (Difco)	0.1%
Glucose (sterilized separately)	0.1%
Tris buffer	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
KNO_3	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
NaCl	0.1%
Sodium glycerophosphate	0.01%
Fe	0.5 mg/liter
Zn	0.3 mg/liter
B, Co, Cu, Mn, Mo	0.1 mg/liter each
Agar	0.25%

The pH is adjusted to 7.5. The medium is autoclaved.

Unlike the other freshwater saprospiras, *S. flammula* is stimulated by 0.3% NaCl. It does not grow on casamino acids alone, but can be cultivated on a medium containing 0.5% tryptone, 0.3% NaCl and 0.25% agar (Lewin, 1965b).

Most saprospiras grow well at 30°C. Many strains even grow at 40°C (Lewin and Lounsbery, 1969). The temperature optimum is near 25°C for (marine) *S. toviformis* (Lewin and Mandel, 1970), 26°C for (freshwater) *S. albida* (the range is still 15–34°C; Dyar, 1947), and about 37°C for (marine) *S. grandis* (Lewin, 1962). *S. grandis* survives 46°C for 30 min (Lewin, 1972). This high temperature optimum of *S. grandis* is somewhat surprising, for the organism usually is found in a relatively cold environment. Thus, strain Sa gl was isolated at Roscoff, Brittany, where the Atlantic Ocean rarely reaches 17–18°C and is much colder most of the year. This strain also grows rather well at 6°C, and thus has a very wide temperature range.

The optimal pH is around neutral, and the range in which growth is possible is 6.5 to 7.5 and occasionally slightly beyond. The doubling time of *S. grandis* is 2 to 3 h at 30°C in SAP1 liquid medium (Lewin, 1962, 1972), that of *S. thermalis* 6 to 7 h at 30°C (Lewin, 1965a). A yield of about 250 mg dry cell mass per liter has been found with *S. thermalis* (Lewin, 1965a). With *S. grandis* in SP5 liquid medium at 30°, after 24 h of cultivation we obtained 12.5 g bacteria per liter by wet weight, corresponding to 2.5 to 3 g dry weight. Aasen and Liaaen-Jensen (1966a) harvested 400 g wet cells, giving 51.5 g freeze-dried bacteria, from 200 liters of culture broth after 20 h at 30°C.

Preservation

Cultures of *S. grandis* strain Sa gl on SAP2 agar remain viable for 2 to 3 weeks at room temperature (21°C). Lysis within 3 days at 30°C, and within a few weeks at 25°C, on all tested liquid and agar media, has been reported for other strains (Lewin, 1962). Dyar (1947) found that her *S. albida* survived on blood agar for about one month but died after a short time in the refrigerator.

Filaments of *S. grandis* strain Sa gl can be suspended in SP5 liquid medium and frozen at −80°C or in liquid nitrogen without any further precautions. We were able to revive both cultures without difficulty after 2.5 years, the longest period tested so far. They will certainly survive for a much longer time. We had no success

with drying in skim milk (H. Reichenbach, unpublished observations).

Sanfilippo and Lewin (1970) tested several freezing techniques with 27 strains of five *Saprospira* species. The longest survival times they found for cultures frozen at −22°C was 2 to 3 weeks (only marine species). In media containing 10% glycerol, all marine strains survived in liquid nitrogen for 1 year, the longest period tested. Of the freshwater species, only some strains of *S. thermalis* could be preserved in this way. Dimethyl sulfoxide (DMSO, 10%) proved toxic for all saprospiras: all strains were killed within 24 h at room temperature.

Characterization

The saprospiras form helical, multicellular, unbranched filaments (see Fig. 1) that move by gliding when in contact with a substrate surface. The length of the filaments is not fixed and varies considerably, even within one culture. The filaments tend to be longer in liquid media than on agar plates. *S. grandis* strain Sa gl, e.g., measures 15–130 μm when grown on a plate, and 20–450 μm when grown in a liquid medium of the same composition. Similar figures have been reported for other strains and species (see Table 1). The diameter of the filaments is relatively constant and usually around 1 μm.

The filaments are composed of cylindrical cells that are 1–5 μm long and closely attached to one another. No constrictions are visible at the surface of the filaments at the sites of the septa. The cells at the ends of the filaments are rounded, but otherwise do not seem different from the rest. (As mentioned above, two dubious species with pointed filaments have been described from oysters; Dimitroff, 1926). The length of the cells within one filament is variable, which suggests that the cells divide along the whole filament and independently from one another. With a good microscope and applying phase or interference contrast, the cross-walls can be seen in the living filament. Also, darkfield microscopy shows the septa clearly (Dyar, 1947; Jarosch, 1967). The cell boundaries become more apparent when the filaments begin to disintegrate (see Fig. 1c). Drying of the filaments to the slide may be sufficient to induce fragmentation (Dyar, 1947). Further, the multicellular nature of the screws can be seen after staining, e.g., with an I-KI solution (Skuja, 1948) or with Heidenhain's hematoxylin (Gross, 1911). Other staining methods often used in the older literature, e.g., with safranine, borax carmine, or methylene blue, also produced chambered filaments in *Saprospira*, but this may sometimes have been an artifact due to shrinkage of the protoplast, for true spirochetes also showed such a chambered appearance (Dobell, 1912; Dimitroff, 1926). Another identified artifact was an "axial filament" in heavily stained mounts of *S. albida* (Dyar, 1947). Of course, the septa are easily recognizable in thin sections under the electron microscope (Lewin, 1962). Also, in the election microscope, a typical Gram-negative cell wall with a thin peptidoglycan layer and an outer membrane can be seen. As usual, the septa consist only of the two cytoplasmic membranes and the peptidoglycan layer. The filaments multiply by breaking in two, apparently sometimes at the sites of necridia, i.e., dead cells (Gross, 1911; see also Chapter 209 on *Herpetosiphon*). It seems that reproduction by simultaneous fragmentation into many short, one- or few-celled pieces can also take place (Gross, 1911, 1912; Dobell, 1912; Lewin, 1962, 1965b). The short pieces move only very slowly if at all, but with growing length their movements speed up again (Dobell, 1912; Skuja, 1948).

The helix of *Saprospira* usually is relatively loose (Fig. 1). Its pitch is 3–10 μm, and its width is 1.5–2.5 μm. Some species show especially lax helices, e.g., *S. thermalis* and *S. (Achroonema) spiroideum.* But pitch and width are not constant features of a strain and can vary somewhat even within one culture. With the freshwater species, the helices appear usually to be coiled sinistrally (Lewin, 1965b), while the marine strains are coiled dextrally (Lewin and Mandel, 1970). The screw shape of the filaments may completely disappear during cultivation, in which case the organism would be difficult to recognize as a *Saprospira* (Dyar, 1947; Lewin, 1965b). Thus, the very long filaments seen in liquid cultures generally form only rudimentary and irregular helices. Also after improper fixation (e.g., with picric acid) the filaments tend to straighten out (Gross, 1911). In chamber cultures when restricted to the narrow space between the agar surface and the cover glass, *S. grandis* grows in the shape of very long, meandering filaments that are completely straight and, interestingly, also nonmotile (Fig. 1d; Reichenbach et al., 1975/1976; Reichenbach, 1980).

In such chamber cultures of *S. grandis,* filaments can sometimes be seen with the end cells inflated into spherical, optically refractile bulbs of 1.4–3 μm diameter (Fig. 2; Reichenbach, 1980). Those bulbs are probably a degeneration phenomenon but should not be confused with the spheroplasts that are found in old cultures all along the filament (Dyar, 1947), or can be produced by adding a 5 to 10% NaCl solution

("blisters"; Dobell, 1912). Filaments of *S. grandis* from liquid cultures appear to be very sensitive to changes in environmental conditions. Simply mounting them on a slide is sufficient to induce a decay of the filament and the formation of spherical protrusions at the surface of the cells (Fig. 1c).

Inside the cells of *S. albida* many highly refractive granules can be seen, sometimes extending from one side of the cell to the other (Dobell, 1912; Dyar, 1947). Dyar distinguishes two types: 1) volutin granules, i.e., polyphosphate, which show metachromatic staining with methylene blue and dissolve in (hot) water or in 0.02% $NaHCO_3$ solution; and 2) lipid globules, which stain with Sudan black B. The latter could well consist of poly-β-hydroxybutyrate. The inclusions are also seen under the electron microscope (Dyar, 1947). They appear to be relatively rare in *S. grandis* (Lewin, 1962; Lewin and Kiethe, 1965).

Gross (1911) mentioned that, in aging cultures of *S. grandis,* series of cells with increased stainability could occasionally be seen within filaments. Such cells later became spherical and surrounded themselves with a heavy wall. He suggested that those cells were spores, although he could not observe any further development. This observation has not yet been corroborated by other investigators.

A DNA phage of *S. grandis* with an icosahedral head, a contractile tail sheath, and rigid tail spines has been isolated and characterized (Lewin et al., 1964). Peculiar rod-shaped particles with long, wicklike tails, found in lysing cultures of *S. grandis* (Lewin, 1963; Lewin and Kiethe, 1965; Reichle and Lewin, 1968) and of the freshwater species *S. albida* and *S. flammula* (Lewin, 1965b) have been described as "rhapsidosomes." The preparations showed no infectivity whatsoever. Initially it was thought that the particles consisted of a ribonucleoprotein containing a highly 2′-*O*-methylated RNA (Correll and Lewin, 1964; Correll, 1968). This, however, turned out to be an analytical error, for the particles are composed exclusively of protein (Delk and Dekker, 1969, 1972; Price and Rottman, 1970). Very similar particles were later found in a myxobacterium and identified as the tails of a defective phage (Reichenbach, 1967). The *Saprospira* particles very probably have the same origin (Delk and Dekker, 1972). In fact, defective phage tails are rather common in bacteria, and many examples are known today. Unfortunately, the term rhapidosome was later also used for rod-shaped particles of a totally different morphology and nature, leading to unnecessary confusion (e.g., Pate et al., 1967; Yamamoto, 1967; Baechler and Berk, 1972).

In autolysed cultures of *S. thermalis,* but not of any other *Saprospira,* cell-wall fragments with a periodic hexagonal substructure are seen under the electron microscope, obviously remnants of a surface layer (Lewin, 1965b).

The helical filaments of *Saprospira* move by gliding when in contact with a suitable substrate surface, e.g., an agar or glass surface. The speed of *S. grandis* at room temperature (25°C) is 50–120 μm/min, and the movement is thus very obvious (Lewin, 1962). A speed of 120–180 μm/min has been determined for *S. thermalis* (Lewin, 1965b), one of 600 μm/min for *S. spiroidea* (Skuja, 1948), and one of only 14 μm/min for *S. flexuosa* (Dobell, 1912). The gliding screws rotate around their long axis, and are also able to bend actively. Bending is particularly conspicuous when the filaments are suspended in a liquid. The helices move equally well in both directions and may quickly and frequently reverse the sense of their movement. As already mentioned, loss of the helical arrangement of the cells leads to a loss of translocation. The locomotory machinery still seems to be operative, however, since tiny particles stuck to the surface of the filaments can be seen to migrate back and forth over a limited distance, presumably the length of a cell (Reichenbach et al., 1975/1976). Obviously, the proper spatial arrangement of the cells in the helix is a prerequisite for their efficient cooperation during movement (Reichenbach, 1980). As with all other gliding bacteria, the mechanism of locomotion is not understood in *Saprospira.* A movement of Indian ink particles on a helical path along the surface of filaments of *S. albida* has been explained by the movement of a helical slime belt that could also be responsible for the translocation of the filaments (Jarosch, 1967), but what moves the slime, is still an open question. A sticky surface is, no doubt, essential for gliding motility, and the surface of *Saprospira* is indeed very sticky (Gross, 1911). Slimy material has been isolated from viscous, nonlysing liquid cultures of *S. thermalis* (27 mg/liter), and various sugars have been provisionally identified in the hydrolysate (glucose, xylose, rhamnose, traces of galactose and mannose; Lewin, 1965a).

The colonies of *S. grandis* on rich agar media are compact with entire edges and of a rather tough consistency (Fig. 2). On dilute media, however, the colonies spread, albeit often slowly and to a much lesser extent than the swarms of many other gliding bacteria. Long, unbranched, rhizoidal tongues may radiate from the edges of those colonies (Fig. 2). In other cases, the swarms are thin and filmlike (e.g., *S. grandis* on SP6 agar). The spreading colonies and, less

so, also the compact ones, show a surprising and very regular pattern of narrow stripes (Fig. 3). The striations are particularly impressive under dark-field illumination. As already mentioned, the phenomenon was first described for *Saprospira* by Dyar (1947) and is also known for other, unrelated, helical bacteria. It may be explained by an exact alignment of the screws (Fig. 3d). The optical pattern seems to arise from differences in light scattering in the rising and falling gyres of the helices, perhaps in combination with an interference effect. Colonies of *S. grandis* Sa gl show a periodicity of the stripes of 7.6 to 8.6 μm, while the pitch of the helices in suspension is 4.9 to 6.5 μm. Apparently, the helices are slightly distorted on the plate.

Relatively little is known about the chemical composition of *Saprospira.* The respiratory chain contains menaquinone MK-7 exclusively (M. D. Collins, personal communication). While initial studies seemed to indicate an *o*-type cytochrome as the terminal oxidase (Webster and Hackett, 1966), it was later found that *S. grandis* has four functional cytochromes, one of which has an unusual maximum at 603 nm (77°K) in the difference spectrum, and that an a,a_3-type cytochrome is most probably the oxidase (Dietrich and Biggins, 1971). NADH-oxidation by *Saprospira* particles is insensitive to antimycin A, rotenone, and amytal, but it is inhibited by cyanide and HOQNO.

The GC content of the DNA of the various species is 35–48 mol% (see Table 1).

The saprospiras are colored in shades of yellow, orange, pink, and red. The pigments are cell-bound. Initial studies provided a preliminary characterization of the pigment patterns by thin-layer chromatography, and of the pigment type by absorption spectra of chromatographic fractions (Fox and Lewin, 1963; Lewin and Lounsbery, 1969). It soon became apparent that the pigments were probably carotenoids, but so far only one, the new xanthophyll saproxanthin (a modified 3,1′-dihydroxy-γ-carotene) from *S. grandis,* has been chemically characterized (Aasen and Liaaen-Jensen, 1966a). The pigment content was 0.02% of the freeze-dried cells. From *S. thermalis,* a single carot-

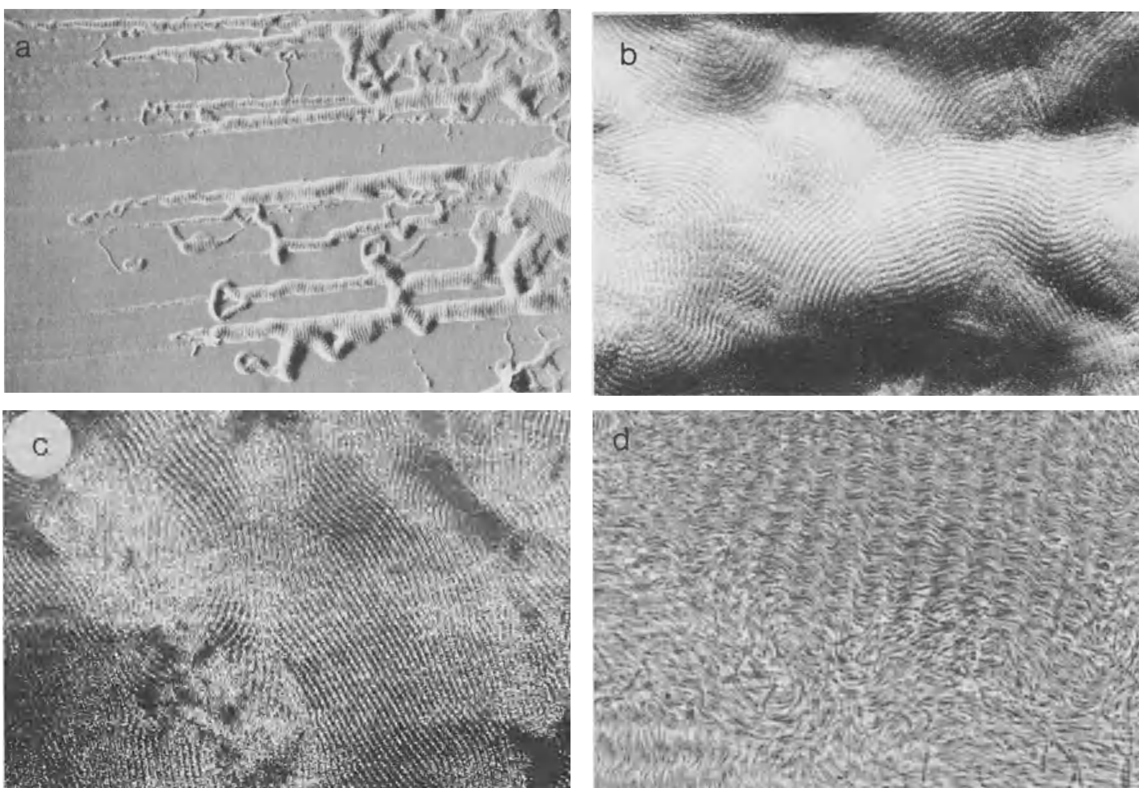

Fig. 3. Striated colonies of *Saprospira grandis.* (a) Colony spreading along grooves in the agar surface; the stripes run almost perpendicular to the direction of the spreading movement; Zeiss Standard Microscope, with oblique illumination; × 60. (b) Surface of a colony under oblique illumination; the filaments are just recognizable as a very delicate striation perpendicular to the stripes; Zeiss Axiomat; × 120. (c) Under dark-field illumination, the pattern of stripes becomes even more distinct; Zeiss Axiomat; × 120. (d) At high magnification it can be seen that in the striated areas the filaments lie strictly parallel, with the screws "in phase"; Zeiss Axiomat; × 580.

enoid, apparently a keto-carotenoid, was obtained, but because of very low yields (0.002% of the dry weight) its chemical structure could not be determined (Aasen and Liaaen-Jensen, 1966b). The early investigators described *S. albida* as completely unpigmented (however, they may have thought mainly of chlorophylls), but when a sufficiently high cell density is obtained, the organism shows a pale yellow tint (Fox and Lewin, 1963). Carotenoid synthesis by *S. thermalis* is stimulated by light, by suboptimal concentrations of cobalamine or tyrosine, and by subinhibitory concentrations of thiamine or sodium thiosulfate (Lewin, 1965a).

As already mentioned, *S. grandis* depends entirely on amino acids for growth (Lewin, 1972). It can be cultivated on various peptones, such as tryptone, casitone, phytone, or yeast extract. It can also be grown on casamino acids, but only if the medium is supplemented with tryptophan, asparagine, and a small quantity of yeast nucleic acid hydrolyzate (the growth factor supplied by the latter could not be identified). In that case, the casamino acids could be replaced by a mixture of essential amino acids, i.e., arginine, histidine, isoleucine, leucine, valine, methionine, phenylalanine, threonine, and perhaps lysine. Curiously, asparagine cannot be substituted by aspartate or glutamine. In nature, *S. grandis* may provide itself with the required amino acids by the degradation of proteinaceous matter. Gelatin liquefaction, the clotting and digestion of milk casein, and the decomposition of bacterial cells by *S. grandis* have been reported. In accordance with the metabolic restrictions outlined above, polysaccharides like starch, alginate, carboxymethyl cellulose, and agar are not hydrolyzed (Lewin and Lounsbery, 1969). Strain Sa gl also does not attack chitin and yeast cells. Marine *S. toviformis* differs from *S. grandis* in that it grows on casamino acids alone and does not require asparagine. Also, it is stimulated by lactate and acetate and depolymerizes carboxymethyl cellulose. It, too, does not utilize any of the tested sugars (Lewin and Lounsbery, 1969).

S. thermalis, the only freshwater species studied in some detail, utilizes glucose but none of the other tested sugars, sugar alcohols, ethanol, or acetate (Lewin, 1965a); Lewin and Lounsbery, 1969). This organism also hydrolyzes starch. *S. thermalis* grows well in a mineral salts/glucose medium with tryptone. The latter can be replaced by either 1) yeast extract; 2) casamino acids plus the vitamins cobalamine and thiamine; or 3) glutamate plus the essential amino acids valine, leucine, and isoleucine and the vitamins. Much better growth is obtained if phenylalanine or tyrosine are also added to the synthetic medium. The main nitrogen source in the latter medium, glutamate, can be substituted by asparagine, methionine, or threonine, to a certain extent also by NH_4^+ or urea, but not by NO_3. *S. thermalis* too is proteolytic. The organism appears to operate a citric acid cycle (Lewin, 1965a). A fructose-bisphosphate aldolase has been purified 240-fold from *S. thermalis* (Willard and Gibbs, 1968). It is a type II aldolase, as is characteristic for bacteria, requiring cysteine, Zn^{2+}, and K^+ for maximum activity.

All saprospiras are catalase negative and produce ammonia from amino acids, none reduces nitrate to nitrite. Only *S. thermalis* produces H_2S and minor amounts of indole.

Taxonomy

An extensive study on the taxonomy of flexibacteria, including several types of saprospiras, was performed in the late 1960s (Lewin, 1969; Lewin and Lounsbery, 1969). The study was based mainly on a set of about 70 morphological and physiological features, but the GC content of the strains was also determined (Mandel and Lewin, 1969; in case of a discrepancy between data in the literature, the GC values given in Table 1 were taken from this study). The data were processed in two different ways: by an Adansonian analysis (Colwell, 1969) and by a recurrent group analysis (Fager, 1969). While in both schemes, the strains of *S. grandis, S. thermalis,* and *S. toviformis* turn out as coherent and clearly separated groups, the connections between those groups, and between them and other flexibacteria, differ substantially. Considering the limitations of such a phenotypic classification it may, however, be too early to conclude that the various saprospiras are not closely enough related to be included in one genus.

The phylogenetic relationship of *S. grandis* with the cytophagas and flavobacteria has already been mentioned. The branching point is, however, relatively low, and the connection to the other members of the group is consequently weak.

At present, only one species of *Saprospira* is accepted, *S. grandis* (Reichenbach, 1989). Although there are no conclusive arguments to prove it, at least some of the various organisms classified as saprospiras in the past (Table 1) may indeed represent other *Saprospira* species. Apparently, a similar situation exists with *Saprospira* as with *Beggiatoa,* for which strains or species of widely different dimensions have also been reported. Unfortunately, with the excep-

tion of *S. grandis*, no other *Saprospira* seems to be currently available for comparison in a public culture collection, so that all planned studies will have to start with new isolates.

Applications

Apart from the remote possibility discussed earlier for using saprospiras for the control of freshwater cyanobacteria, no other practical interest in the saprospiras is obvious at the moment.

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*This film may be obtained on loan as a 16 mm copy from the Institut Nonnenstieg 72, D-3400 Göttingen, Germany. This movie is available free of charge for universities and other educational institutions worldwide; only the mailing costs must be reimbursed.

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The Genus *Haliscomenobacter*

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In the 1st edition of *The Prokaryotes,* the genus *Haliscomenobacter* was considered together with bacteria of the genera *Sphaerotilus* and *Leptothrix,* but *Haliscomenobacter* is not closely related to these other Gram-negative sheathed bactera.

Habitats

Strains of *Haliscomenobacter hydrossis,* the only species of the genus *Haliscomenobacter* isolated so far, are nearly always present in activated sludge flocs, sometimes in large amounts. The conditions for such an abundant development, which gives rise to bulking sludge, are not understood.

Straight, thin, needle-shaped, sheath-forming chains of cells protruding from the sludge flocs (Fig. 1) may interfere with clumping and compacting of the solids.

Isolation

The following procedure (van Veen, 1973) can successfully be used to isolate *Haliscomenobacter* sp. from bulking activated sludge.

I Medium (g/liter of distilled water)

Glucose	0.15
$(NH_4)_2SO_4$	0.5
$Ca(NO_3)_2$	0.01
K_2HPO_4	0.05
$MgSO_4 \cdot 7H_2O$	0.05
KCl	0.05
$CaCO_3$	0.1
Vitamin B_{12}	10^{-5}
Thiamine	4×10^{-4}
Agar (Oxoid)	10

S.C.Y. Medium (g/liter of distilled water)

Sucrose	1.0
Casitone (Difco)	0.75
Yeast extract (Difco)	0.25
Trypticase soy broth without dextrose (BBL)	0.25
Vitamin B_{12}	10^{-5}
Thiamine	4×10^{-4}
Agar (Oxoid)	10

The vitamins are sterilized separately by filtration.

A sample of 0.10–0.50 ml of sludge with a relatively large amount of *Haliscomenobacter* threads is pipetted into tubes containing 10 ml sterile tap water. The tubes are stirred for several minutes with a tube mixer and the flocs allowed to settle. The settling time depends on the characteristics of the sludge and especially on the degree of bulking. This treatment of the sludge is repeated until sufficient filamentous organisms are observed microscopically in the upper layers of the supernatant. During moderate agitation and rotating movements of the dilute floc suspensions, fragments of threads are severed from the protruding filaments. The low total numbers of organisms and the favorable ratio between filamentous and other bacteria in the upper liquid layers permit a direct inoculation of the plates without further dilution of the suspension. The time of agitation of the flocs in the tubes appears to be related to the mechanical properties of the stirrer and to the nature and number of filamentous organisms present in activated sludge.

Very small droplets containing sufficient numbers of filamentous bacteria are transferred to the previously dried surfaces of 30 to 40 agar plates containing I medium. The cells are regularly spread over the whole surface of the agar plates by rubbing firmly with a sterile glass rod. The plates are incubated for 3 to 4 weeks at a temperature between 17 and 20°C.

The small colonies that develop from filamentous bacteria are often difficult to detect with a stereomicroscope, but recognition of the poorly contrasting, filamentous microcolonies on the agar is facilitated by low-magnification (150×) phase-contrast microscopy. Isolation is performed by transferring bacterial cells to S.C.Y. medium using sterile capillary tubes.

Preservation of Cultures

Stock cultures of *Haliscomenobacter hydrossis* can be maintained in the following way. Three milliliters of sterile tap water are pipetted onto the surface of the S.C.Y. agar slopes. The inoculated tubes are incubated at 20–25°C until turbid growth has developed in the liquid layer

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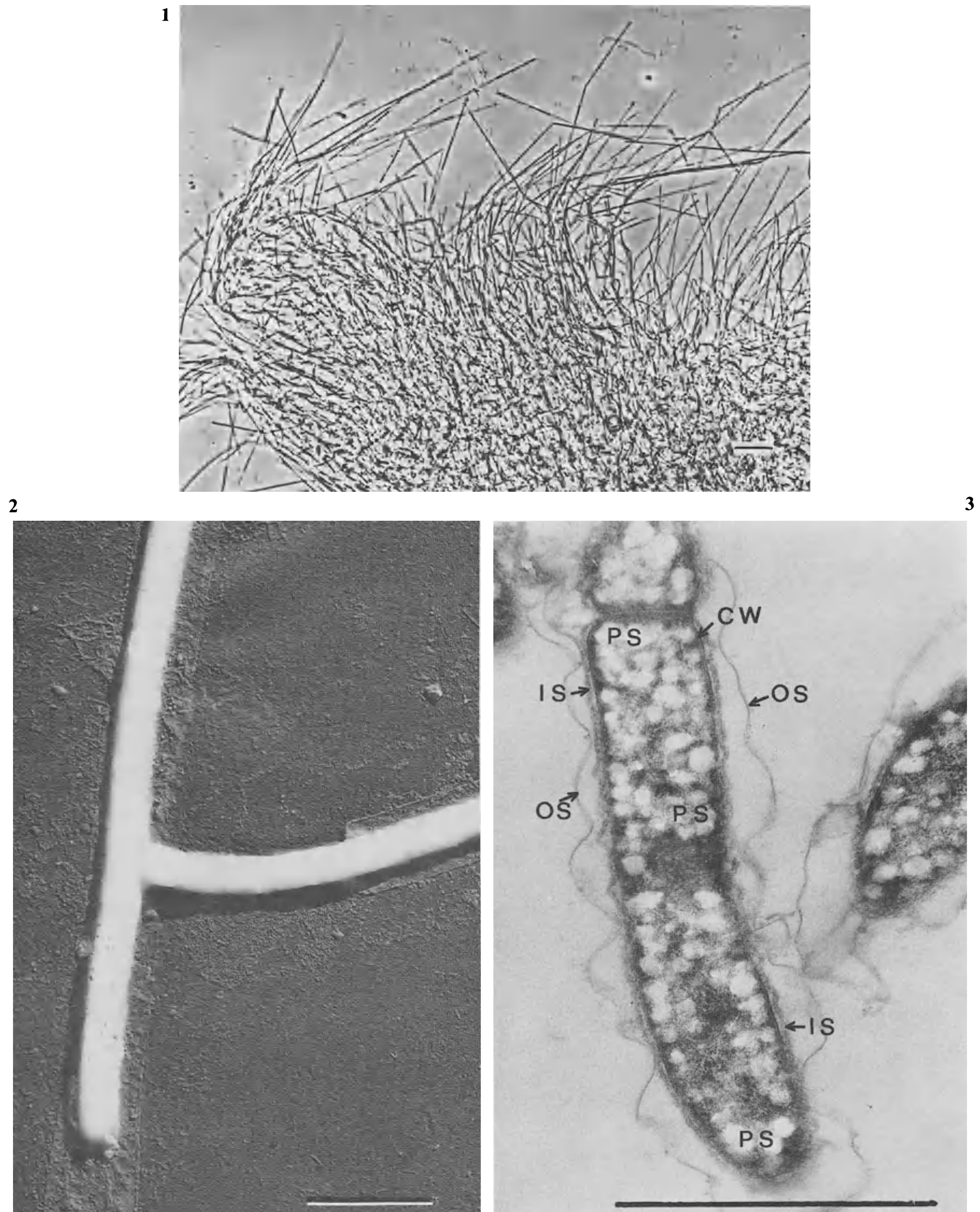
This paper is taken from the 1st edition of *The Prokaryotes.*



Figs. 1–3. *Haliscomenobacter hydrossis.* (1) Activated sludge flocs with many filaments. Bar = 10 μm. (2) Branched filament with thin hyaline sheath. Bar = 1 μm. (3) Fine structure: OS, outer layer of the sheath; IS, inner layer of the sheath; CW, cell wall; PS, polysaccharide globules. Bar = 1 μm.

on the agar. The cells remain viable during 3 months' storage at 4°C.

Identification

The main characteristics of *Haliscomenobacter* spp. are presented in Table 1. Pinkish, smooth, or slightly filamentous colonies about 1–3 mm in diameter are formed on S.C.Y. medium. The cells are nearly always enclosed by a narrow, hardly visible, hyaline sheath (Fig. 2). A holdfast, as may be present in *Sphaerotilus natans,* has never been detected. The sheaths do not attach to glass walls of culture flasks; occasionally single cells are liberated from the sheaths in fast-growing, aerated cultures. Flagella have not been detected by electron microscope examinations, and motility in liquid or hanging-drop cultures has never been observed. Glucose, glucosamine, lactose, sucrose, and starch have been found to be excellent carbon and energy sources. The cells grow very well with $(NH_4)_2SO_4$, KNO_3, glutamate, or casamino acids as the nitrogen source. Both vitamin B_{12} and thiamine are required for growth. Maximum growth was generally obtained after an incubation period of 4–6 days on a rotary shaker at 25°C (Krul, 1977; van Veen et al., 1973).

Instead of poly-β-hydroxybutyrate (present in *Sphaerotilus* and *Leptothrix* spp.), polysaccharide globules are present in cells from media with a high C/N ratio (Fig. 3) (Deinema et al., 1977). Strains of *Haliscomenobacter* isolated so far belong to one species, *H. hydrossis* (Crombach et al., 1974; Eikelboom, 1975; van Veen et al., 1973).

The GC content of the DNA of *H. hydrossis* strains was found to be 49.0 ± 0.7 mol% (Crombach et al., 1974). This value, together with the other properties of this sheathed bacterium (Table 1), confirms the theory that except for the presence of a sheath, bacteria of the genus *Haliscomenobacter* are entirely different from those belonging to the genera *Sphaerotilus* and *Leptothrix.*

Three strains of *Haliscomenobacter hydrossis* have been deposited with the American Type Culture Collection (ATCC 27775–7) in Rockville, Maryland, and with the Deutsche Sammlung für Mikroorganismen (DSM 1100) in Göttingen.

Table 1. Main characteristics of *Haliscomenobacter.*

Cells	
Width (μm)	0.35–0.45
Length (μm)	3–5
Branchings	Real
Structure of sheath surface[a]	Smooth
C source:	
Glucose	+
Sucrose	+
Glycerol	−
Lactate	−
N source:	
NH_4^+	+
NO_3^-	+
Aspartic and Glutamic acids	+
Casamino Acids	+
Vitamin B_{12}	+
Thiamine	+
Optimum pH	7.0–8.0
$Fe(OH)_3$ accumulation	−
Mn^{2+} oxidation	−
Carotenoid pigments	+
Reserve material:	
PHB	−
Polysaccharide	+
GC content (mol%)	48.3–49.7

[a]Electron microscope observations.

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CHAPTER 202

The Genus *Chlamydia*

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Introduction

Members of the genus *Chlamydia* are obligate intracellular prokaryotes whose unique life style has earned them their own order, family, and genus within the kingdom Prokaryotae. The chlamydiae have a unique biphasic developmental cycle that alternates between a spore-like, infectious, metabolically inactive particle, the elementary body (EB), and a noninfectious, metabolically active, replicative form, the reticulate body (RB). The chlamydial outer envelop shares some features with the envelops of Gram-negative organisms but it lacks peptidoglycan, a major structural component of bacterial cell walls. The chlamydiae seem to be completely dependent on their host for high-energy metabolities. However, they are capable of synthesizing their own macromolecules.

The three recognized species of the genus *Chlamydia* cause a wide range of human and animal diseases. *C. trachomatis* is primarily a human pathogen and the causative agent of ocular, genital, and respiratory diseases. *C. trachomatis* causes the most frequent sexually transmitted disease in the industrialized world. Complications of chlamydial infections, and infections in newborns of infected mothers, are a serious health concern. *C. psittaci* is primarily an animal pathogen. Some strains can cause serious respiratory infections in humans as a result of contact with birds. The third, recently described, *C. pneumoniae,* has been shown to cause acute respiratory disease in humans.

Chlamydiae were first observed in 1907 by Halberstaedter and von Prowazek who noted inclusions of the trachoma biovar in conjunctival scrapings (Halberstaedter and von Prowazek, 1909). The organism received worldwide attention in 1929 as the causative agent of a pandemic of psittacosis, an acute pneumonia that resulted from contact with infected parrots. Similar organisms were also identified in association with urethritis and lymphogranuloma venereum infections (LGV). The developmental cycle was first described by Bedson and Bland (1932). Because of the unusual nature of these organisms, early investigators considered this agent first a protozoan (Halberstaedter and von Prowazek, 1909) and then a virus (Miyagawa et al., 1935a). Chlamydiae were considered viruses through much of their early history; they were not generally accepted as bacteria until the 1960s (Moulder, 1964; Storz and Page, 1971).

A major problem in the study of chlamydiae has been the difficulties involved in culturing the organism. The organisms were first cultured in vitro by Miyagawa et al. (1935b) 30 years after they were initially described. Miyagawa et al. cultured LGV isolates in the chorioallantoic membrane of embryogenated hens eggs; this technique was never widely used. It was not until Rake developed a method for in vitro culture of LGV in the yolk sac of embryogenated hens eggs (Rake et al., 1940) and T'ang applied it to the culture of the trachoma strains (T'ang et al., 1957) that chlamydiae were routinely cultured away from the infected host. However, culture in eggs is a tedious procedure, and thus not generally useful, particularly for diagnostic purposes. Two major advancements have facilitated the study of chlamydiae; these were the development of a method for the cultivation of chlamydiae in cell culture (Gordon and Quan, 1965) and the development of recombinant DNA technology. Now, chlamydial DNA can be isolated, introduced into a host organism that is easily manipulated in vitro, and studied, obviating the need to culture chlamydiae for many experiments.

Biology

Taxonomy

Chlamydiae are the only members of the order Chlamydiales, family Chlamydiaceae, genus *Chlamydia* (Storz and Page, 1971; Moulder et al., 1984). There are three recognized species: *C. trachomatis, C. psittaci,* and *C. pneumoniae*

(Moulder et al., 1984; Grayston et al., 1989). Because of the high degree of genetic and biologic diversity within these species, particularly *C. psittaci,* additional species may be designated as our understanding of chlamydiae increases (Table 1).

C. trachomatis is the best characterized of the three species; it has been divided into three biovars based on the nature of disease each group causes. The mouse biovar contains a single nonhuman *C. trachomatis* serotype, MoPn, the causative agent of mouse pneumonitis. The oculogenital or trachoma biovar consists of 12 serotypes (A, B, Ba, C, D, E, F, G, H, I, J, and K) that cause trachoma, conjunctivitis, and genital infections. The third biovar consists of three serotypes (L_1, L_2, and L_3) that are the causative agents of LGV. *C. psittaci* strains are far more diverse. They have a wide range of hosts, including birds, mammals, and reptiles, and are antigenically diverse, factors which so far have precluded any generally accepted scheme for typing. *C. pneumoniae* strains were originally grouped with *C. psittaci.* Although *C. pneumoniae* shares some characteristics with *C. psittaci* studies of DNA similarity show it to be as unrelated to *C. psittaci* as it is to *C. trachomatis* (Table 1). Because a limited number of *C. pneumoniae* isolates have been identified and characterized so far, no statements can be made regarding the potential diversity of this species.

Although there was controversy early in their history as to the nature of chlamydiae, these organisms are clearly bacteria. Chlamydiae have a cell envelope similar to that of Gram-negative bacteria and they have prokaryotic ribosomes; they replicate by binary fission; and they are sensitive to many antibiotics that affect bacteria, including rifampicin, chloramphenicol, erythromycin, and tetracycline. Unlike viruses, chlamydiae contain both DNA and RNA. Like viruses, they depend on the host cell for energy; however, viruses are also dependent on the host for biosynthetic functions. Although chlamydiae require precursors from the host cell, they synthesize their own DNA, RNA, and protein.

Several features differentiate the three species of chlamydiae (Table 1). The EBs of *C. trachomatis* and *C. psittaci* are spherical while *C. pneumoniae* EBs are pear-shaped and, unlike *C. trachomatis* and *C. psittaci,* contain a periplasmic space (Fig. 1) . Inclusions, the phagocytic vacuoles that are the intracellular habitat for these organisms, formed by *C. trachomatis* contain glycogen of bacterial origin whereas *C. psittaci* and *C. pneumoniae* inclusions do not. Also, multiple inclusions may be observed in cells infected with *C. psittaci* whereas cells infected with *C. trachomatis* contain only one inclusion even when grown at high multiplicities of infection, suggesting that phagosomes containing this organism fuse (Fig. 2). *C. trachomatis* is sensitive to sulfadiazine, indicating that it is capable of folate biosynthesis; *C. psittaci* and *C. pneumoniae* are resistant to sulfadiazine. The preferred host and spectrum of diseases also varies with strain (Table 2).

The high degree of structural and genetic heterogeneity among the chlamydiae contrasts with the many functional similarities that are unique to these organisms. This observation raises questions concerning the evolution of these organisms. Convergent evolution is suggested by the very low level of DNA relatedness among the three species (Kingsbury and Weiss, 1968; Cox et al., 1988). Divergent evolution predicts that conserved functions such as enzymes in-

Table 1. Characteristics of the chlamydiae.

Property	Species		
	C. trachomatis	*C. psittaci*	*C. pneumoniae*
Natural host	Humans	Birds Nonhuman mammals	Humans
Inclusion morphology	Round, vacuolar	Variable shape, dense	Round, dense
Glycogen in inclusions	+	−	−
Synthesizes folate	+	−	−
Genome			
Plasmid	+	+	−
GC content[a]	42–45 mol%	39–43 mol%	40 mol%
Sequence relatedness[b]			
Intraspecies	97–100%[c]	20–100%	95–100%
Interspecies	≤10%	≤10%	≤10%

[a]Cox et al. (1988); Gerloff et al. (1970); Kingsbury and Weiss (1968); Moulder et al. (1984).
[b]Cox et al. (1988); Based on DNA-DNA liquid hybridization followed by S1 nuclease protection assays. % is percent of homologous DNA control.
[c]Does not include the mouse pneumonitis biovar.

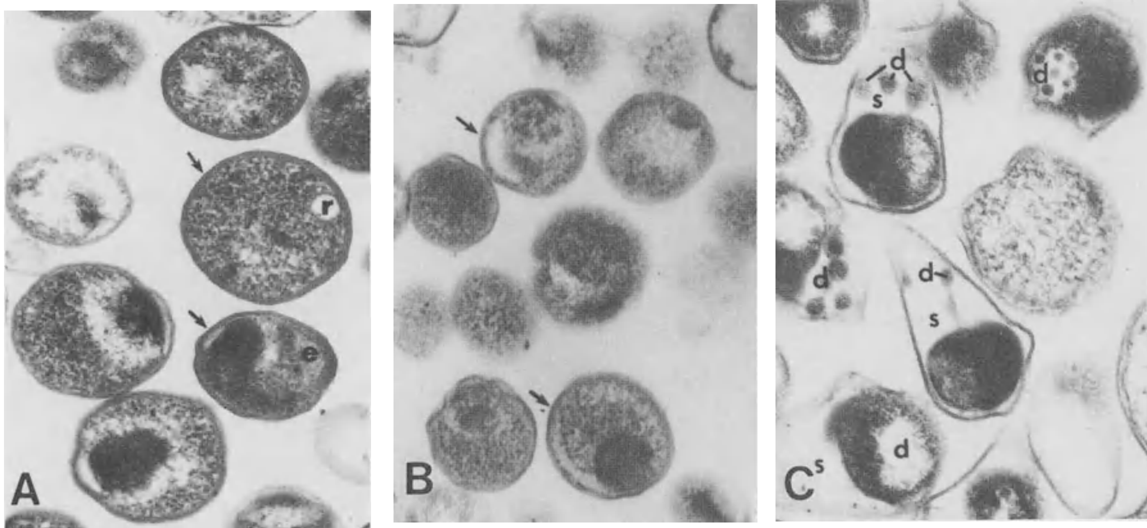

Fig. 1. Electron micrographs of the three species of chlamydiae. (a) *C. trachomatis* serotype B. EBs (e) and RBs (r) are both round. (b) *C. psittaci* meningopneumonitis strain. (c) *C. pneumoniae* strain TW-183. The EB is typically pear-shaped with a large periplasmic space (s) containing electron dense bodies (d). Outer and inner membranes are noted by arrows. Taken from Chi et al., 1987; ASM Publications.

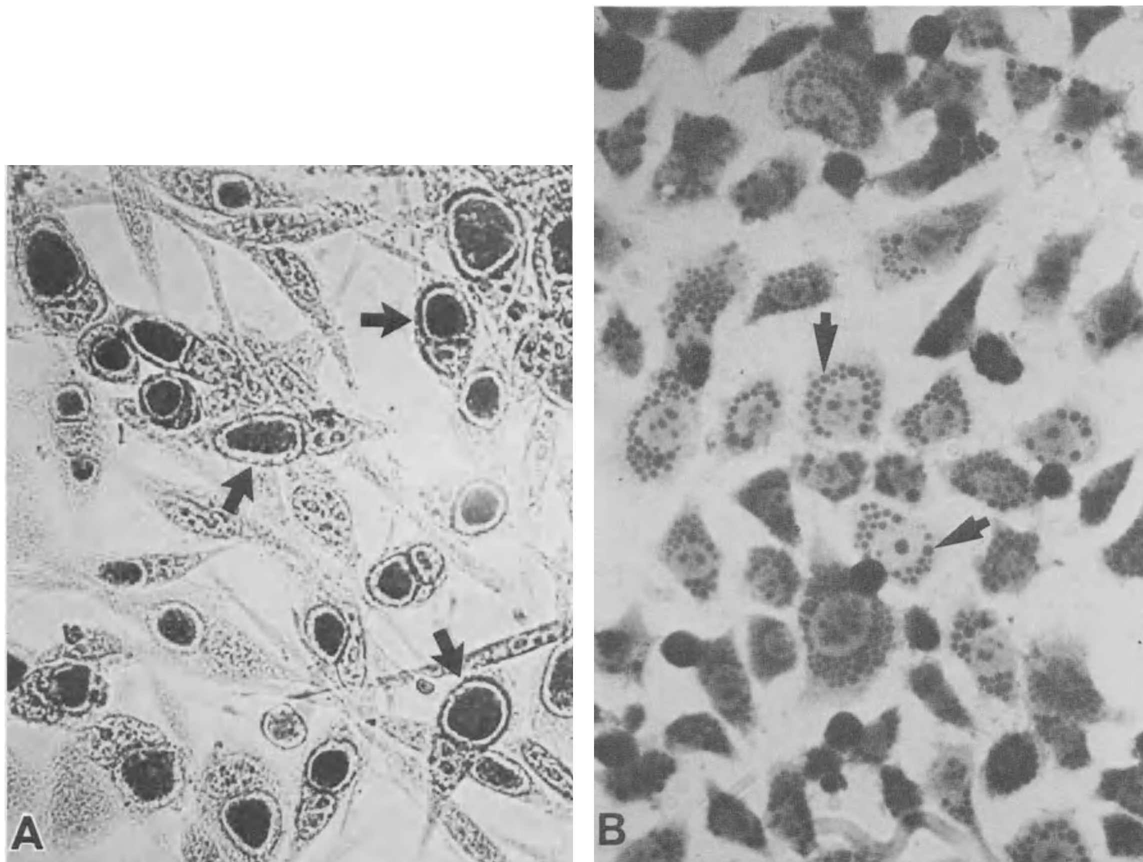

Fig. 2. Light microscopy of *C. trachomatis* and *C. psittaci* inclusions. (A) Single *C. trachomatis* inclusions (arrows) within McCoy cells stained with Jones iodine. (B) Multiple *C. psittaci* inclusions (arrows) within L-929 cells stained with Giemsa. Taken from Hodinka et al., 1988; ASM Publications.

Table 2. Human diseases caused by chlamydiae.

Disease	Causative agent	Serotype	Syndrome
Trachoma	*C. trachomatis*	A,B,B_a,C	Progressive conjunctivitis that may lead to scarring of the cornea and blindness
Inclusion conjunctivitis	*C. trachomatis*	D-K	Self-limiting conjunctivitis transmitted by contact with conjunctivia or genitalia
Urethritis, cervicitis, related infections	*C. trachomatis*	D-K	Inflammation of urethra or cervix, may spread to adjacent organs, conjunctivitis and pneumonia are common infections of newborns exposed to an infected birth canal
Lymphogranuloma venereum	*C. trachomatis*	L_1,L_2,L_3	A sexually transmitted infection that spreads from genitalia to reticuloendothelial system, producing inflammation and suppuration of regional lymph nodes
Ornithosis, psittacosis	*C. psittaci*	Undefined	Acute respiratory infection or pneumonia, bird-to-human transmission
Respiratory infections	*C. pneumoniae*	Unknown	Includes pneumonia, bronchitis, sinusitis, pharyngitis; human-to-human transmission

volved in lipopolysaccharide (LPS) biosynthesis should be similar. A limited amount of data compare DNA sequences from *C. psittaci* and *C. trachomatis.* The genes for the major outer membrane protein (MOMP) from *C. psittaci* and *C. trachomatis* show 60–70% DNA sequence homology (Zhang et al., 1989). The 16S rRNA genes from *C. trachomatis* and *C. psittaci* share 95% DNA sequence homology (Weisburg et al., 1986). These reports contrast with the reported value of 10% overall DNA sequence homology for the two species. The conservation of DNA sequences supports the hypothesis that these species evolved divergently; however, further comparisons of the genetic organization of the three species is required before any definitive conclusions may be made.

Developmental Cycle

One of the unique aspects of chlamydial biology is the biphasic developmental cycle. Chlamydiae exist as two distinct life forms, each of which is adapted to specific environments in a manner not unlike spore formation in *Bacillus* spp. The EB is small (200–300 nm) extracellular, and spore-like. It is infectious but metabolically inactive, and possesses a rigid outer cell wall that may provide protection against environmental stresses. The EB attaches to the host cell, possibly via a receptor/adhesin interaction. Attachment is followed by penetration into a membrane-bound vesicle where the EB differentiates into the larger (700–1000 nm) RB. RBs are noninfectious and relatively fragile but capable of synthesizing macromolecules and replicating by binary fission. After multiple rounds of replication the RBs differentiate into EBs. Throughout the intracellular portion of the developmental cycle, the organisms remain within the phagocytic vacuole, the inclusion, and can be seen as a distinct entity in the cytoplasm of the host cell (Fig. 3). *C. psittaci* infected host cells lyse late in infection and release EBs that can infect other cells. *C. trachomatis* infected cells do not always lyse and must be physically disrupted to release infectious EBs.

Cell Membrane

The cell membrane of chlamydiae exhibits features of Gram-negative organisms, including an inner and outer membrane and LPS. Although peptidoglycan, a major component of most bacterial cell walls, appears to be absent in chlamydiae (Garrett et al., 1974), these organisms have features that are usually attributable to peptidoglycan. For example, chlamydiae are somewhat susceptible to penicillin, an antibiotic that interferes with peptidoglycan synthesis. In the presence of penicillin, EBs differentiate into RBs and replicate; but, the RBs are larger than normal, are irregularly shaped, and cannot differentiate back into EBs (Matsumoto and Manire, 1970; Kramer and Gordon, 1971). In addition, *C. trachomatis* possesses penicillin-binding proteins which are enzymes involved in peptidoglycan biosynthesis in other bacteria (Barbour et al., 1982). The role of these proteins in chlamydiae remains to be determined.

The bacterial cell wall serves to protect the cell from environmental stress. In chlamydiae, rigidity of the EB outer membrane is thought to result from crosslinking of proteins in the outer membrane (Newhall and Jones, 1983; Hackstadt et al., 1985; Hatch et al., 1986a). This crosslinking is lost as the EB differentiates into the more osmotically fragile RB. Thus, the crosslinking of outer membrane proteins may serve the same role as peptidoglycan in other

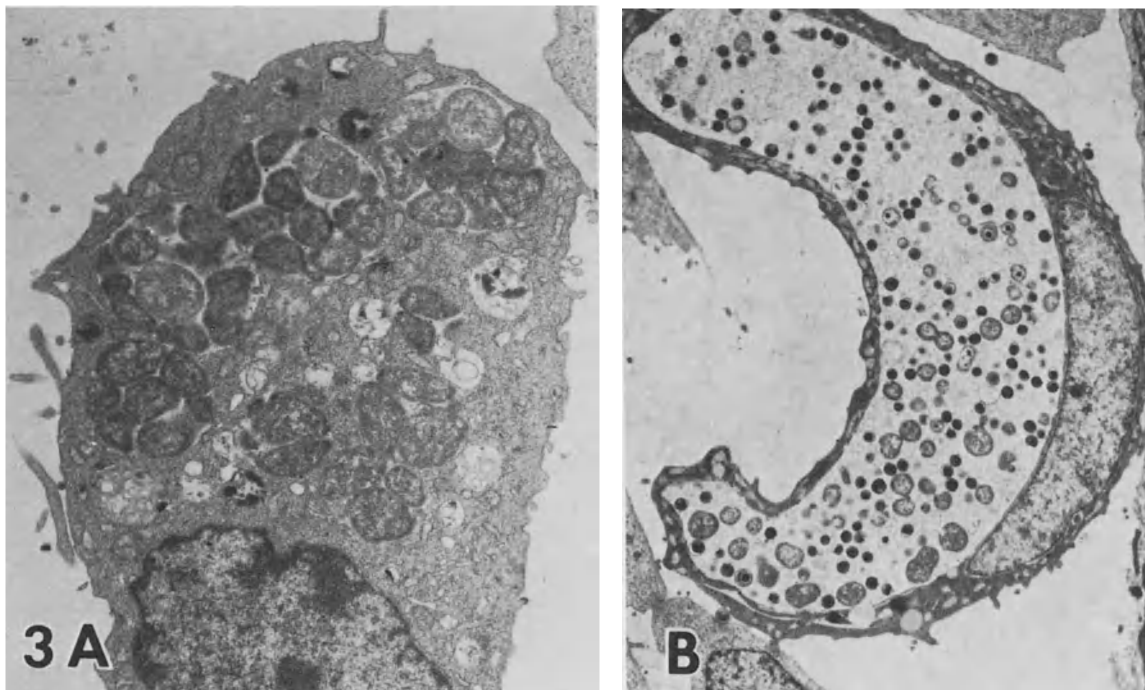

Fig. 3. Electron micrograph of *C. trachomatis* and *C. psittaci* inclusions. (A) *C. trachomatis*. (B) *C. psittaci*. RBs are seen within a membrane bound vesicle in the host cell cytoplasm. Taken from Wyrick and Richmond, 1989; American Veterinary Medical Association Publications.

bacteria; i.e., the formation of a rigid barrier between the cell and its environment.

MAJOR OUTER MEMBRANE PROTEIN (MOMP). The major constituent of the outer membrane and the best-characterized protein in *C. trachomatis* is MOMP. MOMP accounts for approximately 60% of the dry weight of the outer membrane (Caldwell et al., 1981). It has a monomeric molecular weight of approximately 40 kDa; however, it exists in the membrane as a multimeric, transmembrane complex (Chang et al., 1982). Evidence of disulfide-mediated cross-linking between MOMP molecules has been found in the EB but not the RB (Newhall and Jones, 1983). It has been suggested that MOMP functions as a porin in vivo based on in vitro evidence that MOMP confers polar permeability to liposomes (Bavoil et al., 1984).

C. trachomatis has been divided into 15 serotypes based on the reactivity of different strains with monoclonal antibodies (Grayston and Wang, 1975). Much of the heterogeneity seen among the serotypes can be attributed to antigenic differences in MOMP (Stephens et al., 1982). MOMP contains genus-, species-, subspecies-, and serotype-specific epitopes, indicating that there may be conserved and variable regions of this protein (Stephens et al., 1982; Zhang et al., 1987a). DNA-sequence analysis and epitope mapping have supported this hypothesis (Stephens et al., 1987; Baehr et al., 1988). Most DNA-sequence variability among MOMP genes is confined to four domains. Monoclonal antibodies that react with serotype-specific epitopes recognize variable sequences whereas the genus- and species-specific epitopes are encoded by the constant sequences. The DNA sequence of MOMP genes from two strains of *C. psittaci* has also been determined. When compared to *C. trachomatis* MOMP, *C. psittaci* MOMP shows a 60–70% conservation of DNA sequence, and has analogous conserved and variable domains (Zhang et al., 1989). The high interspecies relatedness of MOMP is interesting, given that these species have an overall DNA relatedness of $\leqslant 10\%$.

The temporal expression of the MOMP gene of *C. trachomatis* serotype L2 has been studied (Stephens et al., 1988a). Two mRNA species corresponding to the MOMP gene have been identified. One mRNA is expressed constitutively; the production of the second begins approximately 16 h after infection when RBs are dividing rapidly and presumably need large quantities of MOMP. Analysis of the DNA sequence upstream from the MOMP gene supports this finding; two putative promoters have been identified.

CYSTEINE-RICH OMPs. Cysteine-rich proteins make up a major portion of the outer membrane of *C. trachomatis* strains. Three such proteins have been identified in LGV strains; these have molecular weights of approximately 62, 59, and 12 kDa (Batteiger et al., 1985). The 62-kDa protein is absent in the trachoma strains (serotypes A–K; Batteiger et al., 1985). These proteins are found predominantly in the EB; they are not synthesized until late in the developmental cycle, when RBs are differentiating into EBs (Hatch et al., 1986a; Sardinia et al., 1988). This, combined with the fact that they are highly crosslinked, suggests that they function in the structural integrity of the EB. Like MOMP, there appears to be some serotype heterogeneity in the cysteine-rich outer membrane proteins (Zhang et al., 1987b); this heterogeneity has not been well characterized.

Molecular cloning has revealed that the 62 and 59 kDa cysteine-rich outer membrane proteins are products of a single gene, designated *omp2* (Allen and Stephens, 1989). The different molecular weights are a result of cleavage of the signal peptide at two different sites, resulting in peptides of 507 and 525 amino acids. This is the first description of two-site posttranslational processing of a signal sequence in prokaryotes. The physiologic role of this differential cleavage remains to be determined.

LPS. Chlamydial LPS has not been completely defined chemically; however, immunological studies show that it resembles the short LPS chains of rough *Salmonella* Re mutants (Nurminen et al., 1985). LPS from *Chlamydia* spp. and *Salmonella* LPS share at least two epitopes (Brade, L. et al., 1985). In addition, chlamydial LPS possesses a chlamydia-specific epitope, KDOp (2→8) KDOp (2→4) KDO (Brade, H. et al., 1987), that has been used extensively as a tool for the identification of these organisms. LPS is often referred to as the genus-specific antigen because it was first identified as an immunoreactive moiety common to all chlamydial species and subsequently identified as LPS. A gene encoding an enzyme, presumably a glycosyl transferase, required for the biosynthesis of this epitope has been cloned (Nano and Caldwell, 1985). This epitope is expressed on LPS in *Salmonella* Re strains bearing the cloned gene (Brade, L. et al., 1987).

Genome

Chlamydiae have one of the smallest genomes among the bacteria; estimates of its size range from 5–10 $\times$ 10^5 base pairs (Kingsbury, 1969; Sarov and Becker, 1969; Frutos et al., 1989). This is approximately the size of *Mycoplasma* spp. genomes and one quarter of the *Escherichia coli* genome. The GC content of the chlamydial genome is 40–45 mol% (Kingsbury and Weiss, 1968; Gerloff et al., 1970; Cox et al., 1988). Hybridization studies indicate that the three species of chlamydiae are equally distantly related, each sharing less than 10% DNA-sequence homology with each of the other species (Cox et al., 1988).

Most strains of *C. trachomatis* and *C. psittaci* possess a 7–7.5 kb plasmid of unknown function (Lovett et al., 1980). *C. pneumoniae* strains examined to date do not contain a plasmid (Campbell et al., 1987). The complete nucleotide sequence of the plasmid from several *C. trachomatis* serotypes has been determined (Sriprakash and Macavoy, 1987; Comanducci et al., 1988; Hatt et al., 1988; Black et al., 1989); the sequence of the plasmid is highly conserved among these serotypes. Based on DNA-hybridization experiments, limited DNA-sequence homology exists between the *C. trachomatis* and *C. psittaci* plasmids (Joseph et al., 1986). Eight open-reading frames have been identified on the *C. trachomatis* plasmid; however, this information has not led to any direct insight into plasmid function.

Like other prokaryotic organisms, the chlamydial chromosome appears to be organized in nucleoids. The EB chromosome is highly condensed, perhaps similar to the nucleoid of endospores (Costerton et al., 1976; Popov et al., 1978). In electron micrographs, it is visible as an electron-dense region. The genome in the RB is dispersed, similar to other metabolically active bacteria. DNA-binding proteins that are specific for the EB have been described (Wagar and Stephens, 1988), suggesting that these proteins may serve to hold EB DNA in a specific configuration.

Metabolism

Studies of chlamydial metabolism are complicated by the fact that the chlamydiae are obligate intracellular parasites; biochemical processes of interest must be distinguished from those of the host. The absence of a particular function in uninfected host cells or an altered function in infected cells compared to uninfected cells is helpful; however, the possibility exists that chlamydial infection has altered host metabolism rather than initiated its own. Additionally, since chlamydiae exist as two different forms, the possibility that EB preparations are contaminated with RBs or vice versa must be eliminated in studies of one form or the other.

The metabolic deficiencies that make chlamydiae completely dependent on a host cell for growth are not clear. There is no evidence that chlamydiae make ATP, an observation consistent with Moulder's hypothesis that they are energy parasites (Moulder, 1962). They have their own ribosomes and RNA polymerase (Sarov and Becker, 1971) and synthesize RNA, DNA, and proteins in the absence of host DNA and protein synthesis (Alexander, 1968; Alexander, 1969). These functions are susceptible to bacterial inhibitors such as chloramphenicol and rifampicin (Tribby et al., 1973). Thus, chlamydiae appear to be able to synthesize their own macromolecules; however, the extent of their dependence on the host for the precursors of these macromolecules is unknown.

Host-protein synthesis is not required for chlamydial growth, as evidenced by the fact that they replicate in cycloheximide-treated host cells (Alexander, 1968; Ripa and Mardh, 1977). In fact, chlamydiae grow better when host metabolism is inhibited. Nor is host cell metabolism specifically inhibited by chlamydiae after infection. At low multiplicities of infection, where chlamydial growth has little adverse effect on the host, infected cells continue to divide (Horoschak and Moulder, 1978).

ENERGY METABOLISM. Chlamydiae appear to lack the ability to synthesize high-energy molecules and do not produce cytochromes and other molecules required for aerobic respiration. Enzyme activities associated with energy-producing pathways such as glycolysis (Ormsbee and Weiss, 1963; Weiss et al., 1964; Weiss, 1965), the pentose phosphate pathway (Moulder et al., 1965), and the tricarboxcylic acid cycle (Weiss, 1967) have been found; however, the significance of the presence of only portions of these pathways in chlamydiae is not known. It has been suggested that they acquire ATP from the host via an ATP/ADP exchange mechanism. Host-free *C. psittaci* RBs take up radiolabelled ATP; this activity can be competitively inhibited with ADP (Hatch et al., 1982). Increased numbers of host mitochondria have been observed surrounding chlamydial inclusions (Matsumoto, 1981), suggesting that they may parasitize mitochondrial products.

DNA AND RNA SYNTHESIS. Host-free RBs are capable of both DNA and RNA synthesis if provided with the appropriate precursors and an energy source (Tamura, 1967; Hatch et al., 1986b). In addition, permeabilized EBs may also synthesize RNA (Sarov and Becker, 1971). RNA synthesis in EBs was found to be inhibited by rifampicin, an inhibitor of bacterial RNA polymerase, and actinomycin D, indicating that it is dependent on double-stranded DNA for activity. Unfortunately, this finding could not be reproduced in another study (Hatch, 1988).

It seems clear that chlamydiae draw on host nucleotide pools for RNA synthesis (Hatch, 1975); however, the source of precursors for chlamydial DNA synthesis is uncertain. *C. psittaci* incorporates host guanine nucleotides into RNA and DNA (Ceballos and Hatch, 1979), but does not use thymidine or deoxycytosine of host origin (Tribby and Moulder, 1966). The reason for this is unknown. It has been suggested that chlamydiae synthesize thymidine nucleotides from deoxyuridine monophosphate (dUMP) (Tribby and Moulder, 1966; Hatch, 1976).

PROTEIN SYNTHESIS. The presence of ribosomes in both EBs and RBs, the sensitivity of chlamydiae to antibiotics that inhibit bacterial protein synthesis, and the synthesis of chlamydial proteins in the presence of drugs that inhibit host protein synthesis, all indicate that chlamydiae synthesize their own proteins. In addition, purified RBs have been shown to synthesize a limited amount of protein in vitro; and the proteins synthesized correspond to those produced at the stage of development from which the RBs were harvested (Hatch et al., 1985; Plaunt and Hatch, 1988). However, it is unclear whether chlamydial or host amino acid pools are incorporated into bacterial protein. The fact that host-free RBs are only stimulated by, not dependent on, added amino acids indicates that RBs have intracellular pools of amino acids. However, researchers have been unable to prolong in vitro protein synthesis for more than a few hours (Hatch et al., 1985). This may be due to an inability to replenish depleted amino acid pools by de novo synthesis or a requirement for an unknown host function.

Chlamydial growth in cell culture can be inhibited by the omission of some amino acids but not others from the growth medium (Karayiannis and Hobson, 1981; Allan and Pearce, 1983). Also, amino acid requirements may be strain dependent. However, it is unknown whether the requirement for a particular amino acid is due to the inability of that strain to make the amino acid or to an inadequate pool of the amino acid within the host cell.

There is evidence that chlamydiae synthesize specific amino acids. *C. psittaci* can grow in L cells in the absence of added arginine, lysine, and histidine, all of which are required for L cell growth (Bader and Morgan, 1958). In addition, radiolabelled precursors of arginine are incorporated into chlamydial but not host protein during growth of both *C. psittaci* and *C.*

trachomatis in L cells, indicating that these organisms posses some enzymes of the arginine biosynthetic pathway (Treuhaft and Moulder, 1968).

GLYCOGEN DEPOSITION. A curious aspect of *C. trachomatis* metabolism is the accumulation of glycogen within its inclusion; *C. psittaci* and *C. pneumoniae* do not accumulate glycogen. Although this difference has served as a useful tool in differentiating the species (*C. trachomatis* inclusions are stained with iodine due to the presence of glycogen; *C. psittaci* and *C. pneumoniae* inclusions are not), the physiological basis for the difference is unknown. HeLa cells infected with *C. trachomatis* have a glycogen synthetase activity not found in uninfected cells, suggesting that the glycogen is produced via a chlamydiae specific pathway (Jenkin and Fan, 1971). The enzyme requires ADP glucose, a substrate normally associated with bacterial glycogen synthetases, which distinguishes it from the host cell glycogen synthetase.

Gene Expression

Analysis of gene expression has begun for only a limited number of chlamydial genes. *ompA,* the gene encoding MOMP, is expressed from two promoters in serotype L2 (Stephens et al., 1988a). The DNA sequence upstream from MOMP coding sequences resembles typical bacterial promoters in that they contain identifiable -10 and -35 regions; however, the sequence is only minimally related to *E. coli* consensus promoter sequences. The two promoters are differentially regulated since one is transcribed throughout the developmental cycle whereas transcription from the other begins approximately 16 h after infection when demand for MOMP increases. The chlamydial rRNA genes are also transcribed from two promoters, both of which are expressed throughout development (Engel and Ganem, 1987). A 70-kDa protein is expressed from a single promoter (Sardinia et al., 1988). Comparison of these three promoter sequences indicates that, in addition to being distinct from *E. coli* promoters, there is no developing consensus sequence for chlamydial promoters (Sardinia et al., 1988).

Most clones of chlamydial genes in *E. coli* use vector-supplied promoters for expression of the cloned gene product. This observation has led to the conclusion that chlamydial promoters do not function optimally in *E. coli.* In addition, chlamydial genes that are expressed from their own promoters in *E. coli* tend to be variably transcribed (Sardinia et al., 1988), indicating that *E. coli* RNA polymerase is inefficiently recognizing chlamydial transcription signals. Thus, currently available data suggests that there are some basic differences between gene expression in *Chlamydia* spp. and *E. coli;* however, the physiological basis for these differences remains to be determined.

Ecophysiology: Interaction with the Host Cell

The ecological niche of the chlamydiae is in association with a human or animal host. With the exceptions of mouse pneumonitis and guinea pig inclusion conjunctivitis, there are no animal models for the study of chlamydial disease; thus, studies of chlamydiae in their natural habitat are limited. Most experimentation with chlamydiae has been performed in defined cell lines in vitro. Although the development of in vitro culture systems led to an explosion of research on chlamydiae, the relevance of this information to the understanding of the mechanism of infection remains to be shown.

Cellular Tropism

The oculogenital strains of *C. trachomatis* infect human columnar and squamocolumnar epithelial cells in vivo. These include cells of the conjunctiva, urethra, and cervix. The LGV strains of *C. trachomatis* and *C. psittaci,* which are generally associated with more invasive disease, migrate to the reticuloendothelial system where they reside in macrophages. With the exception of the MoPn strain, *C. trachomatis* infects only cells of human origin. The host and tissue specificities for *C. psittaci* are much broader than those of *C. trachomatis; C. psittaci* strains infect a wide range of organisms including mammals and birds, with tissue specificities that include most major organs of its hosts. Little is known about the host range of *C. pneumoniae.* At present, the only host identified for *C. pneumoniae* is humans.

Host Cell Entry

The obligate requirement for host cells for growth suggests that chlamydiae have efficient mechanisms for attachment to and entry of host cells. Our knowledge of internalization of chlamydiae is taken from in vitro infection experiments. All chlamydiae readily enter nonphagocytic cells that are otherwise impermeable to bacteria, which suggests that they take an active role in the entry of host cells. Live or ultraviolet (UV)-inactivated chlamydiae are readily taken up by nonphagocytic cells, whereas heat-killed

chlamydiae, *E. coli,* and latex beads are not (Byrne and Moulder, 1978), suggesting that a heat-labile moiety is required for uptake. Evidence suggests that chlamydiae can be taken up by different mechanisms depending on the cell system and culture conditions used. Receptor-mediated and nonreceptor-mediated endocytosis and phagocytosis-like mechanisms have been described (Söderlund and Kihlström, 1983; Ward and Murray, 1984; Hodinka and Wyrick, 1986; Hodinka et al., 1988). A recent report shows that the uptake of *C. trachomatis* by polarized epithelial cells is via receptor mediated endocytosis. Furthermore, the mechanism of uptake could be changed by varying culture conditions. When the epithelial cells were grown under nonpolarizing conditions, *C. trachomatis* was not taken up into clatherin-coated pits (Wyrick et al., 1989). Taken together, these findings suggest that chlamydia can enter host cells by multiple mechanisms depending upon the environment in which the EB finds itself.

Evidence for a receptor-mediated endocytic mechanism for uptake of chlamydiae suggests the existence of a specific receptor on the host cell surface and an adhesin on the chlamydial surface. Evidence for a host cell receptor for chlamydiae is circumstantial; however, at least two *C. trachomatis* surface molecules bind specifically to HeLa cell extracts, suggesting that these molecules may be involved in attachment (Hackstadt, 1986; Wenman and Meuser, 1986). Similarly, one such molecule has been identified for *C. psittaci* (Hackstadt, 1986).

Intracellular Survival and Growth

The ability of an organism to survive or replicate within a host cell is a trait limited to pathogenic microbes which have evolved specific mechanisms to evade normal host defense. These pathogens either enter nonphagocytic cells that are incapable of inactivating the intracellular bacteria or evade or inhibit the microbiocidal activities of professional phagocytic cells. The chlamydiae can to varying degrees do both, depending on the strain. *C. psittaci* and, to a lesser extent, the LGV strains of *C. trachomatis* survive and replicate in professional phagocytes, paralleling their invasiveness in vivo; *C. trachomatis* oculogenital strains do not (Kuo, 1978; Eissenberg and Wyrick, 1981). All chlamydiae replicate in epithelial cells. The ability of chlamydia to replicate in host cells has been shown to correlate with the inhibition of fusion between lysosomes and phagosomes that contain chlamydiae, a commonly employed mechanism for intracellular survival among intracellular parasites. In macrophages, lysosomes do not fuse with phagosomes containing *C. psittaci* or LGV strains of *C. trachomatis;* and, these strains replicate. Lysosomes fuse with phagosomes containing the oculogenital strains of *C. trachomatis* and they are inactivated (Yong et al., 1987). PMNs readily kill the chlamydiae in vitro; and, killing is accompanied by fusion of phagosomes with lysosomes (Hammerschlag et al., 1985; Yong et al., 1986). Thus, the inability of chlamydiae to interfere with phagolysosome fusion in PMNs may be the reason they are susceptible to killing by these cells.

The mechanism(s) employed by chlamydiae to inhibit phagolysosome fusion is unknown; however, inhibition of fusion can be prevented by pretreatment of *C. psittaci* with antibody, suggesting that a surface-exposed moiety is involved in this mechanism (Friis, 1972; Wyrick et al., 1978). Also, lysosomes fuse with phagosomes that contain heat-killed *C. psittaci* but not UV-inactivated organisms (Wyrick et al., 1978; Eissenberg et al., 1983), suggesting that the molecule involved is heat labile. Isolated cell membranes from *C. psittaci* EBs inhibit fusion (Levy and Moulder, 1982), whereas purified RBs do not (Brownridge and Wyrick, 1979). This result suggests a functional difference between EBs and RBs in inhibition of phagolysosomal fusion.

Host Response

Studies of the host response to *C. trachomatis* are difficult to interpret because, with the exception of the mouse pneumonitis strain, the only natural host for this organism is the human. As a result, a wide range of animal systems have been used to study chlamydial infection. Conflicting results from the different animal models compounds problems in the assessment of the human immune response to chlamydiae. However, generalizations can be made.

INFLAMMATION. Inflammation at the site of chlamydial infection results in the infiltration of first PMNs then mononuclear phagocytes, cells whose primary function is to phagocytize and kill invading organisms. *C. trachomatis* may inhibit or fail to stimulate specific portions of the respiratory burst in the PMN (Tosi and Hammerschlag, 1988; Tauber et al., 1989). Myeloperoxidase-deficient PMNs are as microbiocidal as normal PMNs (Yong et al., 1986), suggesting that some aspects of oxygen-dependent killing by PMNs are not required to inactivate *C. trachomatis.* Lymphoid follicle formation at the site of infection is common in infections with the oculogenital strains of *C. tra-*

chomatis, whereas granulomatous lesions, most common in lymph nodes, are characteristic of LGV infections.

Inflammation may contribute to the pathologic outcome of some chlamydial infections. Lymphoid follicle formation on the conjunctiva contributes to the mechanical irritation and scarring that ultimately leads to blindness in trachoma. Tubal infertility may be caused by inflammation and scarring that results from repeated or prolonged infection of the fallopian tubes in *C. trachomatis* infection.

IMMUNE RESPONSE. Both humoral and cell-mediated immune responses are stimulated by a chlamydial infection. The relative contributions of each part of the immune system in controlling chlamydial infection is unclear; a complete understanding of the immune response may require the development of better immunologic model systems.

In human infection, seral and mucosal antibodies are produced. In vitro, antibody can neutralize chlamydial infectivity. A similar role for antibody in vivo has not been established; however, secretory immunoglobulin A (IgA) is often produced at the site of mucosal infection and may modulate chlamydial propagation (Brunham et al., 1983). Because chlamydiae are capable of surviving within host cells, cell-mediated immunity may be required for clearing a chlamydial infection. In animal models, passive transfer of *C. trachomatis*- immune T cells can protect a naive mouse against challenge with virulent *C. trachomatis* (Williams, D. M. et al., 1984a; Brunham et al., 1985). Also, mice depleted of B cells are as proficient as control mice at clearing *C. trachomatis* infection suggesting that the T-cell response alone is capable of controlling an infection (Williams, D. M. et al., 1987; Ramsey et al., 1988). Conversely, antibodies administered locally, but not systemically, protected naive mice from challenge with *C. trachomatis* (Williams, D. M. et al., 1984b). Infections in guinea pigs with *C. psittaci* required both humoral and cell-mediated immunity for clearance (Rank et al., 1979, 1989). Together, these results suggest that humoral immunity may play a role in limiting bacterial replication rather than eradicating an infection. Clearing an infection may require cell-mediated immunity; antibody may be important for controlling more invasive infections.

Vaccines

The development of immunity to chlamydiae is complex. In some instances, a single infection or vaccination may produce only short-term immunity, while previous exposure to chlamydiae results in a more severe second infection in others. For example, reinfection plays an important role in the pathogenesis of trachoma, where scarring of the cornea, the major contributing factor to blindness resulting from trachoma, is caused by a hypersensitivity response to reinfection. This may also be the case in tubal infertility, where repeated infection may cause scarring and blockage of the fallopian tubes.

The adverse consequences of delayed hypersensitivity reactions of the host to chlamydiae may hamper the development of whole cell vaccines. In monkeys immunized with whole cells, live organisms or Triton X-100 extracts stimulate a hypersensitivity response whereas formalin-killed or UV-irradiated organisms do not (Taylor et al., 1987). This response suggests that a sensitizing antigen is present within chlamydial cells that must be released either by cell growth or the solublization of EBs. Most of the sensitizing effect of the Triton X-100 extract can be attributed to a 57-kDa protein (Morrison et al., 1989). Thus, a subunit vaccine deficient in the sensitizing molecule that also stimulates protective immunity without stimulating hypersensitivity can be envisioned. Alternatively, antigens that elicit T cell responses could be identified and recombinant or synthetic polypeptides vaccines derived from these antigens. A third approach to vaccine development is the construction of a chlamydial strain deficient in the sensitizing antigen(s) that could be used as a live, attenuated vaccine. Unfortunately, the molecular genetic tools required for the alteration of specific genes are not yet available for chlamydiae.

Diseases

Human Diseases

C. trachomatis and *C. pneumoniae* are primarily human pathogens; *C. psittaci* is primarily an animal pathogen that can be transmitted to humans though contact with infected animals. The most common chlamydial infections affect the eyes, genitalia, and respiratory tract (Table 2). Chlamydial infections are of serious public health concern worldwide. Genital chlamydial infections are the most frequent sexually transmitted disease in developed countries, causing about twice as many urethral infections as *Neisseria gonorrhoeae.* Trachoma is a major cause of preventable blindness in developing countries.

GENITAL INFECTIONS. Genital infection with strains of the oculogenital biovar of *C. trachomatis* account for the majority of chlamydial

infections in North America and western Europe. These infections are caused by *C. trachomatis* serotypes D through K. In men, chlamydia usually causes urethritis; in women, cervical infections are most common, with urethral and rectal involvement occurring in some cases. Cervical and urethral infections may be accompanied by a mucopurulent discharge; however, frequently, these infections are asymptomatic. A major problem in controlling chlamydial infections is that up to one-third of infected men and three-fourths of infected women may be asymptomatic. Women with cervical infection may carry chlamydia without prominent clinical symptoms for more than 1 year; the maximum duration of such carriage is unknown, but may be years. The large reservoir of asymptomatically infected individuals with prolonged carriage and presumed infectivity is responsible for the high prevalence of *C. trachomatis* in sexually active populations.

The diagnosis and treatment of genital chlamydial infections is particularly important because the complications from these infections can be severe. In women, endometritis, salpingitis, and perihepatitis are common sequelae of *C. trachomatis* infection. These infections of the upper female reproductive tract are usually asymptomatic, but may be accompanied by severe lower abdominal pain and tubal inflammation, a syndrome called pelvic inflammatory disease (PID). As many as 300,000 PID infections occur each year in the United States; many PID patients require hospitalization. Both subclinical and overt PID can lead to scarring and dysfunction of the fallopian tubes; chronic chlamydial infections are the most common preventable causes of ectopic pregnancy and tubal infertility.

In men, untreated chlamydial infections may develop into epididymitis. Infection of the epididymis results in acute, painful inflammation of the testicular apparatus which may require hospitalization. Although it is presumed that chlamydial infection may result in male infertility, conclusive studies of this possible complication have not been performed.

Rarely, men or women infected with *C. trachomatis* may develop Reiter's syndrome. This syndrome is a rheumatic disorder of unknown etiology, which consists of chronic inflammation of joints, skin, blood vessels, and mucosal surfaces. Reiter's syndrome is classified as a "reactive arthropathy," and may be seen in increased frequency in individuals of the HLA B27 haplotype following infection with *Chlamydia, Shigella, Yersinia,* and other bacterial species. This suggests that Reiter's syndrome results from some autoimmune phenomena, but this remains to be determined.

NEONATAL INFECTIONS. As many as 60–70% of infants born to mothers with urogenital chlamydial infections acquire some form of chlamydial infection as they pass through the birth canal; 25–50% develop conjunctivitis while 10–20% get pneumonia. Infections are not limited to these sites; localizations to the nasopharnx, rectum, vagina, and ears also occur.

Prophylactic treatment of the newborn's conjunctivae, usually with silver nitrate, is used to prevent neonatal gonococcal conjunctivitis. In spite of this prophylaxis, chlamydial conjunctivitis is seen in a substantial proportion of infected neonates. In addition, ocular prophylaxis does not eliminate chlamydia in the respiratory tract. Thus, the best method to prevent neonatal chlamydial infection is to identify and treat infected mothers prior to delivery.

LYMPHOGRANULOMA VENEREUM INFECTIONS (LGV). LGV is a sexually transmitted disease rarely seen in the western world, but common in tropical areas. It is caused by the more invasive *C. trachomatis* serotypes, L_1, L_2, and L_3. The disease is manifested by a systemic spread of the chlamydial organisms, with production of fever, myalgias, and signs of meningeal irritation. In men, swelling of the inguinal or femoral lymph nodes resulting in buboes is common.

OCULAR INFECTIONS. Trachoma is caused by *C. trachomatis* infection of the squamocolumnar epithelial cells of the conjunctivae. *C. trachomatis* serotypes A, B, Ba, and C are most often associated with this infection. Trachoma is endemic in most developing countries. Most individuals with trachoma become infected in childhood; they develop acute conjunctivitis that progresses to chronic follicular keratoconjunctivitis and pannus (neovascular) formation. Repeated exposures lead to a scarring of the conjunctiva that distorts the eyelid, causing abrasion of the cornea every time the eye is closed. Over time, the irritation causes corneal destruction and eventually blindness.

Uncomplicated conjunctivitis is frequently seen in sexually active adults with genital infections. Inoculation of the eye with infected genital secretions is presumed to be the route of inoculation for this infection. The conjunctivitis is generally self-limited, and does not lead to visual impairment, which may require repeated episodes of active infection as with trachoma.

ORNITHOSIS/PSITTACOSIS. Although humans are not a natural host for *C. psittaci,* human infection can occur after exposure to infected animals, particularly birds. Strains of *C. psittaci* vary greatly in their virulence for humans, with some avian strains being particularly capable of causing human disease. Most *C. psittaci* infections are caused by occupational exposures of poultry or pet-store workers. Because *C. psittaci* infections of humans may come from both psittacine and nonpsittacine birds such as turkeys, the term "ornithosis" is now used naming pneumonic illness caused by avian strains of *C. psittaci.* Symptoms range from mild respiratory infections to severe pneumonia that can be fatal.

C. PNEUMONIAE INFECTIONS. Atypical *C. psittaci* isolates from humans that were not of avian origin were first identified in studies of trachoma (Dwyer et al., 1972; Grayston, 1967). Subsequently, serological studies showed that respiratory infections may be due to similar atypical chlamydial strains (Saikku et al., 1985). A prospective study of university students with acute respiratory disease identified eight more atypical chlamydiae and gave the group the strain designation "TWAR" (Grayston et al., 1986). Characterization of these isolates indicated that they were sufficiently distinct from *C. psittaci* and *C. trachomatis* to warrant a new species, designated *C. pneumoniae* (Grayston et al., 1989). The full spectrum of *C. pneumoniae* disease is not yet known; however, the organism appears to be capable of causing a range of respiratory syndromes. The original isolation of *C. pneumoniae* from trachoma patients is thought to be the serendipitous recovery of respiratory pathogens. No animal reservoir or vector has been described, suggesting human-to-human transmission.

DIAGNOSIS AND TREATMENT. The incidence of chlamydial disease has apparently been increasing for several years; however, a contributing factor to this is the development of simpler tests for the detection of chlamydiae, with the result that infections of unknown etiology in the past are now being diagnosed as chlamydiae. Traditionally, isolation of the organism in cell culture was the only method for detection of chlamydiae. Culture of chlamydiae is technically difficult and requires about 2 days before inclusions can be identified, limiting the usefulness of this technique as a diagnostic test before treatment. Nonculture methods for the detection of chlamydiae have recently become commercially available. These tests allow the much more timely diagnosis of chlamydiae; however, the sensitivity and specificity of the testing are still under debate.

Serologic test are not generally applicable to the diagnosis of chlamydial infections. There is a fairly high frequency of antibody titer to chlamydiae in the general population. In addition, chlamydial infections are not always accompanied by significant increases in anti-chlamydial antibody titer. Significant changes in antibody titer are seen with the systemic infections LGV and ornithosis; a fourfold increase is antibody titer between acute and convalescent sera is considered diagnostic for these diseases. A high anti-chlamydial immunoglobulin M (IgM) titer is often seen in infant pneumonia; therefore, IgM serology may be useful in diagnosing this infection.

Tetracycline or doxycycline are the drugs of choice in treating most chlamydial infections. When tetracycline therapy may not be appropriate, erythromycin is substituted. Topical antibiotic ointments are effective in treating conjunctivitis; however, they do not prevent second site infections. In the case of newborns where multiple site infections are possible, systemically administered antibiotics are more appropriate.

Animal Diseases

C. psittaci includes many different strains that infect a wide variety of animal species resulting in many disease syndromes. The most common *C. psittaci* isolates are of avian and mammalian origin. Avian chlamydioses are of particular interest because of the possibility of transmission to humans. Infections in birds primarily involve the gastrointestinal tract, resulting in organisms being excreted in the feces; however, conjunctivitis and respiratory symptoms are not uncommon. Chlamydial diseases affecting farm animals are of economic importance; they include gastroenteritis, mastitis, and spontaneous abortions. *C. psittaci* can also infect synovial tissues of lambs, resulting in ovine arthritis. Feline pneumonia and conjunctivitis are also common.

Isolation

Animals and Embryogenated Eggs

Until the 1970s, culture of chlamydiae in laboratory animals (Storz, 1971) or embryonated hen's eggs (T'ang et al., 1957; Rake et al., 1940) were the only means available for the propagation of the organism. However, such methods are expensive, tedious, and time consuming. Because of its relative ease and sensitivity, cul-

tivation of chlamydia in defined cell lines has now replaced animal and egg culture for most applications (Gordon et al., 1969); but, animal and egg culture are still used for specific needs. The pathogenicity of chlamydial strains in animal models is useful in differentiating species and determining host range for *C. psittaci* isolates. No reliable substitute has been found for egg-grown chlamydia as a source of antigen for use in complement fixation (CF) assays. In addition, growth in eggs still proves useful for cultivation of fastidious isolates.

Cell Culture

A variety of cell lines have been used for the propagation of chlamydiae in vitro. Most common are L-cells for *C. psittaci,* HeLa and McCoy cells for *C. trachomatis,* and HeLa229 cells for *C. pneumoniae. C. psittaci* and the LGV strains of *C. trachomatis* readily infect a wide range of cell lines, while the oculogenital strains of *C. trachomatis* and *C. pneumoniae* are restricted in host range and infectivity in vitro. The reasons for this difference are unknown; however, it has been suggested that it may be due to a difference in net negative charge on the bacterial surface. The host cell surface is negatively charged; a strongly negatively charged bacterium may be repelled from the host-cell surface under normal circumstances.

Several techniques have been developed to enhance chlamydial infectivity and growth in vitro. Centrifugation of host-cell monolayers after adding the inoculum enhances recovery of non-LGV strains of *C. trachomatis* and *C. pneumoniae;* the reason for this effect is not understood. Although it is logical to think that centrifugation brings the organisms into closer proximity to the host-cell surface, the centrifugal force applied is not great enough to pellet the bacteria onto the host cell. Alternatively, this effect may be due to an alteration of the host-cell membrane. Treatments that inhibit host-cell metabolism increase recovery of chlamydiae, suggesting that they may have to compete with the host for metabolic pools in growing cells. Cycloheximide, a drug that inhibits cukaryotic protein synthesis, is commonly used because it is added at the time of infection. Gamma radiation and treatment with cytochalasin B or iododeoxyuridine (IUDR) have also been used to inhibit host-cell metabolism; however, these inhibitors have the disadvantage of requiring the pretreatment of host cells several days before infection. Treatment of host-cell monolayers with diethylaminoethyl-dextran (DEAE-dextran) enhances the number of inclusion-forming units (IFUs) obtainable with oculogenital strains of *C. trachomatis* and *C. pneumoniae,* perhaps by the modification of charge on the host-cell membrane; but, this appears to have a substantial effect only in the absence of centrifugation.

A General Method for the Culture of *Chlamydia* in Defined Cell Lines

1) Monolayers of an appropriate host cell, such as McCoy, HeLa229, or L-cells, are prepared in flat-bottom vials that can be centrifuged (15×45 mm shell vials are commonly used for this purpose). Growth of the host cells on glass coverslips in the vials will facilitate the subsequent removal and staining of the monolayers. Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 60 mM glucose, 0.1 mM nonessential amino acids, and 10 mM HEPES buffer is commonly used for chlamydial culture. Care should be taken that the cell-culture medium used does not include antibiotics that are inhibitory to chlamydial growth.

2) The confluent monolayers are inoculated by removing the medium and replacing it with 0.1 ml of a clinical specimen or an inoculum of a pure culture diluted in 2SP (0.2 M sucrose, 20 mM potassium phosphate buffer, pH 7.5) or SPG (0.2 M sucrose; 10 mM potassium phosphate buffer, pH 7.5; 5 mM glutamic acid). Clinical specimens should be sonicated or vortexed with glass beads to disrupt any infected cells before adding to monolayers.

3) The vials are centrifuged at 2,500–3,000 × g at 35°C for 1 h. This step is omitted when culturing *C. psittaci* and LGV strains of *C. trachomatis.*

4) The inoculum is removed and replaced with 1.0 ml of chlamydial growth medium containing 0.5–2 μg cycloheximide/ml. Individual lots of cycloheximide should be tested to determine the concentration that is optimally stimulatory for chlamydial growth without being toxic for the host cells. Removal of the inoculum is particularly important when culturing clinical specimens, where inhibitors of chlamydia or host cells may be present.

5) Infected monolayers are incubated at 35°C, in an atmosphere supplemented with 5.0% CO_2 for 48–72 h.

6) Inclusions can be detected by a variety of staining techniques. Alternatively, chlamydial EBs can be recovered by disruption of the host cells by sonication and passed onto new monolayers or purified by differential centrifugation. Commonly, a low-speed centrifugation (approximately 1,000 × g for 5–10 min) is used to remove host-cell debris, followed by a high-speed centrifugation (30,000× g for 30 min) to pellet EBs. EBs can be further purified by sucrose or renografin density gradient centrifugation.

This general procedure can be modified for a variety of culture conditions:

1) Microtiter plates can be used in place of shell vials. Microtiter plates (96, 48, or 24 well) are particularly appropriate for the large-scale screening of clinical specimens (Hayashi and Sheth, 1982; Lees et al., 1988); however, this method may not be as sensitive as that with

shell vials when 96-well microtiter plates are used (Mallinson et al., 1981; Kiviat et al., 1985). Six-well microtiter plates are also useful for the bulk propagation of strains that require centrifugation for optimal infectivity. Microtiter plates should be centrifuged at 1,500× g rather than 3,000× g because the plates may crack under higher g forces.

2) Cell-culture flasks can also be used in place of shell vials for the bulk propagation of chlamydial strains. Monolayers that will be used for the growth of oculogenital strains of *C. trachomatis* should be treated with DEAE-dextran (30 μg/ml in 2SP or SPG) for 30 min before infection when centrifugation is not possible. The DEAE-dextran is removed and replaced with the inoculum after treatment. The volume of the inoculum should be adjusted to the flask size; approximately one ml is appropriate for a 150 cm^2 cell-culture flask. The flasks are incubated at 35°C for 1 h; they should be gently rocked or periodically agitated by hand to keep the inoculum evenly spread over the monolayer and to prevent it from drying during the infection period. The inoculum is then removed and replaced with chlamydial growth medium; and, incubation proceeds as described above. *C. psittaci* and the LGV strains of *C. trachomatis* are particularly suited for culture in flasks because centrifugation is not necessary for optimal infectivity.

3) For clinical specimens, blind passage of cultures that appear devoid of chlamydial inclusions by disrupting the host cells and inoculating a second monolayer may increase recovery of clinical isolates. This technique has been shown to be particularly useful when culturing *C. trachomatis* in 96-well microtiter plates (Jones et al., 1989; Schachter and Martin, 1987).

Identification

Isolation

Because the formation of intracellular inclusions is unique to the chlamydiae, the visual identification of inclusions is the only requirement for positive identification of the organism. To a trained eye, *C. trachomatis* inclusions are easily visible in unstained, infected cells using an ordinary light microscope. *C. psittaci* and *C. pneumoniae* inclusions are not. This difference has not been explained, though it may be due to the presence of glycogen in *C. trachomatis* inclusions. Thus, monolayers are usually stained to confirm the presence of inclusions. This is particularly important for clinical specimens, where numbers of infected cells may be low. Direct or indirect fluorescent antibody stains specific for chlamydia are the most sensitive and commercially available; however, they are relatively expensive and require a fluorescence microscope for visualization of the inclusions. Alternative stains are: 1) Iodine, which stains the glycogen in inclusions, and thus is only applicable for *C. trachomatis* identification. Timing is important in iodine staining because glycogen levels are maximal at 40–72 h postinfection. This is particularly true for the LGV strains that begin to deplete the glycogen stores at about 60 h postinfection. 2) Giemsa stains may also be used. Both inclusions and cellular organelles are stained; thus, Giemsa-stained cells may be difficult to interpret unless the cells are visualized by dark field microscopy where only the inclusion is brightly illuminated.

Nonculture Techniques

Because of the specialized equipment and techniques that are required for the culture of chlamydiae, the expertise for cultivation of this organism is available in only a limited number of clinical and research laboratories. Recently, a number of techniques have been developed that detect chlamydial proteins, carbohydrates, or nucleic acids, thus obviating the need to culture the organism to identify it. These techniques are particularly suitable for clinical laboratories where diagnosis of infection only requires the identification of chlamydiae in a clinical specimen. An obvious drawback of these techniques is that the clinical isolate is not available for further study.

IMMUNOFLUORESCENT STAINING. The fluorescent antibody stains described above for the detection of inclusions can also be used to detect EBs in clinical specimens. Commercially available stains are usually direct stains in which a fluorescent molecule, usually fluorescine isothiocyanate (FITC), is conjugated directly to an antibody that recognizes chlamydial protein or LPS. This method is sensitive and simple; however, a skilled microscopist must interpret the slides.

ENZYME-IMMUNOASSAYS (EIA). EIAs detect chlamydial antigen in a solubilized specimen and can take any of several formats. Generally, the sample is denatured, chlamydial antigens are bound to a matrix or chlamydiae-specific antibody, then incubated with an enzyme-conjugated monoclonal antibody specific for the bound chlamydial antigen. The adsorbed mon-

oclonal antibody can then be detected in several different ways, usually in a colorimetric assay that can be detected spectophotometerically.

NUCLEIC ACID-BASED ASSAYS. The use of specific DNA or RNA sequences to detect a wide range of microbes including those of the genus *Chlamydia* is a new and rapidly expanding technology. Currently, nucleic acid detection takes one of two formats. A nucleic acid probe homologous to a chlamydiae-specific DNA or RNA sequence is linked to an assayable enzyme, a chemiluminescent molecule, or a radioisotope. The probe is then hybridized to denatured target DNA, nonhybridized probe is removed, and the labelled probe is detected by standard methods. Alternatively, new methodologies that employ specific DNA primers to direct the synthesis of DNA to specific sequences allow the amplification of specific DNA sequences in vitro using the polymerase chain reaction (PCR). Published reports of the application of this later technique to chlamydiae are limited at this time; however, the usefulness of this approach to the study of chlamydiae is obvious. PCR technology has potential application both for the detection of chlamydiae in clinical samples and as a research tool where a difficult-to-obtain DNA sequence can be amplified in vitro for further experimentation.

Strain Typing

Fifteen serotypes of *C. trachomatis* have been delineated based on antigenic heterogeneity found predominantly in MOMP (Wang, S.-P. and Grayston, 1971; Wang, S. P. et al., 1985). Monoclonal antibodies have been isolated that react with genus, species, group, and serotype-specific epitopes on MOMP (Ma et al., 1987; Baehr et al., 1988; Stephens et al., 1988b) and other antigens (Caldwell et al., 1975; Hourihan et al., 1980; Zhang et al., 1987b). A strain of *C. trachomatis* can be characterized with respect to its reactivity with a panel of monoclonal antibodies and assigned to specific serogroups. Conversely, the exposure of an individual to a particular serotype(s) of *C. trachomatis* can be assessed by the reactivity of their sera with EBs from the 15 serotypes. This assay, termed microimmunofluorescence (MIF; Wang et al., 1975), has been used for epidemiologic studies but is generally not suitable for clinical applications due to the tedious nature of the assay.

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The Order Planctomycetales and the Genera *Planctomyces, Pirellula, Gemmata,* and *Isosphaera*

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Introduction

The order Planctomycetales comprises a remarkable group of budding bacteria. They and their nearest relatives, the chlamydiae (Weisburg et al., 1986) (see Chapter 202), are the only known cell-wall containing eubacteria that lack peptidoglycan. Furthermore, the planctomycetes are morphologically distinctive because of their budding division, their spherical to ovoid cells with crateriform pits (Figs. 1 and 2), and the nonprosthecate appendages (stalks) produced by some members of the group (Fig. 3). In some species the stalks, which are multifibrillar bundles or fascicles (Fig. 2), are too thin to be discerned by light microscopy. Multicellular aggregates or rosettes are formed by some species that produce polar holdfasts (Fig. 3). One genus, *Isosphaera,* is a multicellular filamentous bacterium that moves by gliding. Other motile members of the group produce flagella. Knowledge of the order is limited due to the relatively few species that have been obtained in pure culture and characterized.

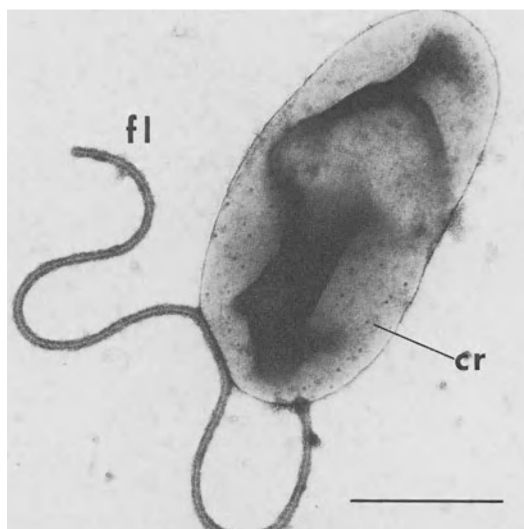


Fig. 1. An electron micrograph of a motile cell of *Pirellula marina* showing flagellum (fl) and numerous circular surface structures referred to as crateriform structures (cr). More distinctive crateriform structures are evidenced on some species (see Fig. 2 below). Bar = 1.0 μm. (Courtesy of Heinz Schlesner.)

The planctomycetes have a typical and unique ribosomal RNA composition. An analysis of their 16S rRNA by oligonucleotide cataloging and sequence analyses places this order as a deep branch within the eubacteria (Stackebrandt et al., 1984) and/or as a group that is undergoing rapid evolution (Woese, 1987). Thus, the planctomycetes differ markedly from the heterotrophic budding and prosthecate bacteria that fall in the alpha group of the purple bacteria. A dendrogram (Fig. 4) of some species has been constructed based upon the 16S rRNA oligonucleotide composition (Stackebrandt et al., 1986a). More recent studies of their 5S rRNA (Bomar et al., 1988) indicate that its length is significantly shorter than that of most eubacteria, ranging from 109–111 nucleotides rather than the "minimal" length of 118 bases of eubacteria and archaebacteria (Erdmann and Wolters, 1986). In addition, position 66 lacks an insertion, and numerous transversions were noted in the secondary structure, features previously unknown for other eubacteria. The evolutionary tree constructed from 5S rRNA data is shown in Figure 5. Another report further indicates that, in contrast to typical eubacteria, the two 16S regions of the ribosomal RNA operon of *Pirellula marina* on the chromosome are separated from the two interlinked 23S-5S rRNA regions by 8.5 and 4.4 kb (Liesack and Stackebrandt, 1989).

Members of the genera *Planctomyces* and *Pirellula* (the latter formerly called *Pirella*) have characteristic eubacterial ester-linked polar lipids rather than the ether-linked lipids of the archaebacteria (Kerger et al., 1988). However, their lipids differ from those of other eubacteria in having large amounts of palmitic, oleic, and palmitoleic acids typically associated with microeukaryotes.

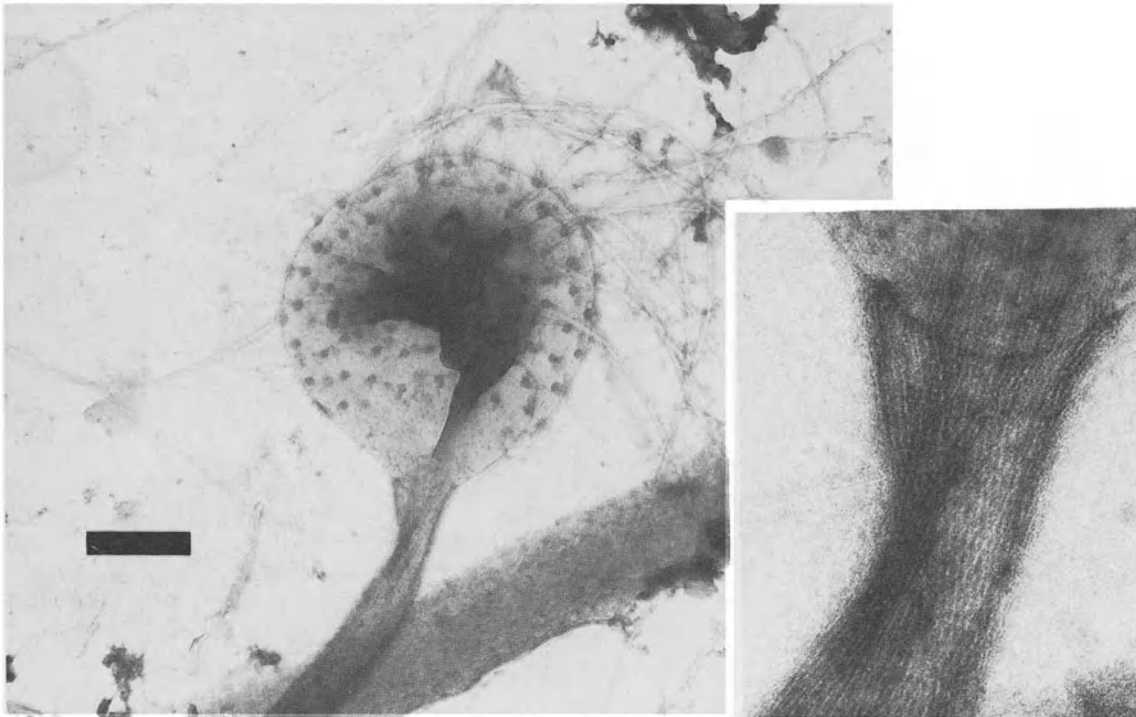


Fig. 2. An electron micrograph of a whole cell of *Planctomyces bekefii* showing many crateriform structures. The inset is an enlargement of the stalk-cell junction, showing the fibrillar nature of the stalk. Sample was collected from University Lake, St. Lucia, Australia, and stained with 1% uranyl acetate in 0.4% sucrose. Bar = 0.5 μm. (Courtesy of John A. Fuerst.)

Because of their unique position within the eubacteria, a new order, Planctomycetales, has been proposed to accommodate members of this group (Schlesner and Stackebrandt, 1986). The order is named for the species *Planctomyces bekefii,* which was first described early in the century by Gimesi (1924), who observed this rosette-forming microorganism in a pond in Hungary. He regarded it as a planktonic fungus, thereby explaining the root "myces" in the genus name. Organisms of this group were also reported by Henrici and Johnson (1935) who observed them in lakes in the northern United States. Neither Gimesi (1924) or Henrici and Johnson (1935) obtained pure cultures. Subsequently several other planktonic, rosette-forming colonial species, including *P. crassus, P. stranskae, P. gracilis,* and *P. guttaeformis* were named but not isolated (see Starr and Schmidt, 1989)

The first reports of pure cultures of this group were published in the 1970s (Staley, 1973a; Bauld and Staley, 1976; Schmidt, 1978). The organisms grew as slow-growing heterotrophs on dilute organic media. Even yet, however, many of the large rosette forms have not been isolated, including the type species of the genus, *Planctomyces bekefii.* All species are aquatic, occurring in freshwater lakes, marine habitats, and salt ponds.

In addition to the unicellular and rosette-forming colonial forms, the order contains filamentous organisms. The filamentous types were initially thought to be cyanobacteria and were named *Isocystis pallida* (Woronichin, 1927). However, strains that have been isolated do not contain chlorophyll *a* or other photosynthetic pigments (Giovannoni et al., 1987b). Isolated strains that were obtained from neutral and alkaline hot springs at temperatures between 35 and 55°C were placed in a new genus as *Isosphaera pallida.*

Table 1 can be used to distinguish the genera of the order Planctomycetales.

Planctomyces

Since the first report of *Planctomyces bekefii,* by Gimesi (1924), limnologists and bacteriologists have periodically reported similar organisms and ascribed several new species and generic names to these bacteria. Isolates of *Planctomyces* spp. were not obtained until the

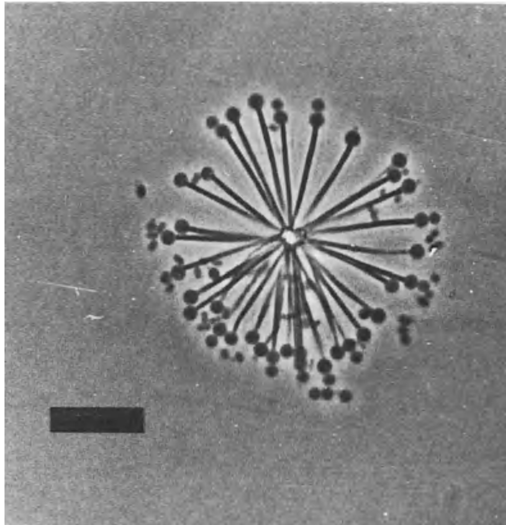


Fig. 3. A phase photomicrograph of a rosette of *Planctomyces bekefii* from University Lake, St. Lucia, Australia. The rosette consists of many spherical cells joined together at the distal tips of their stalks. Bar = 10.0 μm.

late 1970s (Bauld and Staley, 1976; Hirsch et al., 1977; Schmidt, 1978). Even today, *P. bekefii, P. guttaeformis,* and *P. stranskae,* as well as several other unrecognized species, remain unisolated although they are common residents of freshwater habitats.

Habitats

These bacteria occur in freshwater, marine, and saline habitats. Unicellular forms are frequently found attached to other organisms in the environment (Fig. 6). They attach by a holdfast structure located at the tip of the stalk. Species with the most striking morphology, such as *Planctomyces bekefii,* occur in the plankton of freshwater lakes as microcolonies of free-floating rosettes (Fig. 7). It is not known how they can specifically associate with one another to form these rosettes in the natural habitat. The colonial forms do not appear to occur in soft-water habitats, (low osmotic pressure) perhaps because of the peculiar nature of their cell envelop structure. They are most common during the summer and early fall, particularly in eutrophic lakes. For example, in North America they are found in eutrophic lakes in the Midwest (Wintergreen Lake near Kalamazoo, Michigan) and Southeast (University Lake near Chapel Hill, North Carolina, as well as most other lakes of the Carolina and Virginia Piedmont) (J. Staley, unpublished observations). These colonial forms have also been reported to occur in eutrophic ponds in Arizona (Starr and Schmidt, 1984). In contrast, rosette-forming species have not been reported in the mesotrophic to eutrophic soft-water lakes of the Pacific Northwest such as Lake Washington in Seattle, where unicellular forms are found (J. Staley, unpublished observations) (see Fig. 6).

Planctomyces spp. have been enumerated in a variety of Australian freshwater habitats using Most-Probable-Number (MPN) techniques with a dilute peptone broth medium (0.01%). They were found in habitats of all trophic states and their proportion to total viable heterotrophs by this procedure (about 0.025%) was essentially the same, regardless of the trophic state of the habitat (Staley et al., 1980). Thus, higher concentrations were found in eutrophic habitats that contained correspondingly higher concentrations of heterotrophic bacteria than did the less nutrient-rich habitats.

Isolation Procedures

SAMPLE COLLECTION. Water samples (200 ml or more) should be collected aseptically from the environment. If enrichments are to be prepared

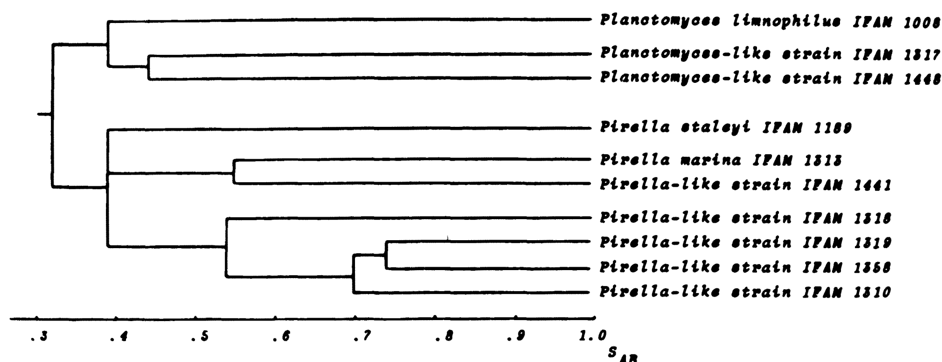


Fig. 4. A dendrogram of similarity coefficients of selected members of the Planctomycetales based on sequence analysis of 16S rRNA oligonucleotides. Note that the genus *Pirella* is now called *Pirellula.* (Courtesy of E. Stackebrandt et al., 1986.)

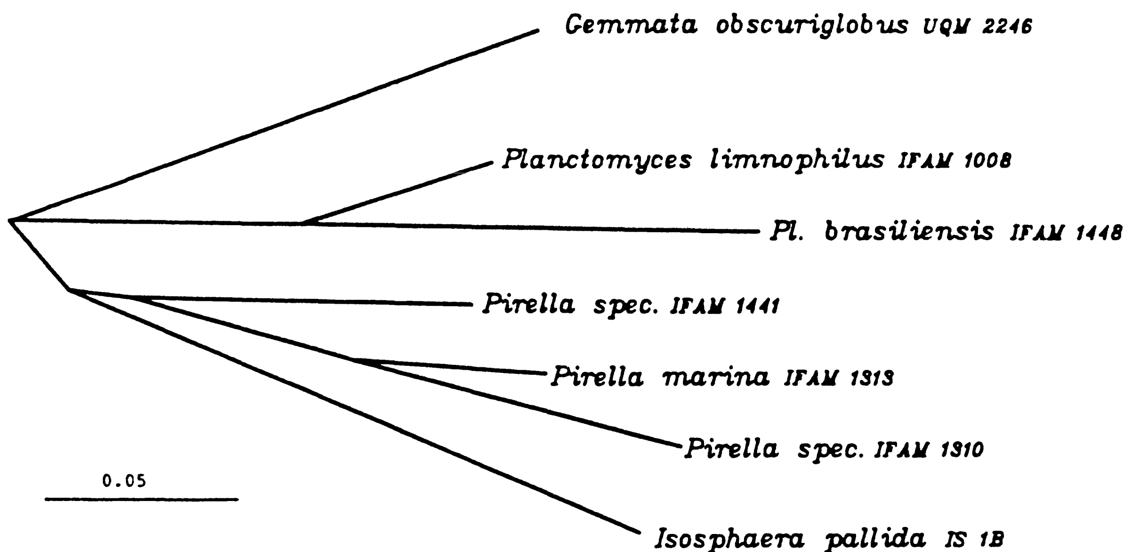


Fig. 5. The evolutionary tree of various members of the Planctomycetales based on 5S rRNA sequences. Scale bar denotes an evolutionary distance of 0.05 D. (Courtesy of D. Bomar et al., 1988.)

Table 1. Characteristics differentiating the genera of the order Planctomycetales.

Genus	Cell arrangement	Stalk	Flagellar type	Crateriform structure	GC content (mol%)
Pirellula	Rosettes in pure culture	−[b]	Single, polar	Reproductive pole	54–57
Gemmata	Single cells	−[a]	Polar bundle	Over entire cell surface	64
Planctomyces	Single cells or rosettes	+	Single, polar	Over entire surface or reproductive pole	50–58
Isosphaera	Filamentous	−	− (gliding)	Over entire surface	62

[a]+, property present; −, property absent.
[b]A rudimentary "stalk" has been reported in ATCC strain 27377 (Starr et al., 1983).

at the day of sampling, samples can be kept at the collection temperature. For periods longer than 12–24 h, it is advisable to refrigerate. In situ temperature and salinity should be known since these parameters will determine the appropriate conditions for enrichment and isolation.

DIRECT ISOLATION AND ENRICHMENT METHODS. Because these organisms occur in low numbers in most natural environments, they usually have to be enriched. Although they are very distinctive morphologically, they have few physiological features that can be used for the development of enrichment and isolation procedures. The addition of penicillin G (1,000–2,000 U/ml) or other antibiotics that inhibit peptidoglycan synthesis, may be used with some success (Schmidt and Starr, 1981), at least at the time of plating, since all isolated strains lack peptidoglycan.

In some cases, it may be possible to isolate strains from natural samples by direct streaking of the sample onto an appropriate agar medium. For example, Hirsch et al. (1977) describe a water agar plating procedure used successfully for the isolation of *Planctomyces limnophilus.* Likewise, *P. brasiliensis* was isolated directly from a salt pit sample from Brazil (Schlesner, 1989) by streaking on a saline medium, M 13 (described in *"Pirellula"*).

A variety of techniques have been applied for the enrichment of *Planctomyces* when numbers from the environment are low. A common procedure is to use the dilute peptone enrichment procedure described for *Prosthecomicrobium* and *Ancalomicrobium* see (Chapter 103). Peptone (Bauld and Staley, 1976) is added to a freshly collected natural sample to a final concentration of 0.01% (or peptone and yeast extract at 0.005% each) and the enrichment incubated at room temperature (or the temperature of the environment from which it was collected). Schmidt (1978) used somewhat lower concentrations of peptone ranging from 0.002 to 0.005% in her enrichments.

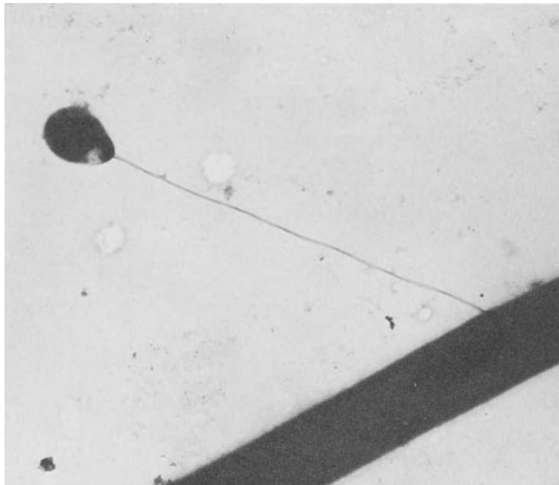
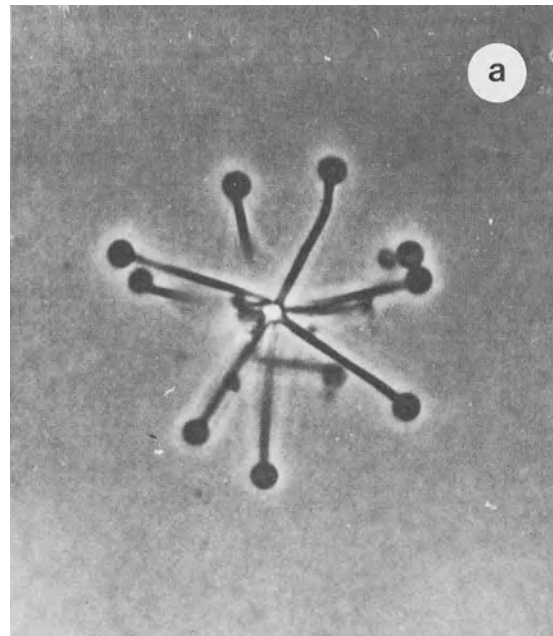


Fig. 6. A lone *Planctomyces* cell attached by its stalk to a sheathed cyanobacterial filament in a sample collected from Lake Washington. Presumably the flexible stalk acts as a tether, allowing the cell to be moved by water currents while the holdfast anchors the cell to the filament. Cell diameter is about 1 μm. Electron micrograph.



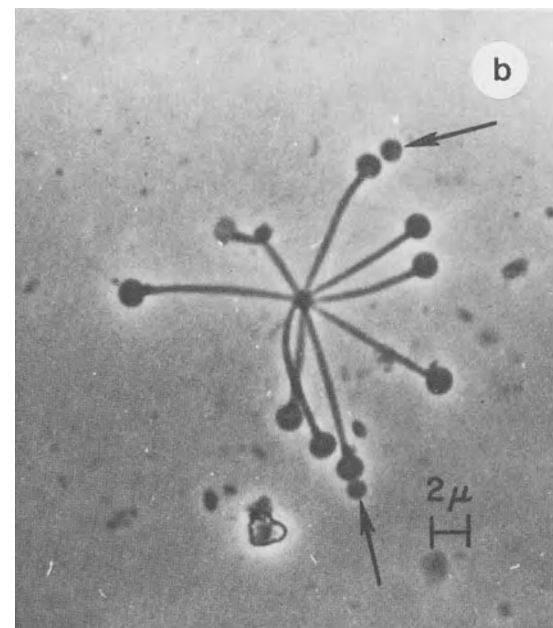


Fig. 7. Phase photomicrographs of a rosette-forming species of *Planctomyces* from University Lake, Chapel Hill, North Carolina. (A) Note the encrustation in the central holdfast region of the rosette due to deposition of iron and/or manganese oxides. (B) Arrows point to buds. Bar = 2.0 μm.

Hirsch and Müller (1986) have described other procedures that have been used successfully in the enrichment of *Planctomyces* spp. 1) In addition to using media with low nutrient concentrations, they have incubated water samples directly with no nutrient addition either in the light or dark for periods of 4 to 16 weeks. 2) Another procedure entails hanging sterile glass slides into fresh or stored water samples and incubating in the light at 20°C. This is reminiscent of the original Henrici procedure in which submerged slides were used to detect periphytic bacteria in lakes (Henrici and Johnson, 1935). When interesting forms appear, material is scraped from the slide with a sterile scalpel and streaked onto an isolation medium. 3) In a different procedure, sterile glass coverslips, some coated with 2% water agar, were inserted vertically into a sterile water agar layer (2 cm deep) in petri dishes, to which was added sufficient sample water to cover the coverslips. Coverslips were examined periodically by phase microscopy for the attachment of budding bacteria. When coverslips contained organisms of interest, replicate ones were removed and material streaked for isolation (Hirsch et al., 1977).

ISOLATION PROCEDURES. Enrichments are examined periodically by phase microscopy to identify types characteristic of the genus: spherical to ovoid cells, oftentimes large (i.e., >1.0 μm in diameter), that attach to detritus or other cells (especially sheathed organisms) and that produce polar to subpolar buds and stalks. The stalks may not be sufficiently thick to be seen by phase microscopy, so to identify these it is advisable to examine samples from prospective enrichments with the transmission electron microscope, by preparing negative stains of enrichment culture material (Fig. 8). Positive en-

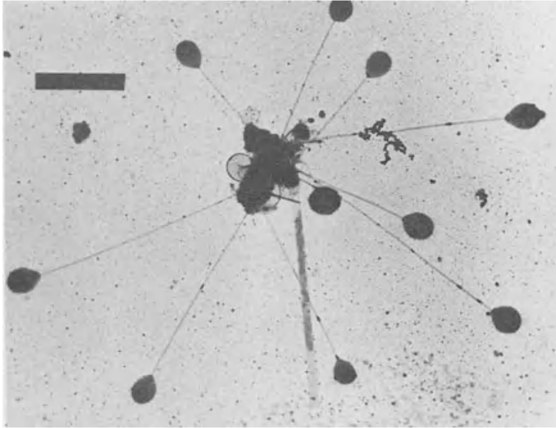


Fig. 8. An unidentified rosette-forming species of *Planctomyces* from a 0.01% peptone enrichment culture of water collected from the Mississippi River in Minneapolis. Bar = 5.0 μm.

richment samples are streaked or dilutions spread (spread 0.1 ml samples from 10^{-2} to 10^{-4} dilutions) on agar media. Media that have been used successfully for this include PYGV medium or PYG medium (same as PYGV, but without vitamins; Staley, 1968).

PYGV medium:

Peptone	0.025%
Yeast extract	0.025%
Glucose	0.025%
Hutner's salts solution (see Chapter 103)	20 ml/l
Vitamin solution (see *Pirellula*)	10 ml/l
Agar	1.5%

For freshwater strains, add distilled water; for marine strains, aged seawater or artificial seawater solution at half- or full-strength should be added (see section on *"Pirellula"*); for strains from other saline or brackish sources, an appropriate salt solution mimicking that of the environment should be used. The pH is adjusted to 7.0 before autoclaving.

Schmidt and Starr (1981) recommend using one of three other media for streaking from enrichment culture material. One medium contains 0.02% peptone, 0.01% yeast extract, 0.1% filter-sterilized glucose or galactose, 10 ml of Hutner's mineral base (Cohen-Bazire et al., 1957), 5 mM $MgSO_4 \cdot 7\ H_2O$ and 1.0 or 1.5% agar. Their second medium contains 0.04% peptone, 0.02% yeast extract, 5 or 10 mM magnesium sulfate, and 1.0 or 1.5% agar made up in either tap water or distilled water to which 10 ml of the Hutner's salts solution is added. The pH of this medium can be adjusted above 7.0 for alkaline samples using 0.005 M Trizma Buffer (Sigma Chemical Co.). Usually a pH of 7.8 or 8.0 is used. Their final medium contains 0.2% peptone, 0.1% yeast extract, 10 ml/l Hutner's salts solution, and 5 mM magnesium sulfate, with 1.0 or 1.5% agar made up with distilled or tap water. Autolysis of osmotically unstable strains can occur in this latter medium.

Colonies of *Planctomyces* spp. develop slowly. Cultures from primary plates need to be incubated for at least one week, and often more than a month is required before they fully develop. One of the most striking characteristics of the colonies is that they continue to develop after most other colony types have stopped growing and are beginning to recede. Typical colonies of the *Planctomyces* types as well as *Pirellula* persist and form impressive mounds about a month after streaking when incubating at room temperature. Colonies may be pigmented (light rose, bright red, or yellow to ochre) or they may be unpigmented.

Repeated restreaking may be necessary to purify some strains. Indeed, some strains cannot be readily isolated even after several restreaking attempts, but some of these can be maintained in monoxenic culture with another heterotrophic bacterium (J. Staley, unpublished observations).

Identification

Because the planctomyces are such distinctive organisms, it is normally a simple matter to determine whether an isolate belongs to this group by microscopic examination alone. If the stalks are sufficiently large, they may be discerned by phase microscopy. However, in the species currently available in pure culture, this is not possible because the stalks are too fine. Thus, the most direct means to verify their identity is by transmission electron microscopy (see Figs. 6 and 8). Whole cell preparations that are negatively stained should show the typical spherical to ovoid cell types with polar stalks. Stalks are the primary characteristic used to distinguish *Planctomyces* from *Gemmata* and *Pirellula.* Although stalks have been infrequently reported from these latter two genera, they are quite rare and often nondistinctive. In contrast, stalks are very common in *Planctomyces* spp. Indeed, in a typical growing preparation of *Planctomyces* spp., all mature, budding cells would be expected to bear a stalk.

Classification of species in the genus has been based largely upon morphological attributes, especially for types that have not yet been isolated. Schmidt and Starr (1977; 1978) and Starr and Schmidt (1989) classified this genus into five morphotypes, I–V. Cells of morphotype I

are spherical, joined together in rosettes, as represented by *Planctomyces bekefii* (Figs. 7 and 9). Cells of morphotype III are ellipsoidal and are represented by the isolated species, *P. maris, P. limnophilus*, and *P. brasiliensis*. Cells of morphotype V are bulbiform-shaped and form rosettes (Figs. 10 and 11). *Planctomyces guttaeformis* and *P. stranskae* are members of this group. No species names have been proposed for morphotypes II and IV, cells of which are ovoid. However, one member of morphotype IV is now classified as *Pirellula staleyi*.

Pure cultures are available of three species, all of morphotype III, including *Planctomyces maris* (Fig. 12), *P. limnophilus*, and *P. brasiliensis*. The stalks on these species are so fine they cannot be discerned by observation with the phase contrast microscope. When observed by the electron microscope, the stalk can be seen to contain a number of fine fibrils bundled together to form a fascicle that emanates at one pole of the ellipsoidal cells. This stalk develops at the pole near the site of the subpolar flagellum. These species are differentiated by 16S rRNA oligonucleotide catalog, salt requirements for growth, and various other morphological and physiological characteristics (Table 2).

All culture isolates have dimorphic life cycles. Reproduction occurs by budding, in which the cell envelope of the daughter bud is formed de novo (Staley, 1973a; Tekniepe et al., 1981). Daughter cells are motile by a polar to subpolar flagellum. Though they lack stalks initially, these are formed as part of their maturation process in a fashion that is analogous to that of *Caulobacter* (although the stalks of *Planctomyces* spp. are not prosthecae).

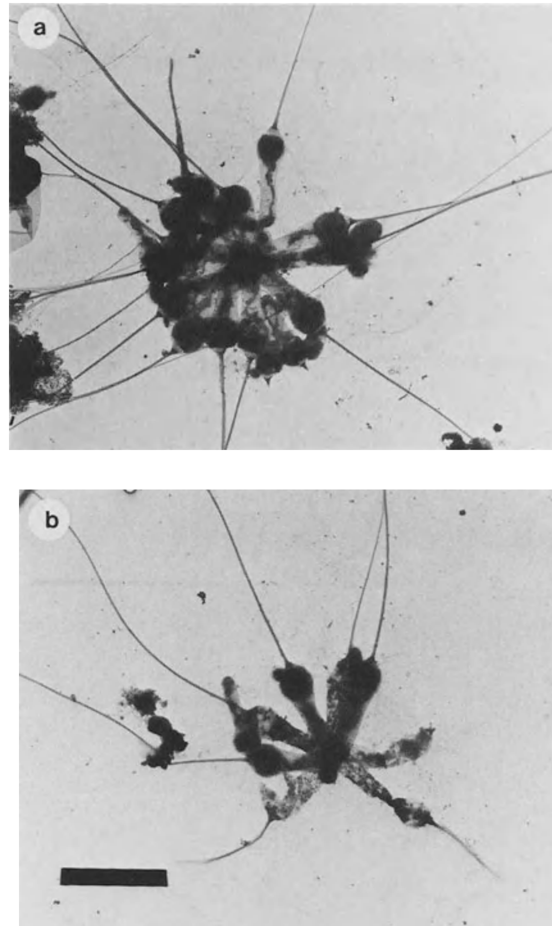


Fig. 10. Rosettes of *Planctomyces guttaeformis*. (A) Note the bulbiform cells that are joined together at their narrow poles. Also note the long spike appendage extending away from the bulbous reproductive pole of mature cells in the rosette. (B) Another rosette from same locale containing budding cells at different stages of development. Buds begin as spherical protuberances but then become bulbiform. Several cells in this rosette are lysed. From University Lake, Chapel Hill, North Carolina. Bar = 5.0 μm.

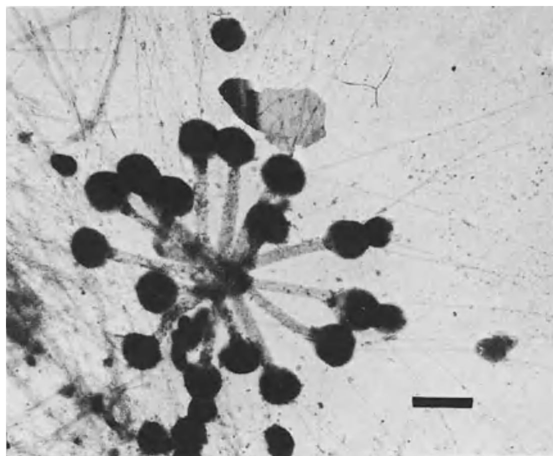


Fig. 9. An electron micrograph of an organism that resembles *Planctomyces bekefii*. Though this organism is similar to *P. bekefii*, note that its cells have two to four or more very long prominent filiform appendages extending outward from each cell of the rosette near the reproductive pole rather than several shorter ones characteristic of *P. bekefii* (see Fig. 2). This type of *Planctomyces* is common in lakes in the southeastern USA. From University Lake, Chapel Hill, North Carolina. Bar = 2.0 μm.

All three of these species are heterotrophic organisms that grow aerobically. Various mono- and dissacharides are used, including glucose, galactose, cellobiose, maltose, and N-acetylglucosamine. *P. maris* and *P. brasiliensis* also use glucuronic acid. Ammonium salts can be used as the sole nitrogen source by *P. maris* and *P. limnophilus*. Vitamins are not required by any of the isolates.

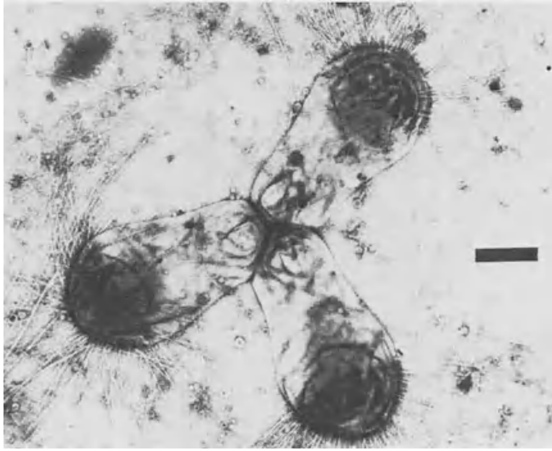


Fig. 11. *Planctomyces stranskae* collected from Kiwanis Park in Tempe, Arizona. Cells of this species do not develop terminal spikes. Bar = 1.0 μm. (Courtesy of Jean Schmidt.)

The characteristics of the other unisolated but currently recognized species are given in Table 3. All of these species produce large, conspicuous rosettes in the natural freshwater habitats in which they occur. Each cell of *Planctomyces bekefii* produces a distinctive thick stalk that terminates in a holdfast. The stalk is a tubular structure comprising many fibrils (Schmidt and Starr, 1980). The holdfasts enable cells to join together in the natural habitat to form rosettes (Figs. 7 and 9). Some colonial forms such as *P. bekefii* can develop stalk encrustations (Fig. 13) containing iron and/or manganese oxides (Schmidt et al., 1981).

The most characteristic feature of *P. guttaeformis* and *P. stranskae* is their bulbiform cells (Starr and Schmidt, 1984), which are joined together at their narrow poles to form rosettes (Figs. 10 and 11). In addition, *P. guttaeformis* produces a long terminal spike from its large pole, a feature lacking in *P. stranskae.*

Pirellula

Habitats

Pirellula spp. and morphologically similar bacteria have been observed in and isolated from a variety of aquatic environments: freshwater (Staley, 1973; Tekniepe et al., 1981; Schlesner, 1989); brackish water (Gebers et al., 1985; Kauolbel-Boelke et al., 1985; Schlesner, 1986);

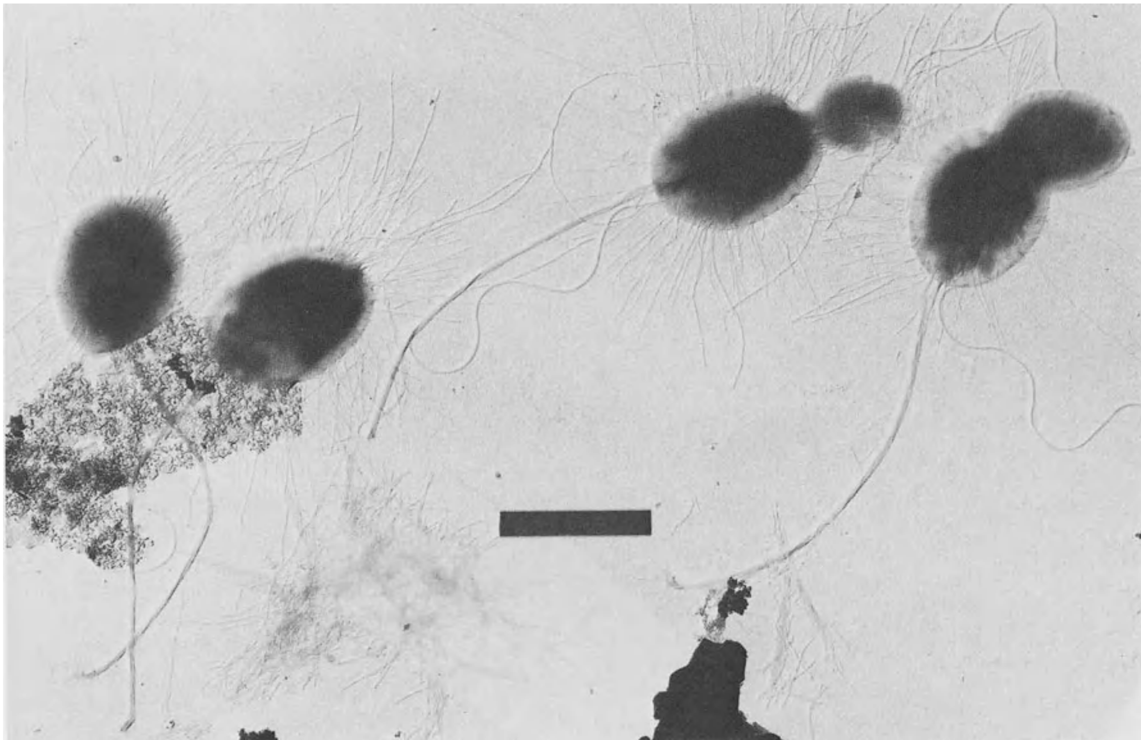


Fig. 12. Shadowed electron micrograph of cells of *Planctomyces maris.* Note the polar to subpolar buds, fibrillar stalks, subpolar flagella, and peritrichous fimbriae. Bar = 1.0 μm.

Table 2. Differentiation among isolated species of *Planctomyces*.

Character	*P. maris*	*P. limnophilus*	*P. brasiliensis*
Cell shape; maximum cell diameter	Ovoid, 1.5 μm	Ovoid, 1.5 μm	Spheroid, 1.8 μm
Source	Marine	Freshwater	Salt pit
Colony pigmentation	Colorless to light rose	Red	Yellow
Salinity growth range	1.5–4.0%	<1.0%	0.7–10.0%
NaCl growth range (mM)	100–>300	ND[a]	100–170
Temperature growth range	6–38°C	17–39°C	ND
GC content (mol%)	51	53	55–58
Genome size (× 10^9 daltons)	3.62	2.67	2.81

[a]ND, not determined.

Table 3. Differentiation among recognized species of *Planctomyces* that have not yet been isolated in pure culture.

Characteristic	*P. bekefii*	*P. stranskae*	*P. guttaeformis*
Cell shape	Spherical	Bulbiform	Bulbiform
Stalk	Tubular	Absent	Absent
Other cell appendages	Fimbriae and larger spikes[a]	Fimbriae only	Fimbriae and a single long tapering spike

[a]At least two variants (species?) exist. One type has several shorter spikes (see Fig. 2) whereas another type has fewer, very long spikes (see Fig. 3).

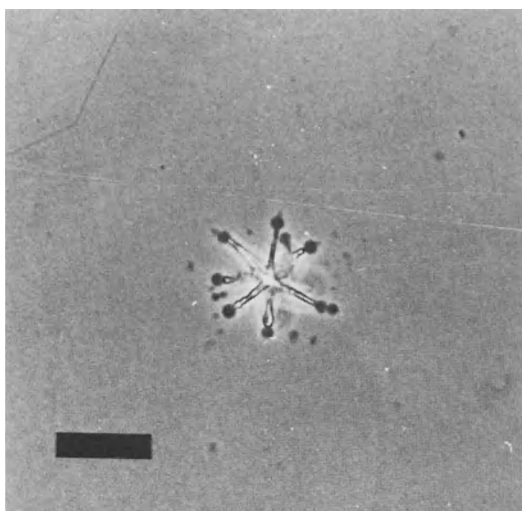


Fig. 13. A phase photomicrograph of a small rosette-forming species of *Planctomyces* from University Lake, St. Lucia, Australia, showing heavy deposition of iron and or manganese oxides on the stalks. Bar = 10 μm.

hypersaline water of a salt pit (Gebers et al., 1985); hot springs of Tiberias in Israel (Kahan, 1961); groundwater (P. Hirsch, personal communication); alkaline water from ponds in a lime pit near Lägerdorf (Schleswig-Holstein, Germany; Schlesner, 1989); lakes rich in Na_2SO_4, Na_2CO_3, and $NaHCO_3$ ("Lacken") in Burgenland, Austria; wastewater aeration lagoons of a sugar factory; marine and brackish water basins in an aquarium (Institut für Meereskunde, Universität Kiel); water basins in a greenhouse in the botany garden of the University of Kiel; and even in the pitcher trap of the insectivorous plant *Nepenthes* sp. (Schlesner, 1989).

In spite of the widespread distribution, *Pirellula*-like bacteria are only occasionally observed in water samples. After storage at room temperature (a few days to some weeks), however, such organisms are often found in high numbers, especially on the bottom or at the wall of the vessel. Obviously, the organisms live attached to surfaces and only the swarmer cells are free in the water column. When the population is very dense, however, *Pirellula*-like organisms may be found in high numbers in the free water.

Isolation

Various methods have successfully been applied for the enrichment of *Pirellula* spp.:

1. Storage of samples in the laboratory.
2. Addition of small amounts (0.005–0.01%) of peptone, yeast extract, or glucose to the water sample.
3. Addition of 0.1% chitin to the sample.
4. Addition of 0.1% KNO_3 and anaerobic incubation.

Isolation procedures take advantage of resistance to antibiotics that affect the biosynthesis of peptidoglycan and, furthermore, the ability to utilize N-acetylglucosamine as sole carbon and nitrogen source. The salinity of the

medium should not differ much from that of the natural environment, since many strains show a rather narrow salinity tolerance (H. Schlesner, unpublished observation). The following media have successfully been applied for isolation of strains of *Pirellula* spp.:

Medium M 1 + A

Solution 1:

$CaCO_3$	5.0 g
$Na_2HPO_4 \cdot 2H_2O$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Hutner's basal salts (see below)	20 ml
Gellan gum Gelrite[a]	9.0 g
Vitamin solution No. 6	10 ml
Distilled water	920 ml

Adjust pH to 9.0; autoclave 121°C for 20 min.

[a]Kelco Inc., San Diego, California, USA.

Solution 2:

N-acetylglucosamine	2.0 g
Ampicillin sodium salt	0.2 g
Distilled water	50 ml

Adjust to pH 9.0, filter sterilize, and add to solution 1.

Hutner's basal salts (Cohen-Bazire et al., 1957)

Nitrilotriacetate (NTA)	10.00 g
$MgSO_4 \cdot 7H_2O$	29.70 g
$CaCl_2 \cdot 2H_2O$	3.34 g
$NaMoO_4 \cdot 2H_2O$	12.67 mg
$FeSO_4 \cdot 7H_2O$	99 mg
Metal salt solution "44" (see below)	50 ml
Double-distilled water	900 ml

The NTA is first dissolved by neutralization with KOH. The other salts are then added. Adjust pH to 7.2 with KOH or H_2SO_4. Adjust volume to 1,000 ml with double distilled water. Store cold (5°C) and clean. The solution is clear.

Metal salts solution "44"

Ethylene diamino tetraacetate (EDTA)	250.0 mg
$ZnSO_4 \cdot 7H_2O$	1,095.0 mg
$FeSO_4 \cdot 7H_2O$	500.0 mg
$MnSO_4 \cdot H_2O$	154.0 mg
$CuSO_4 \cdot 5H_2O$	39.2 mg
$CoCl_2 \cdot 6H_2O$	20.3 mg
$Na_2B_4O_7 \cdot 10H_2O$	17.7 mg
Double-distilled water	1 liter

To retard precipitation add a few drops of H_2SO_4 before making to volume. Store cold (5°C).

Vitamin solution No. 6 (Staley, 1968)

Biotin	4.0 mg
Pyridoxine hydrochloride	20.0 mg
Thiamin hydrochloride	10.0 mg
Ca pantothenate	10.0 mg
p-Aminobenzoic acid	10.0 mg
Folic acid	4.0 mg
Riboflavin	10.0 mg
Nicotinamide or nicotinic acid	10.0 mg
Vitamin B_{12}	0.2 mg
Double-distilled water	1 liter

Stirring of the mixture improves dissolution. Sterilize by filtration only. Store dark and cold (5°C).

Medium M 30 + A

Solution 1:

Hutner's basal salts	20 ml
Artificial seawater	125 ml
Buffer 0.1 M Tris/HCl, pH 7.5	50 ml
Agar	18 g
Distilled water	760 ml

Autoclave at 121°C for 20 min.

Solution 2:

N-acetylglucosamine	2.0 g
$Na_2HPO_4 \cdot H_2O$	0.1 g
Ampicillin sodium salt	0.2 g
Vitamin solution No. 6	10 ml
Distilled water	40 ml

Add filter-sterilized to solution 1.

Artificial seawater (Lyman and Fleming, 1940)

NaCl	23.477 g
$MgCl_2$	4.981 g
Na_2SO_4	3.917 g
$CaCl_2$	1.102 g
KCl	0.664 g
$NaHCO_3$	0.192 g
KBr	0.096 g
H_3BO_3	0.026 g
$SrCl_2$	0.024 g
NaF	0.003 g

Medium 31 + A

Solution 1:

$CaCl_2 \cdot 2H_2O$	0.1 g
$MgCl_2 \cdot 6H_2O$	0.1 g
Hutner's basal salts	20 ml
Buffer 0.1 M Tris/HCl, pH 7.5	50 ml
Agar	18.0 g
Distilled water	880 ml

Adjust pH to 7.5, autoclave at 121°C for 20 min

Solution 2:

N-acetylglucosamine	2.0 g
$Na_2HPO4 \cdot 2\ H_2O$	0.1 g
Ampicillin sodium salt	0.2 g
Vitamin solution No. 6	10 ml
Distilled water	40 ml

Adjust pH to 7.5, filter-sterilize, and add to solution 1.

To minimize growth of fungi, 0.2 g/l cycloheximide may be added to solution 2 of each respective medium.

For culturing or long-term storage of pure cultures, the above media should be enriched by adding 0.25 g of peptone and 0.25 g of yeast extract to solution 1. Good results are also obtained with medium M 13.

Medium M 13 (Schlesner, 1986)

Peptone	0.2 g
Yeast extract	0.2 g

Glucose	0.2 g
Hutner's basal salts	20 ml
Vitamin solution No. 6	10 ml
Buffer 0.1 M tris/HCl, pH 7.5	50 ml
Artificial seawater	250 ml
Distilled water	670 ml

Identification

Pirellula spp. are easily recognized by their morphology. The cells are ovoid, sometimes elliptical, or nearly spherical. They are polarly organized. From the smaller cell pole, holdfast substance is excreted which allows the cells to attach to surfaces or to each other and form rosettes (Fig. 14). Buds develop at the broader pole as a minor mirror image of the mother cell (Fig.15). Immunoferritin labeling experiments have shown that at least the surface components of the new buds are synthesized de novo (Tekniepe et al., 1982). The buds are motile by a single flagellum, polarly to subpolarly inserted, which is about 20 nm thick (Schlesner, 1986) and which inserts at the proximate (the later reproductive) pole (see Fig. 1).

Crateriform structures are scattered over the whole cell surface of the buds in the early developmental stage, while in adult cells their distribution is restricted to the reproductive pole only. Adult cells also have fimbriae, originating from the crateriform structures.

Another type of surface structure, described as "stacked disks" (Schmidt and Starr, 1979), is shown in thin sections through the reproductive cell pole (Liesack et al., 1986).

Colonies are red, pink, or colorless. Pigmentation seems to be of taxonomic significance, as homology groups derived from DNA/DNA hybridization experiments with 54 strains contained either pigmented or unpigmented strains (Bartels and H. Schlesner, unpublished observations).

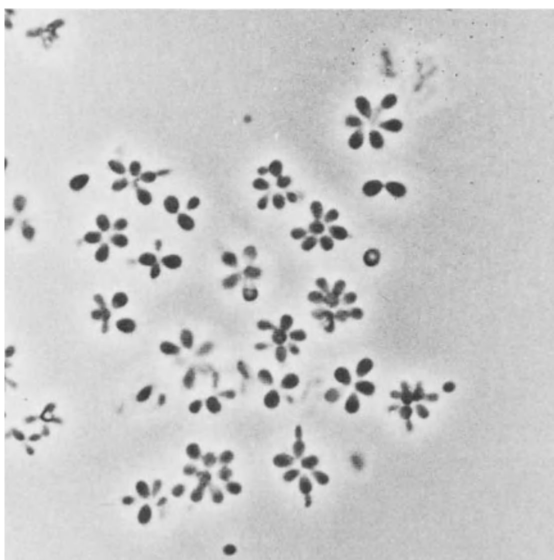


Fig. 14. Phase photomicrograph illustrating rosette formation by *Pirellula marina.*

The phylogenetic position of seven strains of *Pirellula* spp. (including *P. staleyi* and *P. marina*), analyzed by 16S rRNA cataloging showed a great phylogenetic diversity among these strains (Stackebrandt et al., 1986). These findings are supported by analysis of 5S rRNA (Bomar et al., 1988).

Despite their phylogenetic diversity, the strains investigated are phenotypically very similar. As carbon sources, mainly sugars and sugar derivatives are utilized; N-acetylglucosamine serves as both carbon and nitrogen source. Sugar alcohols may or may not be utilized. Some strains are able to reduce nitrate anaerobically. All strains tested so far hydrolyze gelatin; casein, DNA, or starch are hydrolyzed by some strains, but cellulose is not hydrolyzed at all. Additional methods will have to be applied to find more discriminating taxonomic characteristics.

Two species are described. The type species is from freshwater: *Pirellula staleyi* (Schlesner and Hirsch, 1987), syn. *Pirella staleyi* (Schlesner and Hirsch, 1984), syn. *Planctomyces staleyi* (Starr et al., 1983), syn. *Pasteuria ramosa* (Staley, 1973). The other species is marine: *Pirellula marina* syn. *Pirella marina* (Schlesner, 1986).

Gemmata

The genus *Gemmata* is at present represented by one species, *Gemmata obscuriglobus.* The single strain (UQM 2246) representing the single species was isolated from a single source, the surface waters of Maroon Dam, during a study of the microflora of freshwaters in southeast Queensland, Australia (Franzmann and Skerman, 1984). The discoverers of the strain recognized the similarities of this coccoid budding bacterium to a strain of great significance in the history of Planctomycetales systematics, strain ATCC 27377, which at one time was proposed as the neotype of *Pasteuria ramosa,* but later transferred to the genus *Planctomyces* (Starr et al., 1983) and to the genus *Pirella* (Schlesner and Hirsch, 1984), a genus name later rejected in favor of *Pirellula* (Schlesner and Hirsch, 1987; see above "Pirellula" for a detailed account of these changes). Franzmann and Skerman (1984) distinguished *Gemmata obscuriglobus* UQM 2246 from ATCC 27377 by DNA base content, number of flagella on

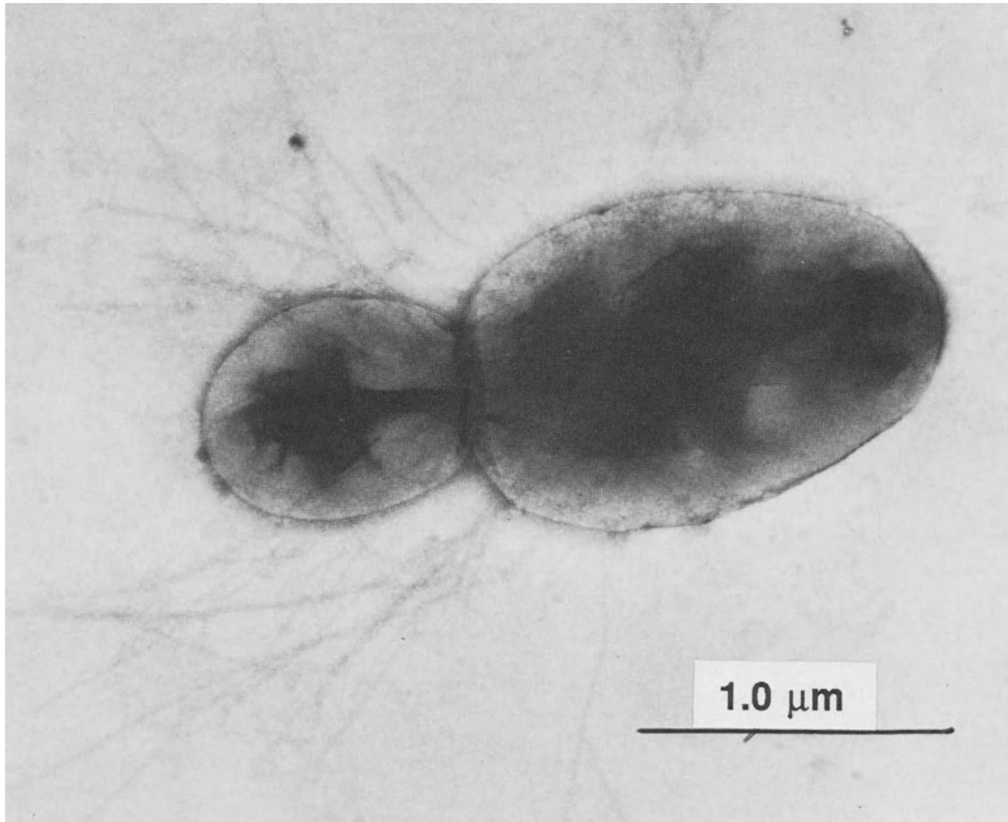


Fig. 15. An electron micrograph of *Pirellula marina* showing a single polar bud. Bar = 1.0 μm.

swarmer cells, lack of a discrete holdfast, and the possession of a phase-dark inclusion in *Gemmata* postulated to contain DNA, in addition to several other phenotypic characters outlined in Table 4. Later molecular systematics investigation of the 16S rRNA and 5S rRNA of *G. obscuriglobus* confirmed its status as a distinct genus and as a member of the phylogenetically distinct eubacterial order Planctomycetales (Stackebrandt et al., 1986; Bomar et al., 1988). *Gemmata* occupies a distinct position within the Planctomycetales phylogenetically, since not only does its 16S rRNA possess its own unique set of signature oligonucleotides among the eubacteria (Table 5), but both 16S rRNA and 5S rRNA sequence similarities with other Planctomycetales genera indicate that the root of the evolutionary tree derived for members of the order lies close to the point at which the ancestor of *G. obscuriglobus* branched off. Thus, this species is the deepest branching organism within the order Planctomycetales (Stackebrandt et al., 1986b; Bomar et al., 1988; see Figs. 4 and 5).

Isolation

The only existing strain of *Gemmata obscuriglobus,* UQM 2246, was isolated from the surface waters of the littoral zone of a freshwater dam in subtropical Queensland, Australia. Micromanipulation using the equipment and methods of Skerman (1968) as applied by Franzmann (1983) was employed in the isolation. This method employs a micromanipulator consisting of a glass microtool attached to a magnet, which is in turn carried on a metal slide that is part of a lens "collar" screwed onto the microscope objective mounting. A Leitz 32× Phaco phase objective is used on a phase contrast microscope (preferably an Olympus CHA microscope equipped with a Reichert Phase contrast condenser or any system compatible with a Leitz 32× Phaco objective). The microtool is focused by moving the lens collar up and down on the objective mounting; once focused, it can be used to transfer cells on an agar surface across the surface and away from other cells by trapping cells in the water film around the microtool and moving the mechanical stage con-

Table 4. Characteristics of *Gemmata obscuriglobus* UQM 2246 and strain ATCC 27377.

Character	UQM 2246[a]	ATCC 27377[b]
GC content (mol%)	64.4 ± 1.0	57.1
Cell shape	"Puckered" spherical to ovoid	Ovoid
Cell size	(1.4–3.0) × (1.4–3.0) μm	(0.5–3.0) × (1.0–5.0) μm
Crateriform structure distribution	Uniform	Polar
Reproduction	Budding	Budding
Gram reaction	−	+
Fimbriae	+	+
Motile swarmer cells	+	+
Flagellation	Multitrichous	Monotrichous
Colony color	Rose	Yellow
Catalase	+	+
Oxidase	−	+
Anaerobic growth	−	−
Generation time	13.1 ± 2.8 h	ca. 13 h
Growth temperature	16.0–35.0°C	17.7–29.6°C
OF test[c]	Oxidative	Oxidative
Carbon source utilization:		
Glucose	+	+
Fructose	−	+
Pyruvate	−	+
Attachment to glass	+	+
Habitat	Freshwater	Freshwater
Rosettes formed	−	+
Phase-dark inclusion	+	−

[a]Data from Franzmann and Skerman (1984) supplemented by unpublished observations (J. A. Fuerst).
[b]Data from Staley (1973b) and Schlesner and Hirsch (1984).
[c]OF test, oxidative-fermentative test.

trols, the agar plate being mounted on the stage. The whole assembly can be placed in a UV-sterilizable Perspex chamber or in a plastic bag with eyepiece holes, to facilitate aseptic handling of plates. 9 cm plastic petri dishes containing 10 ml of lakewater agar (filtered lakewater solidified with 1.5% Noble agar for optical clarity; see below for formula) are inoculated by running an approximately 0.5 ml drop of sample water down the center of a plate which is surface-dried in advance. Storage of samples before inoculation should be avoided. Inoculated plates are allowed to remain horizontal until free moisture is absorbed. Plates are examined after 8 h incubation at 28°C or until microcolony formation is apparent. Cells from microcolonies displaying cell morphology and size consistent with *Gemmata* are manipulated away from the central inoculum line to a position on the surface of the same plate, well separated from the inoculation line. Position of a single cell can be marked using a microtool and an agar block circumscribing that area excised with a sterile scalpel and transferred to a fresh lakewater agar plate or to a fresh lakewater peptone yeast extract agar plate (see formula below). Purity can be checked microscopically at each stage of the isolation. Strains can be maintained on soil extract agar supplemented with 0.1% glucose or on casitone yeast extract agar:

Lakewater Noble Agar (Franzmann and Skerman, 1981)

Filter water from a eutrophic lake or pond through a 2 μm membrane filter and add Bacto Noble agar to 1.5%. Sterilize by autoclaving at 121°C for 20 min.

Lakewater Peptone Yeast Extract Agar (Franzmann and Skerman, 1981)

Bacto peptone	0.1 g
Bacto yeast extract	0.1 g
Bacto agar	15 g

2 μm-membrane-filtered eutrophic lake water 1 liter
Sterilize by autoclaving at 121°C for 20 min.

Casitone Yeast Extract Agar

Bacto casitone	5 g
Bacto yeast extract	3 g
$MgSO_4 \cdot 7H_2O$	2 g
Bacto agar	15 g
Distilled water	1 liter

The pH is adjusted to 7.2, the agar is added and the medium sterilized by autoclaving at 121°C for 20 min.

Soil Extract Agar with 0.1% Glucose

Soil	1000 g
Distilled water	1 liter

Table 5. Oligonucleotide signature for *G. obscuriglobus* and its frequency in other bacteria.

G. obscuriglobus	*Pirellula* (n=7)[a]	*Planctomyces* (n=3)	Other eubacteria (n=450)
CCCCCG	–	–	5
CCUCAG	3	1	15
CCCAUG	–	–	17
ACCUCG	–	–	16
UCCUAG	7	–	20
UACUAG	–	2	24
AAAUUG	1	–	18
CUUUAG	–	–	5
UUUACUG	–	–	5
AUCUUCG	–	–	13
UACACAG	–	–	4
AAUCCCG	–	–	3
CCAUCAG	–	–	10
ACCCCAG	–	–	–
UUAAAAUG	–	–	–
ACUUCUUG	–	–	–
AUUCAUCG	–	–	–
ACCUUUAG	–	–	–
CUAUCAAG	–	–	1
UCAAACCG	4	1	–
AAAAAUAAG	–	–	–
ACCCACAAG	–	–	–
CCAAUCACAG	–	–	–
ACCUCUUCUG	–	–	–
UCCCAUAACG	–	–	1
AUAUCUACAG	–	–	–
CCACCUUCACCG	–	–	–
AAAUUACACCCCAG	–	–	–
AUCUAUCCCAAAACG	–	–	–
AAACCCUUACCUUUCG	–	–	–

From Stackebrandt et al. (1986b).
[a]Where n equals the number of organisms included.

Mix soil with water and autoclave at 121°C for 20 min. Add 10 g $CaCO_3$ and mix. Decant and filter liquid. Autoclave filtrate at 121°C for 20 min. and store as stock solution. Add agar to 1.5% to stock solution and autoclave at 121°C for 20 min. After autoclaving, add filter-sterilized glucose aseptically to the agar medium to give a final concentration of 0.1%.

AXENIC CULTURE MAINTENANCE. *Gemmata* has been successfully stored by lyophilization and under liquid nitrogen; it has been revived after liquid nitrogen storage for eight years. Also, when reviving stored ampoules from liquid nitrogen, soil extract agar supplemented with 0.1% glucose is superior to lake water agar.

Identification

Gemmata obscuriglobus is a budding, nonprosthecate aerobic organism with spherical to avoid cells. Initial appearances from negative-stain transmission electron microscopy and phase contrast light microscopy (Fig. 16) suggest a spherical or ovoid shape. However, scanning electron microscopy of cells prepared by critical point drying reveals that a "puckered" area roughly square in shape is often present on one side of the cell surface, deforming the spherical shape (Fig. 17). Such puckered areas are consistent with the crescent form often seen in thin-sectioned cells (Fig. 18). This feature is unique among the Planctomycetales and may be unique among the bacteria as a whole.

Consistent with the position of the genus within the order Planctomycetales, cells observed by negative staining possess crateriform structures—circular areas on the cell that accumulate negative stain (Fig. 19). These are distributed all over the cell surface, similar to the distribution found in the genus *Planctomyces,* but unlike that in *Pirellula* species (Schlesner and Hirsch, 1984). Such crateriform structures have also been observed in metal-shadowed cell walls of *Gemmata obscuriglobus* (Stackebrandt et al., 1986b). The cell walls of *G. obscuriglobus,* like those of other Planctomycetales, lack pep-

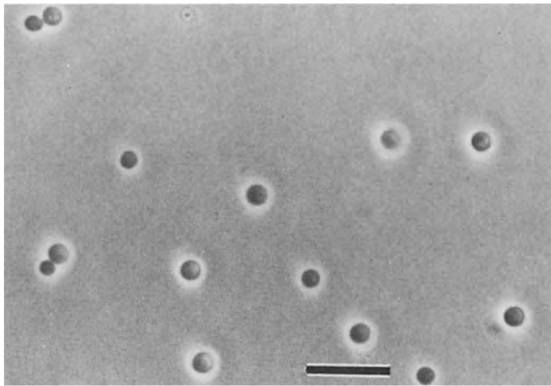


Fig. 16. Phase contrast photomicrograph of cells of *Gemmata obscuriglobus* UQM 2246. Budding is displayed in addition to the superficially spherical cell shape. Bar = 10 μm.

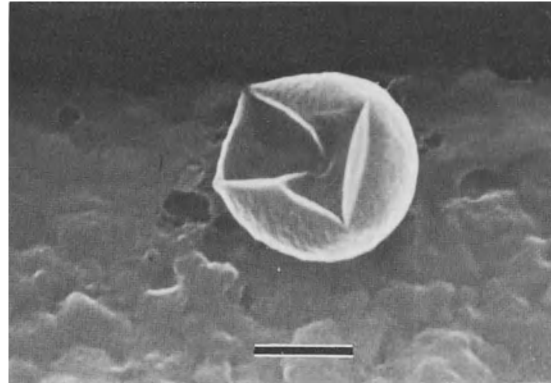


Fig. 17. Scanning electron micrograph of *Gemmata obscuriglobus* UQM 2246, showing the "pucker" in the otherwise spherical cell surface. Cell surface appears continuous inside the "pucker." Cells were prepared by dehydration in ethanol, critical-point drying from amyl acetate, and sputter-coating with gold. Bar = 1 μm.

tidoglycan and diaminopimelic acid, and are also similar to other members of the order in the predominately proteinaceous composition of the wall (Stackebrandt et al., 1986b). In thin sections, the cell wall has the typical appearance expected for the peptidoglycan-less walls of Planctomycetales (Fig. 20) with inner and outer electron-dense layers separated by an electron-transparent layer, the inner layer being more electron-dense, as found for morphotype IV planctomycete ultrastructure by Tekniepe et al. (1981). *Gemmata* thus shares budding reproduction, crateriform structures, and cell-wall composition with other genera of the Planctomycetales, and this relationship is confirmed by the high number of Planctomycetales-specific signature oligonucleotides present in the 16S rRNA oligonucleotide catalog of the genus (Stackebrandt et al., 1986b), and by the presence of the unique Planctomycetales-specific "short" 5S rRNA with lack of insertion at position 66 and numerous characteristic base-pair inversions (Bomar et al., 1988).

In the initial study of *G. obscuriglobus,* Franzmann (1983) noted that in some thin-sectioned cells nuclear material was packaged in a discrete body within the cell, and that this might be correlated with the appearance of phase-dark inclusions in light microscopy preparations. Intracellular membrane material is commonly seen in thin sections, and although in some cases an appearance of membrane involvement with nuclear packages suggests that they may be membrane-bound structures (Fig. 18), this may be artifactual and further studies are required to resolve the nature of the nuclear packages. Such packages often consist of an appearance of fibrillar material within a shell of electron-dense amorphous material separating the material from the rest of the cell (Fig. 18). Several such packages can occur within a single cell.

A stalk structure was not observed in the original examination of the genus, but more recent observations indicate that a multifibrillar stalk-like bundle can sometimes arise from groups of cells (Fig. 21). This bundle is coherent enough to resemble a *Planctomyces* stalk, but further work is required to delineate its significance.

As shown in Table 4, differentiation of *Gemmata obscuriglobus* from the morphologically similar ATCC 27377 is based on the higher GC content of *Gemmata,* occurrence of phase-dark inclusions, flagellation, oxidase test, utilization of fructose and pyruvate, temperature range for growth, and colony color. The GC content for ATCC 27377 has been subject of several determinations, with slightly variable results; thus, Starr et al. (1983) reported a high value of 59.0 ± 0 mol% by buoyant density and Schlesner has noted a lowest value of 56.4 ± 0.4 mol% by thermal denaturation (Schlesner and Hirsch, 1984), while for their comparison, Franzmann and Skerman (1981) used the figure of 57.1 mol% found by Staley (1973) by thermal denaturation. In all cases, however, the GC content of *G. obscuriglobus* is significantly higher. Although stalklike structures are not prevalent in *Gemmata,* and thus confusion with *Planctomyces* is not likely, they are sometimes seen, so that this character does not seem ideal for inclusion in differential keys for the Planctomycetales.

Isosphaera

Isosphaera pallida is an aerobic, heterotrophic budding bacterium that is unusual because it is motile by gliding and is also phototactic. With

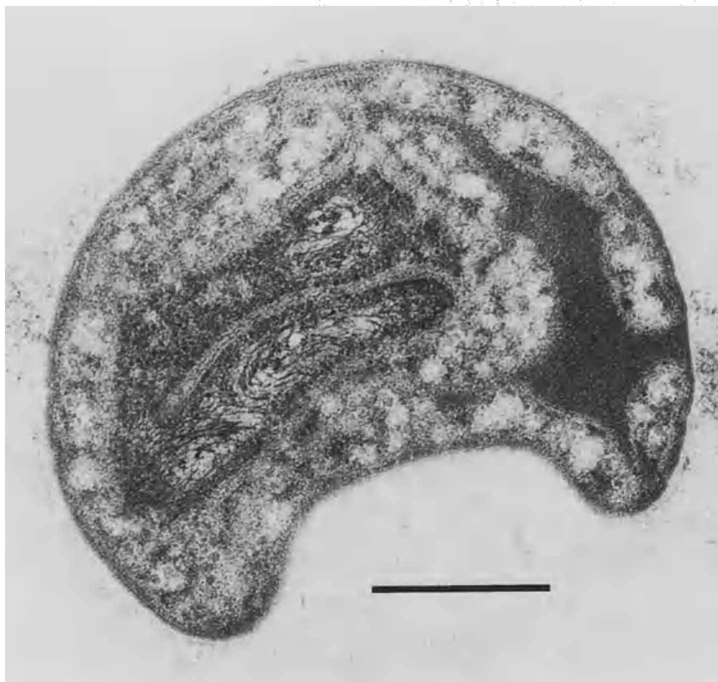


Fig. 18. Electron micrograph of thin section of *Gemmata obscuriglobus* UQM 2246, displaying crescent-cell profile and the package of fibrous nuclear material enclosed within a membrane, folding of which has resulted in a double-membrane profile in the center of the nucleoid. An amorphous electron-dense body with irregular outline but without condensed nuclear fibers is also present in the cell. Cells were fixed with glutaraldehyde and osmium tetroxide. Bar = 0.5 μm.

other members of the Planctomycetales, *Isosphaera* shares characteristics that are of potential evolutionary significance—the presence of proteinaceous cell walls and phospholipids containing beta-hydroxylated fatty acids (Kerger et al., 1984; Stackebrandt et al., 1984; Giovannoni et al., 1987).

Molecular phylogenetic comparisons of 5S rRNA have shown that *I. pallida* is a member of the order Planctomycetales (Bomar et al., 1988), but only distantly related to members of the genera *Gemmata, Pirellula,* and *Planctomyces.* Analyses of 16S rRNA have confirmed the results obtained with the 5S molecule.

Isosphaera pallida, the only recognized species of the genus *Isosphaera,* inspired a debate in the taxonomic literature long before it was first isolated in pure culture in 1979 (Giovannoni et al., 1987b). The organism attracted attention because of its conspicuous habitat and morphology. It commonly occurs in North American and European hot springs at temperatures from 35 to 55°C. The distinctive chains of spherical, budding cells formed by *Isosphaera* (Fig. 22) are readily distinguishable from morphologically similar organisms, such as cyanobacteria of the genus *Pseudanabaena.* However, because of this similarity, *Isosphaera pallida* was originally described by Woronichin (1927) as a cyanobacterium, *Isocystis pallida,* based only on observations of collected field specimens. This early case of mistaken identity was understandable, considering that *I. pallida* is invariably found in the euphotic zone of hot springs in association with cyanobacteria.

Geitler (1955) obtained the first enrichment cultures of an organism resembling *Isosphaera.* These were taken from a water-filled depression in a rotting spruce stump. He concluded that his isolate was a yeast, and assigned it to a novel genus and species, *Torulopsidosira filamentosa.* After examining collected specimens of *Isocystis pallida,* he assigned it also to the genus *Torulopsidosira* (Geitler, 1963). This decision was based on the two observations. The fact that the organisms divided by budding suggested that they were yeasts, since Geitler was dubious that budding occurred in bacteria. Secondly, cytological staining indicated that the nuclear material was sequestered within a small region of the cell—indicating a structure similar to a eukaryotic nucleus. Although it is now clear that *Isosphaera* is a prokaryote, modern staining techniques (e.g., 4′,6-diamidino-2-phenylindole) confirm Geitler's early observation (1963) that the nuclear material is located in a distinct region of the cell.

The micrographs of *Torulopsidosira filamentosa* provided by Geitler show an organism strongly resembling *Isosphaera pallida* in size, shape, and the distinctive formation of intercalary buds within filaments. This suggests that species similar to *Isosphaera* may occur in habitats outside of hot springs. Indeed, in the original definition of the cyanobacterial genus *Isocystis* by Borzi, eight species were named, not

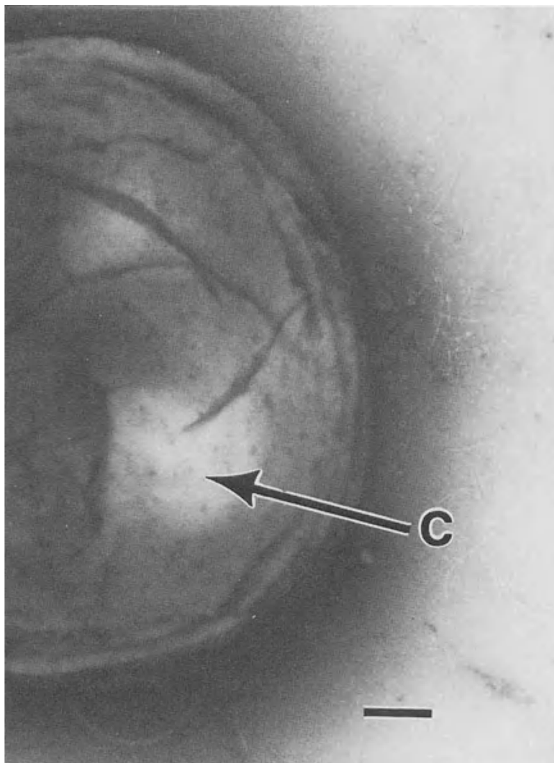


Fig. 19. Electron micrograph of a portion of a negatively stained cell of *Gemmata obscuriglobus* UQM 2246, displaying crateriform structures (C), which are electron-dense pits distributed uniformly over the cell surface. The arrow points to one example of such a structure; many others are visible on the cell surface displayed. Fimbriae are visible outside the cell perimeter. Negatively stained with 1% uranyl acetate supplemented with 0.4% sucrose. Bar = 0.2 μm.

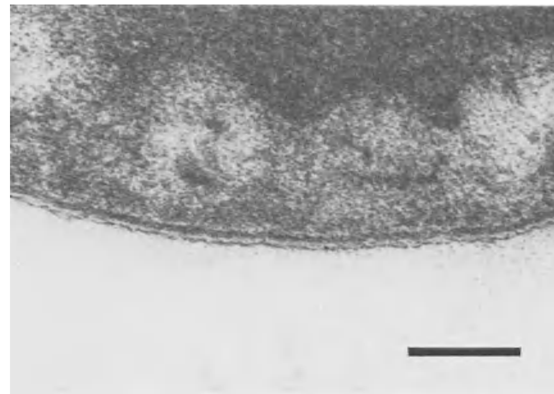


Fig. 20. Electron micrograph of a thin section of *Gemmata obscuriglobus* UQM 2246, displaying cell wall structure. The cell wall consists of an outer electron-dense layer and an inner electron-dense layer separated by an electron-transparent layer. The inner layer is more electron-dense than the outer layer. Cells were fixed with glutaraldehyde and osmium tetroxide. Bar = 100 nm.

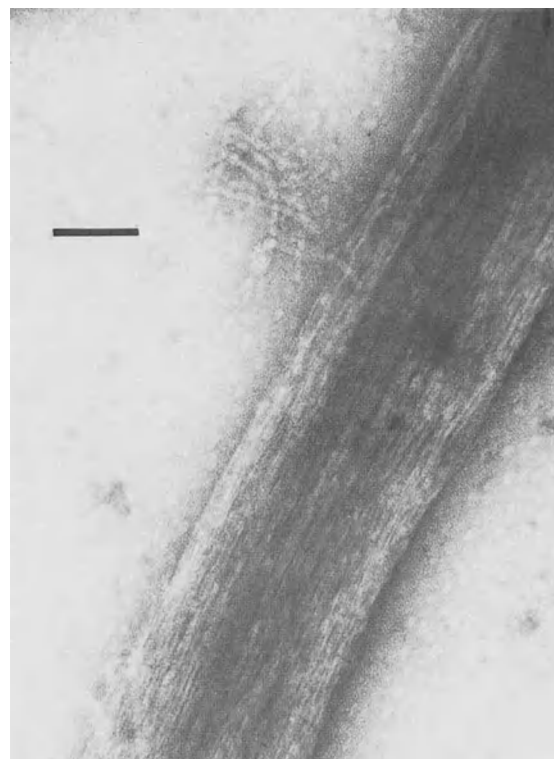


Fig. 21. Electron micrograph of multifibrillar stalklike structure originating from a cluster of cells of *Gemmata obscuriglobus* UQM 2246. Negatively stained with 1% uranyl acetate supplemented with 0.4% sucrose. Bar = 100 nm.

including *I. pallida.* One of these, *I. salina* was described from saline springs and mineral waters. We obtained mixed enrichment cultures of cyanobacteria and *Isosphaera* from a saline hot spring in Utah, U.S.A. (13% NaCl). Attempts to obtain pure cultures failed, even when the *Isosphaera* medium was supplemented with 0.2 M NaCl.

The conclusions of Geitler were later disputed by Anagnostidis and Rathsack-Kunzenbach (1967), who were familiar with *Isocystis pallida* from hot spring collections in Greece. Their extensive description and discussion indicated that *Isocystis pallida* from their cultures and *Isosphaera pallida* are the same. They detected a weak red fluorescence in their cultures, which they attributed to cyanobacterial pigments. We have been unable to confirm this fluorescence with North American collections or cultured material.

Following the first isolation, it immediately became apparent that *Isosphaera* was neither a yeast nor a cyanobacterium, but instead, an aerobic, heterotrophic bacterium. It is a salmon color in culture due to the presence of carot-

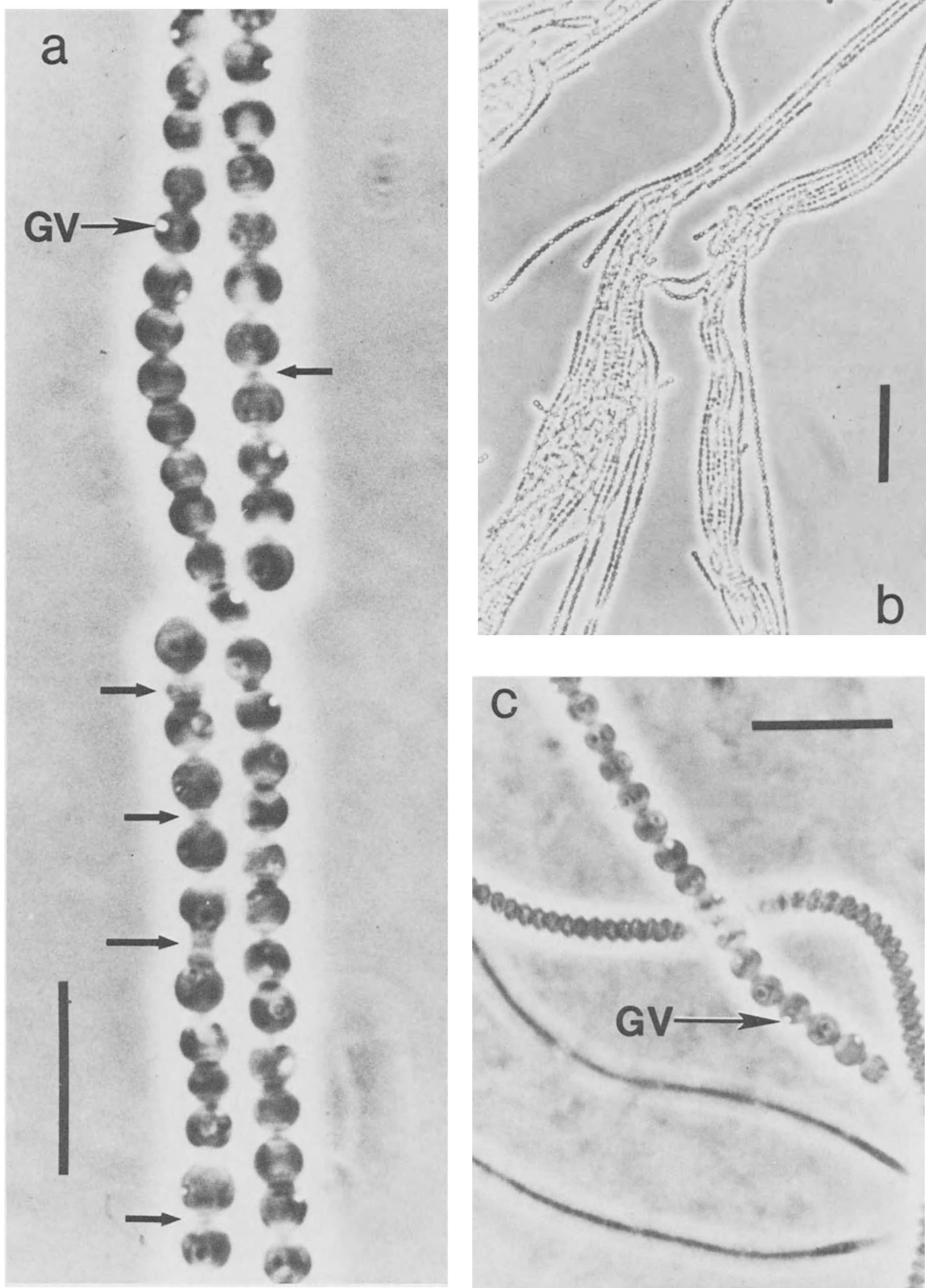


Fig. 22. Phase contrast micrographs of *Isosphaera pallida.* (a) Exponential phase culture (strain IS1). Bar = 10 μm. (b) Exponential phase culture, (strain IS1). Bar = 50 μm. (c) *Isosphaera* from a natural population, Mammoth Hot Springs, Yellowstone National Park, U.S.A., showing *Isosphaera pallida* and *Spirulina.* Bar = 10 μm. Arrows indicate buds. GV, gas vacuoles. Note that the frequency of buds in the exponentially growing culture (b) is low, suggesting that the process of bud development is relatively short compared to the cell cycle.

enoids, but absorbance spectra reveal no maxima corresponding to chlorophylls or phycobiliproteins.

Freshly isolated strains produce gas vacuoles containing gas vesicles with typical conical ultrastructure (Fig. 23). The gas vacuoles are small and usually occur singly in cells.

Colonies of *Isosphaera* are motile. Motility occurs most readily on Gelrite surfaces, though migration is observable to a lesser degree on agar, particularly if it has excess surface moisture. When suspensions of concentrated cells are dropped on plates, they form multiple aggregates of variable size (called comets). These move randomly at about an equal rate in darkness or in uniform light. In the presence of a directional light source, the aggregates are phototactic.

Ultrastructural observations have revealed that the cell surface is uniformly and densely covered with pili. However, there is no evidence for flagella. The mechanism of motility remains unknown. As with other Planctomycetes, surface crateriform structures are present. In *I. pallida* they appear to be uniformly distributed over the cell. Electron-transparent "holes" of similar size and density are found in the isolated SDS insoluble cell wall fraction.

A number of unusual cytoplasmic structures can be present (Fig. 23). Polyhedral structures, similar in appearance to carboxysomes, are often seen. However, there is no evidence for autotrophic metabolism. Also seen are paracrystalline inclusions, and "wall bodies." No functions are associated with these structures.

The first axenic cultures of *Isosphaera pallida* (strain IS1B) were obtained from hot springs located on the Warm Springs Indian Reservation, Oregon, U.S.A. Subsequently, five additional strains were isolated from hot springs in Yellowstone National Park, U.S.A., and Big Spring, Thermopolis, Wyoming, U.S.A.

I. pallida is found either as a component of microbial mats, or planktonically, in which case it may be suspended in the water column by gas vacuoles. The co-occurrence of *I. pallida* with cyanobacteria may be a coincidence. *I. pallida* is an obligate aerobe, and like cyanobacteria, requires neutral or alkaline conditions for growth.

Isolation

I. pallida can be isolated on plates of medium IM incubated in an atmosphere of 5% CO_2/95% air. Medium IM is a dilute mineral medium containing no added carbon sources, and 50 mM bicarbonate. The pH of the medium (7.9) is determined by the bicarbonate/CO_2 buffering system.

Medium IM

Solution A:

$CaCl_2 \cdot 2H_2O$	0.32 g
$MgSO_4 \cdot 7H_2O$	0.4 g
KCl	0.5 g
NaCl	1.0 g
$(NH_4)_2SO_4$	0.5 g
KH_2PO_4	0.3 g
$FeCl_3$	0.292 mg
Trace element solution SL-7[a]	10 ml
Vitamin B_{12}	5 μg
Water	1 liter

Adjust solution to pH 7.6 with NaOH, remove precipitate by filtration through Whatman No. 1 filter paper, store at 4°C.

Solution B:

$NaHCO_3$	42.0 g
Water	1 liter

Autoclave, then bubble vigorously with sterile CO_2 for 1 h.

Final medium:

Solution A	250 ml
H_2O	650 ml

After autoclaving add 100 ml sterile solution B.

[a]Pfennig and Trüper (1981).

In the dark, or in the light with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, an inhibitor of photosystem II, present, medium IM is selective for *Isosphaera.* Plates of medium IM streaked with microbial mat material from a hot spring and incubated in the dark at 45°C usually develop small (1–2 mm) colonies of *Isosphaera* after two weeks. The colonies are firm to the touch of an inoculating loop. In part, the basis for the selectivity of medium IM seems to be the absence of added carbon other than the contaminating organic compounds present in Bacto-agar. However, *Isosphaera* is not agarolytic.

Cultivation

Isosphaera can be routinely cultivated in liquid batch culture on medium IMC sparged with 5% CO_2/95% air.

Medium IMC

Medium IM containing: 0.025% D-glucose, 0.025% casamino acids, 0.5 ml/1 vitamin solution (added after autoclaving). Vitamin solution contains: nicotinic acid, 2 mg/ml; thiamine HCl, 1 mg/ml; *p*-aminobenzoic acid, 0.2 mg/ml; biotin, 0.02 mg/ml.

The minimum doubling time observed (18 hours) occurs at 42°C, but substantial growth is seen at temperatures from 37 to 55°C. A high concentration of CO_2 is probably not required for growth.

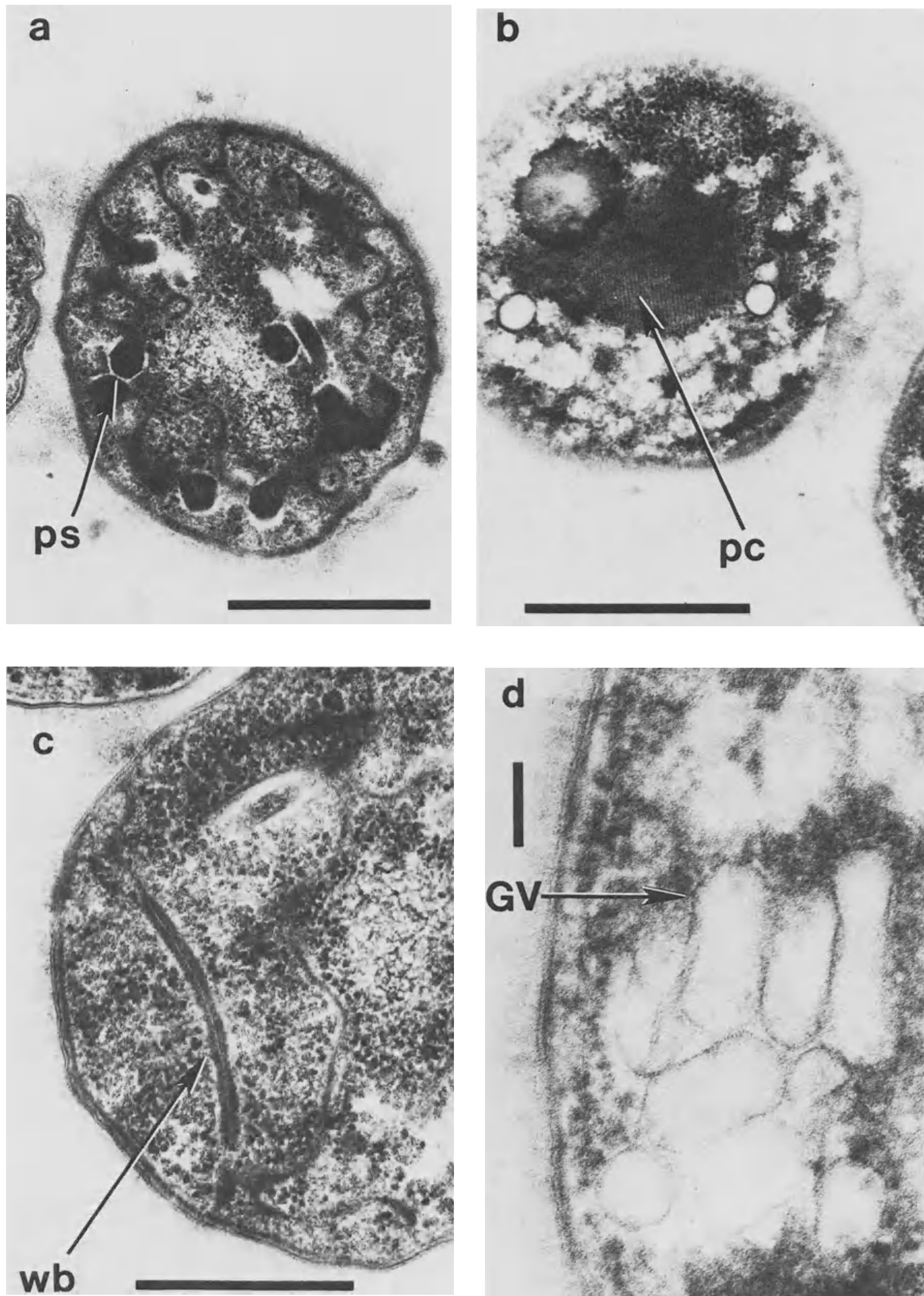


Fig. 23. Thin-sections showing cytoplasmic inclusions of *Isosphaera pallida*. (a) Polyhedral structures (ps). Bar = 1 μm. (b) Paracrystalline inclusion (pc). Bar = 1 μm. (c) Wall body (wb). Bar = 1 μm. (d) Gas vesicles (GV). Bar = 0.1 μm.

Although visible growth occurs on Bacto-agar without organic supplements, the addition of carbon sources at low concentration enhances growth on plates. Only a few compounds, including glucose, ribose, and lactate, are utilized as sole sources of carbon for growth. Growth is more robust in the presence of casamino acids, suggesting that they are assimilated, but are not utilizable as sole carbon sources. D-glucose concentrations of 0.025% are routinely employed because higher concentrations (0.05% D-glucose) inhibit growth. Ribose, which serves as a sole source of carbon for growth in three of four strains investigated, is also inhibitory at concentrations above 0.25%.

The optimal growth temperature of strain IS1B is 42°C. Growth of this strain occurs at temperatures up to 55°C, and as low as 37°C. The reports of Geitler (1955, 1963) suggest that mesophilic strains of *Isosphaera* may inhabit environments other than hot springs, but these have not yet been cultured.

Identification

Isosphaera pallida cells are spherical and typically vary in size from 2.0 to 2.5 μm, although some cells are larger in stationary phase cultures. In wild-type strains the cells are arranged in unbranched filaments of indefinite length. Repeated subcloning results in selection for short chain mutants, such as the type strain, IS1B (S. Giovannoni, unpublished observations).

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CHAPTER 204

The Family Deinococcaceae

ROBERT G. E. MURRAY

The members of the family Deinococcaceae have the remarkable distinction of providing the most radiation resistant of vegetative cells. The Greek root of the generic name, *deinos,* can be translated as both wondrous and strange, which is appropriate because strains have been derived from cells surviving as much as 5 Mrad (50 kGy) of gamma radiation. Despite this strongly selective property, these organisms are not often isolated. Despite some biochemical distinctions, they are not often recognized if isolated incidentally and without knowledge of radiation resistance because of an undistinguished physiology and unreactivity on the commonly used substrates. They masquerade as simple aerobic chemoorganotrophic bacteria; yet they have unusual features, which must be recognized for determination but are not in the routine of most bacteriologists.

The original isolation of an extremely radiation resistant, Gram-positive, tetrad-forming coccus that produced pink to reddish colonies was from cans of meat subjected to supposedly sterilizing doses of radiation in the megarad range. This led to a description of *"Micrococcus radiodurans"* by Anderson et al. (1956). Their original R_1 strain remains the type strain of the species which is now known as *Deinococcus radiodurans* in the family Deinococcaceae (Brooks and Murray, 1981). A number of strains have been isolated since that time and nearly all of them have been obtained with selective assistance by treating the source sample with ultraviolet (UV) or γ-radiation at a dose (1–2 Mrad = 10–20 kGy) that allows few if any other survivors. In this way, radiation-resistant red-pigmented "micrococci" were isolated from diverse sources: Strains from haddock tissue (Davis et al., 1963) subsequently named *D. radiopugnans;* an identifiable species from "Bombay duck" (a dried fish product, *"Micrococcus radiophilus,"* Lewis, 1973) subsequently named *D. radiophilus;* and a species from the feces of *Lama glama* originally named *"M. radioproteolyticus"* (Kobatake et al., 1973) and subsequently named *D. proteolyticus* by Brooks and Murray (1981), who also assigned the new nomenclature because these organisms are not included in the *Approved Lists of Bacterial Names* (Skerman et al., 1980). Unfortunately, Brooks and Murray (1981) included *Deinococcus erythromyxa* (UWO #1045) as a *species incertae sedis* in this study. They and others (Murray and Brooks, 1986; Embley et al., 1987) have subsequently recognized that this was an error because neither the low-radiation resistance nor the chemotaxonomic characters fit either the genus or the family; this culture should be recognized as belonging to the genus *Micrococcus.*

One could argue that most of the extant strains are not "wild type" because they were radiation resistant. However, the isolates from unirradiated sources (e.g., Anderson's U_1 and the Sark strains) have shown an exactly similar extreme resistance to that of the R_1 strain of *D. radiodurans,* which shows virtually no drop in viable count at a dose less than 5 kGy, and, thereafter, a dose for 10% survival (D_{10}) of about 1.5–3 kGy. Because *D. radiodurans* is mutable, albeit with difficulty, by using nitrosoguanidine, and the radiation resistance can be lost (Moseley, 1967); because there are few places on earth with such a large flux of radiation; and because the organisms have been grown from diverse high- and low-radiation sources, we must consider the resistance is incidental to some unknown function important to these organisms. Another utilitarian property that seems to be shared in the family is an extreme capability for surviving desiccation when dried onto non-reactive surfaces (Sanders and Maxcy, 1979b). Because these organisms have been isolated from room dust and air, from textiles as well as from radiated medical instruments (Christensen and Kristensen, 1981; Kristensen and Christensen, 1981), it must be assumed that dessication resistance applies in nature.

The *Deinococcus* strains have a remarkable superficial similarity to *M. roseus* and *M. agilis,* which are red-pigmented species that also suffer from an excess of negative characters in their description but are not radiation resistant. As-

signment of these red-pigmented, Gram-positive cocci to the genus *Micrococcus* (Hill, 1959) was not maintained (Baird-Parker, 1974) because the cell wall and fatty acid composition was inconsistent with that genus. Yet, for some time, *"Micrococcus radiodurans"* was held as *incertae sedis* alongside the red micrococci.

Several biochemical and structural features shared by the species of *Deinococcus* have been identified. These included a lack of the teichoic acids usual in the walls of Gram-positive bacteria and a peptidoglycan type of L-Orn-Gly_{2-3} (Work and Griffiths, 1968; Schleifer and Kandler, 1972); cell membranes without phosphatidyl glycerol and containing phosphoglycolipids and glycolipids unlike the normal membrane phospholipids (Rebeyrotte et al., 1979; Thompson et al., 1980; Counsell and Murray, 1986); loosely bound cellular lipids that were *iso*- and straight chain saturated and mono-unsaturated fatty acids and unlike those of Gram-positive bacteria (Girard, 1971; Brooks et al., 1980; Embley et al., 1987); a complex wall profile in electron microscopy of sections, including an outer membrane (Brooks et al., 1980). These data made it evident that *D. radiodurans* and related species were distinctively different from the red micrococci (Table 1). In fact, on structural and biochemical grounds they are Gram-negative bacteria whose thick peptidoglycan layer in the complex cell wall prevents decolorization (Fig. 1).

The uniqueness of the members of the genus *Deinococcus* was further emphasized by 16S rRNA cataloging which showed (Stackebrandt and Woese, 1979; Brooks et al., 1980) that the radiation-resistant species were related but were very distant from *Micrococcus* and most other eubacterial species. Subsequent sequence analysis and comparison of 16S rRNAs has shown that *Deinococcus* species represent a lineage at least as old if not older than the rest of the 10 phylogenetic lineages of eubacteria (Woese et al., 1985; Woese, 1987). Unique signatures, formed by site-specific nucleotides in the rRNA sequence or specific oligonucleotide sequences in the catalogs, now allow the testing of putative relatives to the Deinococcaceae. A Gram-negative bacillus (Fig. 2) has been so recognized and is assigned to a new genus as *Deinobacter grandis* (Oyaizu et al., 1987). It has a cell wall profile and the biochemical features resembling those of *Deinococcus* species. This organism is as closely related to the *Deinococcus* species as they are to each other (S_{AB}, from 16S rRNA cataloging of 0.55).

The family Deinococcaceae, as now defined, appears to form a stable grouping in both phenotypic and phylogenetic terms and consists of the genera *Deinococcus* and *Deinobacter.* We must suppose that this ancient lineage of cells must have given rise to various related clones at different stages of evolution; they are not obvious and will not be easily recognized even with powerful chemotaxonomic and sequence markers. The first of these are *Thermus* species, which have been identified as distant relatives (Hensel et al., 1986; Weisburg et al., 1989) on the basis of rRNA sequence analysis (S_{AB} 0.22–0.29). They are of the same peptidoglycan and menaquinone type, but the presence of branch chain fatty acids and phosphatidyl glycerol plus its derivatives as membrane components exclude this genus from the family as now defined (Murray, 1986a).

The radiation biology of *D. radiodurans* has been studied intensively and reviewed by Moseley (1983) who, with his co-workers, has shown that this species is naturally transformable using spontaneous or chemically induced mutations (Tigari and Moseley, 1980). All the *Deinococcus* species possess their own plasmids

Table 1. Major distinctions between *Deinococcus* and *Micrococcus*.[a]

Characteristic	*Deinococcus* species	Red *Micrococcus* species
Proteolysis (casein or gelatin)	Yes, usually	No
Cell wall structure in electron micrographs of sections	Complex, layered, thick, and with outer membrane	Thick, homogeneous, and single layered
Survive high doses of UV (about 600 J/m^2) and γ- (about 10 kGy) radiation	Yes, usually	No
Di-amino acid in peptidoglycan	L-Ornithine	L-Lysine
Predominant fatty acids	Straight chain mono-unsaturated	Saturated, branched chain
Presence of phosphatidyl glycerol (PG) or di-PG among membrane phospholipids[b]	No	Yes
Possess lipoteichoic acids	No	Yes

[a]Data from Brooks et al. (1980). Adapted from Murray (1986).
[b]From Counsell and Murray (1986).

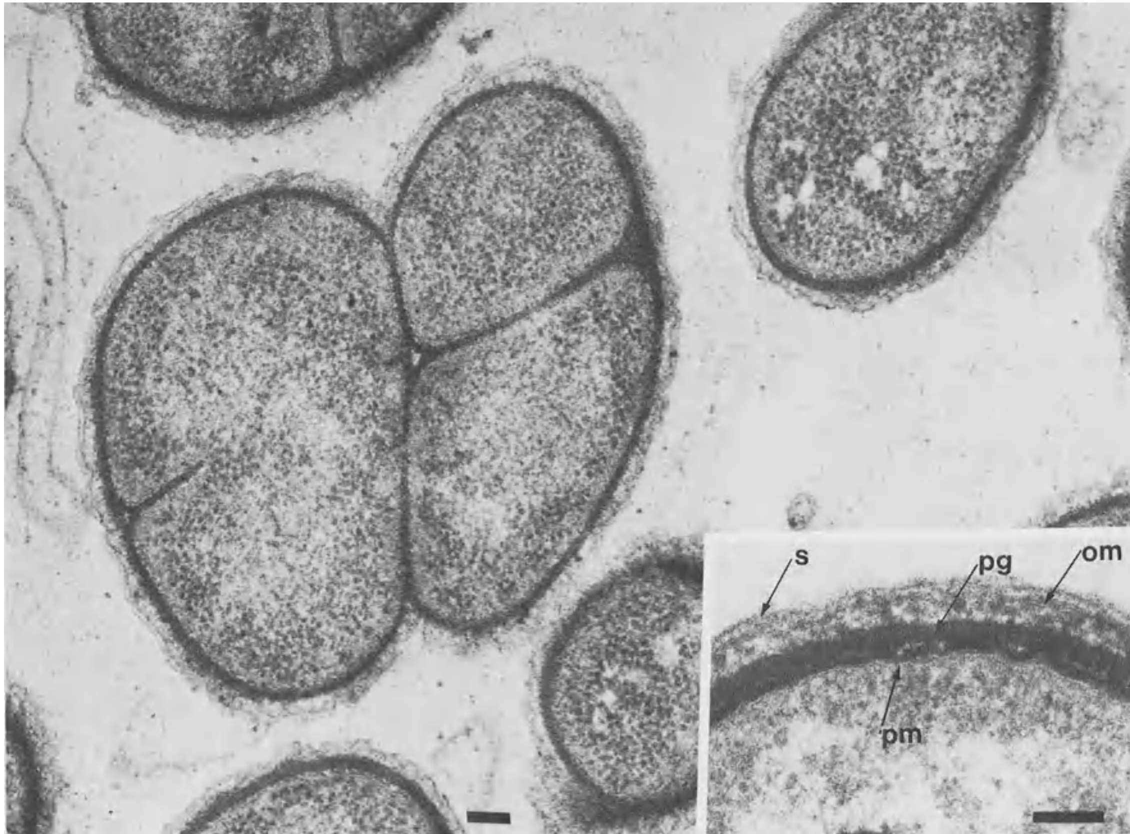

Fig. 1. Section of *D. radiodurans* with inset at high magnification, stained to show the cell wall profile and demonstrating tetrad formation. This species has a protein S-layer (S) outside of the outer membrane (OM), which is well separated from the thick peptidoglycan layer (PG) of the cell wall. The plasma membrane (PM) is heavily stained. Bars = 0.1 μm.

(Mackay et al., 1985; Smith et al., 1988) although they have seemed resistant to accepting plasmids from unrelated organisms.

Habitats

The natural habitat for these organisms is not known because all of the extant species are chemoorganotrophic, require complex media for growth, and have been isolated from a diversity of sites. Their own proteases might allow useful generation of amino acids that are certainly used as are a few sugars. It seems likely that companion microbes would be required in most habitats to process their organic requirements, which may include vitamins, hemin, and methionine, as well as ferric iron (Raj et al., 1960; Shapiro et al., 1977), among other growth factors. It is not surprising that when *Deinococcus* strains have been sought intensively they have been grown from materials, surfaces, and dust contaminated by humans and animals as well as soil, and from soil contaminated by animals, and vice versa (Krabbenhoft et al., 1965; Sanders and Maxcy, 1979a, 1979b; Kristensen and Christensen, 1981; Christensen and Kristensen, 1981; Murray, 1986b), from feces of animals (as quoted above for *D. proteolyticus* and for *D. grandis*), from meat (Grant and Patterson, 1989), and from sewage (Ito et al., 1983). As a result, these strictly aerobic organisms are likely to come from rich organic environments such as soils; a less likely permanent habitat, despite some isolations, is the anaerobic niche provided by feces or intestinal contents. From the beginning, there have been isolations from foods, especially meats at varied stages of processing or packaging, but without evidence of danger to health (Welch and Maxcy, 1979). Some opinions (Ito et al., 1983; Christensen and Kristensen, 1981) incline more to human and animal origins, but the large amount of work on medical bacteriology without recognizable isolations does not give this much support. Certainly the properties of desiccation and radiation (including UV) resistance would allow persistence in dust and lead to aerial contamination at a low

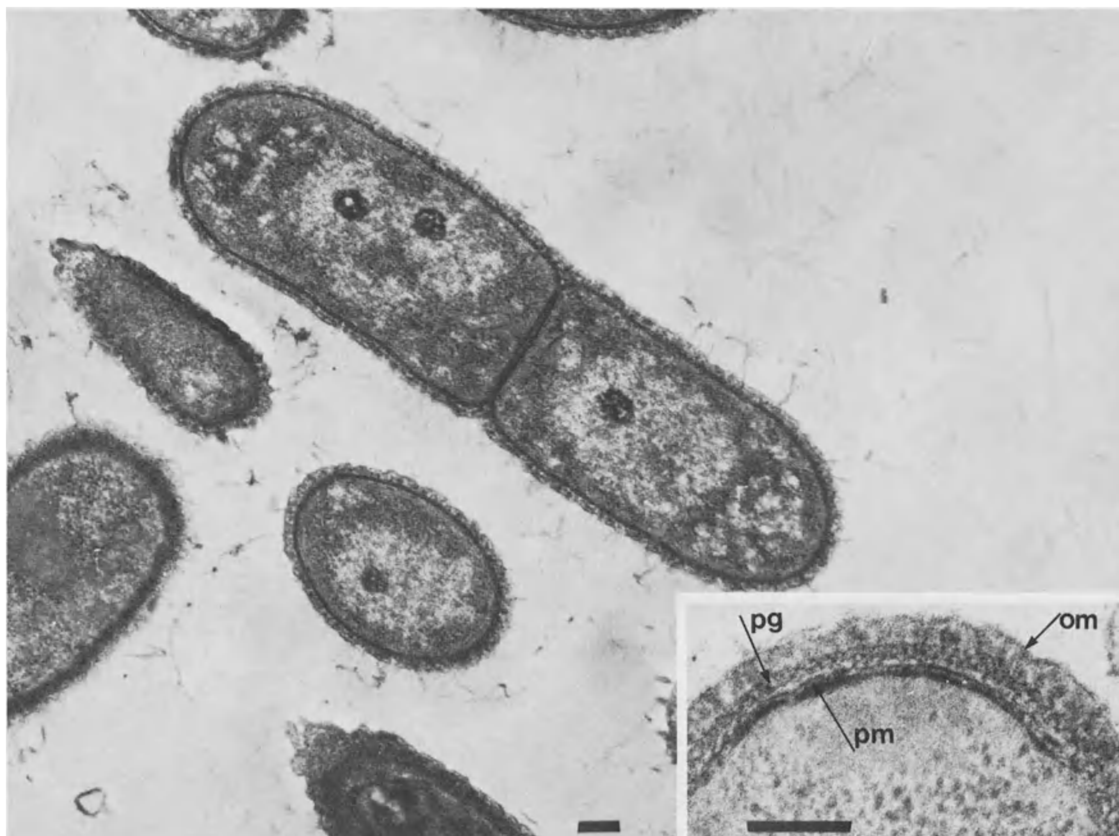

Fig. 2. Section of *Deinobacter grandis* (the fish strain) showing a divided rod and the cell wall profile (inset with abbreviations as in Fig. 1). The OM is hard but possible to resolve as a double-track, as is often the case in this family. The cells show a diffuse nucleoid and low-scattering cytoplasmic inclusions, commonly seen in other family members. The "looped" appearance of the wall is also a common feature. Bars = 0.1 μm.

level in many sites including "clean rooms" (where radiation is extensively used). Dispersal from an as yet unknown habitat subject to desiccation and worldwide distribution in small numbers by turbulence and wind could occur (Murray, 1986b), which would give wide possibilities to explore.

Certainly slower growth than other copiotrophs makes them poor competitors. Wherever they have been found, they seem to occur at low frequency. Few estimates exist of the actual proportions of these bacteria to the total population when isolated direct and without radiation selection. Christensen and Kristensen (1981) stated that the number was one in "several thousand" colony-forming units. There is also an inexplicable patchiness in isolations around positive sites (Krabbenhoft et al., 1965). This has been reinforced by recent experience (I. Masters, personal communication) with a set of isolations from wet (or very damp) soils at an English lakeside but not from nearby dry soils or from the animal excreta thereon. In one study, Ito (1977) grew *D. radiodurans* only from sawdust used for mushroom culture and *D. proteolyticus* from sewage sludges and animal feeds. It is evident that there is still a lot to learn about the biology of the family Deinococcaceae.

Despite extreme radiation resistance and isolation of organisms resembling *D. radiodurans* from shielding liquid pools for radiation sources (Kristensen, 1974), there is no evidence that a major habitat and center of distribution is in a site with a high-radiation flux.

Summary Description of the Family Deinococcaceae (Brooks and Murray, 1981, 356)

Either spherical cells dividing alternatively in two planes showing pairs and tetrads, or rods dividing in one plane to show paired cells. Nonmotile and without differentiated resting forms. Septa may be formed from two sides of the cell

and close as curtains rather than like an iris diaphragm. Gram negative in fine structure but may stain Gram positive. Cell wall profiles show a thick peptidoglycan component, an outer membrane spaced away from the peptidoglycan, and sometimes an external paracrystalline protein S-layer. Most are pigmented pink or orange red to brick red.

Aerobic, catalase positive, mesophilic, chemoorganotrophs with a respiratory metabolism. Although most strains show optimum growth rate at 25–30°C, the temperature limits for growth of some strains may be as high as 42°C and for others as low as 4°C. May require vitamins and accessory growth factors. Generally inactive toward sugars and those that are metabolized cause little if any acid production. Most strains show protease activity, utilize and may require specific amino acids.

Cellular lipids are mainly mono-unsaturated straight chain fatty acids, few with branched chains, and no hydroxy fatty acids (presumably lipopolysaccharide-less). A number of unusual polar lipids with phosphoglycolipids predominating, and absence of phosphatidyl glycerol and derivatives.

Menaquinone type is MK-8 as major and MK-7 as occasional minor component. Peptidoglycan type is L-Orn-Gly_{2-3}.

Most strains are resistant to γ-radiation, UV radiation, and desiccation.

GC content of the DNA ranges from 60–70 mol% (measured from the T_m). Ribosomal RNA signatures allow recognition of relatives.

The type genus is *Deinococcus* Brooks and Murray, 1981, 354. The other genus is *Deinobacter* Oyaizu et al. 1987, 66, which has only been distinguished from *Deinococcus* by being rod-shaped and staining Gram negative.

Isolation

Media

The majority of studies of Deinococcaceae have used either tryptone-glucose-yeast extract or peptone-yeast extract media for both isolation and maintenance. The former derives from that used by Anderson et al. (1956). Raj et al. (1960) performed early nutritional studies, which were supported by the biochemical analyses of Work and Griffiths (1968). They added methionine to their media because it was essential in a chemically defined medium but it seems to be inessential in complex media. They also observed that glucose was readily utilized. Consequently, the following is the most useful basal medium:

TGYM Medium

Tryptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
DL-Methionine	1.0 g
Water (15 g agar, is required	1 liter
Adjust pH to 7.0.	

This may also be used as TGY medium by omitting the methionine. For some purposes, a chemically defined medium may be useful and the simplest is that provided by Raj et al. (1960) for *D. radiodurans:*

Chemically Defined Medium

Ammonium phosphate (dibasic)		50 mg
L-Methionine		1 mg
L-Glutamic acid		50 mg
Biotin		1 μg
Pyridoxine		20 μg
Niacin		25 μg
Thiamin hydrochloride		50 μg
Glucose		500 mg
Salt solution A	0.5 ml	
KH_2PO_4	25 g	
K_2HPO_4	25 g	
Distilled water	250 ml	
Salt solution B	0.5 ml	
$MgSO_4d7H_2O$	10 g	
$FeSO_4 \cdot 7H_2O$	0.5 g	
$MnSO_4 \cdot 4H_2O$	0.5 g	
Distilled water	250 ml	

Make up to 100 ml final volume with distilled water. Adjust pH to 6.8 with NaOH.

Whether or not this defined medium is adequate for all strains or for other species in the genus is not known. A large number of other additives contribute to growth in some smaller degree and these have been assessed and listed by Shapiro et al. (1977). A minimal medium does not seem to have been sought.

For determining characters such as sugar utilization, Brooks et al. (1980) used two standard peptone-based media and obtained no acid production from glucose and sucrose, or a few weak reactions (30°C for 3 weeks) at best, except from *D. proteolyticus.* However, Ito et al. (1983) added 0.1% yeast extract to determinative peptone media and observed acid production from both glucose and sucrose by *D. radiodurans* and *D. proteolyticus* while *D. radiophilus* produced acid from glucose only.

It was evident from the work of Shapiro et al. (1977) that Fe^{3+} was essential for good growth and could be provided by ferric ammonium sulfate (about 50 mg/l). Also of interest is a definite effect of trace amounts of Mn^{2+} (10 μmol) in quenching the formation of thymine dimers, leading to some increase in radiation resistance

(Leibowitz et al., 1976). Some factor of this kind may be responsible for the observations of differences in radiation resistance according to the medium of growth (Krabbenhoft et al., 1967), which might involve differing trace metals, or an increase in resistance after an initial dose of radiation (Tan and Maxcy, 1986). We have also observed that pigmentation is increased by traces of Mn^{2+} (R. G. E. Murray, unpublished observations).

Isolation Methods

Although it is possible to occasionally isolate deinococci by direct plating, isolation is made difficult by low frequency in positive samples. The majority of isolations have taken advantage of the extreme radiation resistance by exposing natural samples, usually suspended in a small volume (2–10 ml) of water or buffer, with or without aeration, to 1–2 Mrad (10–20 kGy) in a ^{60}Co irradiation unit (e.g., Anderson et al., 1956; Kristensen and Christensen, 1981; Ito et al., 1983). At those doses almost but not all spores are killed, but, if the suspension is made in a medium allowing germination, irradiation after allowing a short period of growth reduces the number of surviving spore-formers. A high proportion of the survivors grown after such treatments appear to be deinococci. However, there are other highly radiation-resistant organisms that may be encountered, such as the *Acinetobacter* strains described along with deinococci from chickens (Thornley, 1963; Thornley and Glauert, 1968; Welch and Maxcy, 1979) and from radiation-sterilized cotton tampons (Nishimura et al., 1981).

Selective cultivation and enumeration of radiation-resistant, nonsporeforming bacteria has been attained by Dickson and Maxcy (1985) from a mixture of pasteurized soil (to provide spores) and cultured suspensions of four radiation-resistant bacteria (one of which was *D. radiodurans*) suspended in skim milk or meat serum. They got 50% to complete recovery of the nonsporeforming, radiation-resistant bacteria and little interference from surviving *Bacillus* species, using three 3 kGy doses of γ-radiation preceded and separated by 1 h incubation at 32°C.

The proportion of aerobic organisms collected by slit-sampler from clean laboratories and work areas capable of surviving a γ-radiation dose (1.8–2.4 Mrad or 18–24 kGy) sufficient to reduce the total population one millionfold was estimated by Christensen and Kristensen (1981). Some 20,000 colonies were assessed and 117 of them survived to 10^{-6} (D-6); 35 of these were sporeforming rods (D-6 = 1.8–3.2 Mrad), 50 were cocci of which 10 of the less resistant were nonpigmented (D-6 = 1.8–6.0 Mrad); 32 were nonsporeforming rods, and none were fungi. Some 60% of the strains were pigmented (yellow through orange to red) and the strains resistant to the highest radiation level were salmon pink. The majority of the very resistant cocci resembled *Deinococcus* species (a few have been characterized since then as *D. radiodurans*) and three of the very resistant rods (D-6 = 4.5 Mrad) were, in retrospect, *Deinobacter*-like. A few of the rods isolated in the above study and in a separate set of isolations from textiles and clothing (Kristensen and Christensen, 1981) were tentatively identified as *Nocardia* species. Thus, the proportion of Deinococcaceae in the total from dust populations in relatively clean work rooms, where the organisms must be already selected by desiccation and sometimes UV radiation, is small and about 0.2%.

Selection can also be attained by exposure of a sample in a thin film to UV light. This has been done with soil from an English lakeside (B. E. Moseley and I. Masters, personal communication) using the supernatant of a slurry exposed in a petri dish to 600, 900, and 1200 Jm^{-2} followed by plating 0.1 ml amounts on TGY medium; a number of strains of *Deinococcus* species were obtained.

A procedure for isolation of radiation-resistant bacteria without exposure to radiation (Sanders and Maxcy, 1979b) depends on the extreme resistance to desiccation shown by the vegetative cells of their *Moraxella-Acinetobacter* isolates and "pink micrococci" (including a test strain of *D. radiodurans*) that they studied. They dried the organisms on stainless steel plates (we have done the same on glass coverslips) and survival is almost complete for a year. After 6 years (R. B. Maxcy, personal communication), there is about 10% survival. The procedure of Sanders and Maxcy (1979b) was to suspend the organisms from the specimen in neutral phosphate buffer (they also isolated cultures from nature by shaking 1 g of cattle hair in 99 ml of buffer), storing overnight before smearing 0.01-ml samples on stainless steel plates for storage at 33% relative humidity over saturated magnesium chloride at 25°C for 14 days to 5 months. The smear was then rehydrated for plating and, after growth, radiation resistance was assessed by replica plating. It was noted that the desiccated bacteria recovered slowly, requiring 4–5 days to form visible colonies when sporeformers provide a major problem of overgrowth. A better procedure would include an appropriate growth medium for sus-

pension, a short incubation to germinate the spores, followed by desiccation.

Identification

When the isolation has been made by chance, without using radiation as a selective agent which would be likely to direct attention to this group, one or more of the other traits of the Deinococcaceae outlined above will make one suspect the diagnosis and lead to further characterization. The morphological clues are likely to come first in trying to identify large rods or cocci forming pink to red colonies. Given some degree of suspicion, a rough test for radiation resistance using ordinary eubacteria for reference is helpful (Murray and Brooks, 1986). Because the Gram reaction can be misleading, the wall profile should be defined by electron microscopy as well as the pattern of septum formation and cell division. The most useful chemotaxonomic character is the peptidoglycan type, the pattern of phosphoglycolipids, and the lack of phosphatidyl glycerol and derivatives. Finally, given a high index of suspicion, the culture could be submitted to a laboratory undertaking sequence analysis of rRNA.

THE STRUCTURE OF THE CELL WALL. Cell wall profiles observed in sections by electron microscopy (Fig. 1a, 1b, and 2) show an unusual total thickness of, perhaps, 50–60 nm. A densely staining inner layer, some 14–20 nm thick, is the peptidoglycan component, which alone forms the septa and in some species shows as a fenestrated or "holey" layer (Work and Griffiths, 1968; Fig. 3). The fenestrations are best seen when sections are stained with lead alone rather than the usual lead and uranyl acetate (Thompson and Murray, 1982) and represent areas of different reactivity. The external portion of the wall is a loose, looped, outer structure, which can be resolved with some difficulty as an outer membrane (Brooks et al., 1980; Sleytr and Glauert, 1982). *D. radiodurans* has a remarkably stable, regularly structured S-layer on the outer surface of the outer membrane that is hexagonally arrayed (Baumeister et al., 1982). Often there is relatively little stainable material visible between these major layers but the form is much the same, though the thickness varies for the strains that have been examined. The *Deinococcus* species are mostly tetrad formers, and when they divide alternately in two planes the cell wall septum for the second division usually is initiated before the first septation is complete. Most remarkable (Murray et al., 1983) is the form of the septum, which closes from opposite sides of the cell (Fig. 4a) as a pair of curtains rather than the iris-diaphragm that is usual in most bacteria. *Deinobacter,* on the other hand, has a thinner peptidoglycan layer (about 10 nm), allowing the cells to be Gram negative, and is a rod form with division in only one plane. The septum also completes as a closing pair of curtains (Fig. 4b; R. G. E. Murray, unpublished observations).

Isolation of the peptidoglycan and amino acid analysis of the peptide portion provides indication of the diamino acid and linkage structure, which is unusual enough in Gram-negatives to be helpful: L-Orn-Gly_{2-3} (Schleifer and Kandler, 1972).

Radiation resistance is such an extreme character in the case of the Deinococcaceae (Moseley, 1983) that rather simple testing procedures would be enough to be indicative and the generation of survival curves could be left to later work (Murray and Brooks, 1986). Most isolates have shown 90% survival or better at a dose of 5 kGy in a ^{60}Co-irradiator or at a dose of UV light (254 nm) of 500 Jm^{-2}. These are doses that give survivals at the level of 10^{-4}–10^{-6} or less for most other bacteria. The form of survival curves (Fig. 5) is remarkable for the extent of the shoulder (2–5 kGy of γ-radiation and 300–600 Jm^{-2} of UV radiation) and the subsequent decline in survivors (gives D_{10} values in the range of 1.0–3.5 kGy for γ-radiation and of about. 150 Jm^{-2} for UV radiation) is in sharp contrast to those of most sensitive bacteria.

Phospholipid analysis has shown that representative strains of the species of *Deinococcus* and *Deinobacter* have a most unusual diversity in the number and chromatographic behavior of the polar lipids, which are dominated by glycolipids and phosphoglycolipids (Rebeyrotte et al., 1979; Counsell and Murray, 1986; Embley et al., 1987). A negative but useful character shared by all true members of the family is the absence of phosphatidyl glycerol and diphosphatidyl glycerol or their derivatives, which, together with the complexity (8–14 spots) of the polar lipid pattern, gives a distinctive appearance in both one- (Counsell and Murray, 1986) and two-dimensional (Embley et al., 1987) thin-layer chromatograms.

Fatty acid analysis has shown (Embley et al., 1987; Oyaizu et al., 1987) that the Deinococcaceae show a remarkable range of mono-unsaturated and straight chain fatty acids as major components, few if any branched chains in minimal proportions, and they are unusual for having significant amounts of different isomers of individual fatty acids. Furthermore, both *Deinobacter grandis* (Oyaizu et al., 1987) and *Deinococcus radiodurans* (R. G. E. Murray, unpub-

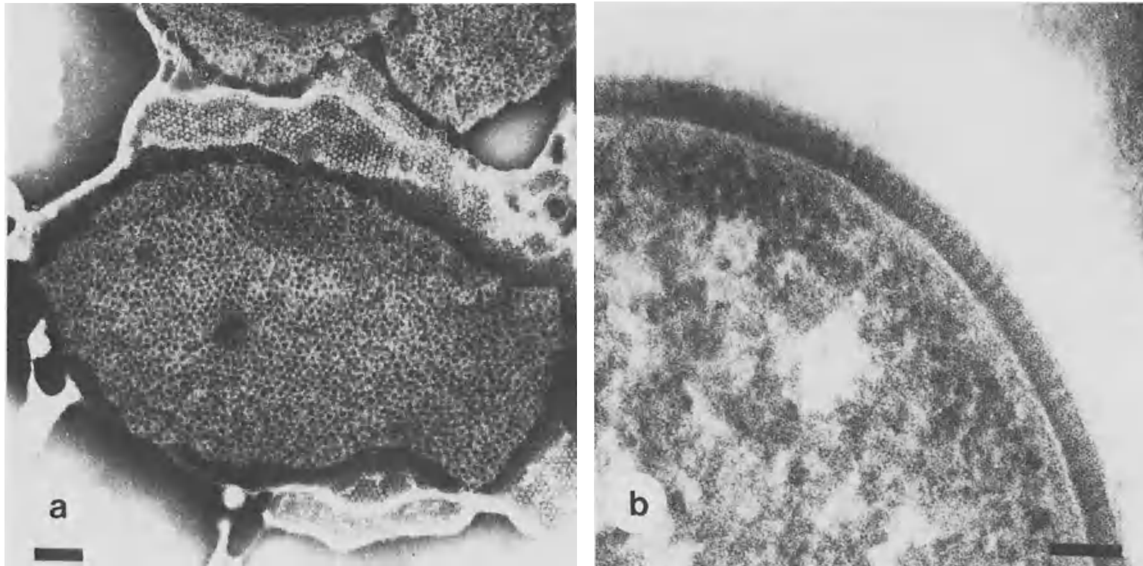

Fig. 3. (a) Negatively stained (Na phosphotungstate) fraction of cell wall of *D. radiodurans* showing a piece of the hexagonally arranged array of units in the S-layer and a piece of the peptidoglycan layer showing the appearance of fenestrations. (b) A section of *D. radiodurans* stained with lead acetate to show fenestrations in the peptidoglycan (see Thompson and Murray, 1982). Bars = 0.1 μm.

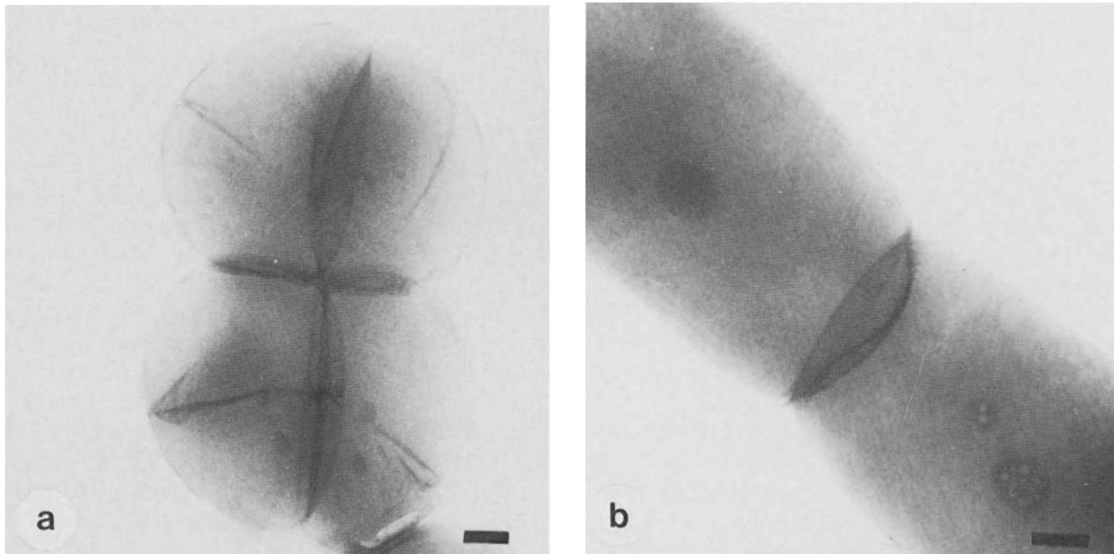

Fig. 4. (a) A dividing tetrad of *D. radiodurans* stained with uranyl acetate after blocking most staining sites with $CoCl_2$ to show the septal curtains. (From Murray et al., 1983); published in the *Canadian Journal of Microbiology*, with permission.) (b) A similar preparation of *Deinobacter grandis* showing two curtain edges in the septum. Bars = 0.1 μm.

lished observations) lack hydroxy fatty acids, from which we assume that these Gram-negative walls lack lipopolysaccharide.

Chemotaxonomic characters give clear direction to the definition and circumscription of the family but give rise to doubts about the validity of some strains assigned to the extant species because of heterogeneity shown by fatty acid and polar lipid analyses (Embley et al., 1987).

Nucleic acid sequence and homology analyses provide an important taxonomic resource. A strain from each of three species of *Deinococcus* (*D. radiodurans, D. radiopugnans,* and *D. radiophilus*) and one strain of *Deinobacter grandis*

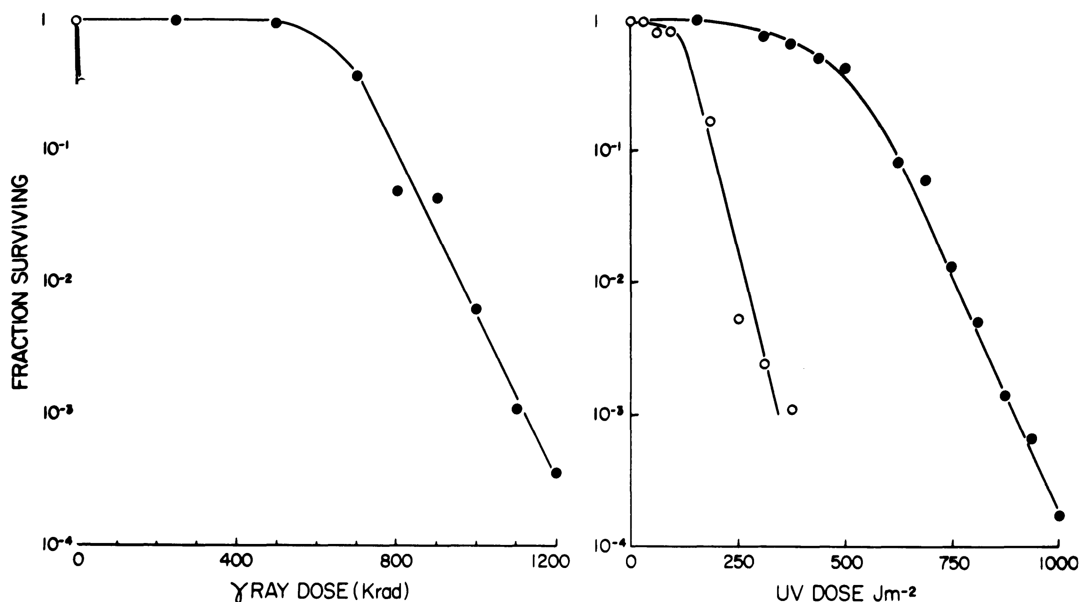

Fig. 5. Survival curves to γ- and UV radiation of *D. radiodurans* (closed circles), showing extreme resistance, and survival of a pink *Micrococcus* strain (open circles) (actually the strain misnamed *D. erythromyxa*, which does not have the chemotaxonomic characters of a *Deinococcus* sp.), showing radiation sensitivity. Note the "shoulders" demonstrating almost complete survival to a rather high dose. (Data kindly provided by Dr. B. E. B. Moseley. Note: 500 Krad = 5 kGy.)

have been compared in respect of catalogs of the 16S RNA oligonucleotides generated with T_1 ribonuclease. There is no doubt of their all being related at a S_{AB} 0.58–0.68 (Oyaizu et al., 1987) and these bacteria are related very distantly to all other eubacteria at a level of S_{AB} 0.22–0.25. Thus, the extant members appear to form a stable phylogenetic group, related to each other, and separated into the two genera by no characters of importance other than morphological division pattern. Oyaizu et al. (1987) consider that until more strains of the species of each group can be studied there is good reason to maintain the genera while recognizing a close relationship. In the meantime, RNA sequencing and the recognition of rRNA signatures (Woese et al., 1985) will remain a powerful technique for recognizing relatives, both close and distant, even in the face of having no concept of where an unknown might be classified. As already stated, this has happened in the case of *D. grandis;* this technique has also allowed recognition of *Thermus aquaticus* and *T. ruber* as distant relatives of the Deinococcaceae. (They also share high GC (59–65 mol%), L-Orn-Gly_2 peptidoglycan, but not polar lipid patterns.) Undoubtedly, other relatives at various levels will be found in the future with such powerful and directive techniques. DNA-DNA reassociation is unlikely to be helpful at the generic level, although not yet attempted between *Deinococcus* and *Deinobacter,* because the homology between the *Deinococcus* species is of a very low order (Brooks et al., 1980).

Identification of Species

There are four described species of *Deinococcus* and one of *Deinobacter.* These can be distinguished on phenotypic grounds as shown in Table 2, and we can say definitely that the type strains of each of the *Deinococcus* species show no significant DNA-DNA homology and must be considered genetically separate species (Brooks et al., 1980; Brooks and Murray, 1981). However, the reliability of characterization using a limited palette of characters may be illusory and will need more careful monitoring by analysis and comparison of the chemotaxonomic and genetic approaches. Embley et al. (1987) provide clear evidence that the representatives of *D. radiodurans, D. proteolyticus,* and *D. radiophilus* (essentially the only species in which more than two strains were available to compare) showed marked differences among the available strains in terms of principal fatty acids and the patterns of polar lipids. A strain reputedly *D. radiophilus* showed the distinctive polar lipid pattern of *D. radiopugnans.* Furthermore, Embley et al. (1987) showed that the R_1 and Sark strains of *D. radiodurans* have considerable heterogeneity in those respects, which may not be unexpected because they were also known to exhibit a 70% difference in DNA-

Table 2. Phenotypic distinctions among *Deinococcus* and *Deinobacter* species.[a]

Character	*Deinococcus*				*Deinobacter*[c]
	D. radiodurans	*D. radiophilus*	*D. proteolyticus*	*D. radiopugnans*	*D. grandis*
GC content (mol%) (T_m)	67	62	65	70	69
Cell shape	Coccal	Coccal	Coccal	Coccal	Rods
Gram reaction	+	+	+	+	−
Lysis by lysozyme[b]	−	+	−	ND	ND
Oxidase reaction	+	+	+	+	−
Fenestrated peptidoglycan in Pb-stained sections	+	−	−	+	+[d]
Growth in 5% NaCl	− (80%)	+	−	−	−
Growth in 3% NaCl media[b]	−	+	+	−	−[d]
Nitrate reduction	− (80%)	−	−	+	+
Acid from glucose in a standard peptone medium	−	−	+	−	−
Acid from glucose in medium + yeast extract[b]	+	+	+	−[d]	−[d]
Acid from sucrose in medium + yeast extract[b]	+	−	+	ND	−[d]
ONPG (β-galactosidase)	−	−	−	+	+[d]
Esculin hydrolysis	−	−	+	−	+

+, property present; −, property absent; ND, no data.
[a]Adapted from Murray (1986), with permission; based on data from Brooks et al. (1980).
[b]Data from Ito et al. (1983).
[c]Data from Oyaizu et al. (1987).
[d]Data from R. Murray (unpublished observations).

DNA reassociation (Brooks et al., 1980). Counsell (1986) has observed that the protein profiles obtained with polyacrylamide gel electrophoresis on 24 strains of *D. radiodurans* showed five or more distinct patterns. Thus, we can expect that a more definitive taxonomic study of *Deinococcus* will undoubtedly lead to further species or subspecies.

These inconsistencies underline the taxonomic hazards involved in working from small numbers of strains, with few positive determinative characters, and with no accurate estimate of the reliability of the characters in use. Difficulties arise, as shown in Table 2, from the choice of media for determining reactions to substrates. The inclusion of yeast extract makes a big difference to reactions in peptone media with sugars (Ito et al., 1983) even if slow growth can occur with utilizable sugars in peptone medium. Possibly anomalous reactions occur because of acid neutralization by-products of dissimilation of amino acids.

Toward the future

Systematic efforts to obtain more strains of *Deinococcus* and *Deinobacter* as well as to find and classify relatives that may belong outside of the present circumscription of the Deinococcaceae, will be important to future understanding. As now constituted, the family has two genera that are so alike in everything but shape and Gram reaction that they could well be fused. There should be a wider range of relatives to be found in nature, if the phylogenetic indications are not misleading, because an ancient lineage of bacteria must have spawned persistent variants during evolution. Furthermore, an expanded effort at retrieval should bring with it an understanding of habitat, which has escaped us up to now because of the diversity of sources (Table 3). To persist in nature for eons, these organisms must have a favorable habitat somewhere.

The identification of *Thermus* species as distant relatives tells us that other distant relatives may have differing selections of the chemotaxonomic characters. Their descriptions might not include radiation resistance because this is already known to be mutable, as pointed out above. Anomalous characters are already a major part of the picture when *Deinococcus* species are recognized as Gram-positive/Gram-negative bacteria; there may be more surprises involving, for instance, more unusual phospholipids (Anderson and Hansen, 1985) and exceptions to the apparent lack of phosphatidyl glycerol and derivatives.

Although these remarkably radiation-resistant bacteria have been used to assess the effectiveness of radiation sterilization, much less at-

Table 3. Sources of Cultures of Deinococcaceae.

Deinococcus species	
D. radiodurans	Irradiated canned meat, cattle hairs and skin, and creek water in Oregon (Anderson et al., 1956). Air contaminant in Canada (Murray and Robinow, 1958). House dust, used towels and underwear, from air in "clean room" laboratories in Denmark, and from radiation facilities (Christensen and Kristensen, 1981; Kristensen and Christensen, 1981). Sawdust culture media for mushrooms in Japan (Ito, 1979). From suture material in Sweden (Osterberg, 1974). Most strains were isolated after high-dose irradiation.
D. radiophilus	Irradiated "Bombay Duck" (*Harpodon nehereus*) in India (Lewis, 1973).
D. proteolyticus	Irradiated feces of *Lama glama* (Kobatake et al., 1973). Irradiated sewage sludge cake, and from animal feeds in Japan (Ito et al., 1983).
D. radiopugnans	From haddock tissue in Massachusetts (Davis et al., 1963). From weathered granite in Antarctica (Counsell and Murray, 1986).
Deinobacter grandis	From the feces of *Elephas maximus* in a zoological garden, from the intestines and skin of a fresh-water fish (*Cyprinus carpio*), and from the skin of an eel (*Anguilla japonica*) in Japan (Oyaizu et al., 1987). From a sample of pork in England (Grant and Patterson, 1988).

Adapted from Murray (1986).

tention has been paid to them as models for the understanding of the resistance mechanisms. Capability for the effective repair of damage to DNA has been assessed and some genetical studies have been accomplished (Moseley, 1983) but much less attention has been paid to the survival of structure and function of the plasma membrane of the Deinococcaceae. The possibility exists that it is not just the high concentration of carotenoids (a feature of all the radiation-resistant bacteria, like the Halobacteriaceae) that play a role, but that the unique polar lipids could also have protective properties including the regulation of ion leakage and a function as antioxidants (Anderson and Hansen, 1985).

The Deinococcaceae provide all sorts of challenges to microbiologists, radiation biologists, biochemists and, indeed, to those interested in general biology, taxonomy, and ecology. Yet, they are familiar to only a few on whom their properties of radiation resistance impinge. There is every reason to encourage a wider awareness and interest.

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The Genus *Thermus* and Related Microorganisms

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Bacteria of the genus *Thermus* have been isolated from many natural and artificial thermal environments throughout the world. The first strains of the type species *Thermus aquaticus* were isolated from neutral and alkaline hot springs in Yellowstone National Park, USA (Brock and Freeze, 1969). Since then, strains have also been isolated from Yellowstone Park (Munster et al., 1986), and from other terrestrial hot springs in Iceland (Cometta et al., 1982b; Hudson et al., 1987a; Kristjansson and Alfredsson, 1983; Pask-Hughes and Williams, 1977), New Zealand (Hudson et al., 1986, 1987b), and Continental Portugal and the Azores Islands (Prado et al., 1988; Santos et al., 1989). In Japan, early isolates were named *"Flavobacterium thermophilum"* (Oshima and Imahori, 1971), and then renamed *"Thermus thermophilus"* (Oshima and Imahori, 1974). Other isolates have been given invalid species names (Saiki et al., 1972; Taguchi et al., 1982). In addition to terrestrial thermal environments, strains of *Thermus* have also been isolated from shallow marine thermal vents off Iceland (Kristjansson et al., 1986).

The isolation of *Thermus* strains from artificial and from natural environments was simultaneous (Brock and Freeze, 1969). They appear to be widespread in neutral, hot aqueous artificial environments, including hot tap water (Brock and Freeze, 1969; Pask-Hughes and Williams, 1975; Stramer and Starzyk, 1981), domestic and industrial hot water systems (Brock and Boylen, 1973), and thermally polluted streams (Brock and Yoder, 1971; Degryse et al., 1978; Ramaley and Bitzinger, 1975; Ramaley and Hixson, 1970). *Thermus* is not usually reported in cold water without obvious thermal inputs, but Stramer and Starzyk (1981) report a low count in rivers and lakes.

Red-pigmented strains of *Thermus ruber,* which have a temperature optimum of 60°C, have been isolated from hot springs in the Kamchatka Peninsula, USSR (Loginova and Egorova, 1975), Iceland (Sharp and Williams, 1988), the island of Sao Miguel in the Azores (R. A. D. Williams and G. Holtom, unpublished observations), and from an aerated fermenter fed with yeast wastes (Hensel et al., 1986). The taxonomic position of *Thermus* relative to other genera remains uncertain, but an oligonucleotide catalog of 16S rRNA has indicated a remote but distinct relationship between *T. aquaticus* and *T. ruber* and *Deinococcus* (Hensel et al., 1986). The sequence of 16S rRNA shows that *"T. thermophilus"* falls in the cluster of the green nonsulfur bacteria along with *Thermomicrobium roseum, Chloroflexus aurantiacus,* and *Herpetosiphon aurantiacus* (Hartmann et al., 1989).

Isolation and Preservation

Thermus Medium (Medium D for *Thermus,* Brock, 1978, 1981)

Basal salts medium D (Castenholtz, 1969) with 0.1–0.3% tryptone and 0.1% yeast extract is generally used for the culture of *Thermus* spp. The salts medium has the following composition (mg per liter of water):

Nitrilotriacetic acid (NTA)	100
$CaSO_4 \cdot 2H_2O$	60
$MgSO_4 \cdot 7H_2O$	100
NaCl	8
KNO_3	103
$NaNO_3$	689
Na_2HPO_4	111
$FeCl_3$	0.28
$MnSO_4 \cdot H_2O$	2.2
$ZnSO_4 \cdot 7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4$	0.016
$Na_2MoO_4 \cdot 2H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.046

The pH is adjusted to 8.2 with NaOH.

The salts medium is made from three stock solutions: 1) the macroelements containing NTA and the salts through Na_2HPO_4 are dissolved in water at 10× the final concentration, 2) the $FeCl_3$ is made up at 100×, and 3) the other trace salts are dissolved separately and then combined at 100× final concentration in water with 0.5 ml H_2SO_4. All three solutions can be stored at 4°C or frozen. To make the final basal salts mixture for

Thermus medium, 1/10 volume of the macroelements, 1/100 volume of the $FeCl_3$ solution, 1/100 volume of the trace salts are added to water, the pH is adjusted to 7.6, and the medium sterilized by autoclaving at 121°C for 15 min. Tryptone and yeast extract may be sterilized separately from, or together with, the basal salts medium.

Isolation Procedures

A low concentration of organic constituents in the medium and an incubation temperature of 70–75°C (to inhibit the growth of bacillus species) were stressed by Brock (1978) as important factors for the isolation of *T. aquaticus.*

Thermus strains are easily isolated by inoculating samples of water, biofilms, mats of biomass or mud into *Thermus* medium and incubating for 1–3 days at 55–65°C for *T. ruber,* or at 70–75°C for yellow to colorless strains. Sample volumes of 0.5–1.0 ml are generally sufficient, but the medium can be made up at 2× concentration to accommodate large inocula. Turbid cultures are streaked on the same medium solidified with 2–3% agar. Plates are sealed, or placed in containers to reduce evaporation, and incubated aerobically for several days at the appropriate temperature. Streaking samples directly onto solidified *Thermus* medium has also been successful (Hudson et al., 1986). Large sample volumes have been concentrated by centrifugation or membrane filtration, and then directly plated (Stramer and Starzyk, 1981). *Thermus* medium has also been used for enumerating *Thermus* cells in water specimens by the most-probable-number method (Brock and Boylen, 1973; Stramer and Starzyk, 1981).

The isolation of the red-pigmented strains at their optimum of 55–65°C often gives cultures overgrown with aerobic sporeforming bacteria. Nevertheless, red colonies can be detected on plates of solidified *Thermus* medium, and isolated by replating.

Other dilute media are sometimes used for the isolation and growth of *Thermus* strains. The basal mineral medium 162 (Degryse et al., 1978) together with 0.25% tryptone, 0.25% yeast extract, and 3% agar was used for the isolation of *Thermus* from samples concentrated by filtration (Kristjansson and Alfredsson, 1983; Kristjansson et al., 1986). Many strains will grow on 0.4% yeast extract, 0.8% polypeptone, and 0.3% NaCl (Oshima and Imahori, 1971), but this is not true of *T. aquaticus.* This medium gives a higher growth yield for strains like *"T. thermophilus"* than more dilute media.

Occasional contamination by thermophilic spore-formers (which occur in some samples of media components) can be overcome by increasing the autoclaving time, or using 131°C. A combination of 0.015% sodium azide and 0.01% lysozyme in *Thermus* agar suppresses the growth of many strains of *B. stearothermophilus,* with little effect on *Thermus* strains (N. D. H. Raven and R. A. D. Williams, unpublished observations). Others have found their strains to grow poorly on such plates, and have instead used gramicidin D at 20μg/ml in plates to suppress sporeformers (H. Nagayama, personal communication).

Preservation of Strains

Most strains of *Thermus* can be stored frozen in *Thermus* medium containing 10–15% glycerol at −80°C, or in liquid nitrogen, for years without loss of viability. Lyophilized strains in *Thermus* medium have been maintained in freeze-dried ampules for 18 years. Strains grown densely on plates of *Thermus* medium survive for about 1 month at 4°C.

Ecology

The original isolations of *T. aquaticus* were from water and algal mats of alkaline hot springs in Yellowstone Park at temperatures of 53–86°C, and pH values between 8 and 9 (Brock and Freeze, 1969). In the same area, 65 sites with temperatures of 30–90°C and pH values from 1.2–10.5 were sampled for strains of *Thermus* (Munster et al., 1986). Isolates were only obtained from sources between 55 and 80°C and pH 6.0–10.5. In Iceland, 55 hot springs from 32–90°C, and pH from 2.1–10.1 were sampled (Kristjansson and Alfredsson, 1983), and *Thermus* spp. were isolated from sites with temperatures ranging from 55–85°C and pH values higher than 6.5. The occasional isolation of strains from a site at 95°C, was felt to be due to transient bacteria washed in from cooler algal mats. Although the temperatures of thermal springs can be quite stable, the effect of rain or snow may be drastic in shallow exposed channels. In Iceland, we have noted that the temperature in shallow pools may drop by as much as 20°C during strong gusts of wind. Such effects may lead to the isolation of *Thermus* from sites with temperatures at which the bacteria cannot grow.

In New Zealand, *Thermus* strains have been isolated from thermal sites as acidic as pH 3.9 (Hudson et al., 1986), but these cells may have originated from more alkaline regions.

Thermus-like strains from shallow submarine hot springs off Iceland (Kristjansson et al., 1986) were generally more halotolerant than

their terrestrial counterparts, but otherwise were similar. The sites yielding these marine isolates contained thin sediment layers where fresh thermal water was mixed with cold sea water. When isolations from the same sites were performed in media with 2–3% NaCl, *Thermus* strains were inhibited and replaced by red-pigmented isolates, later named *Rhodothermus marinus* (Alfredsson et al., 1988). The relationship of this genus to *Thermus* remains to be determined.

The bacteria of the genus *Thermus* are obligate heterotrophs and grow on small amounts of organic materials in the water or derived from photosynthetic or chemolithotrophic organisms growing in biomass accumulations with *Thermus* strains. More studies are needed to clarify the sources of nutrients in natural environments, as well as their uptake and utilization.

Properties of Strains

Morphology and Cellular Composition

The bacteria of the genus *Thermus* stain as Gram negative despite their phylogenetic relationship to the Gram-positive genus *Deinococcus* (Hensel et al., 1986).

Upon initial isolation, many strains form long filaments, but after repeated transfers in laboratory media, most grow as pleomorphic rod-shaped cells and short filaments (Brock, 1978).

Electron microscopy demonstrates that the cell envelope is composed of a thin dense layer, presumably representing the peptidoglycan, surrounded by a highly corrugated outer layer which is closely connected to the peptidoglycan layer only at the indentations (Brock and Edwards, 1970; Hensel et al., 1986; Pask-Hughes and Williams, 1978; Williams, 1975). Unusual morphological structures, such as "rotund bodies" are sometimes seen (Brock and Freeze, 1969; Golovacheva, 1977), and these appear to be of two types. The "aggregation" type of structure consists of several cells bound together by the external layer of the cell envelope. This layer encloses several cells and a large intercellular space (Brock and Edwards, 1970; Kraepelin and Gravenstein, 1980; Becker and Starzyk, 1984). A "vesicular" type of rotund body is seen as developing from an extended bleb on the surface of a single cell (Kraepelin and Gravenstein, 1980). In contrast to other strains, *Thermus filiformis* has a stable filamentous morphology and does not form rod-shaped cells in culture. It possesses an extra coat surrounding the corrugated layer, which runs uninterrupted over zones of septum formation (Hudson et al., 1987b).

All strains examined have peptidoglycan containing ornithine (Pask-Hughes and Williams, 1978; Merkel et al., 1978), a relatively rare peptidoglycan type not found in other Gram-negative bacteria, but which is present in bacteria of the Gram-positive genus *Deinococcus* that are remotely related by 16S rRNA to *Thermus* (Hensel et al., 1986). Ketodeoxyoctulosonate and heptose were not detected in preparations from *Thermus* made by methods for the isolation of lipopolysaccharide (Pask-Hughes and Williams, 1978). A major component of the cell wall of *"T. thermophilus"* HB-8 has been shown to be a calcium-protein complex (Berenguer et al., 1988).

Most strains, including *T. filiformis,* have yellow, orange, or red carotenoids, but some strains are colorless. The isolates from natural habitats exposed to sunlight are generally pigmented, while those from artificial thermal environments lacking illumination are frequently nonpigmented. Only 13% of the *Thermus* strains isolated by Brock and Boylen (1973) and 30% of the strains isolated by Stramer and Starzyk (1981) from domestic and commercial hot-water tanks were colored. Pigmentation, at least in some strains, may be an unstable characteristic. Cometta et al. (1982a) reported the frequent isolation of colorless mutants from *T. aquaticus* YT-1 grown in continuous culture. By contrast, the pigment of strain YS-45 varied directly with the degree of illumination in chemostat cultures (Cossar and R. J. Sharp, unpublished observations), but was never completely lost, and colorless mutants were not detected.

Menaquinone 8 (MK-8) is the predominant quinone detected in all *Thermus* strains examined (Collins and Jones, 1981; Hensel et al., 1986; Williams, 1989), together with minor quantities of MK-7 and MK-9.

The polar fraction of the lipids of several yellow-pigmented and colorless strains comprise a major glycolipid which is a diglycosyl-(*N*-acylglycosaminyl)-glucosyl-diacylglycerol in which the hexose and hexosamine vary from strain to strain (Oshima, 1978; Pask-Hughes and Shaw, 1982; Prado et al., 1988), and a major phospholipid that contains glucosamine. Branched fatty acyl chains (*iso*-C15 and *iso*-C17) comprise the major aliphatic moieties of the polar lipids of *Thermus* spp. (Hensel et al., 1986; Oshima, 1978; Pask-Hughes and Shaw, 1982; Prado et al., 1988).

Physiology and Metabolism

The yellow and nonpigmented strains have an optimum growth temperature, in *Thermus* medium, of 70–75°C. The minimum is about 37–45°C and the maximum about 79°C, although a few strains (such as *"T. thermophilus"* HB-8) grow at temperatures up to about 85°C. The red-pigmented strains related to *T. ruber* have lower growth temperatures with optimum about 60°C, minimum between 35–40°C and maximum close to 70°C (Loginova et al., 1984; Sharp and Williams, 1988). The pH optimum for high-temperature *Thermus* strains is between 7.5 and 8.0, but some strains will grow at pH 5.1 and many grow at pH 9.5 (Hudson et al., 1986, 1987a; Munster et al., 1986).

Metabolic studies of *T. aquaticus* YT1 and four Icelandic strains indicate a complete tricarboxylic acid cycle, together with isocitrate lyase and malate synthase, providing a glyoxylate bypass as well (Pask-Hughes and Williams, 1977). Similar results were reported for the Belgian isolate Z05 (Degryse and Glansdorff, 1976). All of the enzymes of glycolysis have not been systematically measured, but a significant number have been purified and characterized, albeit from several strains, and it seems safe to assume that the Embden-Meyerhof pathway is present. The electron transport chain of *"T. thermophilus"* comprises NADH dehydrogenase, menaquinone, and cytochromes *b, c,* aa_3 and *o* (McKay et al., 1982). These authors found the molar growth yield for glucose to be low relative to comparable mesophiles, and suggest that this is due to a high permeability of the membrane to protons.

Strains of the genus *Thermus* utilize carbohydrates, amino acids, carboxylic acids, peptides, and several other proteinaceous substrates for growth. Most studies have found a nutritional diversity among *Thermus* strains (Alfredsson et al., 1985; Pask-Hughes and Williams, 1977; Santos et al., 1989) which have been reported to correlate with the geographical source of the isolates (Hudson et al., 1989). However, strains with different physiological properties, which have been shown to be different genospecies, coexist in geothermal areas in the USA (Munster et al., 1986) and Europe (Santos et al., 1989). Studies that have involved similar media and test conditions are in broad agreement about the properties of key strains. Where significant discrepancies with particular tests have been found, this may be due to the use of different test procedures. *Thermus* strains may be inhibited by even low concentrations of organic substrates. For this reason, single carbon source utilization is tested with 2–4 g/l of the substrate. Incomplete substrate utilization and growth inhibition have been reported for a number of strains, but no explanation for this phenomenon has been proposed (Sonnleitner et al., 1982).

Many "high-temperature" strains use acetate, pyruvate, proline, and glutamate (Alfredsson et al., 1985; Hudson et al., 1989), while the red-pigmented strains are generally unable to grow on acetate and pyruvate (Sharp and Williams, 1988). On the other hand, polyols are used by many red-pigmented strains but serve as single carbon sources for a minority of the high-temperature strains.

Several monosaccharides are used as single carbon sources by strains of *Thermus,* but pentoses are not usually metabolized at all (Alfredsson et al., 1985; Hudson et al., 1986, 1987a; Munster et al., 1986). Disaccharides, but not necessarily their constituent monosaccharides, may be used. Thus, most strains from Iceland used sucrose and maltose, while only two used glucose and none grew on fructose (Alfredsson et al., 1985).

Proteins such as elastin, fibrin, and casein are hydrolyzed by many isolates, but some strains are incapable of hydrolyzing each substrate (Munster et al., 1986; Hudson et al., 1989; Santos et al, 1989).

Several strains will grow in basal salts medium with ammonium as nitrogen source and an appropriate carbon source. Many strains, nevertheless, require vitamins for growth on minimal medium with single carbon sources (Alfredsson et al., 1985; Sharp and Williams, 1988). Nitrate is not reduced by *T. aquaticus, T. filiformis,* nor most of the red-pigmented strains examined, but serves as a terminal electron acceptor for many high-temperature strains, several of which also reduce nitrite (Brock, 1978; Munster et al., 1986; Hudson et al., 1987b). Moreover, many high-temperature strains grow anaerobically in the presence, but not in the absence, of nitrate. No *Thermus* strain has been shown to be capable of fermentation.

Identification

Most high-temperature strains form yellow or pale to colorless colonies. The low-temperature strains related to *T. ruber* are red pigmented. All strains are Gram negative, and with the exception of *T. filiformis,* form pleomorphic rod-shaped cells and short filaments. "Rotund bodies" present in liquid cultures can be seen by phase-contrast microscopy.

All strains are cytochrome oxidase-positive, nonmotile in liquid cultures, and do not form flagellae. Endospores are not seen in cultures on any medium, including starch agar. Sensitivity to β-lactam antibiotics is reported for all strains of this genus.

The presence of MK-8 as the predominant quinone (Collins and Jones, 1981) and ornithine-containing peptidoglycan are diagnostic characteristics of the members of the genus *Thermus* examined to date (Pask-Hughes and Williams, 1978; Hensel et al., 1986; Sharp and Williams, 1988). The presence of predominantly iso- and anteiso branched fatty acids can be used as a confirmatory characteristic (Hensel et al., 1986; Pask-Hughes and Shaw, 1982, Prado et al., 1988). The mean GC content of the DNA of the strains examined varies between is 57–65 mol%, but analyses of most strains give results above 60 mol%.

Three species of the genus *Thermus* are validly described at present, and there appear to be at least four others that should be recognized (Table 1).

1. *T. aquaticus,* the type species of the genus, has been shown by DNA homology (Williams, 1989) to have a number of representatives (which come from the same site as the type strain) among the numerical taxonomy groups 1a and 1b of Munster et al. (1986). The numerical taxonomy study of Hudson et al. (1989) also shows the type strain YT1 clustered with other Yellowstone isolates.
2. *T. ruber* strains, which are easily distinguished by their red pigment and lower growth temperature optimum, have been isolated from worldwide sources. The DNA-DNA homology is high within the species, but low with yellow and colorless strains (Sharp and Williams, 1988).
3. *T. filiformis,* apparently a single strain which has a stable filamentous morphology and an extra outer cell wall layer. There seem to be no other characteristics that distinguish it clearly from other yellow strains.
4. *"T. thermophilus"* was validly described (Oshima and Imahori, 1974), but subsequently not included in the *Approved Lists of Bacterial Species* (Skerman et al., 1980). The type strain HB8 has a high DNA-DNA homology with *"T. flavus"* AT-62, *"T. caldophilus"* GK24, and *Thermus* strain B (Wil-

Table 1. Characteristics of species of *Thermus* and related organisms.

Species and type strain	GC content (mol%)	Ornithine peptidoglycan	Menaquinone MK-8	Growth temperature		
				Optimum	Maximum	Minimum
T. aquaticus YT1;						
ATCC 25106;	67[a]	+[b]	+[j]	70°C	79°C	40°C
NCIMB 11243;	65[b]					
DSM 625	64[c]					
T. ruber BKMB 1258;	66[d]	+[c]	+[c]	60°C	70°C	37°C
NCIMB 11269;	62[c]					
DSM 1279	61[e]					
"T. thermophilus"						
HB8; DSM 625;	64[g]	+[b]	+[j]	73°C	85°C	40°C
ATCC 2762;	69[f]					
NCIMB 11244	62[c]					
"T. filiformis" Wai						
33A1; ATCC 43280	65[h]	ND	+[h]	73°C	80°C	37°C
"T. brockianus"						
YS-36; NCIMB	60[g]	+[g]	+[g]	70°C	ND	ND
Rhodothermus marinus ATCC						
43812; DSM 4252	64–65[i]	ND	ND	70°C	77°C	54°C

Symbols: +, constituent present; ND, no data.
[a]From Brock and Freeze (1969).
[b]From Pask-Hughes and Williams (1977).
[c]From Hensel et al. (1986).
[d]From Loginova et al. (1984).
[e]From Sharp and Williams (1988).
[f]From Oshima and Imahori (1974).
[g]From Williams (1989).
[h]From Hundson et al. (1987).
[i]From Alfredsson et al. (1988).
[j]From Collins and Jones (1981).

liams, 1989) and there is no doubt that *"T. thermophilus"* comprises a distinct genospecies.

5. A taxon tentatively named *"T. brockii"* was identified by DNA-DNA homology (Williams, 1989) among strains of numerical taxonomy cluster 2 of Munster et al. (1986). Phenotypic characters that allow it to be distinguished from *T. aquaticus* are: the former produces pale-yellow spreading colonies on *Thermus* agar, and commonly grows on fructose and galactose, while the latter forms deep-yellow nonspreading colonies and generally degrades starch, gelatin, and casein. The name *"T. brockianus"* seems likely to be preferred for this taxon, to avoid confusion with *Thermoanaerobium brockii* (Zeikus et al., 1979).
6. A genospecies detected by DNA-DNA homology (Williams, 1989) corresponds to the numerical taxonomy clusters A,B,C, and D of Santos et al. (1989).
7. A genospecies detected by DNA-DNA homology (Williams, 1989) corresponds to the numerical taxonomy clusters E and F of Santos et al. (1989).

Biotechnological Applications of *Thermus*

Restriction and Modification Enzymes

Strains of the genus *Thermus* are sources of several restriction endonucleases that are both thermostable and active at higher temperatures than most other such enzymes (Table 2). Some are isoschizomers, or at least have the same recognition sequences as known enzymes from other microorganisms (Roberts, 1989). Others are important because they are unique in hydrolyzing certain sequences; in particular Taq I is widely used in molecular biology. The corresponding modification enzyme, M.Taq I, which methylates the A residue in the Taq I recognition sequence, has found a minor use in producing large DNA fragments. This depends on the fact that Dpn I will only cut a sequence derived from two adjacent Taq I sites in which both A residues are methylated (McClelland et al., 1984).

DNA Polymerase

The DNA-dependent DNA polymerase of *T. aquaticus* YT1 has optimal activity at 80°C (Chien et al., 1976; Kaledin et al., 1986). It lacks a proofreading 3′to 5′ exonuclease activity and produces base substitution errors at a frequency of 1:9000 (Tindall and Kunkel, 1988). The amplification of selected segments of DNA by the polymerase chain reaction (PCR) involves repeated cycles of: 1) priming with pairs of synthetic oligonucleotides; 2) DNA synthesis; and 3) heat-denaturation to allow the next priming. Originally conceived utilizing thermolabile DNA polymerase (Saiki et al., 1985), the PCR technique has been greatly improved by the use of the *T. aquaticus* enzyme, which survives the heat-denaturation step, and therefore does not need replenishing at each cycle (Saiki et al., 1988). The error rate is not a problem as misincorporated bases are randomly distributed throughout the sequences, and form a fraction of a percent at any one site. Single-stranded DNA can be produced by modification of PCR (asymmetric PCR) and used directly for sequence determination (Gyllensten and Ehrlich, 1988). The *T. aquaticus* DNA polymerase has also been used for the sequencing reactions at 70°C and produced electrophoretic ladders with uniform bands of more than 1000 bases per gel (Innis et al., 1988).

Table 2. Restriction endonucleases of the genus *Thermus*.

Enzyme	Recognition sequence[a]	Isoschizomer	Reference
Taq I	TCGA	Tfl I, TthHB8 I, CviB III	Sato et al. (1977)
Taq II7	GACCGA	None	Barker et al. (1984)
Tth111 I	GACNNNGTC	None	Shinomiyo and Sato (1980)
Tth111 II	CAARCA	None	Shinomiyo et al. (1980)
TspE I	AATT	None	Raven et al. (unpublished observations)[b]
Tsp45 I	GTSAC	None	Raven et al. (unpublished observations)[b]
Tru I	GGWCC	Ava II	Bernal et al. (1986)
Tru II	GATC	Mbo I	Bernal et al. (1986)
TaqX I	CCWGG	Eco II	Grachev et al. (1981)[b]
TspZn I	GGCC	Hae III	Ghufoor et al. (unpublished observations)[b]
TspA I	CASTG	None	Raven et al. (unpublished observations)

[a]Symbols: N = A,C,G, or T; W = A or T; R = G or A; S = G or C.
[b]Cited in Roberts (1989).

Proteinases

Caldolysin, a neutral serine proteinase from New Zealand isolate T351 (Cowan and Daniel, 1982a; Cowan et al., 1987) which is calcium dependent (Khoo et al., 1984) has been immobilized for use in continuous-flow proteolysis (Cowan and Daniel, 1982b). This enzyme, together with a proteinase from an unidentified strain of *Thermus,* were as thermostable as the bacillus proteinase thermolysin, but less resistant to organic solvents (Owusu and Cowan, 1989). A thermophilic alkaline proteinase (aqualysin I) was isolated from strain YT1 (Matsuzawa et al., 1983). The sequence of amino acids deduced from the DNA sequence of the cloned gene (Kwon et al., 1988) showed a high homology to proteinase K and the subtilisins. Aminopeptidase T, which requires cobalt II ions for activity, has also been purified from strain YT1 (Minagawa et al., 1988). It is optimally active at 75–80°C, is of low specificity, and is more thermostable than the *B. stearothermophilus* aminopeptidase. The amino terminal sequence indicates that it is structurally unlike other proteinases discovered to date.

Carbohydrases

Thermus strain YT1 (Ulrich et al., 1972) and New Zealand isolate 4–1A (Cowan et al., 1984) have been used as sources of β-galactosidase. Both enzymes are thiol dependent and have narrow pH optima at 5.0 (YT1) and 6.0 (4–1A), but were different in molecular weights and metal ion activation. The enzyme from strain 4–1A was immobilized, but its value in lactose processing is likely to be limited by its susceptibility to product inhibition.

Thermus strain Z1 produces a thermostable β-glucosidase (Takase and Horikoshi, 1988, 1989), which also has activity against other β-glycosides including β-galactosides. This enzyme has a broad pH optimum from 4.5–6.5 and is also stable within this range. Its optimum activity is above 80°C, but it is more stable at 70–75°C. The ability to hydrolyze cellobiose to completion may render this enzyme useful for cellulose degradation in conjunction with cellulases that are inhibited by cellobiose.

Pullulanase, which hydrolyzes α1:6 links in starch, has been purified from *T. aquaticus* YT1 (Plant et al., 1986). The enzyme has a broad pH profile and a high thermal stability suitable for the debranching process in the saccharification of starch. Strain AMD33 also produces pullulanase which is both activated and stabilized by calcium ions, and also has α-amylase activity (Nakamura et al., 1989). It has been cloned and expressed in *Escherichia coli* (Sashihara et al., 1988) with a view to use in starch processing. Strain AMD33 was described as *Thermus,* and has ornithine in its peptidoglycan, but has a GC content of only 55 mol% and a growth optimum of 65°C. Its taxonomic position is yet to be determined.

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CHAPTER 206

The Family Chloroflexaceae

BEVERLY K. PIERSON and RICHARD W. CASTENHOLZ

The discovery of the photosynthetic flexibacteria was made by Pierson and Castenholz (1971), and *Chloroflexus aurantiacus* was the first genus and species described (Pierson and Castenholz, 1974a). The Chloroflexaceae was proposed as a family (Trüper, 1976) with affinities to the Chlorobiaceae. The similarities with the green sulfur bacteria were signified by grouping both families under the suborder Chlorobiineae. The family was defined as follows: filamentous, phototrophic bacteria with gliding motility, Gram-negative, flexible cell walls, and bacteriochlorophyll (bchl) *a* and bchl *c, d,* or *e.* Included in the family were two other genera, "Oscillochloris" and *Chloronema.*

Chloroflexus aurantiacus (Pierson and Castenholz, 1974a) is still the only genus and species of the Chloroflexaceae that has been described on the basis of axenic cultures. It is also the only organism for which 16S rRNA data are available. *Chloronema* (Dubinina and Gorlenko, 1975) and "Oscillochloris" (Gorlenko and Pivovarova, 1977) are two genera described and assigned to the Chloroflexaceae, but neither description is based on cultured material, and no molecular phylogenetic data are available for either of these genera.

Phylogenetic analysis from 16S rRNA oligonucleotide catalogs and complete sequence data reveal that *Chloroflexus aurantiacus* forms a very deep division within the eubacteria (Oyaizu et al., 1987; Woese, 1987), being grouped with the nonphototrophic gliding filamentous flexibacterium *Herpetosiphon aurantiacus.* Also in this group, but more distantly related, is *Thermomicrobium roseum.* Another thermophile, *Thermus thermophilus,* appears to branch off the same early line of descent (Hartmann et al., 1989). *Chloroflexus* is not closely related to the other phototrophic prokaryotes, including those of the Chlorobiaceae, and forms the deepest division of all the phototrophs in the eubacterial line of descent.

Heliothrix oregonensis has been described on the basis of co-cultures with the nonphototrophic bacterium *Isosphaera pallida* (see Chapter 203) and on the basis of studies with natural populations in the field (Pierson et al., 1984; 1985). Isolation in pure culture has not been achieved and 16S rRNA data are lacking. *Heliothrix oregonensis* is phylogenetically close to *Chloroflexus aurantiacus,* based on 5S rRNA analysis (Pierson et al., 1985), although the description of the family Chloroflexaceae excludes it.

All four of these filamentous phototrophs (*Chloroflexus,* "Oscillochloris," *Chloronema,* and *Heliothrix*) are now included in the grouping called the "multicellular filamentous green bacteria" (Pfennig, 1989). Although *Chloroflexus, Chloronema,* and "Oscillochloris" all have in common the housing of accessory chlorophylls (bchl *c* or *d*) in chlorosomes adjacent to the cell membrane (hence the term "green bacteria"), *Heliothrix* lacks accessory chlorophylls and chlorosomes. Since on the basis of 5S rRNA analysis, *Heliothrix oregonensis* is more closely related to *C. aurantiacus* than to any other phototrophs (Pierson et al., 1985), a new grouping of the filamentous phototrophs may well be in order (Castenholz and Pierson, 1989).

Other filamentous phototrophs not yet in culture but with possible affinities to these organisms will also be described in this chapter. At this time we prefer to consider all the filamentous phototrophic bacteria containing bchl *a* in one category as the phototrophic flexibacteria. Within this group, which may or may not be phylogenetically coherent, we recognize the filamentous green bacteria including *Chloroflexus, Chloronema,* and "Oscillochloris," which contain chlorosomes and accessory bacteriochlorophylls. Also included are the filamentous bacteria lacking chlorosomes and containing bchl *a* only: *Heliothrix* and two other unnamed organisms described below.

Several marine strains of *Chloroflexus*-like organisms have also been observed, but none has been isolated in axenic culture. These will also be briefly discussed in this chapter.

Two other filamentous gliding phototrophs containing bchl *a* and lacking chlorosomes and bchl *c, d,* and *e* have been described. Neither has been isolated in pure culture, and their descriptions here will be brief. Castenholz (1984) first described a red filamentous organism containing bchl *a* from a thermal mat environment in Yellowstone National Park. D'Amelio et al. (1987) reported filamentous "purple" bacteria containing bchl *a* in hypersaline cyanobacterial mats. The two organisms are ultrastructurally identical (D'Amelio et al., 1987; S. Boomer and B. Pierson, unpublished observations). Both contain bchl *a* only and an elaborate system of stacked internal membranes similar in appearance to those of *Ectothiorhodospira* (see Chapter 171). Relatively little is known of the physiology of the hypersaline organism but the thermal strain bears some striking similarities to *Heliothrix oregonensis* (B. Pierson and R. W. Castenholz, unpublished observations).

Habitats

The anoxygenic filamentous phototrophs are found in a wide range of habitats.

Chloroflexus aurantiacus

Chloroflexus aurantiacus was found originally only in hot springs. All isolates were thermophiles capable of growth in the laboratory from an upper temperature limit of 70°C to a lower temperature limit of 30–35°C (optimum, 52–60°C). *Chloroflexus* forms mixed populations with cyanobacteria in "alkaline" hot springs and warm waters with a pH from 5.5 to 10. These populations are found up to temperatures as high as 70–72°C in hot springs of North America. *Chloroflexus* commonly forms a distinct orange mat of filaments below a thin top layer of cyanobacteria. This structure is found over the temperature range from 35°C to about 68°C (Castenholz, 1973a; Doemel and Brock, 1977; Ward et al., 1989). In these associations with cyanobacteria, it appears that the cyanobacteria provide organic carbon that sustains photoheterotrophic and chemoheterotrophic growth of *Chloroflexus* (Bauld and Brock, 1974; Brock, 1978; Castenholz, 1984; Ward et al., 1984; Bateson and Ward, 1988). In the complex habitat of the mat, however, some of the organic carbon used by *Chloroflexus* appears to come from fermentation products produced in the mat (Anderson et al., 1987).

In this type of mat environment, *Chloroflexus* is subject to major diel fluctuations in pH and oxygen levels in response to the oxygenic photosynthetic activity of the overlying cyanobacteria (Revsbech and Ward, 1984). In these mats with very low sulfide levels, it is doubtful that *Chloroflexus* is growing autotrophically to any great extent. The most likely forms of metabolism for *Chloroflexus* in this habitat are aerobic and anaerobic photoheterotrophy and both aerobic and anaerobic chemoheterotrophy. *Chloroflexus* can probably switch among these modes with the fluctuating environmental conditions.

In hot springs with a significant source of primary reduced sulfur (H_2S, HS^-, S^{2-}), *Chloroflexus* often forms mats (green or orange) independent of cyanobacteria (Castenholz, 1973b; Giovannoni et al., 1987). In this situation, *Chloroflexus* is apparently growing photoautotrophically with sulfide as the electron donor. In situ studies in several springs in the Mammoth Group in Yellowstone National Park revealed a dark green mat of nearly pure *Chloroflexus* totally devoid of cyanobacteria and oxygen. At temperatures above 50°C in these springs, cyanobacteria do not grow due to the presence of sulfide (Castenholz, 1988b) so that pure mats of *Chloroflexus* develop from about 50 to 66°C when primary sulfide is available. In situ studies revealed that under these conditions, *Chloroflexus* strains ("green *Chloroflexus*" or GCF strains) were primary producers of organic carbon, performing sulfide-dependent photoautotrophy (Giovannoni et al., 1987). The strains of *Chloroflexus* isolated from this habitat are obligate anaerobes, lacking the ability to grow aerobically by chemoheterotrophy. Thus they are not identical to other *Chloroflexus aurantiacus* strains. In culture they grow best as photoheterotrophs and not autotrophs. Exposure to atmospheric levels of O_2 inhibits growth completely in darkness, but not irreversibly. Even after 72 hours of aerobic exposure in darkness, surviving cells resume growth rapidly when shifted to anaerobic growth conditions in the light. In the light, exposure to O_2 results in an intensity-dependent loss of bchl *c* but an increase in content of carotenoid pigments. Although potentially most active as a photoheterotroph, "green *Chloroflexus*" shows a greater degree of sulfide-stimulated photoautotrophy than do previously characterized strains of *C. aurantiacus* (Giovannoni et al., 1987).

In other sulfide-containing thermal habitats, *Chloroflexus* may be found growing independently of cyanobacteria at higher temperatures upstream from the cyanobacteria (Castenholz, 1973b). In flowing streams such as these, the *Chlorofexus* mats are usually orange. Under these circumstances, *Chloroflexus* is most likely growing as a photoautotroph (Castenholz,

1988b). In some hot springs containing sulfide, the layers of *Chloroflexus* are found above the cyanobacteria in what has been called an inverted mat (Jørgensen and Nelson, 1988). Under these conditions the supposed autotrophic activity of the *Chloroflexus* in the surface layers consumes adequate sulfide from the vicinity of the cyanobacteria to permit their growth at these temperatures (Jørgensen and Nelson, 1988). In this way, a small pocket of oxygenic photosynthesis may actually occur beneath the anoxygenic *Chloroflexus.*

A mesophilic strain of *Chloroflexus aurantiacus* (var. *mesophilus*) was isolated from mat communities in the bottom mud of lowsulfate stratified freshwater lakes containing some sulfide. These strains had temperature optima from 20–25°C and were otherwise identical with the thermophilic strains (Gorlenko, 1976; Pivovarova and Gorlenko, 1977).

Chloroflexus-like Organisms in the Marine Habitat

Chloroflexus-like organisms have been observed in several marine and hypersaline habitats, although none has been isolated in pure culture. Intertidal sand flats at Great Sippewissett Salt Marsh, Cape Cod, MA, rich in biogenic sulfide, were observed to contain recognizable thin mat layers of *Chloroflexus*-like organisms that migrated to the surface in darkness or dim light. The *Chloroflexus*-like filaments were often associated with species of *Oscillatoria, Chloroherpeton, Beggiatoa,* and some purple sulfur bacteria (Mack and Pierson, 1988). Successful enrichment cultures of *Chloroflexus*-like filaments have been obtained and partial purification achieved from sandy intertidal mats on Mellum Island, West Germany (E. Mack and N. Pfennig, personal communication).

Similar *Chloroflexus*-like organisms have been observed as prominent constituents of well-developed mats in hypersaline environments. The presence of these organisms (identified on the basis of electron microscopy) has been reported in mats at Laguna Figueroa, Baja, Mexico (Stolz, 1983, 1984), Solar Lake in the Sinai (Cohen, 1984; D'Amelio et al., 1989), salt evaporation ponds at Exportadora de Sal, Guerrero Negro, Baja, Mexico (D'Amelio et al., 1989), and Shark Bay, Western Australia (John Bauld and Elisa D'Amelio, personal communication). Filamentous bacteria thought to be similar to *Chloroflexus* were observed in Abu Dhabi mats (Cardoso et al., 1978). The presence of *Chloroflexus*-like organisms in the hypersaline mats at Guerrero Negro has been further substantiated by the presence of bchl *c* and γ carotene in particular layers (Palmisano et al., 1989). In some cases, *Chloroflexus* forms a major recognizable mat layer in the hypersaline environment (B. K. Pierson, unpublished observations). Within these mats, *Chloroflexus*-like filaments are frequently found beneath a layer of cyanobacteria and often in association with *Beggiatoa.* Mats containing layers of nearly pure *Chloroflexus*-like filaments were also observed at salinities greater than 10%, and successful enrichments have been made of these filaments in the medium modified from that used by E. Mack and N. Pfennig (unpublished observations) to enrich for *Chloroflexus* from Mellum (B. K. Pierson, unpublished observations). *Chloroflexus*-like organisms were also found in sulfide-containing mats from hypersaline lagoons of Lake Sivash and the White Sea, USSR (Venetskaya and Gerasimenko, 1988; Gorlenko, 1988).

Chloronema

Chloronema has been observed in the plankton in dimictic freshwater lakes with high ferrous iron content and low H_2S (Gorlenko, 1988, 1989b). The organisms are found in the metalimnion and upper hypolimnion of shallow stratified lakes. They are usually below the chemocline in an anaerobic zone containing other species of purple and green sulfur bacteria. *Chloronema gigantea* has been observed to be the dominant phototroph in the plankton of such lakes (Dubinina and Gorlenko, 1975).

"Oscillochloris"

Species of "Oscillochloris" have been observed as yellow-green mats on the surface of hydrogen-sulfide-containing mud in freshwater habitats over a temperature range of 10–20°C and pH range of 7.5–8.5 (Gorlenko, 1989a). Within this habitat they are often found associated with purple and green sulfur bacteria, *Beggiatoa,* and *Oscillatoria* species in algal/bacterial mats (Gorlenko, 1989a). "Oscillochloris trichoides" has been observed in mats on the surface of freshwater mud containing H_2S. It has been seen in microzones below purple sulfur bacteria. "O. chrysea" has been found on the surface of muds in freshwater streams with high organic content from domestic sewage effluents (Gorlenko and Pivovarova, 1977; Gorlenko, 1988). "Oscillochloris"-like filaments have also been observed in mats from hypersaline ponds in Baja, Mexico (D'Amelio, personal communication).

Bchl-*a*-Containing Filamentous Phototrophs

Heliothrix oregonensis is found in conspicuous masses of nearly pure bright orange motile filaments on top of more compact layers of cyanobacteria in some hot springs in western North America. It is found at about pH 8.5 and at temperatures up to 55°C under conditions of high light intensity (Pierson et al., 1984). The layer of *Heliothrix* often forms conspicuous puffs and tufts that are oxic even in their interior (Castenholz, 1988b; B. K. Pierson, unpublished observations). The habitat is lacking in hydrogen sulfide. On the Warm Springs Indian Reservation (Oregon), small alkaline pools are impressively dominated by *Heliothrix,* whereas in a few other alkaline springs in Oregon and in Yellowstone National Park, *Heliothrix*-like filaments are only observed as localized patches.

Other filamentous bchl-*a*-containing phototrophs form a conspicuous deep red layer below layers of cyanobacteria and *Chloroflexus* in alkaline hot springs in Yellowstone National Park (Castenholz, 1984). These layers were seen in springs lacking H_2S. In more translucent mats this dark red layer of filamentous phototrophs can be found more than 1 cm from the surface. It is not yet known how widespread these organisms are in their distribution. The application of *Chloroflexus*- specific antisera to "Chloroflexus" taken from lower layers of mats in Octopus Spring, Yellowstone National Park, revealed that many nonreactive filaments were present (Tayne et al., 1987). Bchl-*a*-containing filaments other than *Chloroflexus* were abundant in this mat and migrated to the surface when it was darkened (B. K. Pierson, unpublished observations). Some of the nonreactive filaments may be phototrophs that have not yet been identified.

The filamentous bchl-*a*-containing phototrophs described by D'Amelio et al. (1987) were observed in hypersaline mats of Solar Lake and the salt ponds of Guerrero Negro where they were found as free-living or intra-bundle-dwelling organisms in association with *Microcoleus chthonoplastes.* These mats contained hydrogen sulfide and the filaments were found at depths of 0.3–1.2 mm, which is below the zone of maximal oxygen production. Hydrogen sulfide was present at fluctuating levels. Filamentous bchl-*a*-containing bacteria were also seen in mats at Laguna Figueroa (Stolz, 1983).

Isolation

Any mat material collected from alkaline hot springs (at least below 65°C) is likely to contain viable filaments of *Chloroflexus.* In general, the mat from a temperature above 45°C will consist primarily of cyanobacteria. The material may be collected with forceps and transported in vials of spring water. Less than 5% of the aqueous volume should be occupied by mat material. The vials will retain live *Chloroflexus* for one to several weeks if stored in darkness and at temperatures from about 12–25°C. Cooler or warmer temperatures usually shorten the survival time. Freezing should be avoided.

Selective Enrichment

Almost all of the published information pertains to thermophilic *Chloroflexus* (Pierson and Castenholz, 1974a; Castenholz and Pierson, 1981; Castenholz, 1989). Present enrichment techniques for *C. aurantiacus* are nonexclusive, but large population densities can nevertheless be obtained by enriching a basic mineral medium such as D medium (see below) or BG-11 medium (Castenholz, 1988a) with yeast extract (0.1–1.0 g/l), and by adding 3,4-dichlorophenyl-1,1-dimethylurea (DCMU) to 5–10 μM in nearly filled screw-cap tubes or flasks. Fluorescent or incandescent lamps may be used (10–100 W/m^2). Many other nonfastidious photoheterotrophic anaerobes may also develop in 45–50°C enrichments, but incubation at 60°C will exclude all known purple bacteria and heliobacteria (see Chapter 90). However, even at 60°C a number of chemoheterotrophic bacterial species will grow, including *Thermus* spp.

Gorlenko (1976) successfully enriched for mesophilic *Chloroflexus* in glass columns that contained samples of lake-bottom water and mud and were filled with a "semiliquid" medium containing mineral salts and Pfennig's mixture of trace elements (Pfennig, 1965; Pfennig and Lippert, 1966) and supplemented with Na_2SO_4 (0.6 g/l), $Na_2S \cdot 9H_2O$ (0.1 g/l), yeast extract (0.025 g/l), and casein hydrolysate (0.025 g/l). The incubation was at 25–30°C under light of 3,000 lux.

C. aurantiacus is a faculative aerobe and is tolerant of at least some oxygen in the medium while bacteriochlorophylls are being synthesized. The use of sodium sulfide or sodium thioglycollate to lower the redox potential and eliminate O_2 has not been a requirement for growth, although either could be helpful.

The obligately anaerobic "green Chloroflexus" (GCF) may also be enriched for using the Hungate-agar-tube method or liquid medium in sealed vessels, with a medium designed for green bacteria that contains acetate and sulfide (see Sirevåg, 1975; Giovannoni et al., 1987). Isolations may be made from such enrichments or directly from field collections following dis-

persion of the material by passage through an 18-gauge needle. A dilution series is plated on DGN medium containing yeast extract (0.05 g/l) using the pour-plate method, followed by incubation in anaerobic (H_2 + CO_2) Gas Paks illuminated by coolwhite fluorescent and/or incandescent lights. Numerous greenish colonies should arise. After picking some and spreading these on fresh plates, single filaments may be isolated and cut out on agar blocks as described later for *C. aurantiacus*. Although "green Chloroflexus" will not grow under aerobic conditions, it is quite tolerant of exposure to O_2 and can be easily manipulated under normal laboratory conditions.

Enrichment of Cyanobacteria Contaminated with *Chloroflexus*

An essentially foolproof method of establishing thermophilic *Chloroflexus* in a mixed culture is to enrich in a mineral medium for cyanobacteria inoculated from a hot spring source. Cyanobacteria from hot springs may be enriched for in a variety of ways (see this Handbook, Chapter 98 and Castenholz, 1988b). Essentially all enrichments or isolations of thermophilic cyanobacteria are contaminated with heterotrophic bacteria, of which *Chloroflexus* is one. A simple technique for culturing *Chloroflexus* is to incubate a sample of the cyanobacterial mat at a temperature appropriate for its growth in a cotton-plugged flask of inorganic D medium (see below). The one or more species of cyanobacteria that invariably grow will excrete organic matter which will support a population of *Chloroflexus* that will persist over numerous transfers. *Chloroflexus* is actually difficult to eliminate in attempts to purify thermophilic cyanobacteria.

Cyanobacteria may also be inoculated on agar-solidified D medium (agar at 15 g/l). Motile cyanobacteria often move out from the initial inoculum spot in large numbers, sometimes forming fascicles or circling aggregates on the agar. These may be transferred to new plates or to similar liquid medium by using watchmaker's forceps to cut out the block with the filaments. These cyanobacterial filaments usually carry *Chloroflexus* as a contaminant. Occasionally, a two-membered culture may be established.

Direct Isolation

Because of its motility, thermophilic *Chloroflexus aurantiacus* is most easily isolated without prior enrichment by a combination of manual techniques. Solid medium is prepared using 1.5% (wt/vol) agar (e.g., Difco or Oxoid No. 3). The nutrient base used is commonly D medium with 0.8 g/l glycylglycine as buffer. The pH is adjusted to 8.2 with 1–2 M NaOH before autoclaving. This medium is excellent for *Chloroflexus* isolation. The addition of very little yeast extract (10 mg/l) may improve it slightly. The plates are allowed to dry inverted at room temperature for 2 days or more.

A small piece of hot-spring cyanobacterial-*Chloroflexus* mat is placed in the center of each plate. A mass of cyanobacteria or *Chloroflexus* grown as an enrichment may be used. Plates can also be inoculated by dragging a piece of the inoculum back and forth over the surface of the agar with a watchmaker's forceps. The plate is then inverted and incubated at 45–50°C for 1–2 days under tungsten or fluorescent lights (e.g., 10–100 W/m^2). The incubator should be humidified with a pan of water at the base.

Plates are then examined under a good-quality dissecting microscope with a magnification of 15–60×. Transmitted illumination should be carefully adjusted for the best resolution of the pale, orange-colored wisps of *Chloroflexus* that may grow and spread out from the inoculation site. With the fastest-known gliding rates, *Chloroflexus* could move out at the rate of about 3.5 mm/day, but probably will be slower.

Quite frequently, *Chloroflexus* may be recognized as pointed, stringy, or recoiled wisps of a few to several filaments that extend as a fringe beyond the edge of the cyanobacteria or other bacteria (Fig. 1; Pierson and Castenholz, 1974a). If the inoculum included rapidly migrating cyanobacteria, the *Chloroflexus* may often be seen extending from the trails of these other gliding bacteria. On the plates in which inoculum has been dragged along the surface, the *Chloroflexus* filaments may have been spread thinner. Smaller colonies of various shapes may then develop and the plate should be examined again after 2–3 additional days of incubation (Fig. 2).

The removal of *Chloroflexus* is the next stage in the isolation. A fine-pointed watchmaker's forceps should be used. The leading edges or tips of the small wisps of *Chloroflexus*-like filaments may be cut from the rest of the inoculum with the forceps. The agar block containing this material is then moved and inverted on a sterile part of the plate or on a new plate. The block is then pushed in a long streak over the top of the agar, leaving a trail of filaments. Some axenic colonies may arise on this first transfer, but usually the process must be repeated after new growth and gliding occur. The agar medium of the first or second transfer should contain a slightly higher concentration of yeast extract (about 50 mg/l).

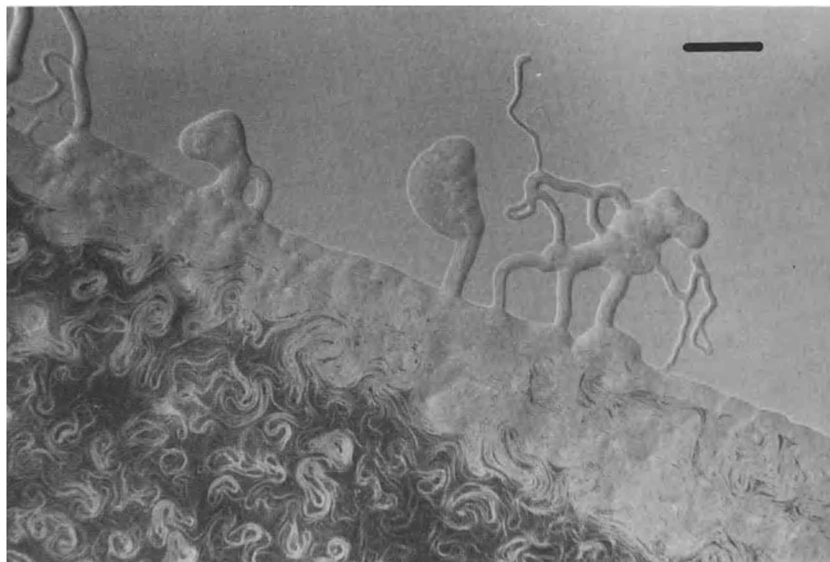

Fig. 1. Dual culture of *Chloroflexus aurantiacus* (NZ-Mar. 62-fl) and the cyanobacterium *Synechococcus lividus* (OH-53-s) on an agar surface (DG medium, 45°C). The dark area at the lower left is the population of *Synechococcus*. The lighter swirls and wisps of filaments advancing toward the upper right are *Chloroflexus*. Bar = 0.2 mm.

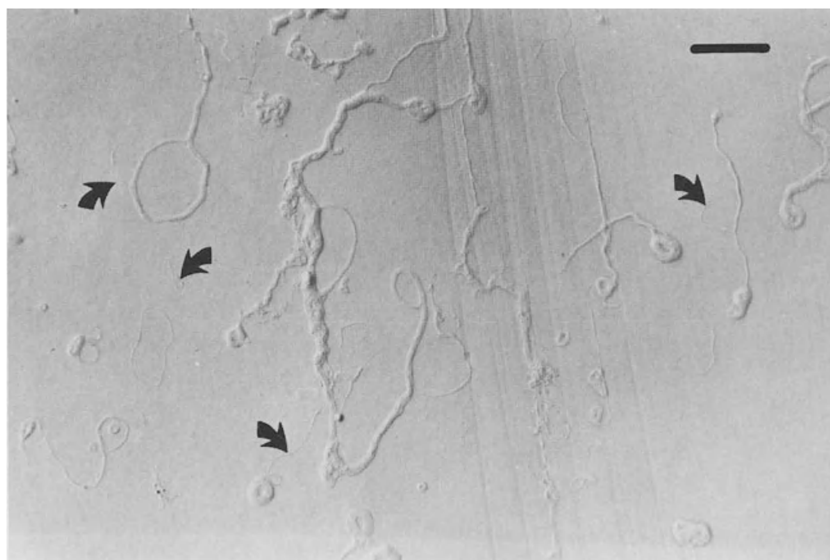

Fig. 2. Culture of *Chloroflexus aurantiacus* (OK-70-fl) on an agar surface at 45°C 10 days after streak dilution. Single and double filaments are indicated by arrows. Bar = 0.2 mm.

An alternative method of doing the first stage of isolation is to pick up the wisp of protruding *Chloroflexus* with the forceps and actually drag it across a sterile area of the same plate. The tip of the forceps should not quite touch the agar. As filaments are moved, some will be left behind (sometimes individually) as a trail.

After each transfer of *Chloroflexus* wisps, a period of a few days for growth and spreading should be allowed before repeating the process. In some strains of *Chloroflexus* presently in culture, the filaments are wide enough (1.0–1.2 μm) to be seen individually under the highest power of a dissecting microscope (Fig. 2). In such cases, blocks of agar with single filaments can be cut from the trail of filaments left by the streaking technique described above. These can then be transferred and inverted on new agar, establishing a definite clone. In the case of thinner filament types (0.5–0.7 μm wide), only pre-

sumptive clones can be established by repeated streakings.

When the colonies isolated appear free of other bacteria (as judged by examination with oil-immersion, phase contrast microscopy at 1,000 ×), colonies may be streaked on plates that contain yeast extract (0.5 g/l) or they may be suspended in liquid medium and serially diluted for introduction into 1.5% agar shake cultures containing yeast extract at 0.5–2.0 g/l. Inoculation of shake cultures should be done when the medium has cooled to about 45°C. Nearly full screw-capped tubes should be used. After inoculation, the tubes are shaken slowly and solidified. *Chloroflexus* grows best as an anaerobic photoheterotroph, and individual filaments will develop into orange to dull green colonies within the agar. The deeper colonies will appear greener due to the higher content of bchl *c* at lower levels of O_2. The addition of a sterile $NaHCO_3$ solution (0.5 g/l from a stock solution of 45 g/l that had been bubbled for 20 min with a stream of pure CO_2) seems to enhance development of colonies.

If contaminating bacteria are still present in the inoculum, this method can also be used for separation and purification by dilution. Tubes or flasks of liquid medium (again with yeast extract at 0.5–2.0 g/l) may be used for culturing, and are particularly useful when searching for possible contaminants. Cotton-plugged Erlenmeyer flasks (e.g., 125 ml) containing 80–90 ml of medium work well when unshaken because both aerobic and facultatively anaerobic contaminants will develop, if present.

Gorlenko (1976) did not explain how he isolated mesophilic *Chloroflexus* after initial enrichment. Presumably, a technique similar to the one for the thermophilic forms could be used.

Cultivation

The culture methods described pertain almost entirely to *Chloroflexus aurantiacus.* A variety of media may be used. The most commonly used base is D medium, (see below) at least for photoheterotrophic or chemoheterotrophic growth. D medium has alternatively been called "Castenholz salts" or Castenholz medium (Claus and Schaab-Engels, 1977). It was originally developed by R. P. Sheridan in Castenholz's laboratory for the culture of thermophilic cyanobacteria.

MEDIA FOR PHOTOTROPHIC CULTIVATION OF *CHLOROFLEXUS.* The following media are currently used in the laboratory at the University of Oregon, for phototrophic cultivation. They are all variations of medium D which is prepared (in order of listing) as a 20-fold concentrated (pH 3) stock that is stored unautoclaved at 4°C. The micronutrient and $FeCl_3$ solutions are included in the D stock. The medium is prepared by diluting the stock, adding other desired chemicals, and adjusting the pH with 1–2 M NaOH. Agar (usually 15 g/l) is added after pH adjustment. Normal medium D is pH 8.2 before autoclaving. After cooling and complete clearing, the pH is 7.5–7.6, when no buffer is used. For growth of *Chloroflexus,* addition of a buffer is needed to maintain the pH from 8.0 to 8.2.

Medium D

Double-distilled water	1 liter
NTA (nitrilotriacetic acid)	0.1 g
Micronutrient solution	0.5 ml
$FeCl_3$ solution (0.29 g/l)	1.0 ml
$CaSO_4 \cdot 2H_2O$	0.06 g
$MgSO_4 \cdot 7H_2O$	0.10 g
NaCl	0.008 g
KNO_3	0.10 g
$NaNO_3$	0.70 g
Na_2HPO_4	0.11 g

Medium DG is medium D to which 0.8 g glycylglycine per liter has been added as buffer.

Medium DGN is medium D to which 0.8 g glycylglycine and 0.2 g NH_4Cl have been added per liter.

Medium ND (lacks combined N except as NTA) is medium D without KNO_3 and $NaNO_3$, but with 0.07 g Na_2HPO_4 and 0.036 g KH_2PO_4 per liter.

Micronutrient solution

Double-distilled water	1,000 ml
H_2SO_4 (concentrated)	0.5 ml
$MnSO_4 \cdot H_2O$	2.28 g
$ZnSO_4 \cdot 7H_2O$	0.50 g
H_3BO_3	0.50 g
$CuSO_4 \cdot 5H_2O$	0.025 g
$Na_2MoO_4 \cdot 2H_2O$	0.025 g
$CoCl_2 \cdot 6H_2O$	0.045 g

For *Chloroflexus aurantiacus* per liter of medium we use 0.8 g glycylglycine (pK′ 8.3) (DG medium) or Tricine (N-tris [hydroxymethyl] methylglycine) (pK′ 8.15) (DT medium) as buffer. For liquid axenic cultures in screw-capped tubes or flasks, we usually include 2.0 g yeast extract and 0.2 g NH_4Cl per liter. When flasks are continuously sparged with any gas mixture, the normal concentration of yeast extract is replaced by 1.0 g yeast extract and 2.0 g casein hydrolysate (e.g., vitamin-free Casamino Acids [Difco]) per liter. The base of D medium has also been modified to exclude nitrate (Castenholz, 1988a) since *Chloroflexus* requires reduced forms of nitrogen (Brock, 1978) and ND medium serves as an adequate substitute for D-based medium. In the German Col-

lection of Microorganisms (Claus and Schaab-Engels, 1977) *Chloroflexus* is usually grown photoheterotrophically in D-based medium with 0.5 g glycylglycine, 0.5 g yeast extract, and 0.4–0.5 g $Na_2S \cdot 9H_2O$ per liter. The latter (for eliminating O_2) is added from a separate sterile solution after autoclaving and cooling. In all cases, the *Chloroflexus* medium is adjusted to pH 8.2 with NaOH prior to autoclaving. For strictly photoheterotrophic growth, tubes or flasks should be filled to the top with preheated medium and inoculum before incubating.

C. aurantiacus, however, is tolerant of O_2 and will produce bacteriochlorophylls even in cotton-stoppered flasks of normal liquid medium if they are not shaken. Presumably, both phototrophic and chemotrophic growth modes are combined under these circumstances.

C. aurantiacus may also be grown as a chemoheterotroph. The same basic medium with yeast extract and Casamino Acids may be used with vigorous shaking or with a sparging system, using sterile filtered line air. Aerobic growth has been sustained in DG medium plus 0.2 g NH_4Cl, 0.5 g yeast extract, 1.0 g Casamino Acids, and 2.0 g sodium acetate per liter (Sirevåg and Castenholz, 1979).

Madigan (in Brock, 1978) and Madigan et al. (1974) have grown *Chloroflexus* with apparently good rates and yields using defined media. A number of sugars, organic acids, and amino acids will serve as carbon sources, but it was found that folic acid and thiamine were required, and the addition of inorganic carbon was needed to sustain normal rates and yields (Madigan et al., 1974; Brock, 1978). Na_2CO_3 or $NaHCO_3$ in water (e.g., 45 g/l) may be autoclaved and cooled, bubbled with pure CO_2 for 20–30 min to lower the pH, and then added to culture medium to make a final concentration of 0.5–2.0 g/l.

Photoautotrophic culturing of *Chloroflexus* is a very slow process, although growth of this type apparently occurs in some hot springs (Castenholz, 1973b; Giovannoni et al., 1987). In the laboratory, $Na_2S \cdot 9H_2O$ (0.5 g/l or about 2 mM final concentration) and inorganic carbon as $NaCO_3$ or $NaHCO_3$ (2.0 g/l) have been used (Madigan and Brock, 1977a; Brock 1978). Photoautotrophic growth has also been achieved with hydrogen as electron donor (Holo and Sirevåg, 1986).

"Green *Chloroflexus*" (GCF) from sulfidic hot springs will grow in the same medium as *C. aurantiacus* providing that anaerobic procedures for preparing and maintaining medium are followed. However, the best medium used for photoheterotrophic growth in liquid was a modification of medium DGN that also contained 0.2–0.5 g yeast extract and 1.0 g D-glucose per liter (Giovannoni et al., 1987). Hungate tubes or other sealed anaerobic vessels may be used.

Mesophilic *Chloroflexus* was cultured by Pivovarova and Gorlenko (1977) within soft agar medium (0.4%) with mineral salts, following the procedure of Pfennig (1965) and Pfennig and Lippert (1966) and supplemented with 0.4 g Na_2SO_4, 0.5 g tryptone, and 0.5 g yeast extract per liter. The pH was adjusted to 7.0.

Maintenance of Stocks

Thermophilic *C. aurantiacus* may be maintained in several ways. Photoheterotrophic cultures are grown at an optimum temperature (52–60°C) in screw-capped tubes that are filled nearly to the top before closing. A tungsten light source (5–10W/m^2 at the tube surface) is used in a table-top incubator with insulated glass door, set at 55°C. Although 45°C is suboptimal for growth, cultures may be maintained at a temperature this low. Transfers of the 55°C cultures should be made at least every 2 weeks, preferably more frequently. As cultures age, total lysis may occur.

Cultures are frequently maintained at 45°C on medium solidified with 1.5% agar in inverted, 20-mm-deep petri dishes. These cultures, although partially chemoheterotrophic, should be illuminated in the same way. Fluorescent lamps (coolwhite or warm white) will also work.

The most dependable method of maintaining *Chloroflexus* is in two-membered culture with a cyanobacterium as the source of organic nutrients. Such cultures can be maintained many weeks (liquid or agar) without transfer. *Synechococcus lividus* (strain OH-53-s), a unicellular cyanobacterium, grows well in DG liquid medium at 45–55°C. Neither the cyanobacterium nor *Chloroflexus* lyses in liquid D medium in cotton-plugged flasks, even when incubated for weeks beyond the regular transfer time of 2–3 weeks. Normally, however, agar plates are used to prevent contamination. These are incubated at 45°C and under coolwhite fluorescent lamps (30 W/m^2). The green-colored lawn of *Synechococcus* is interspersed with nearly macroscopic swirls of *Chloroflexus,* which also form a border or halo around the lawn of *Synechococcus* (Fig. 1). The growth pattern may be easily observed under a dissecting microscope. It is usually impossible to transfer the *Synechococcus* without carrying the *Chloroflexus.* However, *Chloroflexus* may be easily isolated from the dual culture. The normal yeast extract medium is inhibitory to *Synechococcus,* an obligate pho-

toautotroph. If liquid medium containing yeast extract is inoculated with a dual culture and incubated at 60–62°C, the *Synechococcus* will be killed in 4–5 days, leaving an axenic culture of *Chloroflexus*. Another simple method is to cut out with watchmaker's forceps an agar block containing *Chloroflexus* that has spread from the edge of a dual culture. This block can then be inoculated in *Chloroflexus* medium (liquid or agar).

Although O_2 is evolved by the *Synechococcus* in dual cultures, *Chloroflexus* pigment synthesis is not entirely inhibited. The identity of the organic compounds produced by *Synechococcus* in culture and used by *Chloroflexus* has not been determined.

"Green Chloroflexus" (GCF) may be maintained on agar or liquid in Hungate tubes or bottles or other vessels with rubber or soft tops so that they may be flushed or injected with anaerobic gas mixtures (e.g., 99% N_2, 1% CO_2). Plate cultures in "Gaspaks" are less reliable because of frequent failure of the seal. All cultures should be maintained at moderately thermophilic temperatures (45–55°C) in dim light (5–10 W/m^2).

Pivovarova and Gorlenko (1977) have grown mesophilic *Chloroflexus* in soft-agar (0.4%) shake cultures in cotton-plugged tubes that were kept at 3,000 lux (about 30 W/m^2) and 28°C. We have used a similar shake-culture method for maintaining thermophilic *Chloroflexus* but we used screw-capped tubes and 1.0% agar.

Preservation of Cultures

Chloroflexus aurantiacus does not survive air drying or freezing at −15° to −20°C in its own medium. Freeze-drying of *Chloroflexus* is used by the German Collection of Microoorganisms (Claus and Schaab-Engels, 1977). In the culture collection of the University of Oregon, approximately 15 strains of thermophilic *Chloroflexus* have been conserved in plastic cryo-ampules at the temperature of liquid N_2 (−196°C). In one test, all cultures were viable after 2 years of storage. About 0.3–0.5 ml of dense liquid cultures are pipetted directly into the ampules and frozen by direct submergence in liquid N_2. Dual-culture material is scraped off the agar surface with a loop and densely inoculated into an ampule containing sterile DG medium.

Identification

Chloroflexus aurantiacus

Chloroflexus aurantiacus has been isolated from alkaline hot springs in most regions of the world (Pierson and Castenholz, 1974a). It is a narrow (0.5–1.2 μm) (Fig. 3), orange to greenish filament of indefinite length, capable of a slow, gliding motility (0.01–0.04 μm/s). It stains Gram-negative although the chemistry of its wall is not that of a typical Gram-negative organism (see "Cellular Lipids and Cell Envelope"). Under anaerobic conditions it maintains a complement of bchl *a* with larger amounts of bchl c_s (Gloe and Risch, 1978). *C. aurantiacus* var. *mesophilus*, which is very similar in all characteristics except optimum temperature, was isolated from the hypolimnion of a freshwater lake but is no longer in culture (Pivovarova and Gorlenko, 1977).

The bchl *c* of *C. aurantiacus* is contained in chlorosomes that line the cytoplasmic membrane. The bulk of the light-harvesting bchl *a* (B800 and B865 complexes) is located in the cytoplasmic membrane along with the reaction center (Schmidt, 1980; Bruce et al., 1982). A small fraction of the bchl *a* absorbing at 792 nm is associated with the baseplate of the chlorosome at the site of attachment to the cell membrane (Schmidt, 1980; Sprague et al., 1981a). Invaginations of the cytoplasmic membrane occur as mesosomes of rather typical appearance (Pierson and Castenholz, 1974a), but a more elaborate type of membrane of unknown function is sometimes produced (Pivovarova and Gorlenko, 1977). β-Carotene, γ-carotene, and hydroxy-γ-carotene-glucoside comprise 80–95% of the carotenoids for anaerobically grown cells (Halfen et al., 1972; Schmidt et al., 1980). Photosynthesis is anoxygenic, and organic compounds provide the best carbon source for rapid growth. Most strains (but not all) also grow aerobically. Under aerobic conditions, the synthesis of bacteriochlorophylls ceases, but echinenone and myxobactone increase and constitute about 75% of the carotenoids (K. Schmidt, personal communication).

Chloroflexus may be tentatively identified in collected material as fine trichomes (0.5–1.2 μm wide) of indefinite length and essentially colorless as individuals, but generally orange in a mass under a compound microscope. With phase contrast microscopy, thin sheaths may or may not be apparent in *Chloroflexus*. Some members of the flexibacteria (e.g., *Herpetosiphon, Flexibacter, Flexithrix*) are similar in appearance, exhibit gliding motility, and contain somewhat similar carotenoid pigments (see Chapter 199 and Kleinig and Reichenbach, 1977). Neither *Chloroflexus* nor flexibacteria exhibit the characteristic visible red fluorescence of plant-type chlorophyll. Therefore, filamentous cyanobacteria of similar dimensions and behavior may be distinguished under a flu-

orescence microscope. Both *Chloroflexus* and cyanobacteria exhibit infrared fluorescence (Pierson and Howard, 1972).

Large quantities of *Chloroflexus,* collected nearly pure or mixed with cyanobacteria, may be broken by sonication in buffered medium or hot-spring water, cleared by filtration or centrifugation, and tentatively identified by the in vivo absorption maxima at about 740 nm (bchl *c*), 802 nm, and 865 nm (bchl *a*) (Castenholz, 1973b; Pierson and Castenholz, 1974a). Relatively monospecific mats of *Chloroflexus* may be identified as the orange to flesh-colored gelatinous layers in hot springs under a layer of cyanobacteria or occasionally as independent mats in sulfide springs (see Castenholz, 1973b). However, a large number of cyanobacteria in hot springs form extensive mats, yellow to brilliant orange in color, particularly at temperatures below 45–50°C. These may easily be mistaken for *Chloroflexus,* which may be present as only a minor component.

The marine forms of *Chloroflexus* can be initially identified as dark green or olive green filaments often forming a recognizable layer in mats or sediments. Some of the filaments we have observed have been larger than the thermophilic strains, commonly being 1.5 to 2.0 μm in diameter. We routinely identify putative *Chloroflexus*-like filaments on the basis of their olive green color en masse, lack of red fluorescence, and presence of infrared fluorescence. Identification can be substantiated with in vivo absorption spectroscopy. The in vivo absorption maximum for bchl *c* in most marine strains is from 747–755 nm rather than at 740 nm as in the thermophilic strains. Identification can be confirmed with transmission electron microscopy revealing the presence of filaments with chlorosomes lining the cell membrane.

Chloronema and *"Oscillochloris"*

Gorlenko and Pivovarova (1977) suggested that as many as 28 species of filamentous organisms, formerly described as species of *Oscillatoria* or related genera of cyanobacteria, are likely to be anoxygenic phototrophs of the "Oscillochloris" or *Chloronema* type. Several filamentous types have been described from collections in the past as pale, nearly colorless, or yellow-green, rather than blue-green to reddish in color. "Oscillochloris chrysea," for example, was equated to *Oscillatoria coerulescens* described by Gicklhorn (1921).

Samples of "Oscillochloris" and *Chloronema* could be tentatively identified on the basis of habitat, color, and microscopy, but electron microscopy and biochemical analysis would be necessary for more complete identification.

"Oscillochloris" is a larger, rapidly gliding *Oscillatoria*-like trichome (about 5μm wide), which was proposed as a separate genus from *Chloroflexus* on the basis of larger size and lack of sheath. It contains gas vesicles and has an elaborate system of centripetally invaginated partial septa that are lined with chlorosomes (Gorlenko and Pivovarova, 1977). It is yellow-green in color and apparently contains bchl *c* (rather than bchl *d*). Its physiology is thought to be like that of *Chloroflexus.* Two species have been described, "O. chrysea" and "O. trichoides" (Gorlenko, 1989a). "O. chrysea" and "O. trichoides" are distinguished from each other on the basis of filament diameter, cells of "O. chrysea" being wider (4.5–5.5 μm) than those of "O. trichoides" (1.0–1.4 μm). The trichomes of both may contain gas vesicles and are yellow-green in color. Transmission electron microscopy confirms identification of this genus by revealing the characteristic location of chlorosomes along complete and incomplete septa and their absence from the cell membrane parallel to the longitudinal axis of the filament (Gorlenko, 1988).

Chloronema (about 2 μm wide) is similar structurally and possibly physiologically to *Chloroflexus* (Gorlenko, 1989b), but data from cultures are lacking. Its proposed separate generic status is based on the possession of gas vesicles and sheath. The material examined apparently contained bchl *d* rather than bchl *c* (Dubinina and Gorlenko, 1975). The two species described *(Chloronema giganteum* and *C. spiroideum)* were yellow-green in color. Samples from anaerobic zones of lakes with planktonic populations that are predominantly composed of gas vesicle-containing filaments with large amounts of extractable bchl *c, d,* or *e* could be *Chloronema* (Dubinina and Gorlenko, 1975). *Chloronema* is identified by habitat, presence of gas vesicles, and presence of bchl *d* instead of *c* (Gorlenko, 1989b). Strains studied so far were found at lower temperatures (3–15°C) than other species of the Chloroflexaceae. All of these characteristics are of dubious value in distinguishing *Chloronema* as a separate genus from *Chloroflexus,* and studies on pure cultures will be necessary, along with nucleic acid sequence data, to ultimately determine the proper phylogenetic distance of these two genera.

Heliothrix oregonensis

Heliothrix oregonensis is a filamentous gliding anoxygenic phototroph containing bchl *a* as the only chlorophyll. The cells are about 1.5 μm wide and 10 μm long (Fig. 3) and the gliding

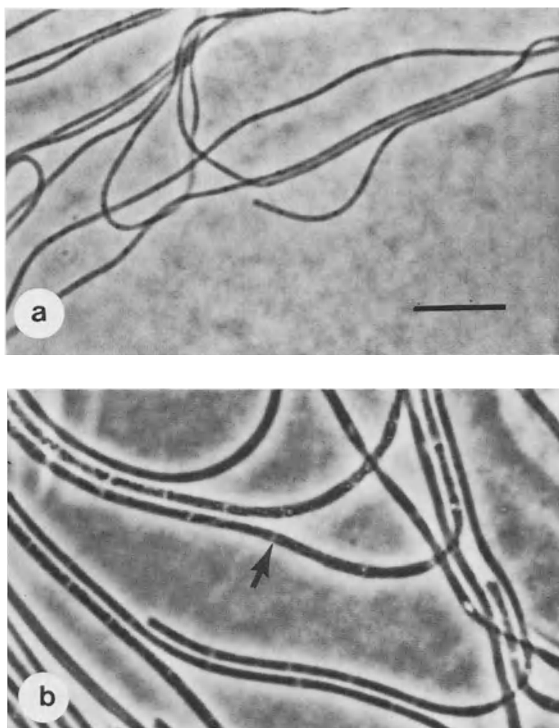

Fig. 3. Phase contrast photomicrographs of wet mounts of *Chloroflexus aurantiacus* (a) and *Heliothrix oregonensis* (b). The narrower filaments are *C. aurantiacus* (strain J-10-fl). The wider *H. oregonensis* filaments have granules (probably PHB) and septa (indicated by the arrow). Septa are also present in *C. aurantiacus* but are not readily visible with light microscopy. (Bar = 10 μm.)

motility is more rapid (0.1–0.4 μm/s) than in *Chloroflexus*. It contains less bchl *a*, and apparently lacks chlorosomes and bchl *c*, *d*, or *e* entirely (Pierson, et al., 1984). Nevertheless, on the basis of morphology, behavior, and types of carotenoid pigments, it appears more closely related to *Chloroflexus* than to other phototrophs. The 5S rRNA analysis confirms this association. First described as "F-1" (Pierson and Castenholz, 1971; Pierson, 1973), *Heliothrix oregonensis* is identified on the basis of the presence of bchl *a* as its only chlorophyll and the absence of chlorosomes and intracytoplasmic membranes.

With the recent discovery of other filamentous bacteria of the same diameter containing bchl *a* as the only chlorophyll and elaborate intracytoplasmic membranes, identification has become more difficult. The identification of these different organisms relies on transmission electron microscopy (TEM) to reveal the presence or absence of intracytoplasmic membranes and in vivo absorption spectra to identify the position of the bchl *a*-protein absorption maxima—factors that could be influenced by conditions of the habitat. Growth in pure or coculture will be essential to determine if the other bchl *a*-containing filaments are really variants of *Heliothrix* or not.

Physiological Properties

Chloroflexus aurantiacus is the only species that has been studied physiologically in pure culture. Thus our discussion of physiological properties will be confined to this species.

Chloroflexus aurantiacus grows in nature primarily as a photoheterotroph. Its best growth rates and yields occur when grown anaerobically in the light on complex organic media. Many strains (but not all) grow well chemoheterotrophically in the presence of oxygen. Some strains (but not all) are capable of photoautotrophic growth. Even those strains capable of photoautotrophic metabolism (such as the GCF strains) grow best as photoheterotrophs. Typically, *Chloroflexus aurantiacus* grows in hot spring mats that are quite oxic during daylight and become anoxic at low-light levels and in darkness. A tolerance for and an ability to use oxygen is definitely advantageous under these conditions. Strains of *Chloroflexus* growing in sulfidic hot springs in the absence of cyanobacteria may never be naturally exposed to oxygen. GCF strains isolated from such conditions could tolerate exposures to oxygen but could not grow aerobically in the dark (Giovannoni, et al., 1987). Light was required for growth.

Autotrophy

Sulfide-dependent photoautotrophy is difficult to demonstrate in the laboratory, yet some strains are most likely using this mode of metabolism in nature as their sole source of energy and carbon (Giovannoni, et al., 1987; Jørgensen and Nelson, 1988). Since there is essentially no competition for light, sulfide, or space in these habitats, the slow growth afforded by photoautotrophy is probably sufficient for dominance of *Chloroflexus*.

Despite the evidence for autotrophy in the field, growth of cultures autotrophically in the laboratory still poses difficulties. One thermophilic strain (OK-70-fl) was successfully grown autotrophically using sulfide while none of the other strains that were tested showed growth (Madigan, et al., 1974). Autotrophic growth was not as good as photoheterotrophic growth. Madigan and Brock (1977a) showed sulfide-dependent CO_2 fixation in autotrophically grown cultures of strain OK-70-fl. Heterotrophic cultures were also able to fix CO_2. Early attempts to de-

termine the pathway of CO_2 fixation produced confusing results (Madigan and Brock, 1977a; Sireväg and Castenholz, 1979). More recently, it has become evident that the confusion was due to the fact that *C. aurantiacus* has a previously undescribed pathway for CO_2 fixation using neither the reductive tricarboxylic acid cycle nor the Calvin cycle (Holo and Sirevåg, 1986). Advances in the study of the autotrophic pathway for CO_2 assimilation were made after developing a method for growing strain OK-70-fl autotrophically with hydrogen (Holo and Sirevåg, 1986). Although photoheterotrophically grown *C. aurantiacus* has doubling times as short as 3–4 h, the lowest doubling time obtained photoautotrophically was 26 h. Using cells grown in this way, Holo and Grace (1987, 1988) found that acetyl-CoA was an important intermediate in autotrophic metabolism. Acetyl-CoA was carboxylated to pyruvate, which could then be reduced and phosphorylated to triose phosphates. Further evidence suggests that 3-hydroxypropionate is an important intermediate in CO_2 fixation and may be involved in a cyclic pathway to regenerate acetyl-CoA (Holo, 1989). More work is needed to completely understand this unique pathway for autotrophy and to discover the basis for the generally poor autotrophic growth obtained in the laboratory.

Heterotrophy

Numerous substrates can support photoheterotrophic growth. In addition to complex substrates such as yeast extract and Casamino Acids, other organic substrates that supported good growth of strain J-10-fl included glycerol, acetate, glucose, pyruvate, glutamate, and lactate (Pierson and Castenholz, 1974a). Aspartate supported good growth of OK-70-fl (Madigan et al., 1974). Other substrates that supported photoheterotrophy, but with significantly lower yields in most strains tested, included glycylglycine, succinate, malate, butyrate, citrate, ribose, galactose, ethanol, and mannitol (Madigan et al., 1974). Growth could be obtained aerobically in the dark in some strains on each of the substrates that supported photoheterotrophy except for glycylglycine (Madigan et al., 1974). Similar results were obtained for three other strains of *C. aurantiacus* (Krasil'nikova et al., 1986). The use of organic substrates was basically the same in the light under anoxic conditions and in the dark under oxic conditions, with acetate, propionate, pyruvate, butyrate, and malate supporting the best growth. Of the sugars tested, glucose, maltose, sucrose, and galactose supported the best growth. Although glycerol supported growth, none of the other 14 alcohols tested, including ethanol and mannitol, was used by any of these strains of *C. aurantiacus* in either the light or the dark (Krasil'nikova et al., 1986). These authors also observed that substantial amounts of poly-β-hydroxybutyrate (PHB) and polysaccharides accumulated in cells growing both phototrophically and chemotrophically. More PHB accumulated in cells grown phototrophically and in cells grown with acetate.

The tricarboxylic acid (TCA) cycle appears to operate under aerobic conditions to supply reductant for respiration and under anaerobic conditions to support the reductive assimilation of organic compounds for photoheterotrophy (Sirevåg and Castenholz, 1979). Activities of the enzymes required for the functioning of a complete TCA cycle were found but the activity of α-keto-glutarate dehydrogenase was low (Krasil'nikova et al., 1986). The activity of citrate synthase increased in cultures grown on acetate. More detailed analyses of the size and activity of the citrate synthase done in crude extracts and with partially purified preparations has revealed that the enzyme from *C. aurantiacus* belongs to the small size class of the enzyme and was not inhibited by NADH or α-keto-glutarate but was inhibited by ATP (Kelly, 1988). The size of the enzyme puts it in the class of those found in Gram-positive bacteria rather than with those found in Gram-negative bacteria. All strains of *C. aurantiacus* also had the two key enzymes of the glyoxylate pathway, isocitrate lyase and malate synthetase, which increased in activity in cells grown on acetate (Løken and Sirevåg, 1982; Krasil'nikova et al., 1986).

C. aurantiacus metabolizes carbohydrates via the Embden-Meyerhof pathway (Krasil'nikova et al., 1986; Krasil'nikova and Kondrat'eva, 1987). When grown on glucose, higher activities of phosphofructokinase and fructose-diphosphate aldolase were detected than when grown on acetate. Enzymes of the pentose monophosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) were also detected (Krasil'nikova et al., 1986). Slow growth can be obtained anaerobically in the dark by fermentation of carbohydrates or pyruvate (Krasil'nikova and Kondrat'eva, 1987). Fermentation products of different strains growing on glucose include acetate, pyruvate, lactate, malate, ethanol, and occasionally formate (Krasil'nikova and Kondrat'eva, 1987). The activities of all enzymes tested were maximal between 45 and 70°C (Krasil'nikova et al., 1986).

Nitrogen Metabolism

The nitrogen metabolism of *C. aurantiacus* has been studied by Heda and Madigan (1986). Ni-

trate did not support growth of the strains tested. Several amino acids (alanine, aspartate, glutamate, glutamine, glycine, and serine) supported good growth of all strains. Cysteine supported good growth of most strains. Tyrosine and valine supported growth of all strains but with lower growth yields. A few other amino acids supported limited growth of some strains. Proline, adenine, and urea did not serve as nitrogen sources for any of the strains tested. All strains grew well using NH_4^+ as sole nitrogen source (Heda and Madigan, 1986).

Nitrogen fixation (diazotrophy) was not detected in any of the strains of *C. aurantiacus.* In addition to not growing with N_2 as sole nitrogen source, none of the strains tested showed nitrogenase activity using the acetylene reduction technique (Heda and Madigan, 1986).

The pathways of nitrogen metabolism in *C. aurantiacus* are partially understood. Ammonia assimilation apparently proceeds differently in *C. aurantiacus* than in other phototrophic bacteria. The only enzyme present for ammonia assimilation is a glutamine synthetase, which is not repressed when cells are grown in high-ammonia culture media (Kaulen and Klemme, 1983). No evidence was found for covalent modification of this enzyme by adenylylation/deadenylylation. The possibility that covalent modification could occur, however, has not been completely eliminated (Klemme, 1989). The absence of glutamate synthase (GOGAT) activity may be due to problems of preparation (Klemme, 1989), and details of this aspect of nitrogen metabolism remain to be clarified.

C. aurantiacus grows naturally in microbial mats, usually in close association with cyanobacteria, and readily uses a wide variety of amino acids both as carbon and nitrogen sources, although it can also grow in minimal medium. Glutamate, alanine, and isoleucine are the main constituents of the intracellular amino acid pool in cells grown photosynthetically in minimal medium (Klemme et al., 1988). Therefore, the metabolic pathways for both the synthesis of and degradation of amino acids are of interest. Two different enzymes with L-threonine (L-serine) dehydratase activity were found in *C. aurantiacus* (Laakmann-Ditges and Klemme, 1986). One was sensitive to inhibition by isoleucine and could be biosynthetic for isoleucine since the other key enzymes for its biosynthesis (asparto-kinase, homoserine dehydrogenase, and acetohydroxy acid synthase) were also present in cells grown on minimal medium (Laakmann-Ditges and Klemme, 1988). However, the intracellular pool sizes for isoleucine, threonine, and valine in *C. aurantiacus* suggest that the enzyme would be inactive in vivo (Laakmann-Ditges and Klemme, 1988). These authors also found that the other L-threonine (L-serine) dehydratase is insensitive to inhibition by isoleucine and is regulated at the gene level by glucose-mediated catabolite repression. The level of this enzyme was greatly increased when cells were grown in the presence of L-threonine, L-serine, or L-isoleucine and L-valine and L-leucine (Laakmann-Ditges and Klemme, 1988). This enzyme may therefore be classified as biodegradative and may be important in the growth of *C. aurantiacus* on amino acids.

Sulfur Metabolism

C. aurantiacus strains can use sulfate as a source of sulfur for biosynthesis, and during photoheterotrophic growth thiosulfate can also serve as a source of sulfur (Kondrat'eva and Krasil'nikova, 1988). Although activities were detected for thiosulfate reductase, rhodanese, thiosulfate oxidase, and sulfite oxidase, the use of thiosulfate by cultures appeared to be very limited. Although thiosulfate may serve as an electron acceptor with the accompanying production of hydrogen sulfide, it does not appear to function as an electron donor for photosynthesis (Kondrat'eva and Krasil'nikova, 1988). A variety of compounds (cysteine, glutathione, methionine, sulfide, sulfate) can function as sources of sulfur during photoheterotrophic growth (Krasil'nikova, 1987). The best growth, however, was obtained with sulfate, and high levels of activity were found for ATP sulfurylase, which exhibited optimal activity at 60–70°C (Krasil'nikova, 1987). Madigan and Brock (1975) and Giovannoni et al. (1987) showed that sulfide was oxidized to elemental sulfur that accumulated outside the cells during both photoheterotrophic and photoautotrophic growth.

C. aurantiacus has a membrane-bound Ni^{2+}-stimulated hydrogenase that is repressed by sulfide (Drutschmann and Klemme, 1985), and autotrophic growth at the expense of hydrogen has been obtained (Holo and Sirevåg, 1986).

Cellular Lipids and Cell Envelope

The biochemical composition of the cell envelope and the composition of cellular lipids in *C. aurantiacus* exhibit some unique properties. The total cellular lipids include the bchl and carotenoid pigments, the polar lipids, and a collection of unique wax esters (Beyer et al., 1983). The polar lipids include phosphatidylglycerol, phosphatidylinositol, monogalactosyl diglyceride, diglycosyl diglyceride, and sulfoquinovosyldiglyceride (Kenyon and Gray, 1974; Knudsen et al., 1982), although the presence of the

latter lipid is subject to question (Imhoff, 1988). The fatty acids are predominantly straight-chain saturated and monounsaturated with C_{16}, C_{17}, and C_{18-20} chain lengths predominating with no detectable hydroxylated or cyclopropane-substituted chains (Knudsen et al., 1982). The major lipid fraction was composed of wax esters (C_{28}–C_{38}) containing fatty acids (C_{12}–C_{19}) and alcohols (C_{16}–C_{19}) (Knudsen et al., 1982), which could be isolated in a particulate fraction called wax oleosomes (Beyer et al., 1983). The presence of the wax esters (rare in bacteria) and the predominance of phosphatidylinositol make the lipid composition of *Chloroflexus* unique among phototrophs. Although the presence of highly saturated and somewhat longer-chain fatty acids is consistent with the thermophilic nature of the organism, it is not clear what the function or role of the wax esters is in these bacteria. It will be most interesting to determine the lipid composition of the mesophilic marine, freshwater, and hypersaline strains.

The cell envelope of *Chloroflexus* apparently lacks the lipopolysaccharide (LPS) characteristic of Gram-negative bacteria. No hydroxylated fatty acids and no saccharides typical of outer-membrane LPS were detected in *Chloroflexus* (Knudsen et al., 1982). The absence of LPS and lipoprotein in the envelope of *C. aurantiacus* and the binding of complex polysaccharides to N-acetyl-muramic acid-6-phosphate in the peptidoglycan via phosphodiester bridges is very unlike the structural organization found in typical Gram-negative bacterial envelopes and more strongly resembles that found in Gram-positive bacterial walls (Jürgens et al., 1987). The abundant complex polysaccharide bound to the rigid peptidoglycan layer contains sugar O-methyl ethers of hexoses and rhamnose, mannose, glucose, galactose, xylose, and arabinose (Jürgens et al., 1987). Although the peptidoglycan of *Chloroflexus* contains *N*-acetyl-glucosamine, *N*-acetyl-muramic acid (about 15% of which is phosphorylated), L-alanine, D-alanine, and D-glutamic acid, the presence of L-ornithine instead of diaminopimelic acid in the peptidoglycan of *Chloroflexus* also distinguishes it from the other Gram-negative phototrophs and substantiates the similarity in wall composition with that of Gram-positive bacteria (Jürgens et al., 1987). The unusual biochemical composition of the envelope of *C. aurantiacus* is particularly interesting since the cells stain Gram-negative, and ultrastructurally the envelope appears to be multilayered. A well-defined outer membrane structure typical of Gram-negative cell envelopes, however, is lacking (Pierson and Castenholz, 1974a; Staehelin et al., 1978). It is of interest to note that cells of "Oscillochloris chrysea" stain Gram-positive and have a very thick peptidoglycan layer with no outer membrane (Gorlenko, 1988, 1989a).

Pigment Synthesis and the Development of the Photosynthetic Apparatus

Bacteriochlorophyll synthesis (both bchl *a* and *c*) in *C. aurantiacus* is via the glutamate-C_5-pathway as in the cyanobacteria and the green and purple sulfur bacteria, rather than via the ALA synthase pathway as in some species of nonsulfur purple bacteria (Kern and Klemme, 1989; Avissar et al., 1989). Little is known, however, about the regulation of pigment synthesis at the molecular level in *Chloroflexus*.

The phototrophic activity of natural populations is dependent on the prevailing light conditions in situ, and populations adapted to low-light intensities may show considerable photoinhibition when exposed to higher light intensities (Madigan and Brock, 1977b). In culture, bacteriochlorophyll (bchl) synthesis decreases in the presence of high-light intensity or oxygen (Pierson and Castenholz, 1974b), and the effects of light and oxygen on the structural development as well as on the pigment composition of the photosynthetic apparatus have been well documented (Sprague, 1981a, 1981b; Schmidt et al., 1980; Feick et al., 1982). The change in ratio of bchl *c* to *a* in cells grown at different light intensities and adapted to reductions in oxygen levels suggested independent regulation of the two pigments (Pierson and Castenholz, 1974b; Sprague et al., 1981b). The increase in bchl content seen in cells adapting to decreased light intensities or oxygen tensions was accompanied by an increase in the number of chlorosomes per cell. The increased ratio of bchl *c* to bchl *a* was accompanied by a general thickening of the chlorosomes (Sprague et al., 1981a).

The chlorosomes contain the bchl *c*, a small fraction of bchl *a* (absorbing at 790nm), and most of the carotenoid pigments of the cells (Schmidt, 1980; Sprague et al., 1981a). Some of the γ- and β-carotene were in the cytoplasmic membrane, but most were in the chlorosome, while the hydroxy-γ-carotene-glucoside was the predominant carotenoid in the membrane and was only a small fraction of the total carotenoid in the chlorosomes (Schmidt, 1980). This glucoside of hydroxy-γ-carotene increased relative to the other carotenoids in cells grown at higher light intensities (Schmidt et al., 1980) and may have a role in protecting the membrane-bound photochemical systems from the damaging effects of high-light intensities.

The effect of light intensity on pigment synthesis may be via an indirect effect on growth

rate. By growing *C. aurantiacus* in a chemostat and controlling the growth rate by regulating the dilution factor while keeping the light intensity constant, Oelze and Fuller (1987) were able to show that the specific contents of bchl *a* and *c* increased at lower growth rates. Since light intensity has a direct effect on growth rate, it is difficult to distinguish the relationships among these parameters without growth in a chemostat. Cell protein levels, as well as specific bchl *a* content, increased linearly with decreasing growth rate, while the specific bchl *c* content increased exponentially with decreasing growth rate.

The nature of the regulation remains undetermined. The relationship of the structural features of the photosynthetic apparatus to the quantities of pigments present, however, has become clearer. By quantifying the number of chlorosomes, their dimensions, and the amount of baseplate-covered membrane, Golecki and Oelze (1987) were able to show that the number of chlorosomes and the percentage of membrane surface area covered by baseplates correlated directly with the cellular content of bchl *a*. Under conditions in which the specific bchl *a* content of cells increased linearly, the bchl *c* content increased exponentially. The large increase in bchl *c* was accomodated by increasing the volume of the chlorosomes, the density of the bchl *c* molecules within the chlorosomes, and the number of chlorosomes (Golecki and Oelze, 1987).

Light Harvesting and Primary Photochemistry

A brief discussion of the light-harvesting and photochemical activities of *C. aurantiacus* is included here because of their relevance to understanding the distant phylogenetic relationship of this organism to all other phototrophs. The photosynthetic apparatus of *Chloroflexus* has very interesting similarities to components within several diverse groups of phototrophs.

The photochemical reaction center of *C. aurantiacus* is very similar to the reaction centers of the purple nonsulfur bacteria. There are also some significant differences. The *Chloroflexus* reaction center is the smallest one known, being composed of only 2 polypeptide subunits instead of the 3 or 4 found in other bacteria (Pierson et al., 1983; Shiozawa et al., 1987). The pigment composition also differs from that of purple bacterial reaction centers. *Chloroflexus* has a photoreactive special pair or dimer of bchl *a* molecules as in the purple bacteria, but it has only one additional bchl *a* molecule and 3 bacteriopheophytin *a* molecules, rather than 2 additional bchl *a* and 2 bacteriopheophytin molecules found in the purple bacteria (Pierson and Thornber, 1983; Blankenship et al., 1984). Furthermore, the *Chloroflexus* reaction center lacks carotenoid pigments (Pierson and Thornber, 1983) and menaquinone is the electron acceptor for photochemistry rather than ubiquinone (Vasmel and Amesz, 1983). Functionally, the reaction center of *Chloroflexus* is very similar to that of the purple bacteria, and the intermediate electron acceptor is bph*a* in both (Blankenship et al., 1983, 1984; Kirmaier et al., 1983). The arrangement of the chromophores appears to be similar to that in purple bacteria, based on spectroscopic measurements (Vasmel et al., 1986). The two subunits of the *Chloroflexus* reaction center are probably structurally similar to those of the purple bacteria since the L subunits exhibit 40% sequence homology and the M subunits 42% (Ovchinnikov et al., 1988a, 1988b; Shiozawa et al., 1989). Within the pigment-binding regions of the two subunits, the homology is higher (59–75%) (Shiozawa et al., 1989). Thus, although *C. aurantiacus* is only distantly related to the purple bacteria, the reaction centers have strong structural and functional similarities. The lack of the H subunit in the *Chloroflexus* reaction center, slight differences in pigment composition, and intrinsic thermal stability have little to do with the apparently highly conserved structural and functional characteristics of the sites directly involved in the primary photochemical act.

The molecular organization of the light-harvesting system is particularly interesting in *Chloroflexus* since it has a complex antenna involving accessory bchl *c* housed in chlorosomes adjacent to the cell membrane. The chlorosomes also appear to contain a small component of bchl *a* (Schmidt, 1980; Sprague et al., 1981a; Feick et al., 1982) that appears to be involved in transferring excitation energy to additional antenna components in the cell membrance (Van Dorssen and Amesz, 1988). The low efficiency of transfer mediated by this component (Brune et al., 1987) and the recently developed preparative procedures that produce chlorosomes lacking this component (Griebenow and Holzwarth, 1989) reveal that the nature of the baseplate bchl *a* complex remains uncertain. Although the size, composition, and ultrastructure of the chlorosomes of *Chloroflexus* differ somewhat from those of the green sulfur bacteria, both contain small-molecular-weight bchl *c* binding proteins. The bchl *c* binding polypeptides among different species of green sulfur bacteria have a high degree of homology and an overall homology of about 30% with the comparable polypeptide from *Chlo-*

roflexus (Wechsler et al., 1985b; Wagner-Huber et al., 1988). The green sulfur bacteria, however, do not have an intramembrane or cytoplasmic membrane antenna system similar to that found in *Chloroflexus* and the purple bacteria. The bchl *a* antenna in the cell membrane of *Chloroflexus* is very similar to that in the intracytoplasmic membranes of purple bacteria. In addition to harvesting light energy directly, however, it also funnels excitation energy from the chlorosomes to the reaction center (Brune et al., 1987; Wittmershaus et al., 1988; Van Dorssen and Amesz, 1988). The absorption properties of this antenna, with maxima near 805 and 865 nm, are similar to those of many purple bacteria, and the small-molecular-weight polypeptides that bind the bchl *a* molecules show some homology to the comparable polypeptides in purple bacteria (27–40%) (Wechsler et al., 1985a, 1987).

Electron Transport Systems

The electron transport chains of both phototrophically and chemotrophically grown cells of *C. aurantiacus* have been characterized (Pierson, 1985; Wynn et al., 1987; Knaff et al., 1988; Zannoni, 1986; Zannoni and Venturoli, 1988). Chemotrophically grown cells contain at least three *c*-type cytochromes and at least three *b*-type cytochromes (Wynn et al., 1987; Zannoni, 1986). These cells lack the high-potential cytochrome c_{-554} that functions as the electron donor to the reaction center in phototrophically grown cells (Wynn et al., 1987). The identity of the terminal oxidase for aerobic respiration remains clouded in controversy. Data from two laboratories support the presence of cytochrome aa_3 (Pierson, 1985; Zannoni, 1986), while others have been unable to detect this cytochrome and suggest that a CO-binding, protoheme-containing cytochrome functions in this capacity (Wynn et al., 1987; Knaff et al., 1988). It seems likely that the terminal oxidase may vary depending on the levels of oxygen in the medium during growth of the cells. While there is some evidence for the presence of a putative cytochrome bc_1 in the membranes of chemotrophically grown cells (Zannoni, 1986), complete confirmation of the presence of cytochrome c_1 and a Rieske center is lacking (Wynn et al., 1987). It appears that the respiratory chains present in cells grown under either oxygen-saturated conditions or with lower levels of oxygen are branched chains (Zannoni, 1986; Zannoni and Fuller, 1988), and identification of all terminal oxidases as well as the complete sequence of carriers in each chain remains to be elucidated.

Phototrophically grown cells also contain multiple *b* and *c* type cytochromes, including the electron donor to the reaction center, cytochrome c_{554} (Wynn et al., 1987; Zannoni and Ingledew, 1985). The cells lack a soluble cytochrome c_2, which is clearly not essential for either photosynthesis or respiration in *Chloroflexus* and in some purple nonsulfur bacteria (Zannoni, 1987). There is strong evidence for the presence of a putative cytochrome bc_1 complex in the membranes of phototrophically grown cells (Zannoni and Ingledew, 1985), as well as for the presence of cytochrome *c*-CO binding activity (Pierson, 1985; Wynn et al., 1987; Zannoni and Ingledew, 1985).

Menaquinone is the only quinone present in both chemotrophic and phototrophic cells of *Chloroflexus* (Hale et al., 1983). The shift from aerobic respiration to photosynthesis requires synthesis of both of the bchls and the cytochrome c_{554} required for photochemistry. Lowered oxygen tension appears to be the only signal needed to induce these syntheses, with light having no role in the process (Foster et al., 1986).

Thermophily

The thermophilic aspects of the physiology of *C. aurantiacus* have been studied in several systems. Soluble enzyme activities determined as a function of temperature were found to be maximal between 45 and 70°C, consistent with the optimum growth temperature in this range (Laakmann-Ditges and Klemme, 1988; Krasil'nikova, 1987; Krasil'nikova et al., 1986). Although *sym*-homospermidine is present in *C. aurantiacus* as in many other thermophiles, its presence in several mesophiles makes it difficult to conclude that this polyamine necessarily has any role in contributing to the thermal stability of components in cells of *Chloroflexus* (Norgaard et al., 1983). The isolated photochemical reaction center of *C. aurantiacus* exhibits thermal stability (Pierson et al., 1983). The functioning of membrane-bound proteins at elevated temperatures, however, is dependent on the properties of the membrane as well as the temperature characteristics of the proteins themselves. Oelze and Fuller (1983) determined the temperature characteristics of growth and the membrane-bound enzyme activities of NADH oxidase, succinate 2,6-dichlorophenol-indophenol reductase, ATPase, and light-induced proton extrusion. Enzymatic activities were maximal at 65–70°C, and a major lipid phase transition occurred near 40°C in cells grown at 50°C. Membrane phenomena were significantly altered at temperatures above

60°C, which may reflect denaturation of bulk membrane proteins (Oelze and Fuller, 1983). Although individual enzyme activities were high in this temperature range, proton extrusion was not thermostable above 60°C and growth rates did not increase in this range. Fluorescence from the membrane-bound bchl *a* antenna complexes increased when the temperature decreased from 50 to about 40°C which corresponded to the region of probable lipid-phase transition (Wittmershaus et al., 1988). Fluorescence from the nonmembrane-bound pigments in the chlorosome did not increase at this temperature and it appeared that energy transfer from bchl *c* to bchl *a* within the chlorosome was not temperature-dependent. Within the membrane-bound parts of the photosynthetic apparatus, however, lipid-phase transitions near 40°C may alter the mobility of quinones in the less fluid membrane. The altered mobility may decrease the rate of photosynthesis and produce the observed enhanced fluorescence of the membrane-bound antenna complexes (Wittmershaus et al., 1988).

Physiological Properties of Other Filamentous Phototrophs

Knowledge of the physiological properties of "Oscillochloris," *Chloronema,* and *Heliothrix* is based on studies conducted in the field or on impure collections, enrichments, or cocultures. Since these observations must be subjected to verification with pure cultures, we have not reported them in detail here. Summaries of the important physiological properties of "Oscillochloris" and *Chloronema* have been compiled (Gorlenko, 1988, 1989a, 1989b). The physiology of *Heliothrix* has been studied in situ (Pierson et al., 1984; Castenholz and Pierson, 1989).

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The Genus *Thermomicrobium*

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Thermomicrobium roseum (ATCC 27502) is the sole representative of a phylogenetically distinct branch of the eubacteria. It is an obligately thermophilic organism originally isolated from the effluent of a hot spring in Yellowstone National Park, WY (Jackson et al., 1973). *T. roseum* has been grouped with the green nonsulfur (GNS) bacteria based on ribosomal RNA sequence comparisons (Oyaizu et al., 1987). *Herpetosiphon* and *Chloroflexus*, the other representatives in this branch (Fig. 1), are markedly different phenotypically from *T. roseum*. Although the GNS bacteria share some common ribosomal characters they are the progeny of a deep phylogenetic divergence (Gibson et al., 1985); *Herpetosiphon* is a mesophile and more rapidly evolving than either *Chloroflexus* or *Thermomicrobium*. The deepest branching in eubacterial evolution is predominantly represented by thermophiles (Fig. 1), and these organisms evolved more slowly than the mesophiles. They have consequently retained more of their ancestral character (Achenbach-Richter et al., 1987).

Isolation and Enrichment

The primary enrichment medium for *T. roseum* was composed of 0.1% tryptone and 0.1% yeast extract (TYE) in Allen's salts (see recipe below) (Allen, 1959). (The final pH was adjusted to 7.8.) Enrichment media were inoculated with samples of bacterial mat or water from various springs in Yellowstone National Park and were incubated at 70, 75, 80, and 85°C. After a suitable time, the enrichments were streaked on TYE agar (3.0%) plates made up in Castenholz's salts (Jackson et al., 1973; Castenholz, 1969) (see also Chapter 206).

The enrichment culture inoculated with bacterial mat sample taken from Toadstool Spring in Yellowstone and incubated at 70°C was the only one that yielded unusual colonies. These were compact pink colonies distinctly different from the other isolates that appeared to be mostly of the genus *Thermus*. Transfer by streaking on the TYE agar medium at pH 8.5 and an increased level of tryptone and yeast extract (to 0.5%) led to a pure culture of *T. roseum* (Jackson et al., 1973).

Allen's Salts (mg/liter)

Component	mg/liter
$(NH_4)_2SO_4$	1,300
KH_2PO_4	280
$MgSO_4 \cdot 7H_2O$	247
$CaCl_2 \cdot 2H_2O$	74
$FeCl_3 \cdot 6H_2O$	19
$MnCl_2 \cdot 4H_2O$	1.8
$Na_2B_4O_7 \cdot 10H_2O$	4.4
$ZnSO_4 \cdot 7H_2O$	0.22
$CuCl_2 \cdot H_2O$	0.05
$Na_2MoO_4 \cdot 2H_2O$	0.03
VCL_2	0.03

Adjust the pH to 7.8.

Castenholz's Salts (mg/liter)

Component	mg/liter
Nitrilotriacetic acid	100
$CaSO_4 \cdot 2H_2O$	60
$MgSO_4 \cdot 7H_2O$	100
NaCl	8
KNO_3	103
$NaNO_3$	689
Na_2HPO_4	111
$FeCl_3$	0.28
$MnSO_4 \cdot H_2O$	2.2
$ZnSO_4 \cdot 7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4$	0.016
$Na_2MoO_4 \cdot 2H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.045

Habitat

Toadstool Spring (no longer active) was located in the Lower Geyser Basin of Yellowstone National Park. *T. roseum* was isolated from a sample taken near the source of the spring where the water temperature was 74°C with a pH of 8–9. The inoculum was from an orange/red bacterial mat attached to a silica substrate. The mat had accumulated under a piece of wax paper debris that may have offered some protection from the high light intensity and rapidly moving

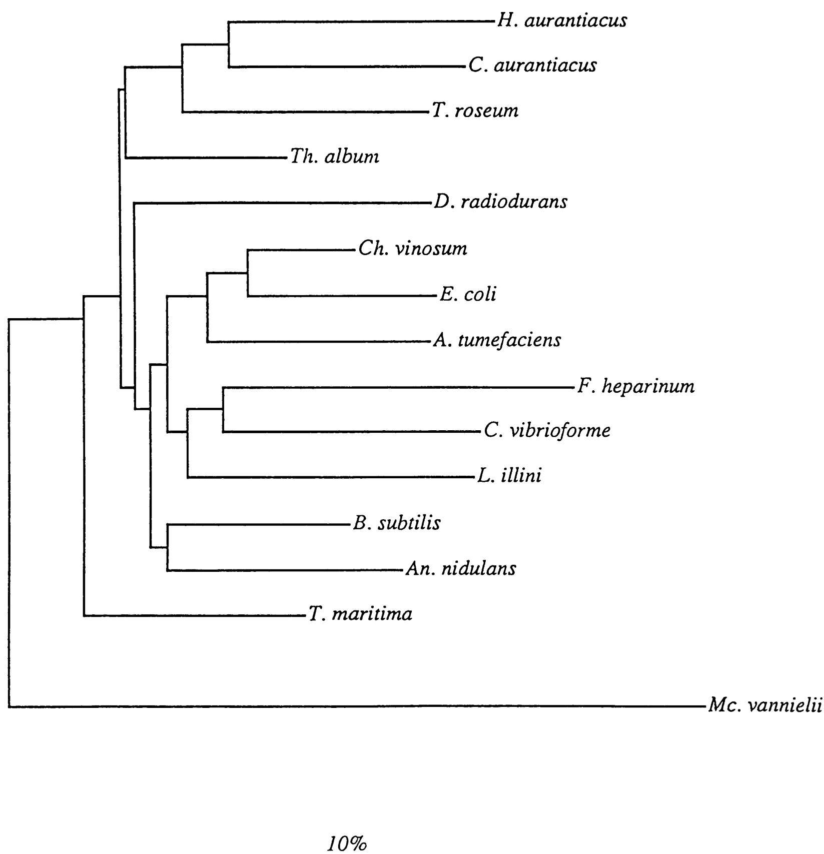

Fig. 1. A phylogenetic tree for the eubacteria, showing also the position of one archaebacterium, *Methanococcus vannielii.* Organisms are: *Herpetosiphon aurantiacus, Chloroflexus aurantiacus, Thermomicrobium roseum, Thermoleophilum album, Deinococcus radiodurans, Chromatium vinosum, Escherichia coli, Agrobacterium tumefaciens, Flavobacterium heparinum, Chlorobium vibrioforme, Lactobacillus illini, Bacillus subtilis, Anacystis nidulans,* and *Thermatoga maritima.* (Personal communication from C. R. Woese.)

water. Although Jackson et al. (1973) suggested that glass slides immersed in the water near the source of the spring exhibited microbial growth that appeared similar to *T. roseum,* microbes have not yet been cultivated from these sources.

A thermophilic organism was isolated from soil by enrichment with *n*-alkanes as substrate and was tentatively assigned to the genus *Thermomicrobium* (Phillips and Perry, 1976). Subsequent DNA-DNA hybridization studies indicated that this isolate should be placed in an entirely different genus (Zarilla and Perry, 1986, 1987.)

Substrate Utilization Patterns

Complex media are the preferred substrate for *T. roseum.* A maximum cell yield was attained with tryptone and yeast extract each added at 0.5%, with a lower level (0.3%) supporting the most rapid growth. Little growth occurred with the substrates added at 1.0%. Levels higher than 1.0% completely inhibited growth. *T. roseum* grew on peptone, casein hydrolysate, brain heart infusion, nutrient broth, trypticase soy broth, and on tryptone or yeast extract (substrate at 0.2%), provided that NH_4^+ (in Castenholz's salts) was incorporated as nitrogen source. Of these, only nutrient broth and yeast extract yielded growth with NO_3^- (in Allen's salts) as the source of nitrogen. *T. roseum* grew on a defined medium with selected substrates and then only on addition of 0.2% glutamate with NO_3^- as nitrogen source. Glycerol and sucrose were adequate carbon sources under these conditions. Sparse growth occurred with glutamate as sole substrate (Allen's salts). Fructose, succinate, mannitol, acetate, and citrate were inadequate as growth substrates (Jackson et al., 1973). Growth with Allen's salts was consistently better than that attained with Castenholz's salts. Apparently, there was some loss of ammonia from the latter at this pH and temperature.

Cultivation

The optimum temperature for growth was 70–75°C at a pH between 8.2 and 8.5. The minimum temperature at which *T. roseum* would grow was 45–48°C, and the pH extreme for growth was 7 to 8.7. Under optimum conditions with tryptone and yeast extract (0.3%) as substrates the organism had a generation time of 5 to 5.5 h.

Identification

T. roseum cells are generally pleomorphic and occur as short, irregular-shaped rods, single or paired, with some cells assuming a dumbbell shape. The organism is 1.3 to 1.8 μm in diameter and 3 to 6 μm in length. The GC content of the DNA is 64.3 mol%. The colonies on agar are compact and pink-pigmented. Although some slight motility was observed in light microscopy, there was no evidence of flagella under electron microscopy, and the organism is considered to be nonmotile (Jackson et al., 1973). Endospores are not visible in *T. roseum* by light, phase contrast, or electron microscopy. The organism is Gram-negative, and thin sections viewed longitudinally have a layered cell wall consistent with this staining character. Electron-dense hexagonal structures were visible in thin sections that may have been bacteriophages, but no lytic activity was observed (Jackson et al., 1973).

DNA hybridization reactions were employed to determine whether there was reassociation of *T. roseum* DNA with DNA from thermophilic *Bacillus* strains, *Thermus aquaticus,* and *Thermoleophilum album.* The results confirmed that *T. roseum* is not related to any of these other organisms (Zarilla and Perry, 1986, 1987). Antibiotic sensitivity tests indicate that *T. roseum* is sensitive to kanamycin, neomycin, and penicillin, but less sensitive to erythromycin and chloromycetin (Jackson et al., 1973).

The pigments were extracted from *T. roseum* with acetone and analyzed spectrophotometrically. The absorbance maxima at 470, 494, and 530 nm indicate that *T. roseum* carotenoid pigments are similar to torulene and 3,4-dehydrolycopene. Extracted pigment from the bacterial mats that served as the enrichment inoculum had equivalent absorptive properties (Jackson et al., 1973).

Physiological Properties

Membrane Lipid Composition

Virtually all eubacterial and eukaryotic cells have membrane lipids composed of fatty acids esterified to α-glycerolphosphate. The membranes of archaebacteria differ markedly in that the lipids of these organisms are composed of isoprenoid chains joined to glycerol by an ether linkage. The membrane lipids of *T. roseum* have neither ester nor ether linkages, but are composed of a series of straight-chain and internally branched 1,2-diols. A typical 1,2-diol is shown in Fig. 2. The carbon length of the diols ranges from C_{18} to C_{23} (Pond et al., 1986).

The cellular lipids account for about 3% of the dry weight of the cell, and, of this, 22.6% is nonpolar neutral lipid and 77.1% is polar glycolipids or phospholipids. Polar lipids were hydrolyzed to remove the polar head groups and the apolar residues were analyzed (Pond et al., 1986). The distribution of the 1,2-diols present in the lipid of *T. roseum* are listed in Table 1. These structures are analogous to the glycerolipids in that the terminal hydroxyl can link to the polar-head group and long-chain fatty acids can be esterified to the secondary hydroxyl. This would provide the hydrophobic chains necessary for a lipid bilayer (Pond et al., 1986). Analysis of the fatty acid constituents of these unusual lipids indicated that they too had normal and internally methylated hydrocarbon chains (Pond and Langworthy, 1987). The distribution of fatty acids in *T. roseum* grown at 70°C is presented in Table 2. Methyl-branched chain fatty acids are also common constituents of eubacterial lipids but generally they are iso- or anteisomethylated fatty acids.

The effect of temperature on the relative amounts of normal to branched chain fatty acids and diols was determined (Pond and Langworthy, 1987) and the results are presented in Table 3. As the growth temperature was increased, the relative percentage of unbranched fatty acids and diols increased, while the rela-

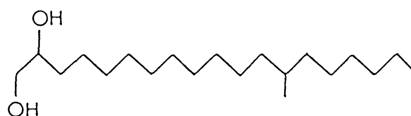

Fig. 2. Typical structure of a long-chain 1,2-diol from the lipid of *Thermomicrobium roseum.* This diol is 13-methyl-1,2-nonadecanediol.

Table 1. Distribution of 1,2-diols in the lipids of *Thermoleophilum roseum* after growth at 75°C.

Compound	Percent of total
11-Methyl-1,2-heptadecanediol	<0.5
1,2-Nonadecanediol	10.6
13-Methyl-1,2-nonadecanediol	21.1
1,2-Cosanediol	6.1
13-Methyl-1,2-cosanediol	2.5
15-Methyl-1,2-cosanediol	0.9
1,2-Eicosanediol	48.5
15-Methyl-1,2-eicosanediol	7.8
1,2-Docosanediol	1.0
1,2-Tricosanediol	1.1
1,2-Tetracosanediol	<0.5

Adapted from Pond et al. (1986).

Table 2. Distribution of fatty acids in *Thermomicrobium roseum* after growth at 70°C.

Compound	Percent of total
n-Hexadecanoic	1.2
10-Methyl-pentadecanoic acid	1.5
n-Heptadecanoic acid	0.5
10-Methyl-hexadecanoic acid	0.7
n-Octadecanoic acid	24.2
12-Methyl-heptadecanoic acid	57.3
n-Nonadecanoic acid	2.6
12-Methyl-octadecanoic acid	1.9
n-Cosanoic acid	8.4
14-Methyl-nonadecanoic acid	1.2

Adapted from Pond and Langworthy (1987).

Table 3. Effect of temperature on the percentage of normal and branched chain fatty acids and diols in *Thermomicrobium roseum*.

Growth temperature (°C)	Percent normal	Percent branched
Fatty Acids		
60	24.1	75.6
75	46.3	52.4
Diols		
60	62.2	37.1
75	87.3	12.5

Adapted from Pond and Langworthy (1987).

tive amount of the methyl-branched constituents decreased (Pond and Langworthy, 1987). The longer chain, normal fatty acids have a higher melting point than the branched homologs, so increased branching as the temperature is lowered may be a mechanism whereby membrane fluidity is retained at lower temperatures.

Cell Wall Structure

Electron microscopy of negatively stained outer cell wall preparations from *T. roseum* indicated that there were regular, repeating structures present in the cell envelope. These structures appear in a consistent mosaic pattern (Ramaley et al., 1978). Analysis of purified cell walls from *T. roseum* suggested that the organism has low levels of peptidoglycan (Merkel et al., 1980), although it is sensitive to penicillin (Jackson et al., 1973). Cell walls were removed from *T. roseum* and purified. This purified cell wall material was readily separated into two fractions (A and B) which were analyzed for amino acid composition (Table 4). Fraction A was composed of an array of amino acids with alanine, glutamic acid, and glycine predominating; lesser amounts of diaminopimelic acid, galactosamine, and muramic acid were detected.

Table 4. Amino acid composition of the two fractions of the cell wall of *Thermomicrobium roseum*.[a]

Component	Fraction A (%)	Fraction B (%)
Threonine	8.1	3.6
Serine	6.2	4.0
Proline	8.1	14.0
Muramic acid	3.1	0
Glutamic acid	12.8	11.9
Glycine	19.3	33.8
Alanine	16.8	11.5
Valine	4.0	1.8
Diaminopimelic acid	1.2	0
Leucine	6.2	3.2
Isoleucine	0	1.8
Tyrosine	4.7	2.2
Galactosamine	3.1	0
Histidine	1.2	3.2
Arginine	3.7	Trace
Lysine	0	Trace
Ornithine	0	7.2
Phenylalanine	0	1.8

[a]Expressed as % of the total amino acid/sugar present.
From Merkel et al., 1978.

Fraction B was a cell wall protein that accounted for 60% of the total cell wall amino-reactive material. It had a M_r of 75,000; the amino acid composition of this protein is presented in Table 4. It is probable that this protein is the principal structural component of the cell wall revealed by electron microscopy (Ramaley et al., 1978; Merkel et al., 1980). How this protein may contribute to the thermostability of *T. roseum* is unknown.

Metabolism

Although *T. roseum* is a strict aerobe, it does not respond to increased oxygen levels by more rapid growth or higher cell yields. This is a common attribute of aerobic thermophiles and particularly of those that grow on hydrocarbon substrates (Allgood and Perry, 1985a, 1985b, 1986). Growth of *T. roseum* under higher O_2 tension did result in increased cellular levels of superoxide dismutase (SOD) and catalase. Addition of methyl viologen to increase superoxide anion generation in cells had little effect on the level of SOD but resulted in increased catalase activity (Allgood and Perry, 1986). That higher oxygen levels do not lead to more rapid growth or cell yield is probably due to factors other than the accumulation of toxic products of oxygen such as superoxide or hydrogen peroxide. Most of the respiratory activity in *T. roseum* is cyanide insensitive. The respiratory pathways in *T. roseum* have not been elucidated further (Allgood and Perry, 1985a, 1985b).

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The Genus *Thermoleophilum*

JEROME J. PERRY

Thermoleophilum is a genus of eubacteria obligate for aerobiosis and thermophily. The genus is distinguished by the ability to grow solely on *n*-alkane substrates ranging in length from C_{13} to C_{20} (Merkel et al., 1978a, 1978b; Zarilla and Perry, 1984, 1986). Mud samples obtained from hot springs and from environmental niches generally considered nonthermal have both yielded isolates of the genus *Thermoleophilum.* Thermal elution profiles of DNA duplexes delineate two species within the genus, *T. album* and *T. minutum* (Zarilla and Perry, 1984, 1986). Sequencing of 16S rRNA from *T. album* strain NM affirms that these organisms should be placed in a separate "phylum" within the eubacteria (C. Woese, personal communication; see Fig. 1, Chapter 1). Organisms in this group are progeny of a deep divergence in the eubacterial line of descent. Isolation of strains from environments separated by considerable geographic distance indicates that *Thermoleophilum* strains may be of fairly common occurrence. Efforts to isolate a mesophilic counterpart of *Thermoleophilum* that would also be restricted to growth on *n*-alkane substrates have been unsuccessful.

Thermoleophilum species are Gram-negative, slow growing, small, nonmotile bacteria. They cannot utilize sugars, amino acids, fatty acids, or any substrate tested (except the *n*-alkanes) as source of carbon or energy. During active growth on n-heptadecane, the organism can incorporate a limited amount of acetate into cell material (less than 10% of total cell carbon). Members of this genus have a unique tetrahydrogenated menaquinone that has not been observed in the respiratory pigments of bacteria of any other species. The catalase from *Thermoleophilum* is a manganese-containing enzyme relatively resistant to cyanide inhibition.

Habitat

Thermoleophilum strains have been isolated from mud samples obtained from both thermal and nonthermal environments. The thermal environments included the outlet stream of hot springs in the states of Wyoming, Arkansas, and New Mexico (Table 1). The nonthermal environments were mud samples from Roanoke Rapids and Beaufort, NC. All mud samples were taken from aquatic environments and the temperature of sampling sites in Yellowstone, WY and Hot Springs, AR were near the optimum for growth of the strains isolated (YS-3, YS-4, and HS-5) (Merkel et al., 1978b; Zarilla and Perry, 1984). The nonthermal environmental samples were obtained from dark mud that was constantly exposed to the sun (strains PTA-1 and RR-D). Solar heating can raise the temperature of such dark surfaces to 60°C or higher (Brock, 1970). Growth of these obligate thermophiles in nonthermal environments would probably occur sporadically when the transient temperatures rose to a suitable level.

Attempts to isolate a mesophilic organism restricted to growth solely on *n*-alkane substrates have been unsuccessful. The presence of fast-growing, *n*-alkane-utilizing organisms in most environments of moderate temperature might well preclude the isolation of organisms with growth characteristics equivalent to *Thermoleophilum* species.

Table 1. Locations where *Thermoleophilum* strains have been isolated.

Strain	Source of inoculum	Temperature
T. album		
HS-5	Hot Spring, AR	61°C
NM	Faywood Hot Springs, NM	>50°C[a]
YS-3	Yellowstone National Park, WY	60°C
RR-D	Roanoke Rapids, NC	Ambient
T. minutum		
YS-4	Yellowstone National Park, WY	63°C
PTA-1	Beaufort, NC	Ambient

[a]Close to the water source and above 50°C.
Adapted from Zarilla and Perry, 1984.

Isolation and Enrichment

The primary enrichment medium for the isolation of *Thermoleophilum* species was prepared by adding *n*-heptadecane at 0.1% (v/v) to a mineral basal medium (L-salts) modified from that of Leadbetter and Foster (1958). The recipe for this medium is given below. Nitrogen was provided in the modified medium by adding 1 g/l of NH_4Cl and $NaNO_3$ (Zarilla and Perry, 1984). Mud samples employed as inocula were obtained from both thermal and nonthermal environments (Table 1). Incubation was at 60°C in stationary culture until turbidity was apparent. After repeated transfer in the liquid medium, a pure culture was obtained by streaking on the modified L-salts medium with 3.0% agar added. The *n*-heptadecane substrate was introduced by inverting the inoculated plate and placing 0.2-ml *n*-heptadecane in the cover. The plates were placed in plastic bags (to prevent desiccation) and incubated at 60°C for 5 days. The colonies were small white, dry, and flat. Axenic cultures were difficult to obtain as the organisms grow poorly on agar surfaces.

Mineral-Salts Medium (Modified L-Salts) for *Thermoleophilum* Species

NH_4Cl	1.0 g
$NaNO_3$	1.0 g
$MgSO_4 \cdot 7H_2O$	200 mg
Na_2HPO_4	210 mg
NaH_2PO_4	90 mg
$CaCl_2$	15 mg
KCl	40 mg
$FeSO_4 \cdot 7H_2O$	1 mg
$CuSO_4 \cdot 5H_2O$	5 μg
H_3BO_3	10 μg
$MnSO_4 \cdot 5H_2O$	10 μg
$ZnSO_4 \cdot 7H_2O$	70 μg
MoO_3	10 μg
Distilled Water	1 liter

Cultivation

The optimum temperature for growth of the isolates was 58–62°C at a pH between 6.5 and 7.5 (Table 2). *Thermoleophilum* species are limited to growth temperatures between 45 and 70°C. The generation time was difficult to determine accurately but exceeded 6 hours in every case. Strains are all strict aerobes but do not respond favorably to increased oxygen availability. Under optimal conditions of temperature (60°C), pH (7.0), and substrate (n-heptadecane), the cell yield ranged from 0.1 to 0.3 g/liter (Zarilla and Perry, 1984, 1986).

Substrate Utilization Patterns

None of the *Thermoleophilum* strains grew on any substrate tested other than *n*-alkanes (C_{13} to C_{20}). No growth occurred on shorter or longer chains of *n*-alkanes, l-alkenes, or alcohols of a chain length equivalent to that of the n-alkanes that did support growth. Homologous ketones and branched alkanes (C_{13} to C_{17}) were not utilized. The following substrates did not support growth: arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, rhamnose, ribose, sorbose, sucrose, trehalose, xylose, glycerol, mannitol, sorbitol, acetate, propionate, butyrate, citrate, pyruvate, succinate, acetone, nutrient broth, peptone, yeast extract, or tryptone plus yeast extract. All of the strains utilized ammonium chloride as a nitrogen source. Strains HS-5, NM, and RR-D could also utilize sodium nitrate as sole nitrogen source. Two strains, HS-5 and NM, could derive their nitrogen from glycine but growth was poor, and none of the carbon of the glycine was incorporated. Neither alanine nor glutamate could serve as source of nitrogen. Addition of growth factors, e.g., B-vitamins or amino acids, to the growth medium did not alter the growth rate or total growth. Compilation and use of a mineral salts medium similar in composition to the hot springs water of the original sampling site in Yellowstone had no effect on growth rate or cell yield.

Identification

Thermoleophilum species are very small, Gram-negative rods (Table 1 and Fig. 1). The organisms are nonmotile and have no pigmentation. They have been isolated only on n-alkane enrichment substrates (C_{13}–C_{20}) and are readily identified by their inability to grow on any other substrate. *Thermoleophilum* strains have a GC content of 68.8 to 70.4 mol%. The major diamino acid in the cell wall peptidoglycan of the genus *Thermoleophilum* is diaminopimelic acid. All strains demonstrated a confluent growth on agar surfaces with few individual colonies formed. Colonies were small, round, and white, and generally dry and flaky. They were often contaminated with pink-pigmented thermophilic strains on original isolation, and separating the slow-growing *Thermoleophilum* species was a tedious process.

DNA hybridization studies indicated that *T. album* and *T. minutum* are of the same genus but different species (Table 3) (Zarilla and Perry, 1986). There was no homology with *Thermomicrobium roseum, Bacillus thermoleo-*

Table 2. Properties of *T. album* and *T. minutum*.

Strain	Cell length (μm)	Generation time (h)	n-Alkane substrate range	GC content (mol%)
T. album				
HS-5	0.9	6.5	C_{13}–C_{20}	70.4
NM	1.0	9	C_{14}–C_{20}	68.8
YS-3	0.7	6	C_{13}–C_{20}	69
RR-D	1.0	7.5	C_{14}–C_{20}	70
T. minutum				
YS-4	1.5	6	C_{13}–C_{20}	70
PTA-1	1.1	7.5	C_{14}–C_{19}	68.8

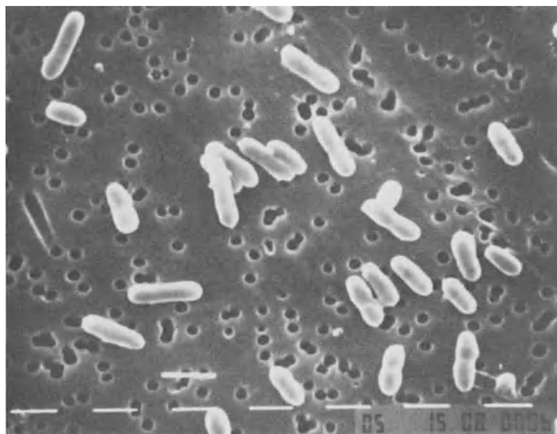

Fig. 1. Electron micrograph of *Thermoleophilum album* strain RR-D after growth on n-heptadecane at 60°C. Bar = 1 μm.

Table 3. Relative binding in reassociation reactions with [α-^{32}P]deoxycytidine triphosphate-labeled DNA from *T. album* (HS-5) and *T. minutum* (YS-4).

	Relative binding (%) with the following source of radiolabeled DNA[a]			
	T. album HS-5		*T. minutum* YS-4	
Strain	62°C	76°C	62°C	76°C
T. album				
HS-5	100[b]	100	44	27
YS-3	81	76	42	27
NM	101	94	42	28
RR-D	86	79	39	28
T. minutum				
YS-4	43	25	100	100
PTA-1	44	25	99	99

[a]Average of duplicate determinations.
[b]Temperature at which reassociation occurred. 62°C is the optimal temperature; 76°C is the stringent temperature.
Adapted from Zarilla and Perry, 1986.

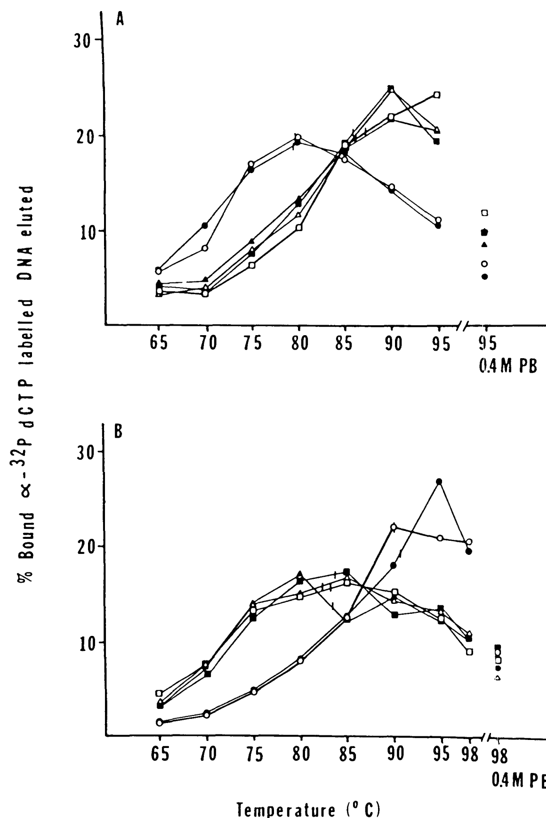

Fig. 2. Thermal elution profiles of *Thermoleophilum* DNA duplexes reassociated at 62°C. One strand of each DNA duplex was made up of [α-^{32}P]deoxycytidine triphosphate (dCTP)-labeled DNA from either *Thermoleophilum album* strain HS-5^T (A) or *Thermoleophilum minutum* strain YS-4^T (B). The other strand was made up of DNA from strain HS-5^T (□), YS-3 (▲), YS-4^T (○), NM (△), RR-D (■), or PTA-1 (●). The homologous duplex is indicated by a thick line. The thermal stability values are indicated by a vertical line on each curve and represent the temperature at which 50% of the double-stranded DNA was eluted from the hydroxyapatite. DNA was eluted with 0.14 M phosphate buffer (PB) containing 0.1% sodium dodecyl sulfate until the maximum temperature was attained, at which point any remaining bound DNA was eluted by using 0.4 M phosphate buffer. Adapted from Zarilla and Perry, 1986.

vorans, or *Thermus aquaticus*. Data presented in Fig. 2 confirm that there are two species within this genus.

Physiological Characteristics

Menaquinones are widely distributed in the plasma membranes of prokaryotes and are generally distinguished by the length of their isoprenyl side chain. The number of isoprenoid units varies from 1 to 15, and there are also species differences in the number of these isoprenoids that are saturated/unsaturated (Collins and Jones, 1981). Generally Gram-negative eubacterial menaquinones are composed of unsaturated isoprenoid units but a few Gram-negative bacterial species have dihydrogenated menaquinones. Examination of the respiratory quinones of *T. album* strain NM led to the isolation of a novel tetrahydrogenated menaquinone, 2 methyl-3-VI, VII-tetrahydroheptaprenyl-1, 4-naphthoquinone, as depicted in Fig. 3 (Collins et al., 1986). The presence of this naphthoquinone in *T. album* readily distinguishes the genus from other Gram-negative, aerobic, obligately thermophilic groups. The genus *Thermus* and *Thermomicrobium roseum* contain saturated menaquinone as their major respiratory quinone.

Superoxide dismutase is induced in aerobic bacteria by high oxygen stress. This enzyme, together with catalase and peroxidase, removes toxic oxygen intermediates generated as by products in the univalent reduction of molecular oxygen. These highly reactive intermediates of oxygen reduction are predominantly superoxide anion, hydrogen peroxide, and the hydroxyl radical. Growth of aerobic organisms at higher oxygen tensions or in the presence of methyl viologen (a superoxide generator) will effectively induce superoxide dismutase (Diguissepi and Fridovich, 1984; Hassan and Fridovich, 1977). Studies suggest that thermophilic bacteria generally react unfavorably to increased oxygen tension (Allgood and Perry, 1985b). As a consequence of this observation, an examination was made of the effect of oxygen stress on the level of superoxide dismutase, catalase, and peroxidase in *T. album* NM. Growth of strain NM in the presence of methyl viologen did not affect the level of cellular superoxide dismutase but caused a marked increase in catalase activity. Peroxidase activity increased a small amount. Increasing the oxygen level also led to higher levels of catalase with a decrease in total growth (Allgood and Perry, 1985a, 1985b, 1986b). The level of oxygen defense enzymes is comparable with that in other aerobic bacteria. It does not appear that products of O_2 reduction are responsible for the negative response to increased aeration.

The catalase from *T. album* NM has been isolated, purified, (>96%), and characterized. The major properties of the enzyme are presented in Table 4. For comparison the properties of the equivalent enzyme from *Escherichia coli, Rhodopseudomonas sphaeroides* and *Lactobacillus plantarum* are included. The catalase from *T. album* is thermoactive and thermostable. It has a low M_r, and the metal present is manganese. This is the only report of a manganese-containing catalase in an aerobic organism. The significance of such a catalase in this ancient thermophile is not for us to understand at this time.

The electrophoretic mobility of several enzymes in crude cell-free extracts from *T. album* strains were markedly different from the equivalent enzyme from other thermophiles (Zarilla and Perry, 1986). Among these enzymes of differing mobility were malate dehydrogenase, catalase, esterase, and superoxide dismutase. Electrophoresis of purified catalase and malate dehydrogenase from *T. album* NM resulted in the same pattern.

T. minutum YS-4 extracts contained all of the enzymes involved with the tricarboxylic acid cycle except α-ketoglutarate dehydrogenase (J. G. McCarthy and J. J. Perry, unpublished observations). Exhaustive attempts to demonstrate this enzyme in any of the *Thermoleophilum* strains have been unsuccessful. An active isocitrate lyase was present in extracts of *Ther-*

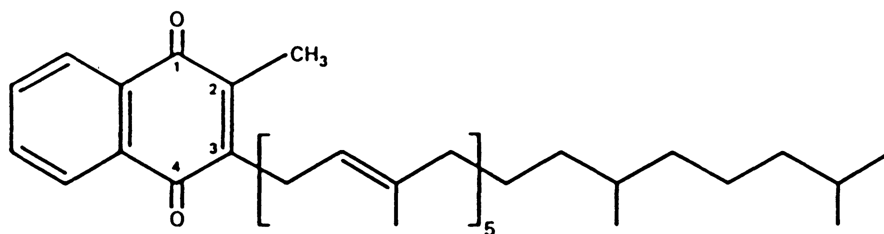

Fig. 3. Structure of the tetrahydrogenated menaquinone from *Thermoleophilum album*. Adapted from Collins et al., 1986.

Table 4. Comparison of the properties of the catalase from *T. album* with that from other bacterial species.

Organism	Metal present	Molecular weight, M_r	Number of subunits
Thermoleophilum album	Mn	141,000	4
Escherichia coli	Fe	337,000	4
Rhodopseudomonas sphaeroides	Fe	232,000	4
Lactobacillus plantarum	Mn	172,000	6

moleophilum, suggesting that an active glyoxylate cycle is present. All strains had NADH and NADPH oxidase activity. *T. album* NM had the large-sized citrate synthase typical of Gram-negative bacteria (K. Weaver and J. J. Perry, unpublished observations). Addition of ^{14}C-acetate to nonproliferating cells of *T. album* NM resulted in little incorporation of acetate. Addition of radiolabeled acetate to a culture during active growth on *n*-heptadecane did result in some incorporation of acetate but at a level of less than 10% of the total cell carbon. Increasing the level of acetate from 0.1 mM to 50 mM did not lead to any increase in incorporation (K. C. Terlesky and J. J. Perry, unpublished observations). The inability of the organism to incorporate more added acetate and the absence of α-ketoglutarate dehydrogenase is reminiscent of these activities in the obligate autotrophs. It may be that the genus *Thermoleophilum* cannot obtain energy from substrates other than the *n*-alkanes utilized.

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CHAPTER 209

The Genus *Herpetosiphon*

HANS REICHENBACH

The *Herpetosiphon* species are aerobic, chemoorganotrophic, filamentous bacteria that are Gram-negative but do not have a typical Gram-negative cell wall. The filaments are very long, unbranched, and multicellular, between 0.6 and 1.5 μm wide and usually 300 to more than 1200 μm long (Fig. 1). Short transparent sections ("sleeves": Fig. 2) are seen at the ends of many filaments. These sleeves are characteristic of *Herpetosiphon*, and they make it possible to recognize the organism easily under the microscope.

Sleeves are also found with related organisms, like *Chloroflexus*. Depending on the strain, the medium, and the age of the culture, the filaments may fragment into much shorter pieces and ultimately into single cells. The cells in the filaments are tightly attached to one another, and in living filaments their boundaries can only be seen with a microscope of high resolution at a high magnification, by phase or interference contrast (Fig. 2). They become clearly recognizable if the filament has been dried to the slide and stained (Fig. 2). The cells have the

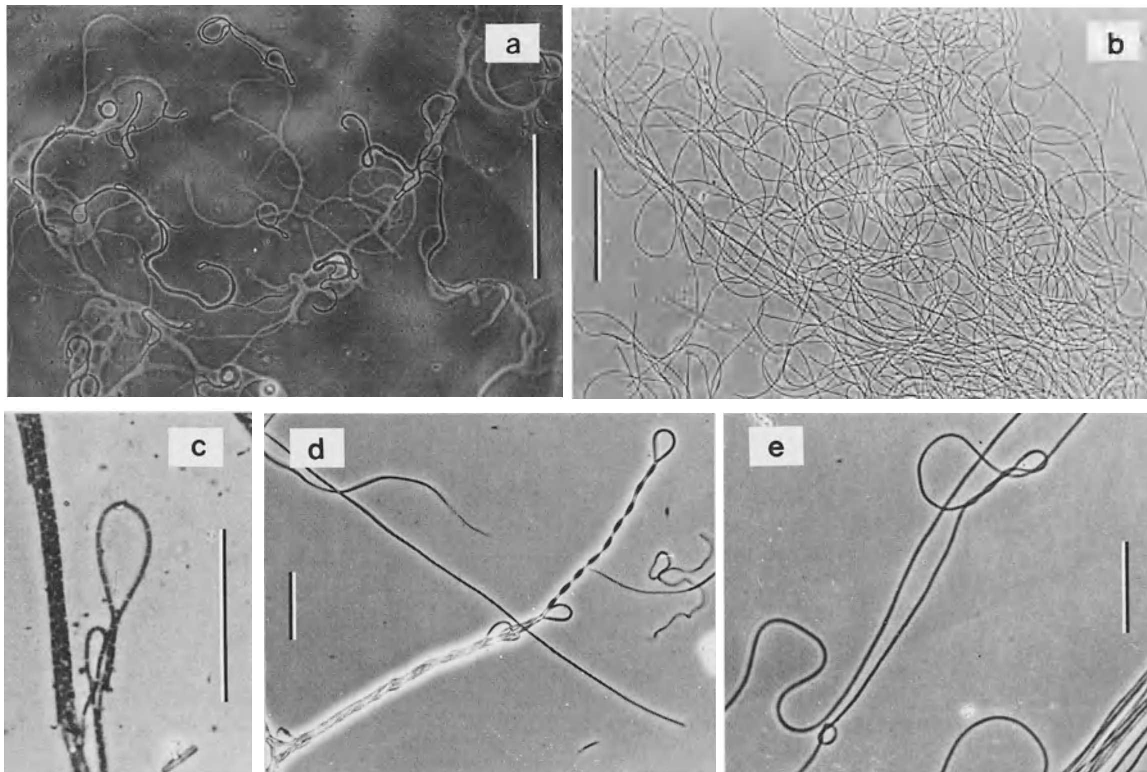
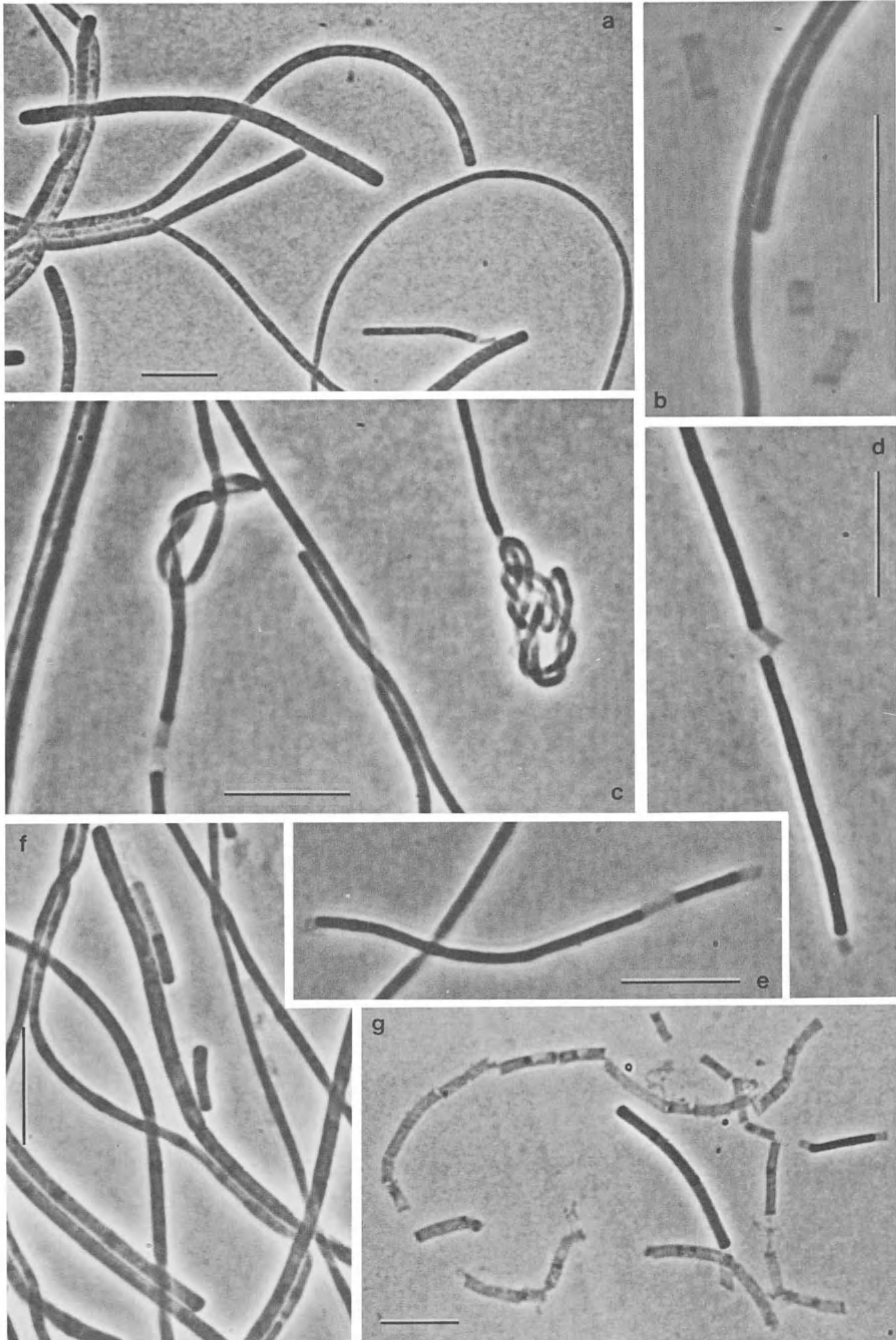


Fig. 1. Filaments of *Herpetosiphon aurantiacus* at low magnifications. (a) Filaments with slime tracks in chamber culture. (b) Filaments from a liquid culture, slide mount. (c) Filaments stained with Loeffler's methylene blue; the individual cells of the filament are clearly visible. (d) and (e) Twisted filaments from a liquid culture. Zeiss Standard Microscope and Axiomat (d, e), in phase contrast. Bar = 100 μm (for a and b). Bar = 25 μm (for c, d, and e).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same width as the filaments and measure 1.5–5 μm in length, rarely more. The filaments may perform slow gliding movements; thus, on suitable substrates, the colonies develop into large spreading swarms (Fig. 3). The surface of the swarm is usually feltlike. Long flamelike projections or protruding strands of curled filaments are seen at the edge (Fig. 3). Normally the colonies are colored in shades of yellow, orange, or brick red, but unpigmented strains do occur. The GC content of the DNA is 45–53 mol%.

Herpetosiphon is a common inhabitant of soil, freshwater, and sewage plants. Some species live in marine environments.

It appears that *Herpetosiphon* was first discussed in the scientific literature under the name of *Flexibacter giganteus* (Soriano, 1945, 1947). Soriano's description of the filaments, the swarm colonies, and their color perfectly matches the cultures now called *Herpetosiphon.* Also, the habitats he studied and the isolation technique he applied must inevitably have led to the discovery of that organism. However, the first valid description of the genus, with one species, *H. aurantiacus,* was provided by Holt and Lewin (1968). The definition was based on three isolates of E. E. Jeffers obtained from the slime coat of a green alga, *Chara* sp., from Birch Lake in Minnesota. Later, a dispute arose about the presence of a sheath in *Herpetosiphon,* and the genus definition was slightly modified to exclude a sheath (Reichenbach and Golecki, 1975). Also, Reichenbach and Golecki (1975) proposed that the species name *aurantiacus* be abandoned in favor of the name *giganteus,* which antedates it. This change could have been done without danger of confusion since Soriano's strain is no longer available. However, there are definitely several *Herpetosiphon* species, and one could argue therefore that Soriano's *H. giganteus* was different from *aurantiacus.* Thus, it may be better to give up the name *giganteus* until we know more about the taxonomy of the genus. Later, five more species were added to the genus (Lewin, 1970). The organism described from Lake Constance (Germany) as a new *Vitreoscilla* species, *V. proteolytica* (Gräf and Perschmann, 1970), is also undisputably a *Herpetosiphon*; strains of this organism are still available and have been studied by the present author. Further, the bacterium isolated from the walls of a sluice of the Neckar river near Heidelberg (Brauss et al., 1969) was most likely also a *Herpetosiphon,* although the authors felt they could rule out that possibility. Also, filamentous organisms connected with bulking sludge in various sewage plants in southwestern Germany have been identified as *Herpetosiphon* and *Herpetosiphon*-related bacteria (Salcher et al., 1982; Senghas and Lingens, 1985; Trick and Lingens, 1984). In the rest of this chapter, the group isolated from sewage will be referred to as *Herpetosiphon,* while those isolated from sludge as *Herpetosiphon*-like bacteria. These organisms are of particular interest because of their practical implications, and because filamentous bacteria in activated sludge have been notoriously difficult to classify (for a summary, see Pipes, 1978).

A comparison of 16S rRNA oligonucleotide catalogs clearly shows a phylogenetic relationship of *Herpetosiphon* to *Chloroflexus* and *Thermomicrobium* (Gibson et al., 1985; Woese et al., 1985). The group is well separated from all other bacteria and represents one of the main branches (a phylum) in the bacterial tree of descent. By the same method, it has been possible to rule out any connection to filamentous, gliding *Vitreoscilla,* as well as to *Beggiatoa* and *Leucothrix,* which belong to the beta and gamma branches, respectively, of the purple bacteria (the class Proteobacteria, as defined by Stackebrandt et al., 1988). Any relationship with the filamentous cyanobacteria (a connection that was discussed for many years) has also been ruled out (Reichenbach et al., 1986; for a summary, see Reichenbach, 1981). Interestingly, the relationship with *Chloroflexus* is also reflected in the very unusual structure and com-

Fig. 2. Filaments of the type strain of *Herpetosiphon aurantiacus* at high magnifications. (a) Filaments from CY agar. Note the enormous variability of the filaments' diameter, which is especially pronounced on peptone media. Slight constrictions at the filaments' surface and dark bands in the interior indicate cell boundaries, which can be particularly clearly recognized in the thick filament in the upper left sector; dark dots seen in many cells may be mesosomelike membrane bodies. (b) The translucent segments seen next to the filaments are empty cell-wall cylinders and correspond to the sleeves often found at the ends of the filaments; here, as in other figures of this table, parallel running filaments approach one another very closely, which seems to exclude the presence of a sheath of any significance. (c) The knots in the filaments testify to their incredible flexibility; the filament on the left contains a necridium; from VY/2 agar. (d) The filament has a short sleeve at the end and a necridium which is almost ready to snap. (e) A short filament with sleeves at both ends and a necridium. (f) In several filaments, cross-walls are recognizable; there is a one- and a two-celled segment, the latter with a living and a dead cell; from CY agar. (g) Decaying filament fragmenting into cell-wall cylinders, each corresponding to one cell. Because the cylinders separate cleanly, there can be no sheath. All micrographs were taken with Zeiss Axiomat, in phase contrast. Bars = 10 μm.

position of the cell walls of the two organisms. Both organisms contain a peptidoglycan in which *meso*-diaminopimelic acid is replaced by L-ornithine, and to which a polysaccharide is covalently bound; and both organisms lack a lipopolysaccharide (Jürgens et al., 1987, 1989).

While some progress has been made, our knowledge of *Herpetosiphon* is still very incomplete (for summaries, see Reichenbach and Dworkin, 1981; Holt, 1989). A film showing the movements of the filaments and the development of the colonies is also available (Reichenbach et al., 1980).

Habitats

Typical *Herpetosiphon* strains are regularly found in freshwater, in soil, and in decaying organic matter, such as rotting wood, dung of herbivorous animals, and compost. *Herpetosiphon* appears to be rather common everywhere in aerobic environments, in the neutral pH range and under mesophilic conditions. Some freshwater habitats have already been mentioned. A study on the distribution of *Herpetosiphon* in the surface layers of Lake Constance, a large, deep, fairly oligotrophic lake in southwestern Germany, demonstrated the organism only in contaminated areas close to cities or at the mouths of rivers, while it was absent in clean stretches of water in the middle of the lake or along uninhabited and undisturbed shores (Gräf and Perschmann, 1970). This suggests that the *Herpetosiphon* population at the lake surface has its origin in the soil of the surrounding country and in contaminated waters running into the lake. The examples mentioned in the introduction show, however, that *Herpetosiphon* also thrives in the benthos of freshwater bodies. therefore, its absence in the surface layers is best explained by a fast sedimentation of the flocs of entangled filaments, which can stay in suspension only in vigorously agitated liquids.

Herpetosiphon and *Herpetosiphon*-like bacteria grow abundantly in the activated sludge of sewage plants (Salcher et al., 1982; Senghas and Lingens, 1985; Trick and Lingens, 1984). In fact, there may be such an explosive development that it leads to the much-feared bulking of the sludge. The reason for mass development is not yet fully understood and is probably not always the same, but one factor could be the presence of a high population of grazing protozoa that remove competing unicellular bacteria (Güde, 1979). Bulking in sludge is not due to the flocs not settling fast enough to become separated from the liquid, since the flocs settle indeed reasonably well. The problem is rather that the flocs show poor compaction properties, so that the sludge removed from the settling tank contains too much water. The cause of bulking sludge is always a microbiological one, and in most cases it is due to the excessive growth of filamentous bacteria. This has been known for many years, and several efforts have been made to classify the various types of filamentous organisms found in sewage (for a summary, see Piper, 1978). Success in understanding bulking was, however, rather limited, mainly because most of those bacteria could not be cultivated (also the case with many *Herpetosiphon* strains, as will be discussed later). *Herpetosiphon* must have been observed many times in sewage material, but it was never recognized as such. Thus, the organism described from a Dutch sewage plant as belonging to group III (van Veen,1973) was almost certainly *Herpetosiphon* (strain Rz in van Veen's Fig. 12 shows the typical empty sleeves at the ends of the filaments). Those bacteria were practically

Fig. 3. Various swarm colony types of *Herpetosiphon aurantiacus.* The colonies of *Herpetosiphon* are very variable and occasionally show striking patterns. (a) Swarm with a rhythmic growth pattern, on VY/2 agar. Bar = 1 mm. (b) Swarm showing surface growth (right), as well as penetration into the agar (light areas). Bar = 1 mm. (c) and (d) Colonies with proboscislike protuberances; (c) survey picture; (d) protuberances at higher magnification. Bar = 1 mm in both (c) and (d). (e) Swarm growing from a streak of autoclaved *E. coli* on water agar; the curly pattern is characteristic for this kind of culture; the cloudy areas in the lower part indicate penetration of the organism into the agar. Bar = 1 mm. (f) and (g) Large swarm on VY/2 agar; the swarm sheet tends to contract and peel off the agar surface, producing large holes surrounded by ridges; this growth pattern is very characteristic for *Herpetosiphon.* Bar = 1 mm in both (f) and (g). (h) Delicately plicated surface of a swarm sheet on VY/2 agar; this is a relatively unusual pattern. Bar = 100 μm. (i) to (l) Knobs; they are sometimes produced in large numbers and arranged in patterns (i, l) that make their resemblance to myxobacterial fruiting bodies even more striking; also, their size is in the range of fruiting bodies. Bar = 1 mm in (i) and (l), 500 μm in (j), and 200 μm in (k). (m) Microcolony showing its composition of long, coiling, and interwoven filaments on CY agar. Bar = 100 μm. (n) Swarm on a streak of autoclaved *E. coli* (center) on water agar spreading in an uncommon pattern of broad, tapelike tongues. Bar = 1 mm. (o) Edge of a swarm growing on a streak of living *E. coli* on water agar; in contrast to the usual pattern on this medium, as shown in (b) and (e), here the swarm ends in a series of compact knobs with delicate, flamelike extensions (swarm center is to the right). Bar = 1 mm. Pictures made with Leitz Aristophot, (a to g); Zeiss Axiomat, (h, j, k, m); and Olympus SZH Stereo Microscope, (i, l, n, o).

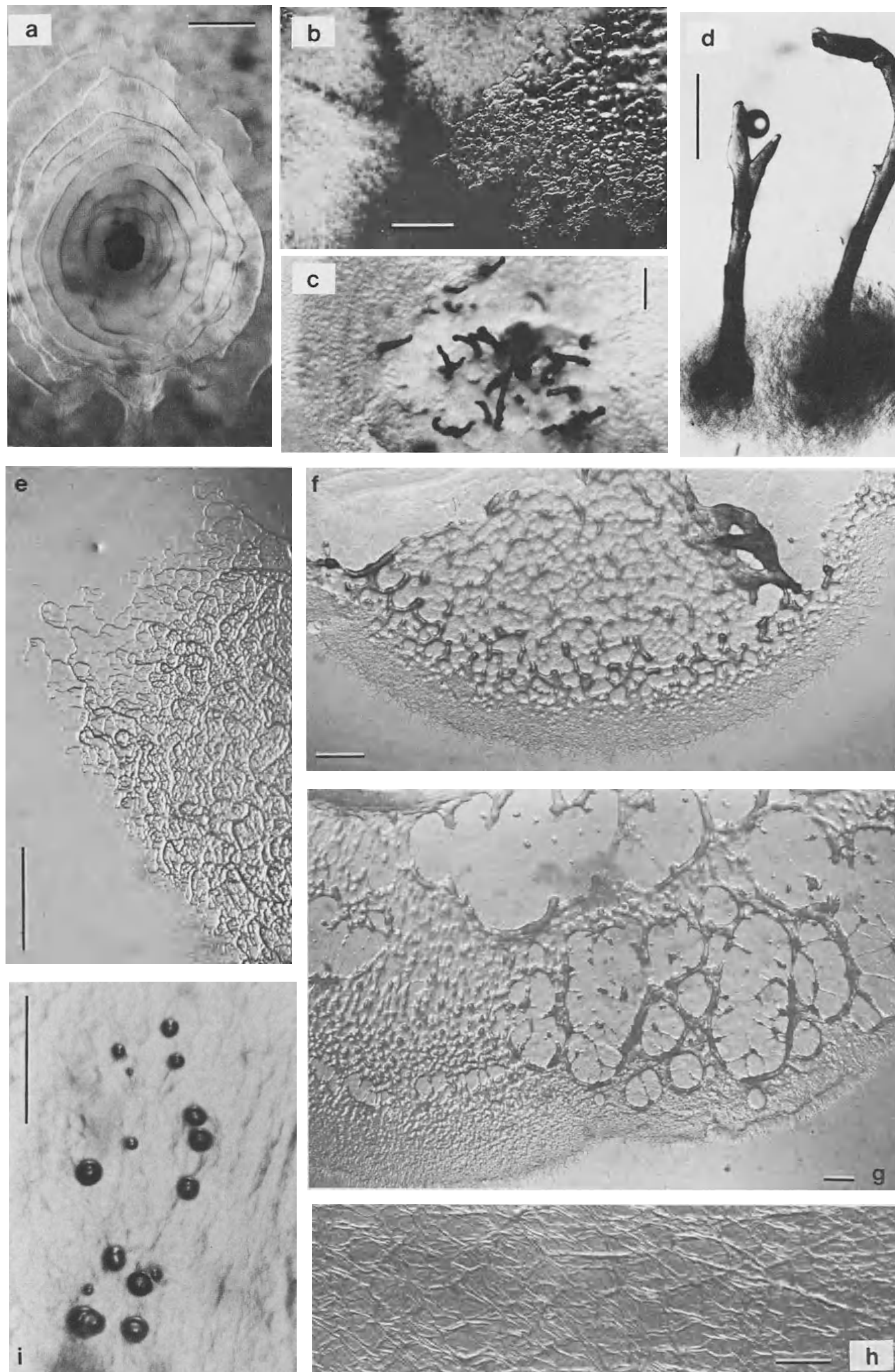

Fig. 3. Parts a to i. (Parts j to o on next page.)

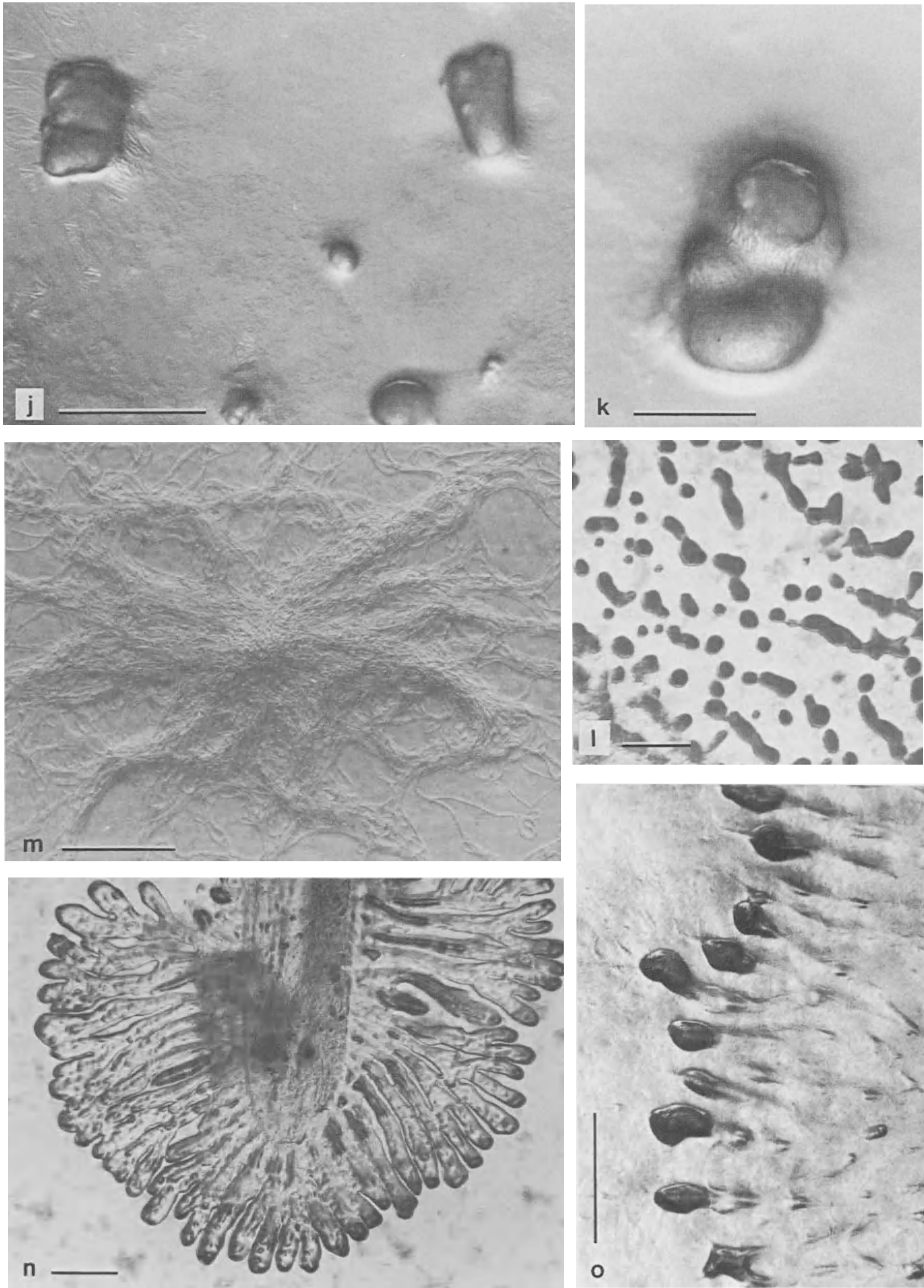

Fig. 3. Parts j to o.

always present in activated sludge and probably also contributed to bulking.

One species, *H. geysericola,* is described from the surroundings of a hot spring (Lewin, 1969, 1970). It is represented by just one strain, which in addition appears to be thermotolerant rather than thermophilic (the literature is completely mute about that important point; but the ATCC catalog lists a growth temperature of 30°C for the strain). The environment from which *H. geysericola* was isolated is of interest insofar as this is the typical habitat of the related *Chloroflexus.* In a study in a Yellowstone hot spring it was shown that the bacterial mat contained two other types of filamentous bacteria besides *Chloroflexus* (Tayne et al., 1987). Unfortunately, the antiserum used to identify *Chloroflexus* was not applied to *Herpetosiphon* out of all bacteria tested for cross-reactions. Perhaps *Herpetosiphon* is more common in that environment than has so far been assumed.

Several *Herpetosiphon* strains came from marine enviroments and were classified as separate species (Lewin, 1969, 1970). The organisms require at least half-strength seawater for growth and thus must be regarded as genuinely marine bacteria. The present author's own experience with isolating gliding bacteria from marine sources shows me, however, that *Herpetosiphon*-type organisms cannot be particularly common in that environment; Lewin also isolated only three marine strains among 90 other gliding bacteria, although in his marine samples he observed a few more *Herpetosiphon*-like organisms, which he could not isolate.

While *Herpetosiphon* obviously is a very common inhabitant of freshwater, it is equally at home in nonaquatic environments. As a byproduct during the isolation of myxobacteria, we have isolated in the course of 20 years more than 200 *Herpetosiphon* strains from soil samples, dung pellets, rotting wood, and similar materials collected all over the world in various climate zones, including decidedly hostile, dry, and hot environments, like semi-deserts in Tunisia and Arizona, steppe habitats in Mediterranean countries, the Canary Islands, and gypsum hills on Cyprus (H. Reichenbach, unpublished observations).

Isolation

Herpetosiphon may be isolated from any of the sources mentioned above. The terrestrial strains usually survive desiccation so that dry samples can also be used. We were able to obtain *Herpetosiphon* from rotting wood after 8 years storage, from filter-paper preserves of contaminated myxobacteria after 6 years, and from soil after 2 years of storage in the dry state at room temperature. It has been noted that strains from sewage plants are also extremely resistant to desiccation (Trick and Lingens, 1984).

No specific enrichment techniques are known. For isolation use is made of the ability of the organism to glide away from the inoculum and to produce a typical swarm pattern. To elicit gliding and to suppress excessive growth of contaminants, media very low in organic constituents are recommended. The simplest medium is plain water agar, to which cycloheximide may be added to prevent the development of fungi:

WAT Agar

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Adjust the pH to 7.2 with KOH. When used for crude cultures, cycloheximide (25 μg/ml medium) may be added after autoclaving, from a filter-sterilized stock solution (yielding WCX agar).

Several small samples of soil or other source of inoculum (size of a lentil) are placed on the surface of the dry plate, a few cm from each other, and the culture is incubated at 30°C. After 2 to 20 days, the typical swarm colonies with long flares and coils of entangled filaments at the edge may be observed (Fig. 3). The very delicate pattern can only be seen, however, if an oblique light beam produced by a tiltable mirror is applied from below. We have had success with WAT agar plates with three thin, parallel streaks of living *Escherichia coli,* each of which is inoculated at one end. *Herpetosiphon* grows in a spreading fashion within the streaks, usually without lysing the *E. coli* cells and often only appears along the edges of the streaks in the form of long flamelike structures or a loose pattern of separate, coiling strands of filaments. Sometimes, however, large and more or less dense swarm sheets are produced that spread over much of the plate. An especially high yield of *Herpetosiphon* can sometimes be obtained if such cultures are incubated at 38°C.

In a similar way, marine strains can be obtained by streaking littoral silt and mud on seawater agar.

Seawater Agar (Lewin and Lounsbery, 1969)

Tryptone (Difco)	0.02%
Agar	1%

In seawater.

After autoclaving, cycloheximide (100 μg/ml) is added from a filter-sterilized stock solution.

The cultures are incubated at room temperature (24°C) and examined over a 2 to 3 week period for the appearance of spreading organisms.

The strains on which the definition of the genus is based were isolated by making a single streak from the slimy coat of *Chara* on a plate with 0.3% peptonized milk (Difco) and 1.5% agar. The culture was incubated at room temperature. Evidently, this relatively rich medium can be used only if the sample contains few other microorganisms (Holt and Lewin, 1968).

For the isolation of planktonic *Herpetosiphon* in freshwater, between 0.5 and 1 liter of lake or river water is passed through a sterile membrane filter (pore size, 0.4 μm). The filter is then cut into pieces, and the segments are placed on a low-nutrient agar medium. In the original study, rabbit dung agar was used, but any other lean medium would certainly also do.

Rabbit Dung Agar (Gräf and Perschmann, 1970)

Dry dung pellets from wild rabbits (20 g) are boiled in 1 liter of distilled water for 20 min. The filtrate is used with 1.5% agar. The pH is adjusted to 7.2.

The cultures are incubated at 33°C. After about 6 days, swarm colonies begin to spread from the edges of the filter.

The strains from activated sludge were isolated by streaking a drop of the sludge sample on BG-11 agar (originally designed for the isolation of cyanobacteria) or on I agar (Salcher et al., 1982; Trick and Lingens, 1984).

BG-11 Agar (Stanier et al., 1971)

$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (Na_2-Mg salt)	0.001 g
Na_2CO_3	0.02 g
Trace element solution A5	1 ml
Agar	15 g
Water	1 liter

The trace element stock solution A5 contains per liter: H_3BO_3, 2.8 g; $MnCl_2 \cdot 4H_2O$, 1.8 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CuSO_4 \cdot 5H_2O$, 0.08 g; $Co(NO_3) \cdot 6H_2O$, 0.05 g.

I Agar (Salcher et al., 1982; from van Veen, 1973, modified)

Glucose	0.15 g
$(NH_4)_2SO_4$	0.5 g
$Ca(NO_3)_2$	0.01 g
K_2HPO_4	0.05 g
$MgSO_4 \cdot 7H_2O$	0.05 g
KCl	0.05 g
$CaCO_3$	0.1 g
Agar	15 g
Water	1 liter

After autoclaving, 10 ml of a filter-sterilized vitamin solution is added. The vitamin solution contains per liter: Ca pantothenate, 10 mg; nicotinic acid, 10 mg; biotin, 0.5 mg; cyanocobalamin, 0.5 mg; folic acid, 0.5 mg; pyridoxine·HCl, 10 mg; *p*-aminobenzoic acid, 10 mg; thiamine pyrophosphate, 10 mg; thiamine, 10 mg; inositol, 10 mg; riboflavin, 10 mg.

As sewage organisms are notoriously fastidious and adapted to very low nutrient levels, I have suggested those complicated media; whether they are really required or could be replaced by simpler recipes has not been determined. While this is consistent with the behavior of the isolated, pure strains, those strains taken immediately from sewage may behave differently from those that have adapted to cultivation. The cultures are incubated at 25°C and examined with a phase contrast microscope over several weeks, because these organisms developed very slowly (Salcher et al., 1982).

Pure cultures can be obtained by transferring filaments from the advancing swarm edge to the same media again, or, as soon as the culture is sufficiently pure, to richer media (see "Cultivation," this chapter). We often have good results with transfers to streaks of autoclaved *E. coli* on water agar, although many *Herpetosiphon* strains do not grow on autoclaved bacteria (see below). As the organism has to be carefully removed from the contaminated plate, transfers are made best by cutting out a piece of the swarm edge on a small agar block using a drawn-out glass rod or, more conveniently, a fine steel injection needle (e.g., on a 1-ml disposable syringe). The procedure is repeated until the strain is pure. Two recently discovered properties of *Herpetosiphon* may substantially speed up purification: I have found that all *Herpetosiphon* strains tested so far grew at 38°C (some even at 40°C), and that all are resistant to 250 mg kanamycin sulfate per liter.

Cultivation

It appears that, in general, *Herpetosiphon* prefers low nutrient levels. Thus, one of our strains, Hp g175, produced good growth over six transfers on plain water agar, obviously living off minute quantities of contaminating material in the agar but without visibly attacking the agar itself. Rich media, like nutrient agar or nutrient broth, are often not suitable at all.

About one strain out of three that is isolated from soil on streaks of living *E. coli* can be

cultivated without difficulty on complex media, such as VY/2 agar and CY agar. Those strains usually produce heavy growth, and often spread within a few days over the entire plate. Some strains tend to penetrate into the agar (Fig. 3). The yeast in VY/2 agar may or may not be lysed.

VY/2 Agar

Bakers' yeast (fresh weight of commercial yeast cake)	0.5%
$CaCl_2 \cdot 2H_2O$	0.1%
Cyanocobalamin	0.5 μg/ml
Agar	1.5%

Adjust to pH 7.2.

CY Agar

Casitone (Difco)	0.3%
Yeast extract	3 g
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Adjust to pH 7.2.

The cultivation of the remaining 70% of those strains is essentially an unsolved problem. A few of them respond favorably to a reduction of the nutrient concentration and can be grown on media like VY/5 agar (as VY/2 agar, but yeast concentration reduced to 0.2%). Others grow more or less poorly on *Escherichia coli* overlay agar, but most of them can barely be kept alive on streaks of autoclaved or living *E. coli* on water agar.

Escherichia coli Overlay Agar

The growth of four culture plates of *E. coli* (on any suitable medium) is suspended in 100 ml of water agar (WAT agar, see "Isolation"). The suspension is autoclaved and poured as a thin layer on top of water agar plates.

Many strains survive only on living bacteria, presumably because they require some labile growth factor. The alternative explanation, that *E. coli* removes some inhibiting component, seems less likely because the *Herpetosiphon* filaments often creep far away from the *E. coli* streak. All our efforts to identify growth factors have been unsuccessful, although many possibilities have been tested (different vitamins, sugars, and amino acids; plant oil; chitin, catalase, cell fractions of *E. coli* and of cultivable *Herpetosiphon* strains; anaerobic and microaerophilic conditions; different agar concentrations, temperatures, pH values; illumination).

The freshwater strains can be grown on the same or on similar media as mentioned above. *H. geysericola* was cultivated on medium 2 (Lewin, 1970):

Medium 2 (Lewin and Lounsbery, 1969)

Casamino acids	0.1%
Na glycerophosphate	0.01%
Tris buffer	0.1%
KNO_3	0.01%
$CaCl_2 \cdot 2H_2O$	0.01%
$MgSO_4 \cdot 7H_2O$	0.01%
Thiamine	1 mg/l
Cyanocobalamin	1 μg/l
Agar	1%
Glucose	0.1% (added after autoclaving)

Adjust to pH 7.5.

Medium 2 was used as a standard medium for the cultivation of many different organisms; thus, not every component may be required by *Herpetosiphon* (e.g., thiamine).

For the sewage and sludge strains, either EC medium or ATCC medium 810 was used:

EC Medium (Enriched *Cytophaga* Medium: Pate and Chang, 1979)

Tryptone (Difco)	0.2%
Yeast extract (Difco)	0.05%
Na acetate	0.02%
Agar	1.5%

ATCC Medium 810 (Myxo 810 Medium)

Skim milk powder	0.5%
Yeast extract	0.05%
Agar	1.5%

The pH is not adjusted.

The seawater organisms grow well on medium 1 (Lewin, 1970):

Medium 1 (Lewin and Lounsbery, 1969)

Tryptone (Difco)	0.5%
Yeast extract (Difco)	0.5%
Na glycerophosphate	0.1%
KNO_3	0.05%
Agar	1%

In seawater.
The pH is adjusted to 7.0.

Most of the cultivable strains also grow in liquid media, in shake flasks, and in fermentors. A good medium for many strains is HP74 liquid medium. Other strains grow equally well or better in peptone-containing media, e.g., in CAS liquid medium or in MDl liquid medium.

HP74 Liquid Medium

Na glutamate	1%
Yeast extract (Difco)	0.2%
$MgSO_4 \cdot 7H_2O$	0.2%
Glucose (autoclaved separately as a 20% stock solution)	1%

Dissolve the ingredients in phosphate buffer (pH 6.5) at 1 mM. The buffer is autoclaved separately as a 50 mM stock solution.

CAS Liquid Medium

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH should be 6.8; do not adjust.

MDl Liquid Medium

Casitone (Difco)	0.3%
$CaCl_2 \cdot 2H_2O$	0.07%
$MgSO_4 \cdot 7H_2O$	0.2%
Cyanocobalamin	0.5 μg/ml

Trace element solution (see below) is added after autoclaving. The pH should be 6.8; do not adjust.

Casitone (Difco) may be replaced by other enzymatically digested casein peptones, but then the pH has to be adjusted to about 7.

Trace Element Solution

$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2$	20 mg
$CuSO_4$	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$ZnCl_2$	20 mg
LiCl	5 mg
$SnCl_2 \cdot 2H_2O$	5 mg
H_3BO_3	10 mg
KBr	20 mg
KI	20 mg
EDTA, Na-Fe_3^+ salt (trihydrate)	8 g
Water	1 liter

Sterilized by filtration, this solution is stable for months at room temperature due to its high EDTA content. The mix is used at 1 ml per 1 medium.

In agitated cultures the organisms may grow as homogeneous suspensions, but often the filaments stick together to form flakes or tight spherules. One of our strains, Hp a2 (DSM 589), forms flakes when inoculated into HP74 liquid medium; the cultures become completely uniform after a few days, but the organism forms flakes again upon transfer into fresh medium. Growth is not particularly fast: the doubling time is in the range of 15–20 h, and the cultures do not enter the stationary phase until after 4–6 days of cultivation (30°C, HP74 liquid medium, shake flasks). Wet-weight yields are in the order of 1–3 g/liter in shake flasks, and 2–4.5 g/liter in fermentors (HP74 liquid medium. The dry weight amounts to roughly 20% of the wet cell mass.

For the cultivation of *Herpetosiphon* from sewage and sludge, EC medium (as above, without agar) is recommended (Trick and Lingens, 1984; Senghas and Lingens, 1985). The organisms were grown at 25°C, their temperature optimum; and the doubling time was 20–25 h. The cell yield after 5–6 days was 2–3.5 g/liter and 0.5–1 g/liter (dry matter), respectively, with the two sets of strains. The phosphate optimum of the sewage strains was at 0.01%.

The marine species were grown in medium 1 (as above without agar). They require a salt concentration between 0.5-fold and twofold that of seawater (Lewin and Lounsbery, 1969).

All known strains are strictly aerobic. The temperature range differs with different strains. In general, the freshwater and marine strains seem to prefer lower temperatures, around 25°C, while the soil and some freshwater strains grow very well at 30°C and above. All soil strains tested by us grew at 38°C, and some strains still grew vigorously at 40°C (e.g., Hp g196). The sewage strains showed a temperature minimum at 15°C, a maximum at 37°C, and an optimum at 25°C. *H geysericola* comes from the vicinity of a hot spring but can be cultivated at 30°C (see "Habitats"). The optimal pH range is 7–7.5, although pH values of 8–9 seem occasionally be tolerated.

Preservation

Most *Herpetosiphon* strains survive on agar media at 30°C or room temperature for up to 3 months. Slant cultures, stored at 4°C, have been successfully subcultured after 14 months. Stock cultures should be transferred every 3–4 weeks. A good medium for such cultures is VY/2 agar.

Herpetosiphon strains may be preserved by any of the standard procedures. A convenient and reliable method is storage at −80°C. The bacteria are suspended in 1 ml of CAS or HP74 liquid medium and simply put into the deep freeze. Thawing should be fast, best accomplished by immersing the tube in cold water, and transfers to a suitable growth medium have to be made immediately after thawing. We have been able to reactivate such preserved cultures with no problems after 8 years of storage, the longest period tested. Alternatively, the bacteria can be frozen in liquid nitrogen. In one study, the filaments were suspended in growth medium (medium 1 or 2 as above, without agar), supplemented with 10% of either glycerol or dimethyl sulfoxide (DMSO) (Sanfilippo and Lewin, 1970). All five strains survived freezing and thawing with both additives. Only the glycerol preserves were tested again after 1 year, and all strains were still viable. When stored in growth medium without additives at −22°C, three out of four strains survived for 21 weeks (the longest period tested) and one for 6 weeks. Strains dried in skim milk could always be reactivated after storage for up to 12 years. We do not freeze-dry the organism itself, but only the skim milk: A few drops of a thick suspension

of *Herpetosiphon* filaments, taken from a young plate culture, are added to a plug of freeze-dried skim milk in an ampule. The plug absorbs the liquid without liquifying itself. The ampule is then dried at room temperature in a desiccator on a vacuum pump for several hours. After filling the desiccator with nitrogen gas, the ampules are sealed.

Characterization

The morphology of *Herpetosiphon* is so distinct that the bacterium can readily be recognized under the microscope. Only the phylogenetically related genus *Chloroflexus* has a similar appearance, but it is a thermophilic, facultative phototroph found only in hot springs. *Herpetosiphon* forms extremely long, unbranched, multicellular filaments of uniform diameter (Fig. 1). The filaments often measure 300 to more than 1200 μm in length. The cells in a filament cohere tenaciously so that the filament does not break easily. Unconstrained filaments in a liquid medium appear straight and stiff, but in fact they are extremely flexible and can sharply bend back and twist around themselves in a ropelike fashion or intertwine into plaitlike masses (Fig. 1). The width of the filaments varies with different strains from 0.5 to 1.7 μm, mostly between 0.7 and 1.2 μm, there is also considerable variation within the same culture (Fig. 2). Thus I have measured filament diameters between 0.7 and 1.6 μm in an 11-day-old culture of the type strain of *H. aurantiacus* on CY agar. In older cultures or under unfavorable conditions, the filaments often swell substantially and may become completely irregular in outline. In old cultures, the filaments also tend to become shorter, especially on peptone-containing media, simply because more and more cells die and the filaments snap at such sites. The fragments can be as short as one cell. In old cultures, particularly on agar, short, optically refractile, irregular filaments are often seen (Fig. 4). As those are the only structures left in old, drying, but still viable cultures, they appear to be desiccation-resistant resting forms. However, their germination has not been directly observed so far.

The filaments consist of cylindrical cells that have the same diameter as the filament, and they are so closely attached to one another that only exceptionally shallow notches can be seen at the surface. Under a microscope with a high resolution using phase or interference contrast, the cells can sometimes be seen in living filaments (Fig. 2). The septa become more conspicuous in older cultures, particularly when the organism is grown on a peptone-rich medium, like CY agar. In such filaments the intracellular membrane bodies (see below) can often also be recognized (Fig. 2). The cells become clearly distinguishable if the filaments are first dried to the slide and then stained, e.g., with Loeffler's methylene blue, crystal violet, or alcoholic fuchsin (rosaniline; Fig. 2). The lengths of the cells vary even within one filament, which suggests that the cells do not divide synchronously. The cell length may also vary with the culture medium, the culture age, and the strain. With many strains, cell lengths between 1.5 and 4.5 μm have been measured. The type strain of the genus appears to have especially long cells, measuring between 3 and 7, and sometimes 10 μm.

A most characteristic feature of *Herpetosiphon* is the presence at the ends of many filaments of transparent sections, which we call sleeves (Fig. 2). The sleeves have the same diameter as the filaments and are 1.4–5.5 μm, usually 1.5–3.5 μm long (the measurements are those of strain Hp a2). The interpretation of those sleeves is still controversial. Some investigators have regarded them as an indication of a sheath (Holt and Lewin, 1968; Skerman et al., 1977) or at least of a microsheath (Trick and Lingens, 1984). We prefer a different explanation (Reichenbach and Golecki, 1975): The filaments appear to multiply by breaking at the sites of necridia, i.e., dead cells along the filament. Such necridia can occasionally be observed in young cultures, and their number increases substantially with the age of the culture (Fig. 2). Their length (1.5–3.7 μm) is in the size range of ordinary cells. Trichome division by breakage at necridial cells is well known from other bacteria and has been described, e.g., for *Oscillatoria* (Lamont, 1969) and *Beggiatoa* (Strohl and Larkin, 1979). When a filament snaps, the empty cell wall cylinder of the necridium remains attached to the end of one daughter filament and gives rise to a sleeve. In strain Hp a2, the outer wall seems to rupture at one of the ends of the necridium near the cross-wall rather than across its middle, for the shortest and the longest sleeves correspond exactly to the lengths of the shortest and the longest cells. This would also explain why only some of the filaments' ends bear a sleeve, although it seems that the sleeves may also be shed; often empty sleeves can be seen lying in between the filaments. With the type strain of *H. aurantiacus,* on the other hand, the sleeves are exceptionally short, with a size distribution corresponding approximately to one-half that of the cell length, so that here the necridia seem to break preferentially in their center. There are,

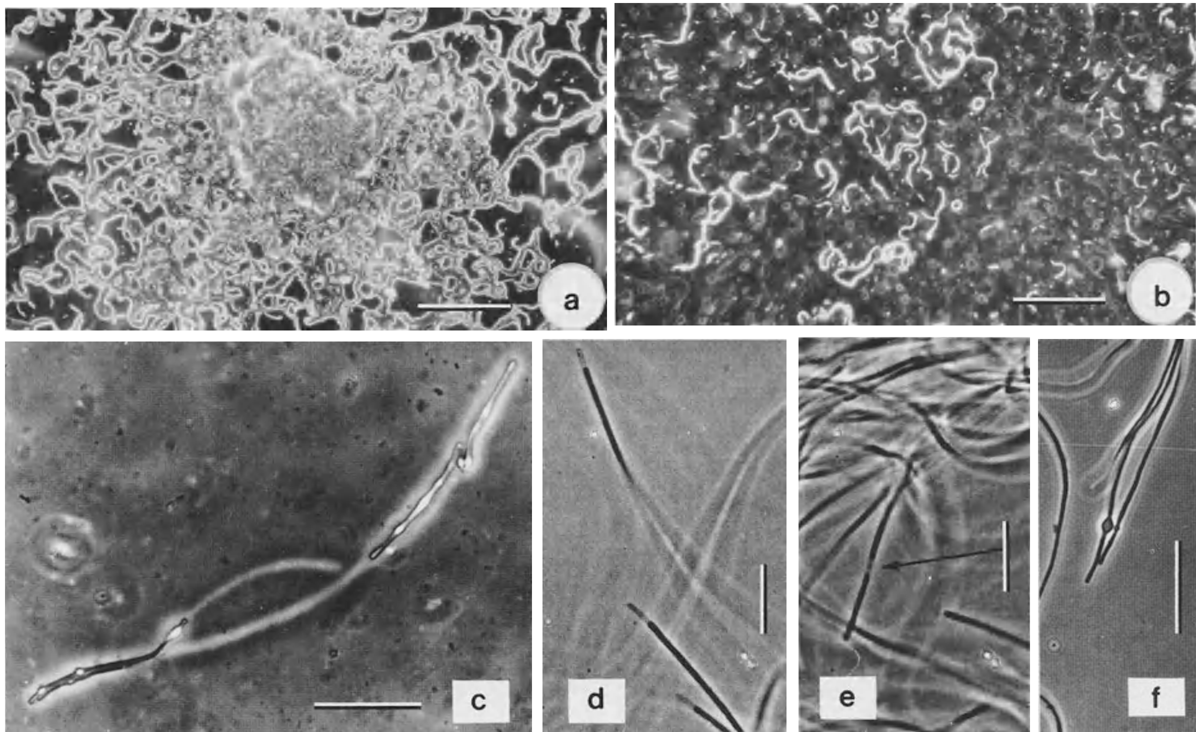

Fig. 4. Special structures of *Herpetosiphon aurantiacus.* (a to c) Desiccation-resistant filaments in a chamber culture. (a) and (b) Survey pictures; dark field; Bars = 200 μm: (a) 9 days old, with the filaments still essentially intact; (b) 32 days old, with only remnants of the filaments left. Bar = 200 μm. (c) At high magnification, the remaining segments of the old filaments appear irregular in outline and optically refractile; there are conspicuous slime tracks. Bar = 30 μm. (d) The empty sleeves that are visible here at the ends of many filaments are easy to recognize, even at a relatively low magnification, and thus help to quickly identify an isolate as a *Herpetosiphon.* Bar = 10 μm. (e) A necridium (arrow). Bar = 10 μm. (f) Bulbs. Bar = 20 μm. Zeiss Standard Microscope, (a) to (c). Zeiss Axiomat, (d) to (f); (c) to (f) in phase contrast.

however, at least six more arguments against the presence of a sheath: 1) The filament glides as a whole, with its empty end-pieces in position, which would seem unlikely if the cells were enclosed in a sheath. 2) There is no movement of cells within the filament, e.g., at the site of a necridium, nor a release of cells at the ends of the filament. This was observed by Holt and Lewin (1968), and also by the present author. But the literature is not in complete agreement on these points. Movement of a filament within its sheath has been described and "verified" by timelapse photography (Skerman et al., 1977); however, it is my impression that what was described as a sheath may really be a slime trail. Also, it was stated that single, gliding cells were released from the filaments (Brauss et al., 1969), but in this case it has not been ruled out that what was seen were simply short fragments that broke off the ends of the filament. 3) If the sleeve were a piece of a sheath, it would be difficult to understand why, as a rule, it does not exceed the length of a cell. 4) If a sheath were present, one could expect an occasional false branching of the filaments. 5) When a whole filament dies and decays, it falls apart into cell-sized pieces (Fig. 2). Obviously, there is no sheath to hold them together. 6) We have never been able to demonstrate anything resembling a real sheath under the electron microscope, with specimens prepared by freeze-etching, negative contrast, metal shadowing, or by thin-sectioning after several different fixation protocols (Reichenbach and Golecki, 1975; see also Gräf and Perschmann, 1970). This is also true for the type strain (Fig. 5). However, the thin, tubelike structure composed of fibrils, which has been described by several investigators and has been interpreted as a microsheath (Skerman et al., 1977; Senghas and Lingens, 1985; Holt, 1989), probably has a different explanation.

How can these differences of opinion about the existence of a sheath in *Herpetosiphon* be reconciled? Differences among strains can be ruled out, for we included the type strain in our studies and found no significant divergence from strain Hp a2. A sheath is a morphologically distinct structure, a hollow cylinder with

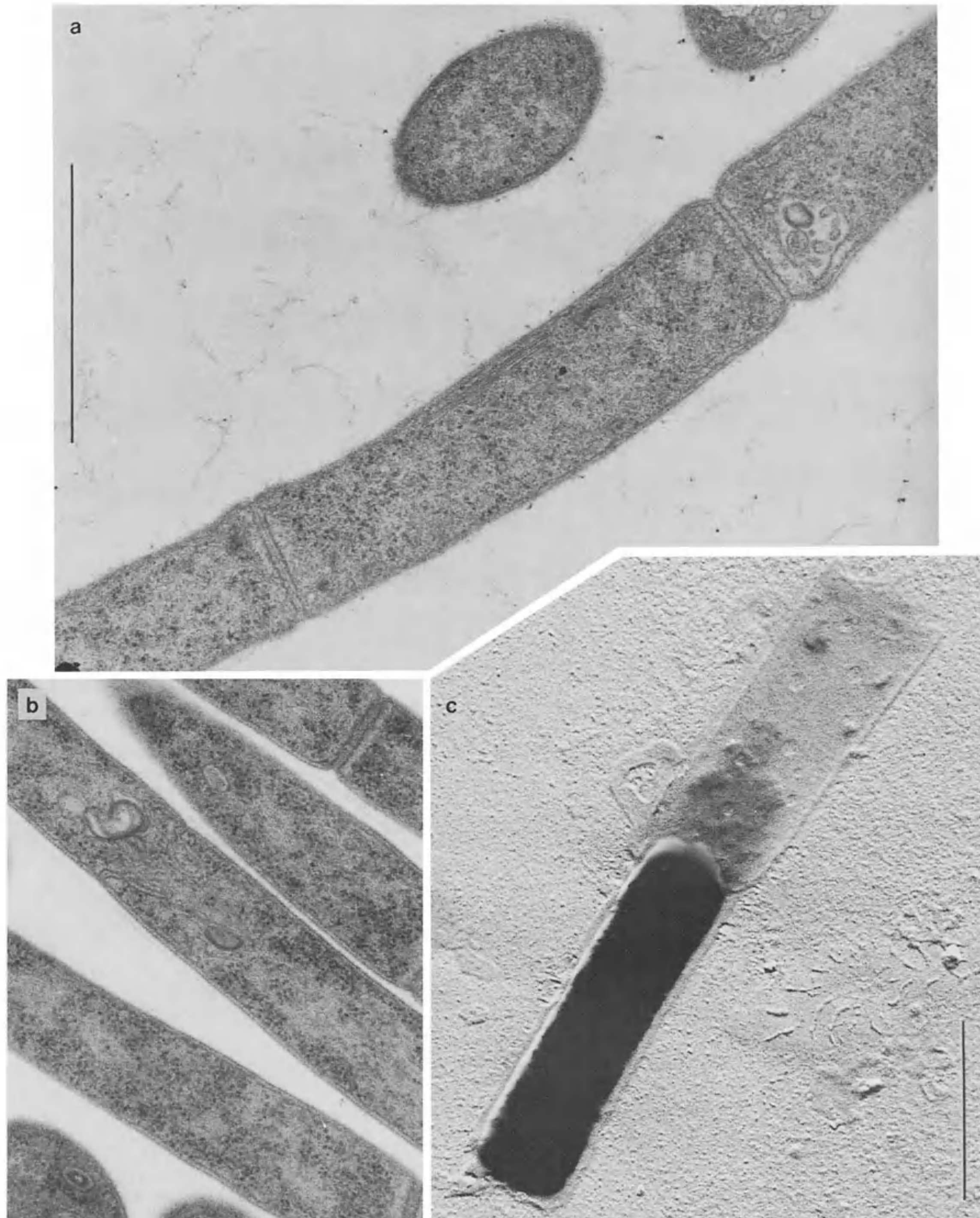

Fig. 5. Electron micrographs of the type strain of *Herpetosiphon aurantiacus.* (a) and (b) Ultrathin sections; the peptidoglycan layer can be clearly distinguished, but while there is plenty of extracellular slime material, no structure resembling a sheath can be located on the surface; also, the close juxtaposition of the filaments in (b) speaks against a sheath. Within the cells, membrane bodies can be seen. (c) Shadowed preparation; an end piece with an empty cell-wall cylinder, or sleeve, is visible; again there is no sign of a sheath. Zeiss EM 10B electron microscope. Bars = 1 μm; the magnification is identical for (a) and (b). (Courtesy of H. J. Hirsch.)

a discrete outer boundary, excreted by the cells and not covalently bound to their surface. Under the light microscope, a sheath should be clearly recognizable, e.g., after negative staining with nigrosin or India ink. Under the electron microscope, a sheath may be difficult to visualize in thin sections, because of poor contrast with the usual techniques, but with negative contrast or metal shadowing, it should be seen. Like all gliding bacteria, *Herpetosiphon* excretes slime, and slime fibrils can always be observed in the neighborhood of the trichomes, but this material is more or less diffuse. Under certain conditions this slime may condense, perhaps as a consequence of dehydration during fixation, and then it may appear as a dense tube. This is probably what has been taken as evidence for a sheath by various investigators (Skerman et al., 1977; Senghas and Lingens, 1985; Holt, 1989). Typically in all those cases cited, a wide gap opens between the filament and the thin, dense tube. More recently, efforts were made to demonstrate a sheath in the sludge organisms by first lysing the cells in the filament with lysozyme and then staining the remains with crystal violet. In that case, indeed, a long thin tubelike structure could be produced (Senghas and Lingens, 1985), but this still is not an unequivocal proof of a sheath, for the technique gives reliable results only if the organism has a normal bacterial cell wall.

Electron microscopy of thin sections reveals, however, that *Herpetosiphon* has an unusual cell wall structure (Reichenbach and Golecki, 1975). Outside the cytoplasmic membrane there is a well-defined peptidoglycan layer, 4–6 nm thick, but no outer membrane can be resolved. In fact, that membrane seems to be absent. Rather there is a thin additional layer, 21–25 nm thick, granular in thin sections, fibrillar in freeze-etch preparations, on top of the peptidoglycan. This layer follows the peptidoglycan into the shallow notches between adjoining cells, but not into the cross-septa where only the peptidoglycan layer can be distinguished. We have never found any other defined layer outside the fibrillar layer and in particular have found no layer running smoothly across the cell junctions, as would be expected for a sheath. This peculiar wall structure is corroborated by chemical analyses of the cell envelope (see below) and may be directly connected with the sheath problem. It appears that the additional layer is considerably more resistant to decomposition than the usual lipopolysaccharide-containing outer membrane, and it may give the peptidoglycan, to which it seems to be covalently bound, a higher stability.

Within the cells there are extensive intracellular membrane systems, some of which originate at the cross-septa, others from the cytoplasmic membrane along the longitudinal walls. They become especially conspicuous when the negative-contrast technique is applied. As mentioned above, under certain conditions they can even be seen in the light microscope.

In old cultures, the filaments are often locally inflated and form large, spherical structures, or "bulbs," with a diameter of 2.5–3.5 μm (Fig. 4; Reichenbach and Golecki, 1975; Trick and Lingens, 1984). Under the electron microscope a membrane body at the end of a septum-like stalk can always be seen within the bulb. Those bulbs seem to be a degeneration phenomenon, and outgrowth or further development has never been observed from them.

In wet mounts, *Herpetosiphon* filaments often can be seen to bend slowly and sometimes to oscillate back and forth. In contact with a suitable interface, i.e., one to which the organism can stick, like a glass or an agar surface, the filaments glide slowly and, during this movement, deposit characteristic slime tracks (Fig. 1). Gliding filaments often bend laterally to form hairpinlike loops (Fig. 1). In liquid media, the filaments apparently move along themselves. They may coil around themselves or one another and produce all kinds of loops and knots (Figs. 1 and 2).

The colonies, or swarms, usually spread quickly over agar surfaces and may completely cover a culture plate within a few days. On poor media, the migrating filaments remain more or less separate and appear in the form of characteristic curls and whirls (Fig. 3). Such colonies may become extremely delicate and are barely recognizable even if oblique illumination is applied. On media that allow good growth, dense and tough swarm sheets arise, with a rough, dry, peltlike surface. Sometimes the swarm surface is completely uniform, with an even, curly, or felt texture. But often elegant networks, massive concentric ridges, a pattern of large and small holes, or large, massive knobs are produced (Fig. 3). These last-mentioned knobs are particularly impressive and have been observed with many different isolates (Holt and Lewin, 1968; Brauss et al., 1969; Reichenbach and Golecki, 1975; Trick and Lingens, 1984). Their diameter varies between 200 and 2000 μm, and, as was noticed repeatedly, they strikingly resemble myxobacterial fruiting bodies, especially since they often are bright red or orange. But the filaments inside those knobs seem not to differ from those in other parts of the swarm, either morphologically or physiologically. Also, the ridges and knobs are not permanent struc-

tures, but shift continuously from place to place and may even dissolve completely again. This can be seen, e.g., in the movie mentioned earlier (Reichenbach et al., 1980). All those structures seem to originate from chance interactions of the long, migrating filaments that locally pull together, tangle, and pile up. The dynamics of the excreted, drying, and contracting slime may also be involved. Even more spectacular are long, sometimes branched, fingerlike protuberances rising up to 5 mm above the swarm surface (Fig. 3). They consist of interwoven filaments and seem to be produced only by certain strains.

Chemically, the cell wall of *Herpetosiphon* (*H. aurantiacus* type strain and strain Hp a2) consists of a peptidoglycan that contains L-ornithine in place of diaminopimelic acid, a very unusual character for a Gram-negative bacterium (Jürgens et al., 1989). A heteropolysaccharide composed of heptose, hexoses, pentoses, and *O*-methyl sugars appears to be covalently bound to the peptidoglycan via muramic acid-6-phosphate. This heteropolysaccharide is probably the material seen in electron micrographs as the granular/fibrillar layer on the surface of the longitudinal walls. The peptidoglycan-polysaccharide complex amounts to 20% of the cell dry weight, with a polysaccharide portion of 70%. No evidence for a lipopolysaccharide was found, which would explain the absence of an outer membrane in thin sections; nor was there evidence for the presence of additional sheath material. The cell wall of *Herpetosiphon* thus closely resembles that of *Chloroflexus,* in both its electron microscopic appearance (Pierson and Castenholz, 1974) and in its chemical composition (Jürgens et al., 1987; Meissner et al., 1988). Incidently, the filaments of *Chloroflexus* also often end with a sleeve, which for this organism also has been taken to suggest the existence of a sheath (Pierson and Castenholz, 1974). Hydrolysates of extracellular slime yielded arabinose and glucosamine as the main constituents (for sewage strains; Trick and Lingens, 1984).

The fatty acid composition has been determined for one of the sludge organisms and for strain Hp a2 (Senghas and Lingens, 1985). It is dominated by $C_{16:0}$, $C_{18:0}$, $C_{16:1}$, and $C_{18:1}$, while branched-chain fatty acids, which are so typical for many other gliding bacteria, are essentially absent. Hydroxy fatty acids are completely lacking. The sludge strain contained, in addition, substantial amounts of $C_{19:0}$ and 8-methyl $C_{17:0}$. The novel sulfonolipids, or capnoids, discovered in gliding bacteria of the *Cytophaga* group, were not found in *Herpetosiphon* (Godchaux and Leadbetter, 1983). The respiratory quinones are exclusively menaquinones (Kleinig and Reichenbach, 1977; Reichenbach et al., 1978; Senghas and Lingens, 1985). The main menaquinone in *H. aurantiacus* is MK-6, and 10% is MK-7.

The harvested cell mass and dense colonies on agar plates may be brightly colored in shades of yellow-orange to brick-red. But some strains are very pale or even totally unpigmented. The pigmentation also depends on the growth medium and is usually more intense with peptone-containing substrates. The pigments are cell bound. Chemically, they are carotenoids (Kleinig and Reichenbach, 1977; Reichenbach et al., 1978). The pigment patterns are remarkably uniform, at least with *H. aurantiacus,* the sewage strains, and the sludge organisms (Trick and Lingens, 1984; Senghas and Lingens, 1985), showing only quantitative but no qualitative variation. The dominant compound has a 4-oxo-α-carotene chromophore, with a hydroxyl in C-1′ to which a disaccharide consisting essentially of glucose is connected via a glycoside bond. One of the sugars bears an ester-bonded fatty acid (Fig. 6). Composite carotenoids of this type are well known from myxobacteria, but in these latter organisms only monosaccharides are found as sugar constituents. Carotenoids with a disaccharide component are unique so far among prokaryotic pigments.

It has been reported that *Herpetosiphon* (sewage and sludge strains) contain granules of poly-β-hydroxy-butyrate (Trick and Lingens, 1984; Senghas and Lingens, 1985), but analytical details have not been given, and I have not seen any evidence for such material either under the light microscope nor in a large number of electron micrographs, including those published by the authors themselves.

The protein patterns as revealed by SDS-electrophoresis correspond rather well between different *Herpetosiphon* types, and they appear to contain elements characteristic for the group as a whole as well as for the various types (Senghas and Lingens, 1985).

The DNA of *Herpetosiphon* of the *aurantiacus* type shows little variation in its content: 48 to 50 mol% (buoyant density). The marine strains range between 45 and 52 mol%. The *Herpetosiphon*-like strains isolated from bulking sludge (Senghas and Lingens, 1985) have a higher GC content: 59 mol%. The base sequence of the 5S rRNA of one of the sludge organisms (strain Wie 2) has been determined (van den Eynde et al., 1987). While the *Herpetosiphon* sequence corresponds, in general, quite well with the model of eubacterial 5S rRNA structure, there are several deviations—in two cases in highly conserved sites not modified in any

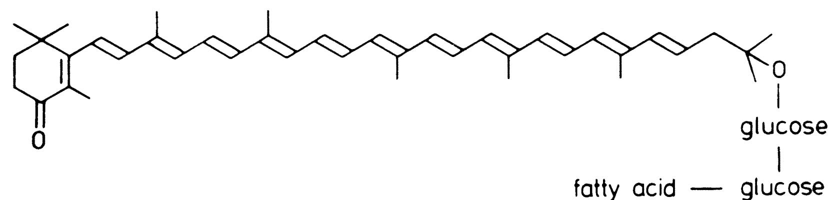

Fig. 6. Chemical structure of the main pigment of *Herpetosiphon aurantiacus*. (From Kleinig and Reichenbach, 1977.)

other eubacterium. Also, the 16S rRNA of *Herpetosiphon aurantiacus* has been completely sequenced (see "Taxonomy," this chapter) (Oyaizu et al., 1987). The unique multicopy single-stranded DNA (msDNA) common in gliding myxobacteria and also found in *Flexibacter* could not be demonstrated in *Herpetosiphon* (Dhundale et al., 1985).

The nutritional requirements, the physiology, and the biochemical capabilities of *Herpetosiphon* are not yet fully understood. *Herpetosiphon* is a strict organotroph, but because it is often able to grow on media very low in organic constituents, the results of substrate-utilization experiments must be interpreted with great care. There seem to be substantial differences among individual strains, and even more among the three main types that can currently be distinguished: 1) the *aurantiacus* group, including "*H. giganteus,*" *H. geysericola,* and the sewage strains (Trick and Lingens, 1984); 2) the *Herpetosiphon*-like organisms isolated from bulking sludge (Senghas and Lingens, 1985); and 3) the marine strains (Lewin and Lounsbery, 1969). The marine strains have not been further studied.

Although all known *Herpetosiphon* strains are aerobic, they seem to prefer microaerophilic conditions. Catalase is positive for the *aurantiacus* group and negative for the sludge and the marine strains. Oxidase is positive with *aurantiacus,* including the type strain for which it originally was claimed to be negative (Holt and Lewin, 1969); in our hands, however, young cultures on VY/2 agar give a strong positive reaction. The sludge organisms are oxidase negative and tolerate up to 2% NaCl, but the other terrestrial strains are rather sensitive to elevated salt concentrations.

It appears that many strains are able to grow on inorganic nitrogen sources (NH_4^+ or NO_3^-), but unequivocal data are often not available. Suitable N sources for the marine strains are tryptone, casamino acids, glutamate, and, for one strain, also nitrate. Nitrate reduction was shown for the sewage organisms, but only under anaerobic conditions, and it did not allow growth in the absence of molecular oxygen. Nitrogen fixation has been ruled out in all cases that have been investigated.

Early reports suggested that carbohydrate utilization by *Herpetosiphon* is poor, but we have found this not to be the case. The sewage strains utilize various hexoses, pentoses, and sugar alcohols as sole C sources, although without gas or acid production. Organic acids, with the exception of β-hydroxybutyrate, do not support growth. The sludge organisms grow on many different sugars and sugar alcohols, mostly with acid production. Some organic acids allow modest growth (H. Reichenbach, unpublished observations).

The *aurantiacus*-type organisms show impressive hydrolytic capabilities, while the sludge and the marine organisms are much less active in this respect. The low activity of sluice strains (Brauss et al., 1969) suggests that they may be similar to the sludge organisms. The data are summarized in Table 1. It should be understood that the results of those digestion experiments sometimes critically depend on the medium and the material used. In the case of pectin, a wide variety of preparations are available that give quite different results. With pectin from apple (38% methyl ester), I found pectin degradation by the (few) *Herpetosiphon* strains I tested. Also, in contrast to reports in the literature, I have found that virtually all *aurantia-*

Table 1. Degradation of biopolymers by *Herpetosiphon.*

Polymer	Organism		
	auranticus type	Sludge	Marine
Starch	+, rarely −	+	−
Chitin	+, some −	−	ND
Cellulose[b]	+, some −	−	ND
Pectin	−	−	ND
Xylan	+, some −	−	ND
Agar	−	ND	−
Esculin	+, some −	−	ND
Gelatin	+	−	ND
Casein	+, rarely −	−	+
Tributyrin	+	−	ND

+, positive; −, negative; ND, not determined.
[b]Crystalline cellulose in the form of filter paper or cellulose powder.

cus-type strains decompose xylan. Cellulose digestion is often seen among *aurantiacus*-type strains, but the type strain and strain Hp s2 are negative (H. Reichenbach, unpublished observations). *H. geysericola* decomposes cigarette paper (Lewin, 1970). In cultures on filter paper placed on a mineral salts agar like ST6 or ST21 agar, [in which $(NH_4)_2SO_4$ is replaced by 0.1% KNO_3], I have found that the filter paper is only very slowly attacked (recognizably after 20–30 days at 30°C). Cellulase activity can, however, quickly and reliably be detected on CEL1 agar (see below), on which large lysis zones are produced within 8–12 days, reaching far beyond the swarm colony. This suggests that cellulose decomposition by *Herpetosiphon* is achieved by diffusible exoenzymes (H. Reichenbach, unpublished observations).

CEL1 Agar

Cellulose powder	0.5%
$(NH_4)_2SO_4$	0.1%
Agar	1%

Adjust to pH 7.2. After autoclaving, the medium is poured as a thin layer on top of ST6 agar plates. A suitable cellulose powder, MN 300, is produced by Macherey and Nagel (Germany).

ST6 Agar

Part A:

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$MnSO_4 \cdot 2H_2O$	0.01%
$FeCl_3 \cdot 6H_2O$	0.02%
Trace elements	

The above ingredients are dissolved in one-third of the water volume.

Part B:

K_2HPO_4	0.1%
Yeast extract	0.002%
Agar	1%

The above ingredients are dissolved in two-thirds of the water volume.

After autoclaving, Parts A and B are combined and poured into plates.

Chitin decomposition becomes clearly recognizable on CT7 or CT agar (see Chapter 176, on *Lysobacter*). With many strains, I have observed DNA hydrolysis on DNA agar, but in some studies (sewage and sludge organisms) tests for DNase were negative. Some, but not all, strains show β-hemolysis (sheep and human erythrocytes; Gräf and Perschmann, 1970; Trick and Lingens, 1984).

Most investigators report the lysis of living and dead bacteria, but not all types of bacteria are equally sensitive. For instance, in contrast to many other enterobacteria, *E. coli* turned out to be particularly recalcitrant (Quinn and Skermann, 1980; H. Reichenbach, unpublished observations). Also, not all *Herpetosiphon* strains show identical lysis patterns. The sludge organisms do not lyse bacteria at all. The autoclaved yeast cells in VY/2 agar are often, but not always destroyed.

The H_2S, indole, acetoin, and phosphatase tests were negative (where looked for), the urease test was only rarely positive. Tyrosine in tyrosine agar is often degraded. It was observed that *Herpetosiphon* (*aurantiacus* type) is coprophilic, i.e., growth was stimulated by fecal extracts, but the specific factors responsible for this effect are not known (Gräf and Perschmann, 1970). Little is known about the antibiotic sensitivity of *Herpetosiphon.* The marine strains are inhibited by low concentrations of penicillin. I have found that practically all strains are resistant to high levels of kanamycin sulfate (250 mg/liter).

Taxonomy

Herpetosiphon is phylogenetically related to *Chloroflexus* and, on a much lower level, to *Thermomicrobium roseum.* This was deduced from 16S rRNA studies, specifically from analyses of oligonucleotide catalogs via binary association coefficients (S_{AB} values) or through oligonucleotide signatures (Gibson et al., 1985); a comparison of the complete base sequence has also been made (Oyaizu et al., 1987). The *Herpetosiphon*-like sludge organisms are clearly related, but not identical with *Herpetosiphon* (S_{AB} values with *Herpetosiphon*: 0.39, for *Chloroflexus*: 0.40, *Herpetosiphon/Chloroflexus*: 0.31). The four genera together form the phylum of the so-called green nonsulfur bacteria.

Among remarkable conclusions that can be drawn from these studies, are the conclusion of a very high age for the group. This high age is reflected in its unique base sequences, e.g., the one from base 607 to 630 which is identical in all eubacteria and archaebacteria but different in *Herpetosiphon.* The group shows a very deep branching point among the eubacteria, which is surpassed only by *Thermotoga.* As the cyanobacteria appear significantly younger, it seems clear that the earliest, 3500 million-year-old stromatolites may have been deposited by *Chloroflexus*-like organisms rather than by cyanobacteria, as had always been previously assumed. In this connection, the unusual cell wall structure of *Herpetosiphon* is also of interest, and it can perhaps be interpreted as a primitive structure. In contrast to the other members of the group, *Herpetosiphon* appears to be a rapidly evolving line, moving away from its ther-

mophilic ancestors. The high thermotolerance of many *Herpetosiphon* strains (growth at 40°C and greater) may reflect those roots.

Table 2 gives the distinguishing characteristics of the five presently recognized *Herpetosiphon* species and the *Herpetosiphon*-like organisms.

Considering the wide distribution of *Herpetosiphon* and its abundance in nature, these organisms must have been observed by many investigators, and in the older literature on indeed occasionally finds morphological descriptions that fit *Herpetosiphon.* A good candidate would be *Oscillatoria angusta* from lakes in central and northern Europe (Koppe, 1924,) later reclassified as *Achroonema angustum* (Skuja, 1956), although neither description mentions the typical sleeves. Since no strains were ever isolated and cultivated, their relation to *Herpetosiphon* can only be decided after a systematic study of the genus *Achroonema.*

As far as one can tell, the *H. aurantiacus* complex includes organisms previously called "*Flexibacter giganteus*" (Soriano, 1945; 1947), *Vitreoscilla proteolytica* (Gräf and Perschmann, 1970), the sewage *Herpetosiphon* (Trick and Lingens, 1984), probably *H. geysericola* (Lewin, 1970; see below), and perhaps also the organism isolated from a river sluice (Brauss et al., 1969; but see comment under "Characterization"). It also includes my own isolates labeled *H. giganteus* (Reichenbach and Golecki, 1975). The species epithet *giganteus* was never meant to name a new species but to replace *aurantiacus* in favor of the older *giganteus.* This does not imply, however, that all these organisms belong to only one species. While they are very similar in some respects, e.g., in their GC content, pigment, and menaquinone patterns, there are also substantial differences, e.g., in the diameter of the filaments, their enzymatic properties, and their growth requirements, which makes it likely that several species may be included in this assemblage.

Also standing on unstable taxonomic ground is the so-called "thermophilic" species *H. geysericola,* which was isolated from the vicinity of a hot spring and is not thermophilic but rather thermotolerant. Such thermotolerance, however, is not unusual for *aurantiacus*-type strains, which often grow very well at 38°C–40°C and probably even higher (H. Reichenbach, unpublished observations). From the published data only one clear difference between *H. geysericola* and *H. aurantiacus* can be seen, cellulose decomposition. Perhaps the cellulose-degrading *aurantiacus*-type *Herpetosiphon* would be better classified as *H. geysericola* (which then would become another misnomer). Originally it was suggested (Lewin, 1970) that *H. geysericola* is identical to *Phormidium geysericola* (Copeland, 1936), but this appears very unlikely, because that organism was found at the rim of a geyser and in various alkaline hot springs in Yellowstone, in water between 60 and 84°C. It thus is definitely a thermophile and would hardly grow at 30°C as does *H. geysericola.* Also, we know today that the bright orange mats found in alkaline hot springs in the western United States may contain a variety of filamentous gliding bacteria, such as *Chloroflexus aurantiacus* (Pierson and Castenholz, 1974) and *Heliothrix oregonensis,* which according to 5S rRNA analyses, may be another relative of *Chloroflexus* and *Herpetosiphon* (Pierson et al., 1985). There still may be un-

Table 2. Characteristics of the *Herpetosiphon* species and related organisms.[a]

Characteristic	*H. auranticus*	*H. geysericola*	*H. cohaerens*	*H. persicus/nigricans*	Sludge organisms
GC content (mol%)	48 (48–50)	48	45	52	59
Pigments	*auranticus* type	ND	Saproxanthin[b]	Saproxanthin/zeaxanthin[b]	*auranticus* type[c]
Fatty acids:					
$C_{19:0}$	−	ND	ND	ND	+
8-Methyl-$C_{17:0}$	−	ND	ND	ND	+
Catalase	+	+	−	−	−
Oxidase	+	ND	ND	ND	−
Seawater required	−	−	+	+	−
Hydrolysis of:					
Starch	+ (some −)	+	−	−	+
Cellulose	− (many +)	+	ND	ND	−
Casein	+ (some −)	ND	ND	ND	−

+, positive; −, negative; ND, not determined.

[a]Parentheses indicate characteristics of strains other than the type strain.

[b]Pigment extracts were examined spectrophotometrically, so that identification is preliminary only. *H. persicus* and *H. nigricans* seem to differ in their pigmentation.

[c]Pigments behave chromatographically and spectrophotometrically like those of *H. aurantiacus.*

known and unnamed species (Tayne et al., 1987). Apparently many of the filamentous organisms in hot springs previously described as blue-green algae (cyanobacterial) are in fact chlorophyll-free, because even when they are collected from the very same spring from which the species was originally reported, they often do not show the red fluorescence characteristic of chlorophyll (Brock, 1968).

The marine *Herpetosiphon* species are poorly understood. *H. persicus* can only be distinguished from *H. nigricans* by pigmentation, the latter producing a diffuse dark pigment on peptone medium, probably a melanin-like compound that may be of little taxonomic relevance (see Table 2). Obviously, more marine strains have to be isolated and characterized.

In conclusion, it is apparent that the taxonomy of *Herpetosiphon* is not yet settled, and further taxonomic studies based on larger collections of strains are needed.

Practical Aspects

The involvement of *Herpetosiphon* and related organisms in the formation of bulking sludge has been discussed above. Considering the hydrolytic capabilities of *Herpetosiphon aurantiacus,* it may be assumed that the bacterium participates in the degradation of macromolecules and, perhaps, in the control of microbial populations in sewage and in nature.

A screening for restriction endonucleases among gliding bacteria demonstrated the presence of such enzymes in nearly all *Herpetosiphon* strains tested (Mayer and Reichenbach, 1978). The enzymes seem to be located in the periplasm. A whole family of 17 enzymes has been more or less completely characterized with respect to their recognition and cleavage specificities (Brown et al., 1980; Kröger et al., 1984). All appear to be class II endonucleases, producing cohesive ends, either in the 5′-strand or, in the cases of endonucleases *Hgi* AI and *Hgi* JII, in the 3″-strands. Most interact with hexanucleotide sequences, and some with pentanucleotide sequences, but virtually all recognition sequences are degenerate. The availability of such a large family of endonucleases is of considerable theoretical interest because it allows a comparative study of the mechanisms of protein-DNA interaction as well as of molecular evolution. The known *Herpetosiphon* enzymes can be arranged in such a way that their recognition sequences overlap and the whole set thus becomes a continuous system (Kröger et al., 1984). One of the enzymes, *Hgi* EII, has a unique recognition sequence of 2 × 3 bases separated by six unspecific nucleotides. When the recognized bases are read in sequence, they give the recognition sequence of *Hgi* CI, and it has been proposed that enzyme *Hgi* EII originated by a inversion of the two enzyme subunits so that the recognition sites are now on the surface rather than in the center of the enzyme. Isoschizomeres can be found for almost all *Herpetosiphon* recognition sequences, e.g., *Sal* I is one to *Hgi* CIII and *Hgi* DII. Interestingly, no hybridization was found between *Sal* I gene probes and *Herpetosiphon* DNA, whereas two other producers of *Sal* I isoschizomeres, *Rhodococcus rhodochrous* and *Xanthomonas amaranthicola,* showed clear DNA homology under stringent conditions (Rodicio and Chater, 1988). While this suggests that the latter organisms and *Streptomyces albus* G have at least partially homologous enzymes, the high GC difference of 20 mol% between *Herpetosiphon* and *Streptomyces* could have obscured, through codon replacement, a structural relationship between their enzymes. A comparison of the base sequences of the genes, or of the amino acid sequences of the various enzymes should answer the question.

The role of *Herpetosiphon* and the *Herpetosiphon*-like bacteria in sewage disposal and sludge bulking has already been discussed.

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CHAPTER 210

The Genus *Verrucomicrobium*

HEINZ SCHLESNER

Introduction

Heterotrophic bacteria with multiple appendages (prosthecobacteria) have been grouped with the genera *Prosthecomicrobium, Ancalomicrobium,* and *Stella* (see Chapter 103 and 104). These genera are mainly defined by morphological criteria such as prosthecae shape, number, length, and location. The members of the genus *Prosthecomicrobium* exhibit 10 to 30 prosthecae per cell, which extend in all directions from the cell surface. They are typically shorter than 2 μm and conical in shape (Staley, 1984). *Ancalomicrobium adetum,* the only species in that genus, normally has less than eight prosthecae, which are tube-like and about 3 μm long (Staley, 1968). Cells of *Stella* are flat, six-pronged stars with prosthecae less than 0.5 μm long (Vasilyeva, 1985). No differentiation of the tips of the prosthecae has been observed. The GC content of the DNA of the above genera ranges from 65 to 73.5 mol% (Staley and Mandel, 1973; Staley, 1984; Vasilyeva, 1985).

New isolates of prosthecate bacteria with bundles of fimbriae extruding from the tips of the prosthecae were placed in a new genus *Verrucomicrobium* with the single species *V. spinosum* (Schlesner, 1987). This species has a significantly lower GC content (57.9–59.3 mol%), thus indicating a low degree of relationship to the previously mentioned genera. This was supported by the determination of the phylogenetic position of *V. spincsum* made by analyzing the 16S rRNA by oligonucleotide cataloging and reverse transcriptase sequencing (Albrecht et al., 1987). Similarity coefficients (S_{AB} values), calculated by comparing the RNase T_1 catalog of *V. spinosum* with about 460 catalogs of eubacterial strains, were found to be as low as those separating individual eubacterial phyla, indicating that *V. spinosum* represents a new division (Albrecht et al., 1987).

Habitats

Prosthecate bacteria with fimbriae have rarely been observed. Such bacteria occurred in an enrichment culture of Lake Plußsee water to which vitamin solution no. 6 (see below) was added (P. Hirsch, personal communication). Lake Plußsee is a small eutrophic lake near Plön, FRG. *V. spinosum* was isolated from Lake Vollstedter See (Holstein, FRG), a shallow (maximum depth, 2 m), eutrophic lake. One strain of *Verrucomicrobium* sp. came from the "Schrevenparkteich," a pond in a public park in Kiel, FRG. This pond is very eutrophic because numerous waterfowl live there and are fed by visitors to the park.

Enrichment and Isolation

Verrucomicrobium spp. can be enriched in Erlenmeyer flasks with 50 ml of enrichment medium over a sediment of $CaCO_3$.

Enrichment Medium Composition

The enrichment medium is composed of:

N-Acetylglucosamine	1.0 g
Hutner's basal salts (see below)	20 ml
Vitamin solution no. 6 (see below)	10 ml

Add distilled water to 1 liter. Adjust pH to 9.7. After autoclaving and cooling to room temperature, add $NaH_2PO_4 \cdot H_2O$ aseptically to a final concentration of 0.65 mM.

Hutner's basal salts (Cohen-Bazire et al., 1957)

Nitrilo triacetate (NTA)	10.00 g
$MgSO_4 \cdot 7\ H_2O$	29.70 g
$CaCl_2 \cdot 2H_2O$	3.34 g
$NaMoO_4 \cdot 2H_2O$	12.67 mg
$FeSO_4 \cdot 7H_2O$	99.00 mg
Metal salts solution "44" (see below)	50.00 ml
Double-distilled water	900.00 ml

The NTA is first dissolved by neutralization with KOH. The other salts are then added. Adjust pH to 7.2 with KOH or H_2SO_4. Adjust volume to 1 liter with double-distilled water. Store in the cold (5°C). The solution should be clear.

Metal salts solution "44"

Ethylene diaminotetraacetate (EDTA)	250.0 mg
$ZnSO_4 \cdot 7H_2O$	1095.0 mg
$FeSO_4 \cdot 7H_2O$	500.0 mg
$MnSO_4 \cdot H_2O$	154.0 mg
$CuSO_4 \cdot 5H_2O$	39.2 mg
$CoCl_2 \cdot 6H_2O$	20.3 mg
$Na_2B_4O_7 \cdot 10H_2O$	17.7 mg
Double distilled water	1 liter

To retard precipitation, add a few drops of H_2SO_4 before making to volume. Store in the cold (5°C).

Vitamin solution no. 6 (Staley, 1968)

Biotin	4.0 mg
Pyridoxine hydrochloride	20.0 mg
Thiamine hydrochloride	10.0 mg
Calcium pantothenate	10.0 mg
p-Aminobenzoic acid	10.0 mg
Folic acid	4.0 mg
Riboflavin	10.0 mg
Nicotinamide or nicotinic acid	10.0 mg
Vitamin B_{12}	0.2 mg
Double distilled water	1 liter

Stirring of the mixture improves solubility. Sterilize by filtration only. Store in the dark and cold (5°C).

Isolation of *Verrucomicrobium spinosum*

When subsequent microscopic checks indicate an increase in prosthecate bacteria, make streaks on the following agar-solidified medium M 13 (Schlesner, 1986):

Agar	18.0 g
Peptone	0.25 g
Yeast extract	0.25 g
Glucose	0.25 g
Hutner's basal salts	20 ml
Vitamin solution no. 6	10 ml
0.1 M Tris HCl, pH 7.5	50 ml
Artificial sea water (see below)	250 ml
Distilled water	670 ml

Artificial sea water (Lyman and Fleming, 1940)

NaCl	23.477 g
$MgCl_2$	4.981 g

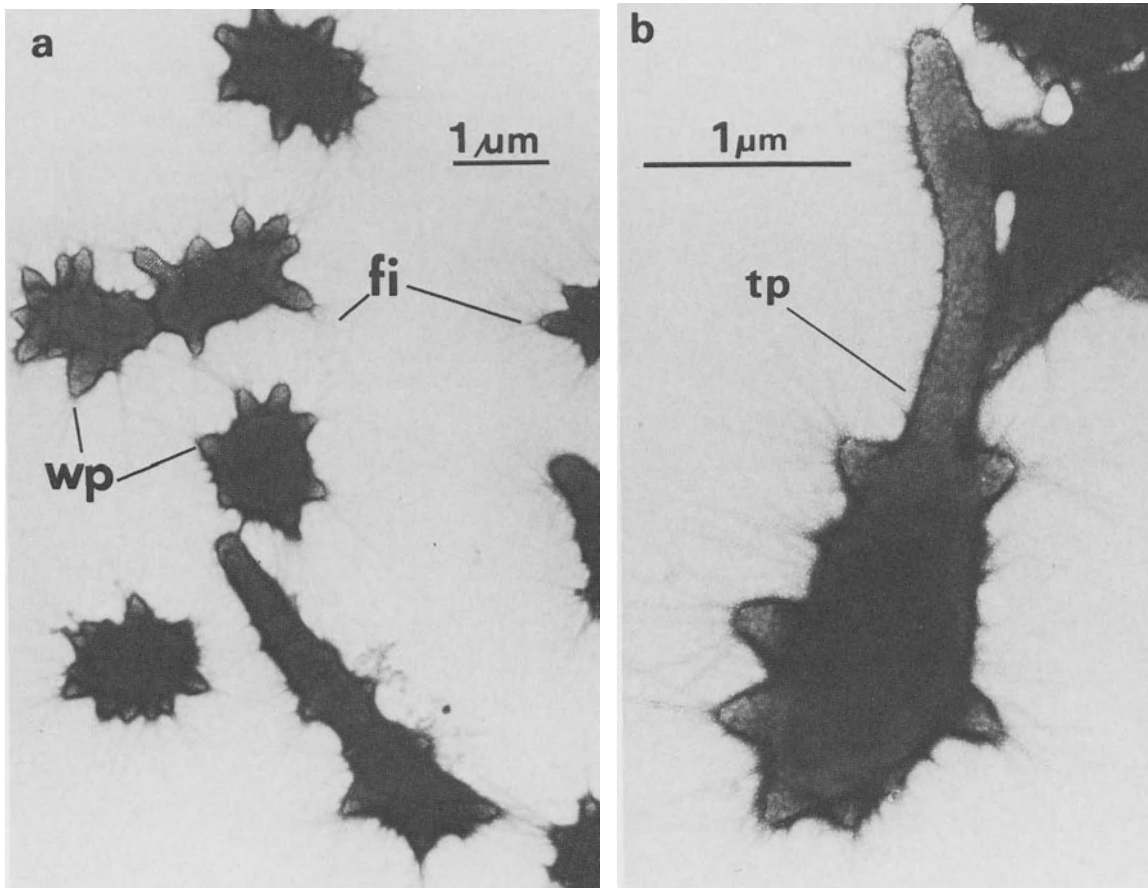

Fig. 1. Morphology of *Verrucomicrobium spinosum.* (a) Cells with wart-like prosthecae (wp), from the tips of which fimbriae (fi) extrude. (b) A cell with a polarly inserted tube-like prostheca (tp). (From Schlesner, 1987).

Na_2SO_4	3.917 g
$CaCl_2$	1.102 g
KCl	0.664 g
$NaHCO_3$	0.192 g
KBr	0.096 g
H_3BO_3	0.026 g
$SrCl_2$	0.024 g
NaF	0.003 g

Colonies that appear should be examined microscopically for the presence of prosthecate cells. This is conveniently done by applying the timesaving toothpick-procedure (Hirsch et al., 1977), which allows the examination of very small colonies: sterile wooden toothpick is stabbed into a colony and then on the agar surface of a petri dish containing the appropriate medium, thus inoculating the agar. To allow inoculation of a single petri dish with bacteria from a large number of colonies, a grid (with squares of about 5 mm in length) can be drawn with a marker on the bottom of the petri dish. After inoculation of the agar medium, the toothpick still generally contains enough bacteria to prepare a smear for microscopic examination. Three specimens can be prepared on one slide.

Identification

When studied with a phase contrast microscope, cells of *Verrucomicrobium* spp. resemble those of *Prosthecomicrobium* spp., i.e., the unicellular organisms have conical prosthecae extending in all directions from the cell surface. These prosthecae are about 0.5 μm long (Fig. 1a). Occasionally, one or two longer prosthecae (up to 2 μm) may occur, and often one of them is polarly inserted (Fig. 1b). Under the electron microscope, bundles of fimbriae of varying number and length extruding from the tips of the prosthecae are visible. The Gram-negative cells are nonmotile. Colonies on medium M 13 are light yellow. Growth of *V. spinosum* is optimal between 26 and 33°C and the maximum growth temperature is 34°C. The organism has a low salinity tolerance. Only 50% artificial sea water and 1% NaCl are tolerated. *V. spinosum* is facultatively anaerobic. Various sugars are fermented without gas formation, but nitrate is not reduced under anaerobic conditions.

Only a limited number of substrates can be utilized as sole carbon and energy source, mainly hexoses, di- or trisaccharides, and derivatives of glucose. One- and two-carbon compounds are not utilized, and neither are fatty acids or amino acids (Schlesner, 1987). Ammonia, urea, nitrate, and *N*-acetylglucosamine are suitable nitrogen sources. *V. spinosum* produces exoenzymes that can be demonstrated by the hydrolysis of gelatin and starch. However, casein and Tween 80 are not hydrolyzed. Catalase, cytochromeoxidase, phosphatase, and urease are produced, but neither H_2S (from thiosulfate) or NH_3 (from peptone) is produced. The cell wall contains *m*-diaminopimelic acid. The GC content of the DNA is 58.6 ± 0.2 mol% (T_m) for strain IFAM 1439^T = DSM 4136 = ATCC 43997.

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CHAPTER 211

The Order Thermotogales

ROBERT HUBER and KARL O. STETTER

The order Thermotogales is a unique group of extremely thermophilic microorganisms, phylogenetically distant from all other eubacteria. 16S rRNA analysis indicates that the Thermotogales represent the deepest branch and the most slowly evolving lineage within the kingdom of eubacteria (Achenbach-Richter et al., 1987; Woese, 1987; see also Chapter 1). A phylogenetic tree based on comparative studies of eubacterial DNA sequences of the elongation factor Tu is in good agreement with the 16S rRNA tree (Bachleitner et al., 1989).

The following common characters are uniform for the order Thermotogales: thermophilic, rod-shaped, anaerobic, fermentative eubacteria with an outer sheath-like envelope ("toga"); nonsporeforming; Gram-negative, but *meso*-diaminopimelic acid is not present in the peptidoglycan; lysozyme sensitive; inhibition of growth by molecular hydrogen; unusual long-chain dicarboxylic fatty acids present in the lipids.

Up to now, the Thermotogales are represented by only one family, the Thermotogaceae, which consists of the genera *Thermotoga* (Huber et al., 1986), *Thermosipho* (Huber et al., 1989), and *Fervidobacterium* (Patel et al., 1985; Huber et al., 1989).

Habitats

Members of the order Thermotogales are widespread and cosmopolitan. They thrive within active geothermal areas but they have not been found in any other sites. The Thermotogales occur in two different biotopes: 1) shallow and deep-sea marine hydrothermal systems; and 2) continental solfataric springs of low salinity. Up to now, they have only been isolated from samples from habitats of high temperature (55–100°C) and slightly acidic to alkaline pH (5–9).

Isolation

Selective Enrichment

To enrich members of the *Thermotoga maritima* group, starch-containing anaerobic seawater medium is used. Selective enrichment is carried out by incubation of the medium at a temperature of 85°C. Selective enrichment methods for *Thermotoga thermarum, Thermosipho,* and *Fervidobacterium* are not known. In contrast to thermophilic archaebacteria, media devoid of sulfur and hydrogen seem to favor growth of these latter eubacteria. Furthermore, carbohydrates, traces of yeast extract, and an incubation temperature of around 75°C are necessary for a successful enrichment.

Isolation Procedures

Various anaerobic culture media are inoculated with 0.5–1 ml samples, incubated at the appropriate temperatures, and examined microscopically over a period of around four weeks. After growth occurs in the enrichment cultures, the bacteria are transferred five times in sequence into fresh medium. The isolates are obtained by serial dilutions (three times) followed by plating (see below).

PLATING. For solidification, 1.5–2% agar is added to the media. After autoclaving, the plates are poured in the anaerobic chamber and dried for 24 hours in a closed pressure cylinder (Balch et al., 1979) at the temperature to be used later for incubation. Serial dilutions from 10^{-1} to 10^{-8} are plated in parallel. The gas phase in the cylinder is exchanged 10 times. The cylinder is then pressurized using the desired gas phase (150 kPa) and 2% H_2S (v/v) is added. The plates are incubated at the appropriate growth temperature (see above) and are examined for

growth at intervals of four days in the anaerobic chamber.

Cultivation

For the cultivation of the Thermotogales, strictly anaerobic growth conditions are necessary; the anaerobic technique described by Balch and Wolfe (1976) is suitable. For cell masses, the isolates are grown in batch cultures at the optimal growth temperature in 15-liter stainless steel fermentors and in 50- to 300-liter enamel-protected fermentors (HTE, Bioengineering, Wald, Switzerland). To increase the cell yields, the fermentors are gassed with nitrogen to get rid of inhibitory hydrogen which is produced by the bacteria.

PRESERVATION OF CULTURES. The bacteria are grown up to the stationary growth phase and are stored afterwards in the growth medium at 4°C. For all isolates, a transfer into fresh medium at three-month intervals is sufficient.

Identification and Physiological Properties

The Genus *Thermotoga*

Morphologically, these bacteria are rod-shaped cells with an average size of 5 μm in length and 0.6 μm in width. They are surrounded by a sheath-like outer structure, "the toga," ballooning over the ends (Figs. 1 and 2). This sheath, visible in all phases of growth, is composed of a regularly arranged outer membrane protein that may have porin-like function (Rachel et al., 1988). Nonmotile and motile species are known. On solid media, white, round colonies are formed. The pH optimum for growth is around 7, and growth occurs in a pH range between 5.5 and 9.0.

Fig. 1. Electron micrograph of a flagellated dividing cell of *Thermotoga maritima*. Bar = 1 μm.

Up to now, three species have been described, which can be placed in two different groups:

THERMOTOGA MARITIMA-NEAPOLITANA GROUP. *Thermotoga maritima* MSB8, DSM 3109 (Huber et al., 1986; Int. J. System. Bact., 1986), *T. neapolitana* NS-E, DSM 4359 (Belkin et al., 1986; Jannasch et al., 1988; Int. J. System. Bact., 1989), and 21 related isolates. This group is characterized by the ability to grow at both high salt concentrations (6.0% NaCl) and low salt concentrations (0.25% NaCl). The growth optimum is around 2.7% NaCl.

Members of this *Thermotoga* group are the most extremely thermophilic eubacteria presently known, with growth up to 90°C and an optimal growth temperature around 80°C. The minimum temperature for growth is around 55°C. *T. maritima* was originally isolated from a geothermally heated, marine sediment at Vulcano, Italy (Huber et al., 1986). The second species, *T. neapolitana* (Jannasch et al., 1988), was obtained from a submarine thermal vent at Lucrino near Naples, Italy (Belkin et al., 1986) and from continental solfatara fields from Lac Abbé, Djibouti, Africa (Windberger et al., 1989). Further members of this group were isolated from shallow marine habitats in Italy (Ischia), the Azores, Indonesia, Kolbeinsey (north of Iceland; depth: 106 m) and from deep-sea sediments from the East Pacific Rise (Guaymas Basin, Gulf of California; depth: 2000 m).

T. neapolitana can be distinguished from *T. maritima* on the basis of its nonmotile character, its rate of growth at optimum growth temperature (*T. maritima:* 75 min; *T. neapolitana:* 45 min), its 5%-lower GC content (*T. maritima:* 46 mol%), and their low DNA homology of around 25%.

These bacteria are fermentative microorganisms metabolizing a variety of carbohydrates, e.g., ribose, glucose, maltose, raffinose, starch, salicin, and cellulose. Main fermentation products on glucose are D(+)-lactate, acetate, CO_2, and H_2. H_2 is a potent inhibitor of growth and H_2 inhibition can be overcome by the addition of sulfur, but not by the addition of sulfate, sulfite, thiosulfate, nitrate, fumarate, or oxaloacetate. The bacteria produce H_2S in the presence of sulfur and under these conditions do not produce inhibitory H_2. The cells are able to fix molecular nitrogen and to use it as a nitrogen source (R. Huber and K. O. Stetter, unpub-

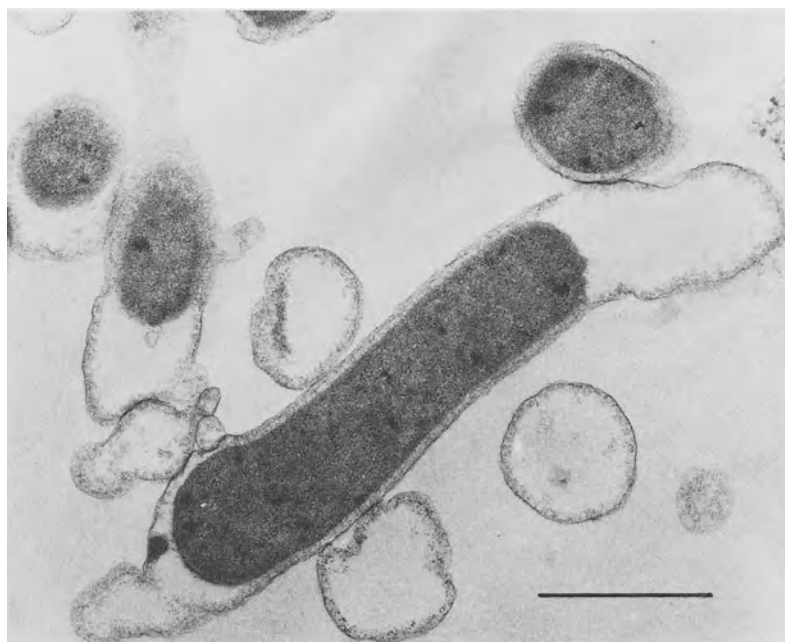

Fig. 2. Thin section of *Thermotoga maritima,* using lead citrate and uranyl acetate for contrast. Bar = 1 μm.

lished observations). Members of the genus *Thermotoga* possess a number of important features atypical for eubacteria: 1) the cells are insensitive to 100 μg rifampicin/ml, and the purified RNA polymerase is resistant to 1 μg rifampicin/ml; 2) growth is not inhibited by 10 μg/ml of aminoglycoside antibiotics and the purified ribosomes are also resistant to this antibiotics (Londei et al., 1988); 3) the peptidoglycan contains D- and L-lysine, which has never before been found in Gram-negative organisms; and 4) the majority of the lipids appear to be of novel structure; also, a new lipid structure was identified in *T. maritima* (Fig. 3; De Rosa et al., 1988).

Culture Medium for *T. maritima, T. neapolitana,* and Related Marine Isolates

The medium contains per liter of distilled water:

NaCl	6.93 g
$MgSO_4 \cdot 7H_2O$	1.75 g
$MgCl_2 \cdot 6H_2O$	1.38 g
KH_2PO_4	0.5 g
$CaCl_2$	0.38 g
KCl	0.16 g
NaBr	25 mg
H_3BO_3	7.5 mg
$SrCl_2 \cdot 6H_2O$	3.8 mg
KI	0.025 mg
$(NH_4)_2Ni(SO_4)_2$	2 mg
Trace mineral solution (see below)	15 ml
Resazurin	1 mg
Starch	5 g
Yeast extract (Bacto Difco)	0.5 g

Trace mineral solution (Balch et al., 1979):

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3 g
$MnSO_4 \cdot 2H_2O$	0.5 g
NaCl	1 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$ZnSO_4$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$KAl(SO_4)_2$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
Distilled water	1 liter

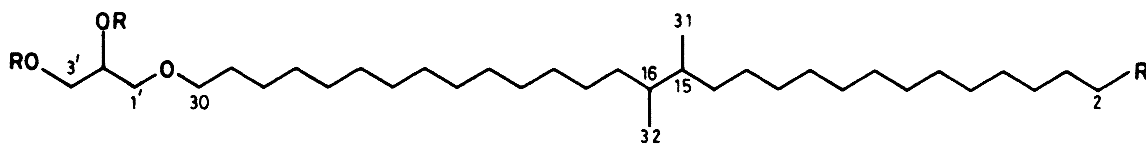

Fig. 3. A new 15,16-dimethyl-30 glyceryloxytriacontanoic acid from *Thermotoga maritima.* (Figure kindly provided by M. De Rosa and A. Gambacorta.)

In a stoppered glass bottle, 1 liter of the medium is flushed with nitrogen for 20 min, 0.5 g $Na_2S \cdot 9H_2O$ is added with a syringe, and the pH is adjusted to 7 by the addition of H_2SO_4. In an anaerobic chamber, the medium is placed in 28-ml serum tubes in portions of 10 ml each. The tubes are closed with rubber stoppers and on a gas station, a gas exchange is carried out (three times). N_2 as the desired gas is pressurized (300 kPa), and the tubes are autoclaved for 20 min. In order to establish the optimal ionic strength for growth, inorganic compounds of the medium are simultaneously increased or decreased by the same ratio.

THERMOTOGA THERMARUM GROUP. *Thermotoga thermarum* LA3, DSM 5069 (Windberger et al., 1989): this isolate is characterized by its ability to grow only at low salinity in a salinity range between 0.2% and 0.55% NaCl. The growth optimum is around 0.35% NaCl.

T. thermarum (Fig. 4) and two strains of *T. neapolitana* were isolated from continental solfatara fields at Lac Abbé, Djibouti (Africa). The isolation of these bacteria demonstrates for the first time the occurrence of the genus *Thermotoga* in low salinity environments also.

T. thermarum is able to ferment carbohydrates, but so far growth has only been observed in medium containing yeast extract. As for the other *Thermotoga* species, molecular H_2 is a potent inhibitor of growth. In contrast, *T. thermarum* is unable to form H_2S from S^0 and cannot grow on substrates when H_2 and S^0 is present in the culture medium. Furthermore, *T. thermarum* differs from all other known *Thermotoga* species by its lower upper temperature limit for growth (max. 85°C), its lateral flagellation pattern, and its high sensitivity to rifampicin (1 μg/ml). The RNA polymerase of *T. thermarum* exhibits subunits significantly smaller in molecular weight than that of the other members of the genus *Thermotoga.* Only partial immunological identity of the RNA polymerase is evident by spur formation of the precipitin lines with antibodies against the purified RNA polymerase of *T. maritima.* Using DNA-DNA hybridization, *T. thermarum* shows no significant homology against *T. maritima* and *T. neapolitana.*

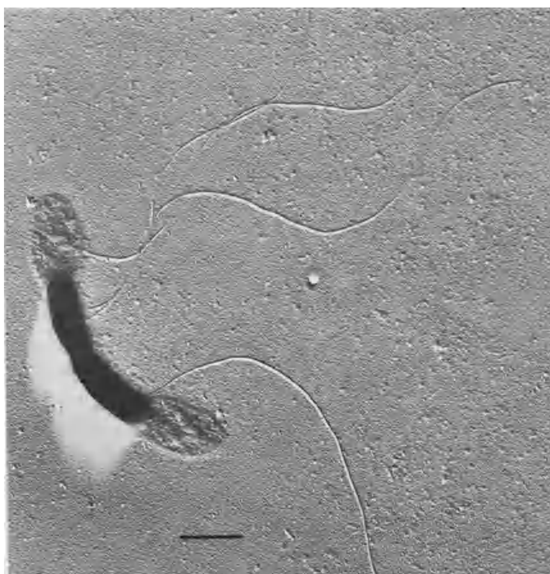

Fig. 4. Electron micrograph of a cell of *Thermotoga thermarum,* showing lateral flagellation. Bar = 1 μm.

Culture Medium for *T. thermarum*

NaCl	3.46 g
$MgSO_4 \cdot 7H_2O$	0.88 g
$MgCl_2 \cdot 6H_2O$	0.69 g
KH_2PO_4	0.5 g
$CaCl_2$	0.09 g
KCl	0.08 g
NaBr	12.5 mg
H_3BO_3	3.75 mg
$SrCl_2 \cdot 6\ H_2O$	1.9 mg
KI	0.006 mg
$(NH_4)_2\ Ni(SO_4)_2$	3 mg
Trace mineral solution	15 ml
Ethylenediaminetetraacetate-tetra-sodium-salt	0.9 g
Resazurin	1 mg
Starch	5 g
Yeast extract (Bacto Difco)	0.5 g
Distilled water	1 liter

The anaerobic culture medium is prepared in the same way as described for *T. maritima.* The trace mineral solution is the same as used for the cultivation of *T. maritima.*

The Genus *Thermosipho*

From marine hydrothermal springs and hot sandy sediments of the Gulf of Tadjoura southwest of Obock (Djibouti, Africa), three isolates of thermophilic eubacteria were obtained. Within the Thermotogales, they represent the genus *Thermosipho* with the type species *Thermosipho africanus* (Ob7; DSM 5309; Huber et al., 1989).

The rod-shaped cells (average size: 3–4 μm; width: 0.5 μm) possess a surrounding sheath with ballooning ends, reminiscent of members of the genus *Thermotoga.* In contrast to *Thermotoga,* however, *Thermosipho* can grow in chains (up to 12 rods) surrounded by the sheath (Fig. 5).

The taxonomic position of *Thermosipho* as a second genus within the Thermotogales is based on 16S rRNA sequencing analysis. The two 16S rRNAs of *T. africanus* (partial sequence deposited in the Gene Bank under accession no. M24022) and *Thermotoga maritima* are 89%

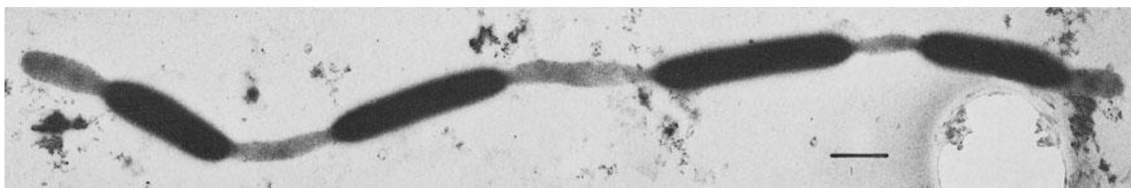

Fig. 5. *Thermosipho africanus.* Four cells within a tube-like sheath. Bar = 1 μm.

similar. Therefore, the bacteria are specific relatives on the level of two different genera. This taxonomic position is reflected moreover in a significant difference in the GC content of the two 16S rRNAs (*T. africanus:* 60 mol%; *T. maritima:* 63 mol%).

Thermosipho can be furthermore clearly distinguished from members of the genus *Thermotoga,* by its ability to grow at much lower temperatures (growth range: 35–77°C), a GC content of only 30 mol%, and an RNA polymerase which shows no serological cross-reaction with the same enzyme from *Thermotoga maritima.*

Thermosipho africanus grows within a pH range from 6.0 to 8.0, with an optimum around 7.2. The shortest doubling time is 35 min at the optimal growth temperature of 75°C. No growth is detected at 32°C or 80°C. Growth is observed between 0.11% NaCl and 3.6% NaCl.

Members of the genus *Thermosipho* are obligately heterotrophic bacteria, unable to grow on defined homogeneous carbon sources. For growth, the bacteria need complex organic materials such as yeast extract, peptone, or tryptone as well as cysteine. Molecular hydrogen inhibits growth but H_2 inhibition can be overcome by the addition of elemental sulfur. Under these conditions, H_2S is formed.

Since *T. africanus* grows at salt concentrations as low as 0.11% NaCl, members of the genus *Thermosipho* may possibly be present in nature also in low-salinity environments. *T. africanus* can grow even within the mesophilic temperature range (35°C: 15-h doubling time) and may therefore even exist in moderately heated environments like black soils and shallow waters.

Culture Medium for *T. africanus* and Related Isolates

The medium contains per liter of distilled water:

NaCl	18 g
$MgCl_2 \cdot 6H_2O$	4.30 g
$MgSO_4 \cdot 7H_2O$	3.45 g
KCl	0.34 g
NH_4Cl	0.25 g
$CaCl_2 \cdot 2H_2O$	0.14 g
K_2HPO_4	0.14 g
$(NH_4)_2Fe(SO_4)_2 \cdot 7H_2O$	2 mg
$(NH_4)_2Ni(SO_4)_2$	2 mg
Trace mineral solution	10 ml
Vitamin solution	10 ml
Sodium acetate	1 g
Yeast extract (Bacto Difco)	2 g
Trypticase peptone (Merck)	2 g
Resazurin	1 mg

Trace mineral solution (Balch et al., 1979):

The same trace mineral solution as for the cultivation of *Thermotoga maritima* is used.

Vitamin solution (Balch et al., 1979):

Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Thiamine hydrochloride	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
DL-Calcium pantothenate	5 mg
Vitam B_{12}	0.10 mg
p-Aminobenzoic acid	5 mg
Lipoic acid	5 mg
Distilled water	1 liter

The medium is prepared in the same way as described for *T. maritima.* As reducing agent, a mixture of 0.5 g $Na_2S \cdot 9\ H_2O$ and 0.5 g cysteine HCl is used. The pH is adjusted to 7.0 with formic acid, the gas phase consists of N_2/CO_2 (80:20; 300 kPa).

The Genus *Fervidobacterium*

The first thermophilic eubacterium known to be a relative of *Thermotoga maritima* (Woese, 1987) was an isolate, now called *Fervidobacterium,* obtained from a continental solfatara field at Hveragerthi, Iceland (strain H21; Huber et al., 1990). Comparison of 16S rRNAs indicates that this bacterium is related to the genus *Thermotoga* and *Thermosipho* at the rank of a third genus. The RNA polymerase of *Fervidobacterium* strain H21 shows no serological cross-reaction with the same enzyme of *T. maritima,* confirming that this isolate is related to *Thermotoga* above the species level.

The rod-shaped motile cells of *Fervidobacterium* (average size: 1.8 μm, width 0.6 μm) can be easily distinguished from those of *Thermotoga* and *Thermosipho* by phase contrast microscopy. *Fervidobacterium* cells do not produce a sheathlike outer structure, but they do tend to form additional spheroids (Figs. 6 and

7). These spheroids occur terminally, and only one spheroid is produced per cell (Figs. 6 and 7). In addition, the bacteria tend to form aggregates and, very rarely, show growth in chains or the formation of rotund bodies (Patel et al., 1985). In their ability to form spheroids, they resemble an eubacterial isolate from a New Zealand hot spring, which was described as *Fervidobacterium nodosum* (Rt-17B; ATCC 35602; Patel et al., 1985). Together with eight further isolates, these organisms were placed in the new genus *Fervidobacterium* (Patel et al., 1985), but a further taxonomic classification was not done. Comparison of the 16S rRNAs of isolate H21 and *F. nodosum* confirmed the presumption that H21 is related to the genus *Fervidobacterium.* Within this genus, however, H21 represents a new species, which is described as *Fervidobacterium islandicum* (isolate H21, DSM 5733; Huber et al., 1990). *F. islandicum* can be clearly distinguished from *F. nodosum* by a 6% higher GC content (*F. nodosum:* 33.7 mol%) and an insignificant DNA homology.

The optimum growth temperature for members of the genus *Fervidobacterium* is 65°C to 70°C, the maximum 80°C, and the minimum greater than 40°C. Growth occurs between a pH of 6.0 to 8.0 with an optimum at 7.0 to 7.5. The optimal doubling time of *F. islandicum* at optimal temperature is 150 min; for *F. nodosum,* doubling time is 105 min.

F. islandicum and *F. nodosum* ferment a variety of carbohydrates. The main products formed during growth on glucose are D(+)-lactate, acetate, ethanol, CO_2, and H_2. In addition, traces of *n*-butyric acid and *n*-valeric acid *(F. islandicum)* or isobutyric acid and isovaleric acid *(F. nodosum)* are formed.

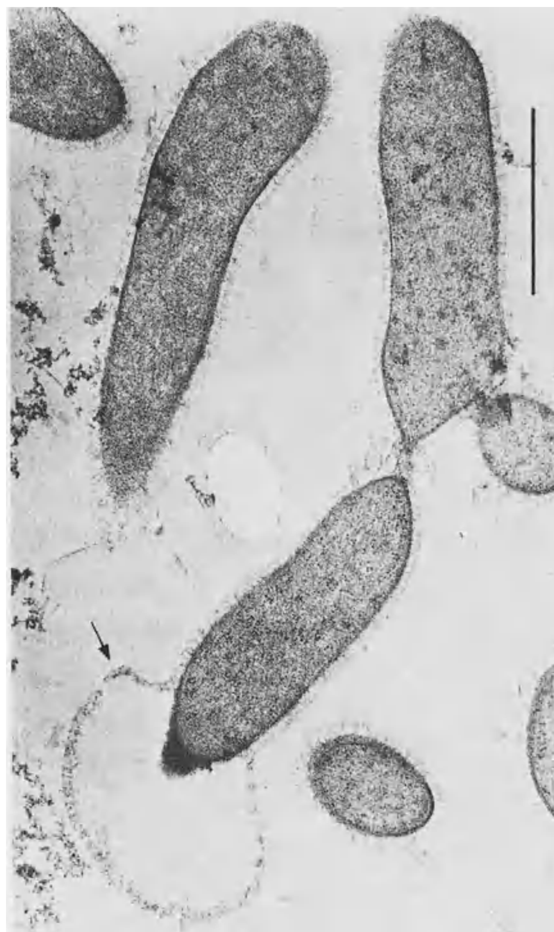

Fig. 7. Thin section of cells of *Fervidobacterium islandicum;* the arrow shows a spheroid. Bar = 1 μm.

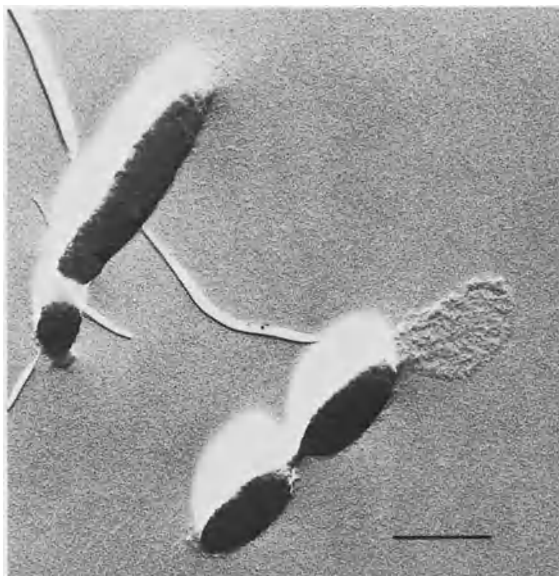

Fig. 6. Electron micrograph of dividing cells of *Fervidobacterium islandicum;* platinum-shadowed. Bar = 1 μm.

Both species can reduce elemental sulfur, forming H_2S, and the addition of molecular hydrogen inhibits growth. In contrast to *Thermotoga* and *Thermosipho, F. islandicum* and *F. nodosum* are unable to grow in the presence of hydrogen when sulfur is added to the medium.

Culture Medium for *F. islandicum* and *F. nodosum*

The medium contains per liter of distilled water:

NH_4Cl	0.90 g
$MgCl_2 \cdot 6H_2O$	0.20 g
KH_2PO_4	0.75 g
$K_2HPO_4 \cdot 3H_2O$	1.97 g
$FeSO_4 \cdot 7H_2O$	3 mg
Trace minerals (see below)	9 ml
Vitamin solution (see below)	5 ml
Glucose	5 g
Trypticase peptone (Merck)	10 g
Yeast extract (Bacto Difco)	3 g

Resazurin	1 mg

Vitamin solution (Balch et al., 1979):
The same vitamin solution as for the cultivation of *T. africanus* is used.

Trace minerals (Patel et al., 1985):

Nitrilotriacetic acid	12.5 g
$FeCl_3 \cdot 4H_2O$	0.2 g
$MnCl_2 \cdot 4H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.017 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$ZnCl_2$	0.1 g
$CuCl_2$	0.02 g
H_3BO_3	0.01 g
$NaMoO_4 \cdot 2H_2O$	0.01 g
NaCl	1 g
Na_2SeO_3	0.02 g
Distilled water	1 liter

The medium is prepared in the same way as described for *T. maritima.* 1 g $Na_2S \cdot 9\ H_2O$ per liter of medium is used as reducing agent. The pH is adjusted to 7.2 with H_2SO_4, the gas phase consists of N_2 (300 kPa).

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PART V

Symbiotic Associations

CHAPTER 212

Cyanobacterial Symbioses

HAINFRIED E. A. SCHENK

Introduction, Terminology, and Overview

The cyanobacteria appear to be an ancient class of organisms, well adapted to most habitats around the earth and often acting as pioneer "plants." They are a large and morphologically diverse group of prokaryotes, containing both unicellular and filamentous forms (some with specialized cells). However, sequence data of 16S rRNA have shown that the other major eubacterial taxa diverged significantly before the diversification of the modern photoautotrophic cyanobacteria (Giovannoni et al., 1988). The chloroxybacteria (prochlorophyta) are included in this chapter, because, in a dendrogram constructed by cluster analysis of either 5S or 16S RNA sequences, they are situated in a cluster containing all cyanobacteria as well as the Prochloron (Van den Eynde et al., 1988; Giovannoni et al., 1988). Obviously, the prochlorophytes branched off after the divergence of the cyanobacteria.

The cyanobacteria together with the prochlorophytes differ from all other prokaryotes by possessing a water-oxidizing photosynthesis like that of higher plants. In addition, many cyanobacterial strains possess an active nitrogenase, an oxygen-intolerant enzyme system. Nitrogen-fixing unicellular cyanobacteria combine within the same cell both photosynthesis, an oxygenic process, and nitrogen fixation, an oxygen-sensitive process. The filamentous nitrogen-fixing cyanobacteria, on the other hand, have solved this problem of oxygen sensitivity by segregation of the nitrogen-fixation apparatus in heterocysts, which are specialized cells with the capacity for nitrogen fixation. The heterocysts are semiheterotrophic and this ability for semiheterotrophic growth seems to be a prerequisite for intimate symbiotic associations (Korinek, 1928). The water-splitting photosynthesis system, in connection with excretion or loss of respiratory useful metabolites and/or the dinitrogen fixation with donation of amino-compounds to the partner, are the main reasons supporting the origin and development of a symbiotic association between cyanobacteria and other uni- or multicellular organisms.

How is the term "symbiosis" defined? Anton de Bary (1879) introduced this term with the phrase " . . . Zusammenleben ungleichnamiger Organismen. . . .," the (permanent or long-lasting) association of differently named organisms (two or more bionts). This broad definition includes both mutualistic and (in contrast to the colloquial usage) parasitic associations. Mutualistic relationships have also been called eusymbioses, and the parasitic ones dyssymbioses (Schaede, 1962; for further definitions of organismic associations, see Ahmadjian and Paracer, 1986; and Smith and Douglas, 1987). It is likely that all or most symbiotic partners are antagonists, and therefore there exist transition forms between eusymbioses and dyssymbioses. Also, in many associations, the relationships are not static and can change directions, depending on environmental conditions or endogenous factors, or on the ontogenetic phases of the symbiotic partners.

One can also distinguish between "ectosymbiosis," including episymbiosis, in which both partners have only external physical contact, and "endosymbiosis," in which one partner lives within the other. For the case that the endosymbiont exists extracellularly within the host soma, I propose the term "endosomabiosis" for extracellular endosymbiosis (see later, Table 2). As an endosomabiont, the guest can live either "intracavitously" (within cavities inside the host tissue), or "intercellularly" between cells in host tissue). If the endosymbiont lies intracellularly within the host cell, it is termed an "endocytobiont" (Schwemmler, 1979, 1989). Likewise, an intracellular endosymbiosis is termed an "endocytobiosis" (Schnepf, 1975). Besides more ecological relations, these four main forms of symbiotic associations (epibiotic, intracavitous, intercellular, intracellular) are found in cyanobacterial symbioses.

Pascher (1914) proposed the name "syncyanosis" for the symbiosis of cyanobacteria with other organisms. Later (Pascher 1929), he differentiated between "endocyanoses" (syncyanoses with intracellular cyanobacteria) and "ectocyanoses" (syncyanoses with extracellular cyanobacteria). The whole consortium was termed a "cyanome," and the symbiotic living cyanobacterium was termed a "cyanelle" (these terms have no taxonomic relevance). Geitler (1959) introduced a more restricted terminology: syncyanoses are symbiotic associations of cyanobacteria with regularly chromatophoreless unicellular host organisms (bacteria, flagellates, rhizopodes, apochlorotic algae, fungi, perhaps also ciliates). More recently, the term cyanelle (cyanella) has been used in a more restricted manner, referring only to unicellular endocytobiotic cyanobacteria (those "living intracellularly") (Reisser, 1984). In addition, it should be mentioned that today the term cyanelle is almost always (and preferably) used for plastid-like cell compartments that show a more or less clear descent from originally endocytobiotic cyanobacteria, but which are, like other eukaryotic cell organelles, unable to grow autonomously. However, it remains questionable whether it would not be better to follow Trench (1982) in applying the term cyanelle(s), or cyanella(e), for "any symbiotic cyanobacteria, without attempting to guess at or speculate on the level of integration achieved by the components of the association."

In this chapter, the term syncyanosis is used in the broadest way for all cyanobacterial symbioses, ectocyanosis for extracellular, and endocyanosis for intracellular syncyanoses. Thus the symbiosis of a cyanophilic lichen or the *Azolla-Anabaena*-symbiosis, would be considered as ectocyanoses, while the cyanome *Cyanophora paradoxa* and the *Geosiphon-Nostoc*-symbiosis may be examples of endocyanoses. The term "cyanobiont" is used for each symbiotic cyanobacterium which has or will have a taxonomic name. Likewise, the term "cyanelle" is used for each (preferable unicellular; Reisser, 1984), at least in principle taxonomically determinable eusymbiotic cyanobiont, which can live intracellularly, is often not virulent, and often no longer able to live outside the host cell. The term "cyanoplast" is reserved here for the intracellular inclusions (organelles) which are obviously derivable from cyanobionts, forming a "symbiogenetic organelle" (new term), characterized by the impossibility of an easy taxonomic classification, and, with regard to some or all of its own proteins, by a real "genetic dependence on the eukaryotic host nucleus" as demanded by Cavalier-Smith and Lee (1985). In the section on "Symbiogenetic Organelles" (see later), the term is used in this sense.

A final terminological question: Since the definition of Pascher (1914), the "main organism" is placed first in the name of a syncyanotic consortium (e.g., *Azolla-Anabaena*-symbiosis). The "main organism" is ordinarily considered to be the host. But who is the host in cyanophilic basidiolichens and in some cyanophilic ascolichens such as *Collema?* It is also apparently not clear in each case whether the "main organism" is an active or passive partner with regard to the establishment of the syncyanosis. In some cases, the cyanobacteria are obviously the actively invading partners. An overview on the broad spectrum of different syncyanotic consortia is given in Table 1. It is not the intention of the author to modify Pascher's nomenclature, but in view of the aims of this Handbook, the names of the symbiotic cyanobacterial strains are placed first in Table 1.

With the help of the references given in Table 1, the interested reader should be able to find reviews or relevant original papers, that will provide more information than that given in this chapter. Additionally, the reader is referred to the reviews of symbiotic cyanobacteria by Reisser (1984) and by Rai (1990b).

The description of interspecific associations in the present paper, is limited to rather mutualistic symbioses (eusymbioses) in which there is intimate contact between the partners (Tables 1 and 2). More ecologically epiphytic and pure ecological relations (see e.g., Gallucci and Paerl, 1983; Korinek, 1928; Mora-Osejo, 1977; Wickstrom and Castenholz, 1978) are usually not discussed in this review. Also not discussed are the known examples of entecy: cyanobacteria within the branchial chambers of marine lobsters (Jennings and Gelder, 1976), *Nostoc* in degraded cells of *Sphagnum lindbergii* and *S. riparium* (Granhall and Hofsten, 1976), nor cyanellae (Nostoc?) in the sporangium of *Andreaea rupestris* (Bryophyta) recently discovered by Filina and Filin (1989). Furthermore, only some very interesting ectocyanoses of epibiotic cyanobacteria on unicellular organisms shall be mentioned, as described by Pascher (1914) (see also Fritsch, 1952), e.g., to a distinct small cyanobacterium epibiotic on *Spirillum* or to the cyanobacterium *Chroostipes linearis* epibiotic on the monad *Oicomonas syncyanotica.* Also not discussed are such associations in which the cyanobacteria are hosts for still-smaller biological systems, such as viruses (cyanophages) (Barnet et al., 1981) and bacteria (Jensen and Bowen, 1970; Wilkinson, 1979; Wujek, 1979).

Table 1. An overview of syncyanotic consortia.

Syncyanosis	Reference[a]
Ectosymbioses and Endosomabioses (Ectocyanoses)	
Cyanophilic lichens	1
Phormidium-Codium-symbiosis	2
Nostoc-Hepaticae-symbiosis	3
Anabaena-Azolla-symbiosis	4
Nostoc-Cycadatae-symbiosis	5
Nostoc-Lemna-symbiosis	6
Nostoc-Gunnera-symbiosis	7
Prochloron-Ascidiaceae-symbiosis	8
Synechocystis-Ascidiaceae-symbiosis	9
Oscillatoria-Ascidiaceae-symbiosis	10
Nostoc-Diptera-symbiosis	11
Cyanobiont-Desmospongiae-symbiosis[b]	12
Endocytobioses (Endocyanoses)	
Richelia-Rhizosolenia-symbiosis	13
Richelia-Hemiaulus-symbiosis	14
Nostoc-Roperia-symbiosis	15
Phormidium-Oedogonium-symbiosis	16
Cyanobiont-Dinoflagellate-symbiosis[b]	17
Nostoc-Geosiphon-symbiosis	18
Nostoc-Trifolium-symbiosis	19
Cyanobiont-Malacophryidae-symbiosis[b]	20
Aphanocapsa-Calacarea-symbiosis	21
Aphanocapsa-Desmospongiae-symbiosis	22
Oscillatoria-Desmospongiae-symbiosis	23
Phormidium-Verongia-symbiosis	24
Synechocystis-Desmospongiae-symbiosis	25
Cyanobiont-Desmospongiae-symbiosis[b]	26
Prochloron-Anthozoa-symbiosis	27
Cyanobiont-*Tridacna*-symbiosis[b]	28
Cyanobiont-Echiurinea-symbiosis[b]	29
Symbiogenetic organelles ("Endocyanoses")	
Cyanelle in *Paulinella*	30
Cyanelle in Bacillariophyceae	31
Cyanoplast in Glaucocystophyceae[c]	32
Cyanoplast in Cyanidiaceae[c]	33
Attempt to induce artificial symbioses	34

[a]Reference citation: (1) Ahmadjian 1967, Rai 1990a, (2) Jacob 1961, Rosenberg and Paerl 1981, (3) Rodgers and Stewart 1977, Enderlin and Meeks 1983, Honegger 1980, Meeks et al. 1985, Meeks et al. 1988, Rai et al. 1989, Riedl 1977, Meeks 1990, (4) see section *Azolla-Anabaena,* (5) Lindblad and Bergman 1990, (6) Cohn 1972, (7) Bonnett 1990, (8) Duclaux et al. 1988, Griffiths and Luong-Van Thinh 1987, Lewin 1984, Lewin 1986, Morden and Golden 1989, Neveux et al. 1988, Olson 1986, Parry 1988, Parry and Kott 1988, Randall et al. 1987, (9) Cox et al. 1985, Lafargue and Duclaux 1979, (10) Larkum et al. 1987, (11) Dodds 1989, Kleinhaus and Kaiser 1988, (12) Rützler 1981, (13) Fogg 1982, Koray 1988, Mague et al. 1974, Martinez et al. 1983, Taylor 1982, Villareal 1988, 1989, Weare et al. 1974, (14) Kimor et al. 1978, (15) Reisser 1984, (16) Verma and Singh 1973, (17) Gaines and Elbrächter 1987, Zaika and Malinochka 1986, (18) see section *Geosiphon,* (19) Bhaskaran and Venkatamaran 1958, (20) Foissner 1980, Klaveness 1984, (21) to (26) Berthold et al. 1982, Rützler 1988, Stoddart 1989, Wilkinson 1980, 1983, 1987, Wilkinson and Evans 1989, Zimmermann et al. 1989, (27) Voskoboinikov et al. 1990, (28) Cowen 1988, (29) Kawaguti 1971, (30) to (33) see section "Symbiogenetic organelles," (34) Gusev and Korzhenevskaya 1990.

[b]The symbiont has not been identified.

[c]cyanoplast (new term, see Table 2 and text).

Table 2 gives an overview of all the cyanobacterial genera containing strains capable of forming syncyanoses. The third column shows abbreviations for the divisions of the eukaryotic partner (macrosymbiont) followed by the lowest taxon, which still comprises the symbiotic strains, species, or genera, of this division. It is not possible in all cases to discriminate clearly between ecto- and endocyanoses, e.g., in *Gunnera* and in some sponges. As shown in Table 2, members of all systematic groups of cyanobacteria are involved in ectocyanoses. But the Pleurocapsales and Stigonematales occur as cyanobionts only in ectocyanotic associations, and that exclusively with lichen fungi. Within the other ectocyanoses, the prochlorophytes (Chloroxybacteria) form a marine association (with ascidians), and the Chroococcales form two marine associations (besides aerophilic lichen associations). Only the Hormogonales have a wider spectrum of symbiotic partners (one association with a marine alga, some associations with land and freshwater plants, and one with invertebrates), although the lichen fungi here too are the dominant group of macrosymbionts. Within the endocytobioses (endocyanoses), the situation is different. Here we find members of many different host families, but only one fungus as partner and host organism *(Geosiphon).* All other endocyanoses, with the exception of the two endocytobioses of *Gunnera* and *Trifolium alexandrinum,* are marine. Only members of the Chroococcales and Hormogonales are found as endocytobionts (as mentioned above). The main cyanobacterial group seems to be that of the Hormogonales, but this impression may be changed in the future by further discoveries of intracellular symbioses. Section 4 in Table 2 lists those endocyanoses in which the original endocytobiotic cyanobacterium has reached the status of a eukaryotic cell organelle, a "symbiogenetic organelle" (new term, see above). These marine or freshwater organisms are all unicellular. Typical representatives are the Glaucocystophyta (Kies and Kremer, 1986b).

There have also been some attempts to construct artificial symbioses (Gusev, 1990). One idea is that cell reconstruction techniques (e.g., cell fusion, micromanipulation) can be used as models to study the putative formation of eukaryotic cells via endocytobiotic events (Bradley, 1979, 1983) using cyanobacteria or cyanoplasts (cyanelles) isolated from *Glaucocystis nostochinearum.* The application of these methods is also considered as one of the new ways for modification of plant cells, e.g., infection of protoplasts of higher plants, such as *Allium cepa, Daucus carota, Nicotiana tabacum,* and *Zea mays,* by Agafodorova et al., 1982 (with

Table 2. Cyanobacterial genera in natural symbioses (syncyanoses).

Cyanobacterial order	Genus	Symbiotic partner division[a]: class/order/family/genus	Number of host species
1. Episymbioses (ectocyanoses)			
Chroococcales:	*Chroostipes*	Chr: *Oicomonas*	1
2. Ectosymbioses and endosomabioses (Ectocyanoses)			
Chloroxybacteria:	*Prochloron*	Cho: Ascidiaceae	
Chroococcales:	*Aphanocapsa*	MAL: Lecanorales	
	Chroococcus	MAL: Lecanorales	25
	Gloeocapsa	MAL: Lecanorales	26
	Synechocystis	Por: Desmospongiae	
	Synechocystis	Cho: Ascidiaceae	
Pleurocapsales:	*Chlorococcidiopsis*	MAL: Lecanorales	3
	Hyella	MAL: Pleosporales	21
Hormogonales:	*Anabaena*	MAL: Lecanorales	1
	Anabaena	Pte: Filicatae	7
	Calothrix	MAL: Lecanorales	19
	Dichothrix	MAL: Lecanorales	20
	Nostoc	MAL: Lecanorales, Sphaeriales	22
	Nostoc	Bry: Hepaticae	12
	Nostoc	Bry: Sphagnaceae	1
	Nostoc	SpG: Cycadatae	40
	Nostoc	SpA: Gunnerales	5
	Nostoc	Art: Diptera	2
	Oscillatoria	Cho: Ascidiaceae	
	Phormidium	Chl: Siphonales	1
	Rivularia	MAL: Lecanorales	
	Scytonema	MAL: Lecanorales, Gyalectales, Erioderma	23
	Scytonema	MBL: Aphyllophorales	
Stigonematales:	*Stigonema*	MAL: Lecanorales, Spilonema	24
3. Endocytobioses (Endocyanoses)			
Chloroxybacteria:	*Prochloron*	Coe: Anthozoa (corals)	
Chroococcales:	*Aphanocapsa*	Por: Calacarea, Desmospongiae	
	Unidentified	Din: Dinophysidacea	
	Unidentified	Cil: Malacophryidae	1
	Synechocystis	Por: Desmospongiae	
	Unidentified	Ech: Echiurinea	
	Unidentified	Mol: Bivalvia	1
Hormogonales:	*Nostoc*	Chr: Bacillariophyceae	2
	Nostoc	Myc: Endogonaceae	1
	Nostoc	SpA: Gunnerales, *Trifolium*	
	Oscillatoria	Por: Desmospongiae	
	Phormidium	Chl: Ulotrichales	
	Phormidium	Por: *Verongia aerophila*	
	Richelia	Chr: Bacillariophyceae	3
	Unidentified	Por: *Phyllospongia lamellosa*	
4. Symbiogenetic Organelles			
	Cyanelle	Rhi: Thecamoeba	1
	Cyanelle	Chr: Bacillariophyceae	6
	Cyanoplast[b]	Gla: Glaucocystophyceae	14
	Cyanoplast[b]	Rho: Cyanidiophyceae	3 genera
	Cyanoplast[b]	Rho: Porphyridiaceae	2

[a]Abbreviations: Art: Arthropoda; Bry: Bryophyta; Chl: Chlorophyta; Cho: Chordata; Chr: Chrysophyta; Cil: Ciliata; Coe: Coelenterata; Din: Dinophyta; Ech: Echiurida; Gla: Glaucocystophyta; Mol: Mollusca; M(yc): Mycophyta (MAL: ascolichens; MBL: basidiolichens); Por: Porifera; Pte: Pteridophyta; Rhi: Rhizopoda; Rho: Rhodophyta; SpA: Angiospermatophyta; SpG: Gymnospermatophyta.

[b]Presumptive proposal with regard to the phylogenetic status of this plastid-like organelle within the given organismic group, formerly called Cyanelle.

Chlorogloea fritschii, Anabaena variabilis); Burgoon and Bottino, 1976 (with *Gloeocapsa*); Bradley and Duke, 1987 (with *Anabaena* sp., *Plectonema* sp., *Gloeothece* sp.); Cheong et al., 1986, 1987 (with *Nostoc muscorum*); Gusev et al., 1984 (with *Anabaena variabilis*), and Meeks

et al., 1978 (with *Anabaena variabilis*). Gusev et al. (1986) have also used whole plants for producing improved agricultural plants and transferring nitrogen-fixing ability from microorganisms into higher plants (*Anabaena variabilis* in the intercellular space of tobacco shoot regenerates). The results of these hopeful investigations have been rather disappointing as yet, because of the instabilities of the synthesized syncyanoses or of their low growth rates.

Ectosymbiosis and Endosomabiosis

In this section all the symbiotic consortia characterized by an ectocyanotic relationship of the symbiotic partners are described. The cyanobionts live extracellularly, in physical or chemical (e.g., mucilaginous shell) contact with the cell surface of the macrobiont. The manner of the symbiosis can be paraphrased as epibiotic or, if the cyanobiont is living inside a multicellular macrobiont, as endosomabiotic (see "Introduction"). It is not possible in all cases to discriminate clearly between ecto- and endocyanoses. In *Gunnera* and in sponges, both types of syncyanoses are usually found, e.g., in *Gunnera* cells the cyanobionts are surrounded with cell wall material of the plant cell. Therefore, *Gunnera* is included in this section (endosomabiosis), whereas the cyanophilic sponges, in which the cyanobionts are within the cytoplasm of the sponge cells, are placed among the endocyanoses. Some ectocyanoses are ecologically very successful and are mostly pioneers in their ecological niches. In this section, the cyanolichens and the *Azolla-Anabaena* association will be described. For information with respect to the other ectocyanoses the reader is directed to the literature cited in Table 1.

Cyanophilic Lichens

Lichens constitute those symbiotic associations between fungi (serving as morphological hosts but metabolic guests) and algae or cyanobacteria as extracellular symbionts (serving as metabolic hosts) in which a new morphological entity is formed, a stable thallus or body of specific structure (different from the original growth form of both partners). This definition rules out all loose lichen-like associations (Ahmadjian, 1965). The morphological transformation (morphogenesis) during the association is controlled primarily by the fungus, the mycobiont, rather than the alga, the phyco- or photobiont (see e.g., Honegger, 1984). Despite the fact that lichens are not a separate systematic taxon, they are classified into three subdivisions (Hawksworth, 1988a, Eriksson and Hawksworth, 1988), into orders, families, genera, and species (in parentheses, nomenclature after Henssen and Jahns, 1974): 1) Ascomycotina (Ascomycetidae) with 80 (43) families and 13,250 lichenized species: 2) Basidiomycotina (Holobasidiomycetidae) with four (three) families and 50 lichenized species; and 3) Deuteromycotina with 200 lichenized species. Within the fungal subdivision of the Mastigomycotina, with more than 1,100 species, only one is lichenized. (The mastigomycete [phycomycete] species *Geosiphon pyriforme,* in a non-lichen-like association with endocytobiotic cyanobacteria, is considered in an own section in this chapter.) Taxonomically, both the myco- and the phycobiont have binary Latin names, but, unofficially, only the name of the fungus is used for the name of the related lichen (Int. Code for Botanical Nomenclature, article 13, 1983). There are about 13,500 (Hawksworth and Hill, 1984; Galun, 1990) to 15,000 (Ahmadjian and Paracer, 1986) named species of lichen-forming fungi. Interestingly, only approximately 30 different types of unicellular or simple filamentous algae and cyanobacteria are reported as lichen-photobionts: one species of the Xanthophyceae, one of the Phaeophyceae, 22 genera of the Chlorophyceae, and 11 to 16 cyanobacterial genera (see Tables 4 and 5). Within 45 (11) orders, representing the 84 (46) known families of lichen fungi, only 18 (14) families of ascolichens and two families of basidiolichens are recorded that include cyanophilic species (Table 3).

Most of the families 7 (10) with cyanophilic species are observed within the order Lecanorales. Thwaites (1849) was the first to regard the unicellular "gonidia" of some members of the Lichinaceae as identical with free-living cyanobacteria; later, Schwendener (1869) identified some phycobionts of the Lichinaceae as species of the cyanobacterial genera *Gloeocapsa* and *Chroococcus.* Since that time, many other families and species of cyanobacteria in lichens have been found about all in ascolichens (Table 4), only few in basidiolichens (Table 5). Although the lists in Table 4 (and considerably less in Table 5) are incomplete, they allow one to estimate the distribution of the different cyanobiont genera within the lichens. Most of the lichen cyanobionts are filamentous (Tables 4 and 5) and belong to the Hormogonales to the common heterocystous genera *Nostoc* and *Scytonema.* In homoiomerous lichens (where the symbionts are distributed more or less uniformly throughout the thallus), the structure of the symbionts is similar to that in culture. In

Table 3. Taxonomy of lichen families (Eriksson and Hawksworth 1988), including the cyanophilic species, and their cyanobacterial type.

Order	Family	Cyanobacterial type[a]
Opegraphales[b]	Opegraphaceae	u, 1
Gyalectales	Stictidaceae[c]	f, 1
Lecanorales	Arctomiaceae	f, 1
	Coccocarpiaceae	f, 1
	Collemataceae	f, 1
	Heppiaceae	u, f, 1
	Pannariaceae	f, 2C
	Peltulaceae[d]	f, 1
	Stereocaulaceae	u, f, (2)C
Lichinales	Lichinaceae	u, f, 1
	Pyrenothrichaceae	f
	Ectolechiaceae	f
Peltigerales	Nephromataceae[e]	f, (C)
	Peltigeraceae	f, 2C
	Placynthiaceae	f
	Lobariaceae[f]	f, 2C
Dothideales[g]	Arthopyreniaceae	u
Uncertain order[h]	Protothelenaceae[i]	f
Aphyllophorales[k]	Clavariaceae	f, (2)
	Corticiaceae[l]	f, 1

[a]Abbreviations: u: unicellular cyanobiont; f: filamentous cyanobiont; 1: the cyanobacterium is the sole phycobiont; 2: the cyanobacterium is the second phycobiont (C: in a cephalodium); (1), (2), or (C) (in parentheses): the occurrence of the given symbiontic type is not obligate for the whole family.
[b]Formerly (Henssen and Jahns 1974) within the Arthoniales.
[c]Formerly (Henssen and Jahns 1974) within the Gyalectaceae.
[d]Formerly (Henssen and Jahns 1974) within the Heppiaceae.
[e]Formerly (Henssen and Jahns 1974) within the Peltigeraceae.
[f]Formerly (Henssen and Jahns 1974) Stictaceae.
[g]Formerly (Henssen and Jahns 1974) Pleosporales.
[h]Formerly (Henssen and Jahns 1974) within the Sphaeriales.
[i]Formerly (Henssen and Jahns 1974) Microglaenaceae.
[k]With the only known cyanophilic basidiolichen families.
[l]Now Dictyonemataceae (see, e.g., Slocum and Floyd 1977).

contrast, in heteromerous lichens (where the symbionts are positioned in a discrete layer, the "symbiont layer"), the filaments of the symbionts are often contorted, appearing as packets. About half of the cyanophilic lichens also contain (eukaryotic) algal symbionts. Here, the algae are mostly found in the symbiont layer and the cyanobacteria are in cephalodia.

The true identity of the cyanobionts has not been established for many cyanophilic lichens, as we can see regarding the new taxonomic identification of the symbiotic cyanobacteria of some members of the lichen family Lichinaceae or the observation of cyanobiont diversity in the Lichinaceae and Heppiaceae, recently reported (Büdel and Henssen, 1983; Bubrick and Galun, 1984; Büdel, 1985). The interaction between the symbionts results in an organism which is mostly morphologically and physiologically distinct from either of the components (Green and Smith, 1974). Cyanobacterial symbionts are often altered to the extent that their origin becomes completely obscured (Marton and Galun, 1976). For example, the symbiotic *Nostoc* of *Nephroma laevigatum* appears in the intact thallus as enlarged, shapeless, and usually separated cells but upon isolation and culturing, *Nostoc* undergoes remarkable modifications and reverts to its typical free-living, filamentous growth form (Kardish et al., 1989). Recent publications have clearly demonstrated that it is not sufficient to identify lichen phycobionts solely on microscopic observations within the lichen thallus. It is essential, therefore, when making a taxonomic determination, first to separate the phycobiont from the mycobiont and to cultivate the isolated phycobiont. By growing free of the mycobiont, the phycobiont is able to change its lichen phenotype to the phenotype of the free-living form. In this state, it may be possible to classify the isolated strain with regard to morphology, fission pattern environmental requirements, physiology, and chemotaxonomical criteria, as a basis for a taxonomic determination. (For definitions of cyanobacterial genera see Rippka et al., 1979; and Chapters 97 and 98. For lichen biology, especially cyanophilic lichens, see the following books and reviews: Ahmadjian, 1965, 1967, 1973; Ahmadjian and Paracer, 1986; Galun, 1988; Hale, 1983; Henssen and Jahns, 1974; Ozenda and Clauzade, 1970; Poelt, 1969; Rai, 1990a; Wirth, 1980).

The basidiolichens (Table 5) represent only a very small group of tropical and subtropical lichens in contrast to the thousands of ascolichens, but their biology is diverse (Oberwinkler, 1970) and seems highly evolved in some fungus species (like *Dictyonema*). The evolution of these organisms may have led from a parasitic to a symbiotic (and partly endosymbiotic) association (Oberwinkler, 1980). The *Dictyonema* association shows a dense hyphal sheath around the cyanobacterial *Scytonema* filaments and an intracellular "central hypha" as an "endosymbiotic organ" (Oberwinkler, 1980). This group contains the only known case of symbiotic eukaryotic guests in prokaryotic host cells.

Lichens occur in most terrestrial habitats, from the equator to the polar regions, but very few species occur in permanently submerged habitats. Only seven truly submerged marine lichen species have been found e.g., the cyano-

Table 4. Cyanobacterial phycobionts and genera or species of cyanophilic ascolichens (incomplete list).

Cyanobacterium	Reference[a]	Properties[b]	Ascolichen[c]	Fungal family[d]
Chroococcales				
Anacystis montana	2	u, e2, F	*Peltula*	Peltu
Aphanocapsa		u, e2, F	*Stereocaulon*	Ster
Chroococcus sp.		u, e, M	*Halographis runica*	Opeg
Chroococcus sp.		u, e, F	*Lichinella*	Lich
Chroococcus sp.		u, e, F	*Phylliscum*	Lich
Chroococcus sp.		u, e, F	*Pterygiopsis*	Lich
Chroococcus sp.		u, e, F	*Cryptothele*[e]	Lich
Gloeocapsa alpina		u, e, A	*Synalissa violacea*	Lich
G. kuetzingiana		u, e, A	*Psorotichia* sp.	Lich
G. kuetzingiana	2		*Pyrenopsis fuliginoides*	Lich
G. kuetzingiana	2		*P. leprosa*	Lich
G. kuetzingiana	2		*Synalissa symphorea*[f]	Lich
G. muralis		u, e, A	*Thyrea pulvinata*[g]	Lich
G. muralis		u, e, A	*Collemopsis schaereri*[h]	Lich
G. pleurocapsoides	2	u, e,	*Phylliscum endocarpoides*	Lich
G. rupestris	2	u, e, F	*Anema moedlingense*	Lich
G. rupestris		u, e, F	*Peccania coralloides*	Lich
G. rupestris	2	u, e,	*Phylliscum endocarpoides*	Lich
G. rupestris	2	u, e,	*Collemopsis schaereri*[h]	Lich
G. sanguinea	2	u, e, A	*Pyrenopsis fuliginoides*	Lich
G. sanguinea	2	u, e, A	*P. grumulifera*	Lich
G. sanguinea	2	u, e, A	*P. rhodosticta*[i]	Lich
G. sanguinea	2	u, e, A	*Synalissa acharrii*	Lich
G. sanguinea	2	u, e, A	*S. phylliscina*	Lich
G. sanguinea		u, e, A	*S. symphorea*[f]	Lich
Gloeocapsa sp.	2	u, e, A	*Gloeoheppia*	Hepp
Gloeocapsa sp.	3	u, e, F	*Jenmania goebelii*	Lich
Gloeocapsa sp.	2	u, e, F	*Phylliscidium*	Lich
Gloeocapsa sp.		u, e, F	*Phylliscum*	Lich
Gloeocapsa sp.	2	u, e, F	*Synalissa conferata*	Lich
Gloeocapsa sp.	2	u, e, F	*S. symphorea*	Lich
Gloeocapsa sp.	3	u, e, A	*Gonohymenia*[j]	Lich
Gloeocapsa sp.	2	u, e, F	*Anema notarisii*[k]	Lich
Gloeocapsa sp.	2	u, e, F	*Thyrea pulvinata*[g]	Lich
Gloeocapsa sp.		u, e2c, A	*Stereocaulon*	Ster
G. as *Xanthocapsa*	2		*Anema*	Lich
G. as *Xanthocapsa*	2	u, e, A	*Gonohymenia*	Lich
G. as *Xanthocapsa*	2		*Peccania*	Lich
G. as *Xanthocapsa*	2	u, e, A	*Psorotichia*	Lich
G. as *Xanthocapsa*	2	u, e, F	*Thyrea*	Lich
Gloeothece		u, e,	*Arthopyrenia*	Arth
Gloeothece		u, e,	*A. areniseda*	Arth
Gloeothece		u, e,	*A. subareniseda*	Arth
Microcystis		u, e,	*Arthopyrenia* sp.	Arth
Pleurocapsales				
Chroococcidiopsis	1	u, e, F	*Anema nummularium*	Lich
Chroococcidiopsis	1	u, e, F	*Peccania* sp.	Lich
Chroococcidiopsis	1	u, e, F	*Psorotichia* sp.	Lich
Hyella caespitosa		f/u, e, M	*Arthopyrenia halodytes*	Arth
Xanthocapsa: see syn. *Chroococcidiopsis* and *Gloeocapsa*				
Hormogonales				
Anabaena	4	f, e2c, A	*Stereocaulon* spp.	Ster
Calothrix sp.		f, e, F	*Calotrichopsis ins.*	Lich
Calothrix crustacea		f, e, F	*Lichina confinis*	Lich
Calothrix pulvinata		f, e, F	*L. confinis*	Lich
Calothrix sp.	7	f, e, F	*L. polycarpa*	Lich
Calothrix sp.		f, e, F	*L. pygmaea*	Lich
Calothrix sp.	7	f, e, F	*L. rosulans*	Lich
Calothrix sp.	7	f, e, F	*L. tasmanica*	Lich

(*continued*)

Table 4. *Continued*

Cyanobacterium	Reference[a]	Properties[b]	Ascolichen[c]	Fungal family[d]
Calothrix sp.	7	f, e, F	*L. willeyi*	Lich
Calothrix sp.		f, e, F	*Porocyphus* sp.	Lich
Dichothrix sp.	3	f, e, F	*Lichina* sp.	Lich
Dichothrix orsiniana		f, e, F	*Placynthium nigrum*	Plac
Nostoc commune		f, e, F	*Collema tenax*	Coll
N. muscorum		f, e, F	*Collema* sp.	Coll
N. laevigatum	7	f, e	*Nephroma laevigatum*	Neph
N. linckia	7	f, e,	*Peltigera praetextata*	Pelt
N. punctiforme		f, e2c, A	*P. aphtosa*	Pelt
N. punctiforme		f, e, A	*P. canina*	Pelt
N. punctiforme		f, e, A	*P. erumpens*	Pelt
N. punctiforme	6	f, e, A	*P. evansiana*	Pelt
N. punctiforme		f, e, A	*P. horizontalis*	Pelt
N. punctiforme		f, e, A	*P. limbata*	Pelt
N. punctiforme		f, e, A	*P. malacea*	Pelt
N. punctiforme		f, e, A	*P. polydactyla*	Pelt
N. punctiforme	6	f, e, A	*P. praetextata*	Pelt
N. punctiforme		f, e, A	*P. pruinosa*	Pelt
N. punctiforme		f, e, A	*Peltigera* sp.	Pelt
N. punctiforme	6	f, e, A	*P. venosa*	Pelt
N. punctiforme		f, e, A	*P. virescens*	Pelt
N. sphaericum		f, e, F	*Collema* sp.	Coll
N. sphaericum		f, e, F	*Hydrothyria venosa*	Pelt
N. sphaericum	5	f, e, F	*Leptogium* sp.	Coll
N. sphaericum		f, e, F	*L. issatschenkii*	Coll
N. sphaericum		f, e, F	*Pannaria*	Pann
Nostoc sp.	3	f, e, F	Arctomiaceae	Arct
Nostoc sp.	3	f, e, F	Collemataceae	Coll
Nostoc sp.	3	f, e, F	*Lempholemma*	Lich
Nostoc sp.	6	f, ec, A	*Lobaria amplissima*	Loba
Nostoc sp.	6	f, ec, A	*L. laetevirens*	Loba
Nostoc sp.	6	f, ec, A	*L. pulmonaria*	Loba
Nostoc sp.	6	f, e, A	*L. scrobiculata*	Loba
Nostoc sp.	6	f, e, A	*L. virens*[1]	Loba
Nostoc sp.	5		*Massalongia* sp.	Pelt
Nostoc sp.		f, e, F,	*Microglaena* sph./le.	Prot
Nostoc sp.	6	f, ec, A	*Nephroma arcticum*	Neph
Nostoc sp.	6	f, e, A	*N. parile*	Neph
Nostoc sp.	3	f, e, F	Pannariaceae	Pann
Nostoc sp.	5	f, e, F	*Parmeliella*	Pann
Nostoc sp.	5	f, e, A	*Polychidium musc.*	Plac
Nostoc sp.	6	f, e2c, A	*Solorina crocea*	Pelt
Nostoc sp.	6	f, e2c, A	*S. saccata*	Pelt
Nostoc sp.		f, e2c,	*Stereocaulon*	Ster
Nostoc sp.	5	f, e, A	*Sticta* sp.	Loba
Nostoc sp.	6	f, e, A	*S. fulginosa*	Loba
Nostoc sp.	6	f, e, A	*S. limbata*	Loba
Rivularia: see *Calothrix*				
Scytonema sp.	3	f, e+I, A,	*Coccocarpia*	Cocc
Scytonema sp.	3	f, e+I, A	*Erioderma*	Pann
Scytonema sp.		f, e+I, A	*Heppia lutosa*	Hepp
Scytonema sp.	3	f, e+I, A	*Koerberia*	Plac
Scytonema sp.		f, e2c,	*Lasioloma*	Ecto
Scytonema sp.	3	f, e+I, A	*Lichinodium ahlneri*	Lich
Scytonema sp.		f, e2c	*Lopadium*	Ecto[n]
Scytonema sp.	4	f, e	*Massalongia*	Pelt
Scytonema sp.		f, e, F	*Pannaria*	Pann
Scytonema sp.	3	f, e+I, A	*Parmeliella*	Pann
Scytonema sp.		f, e+I, A	*Peltula euploca*	Peltu
Scytonema sp.	5	f, e+I, A	*Petractis clausa*	Stic
Scytonema sp.	3	f, e+I, A	*Polychidium*	Plac

(*continued*)

Table 4. *Continued*

Cyanobacterium	Reference[a]	Properties[b]	Ascolichen[c]	Fungal family[d]
Scytonema sp.	4	f, e+I, A	*Pyrenothrix nigra*[m]	Pyre
Scytonema sp.	3	f, e+I, A	*Pyrenothrix riddlei*[m]	Pyre
Scytonema sp.	4	f, e2c	*Stereocaulon*	Ster
Scytonema sp.	3	f, e+I, A	*Thermutis*	Lich
Scytonema sp.	3	f, e+I, A	*Vestergrenopsis*	Plac
Scytonema sp.	3	f, e+I, A	*Zahlbrucknerella*	Lich
Stigonematales				
Fischerella				
(*Hyphomorpha*)[o]				
Mastigocoleus	7	f, e,	*Arthopyrenia*	Arth
Mastigocoleus	7	f, e,	*A. sublitorales*	Arth
(*Sirosiphon*)[p]				
Stigonema		f, e	*Ephebe lanata*	Lich
Stigonema		f, e	*Spilonema*	Cocc
Stigonema		f, e2c	*Stereocaulon*	Ster

[a]Reference: without a number—Ahmadjian 1967, (1) Büdel and Henssen 1983, (2) Bubrick and Galun 1984, (3) Henssen and Jahns 1974, (4) Ozenda 1970, (5) Poelt 1969, (6) Smith and Douglas 1987, (7) other authors.

[b]Abbreviations: A, aerob; c, cyanobiont is located in cephalodia; e, extracellular cyanobacterium; f, filamentous cyanobacterium; F, also freshwater habitat; I, fungus with intracellular haustoria within the cyanobiont; M, marine habitat; u, unicellular cyanobacterium; 2, cyanobiont is the secondary phycobiont; abbreviation in parentheses, see [d] (below).

[c]Other cyanophilic lichen genera are *Collemopsidium, Epiphloea, Euopsis, Leciophysma, Lemmopsis, Leprocollema, Nylanderopsis, Physma, Pseudocyphellaria, Pseudoleptogium, Pyrenocollema, Rechingera, Siphulastrum* and *Xanthopyrenia.*

[d]The families of the fungi are abbreviated, e.g., Hepp = Heppiaceae. See Table 3 for a list of families.

[e]Formerly *Pyrenopsidium.*

[f]Formerly *S. ramulosa.*

[g]Formerly *Pyrenopsis pulvinata.*

[h]Formerly *Pyrenopsis schaereri* = *Psorotichia schaereri.*

[i]Formerly *P. sanguinea.*

[j]Formerly *Thallinocarpon.*

[k]Formerly *T. notarisii.*

[l]Formerly *Nephroma laetevirens.*

[m]*Pyrenothrix,* was formerly *Lichenothrix.*

[n]Family uncertain.

[o]Identified as *Stigonema,* see *Spilonema.*

[p]Syn. is *Stigonema.*

Table 5. Cyanobacterial phycobionts and genera or species of cyanophilic basidiolichens.[a]

Cyanobacterium	Properties[a]	Basidiolichen[b]	Fungal family
Chroococcales			
Chroococcus sp.	u, e, A	*Wainiocora*[c]	Dict
Chroococcus sp.	u, e, A	*Athelia epiphylla*	Dict
Hormogonales			
Scytonema sp.	f, e2+I, A	*Athelia epiphylla*	Dict
Scytonema sp.	f, e2+I, A	*Clavaria* sp.	Clav
Scytonema sp.	f, e+I, A	*Cora pavonia*[d]	Dict
Scytonema sp.	f, e+I, A	*Cora* sp.[e]	Dict
Scytonema sp.	f, e+I, A	*Dictyonema irpicinum*	Dict
Scytonema sp.	f, e+I, A	*Dictyonema ligulatum*	Dict
Scytonema sp.	f, e+I, A	*Dictyonema moorei*	Dict
Scytonema sp.	f, e+I, A	*Dictyonema sericeum*	Dict

[a]See Table 4 for abbreviations and Table 3 for list of families. Adapted from Oberwinkler (1970, 1980, 1984) and Ahmadjian (1982).

[b]Aphyllophorales (overall, Corticiaceae).

[c]Probably synonymous with *Cora pavonia* = *C. montana.*

[d]Obviously also synonymous with *Corella wainio.*

[e]Synonymous with *Rhipidonema.*

philic *Halographis* (Kohlmeyer and Volkmann-Kohlmeyer, 1988; see also Kohlmeyer, 1974).

The range and complexity of symbioses between fungi and algae (or cyanobacteria) can be seen to be still rather complex (Kohlmeyer and Kohlmeyer, 1979; Hawksworth, 1988b). In more recent times, observations have been reported of many fascinating cases of epiphytic, parasymbiotic, symbiotic, or parasitic algae (and cyanobacteria), bacteria, fungi, lichens, or mosses as additional bionts associated with established lichens, which collectively contribute to the century-old debate over the nature of lichen associations, and provide indications about their evolutionary significance (Santesson, 1967). Considering mutualistic and parasitic (see e.g., Duebbeler, 1980) relationships of fungi to the symbiotic partners, Hawksworth (1988b) suggested that symbioses should be discussed only in terms of the number of participating organisms, or bionts, e.g., four-biont symbioses (3 photobionts to one mycobiont) as reported for *Nephroma arcticum*, which contains two morphologically distinct cyanobacteria in addition to the principal one, *Coccomyxa* (Jordan and Rickson, 1971).

Separation and Cultivation of Symbionts

CYANOBIONTS. Cyanobacterial photobionts of some lichen families have been isolated and cultivated in pure culture (some of them are available from culture collections, e.g., *Chroococcidiopsis* sp. SAG 33.84 [Sammlung von Algenkultufen, Universität Göttingen, Göttingen, FRG] from *Psorotichia*). Different methods for separation of the phycobiont from the mycobiont can be used, such as irradiation of thallus fragments with ultraviolet light (Henrickson, 1951) and treatment with antibiotics (Watanabe and Kiyohara, 1963). Detailed instructions are given by Ahmadjian (1967, 1973). Some more recent publications are listed in the next section.

Cultivation of *Chroococcidiopsis* from Lichinaceae (Büdel and Henssen, 1983)

The lichen thalli were washed in a washing chamber (Renner, 1980) for about 30 min. Sections of 30–40 μm thickness were cut with a microtome, placed in 6-cm petri dishes on BG-11 medium containing 1.5% agar (Waterbury and Stanier, 1978) and incubated at 20°C under continuous light at 2,000 lux (Osram, universal white, fluorescent light, 40 W). The recipe for BG-11 medium is given in Table 6. After 6–8 weeks, the first free colonies of the cyanobiont were observed within the disintegrating thallus sections and were transferred to fresh agar plates and incubated under the same conditions. Washed lichen fragements were incubated in 10-ml bottles in liquid Bristol medium (Degelius, 1954) at 20°C and 2,000 lux. From such fragments, colonies of the phycobiont may be harvested after approximately 4 months.

In contrast to the growth of these cyanobionts in ASM-1 medium (Gorham et al., 1954), growth in BG-11 (Stanier et al., 1971) and KM-D (Kratz and Myers, 1955) media was unpredictable, and hence these two media were not routinely used by Bubrick and Galun (1984).

Table 6. Recipe for culture medium BG-11.[a]

	Concentration (g/l)
BG-11 medium:	
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
Na_2Mg EDTA	0.001
Na_2CO_3	0.02
Trace metal solution:	
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$Na_2MoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.0494

Adapted from Stanier et al., 1971.
[a]The trace metal solution is added at 1 ml/l. The pH of the medium is adjusted to 7.1.

Cultivation of *Nostoc* from Peltigeraceae (Boissiere et al., 1987)

Two methods are described, but only one, the more rapid, is given here. A lobe of thallus is carefully and repeatedly washed with water, finally with distilled water. Then the lobe is opened with a vertical and a radial cut and put on a small sterilized glass plate covered with a thin liquid layer of BG-11 medium with 0.14% agar. The plate is placed on sterilized solid agar (1.1% agar in BG-11) in a petri dish (culture conditions: 20°C; 150 μ Einsteins/m²/s; light/dark cycle: 12 h/12 h). After 3 weeks, colonies of *Nostoc* can be observed with a binocular microscope. The colonies are transferred into new petri dishes containing the same medium for further proliferation. It is possible to purify such cultures from both, contaminating bacteria and mycobiont, to an axenic stage. Two further methods have been described. No difference in growth morphology was found between axenic cultures and cultures contaminated with bacteria (Boissiere et al., 1987). *Nostoc* isolates from *Nephroma laevigatum* are described by Kardish et al., 1989.

MYCOBIONTS. Ahmadjian has done pioneering work on the cultivation of lichen fungi (Ahmadjian, 1973) and the experimental synthesis of lichen symbioses (Ahmadjian and Jacobs, 1983). Two strategies are known for obtaining pure cultures of lichen fungi. The best way

seems to be to use spores from lichen ascocarps originating from the mycobiont (Ahmadjian, 1961, 1963; Lallemant, 1985). In the other method, hyphal fragments from the lichen thallus have been used but the results of this strategy seem to be uncertain with regard to obtaining the true mycobiont, because lichen thalli are substrates for many parasitic and parasymbiotic fungi whose hyphae are in intimate contact with the cells of the phyco- and mycobiont (Ahmadjian, 1964; see also Hawksworth, 1988b). Lichen fungi on agar media (2% glucose) form compact, elevated colonies, which vary widely in size, shape, and color (Ahmadjian 1961, 1963, 1964, 1989).

For cultivation and axenic resyntheses of lichens, including questions of recognition and resistance, see Ahmadjian and Jacobs (1983), Ahmadjian (1989), and Stocker-Wörgötter and Türk (1989). Although lectins occur in different lichens (Petit, 1982; Lallemant and Savoye, 1985), the authors doubt that a lectin-mediated recognition system exists in lichens because such systems imply close affinities between the symbionts. On the other hand, studies on lichen synthesis raised the possibility that some type of recognition occurs between algal and fungal symbionts (Ahmadjian et al., 1980; Ahmadjian and Jacobs, 1981; Galun, 1988).

Biochemistry, Physiology, and Ecological Significance

For metabolite exchange, the partners need regular intimate physical contact. Five principal types of contacts have been described (Smith and Douglas, 1987): 1) thin-walled fungal hyphae enter the gelatinous sheath of algal colonies (e.g., *Nostoc*) but do not come into contact with the cell wall layers of the photobionts; 2) close wall-to-wall contact between myco- and phycobiont without apparent formation of specialized attachment structures (nevertheless, the fungus may dramatically influence the structure and cell wall composition of the cyanobiont, as demonstrated by Kardish et al., 1989, with regard to the *Nostoc* of *Nephroma laevigatum*); 3) close contact of the hyphae to the phycobiont through the formation of specialized attachment structures (comparable with appressoria); 4) "intraparietal haustoria" (hyphae form plug-like projections in the phycobiont cell without penetrating the phycobiont cell wall; and 5) "intracellular haustoria" (hyphae penetrate the phycobiont cell wall but not its cytoplasmic membrane).

The best explanation of the relationship between fungus and "alga" may be the view that lichens are examples of a controlled parasitism (Ahmadjian, 1982b; Ahmadjian and Paracer, 1986). The fungus accepts photoassimilated products from the alga (Green and Smith, 1974) and, in the case of cyanobacteria, also fixed nitrogen. The fungus provides the alga with water, minerals, and protection from desiccation and high light intensities by using, in part, secondary metabolites, the "lichen acids" (Culberson et al., 1977). These usually extracellularly in the cell wall material occurring and often crystalline "lichen substances" are usually only produced during symbiosis (Culberson and Ahmadjian, 1980; but see Honegger and Kutasi, 1990). It is not certain whether the respiratory carbon dioxide exchange has evidence for the symbiosis, as discussed by (Coxson et al., (1982). In cyanophilic lichens, the carbohydrate released from the cyanobiont and transferred into the fungus is glucose (Drew and Smith, 1967; Smith and Douglas, 1987), in contrast to the algae which release specific alcohols (polyols) as photosynthates. Free-living cyanobacteria do not excrete glucose (Galun, 1990). The other contribution of the cyanobiont to the fungus is ammonia. All investigated cyanophilic lichens (Millbank, 1984) fix nitrogen and are photoassimilating (Galun and Bubrick, 1984). Where filamentous cyanobacteria are the sole symbionts, the heterocyst frequency is 4–8%, and both photoassimilation and dinitrogen fixation rates are comparable to values for non-symbiotic cyanobacteria. But when the cyanobionts are the second symbionts and restricted to cephalodia, a striking change occurs: heterocyst frequency is higher (14–36%) (Kershaw, 1985), as is the nitrogen fixation rate, because the synthesis of the cyanobacterial enzymes for ammonia assimilation (glutamine synthetase and glutamate synthase) are suppressed (Rai et al., 1981; Stewart et al., 1983) and the rate of photoassimilation is reduced (Smith and Douglas, 1987), e.g., glutamine synthetase is present in high levels in free-living *Nostoc* but only in traces in symbiotic *Nostoc* (see also Champion-Arnaud and Lallemant, 1986). Approximately 50% of the fixed nitrogen is excreted as ammonia and assimilated via glutamate dehydrogenase by the mycobiont (Galun, 1990). An unsolved problem is the regulation of phycobiont/mycobiont relation. It is conceivable that the mycobiont, as an heterotrophic organism, produces urea via the urea cycle including the reversible arginase reaction. The urea is decomposed by the enzyme urease (inducible by urea) into carbon dioxide and ammonia, molecules which are inductors and substrates for metabolic processes in the phycobiont. Via photosynthesis the phycobiont produces more primary metabolites which, partially, move to the

fungus. Here they induce the synthesis of more secondary metabolites including usnic acid, a competitive inhibitor of urease. In this way, the offer of carbon dioxide and ammonia will be reduced for the phycobiont and, in the following, the growth of the phycobiont is diminished. Even, if these "urease theory" could explain (Ahmadjian and Paracer, 1986) how the fungus may control the rate of polyol excretion by chlorophycean photobionts (see also Perez-Urria et al., 1990) and therewith the population density of the phycobiont, its function in the regulation of growth of nitrogen-fixing and ammonia-releasing cyanobionts remains uncertain.

Lichens with cyanobionts are important contributors to the nitrogen economy of various ecosystems. *Lobaria oregana* is abundant in the Douglas fir forests of the Pacific Northwest (USA), and when its thalli fall off the trees onto the ground, they decay and release significant amounts of nitrogen (2.3 kg fixed N/ha). Organic nitrogen compounds may also leach out from lichen thalli during heavy rains (Ahmadjian and Paracer, 1986).

Lichens are very sensitive to air pollutants, especially to sulfur dioxide (e.g., Sharma et al., 1982) but species vary in their sensitivity. Therefore, the species composition and growth forms on substrate can be used as accurate indicators for air pollution (Hawksworth and Rose, 1976). Lichens show a differential sensitivity to heavy metals (Brown and Beckett, 1983). Because lichens are respiratory and photosynthetic consortia, it was to be expected that certain plant-protective substances would influence cyanophilic lichens (Da Silva et al., 1973; Hallbom and Bergman, 1979).

For further information, see reviews by, e.g., Boissiere et al. (1987); Brown (1985); Büdel (1985); Büdel and Henssen (1988); Galun and Bubrick (1984); Hale and Culberson (1966); Lallemant et al. (1986); Lawrey (1984); Millbank (1984); Peveling (1987); Pirocynski and Hawksworth (1988); Smith and Douglas (1987); Stewart (1977); and Tschermak-Woess (1988).

The Azolla-Anabaena Symbiosis

Taxonomy, Morphology, and Development of the Symbiosis

The *Azolla-Anabaena* symbiosis is a eusymbiotic association between the nitrogen-fixing cyanobacterium *Anabaena azollae* and the eukaryotic freshwater fern, *Azolla.* The genus *Azolla* was established by J. B. Lamarck as early as 1783 (Svenson, 1944). It comprises either seven or eight species (Table 7). Whether *Azolla pinnata* var. *imbricata* is a variety or an independent species (Lin, 1980) is not clear. To date, more than 40 fossil species of *Azolla* have been described (Fowler, 1975; Hills and Gopal, 1967). The genus used to be included with *Salvinia* in the Salviniaceae (Smith, 1955), but in the 1960s, it was placed in a separate family, the Azollaceae (Sculthorpe, 1967; Moore, 1969; Konar and Kapoor, 1972, Lumpkin and Plucknett, 1980). The small, fast-growing water ferns of temperate and tropical aquatic ecosystems around the world float freely on the surface of freshwater ponds and marshes. The swimming plant (sporophyte, Fig. 2) has tiny roots and a short, branched stem (rhizome), which is covered with small, overlapping leaves. The sporophyte has a dorsiventral organization (Peters and Calvert, 1983) and each rhizome apex has an upcurved meristem (see Fig. 1). Roots and lateral branches are derived from further divisions of the ventral cells of this meristem whereas leaves are derived from the corresponding dorsal cells. Each leaf is divided into a dorsal and a ventral lobe. The thin ventral lobe (its distal half is only one cell thick) is nearly colorless and floats on the water surface. The mature dorsal leaf lobe is aerial and has a clearly defined multilayered mesophyll as well as adaxial and abaxial epidermal tissues. The abaxial epidermis has many stomata and single-celled papillae. Each mature dorsal leaf lobe has an ellipsoidal cavity formed by an infolding of the adaxial epidermis in the proximal half of its lamina. The cavity normally contains the endophytes, the cyanobacterium *Anabaena azollae,* and in addition Gram-positive, non-nitrogen-fixing bacteria (Gates et al., 1980), identified as *Arthrobacter* spp. (Wallace and Gates, 1986; Petro and Gates, 1987; Grilli Caiola et al., 1988). Vegetative *Anabaena* cells, the "apical *Anabaena* colony," are also found in the pocket of the apical meristem formed by the upcurved meristem and the young overarching dorsal leaf lobe (Fig. 1).

Development of the leaf cavity begins in the young leaves. An area of premesophyll tissue at the base of the dorsal leaf lobe enlarges much less than the more distal mesophyll, causing a depression to form in the adaxial epidermis. As the apical meristem continues to grow and becomes further displaced from each leaf, filaments of the apical *Anabaena* colony become associated with each developing leaf in the area of its forming cavity. The actual partitioning process appears to involve a specialized epidermal hair called the "primary branched hair" (PBH) (Calvert and Peters, 1981). Each leaf has only one PBH. It originates from the axil of the primordial leaf and grows with its terminal cells

Table 7. Taxonomy of the *Azolla-Anabaena* symbiosis.[a]

Cyanobiont[d]	Properties[e]	*Azolla* species
		Section: Euazolla (New World species)
Anabaena azollae	F, e, f, N	*Azolla caroliniana* Wilidenow
A. azollae	F, e, f, N	*Azolla filiculoides* Lamarck (type species)
A. azollae	F, e, f, N	*Azolla mexicana* Presl
A. azollae	F, e, f, N	*Azolla microphylla* Kaulfuss
A. azollae	F, e, f, N	*Azolla rubra* R. Brown[b]
		Section: Rhizosperma (Old World species)
A. azollae	F, e, f, N	*Azolla nilotica* DeCaisne
A. azollae	F, e, f, N	*Azolla pinnata* R. Brown
A. azollae	F, e, f, N	*Azolla imbricata* (Roxb.) Nakai
		Varieties[c]
A. azollae	F, e, f, N	*Azolla pinnata* var. *imbricata*
A. azollae	F, e, f, N	*Azolla pinnata* var. *pinnata*

[a]Cyanobiont: *Anabaena azollae* Strasburger; host: *Azolla* (Azollaceae, Salviniales, Filicopsida, Pteridophyta).
[b]*A. rubra* was also reported as *A. japonicum* (Peters et al., 1979; but see Zimmermann et al., 1988).
[c]Listing is incomplete (see Lumpkin and Plucknett, 1982; Zimmermann et al., 1988).
[d]In the future, the "species" *Anabaena azollae* will probably be split into several different species.
[e]Abbreviations: see Table 4.

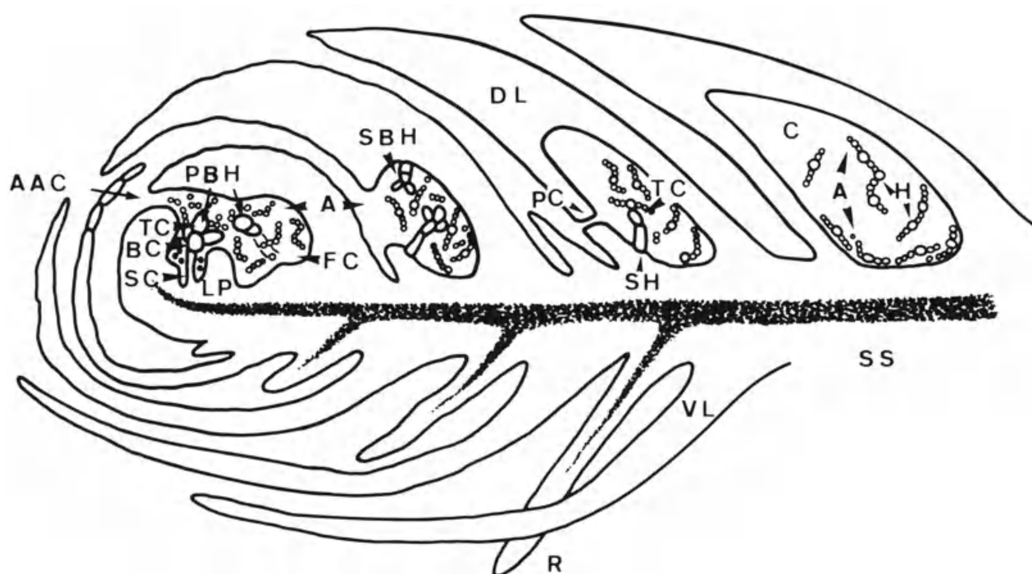

Fig. 1. Anatomy of the *Azolla-Anabaena azollae* symbiosis. Schematic, vertical, longitudinal section of an *Azolla* branch with the apical meristem, illustrating: the apical *Anabaena* colony (AAC) with vegetative *Anabaena* cells (A); differentiation of *Anabaena* cells into heterocysts (H) during maturation of leaves; different stages in the formation of the leaf cavity (C) in the dorsal leaf lobe (DL); and of the transmission of the *Anabaena* cells from the apical colony in the leaf cavity. Abbreviations: BC, body cell; FC, forming cavity; LP, leaf primordium; PBH, primary branched hair; PC, pore closure of the cavity; R, root; SBH, secondary branched hair; SC, stalk cell; SH, single hair; SS, stem stele; TC, terminal cell; VL, ventral leaf lobe. (Drawing based on an illustration from Peters and Calvert, 1983; also using electron micrographs from the same article and unpublished observations of H. E. A. Schenk.)

into the apical *Anabaena* colony. Some *Anabaena* filaments seem to be attracted to the PBH in the apical region and are, in the following development, separated from the apical colony, whereas the PBH is now positioned at the proximal end of the cavity depression. Cells around the rim of the depression become meristematic and produce epidermal cells, which form the

cover of the cavity engulfing the PBH with its associated *Anabaena* filaments. Now, some *Anabaena* filaments begin to differentiate heterocysts, and additionally, many epidermal hairs begin to form on the cavity wall. One of these, the "secondary branched hair" (SBH), is positioned at the back of the cavity. The other hairs (which have, during maturation of the cavity, increased in number up to 25 per cavity), termed "single hairs" (SH), consist of two cells, the stalk cell (SC) and the terminal cell (TC). Both cells (the terminal one, more quickly) differentiate a so-called transfer cell ultrastructure (TCU) as well as the different cell types of the PBH and the SBH. TCU is characterized by the presence of numerous cell wall elaborations and an organelle-rich, dense cytoplasm which contains abundant rough endoplasmic reticulum. For further detail, readers are directed to reviews by Peters and Calvert (1983); Shi and Hall (1988); and Braun-Howland and Nierzwicki-Bauer (1990). Besides biochemistry and physiology, the above-mentioned authors have also comprehensively reviewed the genetics of the *Azolla* system. The capability for high dinitrogen fixation rates is a fascinating characteristic of this symbiosis. The observed production of molecular hydrogen probably will rather remain a limited side effect (Newton, 1976).

Reproductive Cycles of *Azolla*

The Taxonomy of the plant *Azolla* is based above all on characteristics of its reproductive structures. *Azolla* can reproduce either sexually by sporocarp development or asexually by vegetative fragmentation (Rao, 1936; Watanabe, 1982) (Fig. 2). The latter mode was found to be the main method of reproduction. It is rapid in *Azolla,* with a doubling time of about 2 days under optimal conditions (Peters et al., 1980). The less frequently observed type of reproduction is the sexual cycle via sporocarp formation. The most important factors controlling the life cycle are light intensity (Becking, 1979; Watanabe, 1982; Singh and Srivastava, 1985a), temperature (Becking, 1979; Watanabe, 1982), plant density (Becking, 1979; Watanabe, 1982), and photoperiod (Holst and Yopp, 1979; Singh and Srivastava, 1985b). The sexual life cycle enables the plants to overwinter and withstand desiccation (oversummering). The two generalized reproductive cycles of *Azolla* are shown in Fig. 2.

MAINTENANCE OF THE SYMBIOSIS. The symbiotic association between *Anabaena* and *Azolla* is maintained during both sexual and asexual (Fig. 2) modes of reproduction. Akinetes and/or filaments (Becking, 1978) of *Anabaena* (indicated by the asterisk) are apparently present during the development of both the mega- and the microsporocarps (generative life cycle), but are retained to maturity only in the former (Peters and Calvert, 1983). The continuity of the *Anabaena* endophyte throughout the life cycle of *Azolla* does not preclude the possibility of a free-living form of the endophyte, but it does eliminate the necessity for such a free-living form. In this view, the *Azolla-Anabaena* symbiosis is distinct from all other prokaryote-plant associations. Also each plant produced by fragmentation (vegetative life cycle) carries its own *Anabaena* inoculum, because each branch apex of the generative sporophyte contains a vegetative apical *Anabaena* colony. A very interesting problem is the control of the cell cycle of the cyanobiont by the macrobiont. In the growing point of the macrobiont, more rapidly dividing cells of the cyanobiont are observed than in its mature regions. The pattern of photosynthetic carbon and nitrogenase functions change during thallus development. Hill (1989) suggested two operating controls on the cyanobiont cell cycle: 1) nutrient supply for cell maintenance and cell growth; and 2) constraint or stimulation of commitment to divide. The separate operation of these controls leads to cyanobiont cells being in two possible stages: phase I, generative cells associated with growing host tissues and phase II, differentiated cells in the mature regions of the host.

Anabaena azollae

The cyanobacterial endophytes of all *Azolla* species are members of the family Nostocaceae and are collectively referred to as *Anabaena azollae* Strasburger. However, Candales et al. (1988) have stated that because of its life cycle, the cyanobiont in *Azolla* may belong in the genus *Nostoc.* In recent years studies have been made to determine whether the cyanobiont is the same in all *Azolla* species. Immunofluorescence studies (Gates et al., 1980; Ladha and Watanabe, 1982) suggest that the cyanobionts of all *Azolla* species are similar (if not identical). In contrast to these results, studies with lectin hemagglutination tests (McCowen et al., 1987; Mellor et al., 1981) indicate that the cyanobionts are different in at least three *Azolla* species. From restriction enzyme analysis of the *nifDH* region, Franche and Cohen-Bazire (1987) established that all nine symbiotic *Anabaena* strains investigated (from four Euazolla species and five Rhizosperma species) derive from a common ancestral *Anabaena azollae*

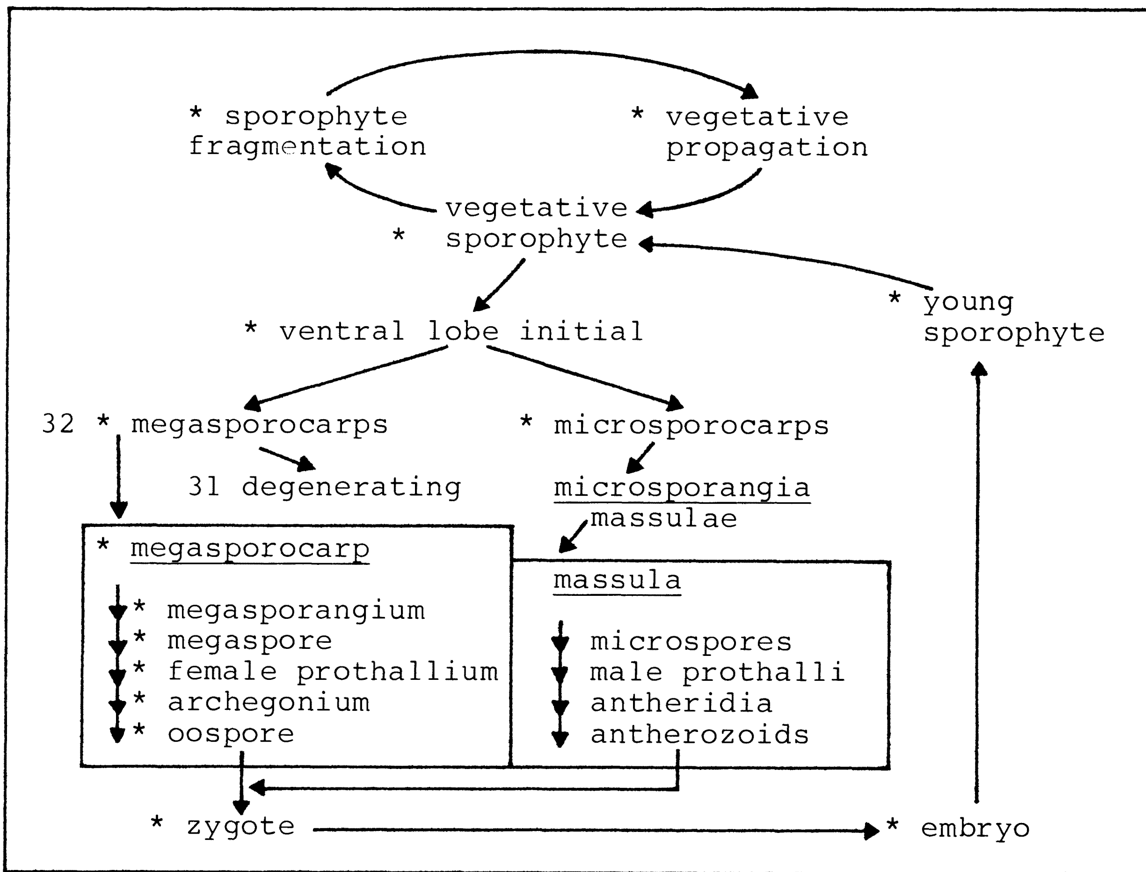

Fig. 2. Reproductive life cycles of *Azolla*. The asterisk indicates that *Anabaena azollae* is present. (Schematic diagram based on Braun-Howland and Nierzwicki-Bauer, 1989; Peters and Calvert, 1983; Shi and Hall, 1988.)

and belong to two slightly divergent evolutionary lines, the Euazolla and the Rhizosperma line. Many authors (e.g., Ashton and Walmsley, 1976) claim to have grown *A. azollae* after isolation, and Newton and Herman (1979) described a new isolation procedure, the "bundles" method. But, although these cultured isolates have exhibited some characteristics different from general free-living cyanobacteria (Newton and Herman, 1979; Newton and Cavin, 1985; Rozen et al., 1986; Shi et al., 1987; Tel-Or and Sandovsky, 1982), there are still doubts. For example, a difference in surface antigenicity appeared to exist between freshly isolated *A. azollae* cells and those obtained from in vitro cultures (Arad et al., 1985; Gates et al., 1981; Ladha and Watanabe, 1982, 1984). Another point of view is the possibility that the major constituent of *Azolla* cavities is an obligate endosymbiont, and that free-living isolates may be minor constituents of the cavity that are selected for during isolation (Meeks et al. 1988; Braun-Howland and Nierzwicki-Bauer, 1990). Identification of strains as effective cyanobacterial symbionts of *Azolla* will require a reinfection of cyanobacteria-free plant material with the in vitro cultivated cyanobiont (Koch's postulates) as was reported by Liu et al. (1984) and Lin et al. (1988).

Cultivation of Symbiotic Consortia and of Partner-Free Symbionts

Cultivation of the *Azolla-Anabaena* Consortium

Kulasooriya et al. (1988) give the following instruction: *A. filiculoides, A. caroliniana,* and *A. pinnata* var. *imbricata* were grown in a phytotron under a light/dark regime of 16:8 h, at temperatures of 27/22°C, respectively. *A. filiculoides* was grown in Hoagland medium diluted 1:8, *A. caroliniana* and *A. pinnata var imbricata* were grown in the medium of the International Rice Research Institute (IRRI) (Watanabe et al., 1977). For *A. caroliniana,* Kaplan and Peters (1988) used the N-free medium of Peters et al. (1980).

Cultivation of *Anabaena*-free *Azolla*

Anabaena-free *Azolla* plants (Lin and Watanabe, 1988) are grown on IRRI's culture solution (Watanabe et al., 1977) containing ammonium nitrate at 50–70 mg/l, placed in a lighted growth cabinet (120 W m^{-2}, (day/night), 75% humidity). The culture solution should be changed every 3 days.

Culture of the Endophyte *Anabaena azollae*

For laboratory culture (Peters and Mayne, 1974), the *Azolla* fronds were grown in Erlenmeyer or Fernbach flasks in a growth chamber in N-free medium or in a medium containing nitrate as N source. Temperature, 23/18°C; 16 h light/8 h dark; illumination with mixed light of cool-white fluorescent and incandescent lamps, 200 or 400 foot candles.

N-free medium: 2 mM KCl, 2 mM $CaCl_2$, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$; each liter contained 1 ml of a stock solution of sesquestrene NaFe (FeEDTA) containing 5 mg Fe/ml and of a micronutrient solution (Allen, 1968), pH 5.4–5.6 (see below).

Laboratory Cultures of *Anabaena* (Boussiba, 1988)

The cyanobacteria (isolate courtesy of E. Tel-Or, Rehovot) were cultivated in 500-ml sterilized glass columns in a circulating water bath (30°C, controlled water temperature); a constant photon flux of 175 μ Einsteins/m_2/s at the surface of the growth vessel by eight cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1.5% carbon dioxide. Under these conditions, the pH was maintained at 6.8–7.0. The standard growth medium was BG-11 (Stanier et al., 1971; see Table 6).

Outdoor Cultures of *Anabaena* (Boussiba, 1988)

2.5-m^2 oval-shaped ponds with two channels forming a single loop were used. The culture (isolate of E. Tel-Or, Rehovot), 250-liter in volume (grown in medium BG-11) and 10-cm depth, was stirred by a paddle wheel. Carbon dioxide was supplied to maintain the pH at a range of 6.5–7.5. Temperature, dissolved oxygen, and pH of the pond were monitored daily. Light intensity ranged from 900 to 1250 μEinsteins/m^2/s, between March to August, respectively. To maintain steady-state growth, excess culture liquid was removed as required. In all outdoor experiments, the biomass concentration was kept between 6–8 mg chlorophyll/liter. Outdoor mass production (maximum yield of ash-free dry weight) *Anabaena azollae* was as follows:

August, 17.9 g/m^2 d; March, 5.6 g/m^2 d.

Separation of Symbionts

Endophyte-free plants rarely occur in nature but can be generated by several techniques (Peters and Mayne, 1974; Hill 1975, 1977; Lin and Watanabe, 1988). Also axenic cultures of *A. azollae* can be obtained (Bai et al., 1979; Newton and Herman, 1979).

Methods for Isolation of the Cyanobacterial Endophyte

1. Peters-Mayne or "Gentle" Method (Peters and Mayne, 1974): A single layer of *Azolla caroliniana* fronds was placed in a shallow porcelain pan, and N-free BG-11 medium (see Table 6) supplemented with 1% PVP-40 (polyvinyl pyrrolidone) was added until the fronds began to float. A teflon roller was used to gently squash the fronds. The resulting mixture was passed through four, then eight layers of cheesecloth, and centrifuged at low speed at room temperature for 2 min. The pellets were resuspended in N-free BG-11 medium, passed through two layers of 100-μm nylon mesh, centrifuged and resuspended in a small volume of the N-free medium. The entire procedure was carried out utilizing sterile techniques.
2. For isolation of the endophytic *Anabaena azollae,* fronds grown in N-free medium were harvested and their fresh weight determined. Fronds were then put in the N-free medium containing 1% soluble PVP-40 (8 ml/g fresh weight) and ground for 30–60 s in a motor-driven, ice-cold, teflon homogenizer. The homogenized material was sequentially passed through 4 and then 8 layers of cheesecloth and a 110 μm nylon mesh, followed by rinsing with the grinding medium. The filtrate was centrifuged at low speed for 20–30 s and the supernatant decanted. The pellet, containing the endophyte filaments, was resuspended in 2 ml of the growth medium without PVP-40 and recentrifuged prior to final resuspension in the same medium for use in specific studies (Peters and Mayne, 1974).
3. Further methods for isolation of the cyanobiont were recently described by Candales et al. (1988).

Two Methods for Preparing Cyanobacterium-Free *Azolla*

1. Antibiotic treatment method (Peters and Mayne, 1974):

Antibiotics were employed individually and in various combinations and concentrations. Cultures were left in nutrient medium with antibiotics for 1 week with one change to fresh antibiotic-containing medium, transferred to antibiotic-free media for 1 week, and then transferred to media containing different antibiotics. This procedure was continued until four different combinations or individual antibiotics had been employed. Three of the sequential treatments were successful and gave fronds which remained free of the symbiont and incapable of acetylene reduction during a 6-month period. For more details the reader is referred to the original publication.

2. Lin-Watanabe method (Lin and Watanabe, 1988):

The apical membranes and the indusium of each megaspore of harvested megasporocarps of *A. microphylla* or *A. filiculoides* to which massulae were attached were decapitated with a razor blade just beneath the level of the apical membrane. The decapitated megaspores were placed vertically on slightly sloping filter paper soaked with water in a petri dish. Sporocarp germination was induced at 24–48°C under fluorescent light (5 W/m^2). When the first leaf emerged, the young *Azolla* plants were transferred to IRRI's culture solution (Watanabe et al., 1977) containing ammonium nitrate at 50–70 mg/liter and placed in a lighted growth cabinet (120 W/m^2, (day/night), 75% humidity). The culture solution was changed every 3 days.

Reinfection of symbiont-free *Azolla* megasporocarps with *Anabaena azollae* and artificial reconstruction of the symbiotic consortium is described by Lin et al. (1988).

Ecological Significance and Agricultural Use of *Azolla*

Growth, mineral nutrition, and agricultural use of *Azolla* has been reviewed by Nierzwicki-

Bauer (1990). Under optimal laboratory conditions, *Azolla* species have a rapid vegetative propagation with biomass doubling time of 2 days or less. The consortium necessarily requires light, air, water, and the same macronutrients as those, essential to other aerobic photoautotrophs (except for nitrogen, which can be supplied in total by dinitrogen fixation). Under natural conditions, their rapid growth enables these ferns to completely cover water surfaces and, depending upon where they occur, the plants can be considered either noxious weeds or a crop plant with agronomic potential. Therefore it seems not surprising that the cultivation of *Azolla(-Anabaena)* has a long history of use as a green manure for rice and as fodder for poultry and livestock in China, Vietnam, and other Far-eastern countries (Tuan and Thuyet, 1979; Liu, 1979; Peters and Calvert, 1983; Shi and Hall, 1988). According to Liu (1979), referred to in Shi and Hall (1988), one of the earliest mentions of *Azolla* seems to be in a Chinese book on agricultural techniques written by Jia Si Xue (540 A.D.), entitled "Qi Min Yao Shu" (The Art of Feeding the People). Within the part of the book on applied plant cultivation, Jia Si Xue describes the cultivation of *Azolla* and its use in rice fields. In Vietnam, the use of *Azolla* was described by a Buddhist monk named Kongh Minh Kongh, who promoted the cultivation of *Azolla* in the 11th century (Tuan and Thuyet, 1979). As the beginning of the 17th century there were many local records of its use as a green manure in China. However, because *Azolla imbricata* (the only species native in China) cannot overwinter and propagates slowly in early spring, the modern use of *Azolla* in China did not begin to expand markedly until 1962 (Liu, 1979). "Perhaps the most significant breakthrough for the improvement of *Azolla* cultivation in China was the importation of an usually cold-resistant variety of *A. filiculoides*" (Lumpkin and Plucknett, 1982), which allowed the cultivation of *Azolla* to expand into northern and northeastern China. Its use currently has been extended to approximately 1.34 million ha (Peters and Calvert, 1983). Therefore it was not until the last 30 years that techniques for cultivation of *Azolla* and its use as an alternative to chemical nitrogen fertilizers were extensively developed.

Numerous factors influence the *Azolla* nitrogen input. Among others, they include the agricultural system used, fertilization requirements of the field, the presence of soluble iron, and problems with pests. Maximum estimated inputs during exponential growth of *Azolla*, assuming unlimited growth, range from 335–670 kg/ha/year (Becking, 1979). If account is taken of more natural growth conditions, such as discontinuous light intensity and variation in temperature, winds, humidity, water depth, and nutrient availability in different geographical areas and with different *Azolla* species, the values may vary from 80–162 (rarely to 500) kg N/ha/year. *Azolla* is rich in proteins, fats, and amino acids, and in addition to its use as a fodder for different animals, above all for pigs, it has even been suggested as food for humans (Singh, 1979). It may serve as a weed suppressor when grown in dual culture with rice (Watanabe, 1982) and might be useful for improvement of water quality (Cohn and Renlund, 1953).

Endocytobioses (Endocyanoses)

This Chapter (see Table 1) summarizes the properties of all facultative or obligate endocytobiotic consortia (eukaryotic host organisms with intracellularly living cyanobionts). Such consortia are composed of genetically independent symbiotic partners. Most of these consortia are marine, and it seems that they are all difficult to cultivate under laboratory conditions, with exception of the terrestrial *Trifolium alexandrinum*. But this consortium, with both *Nostoc* and *Rhizobium* as endosymbionts, must probably be understood rather as an endosomabiosis (ectocyanosis, because obviously most cyanobacteria are living in the intercellular space of the nodules) than as an endocytobiosis. Further studies are necessary to clarify the real relationships of this symbiosis.

A very interesting area for research concerns the cyanophilic sponges. At least four different cyanobacteria occur as symbionts both intra- and intercellularly within marine sponges (this number may expand as other sponges are examined). Two unicellular cyanobionts are described as species of *Aphanocapsa* (Sara, 1971) although they are unlike free-living species of this genus. A multicellular cyanobacterium has been designated as *Oscillatoria spongeliae;* another unicellular form is yet to be named, but it resembles *Synechocystis trididemni* (Rützler, 1981). One of the unicellular species, *Aphanocapsa feldmanni,* is by far the most widely distributed, occurring in at least 14 sponge orders of the classes Calcarea and Desmospongia (Wilkinson, 1980). Cyanobacterial symbionts are only recorded in sponges from warm temperature to tropical habitats and only those growing in relatively shallow water (< 30 m depth). The host species may be divided into two categories on the basis of the number of symbionts and the contribution made to the sponge. Most species have a thin layer of tissue

containing the cyanobionts in those parts exposed to light, whereas some flattened sponges contain numerous symbionts distributed throughout the tissue.

It is not the intention to deal with all the numerous associations (see papers listed in Table 1). However, one very fascinating biological system, *Geosiphon pyriforme,* is based on the work of Mollenhaver (1988).

Geosiphon pyriforme

Geosiphon pyriforme (Phycomycotina, Endogonales, phycomycete of unknown systematic classification, but probably within the relationship of *Glomus* Tulasne), first described by Wettstein (1915), forms a well-known, but rarely observed, unique, facultative endocytobiosis (Fig. 3) between a hormogonal cyanobacterium, related to *Nostoc punctiforme* (Mollenhauer, 1988) and the siphonous fungus *Geosiphon pyriforme* (Knapp, 1933). The only known occurrence at present (Mollenhauer, 1988) is near Biebergemünd, Germany, on ridges of extensively used crop fields (geology: German Trias: Buntsandstein). The fungus colonizes bare soil between liverworts, mosses, and dwarf phanerogams, forming an ecological association called the Centunculo-Anthocerotetum-association. The fungus regularly occurs in intimate association with a moss protonema (not yet satisfactorily identified) which may be a *Dicranella* species (Mollenhauer and Mollenhauer, 1988). It is clear that this endocytobiotic consortium does not represent a lichen ("phycomycotina lichen," as described by Henssen and Jahns, 1974), because the cyanobacterium lives intracellularly within cytoplasmic vacuoles (Schnepf, 1964, 1966) of the fungus and a typical lichen thallus is not formed. Additionally, the synthesis of extracellular lichen substances is unknown, and the morphology of the partners is not strongly modified during the symbiosis, except for the development of the symbiont-containing vesicles of the fungus. The fungus vesicles are observed on the soil surface whereas the uninfected hyphae grow below the soil surface between the soil particles. Besides the dark blue-green vesicles, *Geosiphon* also forms whitish globular bodies, which serve as resting spores (presumably "sporocarps" very similar to those of *Glomus* species), being able to form new hyphae (Mollenhauer, 1988).

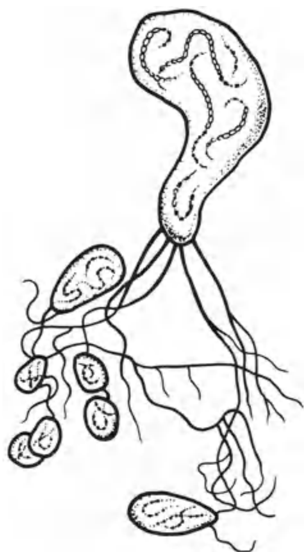

Fig. 3. *Geosiphon pyriforme:* mycelium with vesicles containing the endocytobiotic *Nostoc* filaments. Different times of infection result in vesicles of varying age, and therefore, of varying size. (Based on a photograph in Mollenhauer, 1988.)

It has been possible to cultivate symbiont-free and symbiotic *Geosiphon* (Mollenhauer and Mollenhauer, 1988) and to demonstrate, in part, the mode of the infection of the fungus by motile hormogonia of the cyanobacterium (Mollenhauer, 1988). This very interesting process of active infection attack by the motile hormogonia and the following endocytosis by the fungus (in the region of the apical end of young hyphae) is described in the original papers of D. and R. Mollenhauer (see "Literature Cited").

After colonization of *Geosiphon,* the *Nostoc*-containing filaments develop heterocysts. Besides photo assimilated carbon, the cyanobacterium also contributes fixed nitrogen to the fungal host. A transport system in the plasmalemma of the cyanobiont shows a high affinity for glutamate and a lower one for aspartate (Strasser and Falkner, 1986). Some observations give indication that the symbiosis is a highly balanced equilibrium which, dependent on the environmental conditions, turns from a eusymbiotic into a dyssymbiotic relationship. In the parasitic state, the fungus digests the endocytobiotic cyanobacteria in the vesicles (Mollenhauer, 1988).

CULTIVATION AND SEPARATION OF THE SYMBIONTS. Endocytobiotic *Nostoc* strains from *Geosiphon* were isolated by W. Koch (Göttingen: SAG) and by D. Mollenhauer (Forschungsinstitut Senckenberg, Lochmühle, Biebergmünd), from where strains are also available. For the Mollenhauer strain, batch and agar culture conditions are described by Strasser and Falkner (1986).

Using water exudates of the accompanying moss Dicranella, or, still better, of *Funaria hy-*

grometrica, it is possible to cultivate mycelia of *Geosiphon pyriforme* (Mollenhauer, 1988).

Symbiogenetic Organelles ("Endocyanoses")

Introduction and Taxonomic Overview

Symbioses of cyanobacteria with other organisms as hosts are defined as syncyanoses (Pascher, 1914), and as ecto- or endocyanoses (Pascher, 1929) (see discussion of terminology in "Introduction, Terminology, and Overview"). The endosymbiotic cyanobacterium (e.g., Chroococcaceae or Nostocaceae) of an endocyanome is termed a "cyanelle" in analogy to "zoochlorelle" or "zooxanthelle" (Pascher, 1929). The nonsystematic term "cyanelle" was later restricted to only unicellular endocytobiotic cyanobacteria (Geitler, 1959). However, it is obvious that this cannot be more than a simple microscopic view of classification. Using this more restricted terminology, we shall easily arrive at confusing situations, because it is known that filamentous cyanobacteria can change phenotypically into unicellular forms during symbiosis. Additionally, constructing a phylogenetic tree by using 16S rRNA sequence data, it could be demonstrated (Giovannoni et al., 1988) that the morphotypes of section I (unicellular cyanobacteria) and section III (nonheterocystous, filamentous cyanobacteria) in the classification system of Rippka et al. (1979) are dispersed throughout the tree, indicating multiple evolutionary origins for these species. Therefore, taxonomic classifications of cyanobacteria based principally on morphology do not necessarily reflect phylogenetic relationships. Notwithstanding, for the purpose of this section on the organisms described here, the restricted term for cyanelles given above is used because most of these so-called cyanelles have reached a high degree of genetic adaptation to the "host" cell. At the end of this introduction, a proposal for the further use of the term "cyanelle" is given.

The first discovery of an endocyanosis in a unicellular organism was that of the thecamoeba *Paulinella chromatophora* described by Lauterborn (1895). After this time, more cyanelle-bearing endocyanomes (Pascher, 1914, 1929; Geitler, 1936; Schiller, 1954) were described (Table 8). They are reviewed (also with regard to plastid evolution) by Pringsheim (1958), Geitler (1923, 1959), Echlin (1970), Taylor (1970), Schenk (1973a, 1973b, 1977), Trench (1982), Kies (1984a, 1984b, 1979), Schenk et al. (1987), Smith and Douglas (1987), and Kies and Kremer (1986a, 1986b, 1989). For along time most of these organisms were classified as cyanomes, but some of them, have more recently been proposed as endocyanomes, such as *Cyanidium caldarium* (Kremer et al., 1978; Kremer, 1983). Many endocyanomes were found only one or two times, and, only a few are stored in culture collections. The SAG has: two strains of *Cyanophora paradoxa* (Pringsheim and Kies); *Glaucocystis nostochinearum; Gloeochaete wittrockiana; Glaucosphaera vacuolata;* and *Cyanidium caldarium.* L. Kies (Botan. Inst., Univ. Hamburg has a strain of *Cyanoptyche dispersa.* Researching and understanding the endocyanoms is crucial, since these consortia represent all the steps from facultative to obligate endocyanoses. Therefore it would be desirable to rediscover the formerly described cyanoms (Schiller, 1954; Pringsheim, 1958), to discover new forms and to isolate and cultivate these consortia (certainly an important research field for limno- and phycologists) as sources for a broad research in cell evolution. At this point, it seems necessary to return to the classification problem of regarding the cyanelles as intracellular symbiotic cyanobacteria. By definition, a biological system, a living organism, and therefore also a symbiont, has its own life cycle, its own independent genome, and is a representative of its species as defined by a taxonomic binary name. The difference between a symbiont and an aposymbiotic organism is a more or less strong metabolic or morphological dependence on the symbiotic partner (also in obligate symbioses). Extensive studies with the "cyanome" *Cyanophora paradoxa* have shown that the cyanelles of *C. paradoxa* possess a genome of reduced size comparable to that of chloroplasts (Herdman and Stanier, 1977; Löffelhardt et al., 1980) and nucleus-encoded proteins (Bayer and Schenk, 1986; Burnap and Trench, 1989c; Bayer et al., 1990).

If genes coding (originally) for endogenic proteins of such a cyanelle (endocytobiotic cyanobacterium) are found to be located on the nuclear genome of the "host" cell, the endocytobiont should no longer be characterized as a "biont," but rather as a cyanoplast (Schenk and Hofer, 1972; Schenk et al., 1987b), indicating that the cyanelle has reached more or less the status of a eukaryotic cell organelle, and can hence no longer be considered an endocytobiont qualified to possess its own taxonomic name. Therefore, with regard to Cavallier-Smith's suggestion (Cavallier-Smith and Lee, 1985), such cyanelles cannot be considered to be one partner of a two-biont symbiosis but only a cell organelle (Schenk, 1990) of a new organismic unit, a new species, descended from

Table 8. Unicellular metacyanomes (phylogenetic descendants of endocyanomes; see text).[a]

Cyanelle[b]	Metacyanome formerly endocyanome	Reference[c]
	Rhizopoda (Thecamoeba)	
Cyanelle (*) f, b, C	*Paulinella chromatophora*	1
	Chrysophyta	
	Bacillariophyceae	
Cyanelle (*) f	*Denticulata vanheurcki*	2
Cyanelle (*) f	*Epithemia adnata*	3
Cyanelle (*) f	*Epithemia sorex*	2
Cyanelle (*) f	*Epithemia turgida*	2
Cyanelle (*) f	*Epithemia zebra*	2
Cyanelle (*) f, N	*Rhopalodia gibba*	4
Cyanelle (*) f	*Rhopalodia gibberula*	4
	Glaucocystophyta	5
	Glaucocystophyceae	
	Glaucocystales;	
	Glaucocystaceae	
Cyanelle (#) f	*Glaucocystis bullosa*	
Cyanelle (#) f	*Glaucocystis duplex*	
Cyanelle (#1) f, C	*Glaucocystis nostochinearum*	
Cyanelle (#) f	*Glaucocystis oocystiforme*	
Cyanelle (#) f	*Archaeopsis monococca* (*)	
Cyanelle (#) f	*Glaucocystopsis africana* (*)	6
	Cyanophorales	
	Cyanophoraceae	
Cyanelle (#2) f, C	*Cyanophora paradoxa*	
Cyanelle (#) f	*Cyanophora tetracyanea*	
Cyanelle (#) f	*Peliaina cyanea* (*)	
Cyanelle (#) f	*Strobilomonas cyaneus* (*)	7
	Gloeochaetales	
	Gloeochaetaceae	
Cyanelle (#) f, C	Gloeochaete wittrockiana	
	Glaucosphaeraceae	
Cyanelle (#) f, C	*Glaucosphaera vacuolata*	
Cyanelle (#) f	*Cyanoptyche gloeocystis*	8
Cyanelle (#) f	*Chalarodora azurea* (*)	
	Cyanidiophyceae	9
Cyanoplast a, C	*Cyanidium caldarium*	10
Cyanoplast a, C	*Cyanidioschyzon merolae*	
Cyanoplast a, C	*Galdieria sulphuraria*	

[a]The author recommends changing the term cyanelle to cyanoplast for each listed strain where nucleus-encoded proteins of the cyanelle have been observed (see text).

[b]Abbreviations: *: uncertain affiliation; a: acido- and thermophilic habitat; b: brackish water habitat; C: evidence for photosynthetic carbon dioxide fixation; f: freshwater habitat; N: evidence for nitrogen fixation; #: cyanelles considered as cyanoplasts in this review; #1: *Skujapelta nuda* (Hall and Claus, 1967); but see also #2; #2: *Cyanocyta korschikoffiana* (Hall and Claus, 1963): it is proposed to retain this name for the old and unknown ancestor of the cyanoplast of *Cyanophora paradoxa* (Schenk, 1990).

[c]Reference citation: (1) Pankow 1982, (2) Geitler 1977, (3) Lowe et al. 1984, (4) Drum and Pankratz 1965, (5) all listed as Glaucocystophyta, after Kies and Kremer (1986), (6) Bourrelly 1960, (7) Schiller 1954, (8) Kies (1989), (9) Fukuda (1981), Seckbach et al. (1983), Seckbach et al. (1990), and (10) Kremer et al. (1978) and Maid et al. (1990).

an original endocyanome. Skuja (1950) has argued, in a similar manner in the case of *Glaucocystis* and *Gloeochaete,* that these "associations involve such inseparable integration between the components that they may be considered as one." Pringsheim (1963) agreed and suggested that these "cyanelles should perhaps not be referred to the Cyanophyceae, although

they may well have arisen from them." That was also recognized intuitively by Schenk and Hofer (1972), Herdman and Stanier (1977), Löffelhardt et al. (1980), Trench (1981), Heinhorst and Shively (1983), Bohnert and Löffelhardt (1984), Marten and Brandt (1984), Kies (1984a), and by Kies and Kremer (1986a, 1986b). Without accepting this position, in the future, all plant organisms may be called symbiotic consortia and their plastids renamed with taxonomic binary names. Consequently, I would like to propose that such developed descendants of endocyanomes be called "metacyanomes" (a new, nonsystematic term) and descendants from cyanelles be called "cyanoplasts." The originating "metacyanomic" cell is also no longer the eukaryotic "host" cell. The original host and symbiont cells have coevolved to a new eukaryotic cell type with a new, more complex intracellular, cybernetic system. Today, one could say that it was not correct to consider the species given in Table 8 as symbiotic consortia (what cyanomes really should be by definition) or to call the cyanoplasts of these organisms as cyanelles (what, by definition, these "cyanelles" truly are not). It remains undisputed that, apart from *Cyanophora paradoxa,* this should be demonstrated for each case, in the future. If we wish to retain the term cyanelle, we should use it again in the sense of Pascher (1929), assuming that cyanoplasts are descendants of originally endocytobiotic cyanobacteria, the endocyanelles, and knowing that cyanoplasts are not real cyanelles, but symbiogenetic organelles like the other plastids of plant organisms. But this does not say whether cyanoplasts are representatives of missing links or blind alleys with respect to chloroplast evolution. However, endocyanomes together with their cyanelles, as well as metacyanomes with their cyanoplasts, are clearly important analogous model organisms (Schnepf et al., 1966; Echlin, 1966; Taylor, 1970, Schenk and Hofer, 1972) for studying the mechanisms of plastid evolution with reference to the "(Endo) symbiosis Theory" (Altmann, 1890; Mereschkowsky, 1905, 1910; Geitler, 1923; Sagan, 1967; Margulis, 1970) and the "Serial Endosymbiosis Theory" (Taylor, 1974; Margulis, 1981).

Metacyanomes

THECAMOEBA. *Paulinella chromatophora,* a thecamoeba with two longish cyanobacterial-like entities called cyanelles, was observed in freshwater habitats and in brackish water up to a salt content of ca. 1% (Pankow, 1982). It apparently lives autotrophically (Pascher, 1929) since no food vacuoles could be observed either in the light or electron microscope (Kies, 1974, 1980). All attempts to cultivate this fascinating cyanome or its separated cyanelles have been unsuccessful (Kies and Kremer, 1979). Physiological studies (Kies and Kremer, 1979) have demonstrated the photoassimilation of carbon dioxide (at a rate comparable to free-living cyanobacteria) and an incorporation of up to 22% of the fixed carbon dioxide into glucose, obviously not unsimilar to the behavior found in cyanophilic lichens with one phycobiont. Pascher (1929) observed an elongation by growth of a cyanelle, dividing after isolation, so that it can be assumed that this rhizopode is really still an endocyanome with cyanelles. But the isolated cyanelles have not been cultivated (Kies and Kremer, 1979), and because of the strong internal control of cyanelle number (Kies, 1974, 1984b), a genetic integration seems likely. Therefore, *Paulinella chromatophora* could be one of the phyletically youngest representatives of the known metacyanomes.

DIATOMS. All diatoms listed in Table 8 are found in freshwater habitats in the littoral zone (periphyton) of lakes and ponds. They have been reported from middle Europe, *(D. vanheurcki* and *E. zebra* var. *saxonica),* "Sunda" in Sumatra (Ranau lake), and (USA) *(E. adnata)* in lake Michigan. In addition to the conventional diatom chloroplast, they contain differing numbers of bluish-green inclusions, so-called "sphaeroid bodies" (Hutstedt 1930), which are transmitted from one generation to the other. During cell division, each daughter cell receives at least one spheroid body. The epithemiacean *D. tenuis* is an exception, not bearing such spheroid bodies (Geitler, 1953). Drum and Pankratz (1965) concluded from electron microscopic studies that because they have a thick five-layered cell wall, these bodies presumably are modified coccoid cyanobacteria. The thylakoids are not concentrically arranged as in most other cyanelles, but more radially, and phycobilisomes seem to be absent (Floener and Bothe, 1980). Some characteristics of these bodies indicate their cyanelle nature: 1) The number per cell is not constant; 2) the number is different in the various species, e.g., *E. zebra* bears 1–16 bodies/cell, *E. sorex* normally only 2, and *Rhopalodia gibba* 2–5; 3) the division of the bodies is not synchronized with that of the diatom cell; and 4) their multilayered cell wall, which is comparable to that of aposymbiotic unicellular cyanobacteria. These bodies must have the capability for dinitrogen fixation, because cells of *Rhopalodia gibba* fix nitrogen at a low overall rate, as measured by acetylene reduction: 10 nmoles ethylene formed

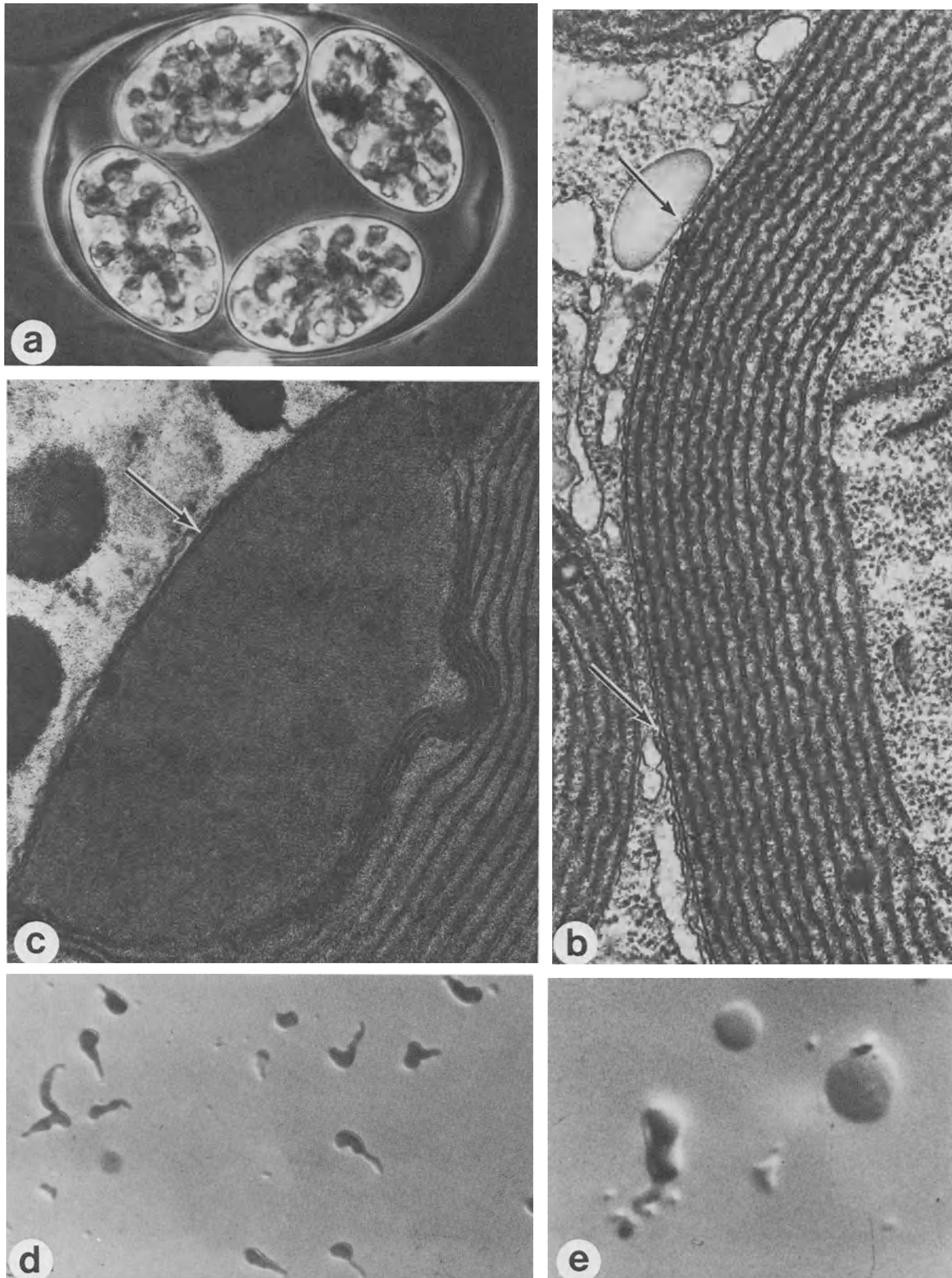

Fig. 4. *Glaucocystis nostochinearum:* (a) four daughter autospores (about 20 μm in length) within an old autospore mother cell wall (phase contrast); (b) crenulating outer membrane surrounding the cyanelle shown by arrows; (c) horizontal striations suggesting a crystalline substance in the lammelar-free region of an immature cyanelle. Electron-dense layer interpreted as peptidoglycan is visible at arrow; (d) cyanelles (about 6 μm in length) isolated in *Glaucocystis* culture medium, 24°C; (e) spheroplast formation of cyanelles in an osmoticum containing sorbitol (0.6 M) and lysozyme (5 μg/ml), 24°C. (From Scott et al., 1984; with permission of H. Bonnett.)

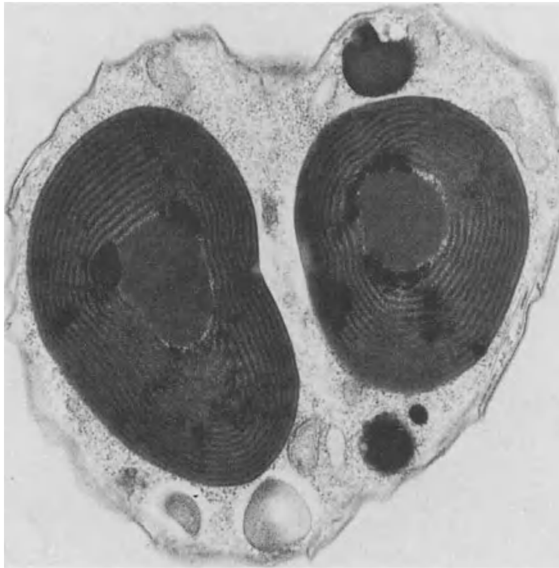

Fig. 5. *Cyanophora paradoxa:* cross-section showing two cyanoplasts, mitochondria, starch granules, and peripheral lacunae. (Courtesy of M. Amiressami.)

per mg protein and per h (Floener and Bothe, 1980). Lowe et al. (1984) could demonstrate that the ability to fix nitrogen offers *Epithemia* and *Rhopalodia* a competitive advantage in nitrogen-poor habitats. The question of whether these bodies really are cyanelles or are a new (dinitrogen-fixing) kind of cyanoplast still has to be examined.

GLAUCOCYSTOPHYTA. Recently, the Glaucocystophyta with one class, the Glaucocystophyceae, have been redefined on the basis of new biochemical and ultrastructural data (Kies and Kremer, 1986b; Skuja, 1954). Some main characteristics of this class are: 1) a system of peripheral lacunae; 2) dorsi-ventrally shaped motile stages; and 3) two subapical inserted heterokont and heterodynamic flagella (see also Heimann et al., 1989), both with nontubular mastigonemes. The Glaucocystophyceae include unicellular or colonial, monadoid, capsalean, or coccoid eukaryotic algae which instead of chloroplasts contain blue-green cell compartments called "cyanelles," which resemble endocytobiotic cyanobacteria. Although it is relatively easy to isolate these cyanelles, it is impossible to cultivate them. Because they are dependent not only metabolically but also genetically on the eukaryotic cell (nucleus), we prefer to call them "cyanoplasts" (see above). These entities are globular or elongated structures, mostly surrounded by remnants of a cell wall (Fig. 4) (except for the cyanoplasts of *Glaucosphaera*). This wall is, as demonstrated for *Cyanophora* (Schenk, 1970, 1977; Heinz, 1973; Aitken and Stanier, 1979) and for *Glaucocystis* (Scott et al., 1984), a murein sacculus consisting of peptidoglycan. This wall around the cyanoplasts (of *Cyanophora, Cyanoptyche, Glaucocystis,* and *Gloeochaete*) is one of the main morphological differences between them and the plastids of other algae and plants. The existence of this wall suggests that cyanoplasts are phylogenetically derived from cyanelles (endocytobiotic cyanobacterial ancestors). The cyanoplasts of the Glaucocystophyceae bear within the centroplasm or assymmetrically located at the smaller end of the plastid *(Glaucocystis),* a dense polyhedral body called a carboxysome (Mangeney and Gibbs, 1987). The carboxysomes of *Cyanoptyche* (Kies, 1989) and *Gloeochaete* (Kies, 1976) cyanoplasts are surrounded by "some sort of membrane which is not a unit-membrane." For more information the reader is directed to the review by Kies and Kremer (1989).

Glaucocystis nostochinearum

This species and other representatives of the genus *Glaucocystis* are found around the world (e.g., Griffiths, 1915; Chodat, 1919; Bourelly, 1960; Prasad, 1961; Fenwick, 1966; Tell, 1979). Colwell and Wickstrom (1976) have investigated the interrelationships between cell division in cyanoplast and "host." They conclude that host cell and cyanelle division mechanisms are partially independent of each other. The first ultrastructural investigation was published by Ueda (1961), followed by those of Schnepf (1965, 1966), Lefort (1965), Echlin (1966), Schnepf et al. (1966), Schnepf and Koch (1966), Hall and Claus (1967), Robinson and Preston (1971a, 1971b), Schnepf and Brown, (1971), Willison and Brown (1978), Kies (1979), and Lefort-Tran (1981). The pigments were analyzed by Chapman (1966): chlorophyll *a*, β-carotene, zeaxanthin, *C*-phyco- and allophycyanin; and by Schmidt et al. (1979): β-cryptoxanthin (perhaps already detected by Chapman, 1966). Typical cyanobacterial carotenoids are absent. The lipid pattern was investigated by Scott (1987). The ^{14}C-incorporation during photosynthetic carbon dioxide assimilation was mentioned by Schenk and Hofer (1972), and demonstrated by Kremer et al. (1979). The latter authors concluded that from a taxonomic point of view, the results support the assumption that *Glaucocystis* may not be regarded as a representative of the Rhodophyceae as suggested by Schnepf and Brown (1971).

Cyanophora paradoxa

This alga (Fig. 5) represents the most extensively and best-investigated species within the

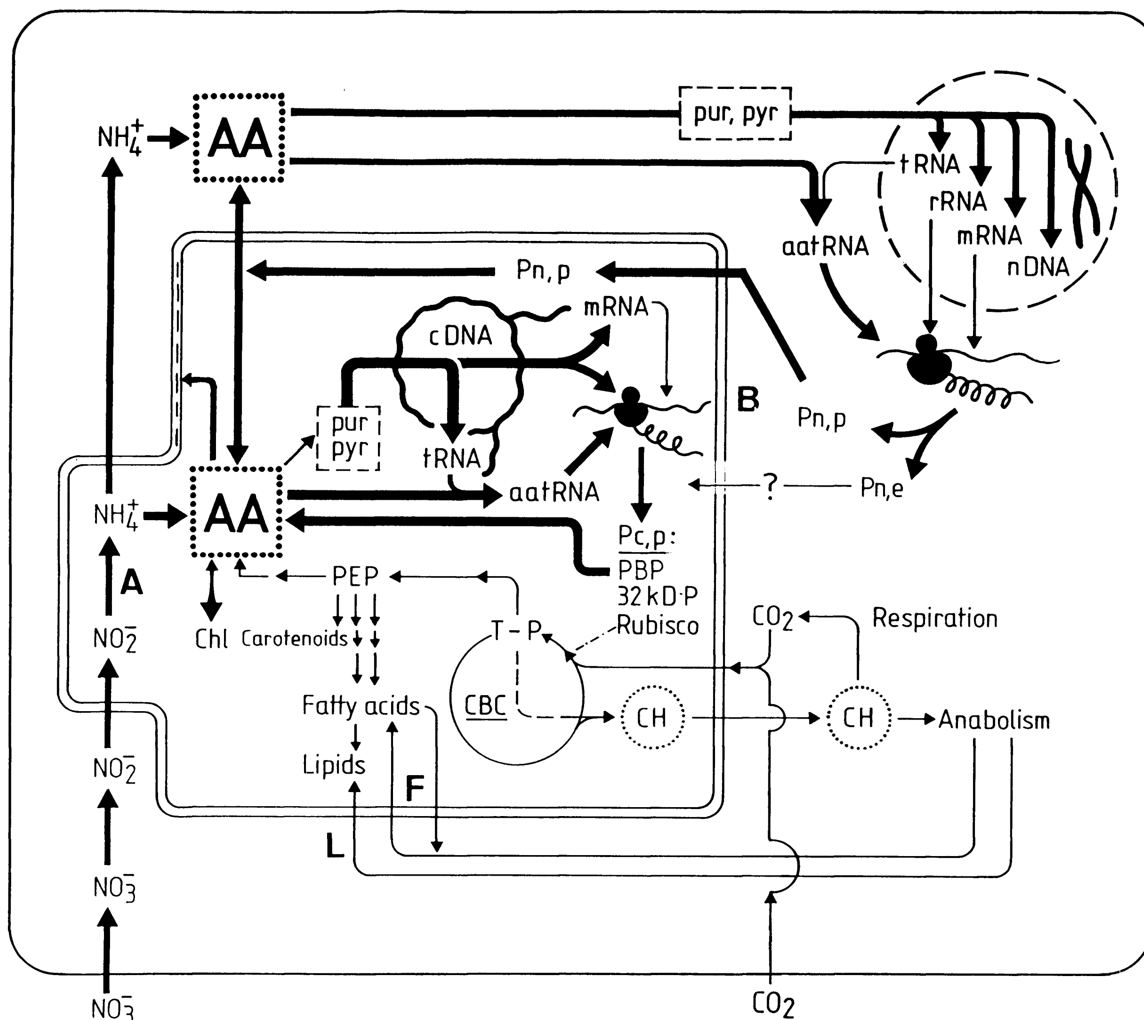

Fig. 6. *Cyanophora paradoxa:* metabolic relationships between the cytoplasmic space (outer rectangle) and the cyanoplast compartment (inner rectangle). Abbreviations: A: nitrite reductase, export of ammonia and amino acids; AA: presumed amino acid pool; B: import of nucleus-encoded proteins; Pn,p: originally prokaryotic proteins—probably as a result of gene transfer; Pn,e: eukaryotic proteins, or protein substitution/not found as yet; CBC: Calvin (-Benson) cycle; CH: carbohydrate pool; F: C_{18} fatty acid export and C_{20} fatty acid import; L: phospholipid (PE) import (for more description see text).

Glaucocystophyceae. In addition it is very interesting that two strains have been found, the Pringsheim and the Kies strains, with different cyanoplast ("cyanellar") DNA (cyDNA) (Löffelhardt et al., 1983; Breiteneder et al., 1988). The cyDNA diversity between the two strains resembles plastid DNA diversity between different species of the green alga *Chlamydomonas.* The inner envelope membrane of the cyanoplasts, observed in freeze-fracture, resembles cyanobacterial plasma membranes and is dissimilar to the chloroplast envelope membranes of red or green algae (Giddings et al., 1983). The pigments were investigated qualitatively by Chapman (1966), Trench and Ronzio (1978), and Schmidt et al. (1979) and seem to be comparable to those of *Glaucocystis;* a method for routine measurements by in vivo visible light spectroscopy with regard to quantitative estimations of carotenoids, chlorophyll *a,* phycocyanin, and allophycocyanin was given by Schenk at al. (1983). Michalowski et al. (1990) were able to detect a third phycobiliproteid, namely, a second allophycocyanin gene.

The metabolic relationships between the cyanoplast compartment and the cytoplasmic space are summarized in Fig. 6. Carbon dioxide photoassimilation (CBC in Fig. 6) was described by Schenk and Hofer (1972), Trench et al. (1978), and Kremer et al. (1979): Besides the incorporation of ^{14}C in the metabolites of the Calvin cycle, it also shows a rapid incorporation into dicarboxylic acids. Kremer et al. (1979) stated that glucose is the main carbohydrate

Table 9. Gene locations of proteins of cyanelles (cyanoplasts) of *C. paradoxa*.

Gene product	Gene location			Reference[a]
	Cyanelle	Nucleus	Chloroplast	
Components of photosystem I				
Apoprotein P700a (psaA)	+		+	1; 2
Apoprotein P700b (psaB)	+		+	1; 2
Ferredoxin-$NADP^+$-oxidoreductase	−	+	−	3
Components of photosystem II				
D-1(herbicide-binding)-protein (psbA)	+		+	1; 2; 4
Apoprotein P680 (51 kDa) (psbB)	+		+	1; 2
Apoprotein (44 kDa) (psbC)	+		+	1; 2
D-2 protein (psbD)	+		+	1
Apoprotein cytochrome *b*-559 (psbE)	+		+	1
Apoprotein cytochrome *b*-559 (psbF)	+		+	1
Protein psbJ	+		+	5
Small polypeptide of PS II (psbK)	+		+	6
Protein psbL	+		+	5
ATP-synthase complex				
α-Subunit, CF_1 (atpA)	+		+	1; 2; 4
β-Subunit, CF_1 (atpB)	+		+	1; 2; 4
ϵ-Subunit, CF_1 (atpE)	+		+	1; 4
γ-Subunit, CF_1	−	+	−	7
Subunit I, CF_1 (atpF)	+		+	8
Subunit III, CF_0 (atpH)	+		+	2; 4
Cytochrome b_6/f complex				
Apoprotein cytochrome *f* (petA)	+		+	1; 4
Apoprotein cytochrome b_6 (petB)	+		+	2
Subunit IV (petD)	+		+	2
Subunit V (petG)	+		+	9
Ferredoxin I (petF I)	+	−	−	10
Ribulose 1,5-bisphosphate carboxylase				
Large subunit (rbcL)	+		+	1; 2; 4; 11; 12
Small subunit (rbcS)	+		−	1; 2; 11; 12
Ribosomal proteins (small subunit)				
Protein S4 (rps4)	−		+	8
Protein S7 (rps7)	−		+	8
Protein S8 (rps8)	+		+	13
Protein S11 (rps11)	−		+	8
Protein S12 (rps12)	−		+	8
Protein S18 (rps18)	+		+	14
Protein S19 (rps19)	+		+	2; 14
Ribosomal proteins (large subunit)				
Protein L2 (rpl2)	+		+	14
Protein L3 (rpl3)	+		−	14; 15
Protein L5 (rpl5)	+		−	13
Protein L6 (rpl6)	+		−	13
Protein L20 (rpl20)	+		+	13
Protein L22 (rpl22)	+		+	14
Protein L33 (rpl33)	+		+	14
Protein L35 (rpl35)	+		−	13
Translation proteins				
Initiation factor IF-1 (infA)	−		+	8
RNA polymerase, α-subunit (rpoA)	−		+	8
Phycobiliproteins				
α-Subunit, C-phycocyanin (pcyA)	+		n.p.	1; 11; 16
β-Subunit, C-phycocyanin (pcyB)	+		n.p.	1; 11; 16
α-Subunit, allophycocyanin (apcA)	+		n.p.	1; 11; 17
β-Subunit, allophycocyanin (apcB)	+		n.p.	1; 11; 17
δ-Subunit, allophycocyanin (apcD)	+		n.p.	18
Linker phycobiliprotein LCM 100 (apcE)	+		n.p.	19
Linker polypeptides (L 1–4)	−	+	n.p.	7

Symbols: +, detected; −, not detected; n.p., proteins not present in chloroplasts.

[a]Reference citations: (1) Lambert et al. 1985, (2) Bohnert et al. 1985, (3) Bayer et al. 1990, (4) Ko et al. 1985, (5) Cantrell and Bryant 1987, (6) Stirewalt and Bryant 1989a, (7) Burnap and Trench 1989c, (8) Wasmann et al. 1987, (9) Stirewalt and Bryant 1989b, (10) Bayer and Schenk 1989, Neumann-Spallart et al. 1990, (11) Starnes et al. 1985, (12) Heinhorst and Shively 1983, (13) Bryant and Stirewalt 1990, (14) Evrard et al. 1990b, (15) Evrard et al. 1990a, (16) Lemaux and Grossmann 1984, (17) Bryant et al. 1985, (18) Michalowski et al. 1990, (19) Bryant 1988.

transferred (from cyanoplast to cytoplasm). Floener et al. (1982) could demonstrate that, similar to the chloroplasts of higher plant cells, only nitrite is reduced in the cyanoplasts (see A in Fig. 6) and not nitrate. A surprising observation was the discovery of polyunsaturated eicosenoic acids, i.e., arachidonic and eicosapentaenoic acid, as main fatty acids besides palmitic acid in the cyanoplasts (Schenk et al., 1985; Kleinig et al., 1986; Zook et al., 1986). These eicosenoic acids have as yet not found in cyanobacteria. They seem to be derived from C18 fatty acids, synthesized in the cyanoplast compartment, and formed by elongation and dehydrogenation of C18 fatty acids in the cytoplasmic space and finally, retransferred to the membranes of the cyanoplast, a process that represents a typical case of "metabolic substitution" (Schenk, 1990). The phycobiliproteids in *C. paradoxa* not only have the function of accessory pigments but also that of nitrogen-storage proteins (Schenk et al., 1983, 1987a) (arrow from PBP to AA in Fig. 6). It seems that the phycobiliproteids of *G. nostochinearum* do not share nitrogen-storage functions (Schenk et al., 1987a). The amino acid sequence of ferredoxin is a typical cyanobacterial one (Stevanovic et al., 1989; Neumann-Spallart et al., 1990). Jehn and Zetsche (1988) reported in vitro synthesis of cyanoplast proteins by isolated cyanoplasts and cyanoplast RNA. Information on the gene localization of cyanoplast proteins is given in Table 9. It seems of interest that the cyanoplast genome, unlike the chloroplast genome, still codes for the SSU small subunit of nibulose-bisphosphate carboxylase (Rubisco) (Heinhost and Shively, 1983), for ferredoxin (Bayer and Schenk, 1989) and for the ribosomal L3 protein (Evrard et al., 1990a, 1990b). Up to 90% (Bayer and Schenk, 1986) or more than 80% (Burnap and Trench, 1989a, 1989b) of the soluble cyanoplast proteins are encoded on the nuclear genome. That could be demonstrated with crossed inhibition experiments of translation and transcription, and in the same manner also for the ferredoxin-$NADP^+$-oxidoreductase (Bayer et al., 1990), a first indication of the existence of "gene transfer." Burnap and Trench (1989c) were able to detect some indication of precursor proteins (with "transit sequences": 1.5–7 kDa) of nucleus-encoded cyanoplast proteins. For further information about this interesting organism, the reader is directed to the reviews by Trench (1982), Wasmann et al. (1987), and Schenk (1990). Cluster analyses with 5S rRNA sequences (Van den Eynde et al., 1988) and with 16S rRNA partial sequences (Giovannoni et al., 1988) suggest that the cyanoplast of *C. paradoxa,* indeed, is not situated within the cyanobacterium-plastid cluster but rather is contained within the cyanobacterial radiation.

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CHAPTER 213

Prokaryotic Symbionts of Amoebae and Flagellates

KWANG W. JEON

Amoebae and flagellates have long been known to be associated with both extracellular and intracellular symbionts (Hall, 1969; Kirby, 1941a; Lee et al., 1985). The presence of prokaryotic symbionts on and in flagellates and in some amoebae, as observed by light microscopy, was reported by several authors during the late 1800s and the early part of this century, as was comprehensively reviewed by Kirby (1941a). Symbiont-bearing flagellates were chiefly found in termite guts, and only a few free-living flagellates were found to have adhering symbionts. Hall (1969) extensively reviewed the literature on symbionts of protozoa published since 1941. Both in flagellates and amoebae, the suspected presence of some of the small bacterial symbionts had to be confirmed later by more sophisticated methods such as electron microscopy and specific staining.

Amoebae and flagellates represent two very diverse groups of protozoa and it is not possible to cover all known prokaryotic symbionts in depth. In this chapter, we shall simply list known symbionts described in the above two reviews in a tabular form (Tables 1 and 2) and then consider newly found symbionts or results of recent studies on earlier symbionts in some detail. The significance of symbiotic relationships remains obscure in most cases and in only a few instances has the host-symbiont relationship been studied in detail. Some disagreements remain about whether the term "symbiosis" should be limited to associations where definite benefits have been proven to exist or not. In this chapter, we shall use the broader definition of symbiosis to include parasitism, commensalism, and mutualism, as did Kirby (1941a) and Hall (1969). Thus, the list of prokaryotic symbionts will include those whose relationships to their hosts are not known or may not be mutually beneficial.

The heightened interest in cellular symbiosis in recent years has been stimulated, in part, by the notion that eukaryotic cell organelles such as mitochondria, chloroplasts, and microtubules may have originated from endosymbionts, i.e., the Serial Endosymbiosis Theory (Sagan, 1967; Margulis, 1970, 1981; Taylor, 1974; papers in Lee and Fredrick, 1987). Some authors have felt that the role of endosymbiosis in the origin of eukaryotic cell organelles has not yet been clearly established (Gray and Doolittle, 1982), but the theory is gaining wider support in view of recent results on the close relationship between the ribosomal RNAs of prokaryotes and those of chloroplasts and mitochondria (e.g., Watson et al., 1987). It should be noted that an opposing view has existed, according to which such organelles evolved as a result of autogenous intracellular differentiation

Table 1. Symbionts of amoebae.

Symbiont name or type	Host species (origin)	Reference
Methanobacterium	*Mastigella*	Goldschmidt, 1907
Methanobacterium	*Pelomyxa*	Gould-Veley, 1905
Schizomycetes	*Pelomyxa*	Penard, 1902
Bacilli	*Acanthamoeba* sp.	Hall and Voelz, 1985
Bacilli	*Acanthamoeba castellanii*	Drozanski, 1956
Bacilli	*Amoeba proteus* (Chicago)	Roth and Daniels, 1961
Bacilli (X-bacteria)	*A. proteus* (Scotland)	Jeon and Lorch, 1967
Cocci	*A. albida*	Nagler, 1910
Cocci	*A. proteus*	Cohen, 1957
Micrococci	*Entamoeba minchini*	Mackinnon, 1914
Micrococci	*E. muris*	Wenyon, 1907
Micrococci	*Sappinia*	Dangeard, 1896

Table 2. Symbionts of flagellates.

Symbiont name or type	Host species (origin)	Reference
Ectosymbionts		
Green bacteria	*Mastigamoeba*	Lauterborn, 1916
Fusiformis	*Caduceia*	Kirby, 1936
Fusiformis	*Devescovina*	Duboscq and Grasse, 1926
Fusiformis	*Foaina signata*	Kirby, 1942b
Fusiformis	*Lophomonas*	Grasse, 1926
Fusiformis	*Mactrichomonas*	Kirby, 1942a
Fusiformis	*Polymastix*	Grasse, 1926
Treponema	*Devescovina*	Duboscq and Grasse, 1926
Bacilli	*Barbulanympha*	Cleveland et al., 1934
Bacilli	*Bullanympha*	Kirby, 1938b
Bacilli	*Chrysostephanosphaera*	Geitler, 1948
Bacilli	*Kalotermes*	Kirby, 1938a
Bacilli	*Lophomonas striata*	Beams et al., 1960
Bacilli	*Macrotrichomonas*	Kirby, 1938b
Bacilli	*Metapolystoma*	Skuja, 1958
Bacilli	*Oxymonas grandis*	Cross, 1946
Bacilli	*Rhynchonympha*	Cleveland et al., 1934
Bacilli	*Streblomastix strix*	Grimstone, 1961
Bacilli	*Trichonympa*	Kirby, 1932
Bacilli	*Urinympha*	Cleveland et al., 1934
Spirochetes	*Devescovina vestita*	Kirby, 1941b
Spirochetes	*Holomastigotoides*	Koidzumi, 1921
Spirochetes	*Hyperdevescovina*	Nurse, 1945
Spirochetes	*Mixotricha*	Cleveland and Grimstone, 1964
Spirochetes	*Rostronympha*	Duboscq et al., 1937
Spirochetes	*Spirotrichonympha*	Sutherland, 1933
Spirochetes	*Spirotrichonymphella*	Sutherland, 1933
Endosymbionts		
Carococcus	*Trichonympha*	Kirby, 1944
Pseudomonas	*Volvox aureaus*	Hamburber, 1958
Bacilli	*Costia pyriformis*	Davis, 1943
Bacilli	*Euglenoids*	Tschermak-Woess, 1950
Bacilli	*Gigantomonas*	Kirby, 1946
Bacilli	*Hyperdevescovina*	Kirby, 1949
Bacilli	*Macrotrichomonas*	Kirby, 1942a
Bacilli	*Volvox carteri*	Kochert and Olson, 1970
Bacilli (bipolar body)	*Strigomonas*	Newton and Horne, 1957
Bacilli (diplosome)	*Blastocrithidia*	Novey et al., 1907

without involving symbionts (Cavalier-Smith, 1975; Raff and Mahler, 1972; Uzzel and Spolsky, 1974, 1981).

Meanwhile, it is interesting to note that *Pelomyxa palustris,* which does not have mitochondria (Daniels et al., 1965; Leiner and Wohlfeil, 1953) contains several types of intracellular symbionts (Daniels, 1973), and the suggestion has been made that such symbionts may carry out metabolic functions in place of mitochondria (Chapman-Andresen, 1971). Bacteria present in *P. palustris* have been found to be methanogenic (van Bruggen et al., 1983, 1985; see also Chapter 33) and may function as electron sinks related to energy production, comparable to mitochondrial function in aerobic eukaryotic cells.

In the case of amoeba-bacteria symbiosis, the D strain of *Amoeba proteus* became spontaneously infected with a large number (60,000–150,000 bacteria per amoeba) of rod-shaped Gram-negative bacteria (Jeon and Lorch, 1967). Initially, the bacteria were harmful and brought about damaging effects to their hosts, called xD amoebae, such as reduced cell size, slower cell growth, increased membrane fragility, sensitivity to starvation, and a poor clonability. When introduced into symbiont-free D amoebae, the bacteria multiplied and killed their new hosts within a few host cell generations. However, adverse effects of infection gradually diminished over a period of about 1 year, and the bacteria became less virulent, bacteria-bearing xD amoebae growing well with near-normal growth

rates. Also, some of the newly infected D amoebae survived, indicating a reduced virulence as compared to earlier infection.

Within a few years, host amoebae became dependent on their endosymbionts (Jeon, 1972). Thus, xD amoebae lost viability when they were deprived of endosymbionts either by nuclear transplantation (Jeon and Jeon, 1976), by treatment with antibiotics (Jeon and Hah, 1977), or by raising the culture temperature (Jeon and Ahn, 1978). Aposymbiotic xD amoebae could be resuscitated only by reintroducing live X-bacterial symbionts (Lorch and Jeon, 1980). Newly infected amoebae became dependent on their symbionts after about 200 cell generations or 18 months. The reason for the hosts' dependence is not known, but preliminary evidence suggests that a symbiont-synthesized protein may be required for the survival of hosts. When xD amoebae are grown in the presence of chloramphenicol (100–700 μg/ml) or rifampicin (125 μg/ml), the synthesis of a unique 29-kDa polypeptide by endosymbionts is instantly suppressed and xD amoebae die much sooner than do symbiont-free D amoebae (Kim and Jeon, 1986, 1987a). The symbiont's gene coding for the xD-specific protein has been cloned (Park and Jeon, 1988) and its nucleotides sequenced (Park and Jeon, 1989). The symbiotic bacteria were found to accumulate host actin selectively (Kim and Jeon, 1987b), as studied using a monoclonal antibody against the amoeba actin. Thus, in this example, the transition of spontaneously infecting parasites to required cell components was observed while it occurred (Jeon, 1980, 1983, 1986, 1987). While the host's dependence on symbionts developed over 200 host cell generations, some physiological characters changed after a few host cell divisions (Lorch and Jeon, 1981, 1982).

In *Blastocrithidia culicis* and *Crithidia oncopelti,* symbiotic bacteria were found to supply their hosts with lysine (Gill and Vogel, 1962, 1963), hemin (Guttman and Eisenman, 1965; Chang and Trager, 1974; Newton, 1956, 1957), and other nutritional factors. An aposymbiotic host, produced by growing symbiont-bearing cells in the presence of chloramphenicol, required exogenous hemin for growth. The ectosymbiotic spirochetes on *Myxotricha* were found to help their host move by their coordinated undulation while the host's flagella functioned only to steer its movement (Cleveland and Grimstone, 1964). Flagella of prokaryotic symbionts attached to *Cryptotermes* were also found to help propel the host cell (Tamm, 1978b).

These are a few examples in which protozoan hosts and prokaryotic symbionts have developed an intimate relationship, and symbiont integration and host-symbiont interactions have been experimentally studied.

Habitats

General

The host-symbiont relationship appears to be somewhat specific for both ecto- and endosymbionts, since certain symbionts are almost always found associated with given hosts (Hall, 1969; Kirby, 1941a). However, the mechanism for specific recognition of the host by symbionts is not known. For ectosymbionts, their only requirement for continuing symbiotic association with their hosts would be to stay attached to their host-cell surfaces. Some symbionts cover the whole surface of their host, while others are limited to certain areas of the host's body (Kirby, 1941a). In some cases, specialized attachment sites such as brackets and underlying network of fibrous strands are present, as in *Myxotricha* (Fig. 1A; Cleveland and Grimstone, 1964). On the basis of extensive electron-microscopic observations, Cleveland and Grimstone reconstructed the attachment complexes as shown in Fig. 1B. Tamm (1978a) also found membrane specialization where two kinds of bacteria, rod-shaped and filamentous, were attached to *Crytptotermes cavifrons.* Thus, the attachment of ectosymbionts is not haphazard but appears to be helped by structural adaptations on the part of host cells.

For endosymbionts, their habitat in the cytoplasm of host cells can be considered to be an extreme environment comparable to hot springs and salt lakes (Moulder, 1979), although most symbionts live within symbiont-containing vesicles and the membranes protect them from direct exposure to the host cytoplasm. These symbiont-containing vesicles have been called "symbiosomes" (Fig. 2; Roth et al., 1988). It appears that endosymbionts have adapted in various ways to overcome adverse effects of the harsh environment, e.g., the possession of rigid cell walls to withstand digestive action of the hosts' hydrolytic enzymes (Drozanski and Chmielewski, 1979; Han and Jeon, 1980) or prevention of lysosomal fusion with symbiosome membranes, thus avoiding exposure to hosts' digestive enzymes (Ahn and Jeon, 1979; Armstrong and Hart, 1971; Jeon, 1983).

Amoebae

In amoebae, all known bacterial symbionts are intracellular. This is understandable since amoebae do not have a firm cortex and ecto-

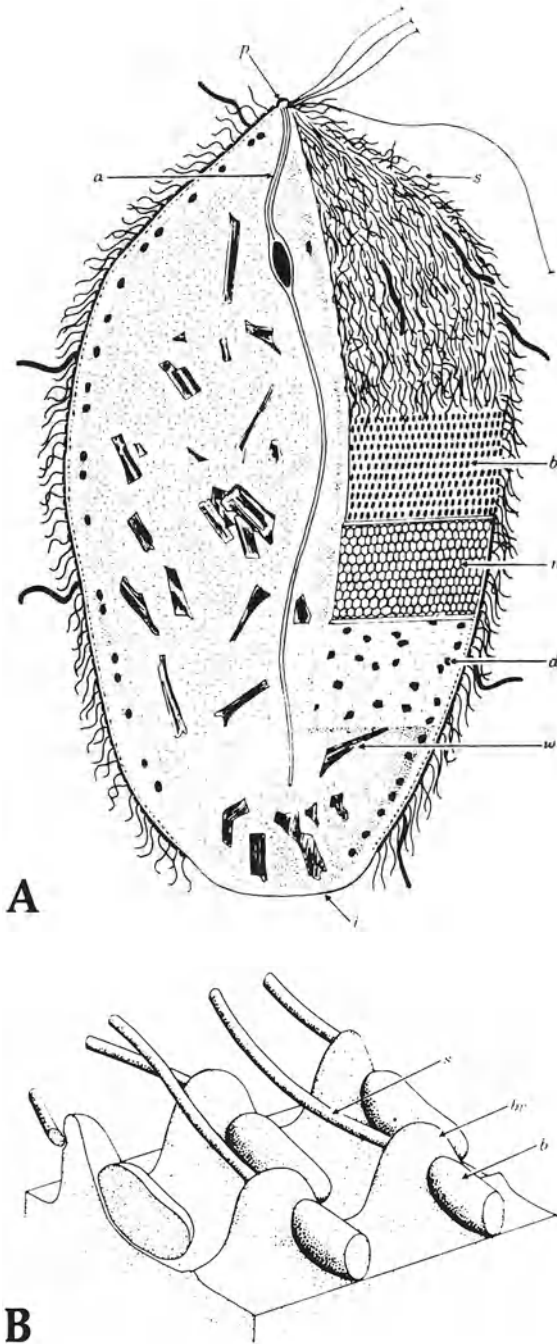

Fig. 1. A diagram of *Myxotricha paradoxa* showing the attachment of ectosymbionts on the cell surface (A) and a reconstruction of a small area of the cell surface showing the attachment complex (B). (A) An optical section is shown on the left and surface structures are shown on the right. b, bacteria; br, brackets; n, fibrous network; s, spirochetes. (From Cleveland and Grimstone, 1964.)

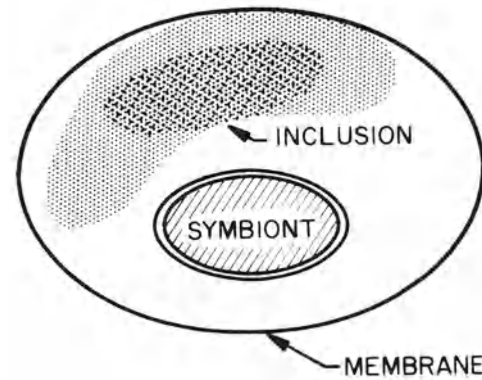

Fig. 2. A schematic diagram of a symbiosome to show its components. Each line represents a membrane and the intrasymbiosome space is shown to contain inclusions. (From Roth et al., 1988.)

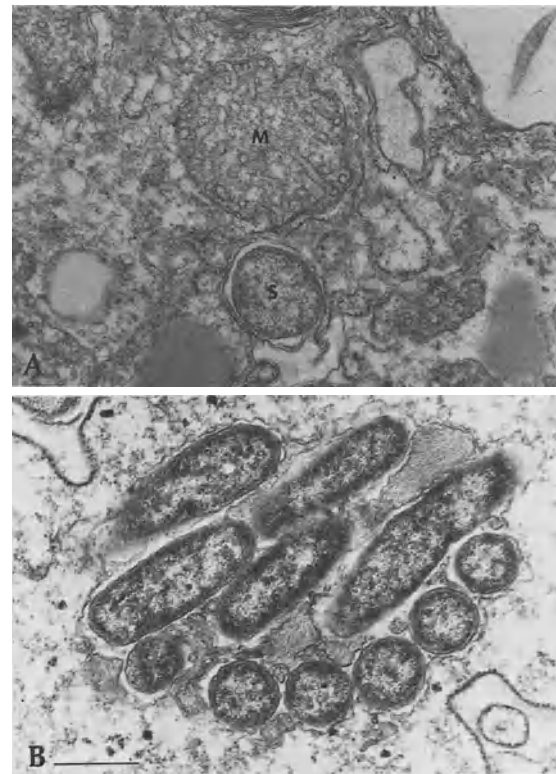

Fig. 3. Electron micrographs of symbiosomes in amoebae. (A) A small symbiosome with a single round symbiont (S) called a "DNA-containing body" and (B) a larger symbiosome with several rod-shaped symbionts. (A) A mitochondrion (M) with tubular cristae is shown for comparison. (B) The interbacterial space is filled with inclusions containing fibrous matter. Bar = 0.5 μm. (Fig. 3B from Jeon, 1987.)

symbionts cannot permanently attach to them as do flagellates. Several different bacterial types have been reported in the cytoplasm of amoebae, mostly enclosed in symbiosomes singly or in groups (Fig. 3). Roth and Daniels (1961) were among the first to confirm by electron microscopy the bacterial nature of previously reported bacteria-like particles (0.5 $\times$ 2 μm) in vacuoles of *Amoeba proteus* (Cohen, 1957). These sym-

bionts could not be eliminated by starvation or penicillin treatment. Chapman-Andresen and Hayward (1963) found rod-shaped bacteria (0.5 × 3–5 μm) in a strain of *A. proteus* (about 4000 bacteria per cell), that could not be detected by light microscopy. Drozanski (1963a) reported Gram-negative rods (0.6–0.8 × 1.3–2.1 μm) that caused a fatal infection in *Acanthamoeba castellanii.* These bacteria first multiplied in food vacuoles and later in the cytoplasm. Other reports followed that confirmed the presence of a large number of bacteria in various strains of amoebae (Jeon and Lorch, 1967; Wolstenholme and Plaut, 1964). In one strain of *Pelomyxa palustris,* Daniels et al. (1966; Daniels and Breyer, 1967) found rod-shaped bacteria (0.3 × 3 μm) within individual vesicles characteristically surrounding the nuclei, while another type of bacteria of similar size was located in other parts of the cytoplasm. In *Acanthamoeba* (Drozanski, 1963a; Hall and Voelz, 1985; Proca-Cibanu et al., 1975), symbionts are found throughout the cytoplasm. In the large, free-living *Amoeba,* all symbionts were enclosed in symbiosomes located in all parts of the cell (Jeon and Lorch, 1967; Wolstenholme and Plaut, 1964). So far, no endonuclear symbionts have been found in amoebae. Many unsuccessful attempts have been made to grow symbiotic bacteria in vitro (Drozanski, 1963b; Jeon and Lorch, 1967; K. Jeon, unpublished observations), and none of the reported bacterial symbionts of amoebae has been grown outside living cells.

The fact that symbionts of amoebae cannot be cultured outside amoebae indicates the symbionts' dependence on their hosts, but in many cases the dependence does not appear to be species-specific. Thus, bacteria isolated from infected *Acanthamoeba castellanii* were able to infect trophic forms of *Hartmannella rhysodes, Schzopyrenus resselli, Didasculus thorntoni,* and other unidentified amoebae of *Limax* type (Drozanski, 1963b). Jeon and Jeon (1982) found that X-bacteria isolated from xD amoebae *(Amoeba proteus)* cross-infected an unrelated species of giant amoeba, *Chaos carolinensis.*

Flagellates

Unlike amoebae, all of whose symbionts are intracellular, flagellates have symbionts in various parts of the cells, some as ectosymbionts and others in the nucleus, in the endoplasmic reticulum, in chloroplasts, or free in the cytoplasm. Most of the prokaryotic symbionts of flagellates were first found as ectosymbionts by light microscopy early in the 20th century (Table 2), and the hosts were mostly flagellates living in termite guts. Some suspected prokaryotic endosymbionts were reported in those flagellates, but confirmation of the bacterial nature of such endosymbionts had to await electron-microscopic observation and advanced biochemical tests. Thus, definitive reports of the presence of endosymbionts in flagellates started to appear in the late 1950s. For example, Roth (1959) found rod-like bacteria within the nucleus of *Peranema,* several hundred per nucleus, while others reported endocytoplasmic symbionts in *Volvox* (Kochert and Olson, 1970). Since then many other flagellates have been found to harbor endosymbiotic bacteria. Gromov (1977) described the presence of Gram-negative bacteria in the surface cortical region of *Trichonympha turkestanica* and suggested that the symbiotic bacteria play a role in the host flagella operation. Gerola (1978) reported inclusion bodies of several bacteria-like elements in *Euglena* cells from an alpine water pool rich in organic residues; the endosymbionts were free in the host cytoplasm, but there was no sign of cytoplasmic reaction around the symbionts detectable by electron microscopy. Endosymbiotic bacteria living inside the endoplasmic reticulum were reported in *Ochromonas monicis* isolated from a saline pool (Doddema and van der Veer, 1983), and these authors thought that the symbionts enabled their hosts to survive in vitamin-poor water. Sousa-Silva and Franca (1985) studied the ultrastructure of two species of dinoflagellates, *Gyrodinium instriatum* and *Glenodinium foliaceum,* harboring bacterial symbionts, some of which were in the nucleus while others were in the cytoplasm, and the third group appeared to live in both.

Chesnick and Cox (1986) presented a summary of results from 17 studies reporting the presence of endosymbiotic bacteria in algal species. In most cases, bacterial symbionts were usually found in small vesicles. However, in the marine alga *Penicillus,* bacteria were contained in one large central vacuole, with occasional presence found in the tip cytoplasm (Turner and Friedmann, 1974). Wilcox (1986) found small bacteria-like symbionts within chloroplasts of the dinoflagellate, *Woloszynskia pascheri,* and suggested that the bacteria may have been in a symbiotic relationship for some time, although no supporting evidence was presented. The author also consider it possible that the "symbionts" may represent regions where chloroplasts' nucleic acids are packaged in membranes. Different species of *Giardia* have been found to have mycoplasma-like organisms attached on the surface of trophozoites while harboring intracellular bacteria in trophozoites and cysts (Feely et al., 1988).

While the roles of many symbionts are not clear, the presence of symbionts has some effects on the structure and physiology of their hosts. Freymuller and Camargo (1981) compared the ultrastructure of symbiont-bearing and symbiont-free species of trypanosomatids and found some differences. For example, paraxial rods of flagella or intraflagellar structures were present only in symbiont-free species, while branching of mitochondria was found exclusively in symbiont-bearing species. Endonuclear symbionts of *Peranema trichophorum* appeared to cause structural changes in their hosts, such as fragmented karyosome and granulation of DNA and RNA within the nucleus (Radchenko, 1983). McLaughlin et al. (1983) found differences in lectin agglutinability between symbiont-bearing and aposymbiotic strains of *Crithidia* and *Blastocrithidia*. Later, it was found that aposymbiotic hosts failed to incorporate detectable amounts of fucose into a major surface glycopeptide (McLaughlin and Cain, 1985a). These authors attributed the differences in lectin agglutinability to different carbohydrate compositions of the flagellates' surfaces. In contrast, McLaughlin and Cain (1985b) failed to detect differences in the incorporation of labeled leucine and methionine between symbiont-bearing and aposymbiotic strains of these hemoflagellates. Krylov et al. (1985) detected many differences between symbiont-bearing (Sym^+) and symbiont-free (Sym^-) strains of *Crithidia oncopelti* and proposed that the two strains be named as different species; observed differences included cell size, flagellum length, colony shape and size, rate of movement, electrophoretic mobility of malate dehydrogenases, growth rate at 32°C, oxygen consumption, and sensitivity to antibiotics. In most other cases, however, host-symbiont relationships have not been clarified.

There have been some reports for the presence of blue-green algae as endosymbionts in various species of dinoflagellates (reviewed by Gaines and Elbrachter, 1987; Steidinger and Baden, 1984). Some of the symbiotic algae appeared to have nutritional importance to their hosts and others were thought to produce toxins.

Isolation

Since all the known endosymbiotic prokaryotes of flagellates and amoebae are obligatory symbionts and do not grow in vitro, the only sure source for their isolation would be symbiont-bearing hosts. Even for facultative ectosymbionts of flagellates that may live free from their hosts at one time or another, their natural host is the best source. *Amoeba proteus* cells are grown in dilute salt solution (Goldstein and Ko, 1976; Jeon and Jeon, 1975). Since these amoebae are strictly phagocytic, they have to be fed live prey organisms, commonly used food organisms for mass culture being *Tetrahymena* cultured axenically in a rich, complex medium containing various vitamins and mineral additives with proteose peptone and liver extract as the main ingredients (Goldstein and Ko, 1976). Amoebae are fed with washed *Tetrahymena* daily or every other day, depending on the desired growth rate. *Acanthamoeba* are cultured axenically in a medium containing proteose peptone, yeast extract, and glucose (Drozanski, 1984). Various media have been used to culture hemoflagellates and other flagellates as hosts of bacterial endosymbionts (e.g., Chang and Trager, 1974).

Bacterial endosymbionts have been isolated in mass from a few species of amoebae and flagellates. No reports are found for mass isolation of ectosymbionts from flagellates. For example, symbiotic bacteria have been isolated from xD amoebae *Amoeba proteus* (Ahn and Jeon, 1982; Han and Jeon, 1980) and *Acanthamoeb castellanii* (Drozanski et al., 1984). In the first example (Han and Jeon, 1980), symbionts were collected by Ficoll gradient centrifugation from lysed amoebae. Later, the method was modified to apply filtration through nylon screens to remove larger pieces of cell debris first (Ahn and Jeon, 1982). Pure bacteria were collected after centrifugation on a sucrose-step gradient. In either procedure, the recovery of symbionts could be greater than 90%. Symbiotic bacteria from *A. castellanii* were obtained by centrifugation from lysed amoebae (Drozanski et al., 1984).

Chang (1975) isolated endosymbionts of hemoflagellates by a sequential treatment of host flagellates by hypotonic shock, complement-dependent immune lysis, and needle passage. Host flagellate cells were incubated in Trager buffer (Trager, 1959) containing guinea-pig serum and rabbit antiserum against flagellates, followed by DNase digestion and differential centrifugation. The final fraction was free of contamination, and the recovery of symbionts was about 20%.

Identification

Since symbionts of amoebae and flagellates cannot be cultured in vitro, the traditional methods of identifying and classifying them (e.g., *Bergey's Manual,* see Buchaman and Gibbons,

1974) cannot be used. Thus, these symbionts have been simply identified as fusiform, rod-shaped, round, or filamentous on the basis of their external morphology based on light or electron-microscopic observations. Most of the bacterial endosymbionts have been reported to be Gram negative, either as a result of Gram staining or by electron microscopic examination for the presence of characteristic surface membranes. More specialized methods are available to identify bacterial species, such as serological tests, bacteriophage typing, and DNA and rRNA sequencing. In particular, the use of specific oligodeoxynucleotide probes, which can be applied in identifying single microbial cells (Giovannoni et al., 1988), should be helpful in dealing with endosymbionts that cannot be grown in vitro. It is also desirable to culture isolated symbionts in vitro for their definitive identification based on their metabolic, physiological, and biochemical characteristics.

Concluding Remarks

This short survey reiterates that many prokaryotic organisms live on and in a wide variety of flagellates and amoebae as symbionts. Their presence is known to bring about various structural, physiological, and biochemical changes in the host. However, many questions still remain unanswered regarding the host-symbiont relationship. For example, it is not clear in many instances how the host and symbionts recognize each other and establish a specific association, how endosymbionts escape digestion by their host, how the symbiont population is controlled, and how the host and symbionts benefit or suffer from their association. Studies of prokaryotic symbionts in flagellates and amoebae have lagged behind those in ciliates (see Chapter 214), where identification and culture of symbionts in vitro and the clarification of the genetic interaction between the host's and symbionts' genomes have been well established. It is hoped that similar advancement will be achieved soon with symbionts in flagellates and amoebae.

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CHAPTER 214

Prokaryotic Symbionts of Ciliates

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Prokaryotes living in ciliates were first noticed over a century ago by J. Müller (1856). Rod-shaped structures were observed in the macronuclei and micronuclei of a number of ciliates, and less commonly, in their cytoplasm. In the beginning, it was not clear whether they were parasites or spermatozoa because the micronucleus was considered to be a testis and the macronucleus an ovary, while chromosome filaments and endonuclear symbionts were mistaken for spermatozoa. This view was corrected by Bütschli (1876), who also wrote the first review on parasites in ciliates (Bütschli, 1889). Accounts of early observations of bacteria in protozoa that followed this initial period were reviewed by Kirby (1941), Wichterman (1953), and Ball (1969).

Interest in prokaryotic endosymbionts of ciliates arose again in the 1950s when it was discovered that a "killer" phenotype is frequently associated with them. The phenomenon that cells of certain ciliate strains may kill cells of other strains by a toxic agent, liberated into the medium, had first been noticed by Sonneborn (1938) in paramecia during experiments on mating types that involved mixing different strains. He found that under certain conditions conjugation could be brought about between killer and sensitive paramecia so that genetic analysis of these traits became technically feasible. Sonneborn (1943) demonstrated that the killer phenotype was an inherited trait that was transmitted via cytoplasmic particles, which he named *kappa*. His findings aroused great interest among geneticists and other biologists, because it furnished one of the first clear examples of a cytoplasmic genetic factor. That *kappa* was an endosymbiont was not known at that time. From data obtained in studies using X rays, Preer (1948a) determined that *kappa* was similar in size to bacteria. He subsequently demonstrated the presence of *kappa* in the cytoplasm of killer paramecia as Feulgen-staining bodies (Preer, 1950). In the following years, cytological, biochemical, and physiological studies by a number of workers established that *kappa* was actually a Gram-negative bacterium. In 1974 it was given a binomial designation—*Caedobacter taeniospiralis*, since changed to *Caedibacter taeniospiralis* (for a detailed review, see Preer et al., 1974, and Preer and Preer, 1984).

After the initial discovery of the first killer paramecia, other types were found. Siegel (1953) described "mate-killers," whose toxins act only during cell-to-cell contact at conjugation, and Schneller (1958) described "rapid-lysis" killers, which may injure sensitives in 10 min and kill them in 30 min, a process that is much more rapid than when *kappa* is the killing agent. In each of these killer paramecia, particles were found that later proved to be prokaryotic symbionts. One only needed to wash the paramecia free of bacteria, crush the ciliates, and observe the resulting preparations in a phase contrast microscope to ascertain the presence of the symbionts. This procedure also revealed, however, that some strains carry endosymbionts without showing any kind of killing ability. The symbionts of nonkiller paramecia were named *nu* (Sonneborn et al., 1959).

Bacterial symbionts have been studied most extensively in the *Paramecium aurelia* species complex (for reviews see Beale et al., 1969; L. B. Preer et al., 1972; Gibson, 1974; Preer et al., 1974; Soldo, 1974; Preer and Preer, 1984). They have also been found and studied in other ciliates, particularly in *Paramecium caudatum* (for reviews, see Ossipov, 1981; Görtz, 1983) and in *Euplotes* species (reviewed by Heckmann, 1983). More recently the presence of endo- and episymbionts of marine and freshwater ciliates living in anaerobic habitats have generated interest among ecologists and microbiologists. In these habitats, many of the symbionts were identified as methanogenic bacteria (Stumm and Vogels, 1989).

None of these symbionts have found commercial or technical applications, and it is unlikely that they will in the future. The symbionts comprise a heterogeneous group of mostly Gram-negative bacteria, which are grouped in

many unrelated genera, but are alike in that their habitat is the cytoplasm or the nucleus of a ciliate. As will become apparent, many of these symbionts appear to be well adapted to their environment; they are no longer free living and have genomes that are reduced in size, indicating a lengthy period of symbiont-host association. Most of the symbionts are not infectious, but a few are. They have developed specific features that guarantee uptake and transport to the sites where they can multiply. In most cases, it is not clear whether the symbionts provide their host with a selective advantage, and under laboratory conditions, most of the symbionts have proved to be dispensable. On the other hand, it is significant that the majority of cells of *Paramecium biaurelia* freshly collected from nature contain symbionts (Beale et al., 1969), and work by Landis (1981, 1987) suggests that paramecia that bear *kappa* have a selective advantage over those that are free of *kappa.* Moreover, *Polynucleobacter necessarius* (formerly called *omikron*) and the closely related *omikron*-like symbionts occurring in several freshwater *Euplotes* species have been shown to be necessary for survival of their hosts (Heckmann, 1975; Heckmann et al., 1983). Since in these cases the hosts depend on their symbionts and these in turn depend upon their hosts—they are no longer free living—the distinction between "symbiont" on the one hand and "organelle" on the other hand becomes blurred.

Ciliates can usually be handled easily, and some of them—particularly species of the *Paramecium aurelia* complex—have been investigated so thoroughly that they can be manipulated in a variety of ways. This has led to a wealth of information about their symbionts that is unparalleled. Since the endosymbionts of the *P. aurelia* complex have been reviewed repeatedly, we will treat them here less extensively than they deserve. We are also not able to list all the types of symbionts of ciliates encountered. Their number is very large, and we are sure that many more will be added to this list. Few of them, however, have been deposited in stock cultures and have been investigated in such a way that they can easily be identified when found again. An even smaller number have been described in keeping with the international rules of nomenclature and have received binomial names. It is however, primarily these last ones that will be discussed in detail in this chapter. We hope that this chapter will generate interest in symbionts of ciliates among microbiologists who have never been very active in investigations of this subject.

Prokaryotic Symbionts of *Paramecium*

With respect to endosymbionts, *Paramecium* is by far the best-studied ciliate genus. In the *Paramecium aurelia* species complex, consisting of 14 sibling species described by Sonneborn (1975) and named *P. primaurelia* to *P. quadecaurelia,* many different types of endosymbionts have been discovered. They have been thoroughly reviewed by Preer et al. (1974) and a detailed description by L. B. Preer appeared in the first edition of this handbook (L. B. Preer 1981). Figs. 1 to 5 and valuable information on their isolation and identification are taken from this chapter. We have added information on endosymbionts of other *Paramecium* species and have enlarged the coverage of symbionts of the genus *Holospora.* The latter differ from most of the *Paramecium* symbionts in being infectious and in their ability to invade nuclei for reproduction. The holosporas are now being investigated to elucidate the mechanisms that allow a prokaryote to invade a eukaryote. Being relatively large, they have the advantage that their route of infection and the changes that they undergo after entering a cell can be followed under a light microscope.

Habitat and Biology of the *Paramecium* Symbionts

Intracellular symbionts may be found in the micronucleus, in the macronucleus, in the perinuclear space, and in the cytoplasm of paramecia. Different types of symbiont invade different parts of the cell and they are moreover often adapted to one *Paramecium* species only. The cell compartment in which a symbiont multiplies and the species in which it occurs are therefore important taxonomic characters.

Most of the endosymbionts are easy to observe. Paramecia are collected from ponds and brought into the laboratory. With the help of a micropipette, a few cells are placed on a microscope slide, covered with a cover slip, and then observed with a light microscope using phase contrast optics. Crushing of the paramecia is sometimes necessary (Preer and Stark, 1953). Staining of the paramecia according to the technique of Beale and Jurand (1966) may facilitate the observations. Many of the paramecia brought in from nature are found to contain symbionts. Usually there are hundreds and sometimes even thousands of symbionts per paramecium (see Figs. 1–5).

Many of these symbionts confer on their hosts the ability to produce toxins capable of killing sensitive *Paramecium* strains of the same spe-

cies and even of other species. If the toxins are liberated into the medium, the toxin producers are called "killers" and their victims "sensitives." If the toxins act only during cell-to-cell contact at conjugation, the toxin producers are called "mate-killers." Different killer stocks of *Paramecium* induce different prelethal symptoms in sensitives mixed with killers. These symptoms include spinning, vacuolization, paralysis, formation of aboral humps, and rapid lysis. The symptoms are valuable characters for identification of a symbiont. In addition to making their hosts capable of producing toxins, the symbionts also confer upon the hosts specific resistance to the toxins produced. When a symbiont is lost from a killer strain the paramecia lose both toxin production and toxin resistance (Sonneborn, 1959). Killer strains have been reported not only for species of the *P. aurelia* complex but also for *P. caudatum* (Schmidt et al., 1987c, 1988), *P. bursaria* (Chen, 1955; Dorner, 1957), and *P. polycaryum* (Takayanagi and Hayashi, 1964). In the latter two species, however, symbionts were not observed although it is likely that they were present and were responsible for the killing properties of the paramecia.

Several symbionts have been shown to require the presence of specific *Paramecium* genes for their maintenance (Sonneborn, 1943; Siegel, 1953; Schneller et al., 1959; Gibson and Beale, 1961). It is not known whether the genes that assure the maintenance of the symbionts are active—e.g., providing the symbionts with some essential metabolite—or whether they are merely inactive alleles, the active ones preventing growth of an "invader" (Preer et al., 1974). In this connection it should be mentioned that, although not all species of the *P. aurelia* complex have been studied with the same thoroughness, the species 3, 7, 9, 10, 11, 12, 13, and 14 have never been found to contain symbionts (Preer and Preer, 1984).

It has been argued that symbionts profit from living inside a paramecium by being better protected from digestion, as compared with free-living species of bacteria, and that symbionts are provided with a convenient and abundant supply of nutrients (Beale et al., 1969). Which metabolites of the host are used, however, is not known. Although many of the symbionts have a smaller genome size than free-living bacteria (Soldo and Godoy, 1973) and some of the associations are probably very ancient (Preer, 1977), no indications for a transfer of genes from symbiont to host nucleus have been discovered, as has been found in the case of mitochondria (Gellissen and Michaelis, 1987). Schmidt (1984), studying the association of *Caedibacter varicaedens* with *P. biaurelia* was unable to obtain evidence for a sharing of the translational systems of host and symbiont. His observations indicate that all major proteins found in *Caedibacter* are synthesized in the symbiont itself.

That the host cells profit from bearing symbionts has been demonstrated in only a few cases; Soldo (1963) and Soldo and Godoy (1973a) found that it was not necessary to provide a *Paramecium* stock bearing *Lyticum flagellatum* (formerly called *lambda*) with folic acid, while the same stock freed of this symbiont required the vitamin. Holtzman (1959) observed that *P. pentaurelia* bearing *Pseudocaedibacter falsus* was more resistant to killer paramecia bearing *Lyticum flagellatum* than *P. pentaurelia* strains that were free of symbionts. And Landis (1981, 1987) showed that under natural conditions paramecia with killer properties have a selective advantage over nonkillers. On the other hand, in the laboratory the symbionts are all dispensable and many of them are lost when paramecia are cultured for some time. The reason for such loss is in most cases a rapid multiplication of the paramecia, resulting in a dilution of the symbionts and then in their loss. Although there is little information about what occurs in nature, it appears highly unlikely that re-infection of paramecia that have lost their symbionts plays a major role. Most symbionts are not infectious and are propagated in the host cells by division only. An exception to this rule is provided by symbionts of the genus *Holospora*. In addition to being propagated during cell division, they develop forms specialized for infection that are released and infect new cells upon being taken up with food. Unlike most other endosymbionts, they tend to harm their hosts and can therefore be regarded as parasites, although it cannot be excluded that under certain conditions the host cells may have advantages from presence of these symbionts (Görtz, 1983).

Isolation of the *Paramecium* Symbionts

The techniques used in collecting and cultivating paramecia have been described by Sonneborn (1950, 1970). Paramecia are easily detected in samples of pond or lake water with the help of a dissection microscope. Paramecia do not form cysts and, unless they have been introduced with the water added, cannot be obtained from infusions of straw or hay. When brought in from nature, the paramecia must be grown slowly at low temperature (about 16°C) without adding any antibiotics, if the purpose is to look for symbionts. Their presence can be

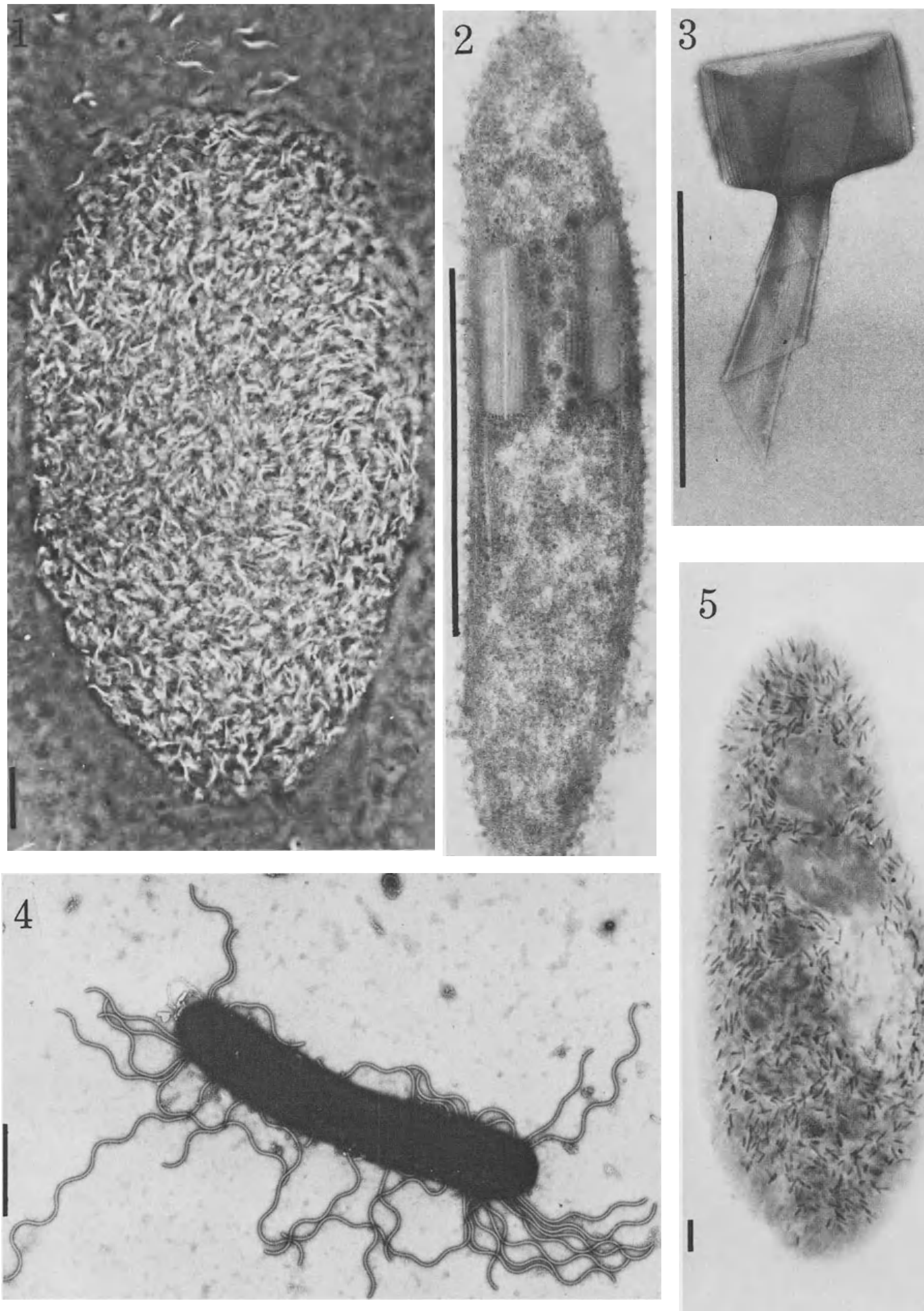

Fig. 1. Vegetative macronucleus of *Paramecium biaurelia* stock 562. The spiral endosymbionts filling the macronucleus are cells of *Holospora caryophila*. A few symbionts are also visible in the cytoplasm. Osmium-lacto-orcein preparation, whole mount, bright phase contrast. Bar = 10 μm. (From L. B. Preer, 1969.)

monitored as described above. If the paramecia are grown at high fission rates, their symbionts often become diluted and may even be lost.

Natural populations of *Paramecium* are infected with symbionts to varying degrees. Most strains carry only one kind of symbiont. A culture containing only one type of symbiont may be obtained, therefore, by isolating a single paramecium and growing it into a clone. If a strain of *Paramecium* does carry more than one type of symbiont, it is often possible to obtain pure cultures by growing the strain at a high fission rate until the symbionts are diluted down to no more than one symbiont per paramecium. Cells isolated at that time and cultured at a low fission rate will grow into populations containing one type of symbiont only. This technique has also been used to separate different types of symbionts (Preer, 1948b).

A simple method of culturing paramecia involves the use of bacterized Cerophyl medium (Sonneborn, 1970). For a stock solution, 75 g of Cerophyl (now available from Sigma under the name "dehydrated cereal leaves") are boiled for 15 min in 1 liter of distilled water. The solution is then filtered, diluted with water (1:30), buffered with Na_2HPO_4, and autoclaved. A day before use, the autoclaved medium is inoculated with *Enterobacter aerogenes, Klebsiella pneumoniae,* or another bacterium suitable as food for *Paramecium.* The medium should have a pH of about 7. The growth rate of *Paramecium* can be regulated by varying the temperature and the amount of bacterized medium that is added to a culture.

An even simpler method is to culture paramecia in autoclaved straw or hay infusions. However, this method is less reproducible than the one using Cerophyl medium. For preparing the straw or hay medium, 3 to 5 g per liter of straw or hay is boiled, filtered, buffered with Na_2HPO_4 (pH 6.8 to 7.0), and then autoclaved. After cooling and a day before use, the medium is inoculated with bacteria.

Unfiltered straw medium, with the straw remaining in the medium, may be used for maintaining stock cultures. In these cultures, paramecia with or without symbionts can be kept for months at low temperatures (6 to 16°C) without special care. Another, even safer, method for maintaining stocks is to freeze the paramecia and to keep them in liquid nitrogen, from which they may be recovered with their symbionts on thawing (Simon and Schneller, 1973). A great number of *Paramecium* stocks bearing symbionts are maintained in the frozen state at the American Type Culture Collection, Rockville, MD, USA.

Paramecia can also be grown axenically. For sterile growth, the paramecia must first be washed free of bacteria. This is achieved by allowing the paramecia to swim through sterile medium for a time sufficient to permit the bacteria in the food vacuoles to be eliminated (Sonneborn, 1950; Van Wagtendonk and Soldo, 1970). A simple method modified from Heatherington (1934) is to place several paramecia at one edge of a depression filled with wash fluid, allow them to swim to the other side, and then to transfer them to a new depression. The procedure should be repeated four times before the cells are left in the fifth wash for an hour. They should then be transferred to a new depression and this should be repeated hourly for another 4 hours. The paramecia must be taken up each time in a new sterile micropipette with as little fluid as possible; slides and wash fluid have to be sterile. The use of antibiotics to obtain bacteria-free cultures is to be avoided, because such substances may harm the symbionts.

A method for culturing paramecia without bacteria involves use of the photoautotrophic alga *Chlamydomonas reinhardtii* as a food organism (L.B. Preer et al., 1974). The medium contains 1 g of yeast autolysate, 0.25 g sodium acetate, 0.625 g Cerophyl, and 0.125 g Na_2HPO_4 in 1 liter of double-distilled water. The medium is dispensed into test tubes, autoclaved, and stored. Before it is used, the medium is inoc-

Fig. 2. Electron micrograph of longitudinal section of *Caedibacter varicaedens,* endosymbiont of *Paramecium biaurelia* stock 7 with an R body (for explanation of R bodies, see "Identification of *Paramecium* Symbionts"). Note the numerous dark-staining phages inside the coiled body. Bar = 1 μm. (From Preer and Jurand, 1968.)

Fig. 3. Electron micrograph of an R body isolated from *Caedibacter taeniospiralis* of *Paramecium tetraurelia* stock 51. The R body begins to unroll from the inside. Negative staining with phosphotungstic acid. Bar = 1 μm. (From L. B. Preer et al., 1972.)

Fig. 4. Electron micrograph of *Lyticum flagellatum* of *Paramecium octaurelia* stock 327. Negative staining with phosphotungstic acid. Bar = 1 μm. (From Preer et al., 1974.)

Fig. 5. *Paramecium tetraurelia* stock 239 bearing endosymbiont *Lyticum flagellatum,* seen as dark-staining rods in the cytoplasm. Osmium-lacto-orcein preparation, whole mount, dark phase contrast. Bar = 10 μm. (From Preer et al., 1974.)

ulated with a small number of *Chlamydomonas* and incubated under light for two days. A single bacterium-free paramecium, put into a tube half-filled with this *Chlamydomonas* medium, usually will multiply and ingest most of the algae within 3 to 4 days.

Most of the axenic media used for growing paramecia are based on a recipe initially designed by Soldo et al. (1966). Recent modifications by Thiele et al. (1980) and Schönfeld et al. (1986) have proved particularly valuable for large-scale cultures of *P. tetraurelia*. A modification (Soldo, 1987) that should be capable of supporting the axenic growth of paramecia and many other ciliates of fresh-water and marine origin follows.

Medium for Axenic Growth of Marine and Freshwater Ciliates

Component	Amount
Proteose peptone	10.00 mg/ml
Trypticase	5.00 mg/ml
Yeast nucleic acid	1.00 mg/ml
Biopterin	0.50 μg/ml
Folic acid	0.50 μg/ml
Nicotinamide	2.50 μg/ml
D-Pantothenate, Ca	7.50 μg/ml
Pyridoxal hydrochloride	2.50 μg/ml
Riboflavin	2.50 μg/ml
Thiamine hydrochloride	0.01 μg/ml
DL-Thioctic acid	0.01 μg/ml
Phospholipid (oleate-containing)	250.00 μg/ml
Stigmasterol	2.00 μ/ml

The medium is prepared in distilled water for freshwater ciliates and in sea water (density 1.015–1.026 g/ml) for marine forms. The final pH is 7.2. Stigmasterol is added from a stock solution (0.5 g stigmasterol dissolved in 100 ml hot absolute ethanol, stored at 4°C in a tightly capped plastic bottle) by injection into the culture medium from a syringe.

It is important to transfer paramecia gradually from a bacterized to an axenic medium. The ciliates need to be allowed to adapt slowly. Several procedures have been proposed for this transfer (see e.g., Van Wagtendonk and Soldo, 1970; Fok and Allen, 1979). An adapting medium (called VS medium) based on that of Allen and Nerad (1978) is one containing all the vitamins of the axenic medium given above plus stigmasterol, but not the other components, the pH being adjusted to 7.0. In VS medium, bacteria (e.g., *Klebsiella pneumoniae* previously grown in a tryptone medium) are suspended and adjusted to $OD_{590} = 3.0$ by dilution. The bacteria are then dispensed in 1-ml portions in screw-cap tubes and placed in the deep freeze. The final medium is prepared, about a week's supply at a time, by adding 1 ml of the frozen bacterized VS medium to 9 ml of unbacterized VS medium. This medium is autoclaved and inoculated with paramecia. The protozoa grow in this medium at a rate of 1/2 to 2 fissions per day. Not all stocks of *Paramecium* can be adapted to an axenic medium. Furthermore, some media were found to support growth of paramecia, but the latter were not able to maintain their symbionts under these conditions.

The purification of symbionts from paramecia (i.e., separation from host cell material) has been achieved in a number of ways: passage of cell homogenates through ion-exchange cellulose columns (Mueller, 1963; Smith, 1961), through filter paper columns (Preer et al., 1966), and centrifugation (Soldo et al., 1970) have all been used. Depending on the type of symbionts, different methods of purification may be necessary. For example the R-body-containing *Caedibacter caryophila* particles can best be isolated with the help of a discontinuous 70% Percoll gradient, while isolation of *Caedibacter caryophila* containing no R bodies, is better achieved by means of an ECTEOLA (anion exchanger) column (Schmidt et al., 1987b, 1988). For explanation of R bodies, see "Identification of *Paramecium* Symbionts."

For the isolation of holosporas, two different approaches have been followed. One method starts with homogenization of the host cells, the other involves separation of the macronuclei from the cytoplasm and then isolation of the symbionts. The latter method involving prior isolation of nuclei (Freiburg, 1985) proved especially useful for preparing clean reproductive forms of the symbionts, because it avoids contamination with bacteria from food vacuoles. For isolation of nuclei, the paramecia are lysed in a buffer containing 10 mM Tris, pH 7.9, 0.25 M sucrose, 3 mM $CaCl_2$, 8 mM $MgCl_2$, plus 0.1 mM phenylmethylsulfonyl fluoride, 0.1% w/v spermidine, and 0.2% Nonidet P40 (Shell Co., FRG) by gently stirring the cells in an ice-bath and subsequently passing the suspension 5 to 10 times through a 20-ml pipette. The nuclei are concentrated on a cushion of 1.6 M sucrose by spinning for 10 min at 700 × g. The purified nuclei are then homogenized in a Mg-free buffer, and the bacteria are pelleted. Two different forms of the bacteria, the infectious and reproductive forms can be separated by sedimentation. The infectious form sediments at 350 × g within 10 min, while the reproductive form remains in the supernatant (Görtz et al., 1988).

Protocols for isolating holosporas directly from cell homogenates have been published by Fujishima and Nagahara (1984), Schmidt et al. (1987a), and Görtz et al. (1988). Infected cells are homogenized in sodium phosphate buffer

with a hand homogenizer. The Teflon pestle should fit tightly. The homogenate is then centrifuged at about 3,000 × g for 10 min. The pellet is resuspended in buffer and centrifuged in a preformed continuous gradient of 70% Percoll for 12 min at 40,000 × g. In order to maintain the gradient, it is advisable to use a centrifuge with an acceleration rate control. For *H. obtusa, H. elegans*, and *H. undulata* the infectious forms then concentrate in a sharp band which is usually uncontaminated with food bacteria and cell debris. The reproductive forms do not form a band in a continuous Percoll gradient. These forms can be obtained with the help of a discontinuous gradient (at 10,000 × g) where—depending on the species—they are found above a step of about 60% Percoll. Most of the cell organelles of *P. caudatum* do not enter at the 50% Percoll step.

The isolation procedures described here have been helpful for studying ultrastructural details and protein composition of symbionts, their R bodies, and their DNA. Attempts to culture endosymbionts outside their hosts have usually been unsuccessful (Preer et al., 1974).

Identification of the *Paramecium* Symbionts

Since endosymbionts do not multiply outside their hosts, characterization based on metabolism and growth, as is customary for bacteria, is not possible. Instead, morphological and biological features and sometimes data on the GC content of the symbiont's DNA, have to be used for identification. Nonetheless, a preliminary identification is often possible with the help of a light microscope equipped with phase contrast. A very useful staining procedure that allows observations in regular bright field is given by Beale and Jurand (1966): Paramecia are placed in a small drop on a slide, as much of the fluid as possible is withdrawn by a micropipette, the paramecia are lightly fixed by exposure to OsO_4 vapor for a few seconds, and immediately stained with a small drop of lacto-orcein (1 g orcein dissolved in 25 ml of hot 45% acetic acid, mixed with 25 ml lactic acid, diluted with water 1:1, and then filtered). A coverslip, with vaseline applied around the edges, is placed over the drop of stained paramecia and is lightly pressed down, flattening but not disrupting the cells. The preparations can then be observed with a 100 × oil immersion objective. In order to remove lipids that sometimes obscure observation of endosymbionts, the paramecia can be treated with a drop of acetone or a 3:1 mixture of ethanol and acetic acid before staining.

In addition to microscopical observations, killer tests should be performed when a stock is brought in from nature. Standard sensitives (e.g., cells of stock 152 of *P. triaurelia*) may be employed in such tests. Equal volumes of the culture to be tested and a culture of sensitive cells are mixed in a depression slide and observed with appropriate controls for prelethal effects. Mate killing can only be detected by mating sensitive cells with symbiont bearers. Any symbiont-free strain that will mate with an unknown strain is usually adequate.

A feature unique to the genus *Caedibacter* is the ability to produce R bodies. These are proteinaceous ribbons, 20–30 μm long, coiled inside the bacterial cell to form a hollow cylindrical structure (Figs. 2 and 3). This structure has a diameter of about 0.4 μm in all species and is about 0.4 μm long, except in *C. caryophila* where the R bodies are approximately 0.8 μm in width and length. R bodies unroll when ingested into a phagosome and also under certain in vitro conditions, e.g., when placed at low pH (Preer et al., 1966). With respect to the shape of the ends and the mode of unrolling, the different types of R body vary (for details see Preer et al., 1974; Quackenbush, 1988; Pond et al., 1989).

It has been suggested that the R bodies play an important role in the killing mediated by *Caedibacter.* Mueller (1963) and Smith-Sonneborn and Van Wagtendonk (1964) demonstrated that only *Caedibacter* particles that contained R bodies were toxic to sensitive paramecia. In addition, R bodies purified from certain strains of *C. varicaedens* have been shown to be toxic to sensitive paramecia (L. B. Preer and Preer, 1964; L. B. Preer et al., 1972). However, neither the toxin itself nor its mode of action have yet been identified, nor is it known how paramecia are protected from the toxic action of their own symbionts.

The genetic determinants of R bodies are probably plasmids or bacteriophages that have lost the ability to lyse their host cells—the symbiotic bacteria—upon maturation of the virions (Preer and Preer, 1967; Preer et al., 1974). Dilts (1976) isolated plasmid DNA from *C. taeniospiralis* 51 and suggested that the extrachromosomal DNA might be the determinant of the R bodies. Further investigation revealed that plasmids are present in all strains of *C. taeniospiralis* and that they are highly homologous, as determined by restriction-endonuclease mapping (Quackenbush, 1983). Evidence that the genetic determinant for R-body synthesis resides on the plasmid was presented by Quackenbush and Burbach (1983), who cloned portions of a plasmid and obtained expression of

the R-body-encoding sequences in *Escherichia coli.* Analysis of various subclones allowed them to determine the approximate location of the R-body-encoding sequence. The DNA required for type 51 R-body synthesis is about 1.8 kbp in size and has been completely sequenced (D. P. Heruth, Ph.D. thesis; see Pond et al., 1989). However, none of the R-body-producing *E. coli* clones was found to be toxic to sensitive paramecia. The DNA sequence required for toxin production has not yet been located. It is believed to also reside on the plasmid (Pond et al., 1989).

Recently Dilts and Quackenbush (1986) have provided evidence that R bodies are required for the killing trait to be expressed but not for resistance of the ciliate host to killing mediated by *Caedibacter taeniospiralis.* They described a mutant strain of *C. taeniospiralis* 169 that simultaneously lost the ability to produce R bodies and to kill sensitive paramecia, but still rendered its host resistant to killing. Investigations of the R-body-encoding plasmid isolated from the mutant revealed that a transposon-like element had been inserted within the R-body-encoding region, thereby eliminating R-body production. Unless two separate mutational events occured in the same cell, one inactivating the R-body-encoding sequence and the other inactivating the toxin-encoding sequence, this shows that R bodies are crucial to expression of the killer trait. Their exact role remains, however, unknown. Evidence indicates that their action probably involves delivery of the toxin to the sensitive paramecium and to its target site by unrolling and penetrating the food vacuole membrane (Dilts, 1986).

The R bodies of other species of the genus were shown to be associated with icosahedral viral capsids (Preer and Jurand, 1968; Grimes and Preer, 1971). The capsids were in most cases found to contain DNA (Preer et al., 1971). The relationships between genomes of different phages of *kappa* and between R-body-encoding plasmids and kappaphage genomes have been studied by restriction-endonuclease analysis and by DNA-DNA hybridization (Quackenbush, 1978; Quackenbush et al., 1986). These studies demonstrate that the R-body-encoding plasmids show little or no homology with kappaphages, and that there is also considerable diversity among the kappaphages.

R bodies have also been reported in the free-living bacteria *Pseudomonas taeniospiralis* (Lalucat et al., 1979) and *P. avenae* (Wells and Horne, 1983). However, with respect to antigencity, genetic determinants, and other features, these R bodies appear to be unrelated to the R bodies of *Caedibacter* species (Bedingfield et al., 1984; Gibson, 1984; Meenaghan et al., 1984; Kanabrocki et al., 1986; Lalucat et al., 1986). R bodies have also been reported for *Rhodospirillum centenum,* a novel nonsulfur photosynthetic bacterium (Favinger et al., 1989). Since R bodies are often present at low frequencies, it appears likely that R-body-producing bacteria are more common than previously suspected.

Species of the genus *Holospora* are host-specific as well as nucleus-specific, i.e., they infect only one type of nucleus, either the micro- or the macronucleus (Ossipov, 1973; Ossipov et al., 1975). They can form short, reproductive rods that undergo binary fission as well as long, infective forms that leave paramecium and infect others very efficiently (Ossipov and Podlipaev, 1977; Wiemann and Görtz, 1989; Wiemann, 1989; Fig. 6). The infectious forms have a structure unique among bacteria. The cytoplasm is condensed and located toward one end of the symbiont, while a voluminous periplasmic area is located mainly at the other end. The periplasm consists of finely granular, strongly osmiophilic material. Some less osmiophilic material is located at the end, distal from the cytoplasm (Fig. 7). When an infective form has been taken up by a paramecium and is on its way into the nucleus, the periplasmic material disappears while the cytoplasm expands (Fig. 6). It is assumed that the periplasm of the infectious form contains substances that interact with host membranes during the infection process (Görtz et al., 1988; Görtz and Wiemann, 1989). The further development of the infectious form into the short, reproductive form is completed in the nucleus.

The infectious form differs from the reproductive form not only in morphology, but also in the protein patterns produced on SDS-polyacrylamide gel electrophoresis (Fig. 8). Some proteins specific for the infectious form of *H. obtusa* are located in the periplasm as shown by means of immune electron microscopy using poly- and monoclonal antibodies as probes against these proteins (M. Wiemann and H.-D. Görtz, unpublished observations).

The fine structure of the reproductive forms of species of *Holospora* resembles that of Gram-negative bacteria. Freeze-fracture studies of *H. obtusa* revealed that the outer membrane of the reproductive form has a density of intra-membrane particles (IMPs) that is similar to that of the inner membrane. In contrast to this, the outer membrane of the infectious form was found to contain only very few IMPs (Görtz et al., 1989). These differences correlate with a difference in the behavior of the two forms during the division of the host nuclei. The infectious

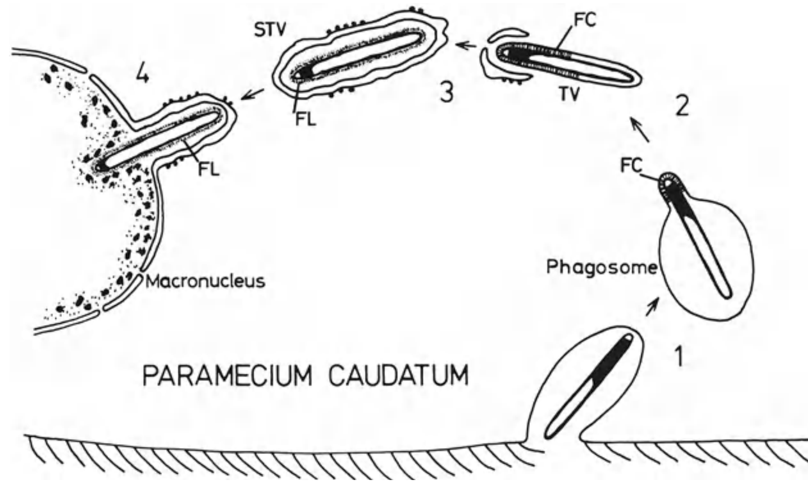

Fig. 6. The route of *Holospora obtusa* into the macronucleus of *Paramecium caudatum*. 1) The infectious form of the bacterium (long rod) is ingested by the paramecium into a phagosome (food vacuole). Fibrillar connections (FC) are established between the bacterial surface and the phagosome membrane. 2) The bacterium is sluiced out of the phagosome. It remains encircled by a host membrane that forms a transport vesicle (TV). 3) Fibrillar connections (FC) are established between the host membrane and the bacterium. Later on, the transport vesicle is surrounded by flat vesicles, some of them bearing ribosomes. These vesicles finally form a secondary transport vesicle (STV). 4) The TV membrane disintegrates or becomes part of a fibrillar lamina (FL) enclosing the bacterium. The STV membranes fuse with the nuclear membranes. Thus, the bacterium is incorporated into the macronucleus. (From Görtz and Wiemann, 1989.)

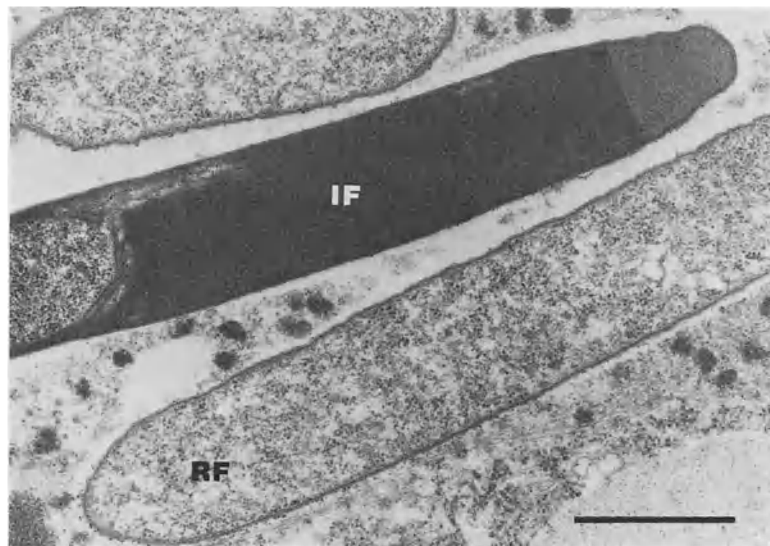

Fig. 7. *Holospora obtusa* in the macronucleus of *Paramecium caudatum*. IF, infectious form; RF, reproductive form. Bar = 1 μm. (From Görtz et al., 1988.)

forms of *H. obtusa* are concentrated in the connecting piece around the separation spindle of the dividing nucleus and are later released from the host cell. The reproductive forms are transported to the poles and in this way get into the daughter nuclei (Ossipov et al., 1975; Ossipov, 1981; Wiemann, 1989; Wiemann and Görtz, 1989). It is of interest that *H. obtusa* makes use of the division apparatus of the host nucleus.

The classification of the endosymbionts of *Paramecium* is based on the following characteristics: morphology; killing activity; the species in which they live; DNA base ratios calculated from the buoyant density (Bd) or from thermal denaturation profiles (T_m), and interstrain DNA/DNA hybridizations. Type strains which have been deposited at the American Type Culture Collection (ATCC), are listed be-

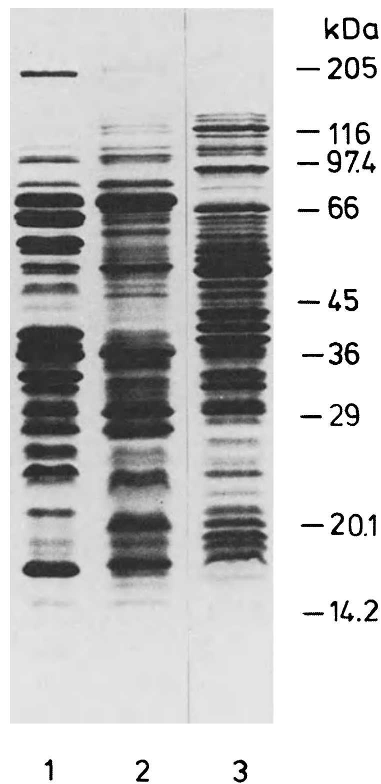

Fig. 8. SDS-polyacrylamide gel electrophoresis of total protein of *Holospora obtusa* and *Escherichia coli*. Lane 1, *H. obtusa*, infectious form (from 2×10^7 cells); lane 2, *H. obtusa*, reproductive form (from 2×10^8 cells); lane 3, *E. coli* (from 5×10^7 cells). The position and size (kDa) of marker proteins are indicated on the right. (From Görtz et al., 1988.)

low by their ATCC numbers. Some type strains have also been deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, Leningrad University, and can be obtained from that source.

GENUS *CAEDIBACTER* PREER AND PREER 1982. Straight rods or coccobacilli, 0.4–1.0 μm in diameter and 1.0–4.0 μm in length. Usually less than 10%, but sometimes up to 50% of the symbionts contain refractile (R) bodies. Cells containing R bodies are usually larger than cells that do not contain R bodies. In addition to R bodies, many spherical phage-like structures or covalently closed circular DNA plasmids are present. The symbionts are Gram-negative and nonmotile. The GC content of the DNA is 40–44 mol%. For a detailed description of this extensively studied genus, see Preer et al., 1974; Preer and Preer, 1984; Pond et al., 1989. The following five species have been described:

1. *Caedibacter taeniospiralis* Preer and Preer 1982. Rods, 0.4–0.7μm in diameter and 1.0–2.5 μm long. The GC content is 41 mol%. Found in the cytoplasm of *P. tetraurelia* only. R bodies unroll from the inside and contain plasmids. Ingestion of R-body-containing symbionts by sensitive paramecia results in the development of clear small blisters on their surface in 2 to 3 hours. During the 4th to 5th hour, a bulge develops first in the posterior part of the oral side and later moves to the posterior aboral side. The position of the bulge gave rise to the designation "hump-killer." Death takes place in 1 to 2 days, the corpses remaining intact for sometime. Type strain: in ATCC strain 30632 (stock 51 of *P. tetraurelia;* see Preer et al., 1974).
2. *Caedibacter varicaedens* Quackenbush 1982. Rods, 0.4–1.9 μm in diameter and 2.0–4.0 μm long. The GC content is 40–41 mol%. Found in the cytoplasm of *P. biaurelia*. Different strains cause either vacuolization, paralysis, or rapid reverse rotation while swimming (spin-killing) of sensitive cells. Vigorous rotation to the right becomes nearly uninterrupted by the 4th to 6th hour and then becomes slower. R bodies unroll from the outside. The outer terminus of the unrolled R body is blunt. The R body is usually associated with bacteriophage capsids. Type strain: in ATCC 30637 (stock 7 of *P. biaurelia;* see Preer et al., 1974).
3. *Caedibacter pseudomutans* Quackenbush 1982. Cigar-shaped rods, approximately 0.5 μm in diameter and 1.5 μm long. The GC content is 44 mol%. Found in the cytoplasm of *P. tetraurelia*. Cause rapid reverse rotations of sensitive paramecia while swimming (spin-killers). R bodies of the *C. varicaedens* type. Type strain: in ATCC strain 30633 (strain 51 ml of *P. tetraurelia;* see Preer et al., 1974).
4. *Caedibacter paraconjugatus* Quackenbush 1982. Small rods. Contain phage-like structures. Less than 1% of the symbionts contain R bodies of the *C. varicaedens* type. Found in the cytoplasm of *P. biaurelia*. Ingestion of symbionts by sensitive strains does not produce any observable toxic effect. Cell-to-cell contact between host and sensitive paramecia is required for toxic effects to be observed in the sensitive paramecia (mate-killers). Type strain: in ATCC strain 30638 (stock 570 of *P. biaurelia;* see Preer et al., 1974).
5. *Caedibacter caryophila* Schmidt, Görtz, and Quackenbush 1987. Rods, 0.4 to 0.7 μm wide and up to 2.5 μm long. Those with R bodies are larger than those without R bodies. The GC content is 34.6 mol%. Found in the macronucleus of *P. caudatum*. R bodies unrolling from the inside, outer terminus blunt, in-

ner terminus acute. R bodies are associated with phages. Width of R bodies after isolation 0.8μm. Sensitive strains are killed by paralysis. Coinfections with *Holospora obtusa, H. undulata* and *H. elegans* may occur in natural populations (H.-D. Görtz, unpublished observations). Type strain: in ATCC strain 50168 (clone C221 of *P. caudatum,* see Schmidt et al., 1987c).

GENUS *PSEUDOCAEDIBACTER* QUACKENBUSH 1982. Rods, 0.25–0.7 μm in diameter and 0.5–4.0 μm long. Do not produce R-body-containing cells. The symbionts are Gram-negative and nonmotile. The GC content is 35–39 mol%. The genus includes some species that confer a killer trait on their hosts, some that render them mate killers, and others that do not produce any killing ability. Four species have been described:

1. *Pseudocaedibacter conjugatus* Quackenbush 1982, formerly *mu.* Rods, 0.3–0.5 μm in diameter and 1.0–4.0 μm long. The GC content is 35–37 mol%. Found in the cytoplasm of *P. primaurelia* and *P. octaurelia* where a mate-killer phenotype of the hosts is produced. Type strain: in ATCC strain 30796 (stock 540 of *P. primaurelia;* see Preer et al., 1974).
2. *Pseudocaedibacter minutus* Quackenbush 1982, formerly *gamma.* Rods, often double; single rods 0.25–0.35 μm in diameter and 0.5–1 μm long. The GC content is 38 mol%. Found in the cytoplasm of *P. octaurelia.* In the host cell, the symbiont is surrounded by an extra set of membranes, apparently continuous with the endoplasmic reticulum of the host. The paramecia which bear these symbionts are very strong killers that cause sensitives to develop vacuoles, accompanied by swelling of the cells, finally becoming spherical and undergoing lysis. Death occurs after about 8 hours. Type strain: in ATCC strain 30699 (stock 214 of *P. octaurelia;* see Preer et al., 1974).
3. *Pseudocaedibacter falsus* Quackenbush 1982, formerly *nu* and *pi.* Rods, 0.4–0.7 μm in diameter and 1.0–1.5 μm long. The GC content of the DNA is 36 mol%. No toxic actions known. Found in the cytoplasm of *P. biaurelia, P. tetraurelia,* and *P. pentaurelia.* Type strain: in ATCC strain 30640 (stock 1010 of *P. biaurelia;* see Preer et al., 1974).
4. *Pseudocaedibacter glomeratus* Fokin and Ossipov 1986. Small rods, about 0.3 μm wide and up to 1.2 μm long. Found in the cytoplasm of *P. pentaurelia.* No toxic actions known. Symbionts are individually enclosed in vacuoles which are tightly associated with the endoplasmic reticulum. Type strain: in strain Bp 171 of *P. pentaurelia* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, Leningrad University, USSR).

A symbiont living in the cytoplasm of *P. bursaria* and found to be antagonistic to symbiotic chlorellae was named *Caedobacter chlorellopellens* by Skoblo et al. (1985), who did not know that the genus name *Caedobacter* had been changed to *Caedibacter. Caedibacter* was then restricted to symbionts that produce R bodies and confer killer traits upon their host paramecia (Preer and Preer, 1982). The symbiont in *P. bursaria* is egg-shaped, rarely rod-shaped, 0.35 μm wide and up to 1.4 μm long. Since no R bodies were observed and no toxic actions on sensitives were observed, the species should be placed in the genus *Pseudocaedibacter.*

GENUS *LYTICUM* PREER AND PREER 1982. Large rods, 0.6–0.8 μm in diameter and 3.0–5.0 μm long. Resemble bacilli in general appearance and bear numerous peritrichous flagella (Figs. 4 and 5), but are not obviously motile. They are enclosed in vacuoles in the cytoplasm of their hosts. They do not contain R bodies. The GC content is 27 mol% in one type and 45–49 mol% in another type. The symbionts are Gram-negative. They produce toxins that kill sensitive paramecia by lysing them within 10 to 20 min (rapid lysis killing). Two species have been described:

1. *Lyticum flagellatum* Preer and Preer 1982 (formerly *lambda*). Straight rods, 0.6–0.8 μm in diameter and 2.0–4.0 μm long. The GC content is 27 mol% in one strain and 49 mol% in another. Found in the cytoplasm of *P. tetraurelia* and *P. octaurelia.* Type strain: in ATCC strain 30700 (stock 299 of *P. octaurelia;* see Preer et al., 1974).
2. *Lyticum sinuosum* Preer and Preer 1982, formerly *sigma.* Curved or spiral rods, 0.7–0.9 μm in diameter and 2.0–10 μm long, sometimes forming chains of 2–3 cells. The GC content is 45 mol%. Found in the cytoplasm of *P. biaurelia.* Type strain: in ATCC strain 30696 (stock 114 of *P. biaurelia;* see Preer et al., 1974).

GENUS *PSEUDOLYTICUM* BOSS, BORCHSENIUS, AND OSSIPOV 1987. Large symbionts with numerous flagella. No motility observed. May contain refractile bodies consisting of polyhydroxybutyric acid. No killer activity and no infectivity could be detected. Only one species known, *Pseudolyticum multiflagellatum* Boss, Borchsenius, and Ossipov 1987. Straight rods,

1.0–2.0 μm in diameter and 3.5–14 μm long. Found in the cytoplasm of *P. caudatum.* The symbionts are individually enclosed in vacuoles. The membrane of these vacuoles forms numerous projections that are continuous with the endoplasmic reticulum. No toxic actions known.

GENUS *TECTIBACTER* PREER AND PREER 1982. The species of this genus are distinguished from other symbionts by a layer of electron-dense material surrounding the outer of the two membranes. The symbionts have sparse peritrichous flagella and show slight motility on occasion. They appear not to be toxic. For a more detailed description see Preer et al., 1974. Only one species described, *Tectibacter vulgaris* Preer and Preer 1982, formerly *delta.* Straight rods, 0.4–0.7 μm in diameter and 1.0–2.0 μm long. Gram-negative. Hosts: *P. primaurelia, P. biaurelia, P. tetraurelia, P. sexaurelia,* and *P. octaurelia.* Type strain: in ATCC strain 30697 (stock 225 of *P. sexaurelia;* see Preer et al., 1974).

GENUS *NONOSPORA* FOKIN, OSSIPOV, SKOBLO, RAUTIAN, AND SABANEYEVA 1987. Rod-like symbionts living in the macronucleus. Flagella have not been observed. No toxic actions known. The symbionts are retained in macronuclear fragments of exconjugants and enter macronuclear anlagen by fusion of old fragments with the anlagen (Fokin et al., 1987a). Only one species described, *Nonospora macronucleata* Fokin, Ossipov, Skoblo, Rautian, and Sabaneyeva 1987. Symbionts rodlike, 0.2–0.3 μm in diameter and mostly about 1.0 μm long, sometimes forming chains up to 10 μm long. The surface of the symbionts appears irregularly wavy in the electron microscope. Found in the macronucleus of *P. caudatum,* often clustered in the center of the nucleus.

GENUS *HOLOSPORA* (EX HAFKINE 1890) GROMOV AND OSSIPOV 1981. Symbionts of this genus live in the micronucleus or the macronucleus of *Paramecium* and exist in two forms: a short rod, 1.0–3.0 μm long and 0.5–1.0 μm wide that can replicate; and a long form 5.0–20 μm long and 0.8–1 μm wide that cannot replicate. The long form is released from the host and can infect other paramecia. The infective form is differentiated into a refractile portion with a less electron-dense, pale tip and a posterior part that contains typical bacterial cytoplasm, stains with DNA-specific dyes, and appears dark in phase contrast. An unnamed symbiont of this type has been observed repeatedly in *Stentor multiformis* and in *S. polymorphus* (Görtz and Wiemann, 1987), and another one has been observed in the peritrich ciliate *Zoothamnium pelagicum* by Laval (1970). This indicates that symbionts of the genus *Holospora* are not restricted to *Paramecium.* Some strains of *Paramecium* were found to be harmed after infection with holosporas, whereas other strains apparently remained unaffected. Six species have been described:

1. *Holospora undulata* (ex Hafkine 1890) Gromov and Ossipov 1981. Lives in the micronucleus of *P. caudatum.* Two forms are seen, a short, spindle-shaped reproductive form, about 0.8 μm in diameter and 1.5–2.0 μm long, and a long, spiral-shaped infectious form that has tapered ends and is 7.0–15 μm long. Type strain: in clone M1-48 of *P. caudatum* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, Leningrad University, USSR).
2. *Holospora obtusa* (ex Hafkine 1890) Gromov and Ossipov 1981. Lives in the macronucleus (Fig. 7). Reproductive form: short rod, 0.8 μm in diameter and 1.5–2.5 μm long. Infectious form: long rod with rounded ends, 0.8–1.0 μm in diameter and 7.0–20 μm long. Type strain: in clone M-115 of *P. caudatum* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, Leningrad Univerity, USSR).
3. *Holospora elegans* (ex Hafkine 1890) Preer and Preer 1982. Lives in the micronucleus. Reproductive form: short rod, 0.8 μm in diameter and 1.5–2.0 μm long. Infectious form: long rod with tapered ends, 0.6–0.8 μm in diameter and 7.0–18 μm long. Type strain: in ATCC strain 50008 (stock C101 of *P. caudatum,* Görtz and Dieckmann, 1980).
4. *Holospora caryophila* Preer and Preer 1982, formerly *alpha.* Lives in the macronucleus of *P. biaurelia;* may also be found in *P. caudatum* (Görtz, 1987). Reproductive form: thin, fusiform rod, 0.4 μm in diameter and 1.0–3.0 μm long. Infectious form: spiral shaped with tapered ends, 0.5 μm in diameter and 5.0–6.0 μm long. Type strain: in ATCC strain 30694 (stock 562 of *P. tetraurelia;* see Preer et al., 1974).
5. *Holospora acuminata* Ossipov, Skoblo, Borchsenius, Rautian and Podlipaev 1980. Lives in the micronucleus of *P. bursaria.* Reproductive form: fusiform rod, 0.6 μm in diameter and 2.0–2.5 μm long. Infectious form: straight fusiform rod with tapered ends, 0.8–1.0 μm in diameter and 4.0–6.0 μm long. Type strain: in stock AC61-10 of *P. bursaria* (deposited in the culture collection of the

Laboratory of Invertebrate Zoology, Biological Research Institute, Leningrad University, USSR).

6. *Holospora curviuscula* Borchsenius, Skoblo and Ossipov 1983. Lives in the macronucleus of *P. bursaria.* Infects only certain strains of three syngens. In other strains the development into the infectious form is not completed. Occasionally, infection of both macro- and micronuclei was observed (Skoblo and Lebedeva, 1986). Reproductive form: fusiform rod, 0.8 μm in diameter and 1.5–2.0 μm long. Infectious form: slightly curved rod with tapered ends, approximately 0.8 μm in diameter and 6.0–10 μm long.

The striking similarity of the biology and cytology of the different *Holospora* species led to their classification in one genus. A comparison of the protein patterns of *H. obtusa* and *H. elegans,* however, revealed great differences. Moreover, the hybridization of the total DNAs and a comparison of DNA banding patterns after digestion with restriction enzymes indicate that the two species may not be as closely related as originally assumed (Schmidt et al., 1987a). The differences at the molecular level suggest that the genus *Holospora* has coevolved with ciliates for a long time.

SYMBIONTS OF *PARAMECIUM* WITHOUT BINOMIAL NAMES. Jenkins (1970) described a Gram-negative bacterium living within bulbous distensions of the outer membrane of the nuclear envelopes of both the micro-and macronucleus of a strain of *P. multimicronucleatum.* It is a very short rod, sometimes appearing nearly coccoid, approximately 0.35 μm in diameter with longer forms reaching 0.7 μm in length. It was named *epsilon.*

Another symbiont, reported to occur in the perinuclear space, has been described by Fokin (1988). It was found inside the nuclear cisternae of the macronucleus of *P. duboscqui.* The symbiont is 0.3 μm in diameter and 0.7–1.4 μm long, looks spindle-shaped and is Gram-negative. No killing activity was observed when symbiont bearers were tested against non-symbiont bearers.

A symbiont occurring in the macronucleus of *P. caudatum* has been studied by Estève (1978). It confers a killer trait on its host. When it was investigated cytologically, a greatly enlarged macronucleus was observed to contain numerous kappa-like bacteria, some of which contained R bodies. Electron micrographs of this bacterium showed spherical phages inside the R bodies. Schmidt et al. (1987c) assumed that the symbiont is identical with *Caedibacter caryophila* recently described and named by them.

A so-far-unnamed symbiont living in the micronucleus of *P. bursaria* was found by Görtz and Freiburg (1984). It is a small rod, 0.5 μm in diameter and up to 2 μm long. Its ultrastructure suggests that it is a Gram-negative bacterium. No flagella were found and no killing capacity of its host was observed.

Another symbiont of the nonkiller type was discovered in the cytoplasm of *P. woodruffi,* a ciliate living in brackish water. The symbiont is a Gram-negative rod, 0.2–0.8 μm in diameter and 0.6–2.5 μm long. It has no flagella. It was found to contain hexagonal viroid particles (Fokin et al., 1987b).

A bacterial endosymbiont of *P. sexaurelia* was repeatedly observed by Görtz (1981) to invade the macronucleus but not the micronucleus. Once the symbiont has entered the macronucleus it tends to disappear from the cytoplasm. The bacterium is slightly curved with a diameter of 0.5–0.8 μm and up to 25 μm long. When present in the cytoplasm, the symbiont tends to be closely associated with food vacuoles. Once the symbiont has entered the macronucleus, it multiplies there without causing nuclear hypertrophy. After autogamy the symbiont is found only in the cytoplasm and not in new macronuclear anlagen. It remains there until a new infection of the macronucleus occurs.

The following key to the prokaryotic symbionts of *Paramecium* only includes symbionts that have been given binomial names. The key is based on the one proposed by L. B. Preer (1981) and on information from articles cited in this chapter.

Key to the Prokaryotic Symbionts of *Paramecium*

I. Host paramecia are killers or mate-killers
- A. 2–50% of symbiont population contain R bodies
  - 1. Host paramecia are killers
    - a. Kill by producing aboral humps on sensitive paramecia; R bodies unroll from inside at low pH, reroll at high pH; found in *P. tetraurelia* .... *Caedibacter taeniospiralis*
    - b. Kill in ways other than producing aboral humps
      - (1) Found in *P. tetraurelia;* R bodies unroll from outside irreversibly when exposed to high temperature or certain detergents .... *Caedibacter pseudomutans*
      - (2) Found in *P. biaurelia;* R bodies unroll from outside irreversibly when exposed to high temperature or certain detergents .... *Caedibacter varicaedens*
      - (3) Found in *P. caudatum,* found in the macronucleus only; R bodies unroll from inside .... *Caedibacter caryophila*

2. Host paramecia are mate-killers
.... *Caedibacter paraconjugatus*

B. Symbiont population does not contain R bodies
1. Host paramecia are killers
a. Rapid lysis killer; symbionts are large flagellated cells
(1) Straight rods found in *P. tetraurelia, P. octaurelia*
.... *Lyticum flagellatum*
(2) Sinuous rods found in *P. biaurelia*
.... *Lyticum sinuosum*
b. Kill by vacuolization; symbionts are very small cells, often doublets
.... *Pseudocaedibacter minutus*
2. Host paramecia are mate-killers
.... *Pseudocaedibacter conjugatus*

II. Host paramecia are nonkillers
A. Symbionts are present only in the cytoplasm
1. Symbionts lack flagella
a. Host: *P. bursaria;* egg-shaped, rarely rod-shaped, up to 1.4 μm long. Antagonistic to symbiotic algae (*Chlorella*) of the host
.... *Caedobacter chlorellopellens*
b. Hosts: *P. biaurelia, P. tetraurelia, P. pentaurelia;* rods, up to 1.2 μm long
.... *Pseudocaedibacter falsus*
c. Host: *P. pentaurelia;* rods, up to 1.2 μm long: the symbiont-containing vacuole is surrounded by endoplasmic reticulum
.... *Pseudocaedibacter glomeratus*
2. Symbionts with flagella
a. Host: *P. caudatum;* rods, 3.5 to 14 μm long; refractile inclusions (polyhydroxy butyric acid); with numerous flagella, but no motility observed
.... *Pseudolyticum multiflagellatum*
b. Host: *P. primaurelia;* rods, up to 2 μm long; Gram-negative with a thick surface layer visible in EM; with sparse flagella, occasionally slight motility; coexists with other symbionts such as *Caedibacter* and *Pseudocaedibacter*
.... *Tectibacter vulgaris*

B. Symbionts are present almost exclusively in the nuclei
1. Symbionts exclusively in the micronucleus, show two morphologically distinct forms, and are highly infectious
a. Long form of symbiont (infectious form), spiral-shaped with tapered ends, 7–15 μm long; host: *P. caudatum*
.... *Holospora undulata*
b. Long form of symbiont (infectious form), straight rod with tapered ends
(1) Host: *P. caudatum;* long form 7–18 μm long
.... *Holospora elegans*
(2) Host: *P. bursaria;* long form 4–6 μm long
.... *Holospora acuminata*
2. Symbionts exclusively in the macronucleus
a. Symbionts are highly infectious, two morphologically distinct forms often observable
(1) Host: *P. biaurelia* (sometimes also observed in *P. caudatum*); long form with tapered ends, 5–6 μm long
.... *Holospora caryophila*
(2) Host: *P. caudatum;* long form, straight rod, with rounded ends, 7–20 μm long
.... *Holospora obtusa*
(3) Host: *P. bursaria;* long form slightly curved, with tapered ends, 6–10 μm long
.... *Holospora curviuscula*
b. Symbionts are weakly infectious, only one morphological form observable. Host: *P. caudatum;* rods small, about 1 μm long, sometimes forming "chains" that are up to 10 μm long; mostly aggregated in the center of the macronucleus; may coexist with *H. obtusa* or *H. undulata*
.... *Nonospora macronucleata*

Prokaryotic Symbionts of *Euplotes*

Endosymbiotic bacteria are also very common in *Euplotes,* a ciliate genus that comprises both freshwater and marine species. So far, only one of the symbionts has been given a binomial name. The others are still referred to by Greek letters, as was formerly customary for cytoplasmic elements. Several *Euplotes* symbionts remained unnamed or were provisionally given Latin letters as names when they were encountered. However, most of these symbionts are not well characterized.

Habitat of the *Euplotes* Symbionts

All except one of the bacterial symbionts of *Euplotes* are confined to the cytoplasm. They can easily be observed with a phase contrast microscope. Fixation and staining of *Euplotes* with aceto-carmin or aceto-orcein can be helpful for the observation and identification of these endosymbionts. When stained, *Polynucleobacter necessarius* (formerly *omikron*) and the closely related "*omikron*-like" bacteria reveal many nucleoids (Heckmann, 1975; Heckmann et al., 1983). It appears that most of the *Euplotes* symbionts cannot grow outside their hosts, although this has been investigated thoroughly only for *P. necessarius.* Like *Caedibacter* and other symbionts of the *P. aurelia* species complex, the *Euplotes* symbionts appear not to be infectious, at least under laboratory conditions. The symbionts found to date have not been observed to be harmful to their hosts, which, however, in some cases are converted by infection into killers or mate-killers. *P. necessarius* and the closely related *omikron*-like bacteria are essential for their hosts (Heckmann, 1983).

The names of the prokaryotic endosymbionts of *Euplotes,* their properties, and the species in which they were found are listed in Table 1.

Isolation of the *Euplotes* Symbionts

Euplotes minuta, E. crassus and *E. vannus* are marine species. They can be isolated from water

Table 1. Characteristics of the prokaryotic endosymbionts of the genus *Euplotes*.

Symbiont designation	*Euplotes* species[a]	Site	Killing type	Reference
Polynucleobacter necessarius	*E. aediculatus* (15)	Cy	NK	Heckmann and Schmidt, 1987
Omikron-like symbiont	*E. eurystomus* (25)	Cy	NK	Heckmann et al., 1983
Omikron-like symbiont	*E. plumipes* (24)	Cy	NK	Heckmann et al., 1983
Omikron-like symbiont	*E. daidaleos* (13)	Cy	NK	Heckmann et al., 1983
Omikron-like symbiont	*E. octocarinatus* (11)	Cy	NK	Heckmann et al., 1983
Omikron-like symbiont	*E. patella* (5)	Cy	NK	Heckmann et al., 1983
Omikron-like symbiont	*E. woodruffi* (22)	Cy	NK	Heckmann et al., 1983
Epsilon	*E. minuta* (K_1,K_3,K_7)	Cy	K	Heckmann et al., 1967
Epsilon-like	*E. minuta* (VF_{17})	Cy	K+MK	Heckmann et al., 1967
Eta	*E. crassus*	Cy	K	Nobili et al., 1976
A	*E. crassus*	Cy	NK	Rosati et al., 1976
B_1	*E. crassus* (C_8)	Cy	MK	Dini and Luporini, 1976
B_3	*E. crassus*	Cy	NK	Rosati et al., 1976
C	*E. crassus*	Cy	NK	Rosati et al., 1976
D	*E. crassus*	Cy	NK	Rosati et al., 1976
Unnamed	*E. crassus*	Ma	NK	Rosati and Verni, 1975
Unnamed	*E. vannus*	Cy	K+MK	Demar-Gervais and Genermont, 1976

Symbols: Cy, cytoplasm; Ma, macronucleus; NK, nonkiller; K, killer; MK, mate-killer.
[a]The typical strain designation is given in parentheses.

samples containing algae or detritus collected at the seashore. All these species can be easily grown in the laboratory in seawater with *Dunaliella salina* as the food organism (for details see Heckmann, 1963). The other *Euplotes* species listed in Table 1 are freshwater organisms that may be collected from ponds. They can be grown in a diluted soil medium (Ruthmann and Heckmann, 1961) or in culture medium (CME) for *Euplotes* (Kuhlmann and Heckmann, 1989) and fed with *Chlorogonium elongatum* or *Chilomonas paramecium*. Most *Euplotes* species can also utilize bacteria as food.

Since the symbionts may differ in different strains of a host species, it is advisable to start *Euplotes* cultures from single cells, so that the cultures maintained in the laboratory form clones. In most cases, this will ensure that one is dealing with homogeneous populations of endosymbionts. However, double and triple infections have also been observed.

Polynucleobacter necessarius is the only endosymbiont of *Euplotes* known so far that has been isolated. In this case, cells of strain 15 of *E. aediculatus* were homogenized mechanically, and the symbionts were then purified by applying the homogenate to an ECTEOLA column, followed by elution with phosphate buffer (Heckmann, 1975; Schmidt, 1982). The procedure basically followed the one developed for the isolation of kappa symbionts from *Paramecium* by Smith-Sonneborn and Van Wagtendonk (1964) and resulted in very clean preparations of symbionts.

Identification of the *Euplotes* Symbionts

The bacterial symbionts of *Euplotes* can be identified by direct observation with a phase contrast microscope, by observation of physiological effects produced on other *Euplotes* cells that lack symbionts, or by a combination of these methods.

GENUS *POLYNUCLEOBACTER* HECKMANN AND SCHMIDT 1987. These are obligate endosymbiotic bacteria living in the cytoplasm of freshwater ciliates of the genus *Euplotes*. The symbionts are characterized by multiple nucleoids, are essential for their host species, are nonmotile, and Gram-negative. The type species is *Polynucleobacter necessarius*.

Polynucleobacter necessarius Heckmann and Schmidt 1987, formerly called *omikron*. Slightly curved rods, about 0.3 μm in diameter and 2.5–7.5 μm long. If stained with DNA-specific dyes, usually 3 to 9 but, in some cases up to 12 intensely stained and regularly spaced dots become visible (Fig. 9). They are considered to be nucleoids. When examined with the electron microscope, these nucleoids differ from those of most free-living bacteria by exhibiting an electron-dense central core that resembles the chromatin of eukaryotes (Fig. 10). Whether this core is formed by proteins associated with DNA or some other material is not clear (Heckmann, 1975).

The symbionts are individually contained in vesicles, to which ribosomes are often attached. *P. necessarius* reproduces by transverse binary fission in a typical bacterial manner. However, the fission products have often been found to differ in size. Frequently, a 7-μm-long rod containing eight to nine nucleoids was observed to bud off a 2.5-μm piece that contained three nucleoids (Heckmann, 1975). The GC content of

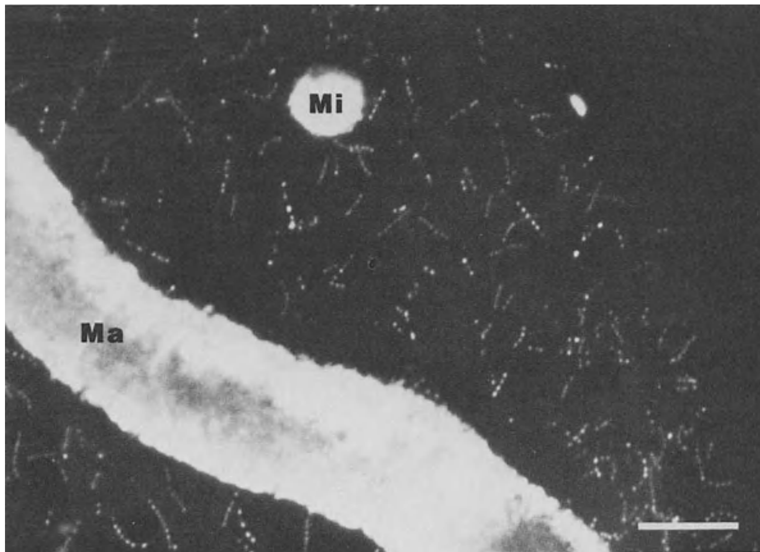

Fig. 9. Fluorescence micrograph of *Polynucleobacter necessarius* in the cytoplasm of a slightly crushed *Euplotes aediculatus* cell after staining with N,N'-diethylpseudoisocyanin chloride. The symbionts are revealed by the DNA-specific yellow fluorescence of their nucleoids. Ma, macronucleus; Mi, micronucleus. Bar = 10 μm. (From Heckmann, 1975.)

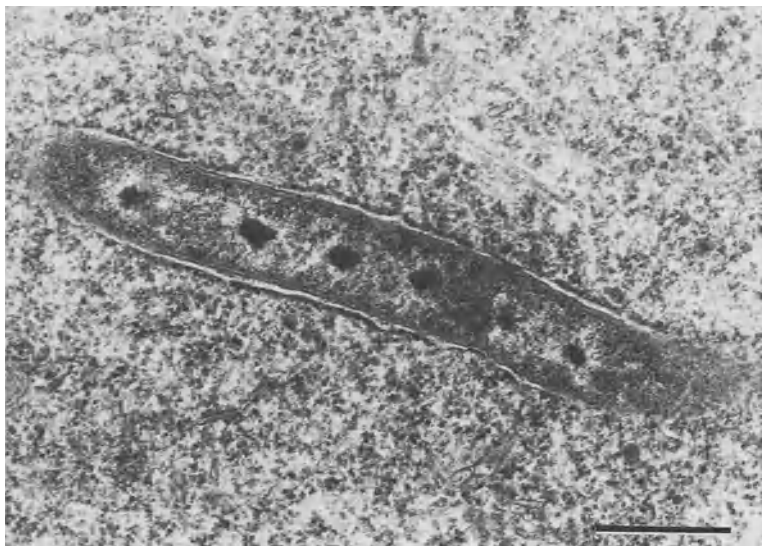

Fig. 10. Electron micrograph of *Polynucleobacter necessarius.* Longitudinal section. The ultrastructure of this symbiont resembles that of Gram-negative bacteria. Bar = 0.5 μm. (From Heckmann, 1975.)

the DNA of *P. necessarius* was 48 mol% by thermal denaturation. A value that was 2.8 mol% lower was found when the GC content was calculated from the buoyant density, which was 1.7036 g/cm^3 (Schmidt, 1982). The differences could be caused by rare bases; however, no DNA chemistry has been done for *P. necessarius.* The average DNA content of the symbiont was determined to be 5.8×10^{-3} pg. Taking into account the average number of nucleoids, a DNA content of 0.5×10^9 daltons per nucleoid was calculated. This value is in close agreement with the value of the kinetic complexity of the DNA, which was determined to be 0.57×10^9 daltons when corrected for the GC content (Schmidt, 1982). Equally small genomes have been reported for *Lyticum flagellatum, Pseudocaedibacter conjugatus,* and *P. falsus,* endosymbionts of the *P. aurelia* species complex (Soldo and Godoy, 1973b), for xenosomes of *Parauronema acutum* (Figueroa-de Soto and Soldo, 1977), and for chlamydiae, rickettsiae, and mycoplasmas (Bak et al., 1969). The genomes of these organisms are the smallest known for cells. They code for only about 700 to 900 proteins.

Heckmann (1975) has shown that it is possible to remove *P. necessarius* from *E. aediculatus* by treating a rapidly growing culture with 100 to 500 units/ml of penicillin for 5 to 6 days. Aposymbiotic hosts may undergo one or two fissions but then stop multiplying and die about 15 to 20 days after the last fission. The same results were obtained with several other antibiotics or with sufficiently high doses of X rays.

Reinfection and rescue of *E. aediculatus* have been achieved either by adding a cell homogenate from symbiont bearers or by injecting symbiont-containing cytoplasm (Heckmann, 1975; Fujishima and Heckmann, 1984). Type strain: in ATCC strain 30859 (clone 15 of *E. aediculatus;* see Heckmann and Schmidt, 1987).

THE *OMIKRON*-LIKE ENDOSYMBIONTS OF *EUPLOTES* These symbionts are very similar in appearance to *P. necessarius.* They were found in six freshwater *Euplotes* species (*E. eurystomus, E. plumipes, E. octocarinatus, E. patella,* and *E. woodruffi*). These species have a 9 type 1 fronto-ventral cirrus pattern like *E. aediculatus,* the bearer of *omikron,* and are considered to be closely related. The symbionts may differ from *P. necessarius* in size, shape, and other features, but they always share with *P. necessarius* the characteristic of multiple nucleoids (Heckmann et al., 1983). Symbionts of this type, but about twice as large as *P. necessarius,* were first noticed by Fauré-Fremiet (1952) in stocks of *E. patella* and *E. eurystomus.* He also observed at that time that small doses of penicillin led to a loss of symbionts and to death of the host ciliates. He therefore suggested that the symbionts might be essential for survival of the ciliate. This was later confirmed when Heckmann et al. (1983) subjected a large number of stocks of *Euplotes* species with a 9 type 1 cirrus pattern to a penicillin treatment of the kind that had earlier been found to remove *P. necessarius.* They found that all the ciliates stopped dividing and eventually died when their symbionts were removed. Heckmann et al. (1986) suggest that all *Euplotes* species with a 9 type 1 cirrus pattern suffer from a common deficiency that arose in a common ancestor of this group of organisms. This ancestor must have lived in symbiosis with a prokaryote that compensated for the acquired deficiency. *P. necessarius* and the *omikron*-like symbionts are considered to be progeny of this prokaryote.

THE *EPSILON* SYMBIONTS OF *EUPLOTES.* The *epsilon* symbionts from killer strains K_1, K_3, and K_7 of *E. minuta,* collected from the Mediterranean Sea at Villefranche-sur-Mer (France), were described by Heckmann et al. (1967). They are small rods, about 0.4 μm in diameter and 0.8–2.5 μm long, when observed in freshly crushed cells with bright phase optics, and appear somewhat darker than free-living bacteria. When the killer strain K_3 was fixed in Schaudinn's fluid, hydrolyzed in 1 N HCl, and stained with basic fuchsin as described by Dippell and Chao (in Sonneborn, 1950), several dozen darkly stained bodies were found scattered in the cytoplasm. Their staining properties, size, shape, and cellular distribution resembled that of *Caedibacter* species and other endosymbionts of *Paramecium.* Since *epsilon* particles were found only in killer strains, it is assumed that they are responsible for the killer phenotype of *E. minuta.*

Siegel and Heckmann (1966) noticed that certain strains of *E. minuta* killed certain other strains of this species when conjugation occurred. It was found that the sensitive cells formed vacuoles, ceased their normal swimming movements, settled to the bottom of the culture vessel, and finally lysed. These changes occurred within 1 to 4 days after the strains had been mixed. No evidence for killing was observed when cells of the three killer strains were mixed together.

A good example of the difficulties encountered in the identification of endosymbionts is provided by the killer particles of stock VF_{17} of *E. minuta.* Heckmann et al. (1967) had noticed that this strain caused symptoms in sensitives slightly different from those observed in mixtures with the other *E. minuta* killers. Here, vacuole coalescence generally proceeded to the point where only one large vacuole was present in the posterior region of the sensitive, giving the whole affected organism a pear-shaped form. Nevertheless, no morphological differences between these symbionts and *epsilon* particles could be detected. Later, when stock VF_{17} was employed for cross-breeding experiments in a study of the genetic control of cortical pattern in *E. minuta* (Heckmann and Frankel, 1968), it was found that this stock not only killed sensitive strains via particles liberated into the medium, but that it also acted as a mate-killer. From a pair of conjugants, one generally developed into a healthy looking exconjugant, whereas the other one failed to develop a large macronuclear anlage, became quiescent, and finally died. The surviving exconjugant clones had the same cortical pattern as stock VF_{17} and were all found to act as killers. They were therefore cytoplasmic descendents of the mate-killer parent. That they were true hybrids, i.e., that they had received a gametic nucleus from the sensitive partner, was shown from presence of certain genetic markers followed in these crosses. Whether VF_{17} was host to a mixed population of symbionts—with one type responsible for the killer trait and the other one for the mate-killer trait, both types being so similar that they could not be distinguished morphologically from each other, or whether a single class of particles present in VF_{17} determined both traits—cannot be decided. Both the killer trait

and the mate-killer trait were lost concurrently with aging of the stock (Frankel, 1973).

THE *ETA* SYMBIONT OF *EUPLOTES*. The *eta* symbiont, which can be observed only by electron microscopy, is round or oval shaped with an average diameter of 0.9 μm. It is bound by three juxtaposed membranes. The outermost membrane has ribosomes attached to it. The interior of the symbiont is granular with a few scattered fibrous strands (Rosati and Verni, 1977). The symbiont was found in strains of *E. crassus* collected by Luporini (1974) from a site on the coast of Somalia on the Indian Ocean. It was named *eta* by Nobili et al. (1976), who investigated the killer trait conferred on its host by this symbiont. Sensitive testers, when exposed to the culture fluid of killer strains or to a homogenate prepared from cells of these lines, develop a large vacuole in the posterior part of the body within 6 to 24 hours. Affected cells are gradually transformed into transparent spheres that eventually "explode."

THE B_1 SYMBIONT OF *EUPLOTES*. A symbiont very similar in morphology to *eta* appears to be responsible for the mate-killer properties of strain C_8 of *E. crassus* (Dini and Luporini, 1976); it was named B_1. When cells of this strain were treated with penicillin, both the mate-killer trait and the symbiont disappeared. Dini and Luporini (1982) provided evidence for the existence of specific host genes required for maintenance of the B_1 particles.

INCONSPICUOUS SYMBIONTS OF *EUPLOTES*. The other symbionts of *Euplotes* listed in Table 1 (A, B_3, C, D, and the unnamed ones) have been discovered by chance during electron microscopic investigations of certain strains. No special functions of these symbionts are known, nor have any effects of the symbionts on their hosts been reported.

The Xenosomes of *Parauronema acutum*

The term xenosome was coined by Soldo to denote the infectious particles that he found in the cytoplasm of the marine ciliate *Parauronema acutum* (Soldo et al., 1974). They were later shown to be small bacteria that possess multicopy genomes and resemble *Caedibacter taeniospiralis (kappa)* and other cytoplasmic symbionts of *Paramecium* in many respects. Two types of xenosomes are distinguished at the present time: killer xenosomes, which inhibit growth when taken up by susceptible ciliates, especially those of the genus *Uronema* (Soldo and Brickson, 1978); and non-killer xenosomes (Soldo et al., 1987). The xenosomes have not yet received binomial names.

Habitat

Parauronema acutum is a small hymenostome marine ciliate that can be maintained axenically. The culture medium is basically that developed for species of the *Paramecium aurelia* complex by Soldo and Van Wagtendonk (1969), distilled water being replaced by seawater (density 1.015–1.026 g/ml) and modified to contain 500 μg/ml asolectin as the sole source of lipid (Soldo et al., 1974). The association with xenosomes is stable. Symbiont-bearing strains have now been maintained for more than 15 years.

Xenosomes released from host cells are capable of infecting symbiont-free strains of *P. acutum.* A single xenosome is capable of infecting a susceptible animal. However, a "threshold" of 100–200 xenosomes appears to be required before a single xenosome can infect a potential host (Soldo, 1983)

Soldo and co-workers measured the oxygen consumption of purified xenosome preparations and reported a rate of oxygen uptake of 1.3 nmoles O_2/min/mg protein, which is higher than the oxygen uptake of *Bdellovibrio* and of the same order of magnitude as that of rickettsiae and *Caedibacter taeniospiralis.* It is about 20 times lower than that of *E. coli.* The rate of oxygen consumption was found to be stimulated by various fatty acids, by intermediates of the glycolytic pathway, and by intermediates of the citrate cycle with the exception of citrate itself, which had no effect. Cyanide was found to be a potent inhibitor of oxygen consumption of xenosomes (Soldo, 1983).

Isolation

Xenosomes can be isolated from *Parouronema acutum* by the same procedures as those used for the isolation of *Paramecium*-symbionts (Soldo and Godoy, 1974). It has been shown that killer xenosomes isolated in this way and purified with the help of Percoll gradients retain their ability to infect *P. acutum* and remain capable of killing sensitive *Uronema* strains (Soldo et al., 1986a). Results obtained from experiments in which isolated killer xenosomes lost their capacity to kill after they had been treated with various enzymes or had been coated with antibodies directed against the xenosomes, indicate that the toxic principles of

the xenosomes is a protein present at or near the surface (Soldo, 1987).

Identification

Rod-shaped, Gram-negative bacteria, 0.3 μm in diameter and 0.8 μm long. The GC content is 33.9 mol%. The symbionts occur in the cytoplasm of the marine ciliate *Parauronema acutum* in numbers ranging from 50 to 300 per cell. Negative staining reveals the presence of flagella, which provide the symbionts with a spinning and darting motility when released from the host (Soldo, 1987). The xenosomes were found to contain inclusions in the form of helical arrays. These structures (called H bodies) are about 0.6 μm long and 0.026 μm wide. They occur singly and in multiples and extend almost the entire length of the symbiont. H bodies were found in over 50% of killer- and nonkiller xenosomes (Soldo et al., 1987).

The genome size of xenosomes was found to be only 515 kbp, which is much smaller than that of free-living bacteria. Analytical measurements and data from sedimentation-rate analyses led to the conclusion that the chromosomal DNA exists in the form of nine circularly permuted double-stranded DNA molecules, each about 515 kbp in length (Soldo et al., 1983). Both the small size and the multicopy nature of the genome are typical of bacterial symbionts rather than for freeliving bacteria (Soldo and Godoy, 1973b; Soldo, 1987).

Killer and nonkiller xenosomes both contain plasmids (Soldo et al., 1986b; Soldo, 1987). The nonkiller plasmids consist of two circular DNA duplexes, each 63 kbp in length. The killer plasmid consists of four circular 63-kbp DNA duplexes. When a ciliate which previously harbored killer xenosomes but had been freed of them, is infected with nonkiller xenosomes, it becomes a killer. Soldo et al. (1986b) report that together with this transformation the restriction pattern of the extrachromosomal DNA is altered. The mechanism by which this transformation takes place is unknown.

Prokaryotic Symbionts of Ciliates from Anaerobic Environments

Ciliates living in an anaerobic habitat, such as the rumen or sewage sludge rich in hydrogen sulfide, show special adaptations. They usually lack mitochondria, bear hydrogenosomes, and are often associated with methanogenic bacteria (Stumm and Vogels, 1989). It has been shown that anaerobic ciliates generate their energy by converting carbohydrates to lactate, acetate, and butyrate and that they can remove reducing equivalents in the form of H_2 (Müller, 1988). Production of H_2 by proton reduction involves the enzyme hydrogenase. However, this enzyme functions well only if the concentration of H_2 is kept low ($<10^{-5}$ atm). This requirement is apparently achieved by methanogenic bacteria, which consume H_2 and tend to be abundant in these habitats.

Habitat

An episymbiotic association with methanogenic bacteria was described for 11 species of rumen ciliates of the family Ophryoscolicidae (Vogels et al., 1980). The attached bacteria were rods 0.9–3.8 μm long and 0.6–0.7 μm wide that occurred as clusters or long chains. They were identified as methanogens (probably *Methanobrevibacter ruminantium*) on the basis of specific fluorescent coenzymes (F_{350} and F_{420}). Experiments with a fistulated sheep revealed a decrease of the association frequency of methanogenic bacteria with rumen ciliates when the hydrogen concentration in the rumen fluid increased and the reverse when hydrogen became scarce (Stumm et al., 1982). This is interpreted in favor of an interspecies transfer of hydrogen. The finding that rumen ciliates have hydrogenosomes, showing strong hydrogenase activity, supports this view (Yarlett et al., 1981). Stumm and Vogels (1989) propose that the attachment of hydrogen-consuming methanogens to rumen ciliates facilitates hydrogen removal, which is advantageous to both the ciliates and to their episymbionts.

Episymbiotic bacteria have been known from sand-dwelling ciliates for many years (Sauerbrey, 1928; Kahl, 1933, 1935; Fauré-Fremiet, 1950a, 1951). They are firmly attached to the cell surface and appear to differ in size, shape, pigmentation, and wall structure from species to species (Fenchel et al., 1977). Fauré-Fremiet (1950b) carried out a unique study of the symbionts of two species of the genus *Kentrophoros* (formerly *Centrophorella*). He investigated their symbionts and found them to be attached to the nonciliated dorsal side of these ribbon-shaped ciliates. The oblong symbionts were densely packed and protruded perpendicularly, so that they appeared like bristles of a brush. They were Gram-negative, nonmotile rods, and were observed to divide by longitudinal fission. Besides containing rather large quantities of polysaccharides, indicated by iodine treatment, they contained dark-refractive sulfur globules, so that the host appeared black. The dark pigmentation is a strong indication that the symbionts are purple sulfur bacteria (Chroma-

tiaceae). Fauré-Fremiet, who found the symbionts exclusively on the ciliates and neither on accompanying sand grains nor on glass slides that had been in the sand in which these ciliates live for several days, considered the symbionts as obligatorily epizoic. Electron microscope studies by Raikov (1971, 1974) confirmed Fauré-Fremiet's observations and led to the discovery that the epizoic bacteria are phagocytized. He studied *Kentrophoros fistulosum* and *K. latum* and found that these ciliates take up the epizoic bacteria by pseudopodia-like cytoplasmic protrusions and enclose them in food vacuoles. This may occur at any place on the nonciliated body side of these ciliates, which have no special mouth structures. The unique form of phagocytosis discovered in these species has been named "random phagocytosis." Based on the content of the food vacuole of these ciliates, their epizoic bacteria appear to be their main nutrition source. 14 *Kentrophoros* species are now known. They all live in the interstices of sandy marine sediments.

Fenchel et al. (1977) examined marine-sediment-dwelling ciliates for cytochrome oxidase activity and for fine-structural details. They found that many of the ciliates of this habitat lacked cytochrome oxidase activity and mitochondria but contained microbodies (membrane-bound organelles, spherical or irregular in shape, 1–2 μm in length, with a homogeneous granular matrix). In addition, these ciliates harbored a species-specific flora of epi- and endosymbiotic bacteria. Large numbers of symbionts per cell were observed, ranging from about 1,000 bacteria per ciliate, corresponding to less than 1% of the host's biomass, to about 100,000 bacteria per ciliate, corresponding to about 20% of the ciliate's biomass. Some of these anaerobic ciliates were found to contain both epi- and endosymbionts, and often more than one type of episymbiont was recognized. Fenchel et al. (1977) hypothesized that the bacteria and the ciliates interact metabolically. This, however, has not yet been investigated. Anaerobic conditions also exist in sediments that are rich in decaying plant material, such as those of freshwater ponds, lakes, ditches, and swamps. This habitat was named "die sapropelische Lebewelt" (life in the sapropel) by Lauterborn (1901). Endosymbiotic bacteria in sapropelic ciliates were first described by Fauré-Fremiet (1909). Later, Liebmann (1937, 1938) noted that all the sapropelic ciliates that he investigated contained rod-like bacteria in their cytoplasm. In recent years, this habitat was thoroughly investigated by a group of Dutch microbiologists (Stumm and Vogels, 1989), and it is now, next to the rumen, the best-studied anaerobic habitat. It is inhabited by large numbers of anaerobic protozoa and methanogenic bacteria. A survey of sapropelic ciliates by means of fluorescent microscopy revealed the presence of methanogenic bacteria inside the cells; they were spread throughout the cytoplasm in considerable quantities (Van Bruggen et al., 1983).

Electron microscopic investigations of sapropelic ciliates revealed the absence of mitochondria and the presence of microbodies. In *Metopus striatus,* a Gram-positive rod-shaped bacterium was regularly found to be in close association with a microbody consisting of a granular matrix surrounded by a membrane (Van Bruggen et al., 1984). The bacterium was isolated and identified as *Methanobacterium formicicum.* A similar or even closer association of microbodies and methanogenic bacteria is reported for *Metopus contortus* (Van Bruggen et al., 1986) and for *Plagiopyla nasuta* (Goosen et al., 1988). Because the methanogens isolated from sapropelic ciliates were found to consume hydrogen it has been hypothesized that the symbiont-associated microbodies are hydrogenosomes (Van Bruggen et al., 1986). A direct proof was recently provided by Zwart et al. (1988) who demonstrated hydrogenase activity in microbodies of *Plagiopyla nasuta* and *Trimyema compressum.*

Isolation

For enrichment and culture of sapropelic ciliates, samples from anoxic natural sediments are introduced into mineral media under reducing conditions. Two media which have successfully been used for this purpose are given by Wagener and Pfennig (1987). They also report the first monoxenic large-scale culture of an anaerobic ciliate. They enriched *Trimyema compressum* from anoxic mud samples and then established a pure culture. The ciliate was fed with a bacterial strain isolated from the enrichment culture that proved capable of serving as food. During continued cultivation, *T. compressum* gradually lost its endosymbionts.

An isolation method for methanogenic bacteria from sapropelic ciliates has been described by Van Bruggen et al. (1986): a small number of ciliates is collected with a pipette and is washed free of potential contaminants in an isolation medium under a dissection microscope. The microscope is covered with a plastic hood flushed with nitrogen. The ciliates are then homogenized in an anaerobic glove box. The homogenate with the symbionts is plated on solid isolation medium containing penicillin G or lysozyme, which do not effect methanogenic bacteria. The homogenate is incubated at 22 to

37°C under an atmosphere of H_2/CO_2 (80:20). In liquid media, growth can be followed by measuring the methane production.

Identification

The symbionts of several sapropelic ciliates have been identified as methanogenic bacteria. The endosymbionts of species of the ciliate *Metopus* (Fig. 11) were first recognized to be methanogens by their autofluorescence when irradiated with short-wavelength blue light (Van Bruggen et al., 1983; 1986). According to Doddema and Vogels (1978), epifluorescence microscopy can detect the presence of the deazaflavin coenzyme F_{420} and the pterin compound F_{342} both of which are specific for methanogens. The methanogenic character of the endosymbionts has subsequently been proven by studies of the isolated symbionts and measurements of their methane production in situ. The symbionts were finally identified as *Methanobacterium formicicum* (Van Bruggen et al., 1984). The methanogenic symbionts of *Plagiopyla nasuta* were also identified as *M. formicicum* (Goosen et al., 1988). This is a bacterium that often occurs in sapropelic habitats; it is a slender, nonmotile rod, with a diameter of 0.4 μm and a length of 2–7 μm. It was identified by colony form, cell morphology, temperature and pH optimum, substrate specificity, DNA base composition, and type of coenzyme F_{420}. It appears likely that *M. formicicum* is preadapted for endosymbiosis, because symbiont-free lines of the ciliate *Trimyema compressum* could also successfully be infected with this bacterium (Wagener et al., 1990).

Methanoplanus endosymbiosus was isolated from the marine ciliate *Metopus contortus* (Van Bruggen et al., 1986). In liquid media the isolated bacteria were irregular, nonmotile, nonsporeforming discs with a diameter of 1.6–3.4 μm. In a side view, they appeared as rods that were sometimes branched. The symbionts were osmotically fragile and lysed immediately when suspended in water. Growth and methanogenesis were observed with H_2/CO_2 or formate as substrates, with generation times of 7 or 12 h, respectively. The temperature range from growth was between 16 and 36°C with an optimum at 32°C. The optimal pH range for growth was 6.8–7.3. Tungsten and NaCl at a concentration of 0.25 M were required for optimal growth. The GC content of the DNA is 38.7 mol%.

For detecting methanogenic endosymbionts, epifluorescence microscopy proved to be a pow-

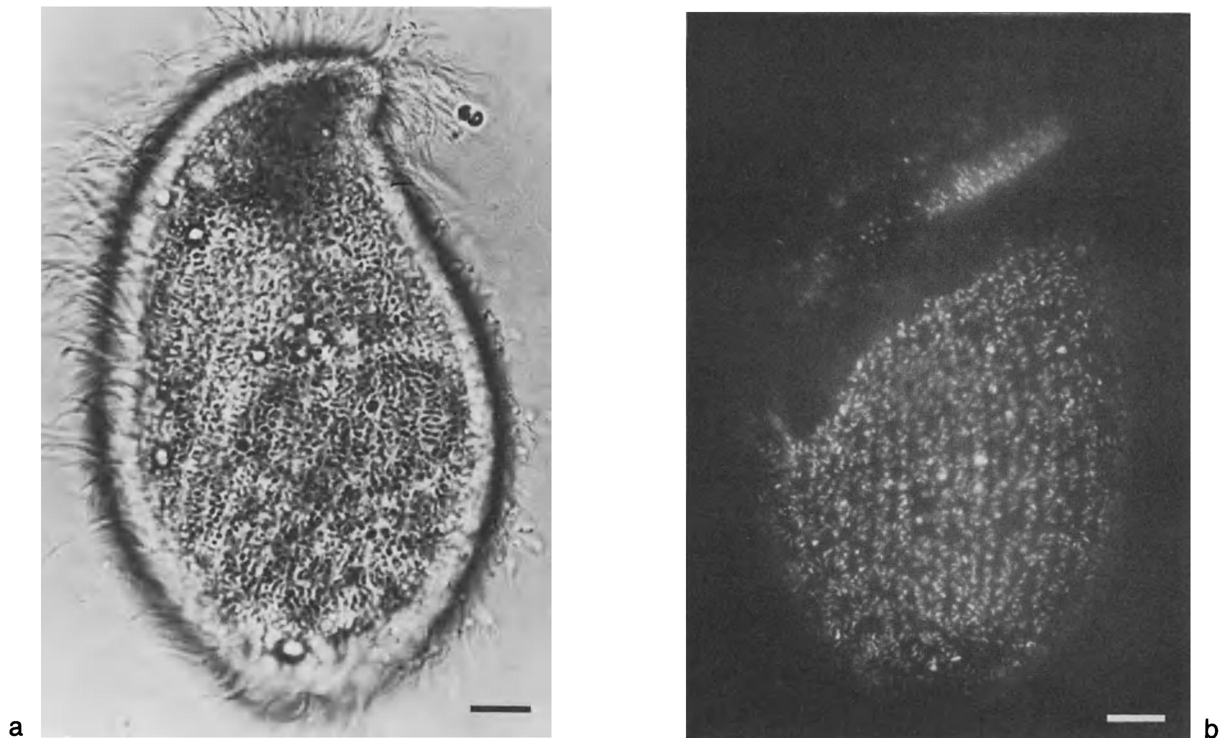

Fig. 11. *Metopus contortus* containing methanogenic endosymbionts. The methanogenic bacteria are located parallel to the kineties (ciliary rows) or the inner side of the cell. Cell fixed with 1.2% formaldehyde and 0.3% glutaraldehyde. (a) Bright field micrograph. (b) Epifluorescence micrograph. Bars = 10 μm. (From Van Bruggen et al., 1986.)

erful method. Even when symbionts are inside cells their blue autofluorescence may be visible. Endosymbionts that fluresce have been observed in the sapropelic ciliates *Bachonella spiralis, Caenomorpha medusula, C. universalis, Lacrymaria cucumis, Metopus es,* and *M. laminarius* (Van Bruggen et al., 1983). They have not been found in ciliates from aerobic habitats.

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CHAPTER 215

Prokaryotic Symbionts of Marine Invertebrates

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The symbiosis of prokaryotic organisms with eukaryotic taxa is a widespread phenomenon that has had profound impact on the physiology, ecology, and evolution of the host organisms. Although these symbioses range from relatively loose coexistence to highly interdependent intracellular associations, in this chapter we will mainly discuss symbionts and symbioses of the latter type, as exemplified by the symbiotic associations of chemoautotrophic bacteria with marine invertebrates. We will also discuss the symbioses of methane-oxidizing bacteria with marine invertebrates and the symbioses of cellulolytic/nitrogen-fixing bacteria with wood-boring marine bivalves (shipworms).

Little is yet known about these symbionts, largely because, except for the symbionts of the shipworms, it has not yet been possible to grow these organisms in pure culture. Therefore, much of what is known about these symbionts comes from studies of the physiology of the intact symbioses. In the following pages we will review what is known about these symbionts based on these studies and on studies of purified symbionts and of purified symbiont macromolecules.

The discovery of the symbiosis between chemoautotrophic bacteria and marine invertebrates was made in conjunction with the discovery of the deep-sea hydrothermal vents (Cavanaugh et al., 1981; Felbeck, 1981). The hydrothermal vents are underwater hot springs that occur near zones of sea floor spreading (Jannasch and Mottl, 1985). In these geologically active zones, the earth's crust is relatively thin. Seawater seeps into the thin crust, is heated by magma beneath the ocean floor, and is ejected again through cracks and fissures in the bottom. During its contact with the hot rocks, the chemical composition of the seawater is radically changed; the water absorbs many minerals and some of its sulfate is reduced to sulfide.

Unlike most deep-sea environments, which are only sparsely populated, some hydrothermal vents are surrounded by large and densely populated animal communities. These communities contain a large variety of invertebrates, including vestimentiferan tubeworms, clams, and mussels. The presence of these large animal populations was extremely puzzling at first because of the absence of any obvious food source for these communities. Soon, however, it was found that bacteria play a primary role in supporting these animal populations (Cavanaugh et al., 1981; Felbeck, 1981). Unlike all previously described communities that obtain their energy directly or indirectly from the sun via photosynthesis, the vent communities are based on bacterial chemosynthesis fueled by sulfide arising from the geothermal reduction of sulfate (Jannasch, 1985). Sulfide from the vent effluent serves as a rich source of energy and reducing power, making possible an abundant growth of free-living chemoautotrophic bacteria. In addition to these free-living chemoautotrophic bacteria, it has also been found that many of the animals in these communities contain chemoautotrophs as endosymbionts within their tissues. These endosymbionts form a potentially important food source for the invertebrate hosts. The complete absence of a digestive tract in the adult vestimentiferans and the greatly reduced digestive systems of the vent bivalves are strong indications that these eukaryotic hosts rely heavily on their endosymbionts to provide them with the energy and nutrients that they require for growth. Therefore, both in terms of ecology and physiology, these vent communities represent a significant departure from previously described environments.

Similar symbioses have also been found in many other animal species, including annelids, pogonophorans, bivalves, and gastropods. The morphology of these symbioses varies widely, as might be expected from the divergent phylogenetic origins of these hosts. A common characteristic, however, is that in these hosts the digestive system is typically absent or reduced in function. The environments that support

these symbioses are also quite varied, both in terms of their geographic location and ecological features. All these environments seem to share some common features, however, including the presence of a redox boundary where a source of reduced sulfur compounds may be found in close proximity to a source of oxygen. Sewage outfalls, eelgrass beds, mangrove swamps, and hypoxic ocean basins are some of the environments that support these symbioses (Felbeck and Somero, 1983; Jannasch and Nelson, 1984; Southward, 1987).

In addition to the sulfur-based chemoautotrophic (thiotrophic) symbioses discussed above, two additional types of symbioses with marine invertebrates have been recently described. The first are the symbioses involving methylotrophic bacteria. These resemble the thiotrophic symbioses, except that the source of energy is a reduced single-carbon compound. This type of symbiosis has been found primarily in areas such as marine hydrocarbon or hypersaline seeps (Cavanaugh et al., 1987; Childress et al., 1986; Fisher et al., 1987). The third type of symbiosis that we will discuss is the symbiosis with cellulolytic/nitrogen-fixing bacteria found in the wood-boring clams of the family Teredinidae. These symbionts allow their hosts to subsist on a diet of wood, using cellulose as their sole carbon source (Gallager et al., 1981; Waterbury et al., 1983). The common feature connecting these symbioses is that, in each case, the eukaryotic host is able to utilize new energy and carbon sources by harnessing the remarkable metabolic flexibility of prokaryotic cells.

Few generalizations can be made concerning these symbionts themselves, partly because these symbionts are diverse in function, and partly because we know very little about these organisms. In the same way as the host organisms have been classified according to their differing modes of nutrition, the symbionts themselves can also be crudely categorized by nutritional modes. These are the thiotrophic, methylotrophic, and cellulolytic/nitrogen-fixing modes. All of the "trophic" endosymbionts so far examined have cell wall structures typical of Gram-negative bacteria. These symbionts range in size and shape from 0.3–0.75μm spheres in *Bathymodiolus thermophilus* (Fiala-Medioni et al., 1986) to extremely large pleomorphic rods of greater than 5 μm in width and 10 μm in length in *Lucinoma annulata* (Distel and Felbeck, 1987). Membrane-bound sulfur inclusions in the periplasmic space, similar in structure and appearance to those seen in *Beggiatoa* and *Thiovulum* species, are frequently observed in the thiotrophic symbionts (Vetter, 1985). The cytoplasm of the sulfur-oxidizing symbionts is typically described as granular in appearance. This granular nature has been ascribed by various authors to the presence of either carboxysomes, ribosomes, or glycogen granules, but the true nature of these granules is yet to be resolved. The methylotrophic symbionts range from cocci of 0.75–2.0 μm in diameter in Florida escarpment mytilids (Cavanaugh et al., 1987) to rods of approximately 1×2 μm in *Siboglinum poseidoni* (Schmaljohann and Flügel, 1987). Stacked internal membranes similar to those seen in type I methylotrophic bacteria (see Chapter 118) are a typical distinguishing feature of the methylotrophic symbionts (Childress et al., 1986; Schmaljohann and Flügel, 1987). A proposed second symbiont lacking these internal membranes has been reported in the Florida escarpment mytilid by Cavanaugh et al. (1987). The cellulolytic/nitrogen-fixing symbionts of the teredinid bivalves are vibrioid rods approximately 0.4–0.6 $\times$ 3–6 μm (Waterbury et al., 1983), typically containing phase-dark material at the apical regions. These symbionts have no flagella when observed in tissue, but when grown in liquid culture they are motile by a single polar unsheathed flagellum. Those symbionts whose phylogeny has been examined (some sulfur oxidizers and the cellulolytic/nitrogen fixer) fall into the gamma subdivision of the protebacteria (see "Characterization Without Pure Culture" in this chapter). The phylogenetic position of the methane oxidizers remains to be elucidated. Only one of these symbionts (the cellulolytic/nitrogen fixer) has been grown in pure culture and has been characterized in considerable detail. Other symbionts have been characterized primarily by their presumed function in the intact symbiotic systems as evidenced by the presence of unique enzyme activities, by examination of symbiont cells purified from host tissue by mechanical means, and by light and electron microscopy of sectioned host tissue.

Because so much of what we know about these symbionts comes from studying the environment where the symbionts live, i.e., the host organisms, we will begin our detailed discussion by describing the intact symbioses. With this as a foundation, we will then move on to discuss the studies of the symbionts themselves.

Morphology of the Symbioses

In the vestimentiferans and pogonophorans, symbionts are a characteristic feature of all members of the phyla. They are embedded

within cells of a specialized tissue called the trophosome, which is the main tissue found in the trunk of the vestimentiferans (Jones, 1985). In vestimentiferans, the trophosome accounts for up to 16% of the animals' wet weight while the percentage is much lower in the pogonophorans, which have a very similar organ (Arp and Childress, 1983; Southward, 1982). The trophosome tissue of the vestimentiferans, which is taken as representative here, is composed of many lobules which are each about 0.15 mm in diameter and are heavily vascularized by the closed circulatory system typical for both of these phyla (Bosch and Grasse, 1984; Hand, 1987). Located in their center are the bacteriocytes containing the symbionts. These bacteria make up 15% to 35% of the volume of the entire trophosome (Powell and Somero, 1986). Within the lobules, as well as from the posterior to anterior end of the trophosome, the phenotype of the bacteria changes (de Burgh et al., 1989): the symbionts in the Vestimentifera range in size from 1 μm to 10 μm (de Burgh et al., 1989) and are very pleomorphic, while those in Pogonophora are typically rods 0.15 μm–0.3 μm thick and 2 μm–4.8 μm long (Southward, 1982). In both cases, they are usually surrounded by a host membrane including one or more bacteria. DeBurgh et al. (1989) describe numerous cytoplasmic inclusions inside of the symbionts of several vestimentiferan species, tubular membrane systems, glycogen-like particles, and poly-β-hydroxybutyrate (PHB) or sulfur bodies. One pogonophoran species, *Sclerolinum brattstromi,* has been described as containing symbionts that are not enclosed in a host membrane but are free in the cytoplasm.

The symbionts in several bivalve and a gastropod species can be found inside of specialized gill cells where they are in close contact with the surrounding seawater. The most detailed description is available for lucinid clams (see Figs. 1 and 2). Here, the bacteriocytes, or bacteria-containing cells, are arranged in tubular stacks whose centers form open cylinders that allow water to flow freely between the mantle cavity and the interlamellar space (Distel and Felbeck, 1987). The symbionts are found in vesicles completely surrounded by a host membrane. They are typically rod- to oval-shaped and rather large (2–5 μm wide and up to 10 μm long) but very pleomorphic. They have numerous inclusions of elemental sulfur in their periplasmic space. These sulfur granules account for up to 2.1% of the dry weight of the gill tissue. Between the bacteriocytes, intercalary cells are located whose thin extensions cover the surface of the bacteriocytes, thus forming the inner lining of the open cylinders

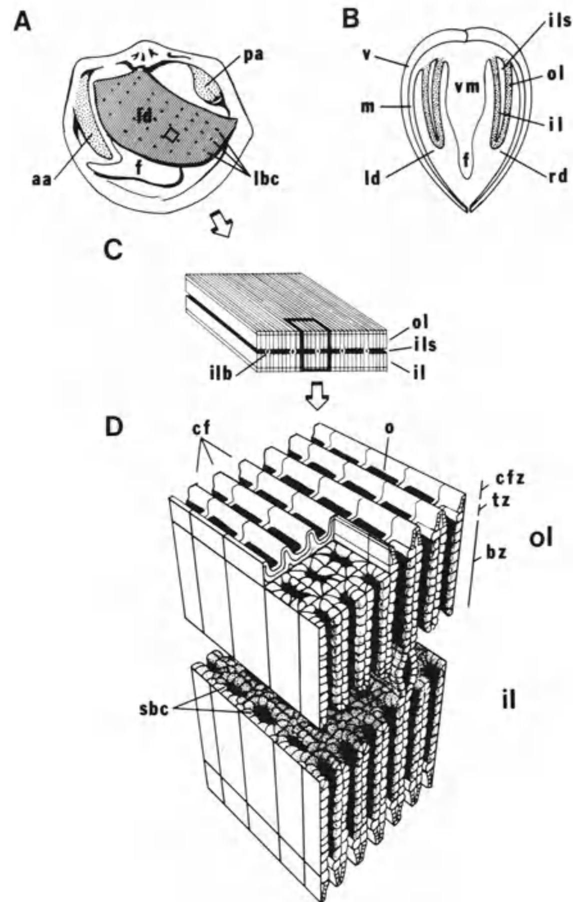


Fig. 1. Structure of the symbiont-containing tissue within the gill of a lucinid clam. (A) A clam (*Lucinoma aequizonata*) with the left valve removed. (B) A transverse section through the entire clam at the median point of the anterior-posterior axis. (C) An enlargement of a block of gill tissue removed from the region indicated by the small box in Fig. 1A. (D) An enlargement of the block of gill tissue removed from the region indicated by the box in Fig. 1C. Symbols: aa, anterior adductor; bz, bacteriocyte zone; cf, ctenidial filaments; cfz, ctenidial filament zone; f, foot; il, inner lamella; ilb, interlamellar bridge; ils, interlamellar space; lbc, large bacteriocyte channel; ld, left demibranch; m, mantle; o, ostium; ol, outer lamella; pa, posterior adductor; rd, right demibranch; sbc, small bacteriocyte channel; tz, transition zone; v, valve; vm, visceral mass. (From Distel and Felbeck, 1987.)

described above. The micromorphology of the gastropod gill is similar (Okutani and Ohta, 1988; Stein et al., 1988).

In the bivalve family Thyasiridae, the symbionts are apparently located between gill cells below the cuticle (Southward et al., 1986). However, other authors describe them as being intracellularly located inside of a large vacuole (Herry et al., 1989; Reid and Brand, 1986). Several different sizes of rod-shaped symbionts have been observed of 0.2–0.5 μm in diameter

and 0.5–2 μm in length, but usually there is only one phenotype in any host species. In two undescribed host species, two distinct and morphologically different symbionts could be detected, which may indicate that these associations are less selective than the intracellular symbioses described above.

Morphologically, the symbioses described in annelids are fundamentally different from the ones described so far. The bacteria are never intracellular but occur either between cells in many oligochaetes or attached to the outside epidermis as a bacterial lawn in some polychaetes. Oligochaetes with symbiotic bacteria have been described mainly in the tubificid subfamily Phallodrilinae. All of the species in two genera, *Inanidrilus* and *Olavius*, are described as having symbionts and being gutless (Erseus, 1984). The bacteria are located underneath the cuticle between irregular and long extensions of the epidermis cells. In *Inanidrilus* (formerly *Phallodrilus*) *leukodermatus*, the best-described oligochaete showing symbiosis, two different shapes of bacteria have been observed, an oval one measuring about 3.5 μm × 2.3 μm with a very "delicate" outer membrane, and a rod-shaped one of about 0.5 μm × 1.7 μm which has a multi-layered thick cell envelope. Both contain inclusions of elemental sulfur and PHB (Giere, 1985; Giere and Langheld, 1987).

Two polychaetes with external bacterial symbionts have been found at hydrothermal vents; both species, *Alvinella pompejana* and *A. caudata* are of the same genus. They usually are found on the outside walls of the chimneys formed by zinc sulfide precipitations from the vent waters in temperatures of up to about 40°C. The bacteria are associated in both cases with the polychaete epidermis. In both species, cluster-like bacterial/associations are located in intersegmentary spaces. The bacteria are rod-shaped, coccoid, or filamentous. In addition, individual bacteria (rod-shaped, prosthecate, spiral, curved, or unsheathed filamentous in appearance) are distributed over the entire animal tegument. In *A. pompejana*, filamentous bacteria are also associated with epidermic expansions, while in *A. caudata* similarly shaped bacteria can be found inserted on the posterior parapods (Desbruyeres et al., 1985; Gaill et al., 1988).

Ecophysiology of the Symbioses

When symbioses of chemoautotrophic bacteria with marine invertebrates were first discovered, all information about the physiology of the bacteria was limited to the activities of a few enzymes that were found in the tissues containing the bacteria: ribulose-bisphosphate carboxylase and ribulose 5-phosphate kinase (both characteristic enzymes of the Calvin cycle), and ATP sulfurylase, and adenosine phosphosulfate (APS) reductase (indicative of sulfur oxidation) (Felbeck, 1981; Felbeck et al., 1981; Southward et al., 1981). These enzymes are normally absent in animal tissues or are found in very small amounts only. Their presence was a strong indication that the bacteria were able to fix CO_2 through the Calvin cycle using energy from the metabolism of sulfur compounds. The abundance of sulfide and oxygen in the environment suggested that the major pathway for energy production was the oxidation of reduced sulfur. Aside from these enzymes involved in carbon and sulfur metabolism, nitrate reductase was detected in some symbiont-containing tissues.

Since then, these initial observations of symbiont physiology have been supported by many more results, such as the measurement of stable isotope ratios, incorporation studies with radiolabelled tracers, and physiological studies of the mechanisms of sulfur and carbon transport and metabolism.

Stable Isotope Ratios

Carbon, nitrogen, and sulfur exist in two or more stable isotopes in significant quantities in the natural environment. Their isotopic ratios are used to determine the source of an element in a biological system. The most widely used ratio is that of carbon ($^{13}C/^{12}C$). The natural abundance of the heavier carbon isotope is approximately 1.11%. When carbon dioxide is first incorporated into organic molecules by autotrophic organisms, the enzymes responsible for this action use the lighter carbon isotope preferentially. Ruby et al. (1987) clearly showed isotope fractionation in two cultures of sulfur-oxidizing bacteria. The degree of preference can provide information about the character of the initial carbon-fixation step. The carbon isotope ratio established in this step then changes only slightly during the further transfer of organic carbon within a food chain.

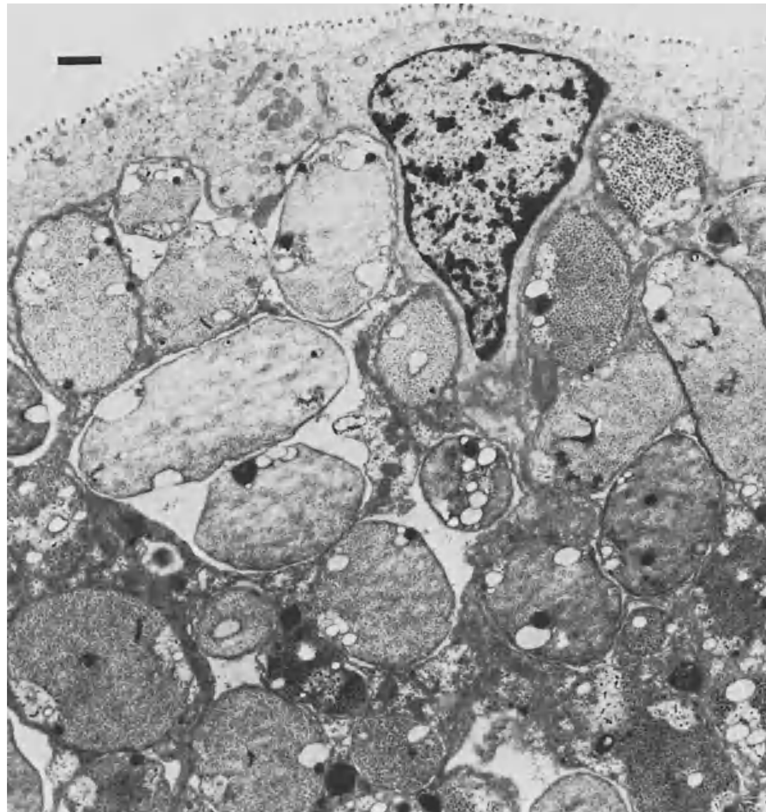
The carbon isotope ratio is usually expressed as a function of the ratio found in the fossil PeeDee belemnite (PDB) standard:

$$\delta^{13}C = \{[(^{13}C/^{12}C)\text{sample}/(^{13}C/^{12}C)\text{ standard}] - 1\} \times 10^3 \text{ (‰)}$$

A sample with a negative number, therefore, is enriched in ^{12}C relative to the PDB standard.

Generally, it has been suggested that an isotope ratio between about −12 to −24‰ indi-

Fig. 2. An electron micrograph of the bacteriocyte tissue in *Lucinoma aequizonata*. A microvilli-covered intercalary cell is visible at the top of the figure, with prominent nucleus and mitochondria. Beneath this cell, a portion of two bacteriocyte cells containing large oval- to rod-shaped symbionts is seen. Bar = 1.0 μm.



cates a photoautotrophic food source, while a value below this to approximately − 45‰ signals a chemoautotrophic source of carbon. When carbon isotope ratios even lower than − 45‰ are found, they are considered to indicate methanotrophy. All of these generalizations, however, have to be viewed carefully because there are many possible sources of error, e.g., if a carbon source is unusually enriched or depleted in one of the isotopes or if an organism is carbon limited the resulting carbon isotope ratios in an organism can be greatly altered.

Stable isotope ratios did, however, provide important clues about the nutritional strategies of the hosts of chemoautotrophic bacteria. The fact that the bacteria-containing tissues and host tissues had similar isotope ratios that were unlike any possible food source nearby indicated the dependence of the host on the symbionts (Rau, 1981a; Rau, 1981b; Rau, 1985). In addition, using carbon isotope ratios, one can also estimate what percentage of the body carbon is from each of two carbon sources whose isotope ratios are known. This calculation is important for those symbioses where the host animal still maintains a functional gut and can, therefore, supplement the carbon supplied by the symbionts by externally obtained food. This calculation has been done for the mud-dwelling clam *Lucinoma aequizonata:* it was assumed that the isotope ratio of carbon supplied by the bacteria stays the same after transfer to the clam and that the only other possible food source is dissolved organic matter in the surrounding mud. Using the measured isotope ratios of these two sources it was calculated that about 70% of the clam's carbon is provided by the symbionts (Cary et al., 1989). Similar studies have been done for the protobranch clam *Solemya velum,* where a bacterial contribution of 98% was estimated (Conway et al., 1989).

Similar investigations and arguments can also be made based on sulfur or nitrogen isotope ratios. In both cases, however, even larger errors than for carbon can arise during the interpretation of an isotope ratio in an animal, because the nitrogen ratio does not stay constant during heterotrophic processes, and the sulfur ratio can change widely due to a wide variability of the isotope ratios of the original sulfur sources for the symbioses. These facts make detailed predictions based on these ratios almost impossible.

Radioactive Tracer Studies

As mentioned earlier, it is generally assumed that the bacteria provide all or part of the host's

nutritional needs. There are two ways this could be accomplished, either as a result of continuous growth followed by digestion of the bacteria by the host, somewhat like an internal chemostat culture, or as a result of excretion of reduced carbon compounds by the bacterial symbionts, followed by incorporation into the host's metabolism.

Several ultrastructural studies have demonstrated that bacteria are indeed digested by their hosts. Bosch and Grasse (1984), deBurgh et al. (1985), and Hand (1987) found a series of degenerative stages of bacteria within the trophosome of the tubeworm *Riftia pachyptila* where, in a gradient from the periphery of the trophosome lobules towards their center, the symbionts degenerate progressively and form myelin-like bodies in the centers of the lobules. Similar observations of lysosomal digestion have been reported for the hydrothermal vent mussel *Bathymodiolus thermophilus* (Fiala-Medioni et al., 1986) and the vent clam *Calyptogena magnifica* (Fiala-Medioni et al., 1990). The quantitative significance of these observations is however unclear. If the bacterial contribution made by this process were significant, one would expect to see large numbers of dividing bacteria to replenish their stock. This, however, has not been observed to date, suggesting that the digestion of the symbionts may simply be due to "housekeeping", as has been proposed by deBurgh (1985) or it may be a way to provide some essential nutrients that cannot be synthesized solely from the excreted products of bacterial metabolism.

The most straightforward way to investigate possible transfer of metabolites between symbiotic partners is to observe the movement of labeled tracers between the partners. This technique has been used in case of the symbiosis with chemoautotrophic bacteria in a variety of studies. Initial studies with the gutless clam *Solemya reidi* and the tubeworm *Riftia pachyptila* (Felbeck, 1983; Felbeck and Somero, 1983) suffered from the lack of a suitable technique to separate the bacteria from host tissue. It was, therefore, difficult to separate the radioactive labeling patterns for metabolic pathways from host or symbiont. Since then, however, a technique has been developed by Distel and Felbeck (1988a) to purify the symbionts quickly from host tissue and to incubate them individually or to separate them quickly after an experiment before further processing of the tissues. This method could be used successfully for sulfur-oxidizing symbionts because these symbionts contain inclusions composed of elemental sulfur (sp. gr. 2.05–2.09), which increase their buoyant density. Thus, sulfur-oxidizing symbiont cells typically exceed 1.1 g/ml in density and can be separated from the less-dense host cells and cell constituents. Symbiont cells purified by this method remain physiologically active for several hours. Distel and Felbeck (1988b) found that purified bacteria from the clam *Lucinoma aequizonata* can incorporate radioactivity from external CO_2 into reduced organic compounds, mainly aspartate and malate.

Fisher and Childress (1986) demonstrated, using autoradiographic techniques, that the initially very high fraction of the fixed radioactivity incorporated into symbiont-containing tissues of the clam *Solemya reidi* decreased substantially after extended chase periods of up to 5 days. Concomitant to this decrease, an increase in labeling of symbiont-free tissues was noted, e.g., in mantle or foot tissue. Their results showed clearly that a translocation of fixed carbon takes place between the bacteria and the host but they cannot distinguish between a translocation through transfer of low-molecular-weight organic material or a lysis of the bacteria. Again, however, it is very difficult to estimate the quantitative importance from these data. Their published value of 45% of the carbon originating from the bacteria can at best be viewed as a rough estimate, because of the large margins of error in the data used for the calculation. Recently, however, it was shown by Anderson et al. (1987) that *Solemya reidi* has the ability for net CO_2 uptake from the environment. Under certain environmental conditions, the concentration of CO_2 in the environment decreased, proving that the clams fixed more carbon from CO_2 than was released due to the sum of all other metabolic processes. This result again supports the conclusion that symbiotic bacteria enable this gutless clam to live by fixing inorganic carbon from the surrounding seawater.

Data on the metabolic interactions of annelid symbioses are relatively scarce. Studies with the symbiont-containing oligochaetes were hampered by the small size of the animals (150 μm thick and about 1 cm long) because they had to be collected individually. Felbeck et al. (1982) demonstrated that this animal/bacteria symbiosis was probably chemoautotrophic and sulfur oxidizing. Results of experiments using radiolabeled CO_2 showed an incorporation of label into organic acids and sugar phosphates. Also, Giere et al. (1988) found an increase in the CO_2 fixation rate of these animals when thiosulfate was added to the incubation medium, suggesting that this sulfur species may be a substrate for the bacteria.

The main problem with experiments using the two polychaete *Alvinella* species is that no method has yet been found to keep them alive after they are removed from their habitat at 2,500 m depth. Thus, experimentation has been restricted to in situ studies and observations on tissues. The characteristic enzyme of the Calvin cycle, ribulose-bisphosphate carboxylase, has been detected in extracts of the episymbiotic bacteria, and incubations with radiolabeled CO_2 showed a significant incorporation in some of the bacterial species. Aside from this indication of autotrophy, however, studies on the uptake of labeled thymidine suggested the existence of facultative heterotrophy (Alayse-Danet et al., 1987). The nature of this symbiosis is not yet clearly understood. Some morphological features of the host appear to be specializations for metabolic interactions between the symbiotic partners, but comparisons of labeling patterns in the *Alvinella* species with a closely related species without symbionts showed no differences (Alayse-Danet et al., 1987).

Physiological Studies

Physiological studies can be used to demonstrate how the intracellular symbionts obtain nutrients and how their metabolism is stimulated by the condition of the external environment. Reduced sulfur compounds and oxygen must be transported to the bacteriocytes, and the symbionts' habitat must be tightly controlled to prevent excessive growth of this bacterial "culture." The bacterial symbionts of the various marine invertebrates are "housed" in several distinctive ways, and different mechanisms are utilized to create environments suitable for the bacteria. We will, therefore, discuss the various symbioses according to the host involved.

VESTIMENTIFERA AND POGONOPHORA. The symbioses in these tubeworms are fairly similar; in both cases, the bacteria are housed in a tissue inside of the worm's body, i.e., everything has to be transported to this organ via the closed circulatory system and the blood (Jones, 1988; Southward, 1982). This fact made the blood of these animals an early subject for investigation of transport mechanisms, especially for a way to carry sulfide from the environment to the "trophosome" (Arp and Childress, 1983). A complication of this transport is that sulfide is extremely poisonous to aerobic eukaryotic organisms because of the inhibition of cytochrome *c* oxidase, the terminal enzyme of the respiratory chain and due to the conversion of hemoglobin to sulfhemoglobin, which is unable to carry oxygen. Oxygen therefore, cannot simply be carried to the tissues in solution but has to be bound or otherwise detoxified to allow its transport through and contact with the host tissues. These animals are unique in that their hemoglobin is able to bind sulfide tightly without undergoing an irreversible chemical reaction, thus preventing its release into the host tissue (Arp and Childress, 1983). This binding is reversible, thus allowing transport from the outside medium to the symbionts. In addition, the blood is able to concentrate sulfide from nanomolar concentrations in the environment to millimolar levels (sum of bound and free sulfide) within the blood. The sulfide is very stable when bound to the freely dissolved hemoglobin in the blood, in contrast to the unbound form of sulfide, which is extremely reactive and is readily oxidized by oxygen. The binding site on the hemoglobin is on the nonheme portion of the molecule. It is not yet understood how the hemoglobin is triggered to release the sulfide again in the symbiont-containing organ, but it has been shown that sulfide is released in the presence of symbiotic bacteria.

This binding and transport mechanism also has several advantages for the bacterial symbionts. Fisher et al. (1988) demonstrated an inhibition of CO_2 fixation in crude symbiont preparations by sulfide concentrations of more than 100 μM, while lower concentrations increased the fixation rate. In the presence of blood, no inhibition of the fixation was observed until the blood-binding capacity was saturated with sulfide. At higher concentrations sulfide was again highly inhibitory. These results suggest that the blood acts as a buffer for sulfide providing a relatively constant controlled level in the immediate environment of the bacteria. The pool of sulfide is high due to the total bound sulfide in the blood but the concentration of free sulfide is in a favorable range for the bacteria.

BIVALVES. Only three bivalve species have been investigated for mechanisms by which suitable environments for their bacterial symbionts are maintained, *Calyptogena magnifica, Lucinoma aequizonata,* and the gutless clam *Solemya reidi.* All three differ from each other in important ways as to how they ensure sulfide, oxygen, and carbon dioxide supplies to the bacteria.

Calyptogena magnifica is found at hydrothermal vents, usually at greater distances from the vents than *Riftia pachyptila.* Frequently, these clams can be observed wedged into cracks in the sea floor where warm water with sulfide is emitted. It has been suggested that the animals concentrate sulfide through their foot,

which they extend deep into the fissures. This behavior enables them to gain direct access to the outflowing sulfide-laden water rather than having to concentrate sulfide from the surrounding water where much lower sulfide concentrations are present. A large fraction of the body weight of the bivalves is formed by blood containing hemoglobin within erythrocytes for oxygen transport. In addition, a large nonheme serum protein is present which is able to bind sulfide and may function as its transport protein. Unlike that of *Riftia pachyptila,* the hemoglobin of *C. magnifica* is easily poisoned by sulfide resulting in the formation of sulfhemoglobin and inability to bind oxygen. Due to the presence of the sulfide-binding protein a high affinity for sulfide, the clam's blood shows no indication of sulfhemoglobin in vivo. Since the bacteria in the gill cells live in close proximity to the environment, no elaborate transport mechanisms for any other substances appear to be necessary.

The bivalve *Lucinoma aequizonata* is collected in the Santa Barbara (CA) channel at a depth close to the interface of a hypoxic basin with the overlying oxygen rich waters. The habitat is unusual because the local oxygen concentrations are very low (<20μM) and almost no sulfide, either bound as metal sulfide or free, is detectable. The bacteria, however, appear to be sulfur oxidizers because they accumulate elemental sulfur in high concentrations. In addition, the clams have no apparent mechanism to concentrate sulfide. This puzzling situation may be explained by the presence of pockets of sulfidic mud in the proximity of the animal; the pockets may be accessible through the clam's long extendable foot (Cary et al., 1989). Sulfide toxicity is avoided in this case through the action of a sulfide oxidase capable of transforming sulfide to the much less toxic form of thiosulfate after sulfide enters the body. Thiosulfate levels in the blood have been observed to be two orders of magnitude higher than the environmental sulfide concentration, suggesting that this compound rather than sulfide may be an energy source for the bacteria rather than sulfide (Cary et al., 1989).

A similar mechanism to maintain a suitable environment for the symbiotic bacteria is used by the protobranch clam *Solemya reidi,* which has only been collected so far from areas around sewage outfalls and at the outflow of a paper mill. In contrast to the previous example, however, both environments are characterized by extremely high concentrations of sulfide (several millimolar). No sulfide-binding protein has been found in the animal. In parallel to the case of *Lucinoma aequizonata,* the sulfide is oxidized to thiosulfate by a sulfide oxidase in the host tissue and then used by the symbionts. Another unusual aspect of this symbiosis is that, in addition to the action of this enzyme, intact host mitochondria are able to oxidize sulfide to thiosulfate, thus producing ATP.

Solemya reidi is the bivalve that has been best investigated for the uptake of substances from the environment. Anderson et al. (1987) have studied in detail the net flux of sulfide, CO_2, and oxygen by live *Solemya reidi* under a variety of conditions. The presence of relatively low levels of sulfide (50–100 μM) or thiosulfate (250–350 μM) stimulated the net uptake of CO_2 from the incubation water, while higher concentrations of sulfide (250–500 μM) inhibited aerobic respiration and halted net CO_2 uptake. This latter phenomenon is most likely due to a partial poisoning of the aerobic metabolism of the host caused by a shortage of oxygen for sulfide detoxification.

Symbioses with Methane-Oxidizing and Cellulolytic Bacteria

Much less is known of the two other kinds of bacterial symbiosis found to date in the deep sea. Two bivalve species (closely related to the hydrothermal vent mussel *Bathymodiolus thermophilus* that has sulfur-oxidizing bacteria) and a pogonophoran species have been shown to contain methane-oxidizing bacteria.

As with the sulfur-oxidizing types, the methane-oxidizing symbionts of the bivalve species are incorporated in the gill cells and have a similar morphology, i.e., they are surrounded by a host membrane and are located in bacteriocyte cells at the surface of the gills. Fisher et al. (1987) describe one type of bacterium seen in the seep mussel, which is spherical and approximately 0.7 to 2.0 μm in diameter. It is characterized by many stacked intracellular membranes like those found in Type I methanotrophs. Cavanaugh et al. (1987) describe two different types of bacteria in a mussel from the Florida escarpment seeps, large coccoid-shaped or rod-shaped cells about 1.6 μm in diameter containing intracellular membrane stacks, and a small coccoid or rod-shaped type without internal membranes.

That these bacteria indeed oxidize methane in situ is shown by the presence of methanol dehydrogenase in cell extracts, a characteristic enzyme of the methylotrophic pathway, and by the incorporation and consumption of labeled CH_4. Cary et al. (1988) could also demonstrate that this mussel species from the Louisiana

coast was able to grow with methane as sole carbon and energy source. In addition, the stable isotope ratios found in both of the mussels were indicative of methane as a carbon source. The methane used in situ originates from several possible sources, and these sources differ in the two sites where the mussels have been found. In the oil fields off the coast of Louisiana, the methane originates mainly from thermogenic processes ($C^{13}/C^{12} > -45‰$), i.e., generated by geological processes, and biogenic sources ($C^{13}/C^{12} < -60‰$), i.e., generated by bacterial sources. For most of the specimens tested the value of C^{13}/C^{12} varied from −40 to −58‰, showing origin from both sources, but thermogenic origin predominated (see "Stable Isotope Ratios," this chapter) (Brooks et al., 1987). In contrast, the mussel found at the Florida escarpment site where brine seeps out of the sea floor uses mostly biogenic methane generated by bacteria in the surrounding muds (Cary et al., 1989).

Since methane is nontoxic, mechanisms like that in the sulfide-oxidizing symbioses to avoid poisoning are not necessary.

One species of pogonophoran tubeworm, *Siboglinum poseidoni* from the central Skagerak close to Denmark, has been shown to have methane-oxidizing symbionts in "trophosome" cells. The Gram-negative rod-shaped bacteria are 1–1.5 μm wide and 2.0–4.0 μm long. They contain numerous membrane stacks and lipid-like storage products. Methylotrophy could be demonstrated by the uptake, metabolism, and incorporation of radiolabeled methane (Schmaljohann, 1987; Schmaljohann and Flügel, 1987).

The third type of bacterial symbiosis with invertebrates covered in this chapter is the one found in several wood-boring bivalve species (shipworms) of the family Teredinidae. Using electron microscopy, Popham and Dickson (1973) observed bacteria in a region of the gills of these species referred to as the gland of Deshayes. The bacteria are Gram-negative vibrioid rods of about 0.4–0.6 μm × 3–6 μm and typically contain phase-dark material at the apical regions. These bacteria provide the enzyme for cellulose digestion, cellulase, and also the nitrogen-fixing enzyme, nitrogenase (Waterbury et al., 1983). In addition, Trytek and Allen (1980) demonstrated, using labeled glucose, that the bacteria synthesize some amino acids that may be essential for the host. These characteristics of the symbionts enable the shipworms to survive on a pure wood diet without any additional carbon or nitrogen source (Gallager et al., 1981).

Culture and Isolation of Bacterial Endosymbionts

To date, the bacteria involved in the symbioses described above have been identified primarily only by their association with the respective eukaryotic host species in which they are found, e.g., the symbionts of the tubeworm *Riftia pachyptila* have been known only as "Riftia symbionts." However, progress is being made toward identifying and studying these symbionts as organisms in their own right.

Before a prokaryotic endosymbiont species can be adequately identified and characterized, the symbiont cells (or informational macromolecules from the symbionts) must be separated from host cells. Two approaches have been successfully applied: the first is pure culture of the symbionts in vitro and the second is characterization of the symbionts or the symbiont's nucleic acid from the host cells and cell constituents after purification by mechanical means.

Pure culture of endosymbiotic bacteria is notoriously difficult. Lack of information relating to the intracellular environment of the symbionts, the evolutionary loss of functions essential for free-living existence, and insufficient knowledge of effective selection factors are some of the obstacles that hinder pure culture of endosymbionts. The most formidable difficulty, however, is the problem of demonstrating the authenticity of the putative symbiont strains once they have been isolated. Traditionally, serial dilution and the presence of diagnostic phenotypic traits have been the best lines of evidence available. Symbionts, which are typically present in the bacteriocyte tissues at densities of about 10^8 to 10^9 cells per gram (Cavanaugh, 1985), should be expected to be isolated consistently from dilutions of 10^{-6} or greater (assuming one symbiont cell in 100–1,000 remains viable after host cell disruption). Furthermore, the same strains would not be expected to be isolated from similar dilutions made from host tissues that do not contain symbionts or from water or sediment samples from the environment surrounding the host organism. The presence of the same phenotypic determinants in both the symbiont-containing tissues and in the cells of the putative symbiont isolates has also been presented as evidence to authenticate putative symbiont isolates. These include unique morphological traits and enzymes diagnostic of specific metabolic pathways. As we will point out below, each of these types of evidence has proven individually unreliable. Therefore, both types of data in concert should be considered

the minimum supportive evidence to demonstrate the authenticity of a putative symbiont isolate. More recently, direct lines of evidence have been employed to determine whether putative symbiont strains are genuine. The most successful of these has been the comparison of molecular sequences of rRNA and, still more recently, the hybridization of specific oligonucleotide probes to rRNA. Such direct evidence, when presented in concordance with traditional data, provides the most comprehensive means of determining if a given isolate is indeed a genuine symbiont species.

A number of bacterial strains have been isolated from homogenates of host bacteriocyte tissues. These hosts include *Riftia pachyptila* (Jannasch and Nelson, 1984), *Lucinoma borealis, Myrtea spinifera, Thyasira flexuosa, T. sarsi* (Wood and Kelly, 1989), *Siboglinum poseidoni* (Schmaljohann and Flügel, 1987), and several wood-boring clams from the family Teredinidae (Waterbury et al., 1983). Of these, only the latter isolates meet the minimum criteria for authenticity previously described.

In the case of *Riftia pachyptila,* Jannasch and Nelson (1984) report isolating from trophosome tissue five bacterial strains at dilutions greater than 10^{-5}. Of these strains, two showed growth enhancement with addition of sulfide, two with addition of thiosulfate, and one (the only isolate with similar morphology to the *R. pachyptila* trophosome symbiont) proved to be a hydrogen oxidizer. However, analysis of the 5S rRNA sequences of the *R. pachyptila* trophosome symbionts and these putative symbiont isolates has demonstrated that none of these isolates are closely related to the authentic trophosome symbiont (Jannasch and Nelson, 1984). Wood and Kelly (1989) have reported isolation of seven bacterial strains from the gills of four bivalve species including: *L. borealis* (family: Lucinidae), *T. flexuosa, T. sarsi,* and *M. spinifera* (family: Thyasiridae). Of the seven isolates, only one (from *L. borealis*) was autotrophic and capable of oxidizing thiosulfate, while the remaining six were methylotrophic. All four of these host species have been shown to contain ribulose-bisphosphate carboxylase (an enzyme diagnostic of autotrophic carbon fixation via the calvin cycle) in their gills. APS reductase has also been detected in all four hosts, and ATP sulfurylase has been found in all but *L. borealis.* This combination of enzymes is strongly indicative of sulfur-based chemoautotrophy. None of these host species have been shown to contain enzymes typical of methylotrophic metabolism in their bacteriocyte tissues.

Methylotrophic symbionts described to date have contained complex arrays of stacked internal membranes. Such membranes are lacking in the symbionts of these four species. Furthermore, serial dilution data are lacking for the isolates from these hosts. No evidence has been provided that these same strains could be consistently isolated from more than a single specimen at a given dilution or that these strains were *not* isolated from symbiont-free host tissues. In fact, a number of different bacterial strains were isolated from one host species when specimens were taken from different locations. Thus, although it is likely that these bacterial strains are associated with the host species in some way, there is currently no evidence to suggest that these isolates are actually the symbionts observed in the gills of these host species. In fact, enzymatic and morphological evidence suggests that they are not. Schmaljohann and Flügel (1987) have reported the isolation of a methylotrophic isolate from the small pogonophoran, *Siboglinum poseidoni.* Again, serial dilution evidence is lacking, but the authors have demonstrated that the host bacteriocyte tissue and intact host animals are capable of incorporating CH_4 and that both the isolate and the bacteria in the host tissue have similar complex membrane stacks and lipid storage deposits. Waterbury et al. (1983) have reported isolation of a single species of cellulolytic/nitrogen-fixing bacteria from gill homogenates from six species of teredinid bivalves. This bacterium could be repeatedly isolated from dilutions in excess of 10^{-7} from a number of different specimens of each species, and it has been shown that cellulase and nitrogenase activities coexist in both the host tissue and in the putative symbiont isolates. These enzyme activities are not known to occur in animal cells and have only rarely been seen to coexist in bacteria. Analysis of 16S rRNA sequences from four species from four morphologically diverse teredinid genera showed that these isolates were identical with respect to this molecular sequence, although the host specimens were taxonomically widely divergent and were collected from the coasts of Massachusetts, Florida, California, and Australia respectively (Distel, 1990). Specific oligonucleotide probes based on the 16S rRNA sequence determined from these putative symbiont isolates were demonstrated by dot-blot assay to hybridize with bulk rRNA purified from intact shipworm gills but not with rRNA purified from three bacterial species known by 16S rRNA sequence to be closely related to the shipworm isolate. Finally, this same oligonucleotide probe was shown to hybridize in situ with the ribosomal RNA of the symbionts within thin sections of host tissue but not with other tissues of the host animal (Distel, 1990).

The teredinid symbiont isolates are the only putative symbiont isolates discussed which we consider to be authenticated.

The shipworm symbiont has been placed in the new genus and species *Teredinibacter turnerae* (Waterbury et al., in preparation), and isolates have been characterized extensively in terms of substrate utilization and growth characteristics. Optimum conditions for growth of the symbionts in pure culture are between 30–35°C, pH 8.5, and NaCl concentrations of 0.3–0.4 M. The isolates are able to grow with cellulose as the sole source of reduced carbon, and with dinitrogen as the sole nitrogen source. The symbionts are microaerophilic. Growth does not occur in vigorously shaken cultures if combined nitrogen is not provided, apparently due to the poisoning of the nitrogenase by oxygen. Growth in liquid cultures is accompanied by a decline in the pH of the medium due to the excretion of substantial quantities of organic acids, including succinate and formic acid (Green and Freer, 1986).

Teredinibacter turnerae cells are Gram-negative curved rods that are motile by virtue of a single polar unsheathed flagellum. Flagella are expressed only in the cultured symbionts and are seen whether these cells are grown in liquid or on solid media. Flagella are apparently not expressed by cells within the host tissue. Analysis of 16S rRNA sequences places *Teredinibacter turnerae* in the gamma subdivision of the proteo bacteria. *Oceanospirillum linum* is among the species most closely related to *T. turnerae* although this relationship is relatively distant (Distel, 1990).

Characterization Without Pure Culture

A variety of methods have been used to attempt to identify and to characterize endosymbionts of marine invertebrates without growing them in pure culture. A prerequisite for these methods was the ability to concentrate or purify symbiont cells or symbiont nucleic acids from homogenates of symbiont-containing host tissues.

Belkin and Nelson (1985) used differential centrifugation to achieve a "partial fractionation" of the gills of *Bathymodiolus thermophilus.* A low-speed (3,000 × g) centrifugation was performed to remove large cellular debris from gill homogenates, followed by a high-speed centrifugation (15,000 × g) to pellet the symbiont cells. This resulted in a preparation that was enriched in symbiont cells. This was determined by the relative enrichment for rapidly renaturing DNA that was higher in GC content than was the genomic DNA from host tissues containing no symbionts. Gross incorporation of ^{14}C-labeled bicarbonate was also increased three-fold in this fraction in comparison to total gill homogenate. Gross incorporation of ^{14}C-labeled bicarbonate by these "bacterial" preparations occurred at a maximum rate at temperatures between 12–15°C. On this basis, the authors suggest that the symbionts of *B. thermophilus* are psychrophilic. Incorporation of ^{14}C-labeled bicarbonate occurred only under aerobic conditions in the symbiont-enriched preparations and ceased when samples were stripped of oxygen. Rates of carbon incorporation were not sensitive to pressures up to 250 atm. Thiosulfate but not sulfide was shown to stimulate carbon fixation in gill homogenate from *B. thermophilus.* No data, however, were presented for the "purified" symbiont preparation. These "purified" symbiont preparations were not examined for physical integrity of the symbiont cells nor for contamination by host cells and cell constituents. Also, the compounds, into which labeled carbon was incorporated, were not identified. Therefore, it cannot be concluded from these results that the incorporation observed was due to a pathway capable of net carbon fixation. In *Lucinoma aequizonata,* a considerable amount of labeled carbon is incorporated by both the host and symbiont cells via equilibrium exchange through pathways that are not capable of net carbon fixation (Distel and Felbeck, 1988b). Such pathways could easily account for all of the label uptake reported here. Finally, it should be noted that the thiosulfate stimulation reported here is based on a single data point. If this single measurement were erroneous, no trend would be seen in the data.

Distel and Felbeck (1988a) purified and separated the symbiont cells from host tissue by means of centrifugation through density gradients. This method is successful for sulfur-oxidizing symbionts because these symbionts contain inclusions composed of elemental sulfur (sp. gr. 2.05–2.09), which increase their buoyant density. Symbiont cells purified by this method were shown by light and electron microscopy, as well as by enzymatic and biochemical criteria, to be intact and largely free of host cell contamination, and to remain physiologically active for several hours. This preparation was used to examine the pathway of carbon fixation in the symbiont cells from *Lucinoma aequizonata* and to determine the individual contributions of host tissue and symbiont tissue to the carbon metabolism of the intact symbiosis (Distel and Felbeck, 1988b).

The compounds into which carbon from ^{14}C-labeled bicarbonate were incorporated by pur-

ified symbionts were aspartate, 3-phosphoglycerate, malate, and citrate, in an approximate ratio of 80:15:5:2. When the symbionts were exposed to labeled bicarbonate while still within the host tissues and then were subsequently purified, they incorporated labeled carbon into the same compounds in approximately the same ratios. The appearance of labeled carbon in 3-phosphoglycerate and the analysis of the position of label incorporation in citrate confirmed that some of the observed carbon fixation occurred via ribulose-bisphosphate carboxylase. This indicates that at least a portion of the observed carbon fixation occurred via Calvin cycle reactions that are capable of net carbon fixation. However, the majority of the carbon fixation detected (carbon fixation into the 4-carbon position in aspartate) occurred via other pathways. Pulse-chase experiments suggest that some of the carbon initially incorporated into aspartate may subsequently enter the Calvin cycle in a manner analogous to that of aspartate/C-4-photosynthetic carbon fixation in plants.

Wilmot and Vetter (1990) have used purified symbiont preparations to demonstrate that the symbionts of *Riftia pachyptila* are unusual among sulfur-oxidizing bacteria in being "sulfide specialists," i.e., these bacteria are capable of metabolizing sulfide but not thiosulfate or sulfite as their sole energy source. The bacteria can utilize sulfide over a broad range of concentrations (55 μM–2 mM), but maximum stimulation of respiratory rate occurred at concentrations above 1.0 mM. The symbionts, which appeared to be microaerophilic, showed no inhibition of oxygen consumption even in relatively high concentrations of sulfide (up to 2.0 mM), which would be inhibitory to most sulfur bacteria. *R. pachyptila* symbionts can oxidize sulfide completely to sulfate, although in short incubations the majority of sulfide is oxidized only to elemental sulfur.

Still another approach to the characterization of as-yet-unculturable symbionts has been the analysis of symbiont nucleic acids. Stahl et al. (1984) characterized the symbionts of three sulfur-oxidizing symbioses by comparing the molecular sequences of the symbiont 5S ribosomal RNA with sequences of known free-living bacteria. The host and symbiont 5S rRNAs were extracted from intact symbiont-containing host tissue and then were separated by electrophoresis on polyacrylamide gels. The symbiont 5S rRNAs were then sequenced by enzymatic and chemical digestion from both termini. Symbionts from *R. pachyptila, Calyptogena magnifica* (from the Galapagos hydrothermal vents), and *Solemya velum* (from tidal flats in Bourne, MA) were examined. These symbionts proved to be members of the gamma subdivision of the proteobacteria. Two-dimensional gel electrophoresis of ^{32}P end-labeled symbiont 5S rRNAs gave evidence of only a single symbiont type in each host species.

Distel et al. (1988) purified 16S ribosomal RNAs from the symbionts of six sulfur-oxidizing symbiotic hosts and used the RNAs to characterize the symbionts phylogenetically (Distel et al., 1988). These hosts included *Riftia pachyptila, Calyptogena magnifica,* and *Bathymodiolus thermophilus* (from the Galapagos hydrothermal vent site), as well as *Lucinoma aequizonata, L. annulata* (from the coast of California), and *Codakia orbicularis* (from the Bahamas). The results of this study are in general agreement with the results of Stahl et al. (1984) based on analysis of 5S rRNA sequences. However, the 16S rRNA molecule (1,500 nucleotides) is considerably larger and has more information than the 5S rRNA (120 nucleotides). Therefore, analyses of 16S rRNAs allow finer resolution of phylogenetic relationships. Also, the scope of the investigation was broadened to include several additional symbionts, and multiple samples of each specimen were examined. This allowed comparison of symbiont populations within individual host species as well as among different host species. A summary of the results is given in Fig. 3. All of the sulfur-oxidizing symbionts examined fall into a restricted cluster within the gamma subdivision of the proteobacteria (Wilmot and Vetter, 1990). No free-living bacterial species are yet known that clearly fall within this symbiont cluster. The closest free-living relative known is *Thiomicrospira* species *L-12* (Ruby and Jannasch, 1982), a sulfur-oxidizing obligate chemolithotroph that was isolated from the Galapagos deep-sea hydrothermal vent site. There is considerable diversity among the symbionts examined. Two distinct subgroups have been identified, one containing the symbionts of the lucinid clams and of *Riftia pachyptila* and a second containing the symbionts of *Bathymodiolus thermophilus* and *Calyptogena magnifica* (see Fig. 3). Each of the sulfur-oxidizing symbiotic hosts examined to date has its own unique symbiont species. When separate specimens of the same host species were examined, they were found to contain symbionts identical with respect to 16S rRNA sequence. These results are in agreement with previous examination of the 5S rRNA sequences of *R. pachyptila, C. magnifica,* and *Solemya reidi,* where only a single symbiont sequence was detected in each host.

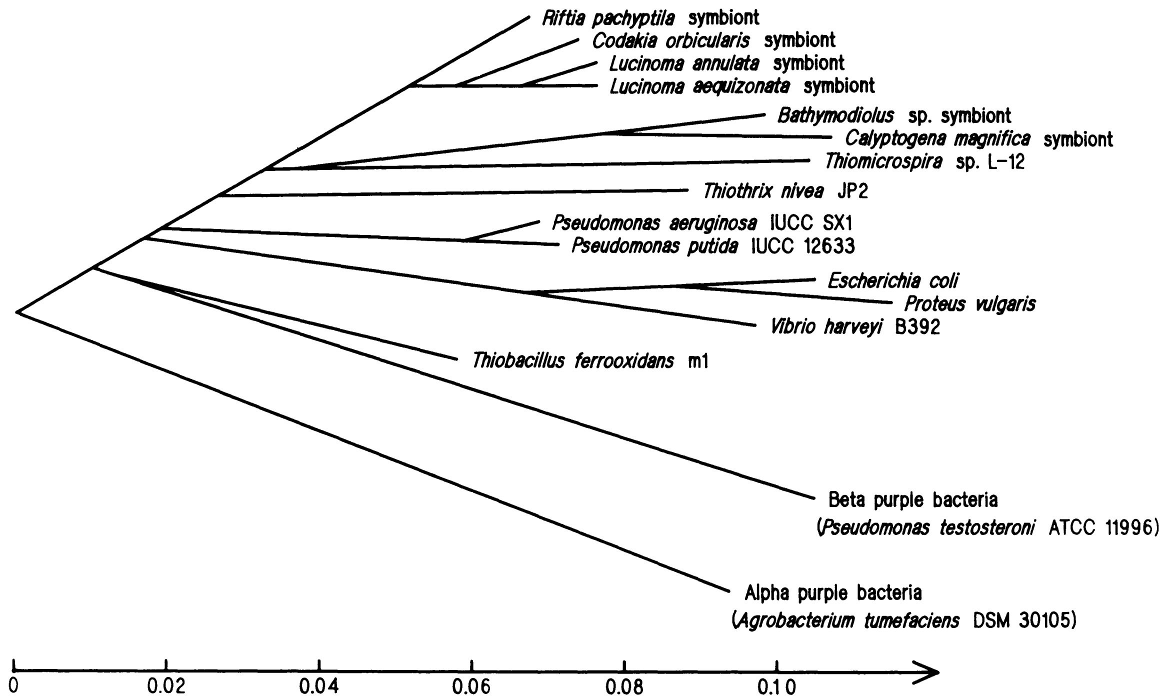


Fig. 3. The 16S rRNA-based phylogeny of invertebrate-associated symbiotic bacteria. All of the bacteria belong to the proteobacteria. This tree is a simplified version of a larger one that included additional, unpublished sequences. The horizontal components of the tree branches represent the estimated number of nucleotide substitutions per sequence position (see the scale on the horizontal axis). (From Distel et al., 1988.)

The symbionts of *R. pachyptila* have been examined by analysis of thermal denaturation kinetics (Nelson et al., 1984) and by DNA-DNA hybridization (Edwards, 1989) of total genomic DNA extracted from trophosome tissue. Thermal denaturation kinetics are consistent with a symbiont population in the trophosome of *R. pachyptila* that is homogeneous and composed of a single type of prokaryotic cell. The symbiont DNA was estimated to have a GC content of about 58 mol% and a genome size of about 2.1×10^9 Daltons (Nelson et al., 1984). Edwards (1989) used CsCl gradients to separate the genomic DNA of the symbionts of *R. pachyptila* from that of the host cells. This was possible because of the large difference in GC content between the symbionts (58 mol%) and the host (34 mol%). DNA-DNA hybridization experiments indicated that there was little variation between the symbionts of *R. pachyptila* specimens from a given site. Estimates of sequence relatedness were based on relative binding ratios (RBR) of total genomic DNA. The RBR was defined by comparison to the degree of binding of the reference DNA to itself, which was considered to be 100%. RBRs for specimens collected within each vent site were from 91–102%. These values are well within the range typically associated with a single prokaryotic species indicating that only a single symbiont species was present in all specimens. *R. pachyptila* symbiont DNA from specimens from distantly separated sites showed a degree of sequence similarity that was not significantly different from that found in specimens from within any single site. The sites examined were the Galapagos, 13°N, and Guaymas Basin hydrothermal vent fields. Each site is separated by at least several hundred miles. Thus, in agreement with rRNA-based analyses (Distel et al., 1988; Stahl et al., 1984), the symbionts of *R. pachyptila* appear to represent a single species that is specifically associated with this host regardless of local environment.

The symbionts of two other vestimentiferans, *Tevnia* species and *Lamellibrachia* species were also compared to *R. pachyptila* symbionts in this study. The *Tevnia* symbionts appear to be very closely related to the *R. pachyptila* symbionts, differing at a level (RBR = 76%) high enough to be attributed to strain differences within a single species. *Lamellibrachia* symbionts, on the other hand, had RBR values considerably lower than this (RBR = 11%) indicating that this symbiont is not closely related to those of *Ternia.*

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CHAPTER 216

Prokaryotic Symbionts of the Aphid

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All aphids have an intimate association with prokaryotic symbionts (Buchner, 1965; Houk, 1987). These symbionts are housed in specialized cells termed mycetocytes, which aggregate to form a subcellular organelle, the mycetome (Houk and Griffiths, 1980). While mycetocytes generally contain only one type of symbiont, the entire mycetomal population may consist of two or at most, three different symbionts (Houk, 1987). For example, the pea aphid (*Acyrthosiphon pisum* Harris) contains two endosymbionts designated the primary (P) symbiont and secondary (S) symbiont (Griffiths and Beck, 1973; McLean and Houk, 1973; Fig. 1). The P symbiont is the predominant organism and is located in the mycetocyte while the S symbiont, when present, is located in the sheath cells surrounding the mycetome. Within the mycetocyte, the P symbionts are found exclusively in vacuoles (Houk and Griffiths, 1980).

Evidence as to the prokaryotic nature of the endosymbiont population stems from morphological (Buchner, 1965; McLean and Houk, 1973; Griffiths and Beck, 1973), chemical (Houk and Griffiths, 1980; Houk, 1974a; Houk et al., 1977), nutritional (Dadd et. al., 1967; Dadd and Mittler, 1966), biochemical (Houk et al., 1976; Ishikawa, 1982a, 1982b, 1982c), and molecular biological studies (Houk et al., 1980; Ishikawa, 1982a, 1984a, 1987; Unterman et al., 1989).

Almost all types of aphid symbionts have two peripheral membranes. In addition, they all appear to reproduce by binary fission. The outer membrane is a fluid structure (Hinde, 1971b) capable of forming the characteristic blebs observed on the cell surface of typical Gram-negative eubacteria. Moreover, when pea aphids were fed an artificial diet supplemented with penicillin, this led to alternations in the outer envelope of both P and S symbionts, resulting in the accumulation of an electron-opaque material in the putative periplasm (the space between the two membranes) (Griffiths and Beck, 1973). Finally, peptidoglycan constituents were demonstrated both microscopically and by chemical analysis (Houk et al., 1977). In addition to the two membranes, both P and S symbionts frequently exhibit an outermost membrane, which is an extension of the host-cell endoplasmic reticulum (Griffiths and Beck, 1973).

One fascinating feature of the microbial symbiotic relationship within aphids is the transmission of the symbiont transovarially into the developing eggs/embryos from the mycetocyte (Buchner, 1965; Lanham, 1968). The precise mechanism of this transmission from generation to generation is unknown. Microscopic studies have indicated that as the embryo reaches the blastula stage the symbionts are released from mycetocytes and enter the blastula via the blastopore (Buchner, 1965). These symbionts are devoid of the host membrane during and immediately after entry. In addition, during this process both the P and S symbionts mix together and are extracellular (Hinde, 1971c). The actual infection process itself has not been visualized microscopically, suggesting that it must occur rather quickly (Hinde, 1971c).

Aphids can be grown on artificial diets that lack required nutrients such as sulfur-containing amino acids, B-type vitamins, and sterols. (Dadd et al., 1967; Dadd and Mittler, 1966; Henry, 1962; Hinde, 1971a; Krieger, 1971; Tokumitsu and Maramorosch, 1966; Turner, 1971, 1977). Since these materials are not normally produced by the aphid, the inference is that they are produced by an endosymbiont. The administration of antibiotics to aphids growing on such artificial diets generate aposymbiotic aphids which become nutritionally deficient very quickly, lose their fecundity, and eventually die off (Griffiths and Beck, 1974). Among the antibiotics that have been used are the tetracyclines (Koch, 1968); penicillin (Ehrhardt, 1968a; Ehrhardt and Schmutterer, 1966; Mittler, 1971; Srivastava and Auclair, 1976); streptomycin (Ehrhardt and Schmutterer, 1966; Ehrhardt, 1968a; Mittler, 1971; Srivastava and Auclair, 1976); chloramphenicol (Ehrhardt, 1968a; Mittler, 1971a, 1971b; Srivastava and

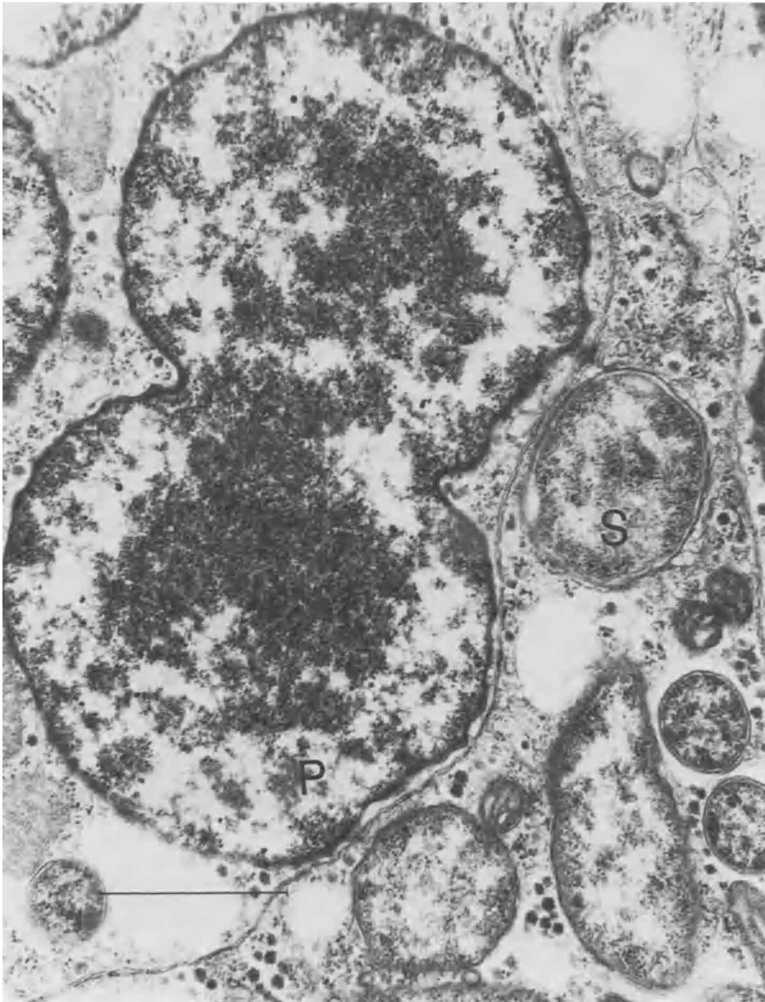

Fig. 1. Primary (P) and secondary (S) endosymbionts from the pea aphid *Acyrthosiphon pisum*. Bar = 1 μm. (Courtesy of E. J. Houk.)

Auclair, 1976); neomycin (Srivastava and Auclair, 1976); and erythromycin (Ehrhardt, 1968a; Mittler, 1971a, 1971b).

Once techniques for the isolation of endosymbionts were developed (see "Isolation," this chapter) the capacity of the endosymbionts to synthesize marcromolecules was shown to be sensitive to such antibiotics as nalidixic acid (Ishikawa, 1982a), rifampicin (Ishikawa, 1982a, 1982b), and chloramphenicol (Ishikawa, 1982a, 1982b). The RNA synthesized in the endosymbiont was shown to consist primarily of tRNA, 5S RNA, and 16S and 23S RNA (Ishikawa, 1982b). In addition, Houk et al. (1980) reported the GC content of the DNA of pea aphid endosymbionts to be as low as 31mol% and concluded that this low GC composition was a reflection of its intracellular habitat. Similar results were obtained by Ishikawa (1987) and by Unterman and Baumann (in press). However, it has recently been shown by the last authors that this GC composition is identical to the GC content of the aphid DNA itself. Thus, determination of the GC composition of isolated DNA is insufficient to establish proof of purity, and more sensitive molecular biological techniques, such as probing with suitable probes, may be required to identify the isolated DNA (see "Isolation," this chapter).

Isolation

Method of Houk and McLean (1974).

The isolation of the pea aphid symbionts was achieved by Houk and McLean (1974). Mycetocytes were isolated from homogenized adult pea aphids in a hypertonic medium and disrupted in a lysis medium (Bruening et al., 1971) by gently bubbling air through them. The medium was supplemented with 1% polyvinyl pyrrolidone (PVP-40) to prevent cross-linking of proteins due to oxidation of any polyphenolic materials. Aphids were wrapped in cheesecloth and ground in a mortar and pestle containing 3 volumes of isolation buffer per unit weight of aphids. The filtrate was collected, passed through Miracloth, and homogenized in a Teflon homogenizer. The suspension was then centrifuged at 5,000 g for 15 min.

The pellet was redissolved in isolation medium (diluted 1:1000). The suspension medium was added at a ratio of 3:2 (v/w) of the original aphid mass. The symbionts were isolated from the suspension by density gradient centrifugation using a preformed, discontinuous Ficoll gradient (2 to 10%). The gradients were formed by layering four different concentrations of 2, 4, 6, and 10% Ficoll respectively in 0.01M phosphate buffer (pH 7.2) in the bottom of 50-ml tubes (Houk and McLean, 1974). Three ml of suspension medium were used for every 2 g of aphids and layered carefully on top of the Ficoll gradients and centrifuged at 400 g for 10 min. The amount of material layered on the gradient was typically equivalent to 2 g of aphids. The region of the gradient containing the symbiont fraction was readily detected as a sharp opaque band that could be isolated by aspiration of a 3-ml aliquot with a no. 20 hypodermic needle into a sterile syringe. The primary symbiont population was found to sediment with a relative mobility (R_m) of 0.60 (calculated as the distance from the meniscus travelled by the microbial population divided by the distance from the meniscus to the bottom of the tube). By comparison, *Escherichia coli* was found to sediment at an R_m of 0.36 while *Streptococcus faecalis* was even smaller and travelled with an R_m of 0.20. The gradient fractions were sedimented by centrifugation (15,000 g) and examined by electron microscopy. Houk and McLean (1974) observed that the symbiont fractions prepared as above were contaminated to some extent (between 4.5–6.5% contamination as judged microscopically) with secondary symbionts, mitochondria, and membrane fragments. This contamination was dependent to some extent on the volume of sample removed from the Ficoll gradient. It was therefore recommended that the maximum sample size be no more than 3–4 ml from such a gradient.

Method of Ishikawa (1982a)

This is a modified version of the original symbiont isolation procedure described above. Aphids were lightly homogenized in buffer A (.035M Tris-HC1, pH 7.6; 0.25M sucrose, 0.025M KCl, 0.01 M $MgCl_2$, 1 mM dithiothreitol, 5mM phenylthiourea). The homogenate was passed through cheesecloth and centrifuged at 2,500 rpm for 25 min in a swinging bucket. The pellet was resuspended in buffer A, layered over a solution of buffer A containing 2.2 M sucrose, and centrifuged at 160,000 g for 30 min. The pellet containing the isolated mycetocytes was then homogenized in a minimal volume of buffer A containing 0.3% (v/v) Nonidet P-40 (BDH).

Aliquots of the homogenate were layered over a linear Percoll gradient (10–90%) and centrifuged for 15 min at 7,000. The Percoll mixture contained 5 g polyethylene glycol 6000 (Sigma), 1 g bovine serum albumin, 1 g Ficoll 400 (Pharmacia) and 8.6 g sucrose in 100 ml Percoll (Pharmacia). The Percoll mixture was diluted in Buffer A before the gradient was formed. In this system, the band of symbionts was readily observed in the gradient (Ishikawa, 1982a). However, cells of *E. coli* were found to move only slightly slower than the isolated symbionts in the Percoll gradient, as opposed to the results with the Ficoll gradient in which the cells of *E. coli* were much smaller and showed a considerably lower R_m (Houk and McLean, 1974). In this method, unbroken mycetocytes pelleted at the bottom of the gradient while mitochondria and membrane fragments were found scattered above the symbiont band.

Cultivation

It is commonly accepted that the prokaryotic symbionts of aphids cannot be cultivated outside the insect (Houk, 1987). However, conditions for the maintenance of isolated symbionts for periods of up to 12 hours have been described (Houk, 1974a). An optimal temperature for protein and nucleic acid synthesis was found to be 28–30°C. The only medium tested for these experiments was Grace's insect tissue culture medium (Tokumitsu and Maramorosch 1966; Houk, 1974b). Under these conditions there was no detectable dependence on supplementation with fetal calf serum. It should be noted, however, that no other systematic approaches to cultivating the symbionts in vitro on more "classical" media have been reported.

Identification

A number of aphid hosts have been examined for their symbiont populations (for references, see Houk and Griffiths, 1980). In the majority of cases the symbiont populations were found to be Gram negative (see Fig. 1).

Unterman et al. (1989) analyzed the sequence of genes in the 16S rRNAs of both primary and secondary symbionts of pea aphids. In these experiments, DNA was isolated from both the intact insect and an isolated mycetomal preparation. Although identical restriction fragments were obtained with either preparation, the DNA from the whole aphid was used since the mycetomal preparation was more readily susceptible to nuclease digestion. The identification of P (primary) symbiont DNA and S (secondary) DNA was on the basis of the relative intensities of restriction endonuclease fragments using a cloned gene for 16S RNA from *E. coli* as a probe. For example, EcoR1 digestion of aphid DNA yielded two fragments of 10 kb and 2 kb, respectively. After hybridization the 10 kb fragment was much more intense than the 2 kb band. Since the P symbiont is present in much larger numbers than the S symbiont in the pea aphid (McLean and Houk, 1973), the 10 kb fragment was assumed to code for the rDNA sequence of the P symbiont. However, the authors did not include controls using DNA from aposymbiotic aphids prepared after growth in the presence of antibiotics (Mittler, 1971b). When the sequences of the two 16S RNA genes

were compared with the sequences of selected prokaryotes and analyzed for evolutionary relatedness (Ristel et al., 1988; Olsen et al., 1986; Woese, 1987) with selected eubacteria, Unterman et al. (1989) were able to place both the primary and the secondary symbionts within the γ-subdivision of the proteobacteria (Stackebrandt et al., 1988; Woese, 1987). The analysis showed the S symbiont to be a member of the Enterobacteriaceae. The P symbiont, which is closely related to the Enterobacteriaceae apparently diverged from *E. coli* about 420 million years ago, while the S symbiont diverged from same organism some 250 million years ago. It is interesting to note that both the P and the S symbionts are clearly distinct from the obligate intracellular parasites, the chlamydiae, and the mycoplasmas. The chlamydia and mycoplasmas belong to the Gram-positive branch of the eubacteria. Unterman et al., (1989) speculate that the initial divergence of the P and S symbionts may have been the result of adaptation of the P symbiont to the intracellular environment followed by a later infection of the of the aphid host by the S symbiont. One interesting observation from this work is that the pea aphid symbionts contain only a single copy of the rRNA operon (Unterman et al., 1989). Similar results (Unterman and Baumann, in press) were obtained with the symbionts from the peach aphid *Myzus persicae* and the sorghum aphid *Schizaphis graminum* as well as with one member of the family Coccoidae *(Peudococcus citri)*. It is interesting to note the comparison with *E. coli* which has seven rRNA operons (Boros et al., 1979), and *B. subtilis* which appears to have ten (Rudner et al., 1988). Since the presence of a single copy of rRNA operons has been correlated with a very slow growth rate (Neumann et al., 1983; Razin, 1985), it is not surprising that attempts at in vitro cultivation have failed. If the growth of the endosymbiont is correlated with the growth of the host, the expected doubling time ould be about two days (Srivastava et al., 1980).

Biochemical Activities

Two general approaches have been used to study metabolic processes by aphid symbionts. In the first approach, the ability of the whole aphid to incorporate a radioactive precursor into product subsequently detected by autoradiography is compared with the activity in aposymbiotic aphids prepared by treatment with antibiotics. This approach has been used to study such processes as: 1) incorporation of sulfur into amino acids (Ehrhardt, 1968a, 1968b.); 2) the synthesis and incorporation of lipids Houk, 1974b); and 3) the incorporation of [^{14}C] acetate into sterols (Ehrhardt, 1968b) and other classes of lipids. In sharp contrast, it was shown (Campbell and Nes, 1983) that aphids of *Schizaphis graminum* could not incorporate [2-^{14}C] mevalonic acid into either sterols or into sterol intermediates, such as squalene and 2,3-oxidosqualene, when reared on an artificial diet. It is possible that the family Aphididae could be divided into two groups, one that is capable of de novo synthesis of sterols via their endosymbionts, and one that is not.

The second approach involves similar types of labelling experiments except that isolated symbionts are used. Houk et al. (1976) showed that isolated symbionts of the pea aphid incorporated [^{14}C] acetate into most of the common classes of lipids. The same radioactive precursor, as well as [^{3}H] mevalonate, were also incorporated into sterols, with cholesterol being the major product.

DNA, RNA, and protein synthesis have been extensively studied in isolated symbionts of pea aphids (Ishikawa, 1982a, 1982b, 1984a, 1984b 1985, 1987) using [methyl-^{3}H]thymidine, [5-^{3}H]uridine, or L-[^{35}S]methionine respectively. DNA synthesis was inhibited by nalidixic acid, RNA synthesis by rifampicin, and protein synthesis by both rifampicin and chloramphenicol. This analysis led to results indicating that the proteins synthesized by the isolated symbionts in vitro are apparently quite different from those associated with the symbionts inside the insect. In fact, it has been asserted that the synthesis of only a single symbiotic protein species, symbionin (MW 63,000), is sensitive to chloramphenicol in the intact insect (Ishikawa et al., 1985). This protein was not synthesized by the isolated symbionts in vitro, suggesting that either the gene or the messenger RNA encoding this protein do not reside in the symbiont itself, but are imported! In support of this conclusion was the finding that symbionin (identified by two-dimensional gel electrophoresis) was the only protein isolated from the whole insect whose synthesis was apparently resistant to cycloheximide and sensitive to chloramphenicol (Ishikawa, 1982b). Evidence has been presented suggesting that symbionin is present in much higher amounts in the symbionts from nymphal tissue than in those of adult aphids (Ishikawa et al., 1985). While no direct evidence for enhanced turnover was presented, a number of low molecular-weight molecules have been isolated that actually inhibit protein synthesis in symbionts (Ishikawa, 1984). One intriguing possibility is that the aphids produce some materials during their development which turn off

macromolecule synthesis as the insects age. The hypothesis was advanced that symbionin was required for embryonic development following fertilization. The interpretation of the results are complicated by the possibility of contamination with bacteria, mitochondria, or membrane fragments during the isolation of the symbionts. Controls using aposymbiotic hosts and specific immunological probes would be useful in linking a particular biochemical reaction to a specific isolate.

The Basis of Symbiosis

The function of the symbionts in aphid survival has remained a subject of some controversy. The various hypotheses can be grouped as follows: biosynthesis—for sulfur-containing amino acids, tryptophan, vitamins, and sterols, and other lipids (Buchner, 1965; Houk and Griffiths, 1980); energy production (Lanham, 1968); osmoregulation (Houk and Griffiths, 1980); and the production of polysaccharide-degrading enzymes (Campbell and Dreyer, 1985; Dreyer and Campbell, 1987). Evidence for nutritional and biosynthetic activities stems from the ability of different aphid populations to survive for many generations on artificial diets in the absence of specific nutrients that are not normally synthesized by insects. In the presence of antibiotics, such diets are inadequate and the aphids lose their fecundity and subsequently die off. Sterol synthesis has received the most attention, since as a group, insects do not synthesize these compounds de novo. Both biochemical (Houk et al., 1976) and morphological evidence supporting the notion that the symbionts provide the host with endogenous sterols (Griffiths and Beck, 1977a, 1977b) have been presented. Moreover, the fact that the aphids can be reared on an artificial diet without sterol supplementation provides additional support (Akey and Beck, 1972; Dadd and Mittler, 1966). However, the possibility of fungal contamination during feeding experiments cannot be ruled out as a source of sterols in the diet. In addition, Campbell and Nes (1983) pointed out that the specific sterols or other essential nutrients provided by the symbionts may be specific for only one class of aphids but not for all classes.

Recently, the hypothesis was advanced that the prokaryotic symbionts furnish the aphid host with polysaccharide-degrading enzymes such as pectinases, cellulase, and hemicellulases (Campbell and Dreyer, 1985). Such enzymes could be used by the aphids for plant tissue penetration and phloem feeding. In this regard, it has been shown that the more susceptible a particular sorghum variety is to the aphid *Schizaphis graminum* (the greenbug), the greater the rate of pectin hydrolysis. Moreover, aphid biotypes that can overcome resistant species of sorghum appear to have a different array of polysaccharidases from aphids that cannot overcome resistant species. Substantial activities for these enzymes were found in aphid homogenates (Campbell and Dreyer, 1985), even though such enzymes are generally only associated with microorganisms, either bacteria or plant-pathogenic fungi (Bateman and Basham, 1976). Different regulatory patterns for enzyme synthesis coupled with associated mutations could explain the ability of the aphid to overcome the resistance to aphid infection of specific plant varieties. Thus far, no direct evidence has been forthcoming suggesting that symbionts in fact either produce these enzymes or contribute to their production in the insect host. However, it has recently been shown (Eisenbach and Mittler, 1987), by crossing resistant strains of greenbugs *(Schizaphis graminum)* with sensitive strains, that the ability to overcome the resistance of a particular sorghum biotype was maternally inherited. This non-Mendelian pattern of inheritance may result from symbiotic transfer and subsequent expression of the resistance characteristics by the prokaryote (Eisenbach and Mittler, 1987). Ideally, one should be able to probe symbiotic DNA with genetic probes for the degradative enzymes. The use of specific monoclonal antibodies for this purpose should also prove useful.

Applications

Aphids are among the most important agricultural pests in modern agriculture (Minks and Harreweijn, 1987). This stems not only from the damage they cause through sap-sucking, but also as a result of their ability to serve as vectors in transmitting viral diseases (Raccah, 1986). A better understanding of the basis of aphid symbiosis should lead to new experimental models for the manipulation of the symbiont population, which in turn may lead to a unique approach to the biological control of these important agricultural pests.

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PART VI

Phylogenetically Unaffiliated Bacteria

CHAPTER 217

Thermophilic, Aerobic, Hydrogen-Oxidizing (Knallgas) Bacteria

MICHEL ARAGNO

Introduction

Geothermal fluids and gases often contain significant concentrations of molecular hydrogen ($\geqslant 1$ % v/v). This suggests that hot springs and other geothermal manifestations might harbor thermophilic, aerobic, hydrogen-oxidizing (Knallgas) bacteria. It is therefore surprising how few attempts were made before 1980 to isolate thermophilic hydrogen bacteria from these environments (Goto et al., 1977), although they were found in cold nongeothermal habitats (McGee et al., 1967; Emnova and Romanova, 1977; Aragno, 1978; Schenk and Aragno, 1979).

The discovery of strains of the highly thermophilic, obligately chemolithoautotrophic *Hydrogenobacter* (Kawasumi et al., 1984; Kryukov et al., 1983), of *Bacillus tusciae* (Bonjour and Aragno, 1984), and of the geothermal habitat of *B. schlegelii* (Bonjour et al., 1988) recently confirmed that geothermal sites were the source of several new, highly thermophilic, hydrogen-oxidizing aerobes, and that these could play a role in the primary production of organic matter in such environments. Moreover, these organisms, which often have a high growth rate and produce large numbers of cells, certainly present an interesting potential for biotechnological applications.

Ecology

Origin of Geothermal Manifestations

The total heat flux of internal origin on earth amounts to about 3×10^{10} kW, corresponding to about 0.2% of the heat flux reaching the earth as solar energy. The internal heat probably is composed partly of a residue of the initial energy (due to the impact and the early decomposition of short-lived radioactive elements), and partly from the decomposition of still-present long-lived radioactive elements.

In normal conditions, this flux generates a temperature gradient under the surface (starting 10 m below ground level, where solar energy becomes insignificant), of about 3°C/100 m. Anomalies in this gradient generally occur near the limit of lithospheric plates, where fluid magmatic masses come close to the surface. In regions where the Earth crust is fractured by active tectonism, convective circulation of warm fluids is more probable and thus may generate hot springs or fumarolic fields. Water, the main fluid present in this case, in liquid or gaseous form, is almost exclusively of superficial origin: the deuterium/hydrogen (D/H) ratio of geothermal waters is identical to that of cold interstitial waters of the same region (Panichi and Gonfiantini, 1978); the increased $^{18}O/^{16}O$ ratio of these waters is due to isotopic exchange between waters and rocks in the deep reservoir (Panichi et al., 1974; Panichi and Gonfiantini, 1978).

Consequently, hot springs and other geothermal manifestations are present mainly in volcanic zones, even where there has been no volcanic activity for a long time. Rain water penetrating geothermal fields is heated by contact with hot rocks. Ions and gases dissolved in geothermal fluids occur mainly by the reaction, at high temperature (>800°C), of water and hot rocks or magma. During their climb along fissures, geothermal fluids lose heat by adiabatic expansion and conduction of the neighboring rocks. Finally these fluids may reach the surface, giving rise to hot springs, solfataras, or fumaroles.

Submarine hot springs are perhaps more abundant on earth than terrestrial ones. Some are situated in shallow waters near volcanic islands like Vulcano (Eolian Islands, Sicily), Santorini (Aegean arc, Greece), and S. Miguel (Açores) where the mixing of sea water with hydrothermal fluid probably occurs. A borderline case would be seaside hot springs, such as in Porto di Levante (Vulcano Island, Sicily) or the Izu Peninsula (Japan).

Other hydrothermal vents are situated in the mid-oceanic ridges, where continental plates separate, at a depth of 2,000 to 3,000 m (Jan-

nasch and Mottl, 1985). Two main types are recognized: the so-called "black smokers", or hot vents, where pure hydrothermal fluid reaches the crust surface at 350 ± 2°C at a flow rate of about 1 to 2 $m \cdot sec^{-1}$; and "warm vents," which typically open through submarine basaltic pillow-lavas, at a temperature of 2 to 20°C over ambient and a flow rate of 0.5 to 2 $cm \cdot sec^{-1}$; in these, the hydrothermal fluid mixes with normal seawater at a depth of 10 to 200 m.

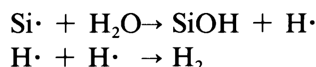
The neighborhood of deep-sea vents is known for harboring a rich animal community, mainly worms, mussels, and crustaceans, as well as a rich bacterial biomass. Some of these chemolithoautotrophic prokaryotes are the primary producers of the whole nutrient chain in these ecosystems. H_2S appears to be the main primary source of energy and electrons, but H_2 and other reduced compounds might also play a significant role (Jannasch and Mottl, 1985). Although hydrogen is present in fairly high concentrations in the hot vents, its concentration in the low-temperature, warm vents appears to be very low.

Very little is known so far about the mechanisms of aerobic oxidation of hydrogen in marine springs. Jannasch and Nelson (1984) have reported the isolation of an aerobic, hydrogen-oxidizing bacterium from a *Riftia* trophosome, whereas Nishihara et al. (1990) have isolated an organism related to *Hydrogenobacter* from a seaside saline hot spring in Japan. Further research on the occurrence of hydrogen-oxidizing bacteria in marine hydrothermal vents is therefore necessary and seems promising.

Origin of Molecular Hydrogen and of CO_2 in Geothermal Manifestations

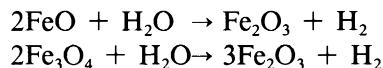
Molecular hydrogen occurs frequently in the gas phase of geothermal manifestations. It can reach concentrations of the order of several percent v/v of the total dry gas, for example 4.6–4.8% in S. Federigo solfatara, in Tuscany, Italy (Bonjour and Aragno, 1984; Conrad et al., 1985).

Several mechanisms have been suggested to explain the occurrence of H_2 in geothermal gases. The following reaction may be possible at high temperature between water and silicon (Si) radicals (Kita et al., 1982):



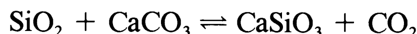
$$Si\cdot + H_2O \rightarrow SiOH + H\cdot$$
$$H\cdot + H\cdot \rightarrow H_2$$

The following reactions for the reduction of water by hot (>800°C) ferrous rocks (d'Amore and Nuti, 1977) have also been proposed:



$$2FeO + H_2O \rightarrow Fe_2O_3 + H_2$$
$$2Fe_3O_4 + H_2O \rightarrow 3Fe_2O_3 + H_2$$

CO_2 is generally the most abundant gas in the uncondensable fraction of geothermal gas. In S. Federigo (Bonjour and Aragno, 1984; Conrad et al., 1985), it makes up 80–90% (v/v) of the total. It can originate from metamorphic reactions between silicic and carbonate rocks, the simplest equation being:



$$SiO_2 + CaCO_3 \rightleftharpoons CaSiO_3 + CO_2$$

A small part of the CO_2 could also originate from the atmospheric CO_2 and from that produced by the decomposition of organic matter that is transported by water (Gunter and Musgrave, 1966).

The gases directly sampled from melted magma at 1,050 to 1,150°C were CO_2, H_2, CH_4, NH_3, SO_2, and some H_2S.

Ecological Factors Governing Bacterial Growth in Terrestrial Geothermal Habitats

The ecology and evolution of bacteria of superficial geothermal habitats are governed by several factors unique to these environments. The most important are listed below:

1. The very small area of these habitats and the often-considerable distances between one geothermal region and another (≈ thousands of km).
2. The temperature varies between the ambient temperature and the boiling point of water.
3. The pH, even in a given geothermal region, may vary considerably from one manifestation to another. Values from pH 1.5 to 10.5 have been reported (Brock, 1978; Aragno, 1981)
4. Potential electron donors present in the geothermal gases often include significant concentrations of H_2, H_2S, S, NH_3, or CH_4 (Tables 1 and 2), all of which may serve as electron donors for chemolithotrophs.
5. Presence of CO_2 in the geothermal gases.
6. Oxygen is limiting, since most of the oxygen reaching these waters simply diffuses from the air. The oxygen concentration in geothermal waters is generally very low; not only does oxygen solubility decrease with increasing temperature, but the waters are contin-

Table 1. Composition of the uncondensable fraction of endogenous gases in S. Federigo solfatara.

Gas	Percentage (v/v)
H_2	3.5–5
N_2	5.5–14
CH_4	0.7–1.8
CO_2	79–88
H_2S	0.2–4.3

From Bonjour (1988).

uously bubbling with oxygen-free geothermal gases, and there is a high concentration of compounds with a high reducing power, such as H_2S.

7. Scarcity of organic matter is common. With the exception of airborne allochthonous material (insect and plant fragments), the only organic matter in geothermal environments above 70°C is that produced by chemolithoautotrophs, as photosynthesis does not occur above that limit.
8. Mineral salts are generally present in geothermal waters at relatively high concentrations, often up to 2,000 mg/l of total dissolved solutes or more. The composition of the principal salts varies considerably from one site to another. Table 2 gives examples of the mineral composition of waters from six geothermal sites. Among the compounds present, some can have exceptionally high local concentrations, such as boric acid in the geothermal fields of Tuscany.

It should be noted that in some hot pools or hot springs, the above physicochemical characteristics may vary strongly and suddenly for several reasons. Some variations are caused by the influence of surface rain waters. Other may be ascribed to changes in the underground channels due to clogging by salt deposits or to microseisms, frequent in these regions.

Consumption of Hydrogen in Geothermal Waters

Hydrogen consumption in natural environments is a widespread phenomenon (Conrad, 1988). In fresh water and in soils, nevertheless, H_2 uptake cannot be explained by the activity of the "classical" aerobic, hydrogen-oxidizing bacteria, because their apparent K_m for H_2 is much higher than that measured in water and soil samples (Conrad and Seiler, 1979, 1981; Conrad et al., 1983a), and because they are unable to utilize H_2 at the concentrations usually encountered in these environments (Conrad et al., 1983b).

The situation is different in geothermal manifestations. The first-order kinetics reported for H_2 consumption in two geothermal samples from the S. Federigo solfatara in Tuscany (Conrad et al., 1985) showed an apparent K_m greater than 1 μM H_2, which is of the same order as the kinetics of H_2 consumption by aerobic, sulfidogenic, acetogenic, or methanogenic hydrogen-oxidizing bacteria.

Whether aerobic or anaerobic bacteria are involved in H_2 consumption seems to depend on the temperature of the sampling site. Even though consumption occurred under both conditions in all the samples tested, Conrad et al. (1985) showed that anaerobic H_2 consumption was much higher in the sample taken at 95°C,

Table 2. Concentrations of chemicals in waters from six superficial geothermal sites.

Chemical	Concentration (mg/l)[a]					
	1	2	3	4	5	6
NO_3^-	3.25	38	32	0.3	0.0	0.0
NO_2^-	0.0	0.0	0.0	0.03	0.0	0.0
NH_4^+	29.2	334.4	1052	0.1	3.1	5.4
SO_4^{2-}	315.6	2400	8500	178	602	501
S^{2-}	20	0	0	2.35	32.94	2.45
PO_4^{3-} (total)	10.42	0.306	Tr	0.14	6.1	0.8
Ca^{2+}	126	533	533	2	64	272
Mg^{2+}	13	5.9	127.7	0.5	31	68
Na^+	30.9	5.36	82.9	156	22	129
K^+	9.89	4.87	86.6	15	7.1	69
Fe (total)	0.45	3.12	585	0.21	28	0.06
H_3BO_3	2014	61	3170	3.88	325.47	24.55
As	2.75	0.092	ND	0.5	1.9	0.5
Cl^-	34	9	70	63	169	170
Mn^{2+}	0.08	0.8	13.5	0.0	0.3	0.03
SiO_2	151.9	146.8	317.5	609	147	60
HCO_3^- (titratable)	64.0	15.2	ND	112	0	667
pH	6.7	6.0	2.2	8.7	1.5	6.6

[a]The numbers indicate the site of the water. Nos. 1–3: Tuscany, S. Federigo solfatara. *Hydrogenobacter* spp. were isolated from sites 1 and 2, *Bacillus tusciae* from site 3. The data for nos. 1–3 from Bonjour (1988). Nos. 4–6: geothermal waters from Iceland, Japan, and Yellowstone (USA), respectively. The data for nos. 4–6 are from Castenholz (1969).
Tr, trace; ND, not determined.

Table 3. Temperature, pH, and rate constants of H_2 consumption activities in geothermal spring water incubated at 60°C under aerobic and anaerobic conditions.

Site	Original water temperature	pH	Rate constant of H_2 consumption (h^{-1})	
			Aerobic	Anaerobic
Number 1	95°C	6.2	1.9	11.5
Number 2	79°C	3.2	9.7	1.7

From Conrad et al. (1985).

while aerobic consumption dominated in the sample taken at 79°C (Table 3). This fits well with the decrease of oxygen solubility with temperature. Most environments above 90°C are almost totally anaerobic; they are indeed the habitat of the strictly anaerobic, sulfur-reducing, and methanogenic, hyperthermophilic archaebacteria (see Chapters 28 and 33)

The above considerations suggest the following picture: geothermal habitats are likely to harbor aerobic, hydrogen-oxidizing bacteria, provided molecular hydrogen is present in the geothermal gases at a significant concentration. The most severe limitation is oxygen concentration, which in turn depends on the temperature. The ecological extreme for aerobes is about 85 to 90°C. This agrees with the upper limits for growth of the most extreme thermophilic aerobes, such as *Sulfolobus* (Brock, 1978) and *Hydrogenobacter* spp. (see below).

Habitats of Thermophilic, Hydrogen-Oxidizing Bacteria

These bacteria have been found in many different habitats, both geothermal and nongeothermal.

GEOTHERMAL HABITATS. These include water from hot and warm springs (≥50°C), sediments from hot and warm pools and creeks, and moist fumarolic soils (≤100°C). Temperature and pH appear to be the major ecological factors determining the presence or absence and the type of knallgas bacteria in a given spring. Fig. 1 shows the distribution of different types of thermophilic knallgas bacteria from Tuscany (Italy) and S. Miguel (Azores) in a pH versus temperature plot. *Hydrogenobacter* spp. and *Bacillus schlegelii* were found in neutral to slightly acidic sources (pH ≥ 5.0). Both genospecies from Tuscany are distinctly adapted to different temperature ranges. *Bacillus tusciae* was isolated from moderately acidic ponds (pH 2.5–5.5) at temperatures below 60°C. Schink et al. (1983) reported the isolation of strongly acidophilic knallgas bacteria from Lemonade Pool (Yellowstone) at pH 2.5 and 40°C. The isolate grew well at pH 2.5, but not at pH 5.5. Unfortunately, this strain (Gram-negative, nonsporeforming rod) was then lost. Obviously, more attempts to isolate strongly acidophilic knallgas bacteria are necessary. No acidophilic knallgas bacteria were isolated from muds that were both hot and acidic (T>60°C, pH<4, see Fig. 1).

NONGEOTHERMAL HABITATS. Surprisingly, several strains of thermophilic knallgas bacteria have been isolated from cold, nongeothermal habitats. This is the case for the moderately thermophilic *"Pseudomonas thermophila,"* isolated from soil (McGee et al., 1967) and from drainage (Emnova and Zavarzin, 1977), and for *Bacillus schlegelii* (Aragno, 1978; Schenk and Aragno, 1979; Bonjour et al., 1988). Although *B. schlegelii* is a strict thermophile, with a minimum temperature of approximately 45°C and an optimum at 70°C, it was originally isolated from a freshwater sediment where the maximum temperature in summer was about 8°C. Relatively high most-probable-number (MPN) counts (10–100 per g dry sediment) were obtained from various lakes and canals. The organism was later also isolated from glacier ice (Bonjour et al., 1988) and from the air in Switzerland. This clearly indicates the allochthonous origin of this sporeformer in low-temperature environments, as well as its relative abundance as air-borne endospores. Thus, if moderately thermophilic, nonsporeforming knallgas bacteria are likely to be found in nongeothermal environments, the presence of strict thermophiles is clearly associated with the requirement for the possession of endospores allowing transport by air. In contrast to *B. schlegelii, Hydrogenobacter spp.* and *Bacillus tusciae* have never been isolated from nongeothermal habitats.

Warm, nongeothermal habitats, such as compost piles or waste masses during the transient phase between aerobic and anaerobic decomposition, are likely to harbor thermophilic knallgas bacteria because significant concentrations of hydrogen are produced via fermentation reactions (Dugnani et al., 1986). So far, few attempts have been made to isolate thermophilic knallgas bacteria from these environments, and in cases where a positive enrichment culture was obtained, only *B. schlegelii* was isolated (M. Marchiani, T. Beffa, and M. Aragno, unpublished observations).

Sampling, Enrichment, Isolation, and Cultural Methods

General methods for isolating and cultivating aerobic, hydrogen-oxidizing bacteria, as well as the indispensable precautions that are essential

for handling explosive gas mixtures are described in Chapter 15. The following considerations are specific to the thermophilic hydrogen-oxidizing bacteria.

Sampling

The minimal equipment for sampling in geothermal areas for knallgas bacteria consists of sterile tubes or flasks that close hermetically, a holder for the sampling tube or bottle, an electrical thermometer (or thermistor) with a range of at least 0–100°C, and a portable pH meter. No special precautions are required for conditioning the samples, which are not harmed by exposure to air. If the samples cannot be tested the same day, it is preferable to keep them refrigerated.

Gas Analysis

Semi-quantitative determinations of the H_2 content of the geothermal gases can be made using Draeger tubes for 0.5% H_2 (Draegerwerk AG, Lübeck, FRG). This allows direct field detection and estimation of concentrations above 0.5% H_2 v/v. Gases bubbling through small pools are best suited for such analyses. The procedure is as follows:

Field Measurement of H_2 Concentration

A funnel (minimum volume, 600 ml) fitted with a tube of sufficient length is dipped into the pool water beside the bubbling zone. The tube should be made of natural rubber, polyvinyl chloride (PVC), or neoprene, but not of silicone, which is highly permeable to gases. 25% of the funnel volume should be filled with air: the reaction in the tube is the oxyhydrogen reaction, and the gas analyzed must contain at least 5% O_2. Then, the funnel must be transferred to the bubbling zone and filled with gas. The gas will then be pumped through the tube. The length of the pink zone indicates the H_2 concentration. *Follow the manufacturer's instructions exactly.*

Gas for laboratory analysis may be sampled using the procedure described below, which is diagrammed in Fig. 2.

Gas Collection Procedure

A 100-ml serum bottle is filled with CO_2-saturated water, and 1–2 ml of the carrier gas to be utilized for the gas chromatographic (GC) analysis is added to avoid an explosion due to expansion. The tube from the funnel is first completely purged with bubbling gas. It is then connected to the serum bottle through a syringe needle while another needle connects the bottle to a syringe with two valves. While the bottle neck is held down, the syringe is pumped slowly until the gas has almost completely replaced the water in the bottle, after which the needles are withdrawn.

Enrichment Procedures

The general procedures described in Chapter 15 are applicable, with the following modifications and precautions.

ENRICHMENT MEDIA. Schlegel's basal mineral medium (Chapter 15) was used with success for enrichment of thermophilic knallgas bacteria from geothermal sources. Used without modification, it permitted the isolation of several genospecies of *Hydrogenobacter* and *Bacillus schlegelii.* As its phosphate content is high compared to most natural habitats, a medium of similar composition, but with only 10 mM phosphate, can also be used.

Other basal mineral media were used by Goto et al. (1977) Kawasumi et al. (1980), Kristjansson et al. (1985), and Kryukov et al. (1983) for enrichment of several types of thermophilic, neutrophilic knallgas bacteria.

Enrichment of *Bacillus tusciae* (Bonjour and Aragno, 1984) was achieved on Schlegel's basal mineral medium modified as follows: phosphate concentration was lowered to 10 mM, and HC1 was added to lower the pH to about 3.5. Note that after good growth occurred, the pH increased to 5.5–6.0.

For enrichment of halophilic *Hydrogenobacter* (Nishihara et al., 1990), the same basal medium can be used as for mesophilic, halophilic, hydrogen bacteria (Nishihara et al., 1989; see also chapter 14)

GASSING AND INCUBATION CONDITIONS. The composition of the gas phase used is different depending on the author. In general, thermophilic, hydrogen-oxidizing bacteria are microaerophilic. In our experience, the best enrichments are obtained using shallow, unshaken cultures (e.g., 10 ml in 50-ml bottles, or 20 ml in 100-ml bottles) placed in desiccators or Witt's vessels, filled with 5 KPa O_2, 10 KPa CO_2, and 30–40 KPa H_2 (partial pressures measured at normal laboratory temperature). This underpressure of 45–55 KPa is necessary to avoid overpressure when the vessel is warmed to an incubation temperature over 60°C.

Other authors (Goto et al., 1977; Nishihara et al., 1990) used bottles gassed with a H_2:O_2:CO_2 mixture in volumetric proportions of 7:1:1 and placed on a shaker at the desired temperature.

Incubation temperature depends on the type of organism to be isolated. *Hydrogenobacter* spp. and *Bacillus schlegelii* grow well at 65°C. However, the latter tends to predominate at this

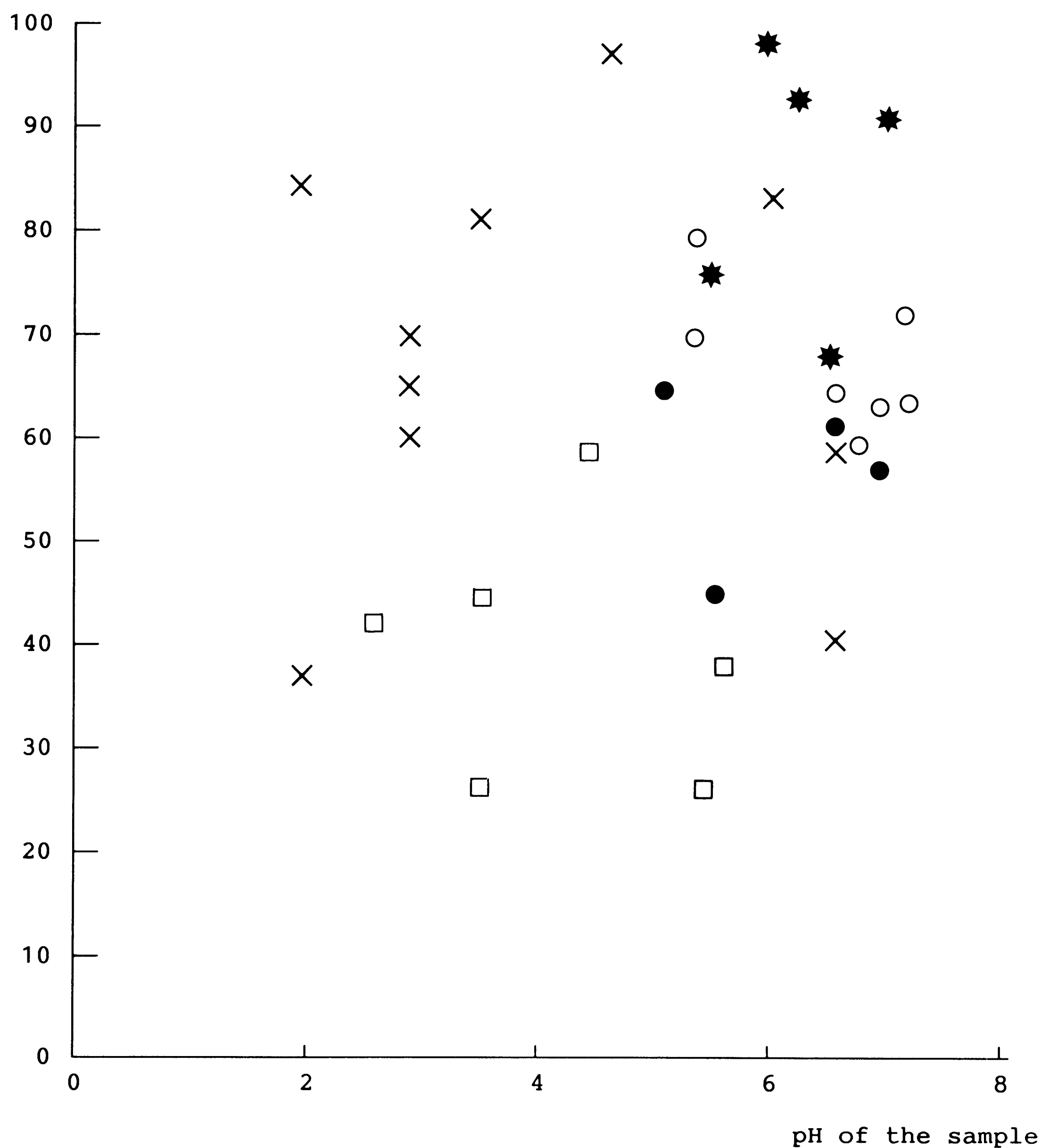


Fig. 1. Distribution of different types of thermophilic, hydrogen-oxidizing bacteria from Tuscany (Italy) and S. Miguel (Açores) in a pH versus temperature plot. Each point represents a single sample. ✸ *Hydrogenobacter*, homology group 1; ● *Hydrogenobacter*, homology group 2; ○ *Bacillus schlegelii;* □ *B. tusciae;* × no bacteria found.

temperature but incubation at 73–75°C should favor the specific enrichment of *Hydrogenobacter* spp. Incubation at 80°C will permit enrichment specifically of the most thermophilic *Hydrogenobacter* spp. (e.g., genospecies 1, see below). In that case, it is preferable to preincubate the culture overnight at 75°C before increasing the temperature to 80°C, as this temperature is over-optimal for this genospecies as well. Halophilic *Hydrogenobacter* were obtained after enrichment at 70°C (Nishihara et al., 1990).

Normally, it is preferable to repeat the enrichment procedure once or twice, each time using one loopful of the culture as inoculum. This increases the proportion of knallgas bacteria and prevents nonautotrophic growth at the expense of compounds present in the original inoculum. Cultures showing a good growth after the third enrichment (a control can also be run,

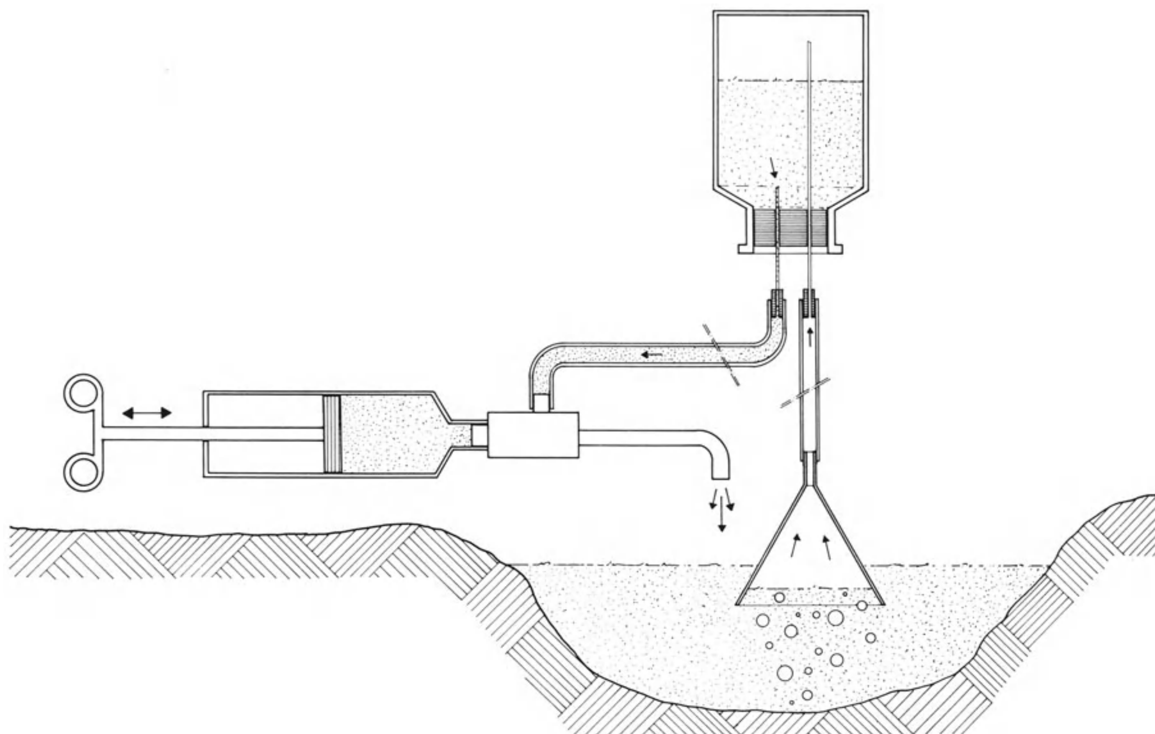


Fig. 2. Sampling system for collecting underground gases from geothermal sources.

incubated under air or N_2:O_2:CO_2 mixture) can then be subjected to the isolation procedure.

Isolation Procedures

When isolating thermophilic knallgas bacteria, it is often difficult to obtain well-separated single colonies on solidified media. One often observes a spreading of the cultures on the entire medium surface, or no growth at all. This generally happened when *Hydrogenobacter* spp. has been cultivated autotrophically with H_2 as the sole electron and energy source.

Increasing the agar concentration and air drying the surface of the plate prior to incubation often helps the formation of single colonies. Several other methods have also been proposed: Alfredsson et al. (1986) found that Icelandic *Hydrogenobacter* strains grew well on an agar medium supplemented with thiosulfate (3.92 g/l $Na_2S_2O_3 \cdot 5H_2O$) under air plus 10KPa CO_2, and even better under air with 10KPa H_2 and 10 KPa CO_2, but not with H_2 as the only electron and energy source.

Ishii et al. (1987b) achieved colony formation by *Hydrogenobacter thermophilus* on media solidified with Gelrite, an agar-like bacterial polysaccharide (Merck & Co, Darmstadt, FRG). The composition of the culture medium was as follows:

Isolation Medium for *H. thermophilus*

Component	Amount
$(NH_4)_2SO_4$	3 g
KH_2PO_4	1 g
K_2HPO_4	2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.25 g
$CaCl_2$	0.03 g
$FeSO_4 \cdot 7H_2O$	0.014 g
$CuSO_4 \cdot 5H_2O$	0.010 g
Trace mineral solution	0.5 ml
Gelrite	10 g
Water	1 liter
Trace mineral solution:	
MoO_3	4 mg
$ZnSO_4 \cdot 7H_2O$	28 mg
H_3BO_3	4 mg
$MnSO_4 \cdot 5H_2O$	4 mg
$CoCl_2 \cdot 6H_2O$	4 mg
Water	1 liter

The addition of 10 mg/l $CuSO_4$ as shown above, was necessary for colony formation by *H. thermophilus.* At that concentration, the copper salt inhibited colony formation by the halophilic *Hydrogenobacter* (Nishihara et al., 1989b), which requires copper at a much lower concentration (2 mg/l $CuSO_4 \cdot 5H_2O$ in the trace mineral solution).

If it is not possible to isolate single colonies by streaking on solidified media, it is possible to proceed by serial dilution of the last enrich-

ment culture. This should be repeated several times, using the last positive tube, in order to obtain pure cultures. Incubating the cultures at about 75°C rather than at 70°C or below would favor *Hydrogenobacter* spp. over *B. schlegelii.*

Purity can be checked by microscopical observation, confirming morphological homogeneity and the absence of *B. schlegelii* spores (the most probable contaminant), and by the presence of a single inflexion point in the DNA denaturation curves.

Culture Procedures

CULTURE MEDIA. Schlegel's basal mineral medium (see Chapter 15) is well suited for the cultivation of most of the neutrophilic strains isolated so far. To prevent inhibition by phosphate, the concentration of this anion can be decreased to 10 mM. For halophilic *Hydrogenobacter,* the medium described by Nishihara et al. (1990) has been used. For *Bacillus tusciae,* rather than using the enrichment medium proposed above, it is preferable to use a low phosphate ($\leq$ 10 mM) medium, using as buffer a compound with good buffering capacities around pH 4.5. For the type strain, L(+)-tartrate at a concentration of 50 mM was added successfully as a buffer at pH 4.5. For other strains, the buffer compound must be checked for both nonutilization and nontoxicity.

GAS MIXTURE COMPOSITION. The composition of the gas mixture may vary according to the experimental conditions. Oxygen concentration is often critical because of the pronounced microaerophily of many strains. The culture should be started with a very low partial pressure of oxygen (1–5 KPa). Due to respiratory protection, actively growing cells are more oxygen resistant: this makes it possible to increase the O_2 concentration progressively during growth. Bonjour and Aragno (1986) have shown that in *Hydrogenobacter* spp., the level of microaerophily depends on the electron source. It is more pronounced when thiosulfate is used than under H_2. The mixolithotrophic cultures (H_2 + thiosulfate) are the most resistant to oxygen. See Chapter 15 for safety precautions when using gas mixtures containing H_2.

CULTURE CONDITIONS. Most strains of *Bacillus schlegelii* do not grow well under agitation. Good cell yields may be achieved in shallow, static cultures—for example, in Fernbach vessels placed in desiccators filled with the proper gas mixture. Fermenter cultures of *Hydrogenobacter* and of *B. tusciae* are also possible, provided the desired temperature can be reached.

Identification of Thermophilic Knallgas Bacteria

Key to the Identification of Thermophilic knallgas Bacteria

The following organisms are all able to grow chemolitho-autotrophically under a $H_2/O_2/CO_2$ mixture. Organisms belonging to the same species, but lacking the ability to oxidize H_2 (though they may be able to grow either autotrophically with reduced sulfur compounds or heterotrophically) could exist in nature, although none have been isolated so far. As is the case for several mesophilic knallgas bacteria (see Chapter 15), H_2-chemolithoautotrophy is not necessarily an important taxonomic marker at the species or genus level.

1. Sporeformers, Gram positive to variable 2
   Nonsporeformers, Gram negative 3
2. Growth at pH 7.0, no growth at pH 4.0. Endospores terminal, spherical, deforming the sporangium. GC 66–68 mol% .... *Bacillus schlegelii*
   Growth at pH 4.0, no growth at pH 7.0. Endospores subterminal to terminal, oval, deforming the sporangium. GC 57–58 mol% .... *B. tusciae*
3. Growth at 70°C. GC 37–46 mol% 4
   No growth at 70°C. Growth at 50°C 5
   Growth only below 50°C: see Chapter 15: key to the mesophilic, knallgas bacteria
4. Halophilic, growth with 0.5 M NaCl .... Halophilic *Hydrogenobacter* spp.
   Not halophilic, no growth with 0.5 M NaCl .... *H. thermophilus,* as well as several genospecies almost undistinguishable phenotypically, but showing low DNA homology with *H. thermophilus. Calderobacterium hydrogenophilum* should be included among these genospecies of *Hydrogenobacter.*
5. Three species of facultative knallgas bacteria, all moderate thermophiles, were described on the basis of 1–2 strains isolated. So far they have not been validly described:
   No carotenoid pigment formed; colonies light brown .... *"Pseudomonas thermophila"*
   Presence of carotenoid pigment; colonies dull yellow:
   Motile .... *"P. hydrogenothermophila"*
   Nonmotile .... *"Flavobacterium autothermophilum"*

These organisms need further characterization (see below).

The Thermophilic, Sporeforming Knallgas Bacteria

Table 4 lists the characteristics of *Bacillus schlegelii* and *B. tusciae.* Both species of thermophilic, sporeforming knallgas bacteria have a relatively high GC content, compared to most other members of the genus *Bacillus.* Other

Table 4. Characteristics of the thermophilic, hydrogen-oxidizing bacilli.

	B. schlegelii	*B. tusciae*
GC content (mol%)	66–68	57–58
Spore shape	Spherical	Oval
Gram reaction	Variable	+
Flagellation	Peritrichous	Lateral
Optimum pH	6–7	4.2–4.8
Optimum temperature	70°C	55°C
PHA inclusions[b]	+	−
Carotenoid pigment	−	−
Electron sources:		
H_2	+	+
CO	+	−
Thiosulfate	+	−
Organic carbon sources:		
Organic acids	Some	Some
Sugars	−	−
Growth factors requirement	−	−
Nitrogen sources:		
N_2	−	−
NO_3^-	−	−
NH_4^+	+	+
Urea	+	+
Asparagine	+	+
Growth in presence of:		
1% glycine	−	+
3% NaCl	+	−
Penicillin G, MIC[c] (units/ml)	0.005	0.04
Hydrolysis of:		
Starch	−	−
Casein	Weak	ND
Gelatin	−	ND

Symbols: +, property present; −, property absent; ND, no data.

[b]PHA, polyhydroxyalkanoate.

[c]MIC, minimum inhibitory concentration.

thermophilic *Bacillus,* among which are *B. acidocaldarius, B. thermoruber,* and *B. thermocatenulatus* (Schenk and Aragno, 1979; Aragno, 1981), have a GC content > 60 mol%. Nevertheless, this property is probably not related to thermophily, since the even more thermophilic *Hydrogenobacter* spp. have a low GC content, whereas most of the mesophilic Knallgas bacteria (so Chapter 15) have a GC content of 60 mol% or higher. Phylogenetically, Gram-positive bacteria, including the sporeformers, are separated in two groups, one comprising the low GC organisms (including the genus *Bacillus*), the other the high-GC organisms. The phylogenetical position of the thermophilic bacilli with high-GC content is still unclear (Woose, 1987).

SHORT DESCRIPTION OF *BACILLUS SCHLEGELII* SCHENK AND ARAGNO (1979). This species has long straight rods, 0.6 × 2.5–5 μm, with peritrichous flagella. Spherical, terminal endospores, 0.8–1 μm in diameter, distinctly distend the sporangium. It is Gram variable, and the cell wall is covered by a regular array of protein units (Schenk and Aragno, 1979; Krüger and Meyer, 1984).

It has a strictly respiratory metabolism, with oxygen as the final acceptor. It cannot grow anaerobically with nitrate although nitrate is reduced to nitrite. Either chemolithoautotrophic growth (using H_2, CO, or reduced sulfur compounds as the electron donor and CO_2 as the carbon source), or chemoorganoheterotrophic growth occurs. Hydrogenase is constitutive, membrane-bound, and does not reduce NAD(P). The optimum temperature for hydrogenase is above 90°C (Pinkwart et al., 1983). Phenol, 1-propanol, and a small number of amino and organic acids can serve as sole carbon source. Sugars are not metabolized.

Its optimum temperature for growth is 70°C, with no growth at 37 or 80°C. Optimum pH for growth: 6 to 7. It is widespread as air-borne spores, originally isolated from a freshwater sediment, later also from other nongeothermal and geothermal habitats (Bonjour et al., 1988; see "Habitats of Thermophilic, Hydrogen-Oxidizing Bacteria"). The type strain is DSM 2000.

FURTHER STUDIES OF *B. SCHLEGELII*. *Bacillus schlegelii* is able to grow chemolithoautotrophically with thiosulfate (Hudson et al., 1988) as electron donor. The organism named *"Thiobacillus thermophilica"* (Egorova and Deryugina, 1963), a sulfur-oxidizing, thermophilic sporeformer, may have been identical to *B. schlegelii,* but apparently the original strain was lost. Krüger and Meyer (1984) isolated several sporeformers after enrichment under thermophilic conditions, using a mineral medium under 5% CO_2 + 35% CO + 60% air. These isolates were identified as *Bacillus schlegelii,* except strain OMT2 (see below). The type strain of *B. schlegelii* was then shown to share with the new isolates the ability to grow even faster with CO as the electron and energy source than with H_2. There was no immunological cross-reaction detected between the CO oxidase (CO dehydrogenase) from *B. schlegelii* and from *Pseudomonas carboxydovorans.* Other strains of *B. schlegelii* have not been tested so far for the utilization of CO as an electron source.

Hudson et al. (1988) isolated a strain of *B. schlegelii* from geothermally heated Antarctic soils by enrichment under air in a thiosulfate-containing mineral medium. The assignment of their isolate to *B. schlegelii* was later confirmed by the high DNA homology with the type strain (F. Bonjour, unpublished observation).

All strains tested but one (Bonjour et al., 1988; Krüger and Meyer, 1984) share a DNA-DNA homology of 78% or higher, regardless of their ecological or geographical origin and the mode of enrichment followed. Strain OMT2 (Krüger and Meyer, 1984) resembled *B. schlegelii* strongly (spherical spores, thermophily, ability to grow chemolithoautotrophically with H_2 or CO), but had a somewhat higher GC content of its DNA, a lower DNA homology with the type strain (59%), and differed in a few phenotypical characteristics.

The hydrogenase of *B. schlegelii* was compared to those of mesophilic, Gram-negative knallgas bacteria by Pinkwart et al. (1983). The *B. schlegelii* enzyme was more oxygen-sensitive and more stable under H_2 and in the presence of thioglycolate. Its molecular weight is about 100 kDa (110–125 kDa according to Krüger and Meyer, 1984, with possible dimers of 220–250 kDa). No immunological cross-reaction was detected with the hydrogenases from other Gram-negative, mesophilic knallgas bacteria.

SHORT DESCRIPTION OF *BACILLUS TUSCIAE* BONJOUR AND ARAGNO (1984). The cells of this species are straight rods, 0.8 × 4–5 μm, with a single lateral flagellum (see table 4). Oval, subterminal endospores, 1.2 × 1.8 μm, distinctly swell the sporangium. They are Gram positive in young cultures. No protein coat is outside the cell wall. PHA inclusions were observed in cells grown autotrophically under nitrogen starvation. Colonies on agar are spreading, white to cream, with a chalky surface. A white pellicle forms on the surface of liquid cultures at the end of the growth phase.

B. tusciae has a strictly respiratory metabolism, with oxygen as the final acceptor. It cannot grow anaerobically with nitrate but nitrate is reduced to nitrite. It is either chemolithoautotrophic, using H_2 as electron donor and CO_2 as carbon source, or chemoorganoheterotrophic; hydrogenase inducible, non-NAD-reducing, found in the soluble fraction after ultracentrifugation of the crude extract. CO_2 is fixed via the reductive, ribulose-1,5-bisphosphate cycle. A few amino acids and organic acids and several alcohols can serve as sole carbon source. Sugars are not metabolized.

The optimum temperature for growth is 55°C, with no growth at 35 or 65°C. The optimum pH for growth is 4.2 to 4.8. Growth is weak at pH 3.5 and at pH 6.0. *B. tusciae* has been isolated so far only from acidic geothermal pools, subject to short-term variations in temperature and pH.

Unlike *B. schlegelii, B. tusciae* has never been isolated from nongeothermal habitats, despite repeated attempts with freshwater sediments. Although authentic *B. tusciae* has so far been isolated only from Tuscany, similar strains have been isolated from the Furnas hot springs in S. Miguel (Azores) (M. Marchiani, personal communication). The presence of a soluble, non-NAD-reducing hydrogenase is unusual among knallgas bacteria and deserves further investigation.

The Highly Thermophilic, Nonsporeforming Knallgas Bacteria

From geothermal regions all over the world, various research groups have isolated several knallgas bacterial strains sharing the following basic properties:

Gram negative
Strict thermophiles (optimum temperature ≥70°C)
Habitat, exclusively geothermal
Strictly aerobic, although more or less microaerophilic
Autotrophic
Lithotrophic, using molecular hydrogen, elemental sulfur, or thiosulfate as electron and energy sources
Nonsporeforming
Low GC content (37–46 mol%)

Most strains isolated have been obligate autotrophs, although at least two strains, strain Z-809 (Savel'eva et al., 1982) and strain MF3 (Bonjour, 1988), were able to grow heterotrophically with a limited number of organic compounds (see below).

TAXONOMY OF EXTREME THERMOPHILES. Two genera and species were described almost simultaneously and independently by Japanese and Russian authors: *Hydrogenobacter thermophilus* (Kawasumi et al., 1984) and *Calderobacterium hydrogenophilum* (Kryukov et al., 1983). These organisms resemble each other strikingly, so they should be assigned at least to the same genus. In that case, the genus name *Hydrogenobacter* must have priority over *Calderobacterium,* since the Japanese authors published the original description in issue no. 1 of vol. 34 of the *International Journal of Systematic Bacteriology,* whereas the validation of the publication by the Russian authors appeared in the next issue.

Later, organisms similar to *Hydrogenobacter* and *Calderobacterium* have been isolated in other parts of the world: Iceland (Kristjansson et al., 1985) and Italy (Bonjour and Aragno, 1986; Bonjour, 1988). A halophilic strain clearly belonging to *Hydrogenobacter* was described by

Nishihara et al. (1990). Further isolates from the Azores, the USA, and New Zealand are at present under study in our laboratory. The phenotypical characteristics of all these organisms are very similar and allow a precise definition of the genus *Hydrogenobacter*.The cells are: long, straight to slightly curved rods, 0.3–0.5 × 2–4 (−8) μm; filaments are sometimes formed at supra-optimal temperatures; nonmotile; flagella, endospores, PHB granules, carboxysomes, or intracellular membranes have never been observed; Gram negative (by staining and cell wall ultrastructure), although the peptidoglycan layer appears to be relatively thick (Kristjansson et al., 1985).

Isolates of *Hydrogenobacter* are all: strictly respiratory, with oxygen as the terminal acceptor; microaerophilic; cannot grow anaerobically with nitrate; nitrate is not reduced; chemolithoautotrophic, using H_2 or reduced sulfur compounds as electron donors and CO_2 as carbon source; CO_2 is fixed via the reductive TCA cycle (Shiba et al., 1985). Most strains are unable to grow heterotrophically with any of a variety of organic compounds (organic and amino acids, alcohols, sugars) tested. Hydrogenase is membrane-bound and does not reduce NAD(P). Ammonium salts and/or asparagine can serve as sole nitrogen sources. Dinitrogen is not fixed.

These strains are: sensitive to penicillin and other eubacteria-specific antibiotics; have a GC content of 37–46 mol%; are oxidase positive and catalase negative; contain cytochromes *b, c,* and *o,* but no cytochrome a/a_3 (Kawasumi et al., 1984; Pusheva et al., 1988). The optimum temperature for growth is 70–75°C and no growth occurs at 45°C or at 90°C. Optimum pH for growth is 6–7 and the pH limits are 4.0 and 7.5. So far, they have been isolated exclusively from geothermal environments.

Several characteristics have only been studied in strains from one geographical origin: they should be tested on the other strains. They include: 1) an unusual profile of cellular lipids in the Japanese strains, with linear $C_{18:0}$ and $C_{20:1}$ as the major lipids (Kawasumi et al, 1984); 2) a unique quinone, 2-methylthio-1, 4-naphthoquinone, as the main quinonic component, in the type strain of *H. thermophilus* and in the halophilic *Hydrogenobacter* (Ishii et al., 1983; Nishihara et al., 1990); 3) a carotenoid pigment reported only in the Icelandic strains (Kristjansson et al., 1985); 4) an external coat consisting of regular arrays of proteic subunits in Icelandic strains (Kristjansson et al., 1985); and 5) the genome size of the Russian strains is very small (1.4–1.6 × 10^9 Dalton) compared to that of other eubacteria. The kinetics of DNA renaturation is similar for all strains examined, but differs from other bacteria tested (Lysenko et al., 1985)

So far, it has been very difficult to establish clear phenotypical distinctions among the organisms described. This is due in part to their striking similarity to each other, but also to the absence of most of the "classical" phenotypical characteristics (carbon substrate utilization spectra, many catabolic enzymatic activities) since they are obligate autotrophs.

A comparison by DNA-DNA homology analysis of strains of Hydrogenobacter from various parts of the world has yielded interesting results (Bonjour, 1988; Lysenko et al., 1985). The results shown in Table 5 indicate that there are five very clear-cut homology groups. Groups 1 and 2 were isolated from Italian sources. While all the organisms belonging to group 1 were isolated from the S. Federigo solfatara (Tuscany), two of the strains from group 2 originate from regions distant from Tuscany: strain 102 from Agnano Terme (near Naples, 250 km SE of S. Federigo) and strain MF3 from a fumarole on the Etna volcano (Sicily, 250 km SE of Naples). Group 3 comprises two strains isolated in Iceland by Kristjansson et al. (1985), and group 4 comprises two Japanese strains described by Kawasumi et al. (1984); strain TK-6 is the type strain of *H. thermophilus.* The strains in group 5, from the USSR, were studied by Kryukov et al. (1983) and by Lysenko et al. (1985). Among them, strain Z-829 is the type strain of *Calderobacterium hydrogenophilum.* DNA homology measurements between the halophilic *Hydrogenobacter* (Nishihara et al., 1990) and the other homology groups are still lacking.

Due to their very low intergroup homology, these five groups should be considered as different genospecies. However, following the recommendation by the "Ad hoc committee on reconciliation of approaches to bacterial systematics" (Wayne et al., 1987), we will not propose new species names for these genospecies unless clear-cut phenotypic properties can be associated with the DNA homology groups.

It appears from these results that DNA homology grouping partially reflects the geographical distribution of the organisms. The two groups isolated from Tuscany and other Italian sources differ by their maximum temperature, group 1 being more thermophilic (maximum, 86°C) than group 2 (maximum 80°C). Although strains of both groups were isolated from the same geothermal field (S. Federigo), they appear to be thermally diverse (see Fig. 1).

Surprisingly, strain MF3, which is facultatively autotrophic, clearly belongs to group 2, whereas all the other strains are obligate auto-

Table 5. DNA-DNA homology values (%) between strains of *Hydrogenobacter* from different geographical origins.

	Italy												Iceland		Japan		Kamchatka (USSR)			
Strain no.	T3	T4	P4	P9	P10	T5	T13	T171	T181	T191	102	MF3	HvH27	H-1	TK-6	TK-H	Z-829	Z-1803	Z-121	Z-7
T3	100																			
T4	89	100																		
P4	74		100																	
P9			80	100																
P10		93	85		100															
T5						100														
T13	0	0					100													
T171								100												
T181			0				95		100											
T191										100										
102							96	83			100									
MF3	0					87	88	100	84	93	88	100								
HvH27													100							
H-1	0					0					0		86	100						
TK-6	14							0			0			15	100					
TK-H		10						0							86	100				
Z-829	0						0				0			8	15	13	100			
Z-1803																	103	100		
Z-121																	92	89	100	
Z-7																	71	64		100
Homology group	1	1	1	1	1	2	2	2	2	2	2	2	3	3	4	4	5	5	5	5

Data from Bonjour (1988) and Lysenko et al. (1985).

trophs. The other facultative autotroph described (strain Z-809, Savel'eva et al., 1982) has been lost, and did not appear in the paper by Lysenko et al. (1985).

In short, at least five genospecies of *Hydrogenobacter* exist, reflecting wide geographic and temperature distributions. Contrasting with clear-cut genomic separation, they show striking morphological and metabolic similarities that so far do not permit distinction on a phenotypical basis. Thus, specific names cannot be given at this time. Rare in the group, facultative heterotrophy is only a strain-specific property without taxonomic significance.

FURTHER STUDIES OF THE EXTREME THERMOPHILES. *Hydrogenobacter* spp. are able to grow chemolithoautotrophically with thiosulfate and elemental sulfur (Bonjour and Aragno, 1986; Alfredsson et al., 1986) as well as with H_2 as electron donor. When thiosulfate and H_2 are given simultaneously, both are utilized concomitantly (mixolithotrophy), allowing a higher growth rate than with only one electron donor. Growth with thiosulfate only is very sensitive to oxygen, whereas mixolithotrophic growth is the least sensitive to oxygen. During growth on thiosulfate, elemental sulfur accumulates transiently.

Although obligately lithotrophic, *H. thermophilus* TK-6 could incorporate limited amounts of organic compounds, like acetate, pyruvate, and glucose, but only when $H_2 + O_2$ were present (Shiba et al., 1984). The addition of small amounts of pyruvate, acetate, or malate stimulated the autotrophic growth of this organism.

The respiratory chain was investigated by several authors. The hydrogenase activity of strain Z-809 was studied by Pusheva and Savel'eva (1982), who noted the thermophily of the enzyme, as well as its resistence to O_2. The hydrogenase of *C. hydrogenophilum* Z-1215 was purified (Pusheva et al., 1987) and its kinetic parameters determined. The hydrogen uptake activity by whole cells of strain Z-829 showed no measurable H_2-concentration threshold ($\leq$0.01 ppmv) whereas a distinct threshold was observed with cell-free extracts of the same organism (10–100 ppmv) (Kryukov and Bodnar, 1987). Cytochrome c_{552} from *H. thermophilus* TK-6 was purified and characterized (Ishii et al., 1987a). It reacted directly with the purified hydrogenase from the same organism (Ishii et al., 1987c). These authors propose a model for the respiratory chain of this organism, including 2-methylthio-1,4-naphthoquinone in the reverse electron transport pathway to NAD. The cytochromes of *C. hydrogenophilum* Z-829 were investigated and its cytochrome c_{552} was purified (Pusheva et al., 1988). It reacted with bovine cytochrome oxidase.

The elongation factor EF-Tu from *C. hydrogenophilum* was isolated and purified (Mikulik et al., 1988). In contrast to EF-Tu from *Thermus thermophilus,* it cross-reacted with antibodies against EF-Tu from *Escherichia coli.* In spite of this similarity, the temperature relationships of EF-Tu from *C. hydrogenophilum* and from *E. coli* were quite different: optimal at 40°C and complete loss after 5 min at 60°C for the latter, optimal at 70°C and complete loss after 5 min at 95°C for the former.

The Moderately Thermophilic, knallgas Bacteria

Three species of moderately thermophilic knallgas bacteria have been described, although at present they are not recognized as validly published. Surprisingly, they were the first thermophilic knallgas bacteria isolated. All have an optimum growth temperature of about 50°C, whereas their minimal temperature extends into the mesobiotic range.

"PSEUDOMONAS THERMOPHILA." McGee et al. (1967) described a moderately thermophilic knallgas bacterium as *"Hydrogenomonas thermophilus"* (sic). Further studies on this organism described conditions for colony formation (Tischer et al., 1975) and the ultrastructure of the cell wall (Wang and Tischer, 1975). The strain was not deposited in an official culture collection and apparently was lost. Krasilya et al. (1973) isolated an organism (strain K-2) they attributed to *H. thermophilus.* After the rejection of *Hydrogenomonas* by Davis et al. (1969), Emnova and Zavarzin (1977) transferred this organism to *Pseudomonas,* as *"P. thermophila."* However, neither the latter nor the former description was acknowledged as valid (Skerman et al., 1980). As the description by McGee et al. (1967) is rudimentary, the following description is based, unless otherwise stated, on the paper by Emnova and Zavarzin (1977) for strain K-2.

The cells of this organism are: short rods, 1.3–1.75 × 0.5–0.6 μm; motile by means of one polar flagellum; Gram-negative (Gram reaction and cell wall structure); neither endospores nor capsules are formed; PHB inclusions are present (Kostrikina et al., 1981). Strain K-2 contains carboxysomes and extensive intracellular membranes (Kostrikina et al., 1981; Romanova et al., 1982). Colonies are light brown. No carotenoid pigment is formed.

This organism has a strictly respiratory metabolism, with oxygen as the terminal acceptor. It cannot grow anaerobically with nitrate, al-

though nitrate is reduced to nitrite. It is a facultative chemolithoautotroph, using H_2 as electron donor and CO_2 as carbon source. Hydrogenase is membrane-bound and not NAD-reducing. Cytochromes *a, b, c,* and *o* are present (Emnova and Zavarzin, 1979). CO_2 is fixed via the ribulose-bisphosphate cycle (Romanova et al., 1980). It grows under air on a variety of organic and amino acids. Sugars and alcohols are not utilized, and it is neither proteolytic (on gelatin) nor lipolytic (on Tween 80). Ammonium salts, nitrates, and several amino acids can serve as N sources, but nitrites and urea cannot. Dinitrogen is not fixed. The optimum temperature for growth is 50°C, with slow growth at 36 and 56°C. Strain K-2 was isolated from a drainage ditch.

The presence of carboxysomes and intracellular membranes is unusual in knallgas bacteria (Walther-Mauruschat et al., 1977), indicating a possible relationship of *P. thermophila* with thiobacilli or nitrifying bacteria. Its aptitude to utilize other electron donors (reduced sulfur or nitrogen compounds) has not been tested so far. Wang and Tischer (1975) noted the presence, in the original strain, of a proteinaceous coat consisting of a hexagonal array of subunits. This was not observed in strain K-2 (Kostrikina et al., 1981).

Strain K-2 has been extensively studied by Russian microbiologists as a potential biomass producer (Emnova and Romanova, 1982). The published papers deal mainly with the activity and localization of the hydrogenase (Emnova and Romanova, 1977; Emnova et al., 1979), the respiratory chain and membrane function (Emnova and Zavarzin, 1979; Romanova and Emnova, 1980; Kuznetsov et al., 1984), the temperature and pH dependence of the ribulose-bisphosphate carboxylase (Romanova et al., 1980), the ultrastructure (Kostrikina et al., 1981; Romanova et al., 1982), enzyme production (Emnova et al., 1982), and the pattern of RNA extraction from cell biomass (Emnova, 1978).

"PSEUDOMONAS HYDROGENOTHERMOPHILA" AND "FLAVOBACTERIUM AUTOTHERMOPHILUM." These two species were described on the basis of four strains isolated from soils around hot springs in Japan (Goto et al., 1978). In fact, they appear to be very similar to each other, differing only by the motility of the former and the occasional observation of round "cysts" in cultures of *F. autothermophilum.* Genomic relationships between both types and a deeper taxonomic approach are thus needed before these organisms can be accepted as valid species. The following description fits both "species":

The cells are: long, straight rods; 2–6 × 0.6 µm; motile by means of one polar flagellum *(P. h.)* or non-motile *(F. a.);* Gram-negative; endospores are not formed; colonies dull yellow, due to the presence of a carotenoid pigment.

Both species display a strictly respiratory metabolism, with oxygen as the terminal acceptor. They are either chemolithoautotrophic, using H_2 as electron donor and CO_2 as carbon source, or chemoorganoheterotrophic. A few organic acids, but neither sugars nor alcohols, are utilized. Ammonium salts, nitrate and urea, but not nitrite, can serve as N source. Dinitrogen is not fixed. They are catalase and urease positive. Their GC content is 63.5 mol% *(P. h.)* and 64.9 mol% *(F. a.).*

The optimum temperature for growth is 50–52°C, with extremes of 30 and 55°C. The pH optimum is 7.0; ranging from pH 5–8. They were isolated from soil around hot springs in Japan. (The paper by Goto et al. (1977) deals mainly with the optimization of the culture medium.)

Biotechnological Potential of Thermophilic Knallgas Bacteria

The thermophilic knallgas bacteria present a number of advantages for utilization in biotechnological applications: they combine the characteristics of knallgas bacteria discussed in Chapter 15 (lithoautotrophy, use of H_2, a potentially important energy converter, high growth yields) with those of thermophiles. Among these, one could mention the lower risk of spontaneous contamination and the production of structures, particularly enzymes, that are more resistant, not only to heat, but also to other external factors, and that are often more stable. Thermophiles do not normally require cooling of the culture. An other advantage of thermophilic knallgas bacteria over other types of thermophiles is their high growth rates and the high cell densities they reach in fermenter cultures. For these reasons, *Hydrogenobacter* spp. appear particularly promising for biotechnological applications.

A deeper knowledge of the biochemistry and the genetics of these organisms is thus urgently needed, particularly regarding the possibility of using them as recipients of genes from other thermophiles, or of using enzymes and/or genes from these organisms as tools for a variety of applications.

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Morphologically Conspicuous Sulfur-Oxidizing Eubacteria

JAN W. M. LA RIVIÈRE and KARIN SCHMIDT

This chapter deals with the genera *Achromatium, Macromonas, Thiobacterium, Thiospira,* and *Thiovulum* (see Fig. 1). They all belong to chemotrophic microbial populations generally encountered in natural habitats that are characterized by the simultaneous presence of H_2S and O_2, i.e., at the border between aerobic and anaerobic zones in surface waters and in the outflows of H_2S-bearing springs. It will be useful to introduce this discussion with some general remarks on the special nature of this ecological niche to help explain the embarassing paucity of knowledge we possess about its inhabitants.

In nature, the coexistence of H_2S and O_2 can only be sustained in systems subject to continuous inputs of both substances, because H_2S is not stable in the presence of O_2. H_2S can be rapidly oxidized without the intervention of living organisms, the rate of oxidation depending on pH, temperature, and the presence of catalysts and/or inhibitors (Chen and Morris, 1972a, 1972b); reaction products range from sulfur and polysulfides to thiosulfate, sulfite, and sulfate. Thus, this habitat contains an array of reduced sulfur compounds that are all potential substrates for chemolithotrophic oxidation but that differ greatly in their stability in the presence of O_2.

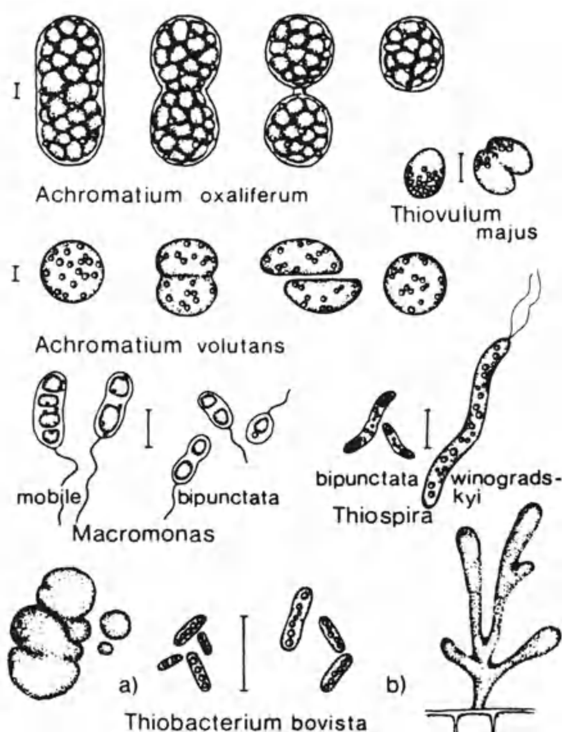


Fig. 1. Composite drawing of the described organisms, redrawn from the original literature. Bars = 10 μm—except for the typical colonies of the *Thiobacterium* species: (a) "puffball" shape, (b) dendroid shape.

The chemotrophic segment of the microbial population occupying this special niche includes—in addition to accidental "interlopers" such as H_2S-tolerant microaerophilic heterotrophs—a group of organisms called *colorless sulfur bacteria,* which appear to interact directly with the reduced-sulfur compounds characterizing this habitat.

These colorless sulfur bacteria include in the first place *Sulfolobus, Thiobacillus,* and *Thiomicrospira* (see Chapters 28 and 138), which have been shown to possess the capacity to chemolithotrophically oxidize reduced-sulfur compounds. Secondly, the group includes the five genera to be discussed here—*Achromatium, Macromonas, Thiobacterium, Thiospira,* and *Thiovulum*—as well as *Beggiatoa, Thiothrix,* and *Thioploca* (see Chapter 16 and 166). These eight genera are included among the colorless sulfur bacteria because the observed appearance and disappearance of sulfur inclusions suggest the possession of at least the capacity to oxidize sulfide and sulfur. The nutritional status of these genera is by no means certain and their relationship to reduced-sulfur compounds may range from obligate chemolithotrophy to protective, detoxifying sulfide oxidation, or to merely gratuitous sulfide oxidation. Another argument for inclusion of this group is the observed absence of these genera in habitats devoid of H_2S. Finally, there are an indefinite number of colorless sulfur bacteria that are not recognized in the eighth edition of *Bergey's*

Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) or discussed in this Handbook because they have been described poorly or no more than once. The works of the old masters, such as Gicklhorn, Hinze, Kolkwitz, Lauterborn, Molisch, Nadson, Warming, and Winogradsky, offer clear indications that several genera and even more species of colorless sulfur bacteria exist that cannot now be recognized usefully.

Thus the 11 genera discussed in this book represent only the most accessible part of the inhabitants of this ecological niche; even so, only five of them exist at the moment in pure culture: *Thiobacillus, Sulfolobus,* and *Thiomicrospira,* which can be cultivated with the stable compounds thiosulfate and/or sulfur as oxidizable substrates, *Macromonas,* of which only heterotrophic strains exist in pure culture, and *Beggiatoa,* from marine and freshwater habitats. Only the marine *Beggiatoa* strains seem to be facultative autotrophs (Nelson, 1989). The other six genera have not yielded pure cultures most probably because they are obligate sulfide-oxidizers, which, in view of the autoxidizability of H_2S, renders the use of solid media impossible and that of liquid media extremely cumbersome, since continuous inputs of H_2S and O_2 have to be maintained. Thus, for the moment, these six genera exist only by virtue of their morphological recognizability. The morphological characteristics are the only basis for the division into different genera.

In nature, quite often the predominance of one or a few colorless sulfur bacteria can be observed. Knowledge of the factors determining such predominance would obviously be of great help in isolation studies, but so far we have no clue as to the determinants of competition and survival of these diverse organisms, which have to compete not only among one another, but very likely also against the chemical oxidation of H_2S.

Thus we are faced with a vicious circle: lack of knowledge of the physiology of these organisms impedes the design of effective isolation procedures, which, in turn, are the very requirement for obtaining such physiological knowledge through pure culture study. The riddle they pose is further complicated by the complexity of their natural habitat. In addition to natural aeration, when exposed to light, the medium also receives O_2 from photosynthesis, subject to diurnal fluctuations. Furthermore, H_2S can originate from groundwaters poor in organic matter, as well as from sulfate reduction in mud layers, in which case it is invariably accompanied by organic compounds. The mineral contents of the water also appear to play an important role. A relatively high Ca^{2+} concentration or the presence of solid $CaCO_3$ characterizes many of the habitats in which the organisms are found; the relationship is most obvious for *Achromatium* and *Macromonas,* which both can have $CaCO_3$ inclusions.

Enrichment and Cultivation Methods

Imitation of the natural conditions in the laboratory appears to be the best approach to enrichment, cultivation, and isolation of *Achromatium, Macromonas, Thiobacterium, Thiospira,* and *Thiovulum,* the five genera under discussion in this chapter. The enrichment methods consist essentially of using Winogradsky columns (Winogradsky, 1888) that are kept in the dark and that contain sediments in which H_2S is generated through sulfate reduction. Hence, adequate, i.e., slowly decaying, organic material—and in the case of freshwater enrichments, $CaSO_4$—has to be included in the sediment; in seawater enrichments, a considerable amount of sulfate is already present in the water phase. Besides stationary columns, flow-through variations have been used in which the water phase is continuously and slowly replenished, which provides a means of controlling the position of the H_2S/O_2 border zone and of eliminating contaminants. This method has led to reproducible enrichment procedures for *Thiovulum,* while a more classical enrichment method could be used for the heterotrophic *Macromonas bipunctata.* For the other three genera, enrichment is still extremely difficult and at best capricious.

In the design of effective enrichment and cultivation methods, some guidelines can be further derived from specific ecological attributes of the organisms themselves:

1. The presence of elemental sulfur inclusions, a stored energy source, implies that the culture can survive periods of absence of H_2S but not of O_2 (all genera).
2. Possession of strong chemotactic properties introduces the possibility of exerting selection through water flow *(Thiovulum, Thiospira).*
3. Limited motility, or its absence in some genera, calls for stability of the position of the H_2S/O_2 border zone in the place where the organism is expected to accumulate (*Achromatium:* at the bottom; *Thiobacterium:* at the surface).
4. Capacity for attachment to solid surfaces permits selection by water flow, provided the

water contains the proper mix of H_2S and O_2 *(Thiobacterium, Thiothrix).*
5. The organism permitting, application of low temperatures (10–17°C) appears favorable, as this slows down chemical H_2S oxidation and thus leads to greater stability.

In conjunction with these methods, purification of cell material has been undertaken by taking advantage of chemotaxis *(Thiovulum)* and of selective sedimentation based on high specific gravity *(Achromatium).* Suspending the cells in sterile solutions followed by transfer to fresh sterile solution after separation had taken place could be carried out repeatedly, the "washing efficiency" depending on the initial degree of contamination and the endurance of the cells to repeated washings.

Such purified material, derived from nature or enrichment cultures, can then be subjected to cultivation in aseptic, controlled systems in which constant inputs of H_2S and O_2 are maintained. A great deal of ingenuity has gone into the design of such systems, based, for instance, upon the use of gas mixtures, semipermeable membranes, controlled pumping of sterile nutrient solutions, and solid/liquid phase systems in which the solid phase provides H_2S. So far, the use of sulfide-charged ion-exchangers or badly soluble sulfides as a constant source of H_2S has not been found practicable, nor has the use of inhibitors of chemical H_2S oxidation. Important examples of devices successfully used in specific instances are presented by Devidé (1954), Keil (1912), la Rivière (1963, 1965), and Perfil'ev and Gabe (1961). Wirsen and Jannasch (1978) described a variety of cultivation systems for *Thiovulum* (see below) in which full advantage was taken of modern materials and equipment. It is likely that these systems are also suitable for cultivation of other recalcitrant sulfur bacteria.

Presently, however, our knowledge is still restricted to such data as can be obtained from observations on natural populations and on enrichment cultures in the laboratory, both macro- and microscopically. In addition, laboratory cultures have in several cases produced sufficient quantities of cell material, sufficiently free from contamination, to make electron microscopy of whole cells and thin sections possible. Similarly, such cell material has permitted the determination of sometimes important physiological data. The main target of studies in complex culture systems remains, however, the acquirement of knowledge about the organism that will lead to the design of simpler cultivation methods. After all, one should keep in mind that one successful petri dish culture offers more promise for isolation and subsequent work than any of the culture systems mentioned above.

Significance of the Colorless Sulfur Bacteria

This group of organisms poses the question of how such a great morphological diversity has evolved in a very specialized and restricted niche in competition with chemical sulfide oxidation. One might well speculate that this niche must have been much larger at the time when, during the evolution of the present biosphere, the first oxygen appeared on an initially anaerobic stage and that, for a long time since, a much smaller O_2 concentration prevailed in the atmosphere than at present. However this may be, it is certain that microbial activity, including that of colorless sulfur bacteria, has played and is still playing an important role in the biogeochemical cycle of sulfur (Jørgensen, 1989; Kuenen, 1975; SCOPE 19, 1983; SCOPE 39, 1989; Kuenen and Bos, 1989; la Rivière, 1966). It is, for instance, generally accepted that the sulfur deposits that are mined today are biogenic and have been formed by sulfate reduction followed by an oxidation step that may have involved colorless sulfur bacteria. The well-known *sulfuretum,* in which sulfur is still accumulating today, offers an opportunity for determining the additional contributions of chemical and photosynthetic sulfide oxidation. Furthermore, sulfide oxidation to sulfate is an important step in the regeneration of oxidizing power within water bodies where sulfate plays a role in anaerobic mineralization. Also, the release of H_2S into the atmosphere from oceanic, marshy, and estuarine areas is at the moment a matter of concern on a global scale in judging the impact of artificial SO_2 emissions that are superimposed on it (Svensson and Söderlund, 1976). The magnitude of the biogenic H_2S emissions is presently not well known, but it is certain that the colorless sulfur bacteria codetermine it to a large extent. Finally, biological sulfide oxidation is responsible for the generation of certain types of acid soils and the formation of acid mine effluents. In the mining industry, so-called microbial leaching is presently being practiced for improving the yield of various metals from sulfide ores. For this purpose, *Thiobacillus* species are frequently being used (see Chapter 32). Furthermore, the microbiological methods for removing sulfur from coal, and removing hydrogen sulfide from biogas, generated by anaerobic digestion, are growing in industrial importance.

In the situation as outlined in this introduction, more detailed presentation of available information that follows can offer no more than some springboards and roadsigns for those who want to penetrate further into a fascinating and as yet unconquered territory of the microbial kingdom. Such endeavors also offer the rare sensation of reading the works of the old masters as valuable guidebooks of undiminished contemporary relevance. In order to facilitate such reading, pertinent references as well as earlier used names of organisms have been included.

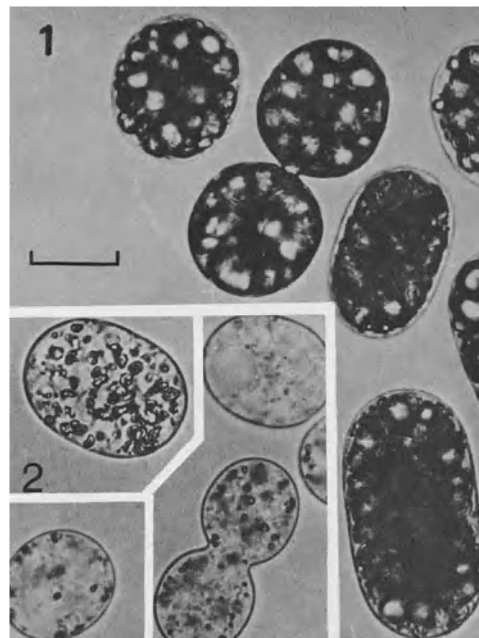
The Genus *Achromatium*

Two species of Achromatium are known (La Rivière and Schmidt, 1989): *Achromatium oxaliferum,* which occurs in fresh waters and marine environments, and *Achromatium volutans,* which is less well known and has been found in marine muds only.

Achromatium oxaliferum

Achromatium oxaliferum (Figs. 1 and 2) was described for the first time by Schewiakoff (1893). Since then the organism has temporarily also carried the names *Hillhousia mirabilis* (West and Griffiths, 1909), *Hillhousia palustris* (West and Griffiths, 1913), and *Achromatium gigas* (Nadson, 1913). Its cells are conspicuous because of their massive $CaCO_3$ inclusions occurring together with much smaller sulfur globules. In addition, their large size immediately attracts attention: the cells are spherical, ovoid, or cylindrical with a minimum length of the small axis of 5 μm and a maximum length of the long axis of 100 μm. Division is by constriction. The cells are slowly motile on solid surfaces only. It is, therefore, not surprising that the organism has tantalized numerous observers, none of whom, however, has so far succeeded in developing an effective enrichment method.

ECOLOGY. In all cases, the cells were encountered in or on the bottom mud of fresh and saline waters, as is to be expected from the high specific weight of these organisms which prevents them from moving vertically in the water. Since they are not at all commonly found among the macroscopically recognizable white masses that usually guide the investigator most effectively to natural populations of colorless sulfur bacteria, it appears useful to list here some of the habitats in which the organism was found, all the more so because no useful common characteristic of these habitats has as yet emerged.



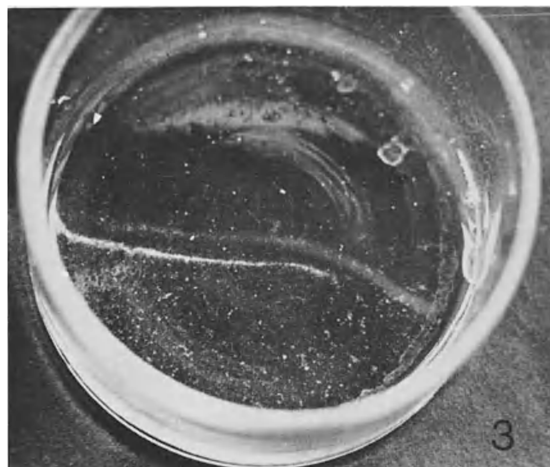


Fig. 2. *Achromatium oxaliferum* (from a garden pond near The Hague, The Netherlands). (1) Cells filled with $CaCO_3$ crystals. Bar = 20 μm. (2) Cells after treatment with 0.05 N acetic acid; sulfur globules can be clearly seen. (3) Topside view of a beaker with a band of "enriched" *Achromatium* cells.

Gicklhorn (1920), Bersa (1920), and Bavendamm (1924) found *Achromatium* in ponds in the botanical garden in Graz where the alga *Chara* also occurred in large numbers. The bottom mud of Swedish lakes was the source for Skuja (1948), and that of Lake Windermere for Skerman (Starr and Skerman, 1965). Lackey and Lackey (1961) found the organism in fresh and brackish waters in Florida as well as in salt marshes at Woods Hole. It was also found in a small concrete garden pond in Pacific Grove, California, and in 2 out of 14 garden ponds examined near The Hague (J. la Rivière, unpublished). These were made of concrete, had

a depth of 40 cm, and contained very loosely packed mud with much organic matter as well as cells of algae and cyanobacteria. Nadson and Visloukh (1923) found *Achromatium* in marine muds. Some of these waters were high, others low, in Ca^{2+} concentration.

Since all authors agree that the organism is an obligate aerobe and the high weight of its cells restricts it to the bottom habitat, it seems obvious that in all of the above-mentioned locations the borderzone between H_2S and O_2 must have resided in the mud or on top of it, indicating that the H_2S generation in the sediment must have been relatively weak, or, alternatively, aeration consistently strong. This appears to make *Achromatium oxaliferum* the counterpart to *Thiobacterium* which (as a "puffball" type) floats at the surface. There is, however, one important difference in that *Achromatium* possesses some means of locomotion while *Thiobacterium* does not. This motility is very slow and restricted to jerky rolling movements on solid surfaces, most probably effected by means of peritrichous filaments moving about in the slime layer that surrounds the cells (de Boer et al., 1971). In this way, the cells are capable of migrating chemotactically through the mud, where, depending on the distribution of localized microsources of H_2S, concentration gradients exist over short distances that are manageable by the organisms.

Lauterborn (1915) noted that cells were packed with $CaCO_3$ and had few sulfur inclusions when encountered on top of the mud, while cells below the surface contained little or no $CaCO_3$ and much more sulfur. This suggests that the restricted capacity for chemotaxis is somewhat complemented by the storage of sulfur but does not explain the significance that $CaCO_3$ storage could have for the organism beyond keeping it in the bottom layers by sheer weight. In this respect, any possible interaction with surrounding photosynthetic organisms appears important, as these exert a considerable influence not only on the O_2 concentration but also on the $CO_2/CaCO_3$ equilibrium, which are both subject to diurnal changes. In addition, $CaCO_3$ might exert a buffering effect with respect to H_2SO_4 formed by sulfur oxidation.

ENRICHMENT AND CULTIVATION. Most observations on the organism have been done on material taken directly from nature. No effective laboratory enrichment culture methods are available; at best the natural populations can be kept alive in the laboratory for 6–10 months. Schewiakoff (1893) used a watch glass containing mud, *Achromatium* cells, and some supernatant water from the natural habitat. After placing it in sunlight he observed "energetic multiplication" (perhaps chemotactic accumulation?) and formation of white agglomerations of 50–100 cells each. West and Griffiths (1913) placed the mud with overlaying tap water in small cylinders (15 cm wide, 5 cm high) and stirred the mud from time to time; in this way they kept the organisms alive for 9 months. They stated that the cells had a doubling time of 24–48 h under these conditions and that death resulting from O_2 deficiency was accompanied by the loss of the $CaCO_3$ inclusions. De Boer et al., (1971) maintained natural populations for 10 months by placing the loosely packed mud from a pond in closed plastic bottles at 5°C. The overlaying pond water column was about four times higher than the mud layer. The same authors used a very simple method, suggested to them by C. B. van Niel, for concentrating the cells in such a mud-water system. It is based on the heavy weight and white color of the *Achromatium* cells:

Collecting and Concentrating *Achromatium* Cells Found in Nature (de Boer et al., 1971)

Material from the upper part of the sediment was collected. Lightweight particles were removed by repeated decanting in tap water. Layers of heavier mud particles, approximately 0.5 cm thick, were subjected to gentle swirling in a tilted beaker that at the same time was rotated slowly along its vertical axis (Fig. 2). This treatment led to further fractionation of the particles according to their specific gravity and the heavy *Achromatium* cells soon became visible as a narrow white band very close to that of the sand-particle fraction. The cells were pipetted off and transferred to a smaller beaker and further purified by the same method. This procedure was repeated several times until a dense mass of *Achromatium* cells was obtained. This no longer contained cells of algae, protozoa, or sand particles, and the number of smaller bacteria present had been reduced to a very small minority, as shown by microscopic examination.

The method was obviously only successful for *Achromatium* cells that actually contained appreciable quantities of $CaCO_3$ inclusions.

At least 50% of the cells obtained in this way were viable, as shown by their motility observed under the microscope. They proved to be quite suitable for electron microscopy and some elementary physiological tests.

FURTHER CHARACTERISTICS. The organism is very easy to identify by the morphological characteristics already mentioned. Healthy cells are almost completely filled with highly refractile $CaCO_3$ crystals, which makes it difficult to observe the sulfur globules. These are best seen after selective removal of the $CaCO_3$ by treating

the cells with 0.05 N acetic acid (de Boer et al., 1971) (Fig. 2).

Cells collected by the method described above remained contaminated by heterotrophs even after repeated washings with sterile water. Nevertheless, indications were obtained that the cells moved and multiplied more actively in the presence of organic acids, casamino acids, or both. The experiments, however, could not be pursued further because of overgrowth by more rapidly multiplying heterotrophs (K. Schmidt, unpublished). The cells could be lysed by lysozyme and proved to be Gram-negative and catalase-negative.

Achromatium volutans

Achromatium volutans (Fig. 1 and 3) was first described by Hinze (1903) under the name of *Thiophysa volutans* and later by Nadson (1913, 1914) under the name of *Thiophysa macrophysa*. The organism was found in saline waters only: in association with mud and decaying seaweeds from the Bay of Naples (Hinze, 1903, 1913), in the saline sulfur spring "Solgraben von Artern" (Kolkwitz, 1918), and in a saline spring in Florida (Lackey and Lackey, 1961). The best description is probably that of Hinze (1903), who obtained his cell material from marine mud placed in a flat dish; within 1 h, many cells were found at the mud surface, concentrated through chemotaxis into a thin white layer.

The organism was very similar to *Achromatium oxaliferum* with respect to cell shape, motility, and mode of division. A striking difference was the total lack of $CaCO_3$ inclusions and the presence of far more and larger sulfur globules. Also the cells were consistently smaller, ranging from spheres with a diameter of 5 μm, when young, to ovals up to 40 μm in length. Right after division cells were flat at one end, which was never observed in *Achromatium oxaliferum* (Fig. 1).

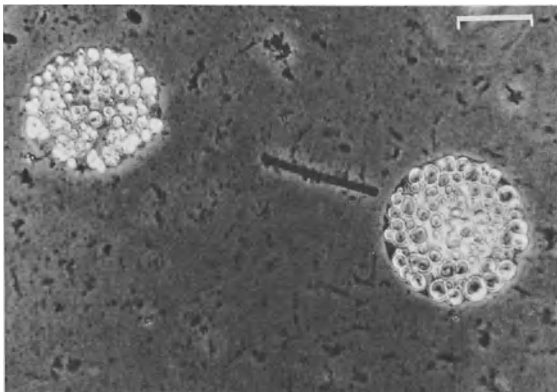


Fig. 3. *Achromatium* (=*Thiophysa*) *volutans* (from Solgraben in Artern, Germany). Bar = 20 μm.

The observations of Hinze (1903) suggest that this organism is more sensitive to environmental conditions like H_2S and O_2 concentrations than is *Achromatium oxaliferum*. This appears plausible because its lower specific gravity is likely to permit more rapid chemotactic response.

The Genus *Macromonas*

Cells of *Macromonas* are often found in habitats where *Achromatium oxaliferum* also occurs (Bavendamm, 1924; Gicklhorn, 1920; Lauterborn, 1915; Skuja, 1956). To the knowledge of the authors, only one attempt at enrichment of these organisms has been described (Dubinina and Grabovich, 1984). Hence, most available data originate from observations on material directly taken from natural sources.

The cells are colorless, cylindrical or bean shaped, and sluggishly motile with one polar flagellum. Since flagellation was observed in the light microscope, presence of a tuft of flagella is more likely. Their typical characteristic is the presence of several large refractile spherules, which have been thought to consist of $CaCO_3$; definite proof for this is lacking, however. In addition, small sulfur globules may be present.

Two species have been recognized (la Rivière and Dubinina, 1989).

Of the two species *Macromonas mobilis* (Fig. 1) was first described by Lauterborn (1915) under the name *Achromatium mobile* and later by Gicklhorn (1920) as *Microspira vacillans*. The cells are ellipsoidal or cylindrical and slightly curved, measuring usually 9 by 20 μm. Smaller cells are often present and are believed to be daughter cells (Gicklhorn, 1920). The single polar locomotor organ (tuft of flagella?) measures 20–40 μm and can be observed by light microscopy. It provides for motility at a rate of 800 μm/min. Besides small sulfur inclusions, usually between 5 and 15, one to four large refractile bodies occur, which closely resemble the $CaCO_3$ inclusions of *Achromatium oxaliferum* and, therefore, are provisionally considered to consist of $CaCO_3$. No methods for isolation are known.

Macromonas bipunctata (Fig. 1) was first described by Gicklhorn (1920) as *Pseudomonas bipunctata* and renamed *Macromonas bipunctata* by Utermöhl and Koppe (Koppe, 1923). Dubinina and Grabovich (1984) reported the isolation of heterotrophic bacteria answering the description of *Macromonas bipunctata* by these authors. The cells are single or in pairs,

pear-shaped, cylindrical or curved; they measure 2.2–4×3.3–6.5 μm. They are motile by a polar tuft of flagella, Gram-negative, strictly aerobic and catalase-positive. Sulfur inclusions are produced when the cells are grown in media containing sulfide, which is oxidized by hydrogen peroxide and thus offers no useful energy to the cell.

While recognizing the valid description of *Macromonas bipunctata* by Dubinina and Grabovich, la Rivière and Dubinina (1989) still maintain *Macromonas mobilis* as type species because " . . . now that the way has been paved, it is felt that isolation of *Macromonas mobilis* may take place in the near future and thus complete the taxonomy of the genus with a minimum of alterations."

Enrichment and Isolation of *Macromonas bipunctata* (Dubinina and Grabovich, 1984)

The white mat found on the surface of bottom sediments in a sewage treatment plant was used for inoculating 10 ml portions of the following semisolid medium contained in test tubes:

Sodium acetate	1 g/l
Calcium chloride	0.1 g/l
Casein hydrolysate (Difco)	0.1 g/l
Yeast extract (Difco)	0.1 g/l
Agar (Difco)	1 g/l

After sterilization, a mixture of vitamins and of trace-elements (Pfennig and Lippert, 1966) was added, as well as 0.2 mg freshly prepared FeS per 10 ml medium as source of sulfide. The pH was adjusted to 7.2–7.4.

After two to three days at 28°C, a white surface film appeared, which contained a large number of typical *Macromonas* cells. Streaks were made from a suspension of this film on agar plates containing the medium described above solidified with 1 g agar/l. After two to three days flat *Macromonas* colonies appeared with a diameter of 1 to 4 mm and a finegrained structure. These colonies were used for subsequent purification by restreaking on solid medium.

The Genus *Thiobacterium*

The organisms of this genus are nonmotile rods that form sulfur inclusions. The cells are embedded in gelatinous masses that are either "puffball"-shaped or dendroid. Spherical, "puffball"-shaped gelatinous masses may be found freely floating in stagnant, H_2S-bearing waters (Fig. 1; *Thiobacterium* a). Dendroid gelatinous masses may be found attached to solid surfaces that are exposed to flowing, H_2S-containing waters (Fig. 1; *Thiobacterium* b).

Their lack of motility prevents these organisms from following chemotactically the spatial fluctuations of the zone of optimal H_2S and O_2 concentrations. This suggests that they are either capable of growing under a wider range of H_2S and O_2 concentrations than acceptable to most of the motile colorless sulfur bacteria, or, alternatively, that they are confined to growth in locations where their optimal conditions more or less continuously prevail. The latter alternative appears to be supported by the fact that they are found floating at water-air interfaces or attached to solid surfaces. The first case is likely to occur when H_2S generation is consistently so high that the zone of optimal conditions is kept automatically at the top of the anaerobic water column. The second case arises when a water stream with more or less constant H_2S and O_2 concentrations is maintained for longer periods of time; this is the situation one finds in certain sections of the outflows of sulfur springs.

In *Bergey's Manual* (Buchanan and Gibbons, 1974; la Rivière and Kuenen, 1989a), only one species, *Thiobacterium bovista,* was recognized. It comprised the bacteria embedded in spherical, gelatinous masses, first described by Molisch (1912) under the name of *Bacterium bovista,* as well as those embedded in dendroid structures, first described by Lackey and Lackey (1961) under the name of *Thiodendron mucosum.* Observations in nature of *Thiobacterium bovista* have been reported by Scheminsky, et al. (1972), and by Caldwell and Caldwell (1974).

Molisch obtained the spherical colony type (Fig. 1) of *Thiobacterium* at the surface of Winogradsky column enrichments prepared with seawater plus a sediment consisting of black mud mixed with decaying algae, all ingredients originating from the harbor of Trieste, in which the organism was also directly observed. The diameter of the spherical structures ranged from microscopic dimensions to about 4 mm and the structures were sometimes clustered together. The lengths of the cells were between 2 and 5μm, their width between 0.6 and 1.5μm. They usually contained four sulfur globules per cell. These cells were embedded in the gelatinous skin of the spherical masses, the inside of which was filled with water. The nature of the mucoid matrix was not elucidated.

The dendroid forms (Fig. 1) were found by Lackey and Lackey (1961) in saline, H_2S-bearing waters in Florida at temperatures between 13 and 30°C. The dendroid matrix, of as yet unidentified material, had the shape of branching filaments with a varying thickness of up to 20μm, individual branches sometimes being clubshaped. Thus, the colonies acquired treelike structures, reaching a width of up to 3 mm. Most of the colonies were found attached to algal and cyanobacterial cells, (generally of the

genus *Lyngbya*) which raises the question of a possible interaction involving O_2.

The cells were randomly arranged throughout the branches and consisted of rods, normally 3–5 μm long and 1.5 μm wide; in the swollen ends of the branches, however, cells three times that size were found. Most cells contained three to eight sulfur globules arranged on a longitudinal axis.

The organism could not be grown in enrichments in the laboratory but was kept alive for 3 months in jars with water of the original habitat at 20°C.

The Genus *Thiospira*

Thiospira cells consist of typical colorless spirilla with sulfur inclusions. Their polar flagella are in some cases united in a tuft, visible in the light microscope. The cells are chemotactic with respect to O_2 and H_2S. They can arrange themselves in characteristic agglomerations located in regions of optimal H_2S and O_2 concentrations, forming "Bakterienplatten" in liquid columns and "Atmungsfiguren" in covered slide preparations (Fig. 4). The members of the genus have been found in fresh waters and marine environments in which H_2S was present.

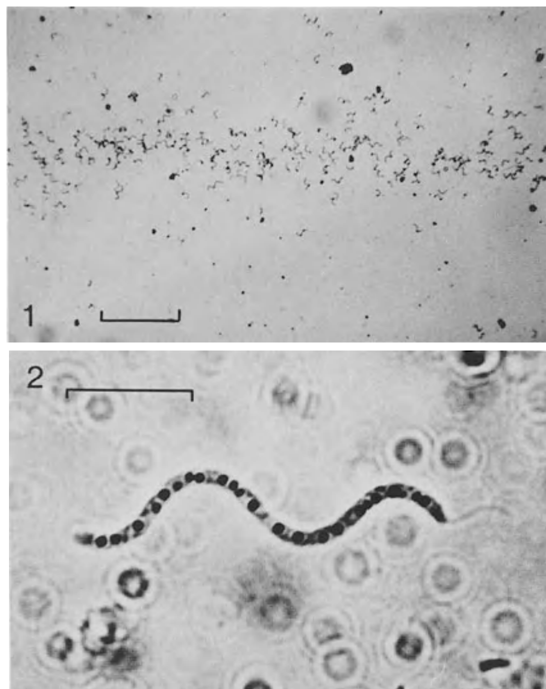


Fig. 4. *Thiospira* sp. (from upwelling groundwater near Delft, The Netherlands). (Courtesy of J. Klein, IHE, Delft.) (1) Cells accumulating at the optimum O_2/H_2S-zone. Bar = 100 μm. (2) Dividing cell with a flagellum. Bar = 10 μm.

At the moment, two species are recognized (la Rivière and Kuenen, 1989b).

Thiospira bipunctata (Fig. 1) was first described by Molisch (1912) and at that time named *Spirillum bipunctatum.* Its cells are slightly twisted rods forming short spirilla with a width of 1.7–2.4 μm and a length of 6.6–14 μm. They are most probably polarly flagellated. Each end of the cell typically contains a large granule of some storage material, described as volutin, leaving a clear space in the middle normally filled with two or three sulfur globules. The latter are sometimes also found at the ends.

Thiospira winogradskyi (Fig. 1) was first described by Omelianski (1905) and at that time named *Thiospirillum winogradskii.* It is a colorless spirillum containing numerous sulfur globules that may disappear when the H_2S in the medium is exhausted. It is 2–2.5 μm wide and can reach a length of 50 μm. "Volutin" bodies are absent. The cells have polar flagella and are strongly chemotactic.

Dubinina and Grabovich (1983) reported the isolation of heterotrophic spirilla under the name of *Thiospira bipunctata.* These were morphologically similar to *Thiospira bipunctata* as described by Molisch since they formed sulfur inclusions when grown in media containing sulfide. The sulfur was formed by nonspecific sulfide oxidation by metabolically produced hydrogen peroxide. Since other heterotrophs have also been found to form sulfur inclusions in sulfide-containing media (Skerman et al., 1957), further study is required for determining the proper taxonomic status of these isolates, which may well prove to be identical to existing *Spirillum* species.

Enrichment Procedures

All enrichments for *Thiospira* have been carried out in Winogradsky columns (Winogradsky, 1888) in which H_2S was released from a sediment.

Molisch (1912) enriched *Thiospira bipunctata* from mud from a Black Sea estuary (a liman) in cylinders (5–15 × 10–30 cm), filled with seawater and a sediment of mud mixed with dying or dried algae. The columns were kept for several weeks at room temperature in the dark or under dim light.

Omelianski (1905) enriched *Thiospira winogradskyi* using tap water and a mixture of liman mud, $CaSO_4$, and fragmented rhizomes in cylinders 7 cm wide and 40 cm high. After some months' incubation at room temperature in the dark, "Bakterienplatten" appeared in the lower part of the columns, which moved up and down with the prevailing H_2S concentration. When

the cells had settled to the bottom, the sediment was stirred up, which apparently led to renewed H_2S production and prolongation of the lifetime of the culture, which in this way could be kept for 2 years. The present authors had occasion to observe a *Thiospira* species among the colorless sulfur bacteria at 7°C in the outflow of an upwelling, H_2S-bearing groundwater near Delft, The Netherlands (Fig. 4). The cells formed "Atmungsfiguren" in slide preparations and could be kept viable for at least 6 weeks at 4°C in the dark, in cylinders containing groundwater and some mud from the location of origin.

The Genus *Thiovulum*

The cells of this spectacular genus (Figs. 1 and 5) are ovoid and measure 5–25 μm in length. They normally contain sulfur inclusions, often concentrated at one end. The cells multiply by longitudinal fission preceded by constriction. The cells have peritrichous flagella that provide strong chemotactic motility, the cells rotating around an axis coinciding with the path of travel; they never reverse direction and concentrate at places of optimum O_2 and H_2S concentrations in sharply defined characteristic veils or webs consisting of separate, ever moving, individual cells, to some extent held together by a very loose slime matrix (Fig. 5).

Because of this strong chemotaxis, the development of methods for enrichment and cultivation has been more successful for *Thiovulum* than for the other genera discussed in this chapter.

Only one species, *Thiovulum majus,* is recognized (la Rivière and Kuenen, 1989c). This includes the former species *Thiovulum minus* (Hinze, 1913). Other obsolete names are *Monas muelleri* (Warming, 1875) and *Thiovulum muelleri* (Lauterborn, 1915).

Ecology

Thiovulum has been observed in marine environments and saline springs by Warming (1875), Hinze (1903, 1913), Molisch (1912), Bavendamm (1924), Fauré-Fremiet and Rouiller (1958), Lackey and Lackey (1961), and la Rivière (1963). Freshwater forms were reported by Lauterborn (1915) and Lackey and Lackey (1961). In all instances, *Thiovulum* is found in very sharply localized white masses in situations where H_2S meets with O_2. Since it appears restricted to low concentrations of each substance for optimal growth, the patterns it forms provide a macroscopic visualization of this borderline, even in slowly flowing waters,

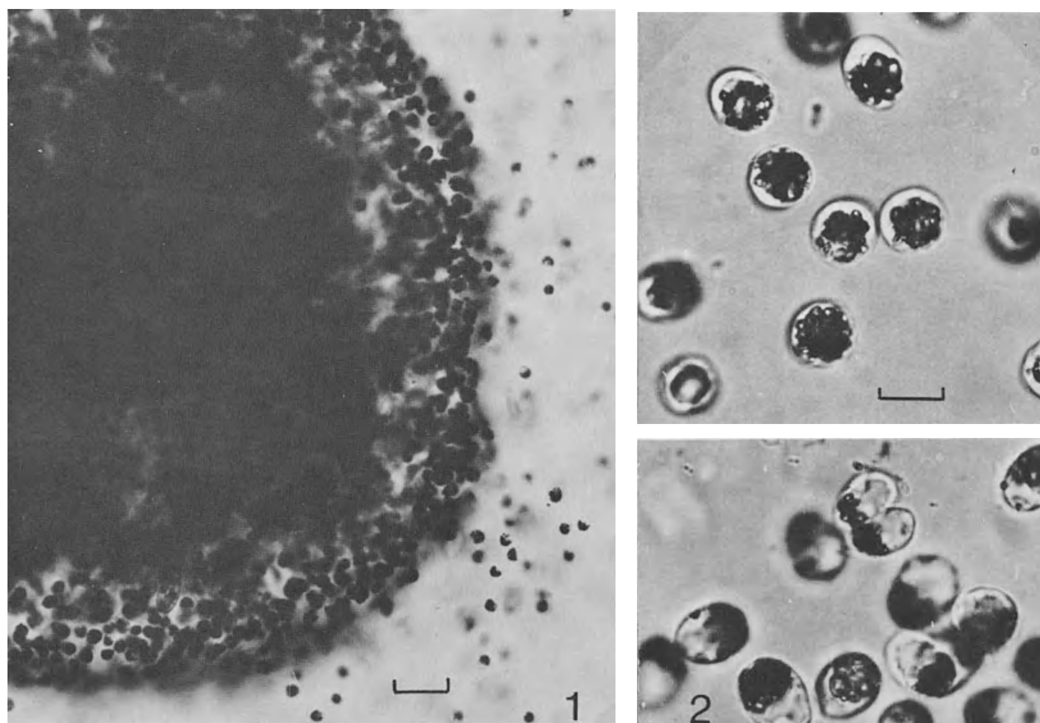


Fig. 5. *Thiovulum majus* (from Bergen op Zoom, The Netherlands). (1) Periphery of a veil of *Thiovulum majus.* Bar = 50 μm. (2) Swarming cells. Bar = 20 μm. (From Schlegel, 1976.)

as the cells rapidly follow changes of the concentration gradient. This makes it sometimes possible to identify *Thiovulum* accumulations in nature by their characteristic white patterns observed above black, sulfide-releasing muds in otherwise clear water. Similarly, such veils may be observed over decaying organic matter in flowthrough marine aquaria.

Measurements with microelectrodes in natural and laboratory systems (Jørgensen and Revsbech, 1983) have established that these veils are about 100 μm thick and are formed at the interface of the oxygen and sulfide zones. The veils create an unstirred boundary layer preventing mixing of oxygen and sulfide and thus also preventing chemical sulfide oxidation. The veils thus create a situation in which the *Thiovulum* cells are fed by diffusion of both substrates, which each penetrate over a distance of 50 μm into the veil. In oxygen-rich water the veils take the shape of small spheres which are made anaerobic in the center by the rapid oxygen utilization of the cells, which thus create the gradient required for optimum growth. Jørgensen and Revsbech (1983) provide further fascinating evidence showing that the chemotactic behavior of *Thiovulum* is extremely sensitive to environmental influences and is an important instrument, by "concerted action" of many cells, in the creation of the precarious niche in which *Thiovulum* lives.

Enrichment

Unpublished work by van Niel, Wijler, and Lascelles (van Niel, 1955) led to the development of a simple enrichment method, described by la Rivière (1963, 1965). The method has been successfully used in Pacific Grove (California), Delft (The Netherlands), and also in Woods Hole (Massachusetts) (Wirsen and Jannasch, 1978).

Enrichment of *Thiovulum* (Wirsen and Jannasch, 1978)

A layer of decaying seaweed *(Ulva)* mixed with some marine mud is placed on the bottom of a jar of 1–10 liters filled with seawater, the thickness of the compressed algal layer is kept to less than half the height of the jar (Fig. 6A). A continuous, slow flow of seawater is introduced near the surface of the sediment, and the jar is allowed to overflow. The layer of *Ulva* via sulfate reduction as a source of H_2S while the flowing seawater provides a continuous supply of O_2. By means of chemotaxis, the cells of *Thiovulum* seek out the regions of optimal concentration, where growth takes place in characteristic veils. The flow of seawater has also the essential functions of supplying the sediment with SO_4^{2-} as a source of H_2S, and of flushing out contaminating organisms that are not chemotactic; as a rule the *Thiovulum* veils are surrounded by clear water.

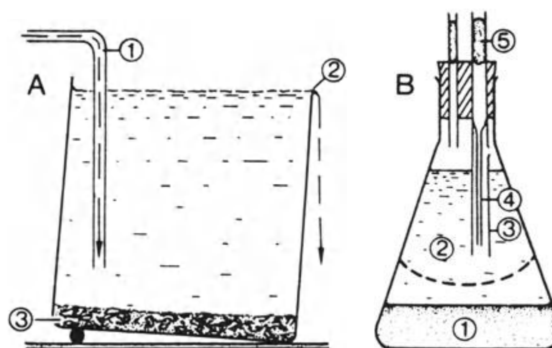


Fig. 6. Culture vessels for enrichment of *Thiovulum:* (A) by Wijler's method and (B) for stationary cultivation of *Thiovulum.* (A): 1, inflow of seawater; 2, overflow; and 3, layer of decaying *Ulva.* (B): 1, sulfide-containing agar layer; 2, seawater; 3, protective tube for localized aeration; 4, Pasteur pipette; and 5, cotton plug. The broken line indicates the zone of growth of *Thiovulum.*

When fresh *Ulva* is used, one should wait ca. 2 weeks before starting the seawater flow; a thick film of bacteria usually has developed at the surface at that time. After the flow of seawater is started, growth of *Beggiatoa* and/or *Thiothrix* may be observed in the upper part of the jar. In that case, the flow rate should be increased somewhat; the filamentous bacteria move to lower regions and eventually to the bottom while *Thiovulum* appears in the top layer usually 2–3 weeks after the start of the experiment. This indicates that the optimum sulfide concentration for *Thiovulum* is definitely lower than that for *Beggiatoa* and *Thiotrix.* When the flow rate is further increased, the veils of *Thiovulum* move further downwards.

At all times the culture should be kept in the dark, and relatively low temperatures (15°C) appear to be favorable. The layer of *Ulva* can be replaced by decaying marine animals like starfishes and sea urchins; a layer of fishmeal has also given positive results.

Wirsen and Jannasch (1978) found that an initial addition of neutralized Na_2S led to an earlier appearance of *Thiovulum* veils and to larger enriched populations.

Enrichment cultures of *Thiovulum* can be used as a source of crude cell material for several weeks until the H_2S-generating power of the sediment is exhausted. Lateral illumination with a flashlight greatly facilitates observation and harvesting of the *Thiovulum* veils.

Purification of Crude Cell Material

Besides filtration, methods based upon the chemotaxis of *Thiovulum* have been used, singly or in combination. la Rivière (1963, 1965) used the following method:

Purification of *Thiovulum* (la Rivière, 1963, 1965)

A total of 50–100 ml of a cell suspension carefully harvested by pipetting from an enrichment culture is rapidly filtered through a thin layer of cotton or of cheese-

cloth into a 100-ml glass cylinder. After 5–10 min, the cells settle chemotactically near the bottom, from where they are transferred by means of a pipette in as small a volume as possible into a test tube containing 15 ml sterile, aerobic seawater, cooled to about 15°C, in which they again form a veil near the bottom in 5–15 min. Repeated transfers in a similar manner to fresh tubes with sterile seawater lead to highly purified cell suspensions, as the initial filtration removes debris, protozoa, and other larger organisms while subsequent "washings" decrease the number of smaller contaminants. Plating on media for *Thiobacillus* and for heterotrophs permits following the effectiveness of the purification steps.

The success of the method depends entirely on the degree of initial contamination of the raw material and the capacity of the cells to survive successive purification steps. In experiments in Pacific Grove, "washing" could be performed 6–10 times in succession, leading to suspensions that were consistently free from contaminants. However, similar pure suspensions were not obtained in experiments in The Netherlands (la Rivière, 1965) or in Woods Hole (Wirsen and Jannsch, 1978), which is probably caused by differences in vigor between the enriched populations and in properties of the seawater used, such as temperature, degree of pollution, and microbial contamination.

Wirsen and Jannasch (1978) used the following modifications of the method described above:

1. Repeated chemotactic "washing" by pipetting or decantation in sterile aerobic seawater without an initial filtration step; this could be done only when freshly formed veils, relatively free from larger organisms, were available in the enrichments.
2. Chemotactic "washing" in deoxygenated sterile seawater, in which case the cells migrate to the top layer.
3. Concentration and washing of the cells by means of a membrane filter with a porosity of 8μm. Initial cell suspensions are concentrated to about 2 ml by suction, whereupon fresh sterile seawater is added, followed again by concentration by suction, *Thiovulum* cells being retained by the filter while contaminants are passed through it. Three to five such "washings" are possible.

Application of these methods combined with subsequent cultivation procedures provided Wirsen and Jannasch (1978) with highly purified cell suspensions suitable for electron microscopy and for the performance of some important physiological experiments. The various treatments, however, could not be repeated a sufficient number of times to obtain suspensions that were entirely free from contaminants.

Cultivation of Purified *Thiovulum* Populations

STATIONARY CULTIVATION OF THIOVULUM SUSPENSIONS FREE FROM CONTAMINANTS (LA RIVIÈRE, 1963, 1965). In a 500-ml Erlenmeyer flask, 250 ml of the following sterile agar medium is sterilized at 120°C for 10 min: 250 ml seawater, 2% agar (Difco), 0.01% NH_4Cl, 0.01% KH_2PO_4. Just prior to solidification of the agar, 2.5 ml of a separately heat-sterilized solution of 1% Na_2CO_3 in seawater is added, followed by 8 ml of sterile seawater previously saturated by gaseous H_2S. The pH does not need adjustment; it should be around 8.0. After setting of the agar, 250 ml of seawater are added and the flask is closed with a steam-sterilized stopper allowing for local aeration by means of a Pasteur pipette surrounded by a vertical glass tube which is open at the lower end (Fig. 6B). In this way, aeration is restricted to the inside of the protective glass tube, the dissolved oxygen slowly diffusing downwards to the bulk of the seawater layer. Thus, a gradient between the tube and the agar block is established which can be easily demonstrated with a redox dye; e.g., thionine. Without aeration, the dye is in its reduced form throughout the liquid; with conventional aeration without protection tube, the turbulence distributes the oxygen uniformly and the dye is in its oxidized form throughout the liquid. With localized aeration we obtain a sharp boundary between a "reduced" and an "oxidized" zone, which can be maintained for weeks.

The flask is placed at a temperature of 10–15°C and kept under constant aeration with cotton-filtered air. After equilibration overnight, the flask is ready for inoculation with purified cell material, and soon the individual cells of the inoculum can be seen to settle in one plane somewhere between the agar surface and the aeration tube.

Within days, the cells increase in number until a sharply defined web is formed at about 1 cm above the agar surface in an otherwise clear liquid. On prolonged incubation, the web rises slowly because of increasing O_2 demand; later on the web moves downward as H_2S becomes exhausted. By this time, signs of decay become apparent: White strands consisting of dead cells adhering to each other hang down from the sides of the flask. The life of the culture lasts for about 3 weeks.

This procedure has been successfully used only in Pacific Grove, where purification of the inoculum could be pursued to the point where

all contamination had been eliminated. It was also used—with some modification—by Pringsheim and Kowallik (1964) in their study of H_2S oxidation by *Beggiatoa.*

It should be pointed out that the method is unsuitable when impurities are present, as *Thiovulum* is rapidly overgrown in any closed stationary system. It should further be realized that even when successful the method yields only very small amounts of cell material.

CULTIVATION IN OPEN, FLOW-THROUGH SYSTEMS. Such systems are based upon the same principle that underlies the enrichment culture method. Besides the use of sterilized, aerated seawater fed into the culture vessel by controlled pumping, different sources for input of H_2S have been applied. These include periodical addition of H_2S or portions of saturated solutions of H_2S once or twice daily (la Rivière, 1963, 1965). Wirsen and Jannasch (1978) developed several reliable devices in which continuous H_2S provision was assured from different sources:

1. Decaying *Ulva* kept separately from the culture vessel by a nylon membrane of 0.45-μm porosity.
2. A pure culture of *Desulfovibrio aestuarii* separated from the culture vessel in the same way, H_2S generation being kept up by periodic addition (every 2–4 weeks) of fresh sodium sulfate/lactate medium. This system (Fig. 7) provided the best results permitting the maintenance of active *Thiovulum* populations for 220 days without reinoculation; during this time cells were frequently harvested, leading to rejuvenation of the veils.
3. A neutralized solution of Na_2S in sterile seawater separated from the culture vessel by a dialysis membrane.
4. A coil of silicone tubing, permeable to H_2S, through which a gas mixture of N_2 and H_2S (1%) was kept circulating. The coil was placed on the bottom of the culture vessel.

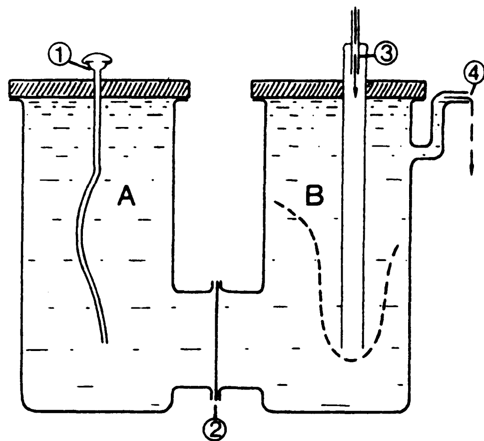


Fig. 7. Double culture vessel for the maintenance of purified cell suspensions of *Thiovulum* sp. (after Jannasch and Mateles, 1974; and Wirsen and Jannasch, 1978). (A) Culture vessel with *Desulfovibrio estuarii.* (B) Culture vessel for *Thiovulum.* 1, lactate supply; 2, semipermeable membrane; 3, inflow of air-saturated seawater; 4, overflow. The broken line indicates the zone of growth of *Thiovulum.*

The same authors, furthermore, used closed-flow systems based upon recirculation rather than open systems with overflow. In the closed system, no *Thiovulum* cells are lost, but contaminants are not flushed away; hence they are only useful for specific short-term experiments.

Finally, it should be pointed out that none of the authors who studied *Thiovulum* has been successful in using solid media. Also H_2S could not be replaced by thiosulfate, nor could nitrate be substituted for O_2.

State of Present Knowledge on *Thiovulum*

Besides the properties mentioned earlier, further characteristics of the organism have been determined by study of cell material derived from enrichments and from mass cultivation of purified material.

Thiovulum is Gram-negative and catalase-negative (la Rivière, 1965); the cells die quickly at high O_2 concentrations, as can be easily seen when they are trapped at the edges of a slide preparation. They are also immediately killed by anaerobic conditions. Its internal structure has been studied by electron microscopy by Fauré-Fremiet and Rouiller (1958), de Boer et al. (1961), Remsen and Watson (1972), and Wirsen and Jannasch (1978). Among its most striking features are Fauré-Fremiet's polar fibrillar organelle, the thin cell wall, and the slime excretion, which plays a role in structuring and maintaining the veils in which, nevertheless, the cells are in continuous motion. A discussion of the earlier results is presented by Starr and Skerman (1965).

Wirsen and Jannasch (1978) performed uptake experiments with CO_2 and some organic compounds labeled with $^{14}CO_2$ on highly purified cell suspensions, which permitted correction for uptake by contaminants. They showed substantial CO_2 uptake in *Thiovulum* which was optimal at H_2S concentrations of 1 mM in initially air-saturated seawater. No significant carbon uptake was registered for casamino acids, acetate, glutamate, mannitol, and some vitamins. These results provide strong evidence for the chemolithotrophic nature of the *Thiovulum* strains examined.

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CHAPTER 219

The Genus *Propionigenium*

BERNHARD SCHINK

The genus *Propionigenium* consists so far of one single species that comprises four strains of physiologically and morphologically similar isolates from various origins (Schink and Pfennig, 1982). This genus was created to house strictly anaerobic bacteria that are able to grow by decarboxylation of succinate to propionate. Enrichment cultures, which were set up originally to enrich for syntrophic succinate degraders from marine and freshwater sediments, developed unexpectedly fast growth of small, coccoid bacteria that did not depend on cooperation with hydrogen-scavenging partners and formed propionate as the sole fermentation product. Pure cultures could only be obtained with enrichment cultures from marine sources; the freshwater enrichments grew much slower, and pure cultures were finally isolated when the sodium chloride concentration of the medium was enhanced to 100–150 mM. This finding gave the first hint on a sodium dependence of this new type of energy conservation.

Succinate is formed in large amounts in the rumen of cows and sheep, but the animal host cannot take up this valuable fermentation product (Wolin, 1979). Conversion of succinate to propionate is therefore an important function of the rumen microflora, and succinate decarboxylation appears to be a rather common capacity of several propionate-forming fermenting bacteria, e.g., *Selenomonas* (Scheifinger and Wolin, 1973), *Veillonella* (Yousten and Delwiche, 1961), or certain *Propionibacterium* spp. (Yousten and Delwiche, 1961). Initially these conversions were regarded as "cometabolic" activities, which did not provide any advantage to the bacteria. Many years later, it was shown that the methylmalomyl-CoA decarboxylase of *Veillonella alcalescens* was a membrane-bound enzyme that coupled the decarboxylation reaction with the transfer of sodium ions across the cytoplasmic membrane (Hilpert and Dimroth, 1982). The sodium gradient thus established helps to provide energy for transport of dicarboxylic acids across the membranes, but it cannot supply energy for growth of these bacteria. It was quite a surprise, therefore, when a bacterium was isolated from sediments that can run its entire energy metabolism for growth on this decarboxylation reaction. This unusual capacity explains why these isolates were placed in a new taxonomic unit, apart from other propionate-forming bacteria.

Today we know of several other bacteria able to grow by conversion of succinate to propionate. Among these are Gram-negative mesophiles and also thermophiles (C. Guangsheng and A. Stams, unpublished observations). The coupling between sodium-extruding decarboxylation and ATP synthesis varies to some extent between these isolates, and the direct sodium coupling between a methylmalonyl CoA decarboxylase and a sodium-dependent ATPase as found in *Propionigenium modestum* (see below) is not the only solution to this problem. Further research in this area will discover whether *P. modestum* should be maintained in a genus of its own, or whether it should be associated with the genus *Veillonella* (Mays et al., 1982; Delwiche et al., 1985), with which it shares many morphological and physiological properties.

Habitats

P. modestum was originally isolated from a black, anoxic, marine sediment sample taken from the Grand Canal in Venice, Italy, where it occurred in numbers of 100 cells/ml sediment (Schink and Pfennig, 1982). Similar strains were isolated later from many other marine habitats and also from human saliva. Enrichments from freshwater sediments sometimes produced cells of similar morphology as the type strain GraSucc2, and could be cultivated only in media with enhanced (100–150 mM) sodium chloride concentrations. Later enrichment experiments with freshwater sediments from many different sites in Germany, Italy, and the USA yielded different, vibrioid, Gram-negative bacteria (Denger and Schink, 1990). No isolates able to grow with succinate as the sole energy

source have yet been obtained from rumen ecosystems.

It has to be assumed that anoxic marine sediments are the typical habitats of *P. modestum.* Its energy metabolism is based entirely on sodium ions as coupling ions in energy conservation. With this ability, it is well adapted to a marine environment. Many marine bacteria have recently been found to use sodium ions as energy couplers in various functions, e.g., respiration (Skulachev, 1985; Dibrov et al., 1986a) or motility (Brown et al., 1983; Dibrov et al., 1986b).

Isolation

Medium for Isolation and Cultivation

A strictly anoxic, sulfide-reduced mineral medium with 20-mM succinate as the sole organic carbon and energy source, and incubation at 27–30°C has proved to be highly selective for the enrichment of *P. modestum* if marine sediment samples of about 5-ml volume are used as the inoculum. The carbonate-buffered standard medium used for enrichment and isolation is described below in detail (after Widdel and Pfennig, 1981; Schink and Pfennig, 1982).

Carbonate-Buffered Standard Medium

Dissolve in 1 l of distilled water:

KH_2PO_4	0.2 g
NH_4Cl	0.5 g
NaCl	20.0 g
$MgCl_2 \cdot 6H_2O$	3.0 g
KCl	0.5 g
$CaCl_2 \cdot 2H_2O$	0.15 g

Autoclave the complete mineral medium in a vessel equipped with a filter inlet to allow flushing of the headspace with sterile oxygen-free gas and use screw cap inlets for addition of thermally unstable additives after autoclaving. Connect a silicon tubing from the bottom of the vessel to a dispensing tap (if possible with a protecting bell) for sterile dispensing of the medium (do not use latex tubing; it releases compounds that are highly toxic to many anaerobes). A stirring bar is also required.

After autoclaving, connect the vessel with the still hot medium to a line of oxygen-free nitrogen: carbon dioxide mixture (90% N_2:10% CO_2) at low pressure (<100 mbar), flush the headspace and cool it under this atmosphere to room temperature, perhaps with the help of a cooling water bath.

The mineral medium is amended with the following additions from stock solutions that have been sterilized separately (amounts per liter of medium): 30 ml of 1 M $NaHCO_3$ solution (autoclaved in a *tightly closed* screw cap bottle with about 30% headspace. The bottle should be autoclaved in a further protecting vessel, e.g., a polypropylene beaker, to avoid spills of carbonates if the bottle breaks in the autoclave); b) 2 ml of 0.5 M $Na_2S \cdot 9H_2O$ solution (autoclaved separately under oxygen-free gas atmosphere as above); c) 1 ml of trace element solution, e.g., SL 10 (Widdel et al., 1983); d) 0.5 ml of 10–fold concentrated, filter sterilized vitamin solution (Pfennig, 1978); and e) adequate amounts of sterile 1 M HCl or 1 M Na_2CO_3 to adjust the pH to 7.1–7.3.

The complete medium is dispensed into either screw-cap bottles or screw-cap tubes which are filled to the top, leaving a lentil-sized air bubble for pressure equilibration. Enrichment cultures usually produce gas in the first enrichment stages and are better cultivated in half-filled serum bottles (50–100 ml volume) under a headspace of nitrogen: carbon dioxide mixture (90% N_2:10% CO_2). The vitamin mixture is not needed by all strains.

For mass cultivation of *P. modestum* strain GraSucc2 in fermenters, a phosphate-buffered medium is easier to handle. The following medium has been applied successfully (Hilpert et al., 1984).

Phosphate-Buffered Medium

Distilled water	940 ml
KH_2PO_4 (= 30 mM)	4.2 g
NH_4Cl	0.5 g
NaCl	20.0 g
$MgCl_2 \cdot 6H_2O$	3.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g

Add sulfide, trace elements, and vitamins as above, as well as 60 ml of a neutralized 1 M sodium succinate solution, and adjust the pH to 7.0 before inoculation.

Isolation of Pure Cultures

After two to three transfers in liquid medium, gas should no longer be formed by the enrichment cultures, and a dominant population of short, coccoid rods should be established. These bacteria can be isolated easily in anoxic agar deep dilution series (Pfennig, 1978) or in roll tubes (Balch et al., 1979). Streaking on petri dishes in an anoxic glove box has not yet been tried with these bacteria, but there is no reason why this method should not be applicable as well. Preparation of pure cultures requires two subsequent dilution series; purity should be checked after growth in selective mineral medium as well in complex medium, e.g., AC medium (Difco Laboratories, Ann Arbor, MI, USA). AC medium prepared according to the original recipe appears to inhibit several sediment bacteria. Therefore, a 1:10 or 1:5 diluted variation (in sea water as well as in freshwater medium, with corrected agar concentration) of this medium should also be used to ensure that the cultures are pure.

Identification and Further Properties

In phase contrast microscopy, cells of *P. modestum* appear as short, coccoid rods with a diameter of 0.5–0.6 μm and a length of 0.5–2.0 μm, often in short chains (Fig. 1). They are Gram negative and do not form spores. The GC content of the DNA is in the range of 32–36 mol%.

P. modestum is strictly anaerobic and does not stand increased oxygen tensions. The optimum temperature for growth is 30–33°C and under these conditions, doubling times of 2.5–4.5/h are obtained, depending on whether yeast extract (0.1% wt/vol) is provided as source for assimilatory metabolism. Yeast extract is not required and does not support the energy metabolism, but it enhances the growth yields by about 35%.

In addition to succinate, fumarate and pyruvate also support growth and are fermented stoichiometrically to acetate and propionate. No sugars, alcohols, or any other organic substrates are utilized. No cytochromes have been detected, which is consistent with the absence of electron transport phosphorylation. The growth yields with fumarate and pyruvate are small (3.8 and 6.2 g/mol on fumarate and pyruvate, respectively.

The biochemical basis of energy conservation with succinate as sole substrate has been studied in detail (Hilpert et al., 1984). Succinate exchanges in the cell with propionyl-CoA to form succinyl-CoA and free propionate, which leaves the cell. Succinyl-CoA is rearranged to methylmalonyl-CoA and decarboxylated by a membrane-bound decarboxylase that couples this reaction with the transport of sodium ions across the cytoplasmic membrane. The sodium ion gradient thus established, drives ATP synthesis via a membrane-bound, sodium-dependent ATP synthase. The latter enzyme has been characterized thoroughly (Laubinger and Dimroth, 1987; Laubinger and Dimroth, 1988). It is composed of an F_1 and an F_0 moiety, both of which have subunits comparable to those of a "classical" bacterial proton ATPase. The enzyme of *P. modestum* can also pump protons across the membrane, but only if the sodium concentration is very low (< 1 nM). Under physiological conditions, ATP synthesis is driven entirely by sodium ions.

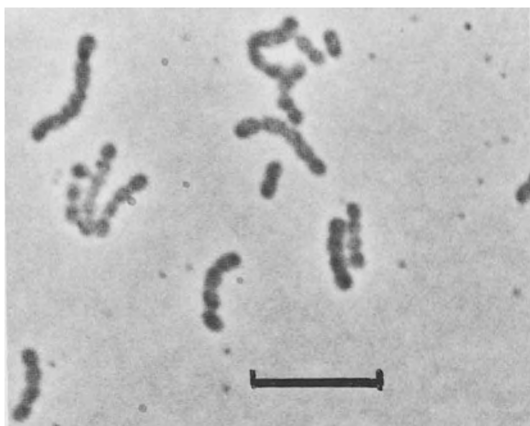

Fig. 1. Phase contrast photomicrograph of cells of *Propionigenium modestum* GraSucc2 (type strain) after growth with 20 mM of succinate. Bar = 5 μm.

This unusual coupling of decarboxylation and ATP synthesis was the first case of an energy metabolism found that was based entirely on sodium as coupling ions, and which did not involve classical substrate-linked phosphorylation or electron transport phosphorylation steps. Today we know of several more cases of bacteria that can base their total energy metabolism on such decarboxylation reactions. The biochemical basis for energy conservation may be quite different in each case: e.g., anaerobic bacteria that ferment oxalate to formate and carbon dioxide employ a decarboxylase enzyme which is soluble in the cytoplasm, and energy conservation occurs via substrate import and product export by an antiporter system (Anantharam et al., 1989).

Conservation of decarboxylation energy is of special interest because the free energy change of such reactions is small and yields only about 20–25 kJ/mol. If such reactions drive ATP synthesis (free energy expense 70–75 kJ/mol ATP; Thauer et al., 1977), several decarboxylation reactions have to be coupled with one ATP synthesis reaction. Although the exact stoichiometries of sodium ion translocation by the methylmalonyl-CoA decarboxylase and the ATP synthase have not yet been determined, it is obvious from the free energy calculations that at least three decarboxylations are necessary to allow synthesis of one ATP. This calculation agrees well with the cell yield obtained with *P. modestum* GraSucc2 (2.0–2.5 g/mol succinate). Bacteria that base their whole energy metabolism on a decarboxylation reaction represent the most "modest" ways of energy conservation, i.e., they operate with the lowest amount of energy a living cell can convert into ATP (Thauer and Morris, 1984).

Applications

So far there is no commercial application for *P. modestum*. The fact that its unique energy conservation mechanism can also be reversed and

allows carboxylation reactions without the expense of ATP (Dimroth and Hilpert, 1984), can possibly render such systems as interesting for new ways of biochemical syntheses.

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CHAPTER 220

The Genus *Zoogloea*

PATRICK R. DUGAN, DAPHNE L. STONER, and HARVEY M. PICKRUM

Bacteria of the genus *Zoogloea* have historically been considered members of the family Pseudomonadaceae but have been differentiated from other obligately aerobic, Gram-negative, nonsporeforming, rod-shaped bacteria that grow in aquatic habitats on the basis of their production of a characteristic gelatinous matrix. The "zoogloeal matrix" surrounds clumps of cells found in natural aquatic habitats or when grown in unshaken liquid culture in the laboratory (Butterfield, 1935; Butterfield et al., 1937; Wattie, 1943). The name *Zoogloea,* which was derived from the Greek word meaning animal glue, refers to the primary trait, the zoogloeal matrix, that is used to distinguish *Zoogloea* from other metabolically similar bacteria.

The two species of *Zoogloea, Z. ramigera* and *Z. filipendula,* have been isolated from sewage or from sewage-treatment systems that employ oxidative methods, i.e., activated sludge systems, trickling filters, or oxidation ponds (Butterfield et al., 1937; Butterfield and Wattie, 1941; McKinney and Horwood, 1952; McKinney and Weichlein, 1953). Both species produce branched, finger-like projections or outgrowths from the floc (i.e., clumps of cells that grow in an aggregated form that settles or "flocs" from aqueous suspension) when grown under quiescent culture conditions but have been differentiated from each other on the basis of cell size, biochemical reactions, and appearance of the zoogloeal matrix.

Characteristics of the Zoogloeal Matrix

The zoogloeal matrix is a capsular envelopment that surrounds several cells and commonly results in a flocculent growth habit in liquid media. That is, cell flocs settle from suspension, leaving a relatively clear supernatant. Unz and coworkers (Unz, 1974; Farrah and Unz, 1975; Unz and Farrah, 1976a) have convincingly demonstrated that floc formers of the *Zoogloea* type grow in an aggregated state and that the individual cells are entrapped within the zoogloeal matrix while cell division occurs. Floc formation of the zoogloeal type implies cell growth in an aggregated form (Friedman and Dugan, 1968b), whereas the term "flocculation" (Peter and Wuhrmann, 1971) has been defined as the aggregation of suspended bacterial cells after growth of cells has occurred, i.e., the "flocculation" of colloidal material. Biologically, the two processes are considerably different although the same physical and chemical laws apply to both.

Although there can be confusion relative to the distinction between zoogloea formation and flocculent growth, there is a consensus that microbial floc formation results from the presence of adherent extracellular fibrils (Busch and Stumm, 1968; Deinema and Zevenhuizen, 1971; Finstein, 1967; Friedman and Dugan, 1968b; Friedman et al., 1968, 1969; Tago and Aida, 1977). The presence of polysaccharide and the ability to flocculate has been demonstrated with mutants lacking polysaccharide and which do not exhibit flocculent growth; recombinant bacteria with restored polysaccharide production also simultaneously show restored floc formation (Easson et al., 1987a, 1987b). All floc-forming bacteria appear to posses extracellular fibrillar strands, but not all Gram-negative floc-forming bacteria produce the characteristic zoogloeal matrix, such as that for *Zoogloea ramigera* strain 115 seen in Figs. 1 and 2.

The polysaccharide of *Zoogloea* may remain as a loose slime layer in the vicinity of the cells, a well-defined capsule, or a zoogloeal matrix around the cells which synthesized it or it may be dispersed in the medium as a colloidal suspension or in solution. Increased viscosity of the surrounding medium may result from extensive polymer synthesis by the cells. The key feature in bioflocculation appears to be the synthesis of relatively insoluble extracellular polymer strands that remain within the vicinity of cells and do not disperse. The photographs

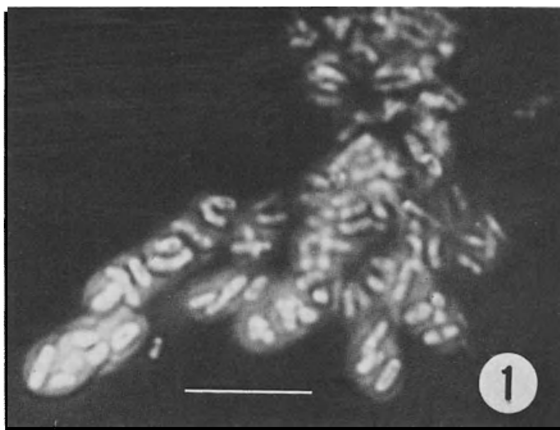

Fig. 1. Photomicrograph of *Zoogloea ramigera* strain 115 floc negatively stained by the Maneval method and photographed through the light microscope. The cells are shown embedded within the zoogleal matrix and with the characteristic finger-link projections. Bar = 10 μm.

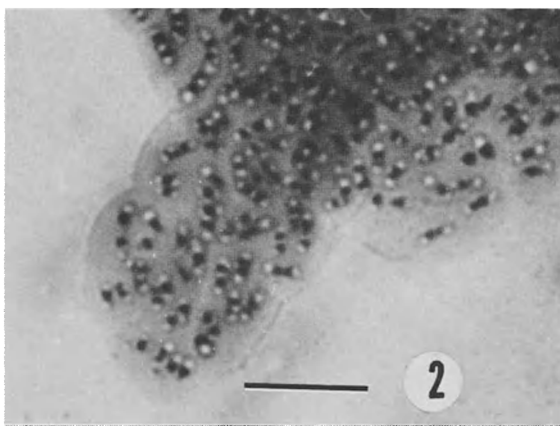

Fig. 2. Photomicrograph of *Zoogloea ramigera* 115 floc stained with 1% aqueous crystal violet. The gelatinous zoogloeal matrix in which cells are embedded is shown. The variable staining appearance is due to polybetahydroxy butyric acid (PHB) granules that accumulate in the cells. Bar = 10 μm.

shown in Figs. 3, 4, 5, 6, and 7 show the insoluble polysaccharide strands found around flocs of these bacteria. The polymer strands shown in Fig. 6 were photographed under ultraviolet illumination after the flocs were stained with the fluorescent dye Paper White-BP as previously reported (Dugan and Pickrum, 1973; Friedman et al., 1969).

The chemical composition and structure of the extracellular polysaccharides determine its physical and chemical properties. Polysaccharides in solution or suspension vary in water solubility, rheological properties (e.g., viscosity, viscoelasticity), chemical and thermal stability, surfactant properties, the ability to bind water, etc. *Zoogloea* strains vary in the composition of the in polysaccharide (Table 1), but information concerning the structure of some isolates is limited.

The polysaccharide of *Z. ramigera* 115 has been examined in detail. This weakly acidic polysaccharide is slightly soluble in water, stable in a pH range of 3 to 10, and is not precipitated in the presence of salt (Stauffer et al., 1980). Stauffer et al. (1980) reported that the polysacchride of *Z. ramigera* 115 is stable to temperature cycling from $-15°$ to 90°C. However, Norberg and Enfors (1982) reported that the first temperature cycle from 25° to 90°C did increase the viscosity of the polymer at the lower temperature. The polysaccharide of *Z. ramigera* 115 is highly viscous and lowers the surface tension of water. The polysaccharide exhibits Newtonian flow at lower concentrations and, as the concentration increases, the polymer becomes increasingly pseudoplastic and viscoelastic (Stauffer et al., 1980). Similar flow behavior was observed in relation to growth and polysaccharide production in a fermentor (Norberg and Enfors, 1982).

The polysaccharide of *Z. ramigera* 115 contains glucose, galactose, and pyruvic acid, the ratios of which may vary with culture conditions and growth phase of the culture (Norberg and Enfors 1982; Franzen and Norberg, 1984; Ikeda et al., 1982). Growth on different sugar substrates did not alter the composition of sugars in the polymers (Parsons and Dugan, 1971). A ratio of glucose:galactose of 2:1 with trace amounts of terminal pyruvylated glucose (Franzen and Norberg, 1984; Friedman et al., 1969; Parsons and Dugan, 1971; Sinskey et al., 1986) as well as a ratio of 11.0:3.1:1.5 for glucose:galactose:pyruvic acid (Ikeda et al., 1982) has been determined. Uronic acids, pentose, or amino sugars have not been detected in the polysaccharide of *Z. ramigera* 115 (Franzen and Norberg, 1984; Friedman, et al. 1969; Ikeda et al., 1982). The polysaccharide of *Z. ramigera* 115 is a highly branched structure with β-1-4-linkages and a molecular weight of approximately 10^5 (Friedman et al., 1968, 1969; Ikeda et al., 1982;). A structure with repeating units has been proposed (Ikeda et al., 1982), although there is conflicting evidence that the polysaccharide of *Z. ramigera* 115 has an irregular structure (Franzen and Norberg, 1984).

A variety of sugars have been identified as components of the polysaccharides of *Zoogloea* cultures (Table 1). Polymer from *Z. ramigera* MP6 contains the amino sugars, glucosamine, and possibly methyl-pentose amine, hexose

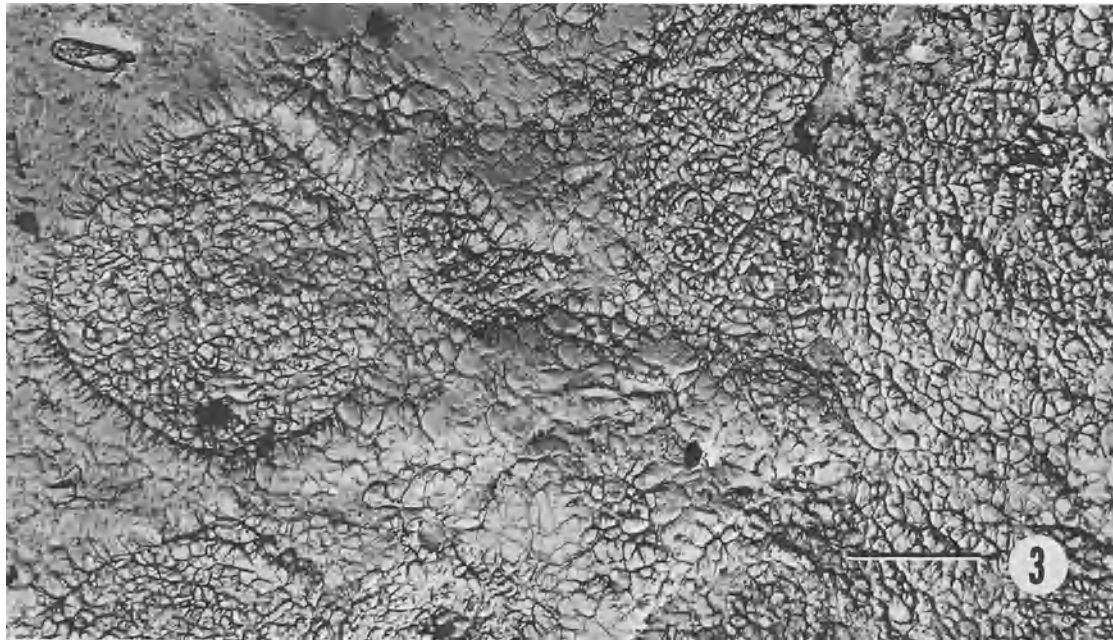

Fig. 3. Electron micrograph of a freeze-fractured specimen of *Zoogloea ramigera* 115. The fibrillar network of extracellular polysaccharide strands from which the zoogloeal matrix is formed is shown. In the living specimen, water is bound in the interstices of the network, which resembles a ball of chicken wire. Bar = 10 μm.

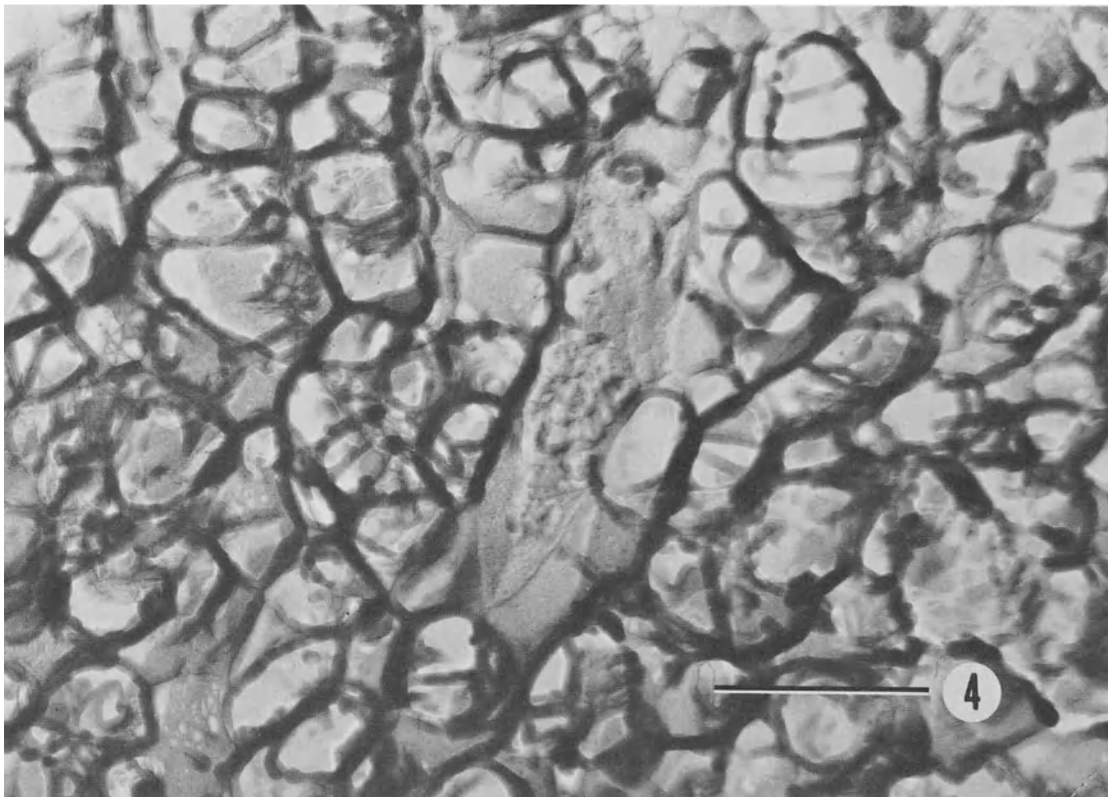

Fig. 4. Enlargement of the floc shown in Fig. 3. A cell embedded within the zoogloeal matrix is shown. Bar = 10 μm.

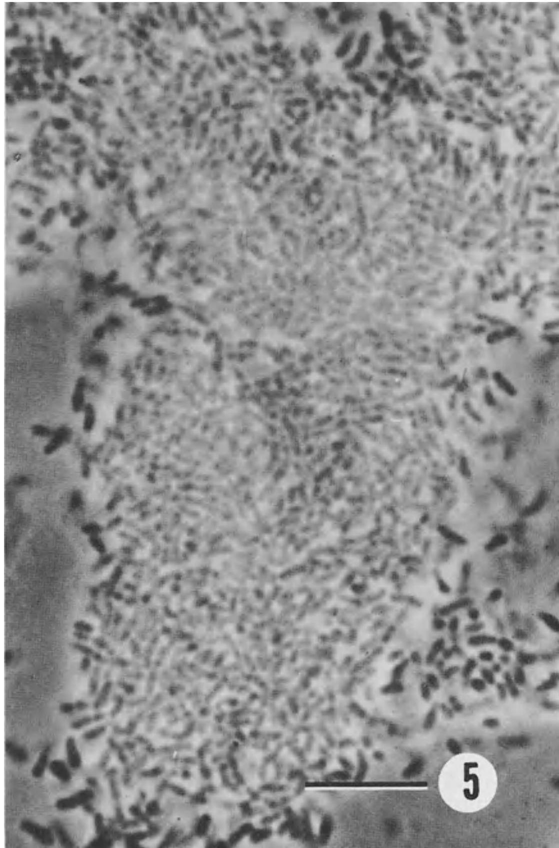

Fig. 5. Photomicrograph of a floc of the *Zoogloea* isolate designated CT-2 originally isolated by K. Crabtree. The floc was stained by a water-clear solution of the fluorescent dye Paper White-BP, and photographed under phase-contrast optics. Some of the bacteria are in the focal plane whereas others are not. Bar = 10 μm.

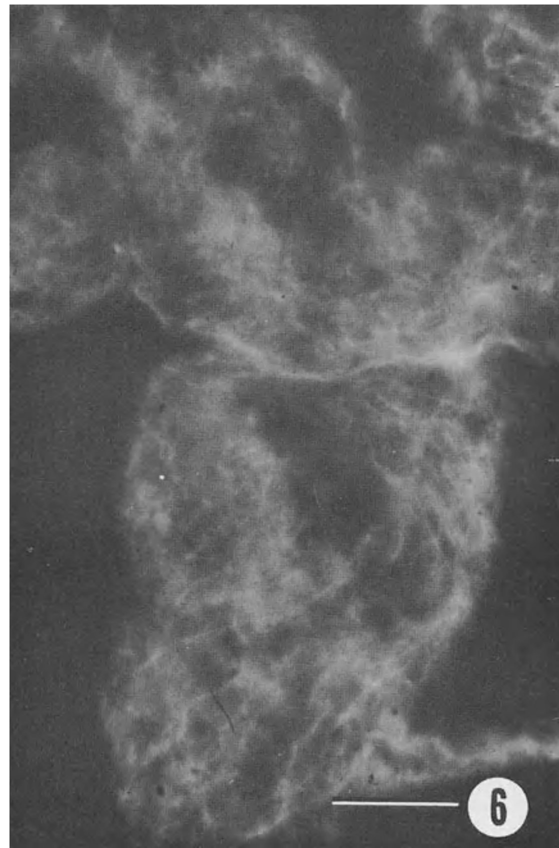

Fig. 6. Photomicrograph taken under ultraviolet (UV) illumination of the identical field shown in Fig. 5. Only material stained by the polysaccharide-specific fluorescent stain Paper White-BP can be seen under UV illumination. This procedure demonstrates that the cell floc shown in Fig. 5 was interspersed by a network of fibrous polysaccharide strands analogous to a "cobweb" which appears to hold the floc intact. Bar = 10 μm.

sugars, and uronic acid (Farrah and Unz, 1976). Tezuka (1973) identified glucosamine and possibly fucosamine as components of the polymer of a *Zoogloea* culture isolated from activated sludge, but did not detect neutral sugars or uronic acids. Polymer of *Z. ramigera* NRRL B-3669M contains glucose, mannose, and galactose, while isolate NRRL B-3793 contained rhamnose, mannose, and possibly galactose (Wallen and Davis, 1972). *Z. filipendula* P-8-4 and *Z. ramigera* contain glucose, galactose, and mannose in the polysaccharide, while an unidentified *Zoogloea* species designated C-1 contained glucose, galactose, mannose, arabinose, and rhamnose (Pickrum, 1972). It is possible that those isolates that have been shown to produce extracellular polysaccharides with only glucose, galactose, and mannose are more closely related to *Pseudomonas dentrificans* than to the *Z. ramigera* 115 isolate (see Table 1). Research on *Zoogloea* isolates has increased significantly because of its potential use in applications for removal of hazardous metal contaminants from waste streams.

Habitats

Zoogloea species have been isolated from organically enriched oxygenated water, particularly domestic sewage and aerobic sewage-treatment systems, such as trickling filters, activated sludge tanks, or oxidation ponds (Amin and Ganapati, 1967; Dugan and Lundgren, 1960; Unz and Farrah, 1972). These are all continuous-flow systems that are rich in dissolved or particulate oxidizable organic materials, which provide for the enrichment of *Zoogloea* and closely related bacteria. Zoogloeal masses may also be found adhering to solid objects suspended or floating in lakes and ponds. For example, 19 of 36 Gram-negative bacteria isolated

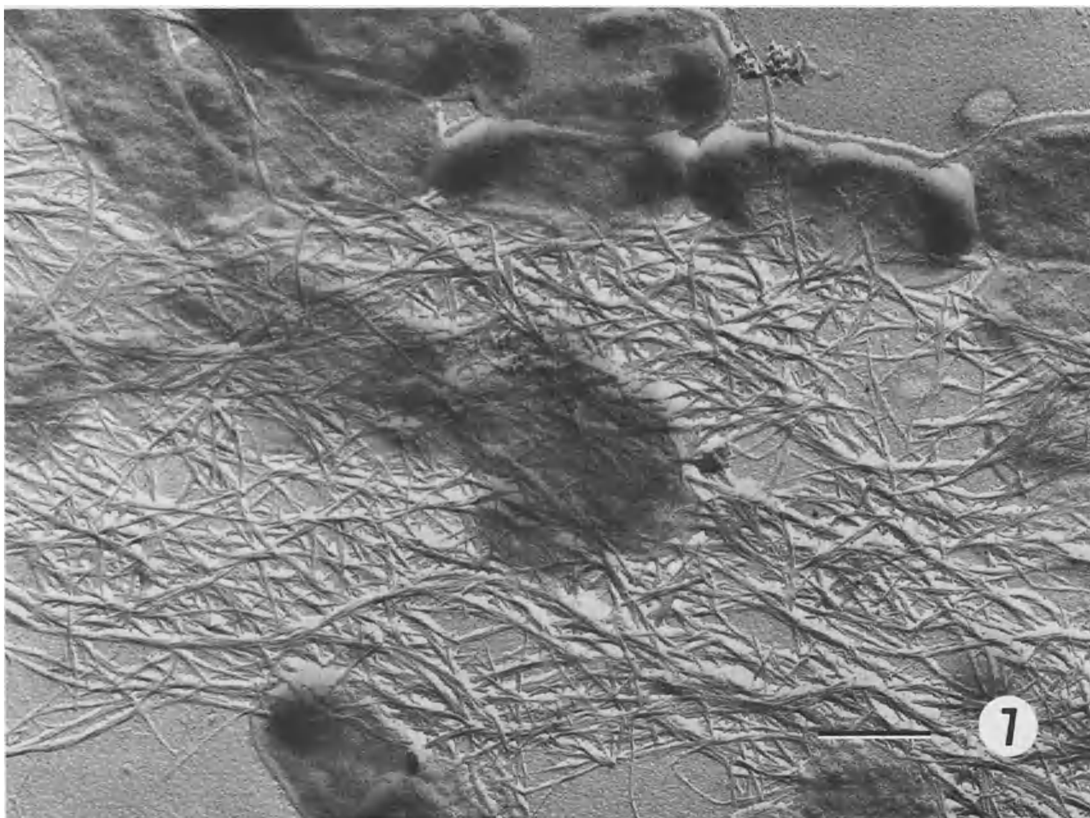

Fig. 7. Shadow-cast electron micrograph of *Zoogloea ramigera* isolate I-16-M after treatment with 1 N NaOH for 24 h at 28°C. Extracellular fibrillar strands and cell ghosts are shown. I-16-M produces flocs but no observable gelatinous zoogloeal matrix. Bar = 10 μm.

from Lake Erie formed flocs in the laboratory, and they all clustered with previously identified isolates of *Zoogloea* when subjected to a computer taxonomic program. Therefore, *Zoogloea* appears to be ubiquitous in aquatic environments.

Isolation

Zoogloeal masses obtained from organically rich natural aquatic systems, sewage treatment plants, wastewater, etc., may be used in isolation procedures to obtain pure cultures of *Zoogloea.* These organically rich sources may also be used to inoculate enrichment media from which strains of *Zoogloea* may be isolated. Both the zoogloeal matrix found around the characteristic isolates of *Zoogloea* and the nongelatinous fibrillar strands associated with the nonzoogloeal *Zoogloea* isolates are a deterrent to pure-culture isolation because other bacteria adhere to or become entangled within the floc. Successful isolation of *Zoogloea* has been achieved with methods employing ultrasonic methods to disrupt zoogloeal masses, micromanipulation to manually separate individual cells from the zooglea matrix, the inclusion of inhibitory agents in culture media to prevent the growth of unwanted microorganism and the use of carbon growth substrates which may enrich for *Zoogloea.* These methods are used in conjunction with conventional isolation precedures to obtain isolates of *Zoogloea.*

Enrichment

Several methods can be used to establish enrichments of zoogloeal bacteria. The simplest method is the incubation of raw sewage, sewage sludge, treated sewage effluent etc., in containers at room temperature until a surface film or pellicle is formed (Amin and Ganapati, 1967). Alternatively, mineral salts solution overlaying a nutritive agar plug may be inoculated and incubated until a surface pellicle develops (Unz, 1984).

Mineral Salts Solution

The overlay medium is prepared from stock solutions and adjusted to pH 8.5 with 0.5 N NaOH. The mineral salts solution contains:

Table 1. Composition of purified extracellular polysaccharides of *Zoogloea*.

Strain	Composition	Reference
Z. ramigera 115	Glucose, galactose, pyruvic acid	Ikeda et al., 1982; Franzen and Norberg, 1984
Z. ramigera I-16-M	Glucose, galactose, mannose	Pickrum, 1972
Z. ramigera MP6	Glucosamine, methylpentose amine, hexose sugars, uronic acids	Farrah and Unz, 1976
Z. ramigera NRRL B-3669M	Glucose, galactose, mannose	Wallen and Davis, 1972
Zoogloea species NRRL B-3793	Rhamnose, mannose, galactose	Wallen and Davis, 1972
Zoogloea species C-1	Glucose, galactose, mannose, arabinose, rhamnose	Pickrum, 1972
Zoogloea species	Glucosamine, fucosamine	Tezuka, 1973
Z. filipendula P-8-4	Glucose, galactose, mannose	Pickrum, 1972
Zoogloea isolate P-95-5[a]	Glucose, galactose, mannose	Crabtree et al., 1965; Crabtree and McCoy, 1967

[a]The isolate is probably actually *Pseudomonas denitrificans*.

$(NH_4)_2SO_4$	0.3 g
NaCl	5.85 g
$CaCl_2 \cdot 2H_2O$	0.2 g
K_2HPO_4	0.1 g
$MgSO_4 \cdot 7H_2O$	0.14 g
$FeSO_4 \cdot 7H_2O$	0.3 mg
$MnCl_2 \cdot 4H_2O$	6.3 mg
$CoSO_4 \cdot 7H_2O$	0.11 mg
H_3BO_4	0.6 mg
$ZnCl_2$	0.22 mg
$CuSO_4 \cdot 5H_2O$	0.08 mg
Distilled water	1 liter

Nutrient-Enriched Agar

The nutritive agar contains 20 g agar per liter of mineral salts solution. The carbon source is any *one* of the following added per liter of mineral salts.

Starch	2.4 g
m-Toluic acid	1.35 g (neutralized)
n-butanol	1.5 ml
Lactic acid (85%)	1.35 g
Ethanol (95%)	1.5 ml
Glucose	2.4 g

After the addition of the carbon source, the pH is adjusted to 8.5 with 0.5 N NaOH.

Isolation

Isolations may be made from surfaces covered with zoogloeal masses and/or effluents taken from organically polluted water. Material on surfaces is scraped off and placed into 0.05% proteose peptone-yeast extract broth (PPYE). The gelatinous material is disrupted by ultrasound (20 kc output, Branson) in sterile PPYE, samples serially diluted and incubated in PPYE (28°C). After incubation for 3 days, the highest dilutions showing growth (10^{-7} to 10^{-10}) are streaked onto PPYE agar and tryptone-glucose extract agar (TGE, Difco). When sufficient growth has occurred, colonies of different morphological types are transferred to duplicate tubes of PPYE broth. Tubes are incubated with and without agitation. After incubation, tubes containing a pellicle (stationary) or a floc (shaken) are selected, and the entire isolation procedure is repeated until pure cultures are obtained. In general, isolations can be most readily achieved with the use of organically dilute media.

Micromanipulation may be used to separate bacterial cells from zoogloeal matrices in order to obtain pure cultures of *Zoogloea* (Unz and Dondero, 1967a). In this method, flocs or slimes from enrichment cultures are dispersed in liquid medium and observed at 100× magnification. To remove debris and microorganisms, fingered zoogloeal projections are transferred individually by micropipettes through successive drops of medium. Washed zoogloea are placed upon nutritive agar films coated on the underside of coverslips and individual cells dissected away from the zoogloeal matrix with microneedles. Slides are incubated and the developed microcolonies transferred with sterile micropipettes to broth medium. Alternatively, washed zoogloea may be collected and briefly sonicated prior to streaking on growth medium (Unz, 1984). Media appropriate for the above methods are casitone-yeast (CY) broth (see below), nutrient broth, and the respective agar media (Unz, 1984; Unz and Dondero, 1967a).

CY Medium

CY medium contains per liter of distilled water:

Casitone	5.0 g
Yeast autolysate	1.0 g

The addition of 0.001% crystal violet to isolation media, either broth or agar, has also been used successfully to inhibit many undesirable bacteria that tend to adhere to the flocs. Agar

plates that contain crystal violet in the growth medium also have differential value. On these plates, the isolates listed in Table 1 can be identified as follows: *Z. ramigera* 115 decolorizes and degrades crystal violet and forms straw-colored colonies; isolate C-1 fails to develop colonies; and isolates I-16-M, P-8-4, and P-95-5 absorb the dye and produce violet colonies. Since isolate C-1 is different from the known *Zoogloea* isolates, the use of the crystal violet is considered to have value in the identification of *Zoogloea* species, but such use has not been well studied (Friedman and Dugan, 1968b; Dugan, 1975).

Aromatic compounds utilized for growth may be used for the isolation of some *Zoogloea* from environmental samples (Unz and Farrah, 1972). *Zooglea* isolates may be obtained from activated sludge using solid basal medium with 1% *m*-toluate as the carbon source by streaking sludge or other inocula directly on the media and incubating. Solid samples, i.e., soils and feces, may be suspended with equal volumes of sterile distilled water then streaked onto media. Developed colonies are transferred to CY medium (see above) disrupted by agitation, and streaked for isolation on *m*-toluate medium. *Zoogloea* grown on *m*-toluate medium form large colonies with yellow-green coloration indicative of *meta* cleavage products. Colonies are usually difficult to remove from the surface of the plates. This procedure will not isolate *Zoogloea* that do not utilize *m*-toluate as a growth substrate.

Zoogloea colonies on agar plates have a glistening, viscous appearance (Fig. 8). Colonies are straw-colored on media containing yeast extract or protcose-peptone and white on mineral salts media supplemented with alanine and argenine as the carbon source. The color differences are also observed in liquid media. Colonies have a leathery consistency, and the entire colony can frequently be removed with an inoculating loop (Dugan and Lundgren, 1960; Friedman and Dugan, 1968b). This distinctive characteristic is probably related to the formation of extracellular polysaccharide. Growth of *Zoogloea* in shaken liquid culture is often flocculent but flocs are tight and do not have a typical zoogloeal matrix.

Identification

As the name of the genus implies, *Zoogloea* species have been identified primarily on the basis of the presence of the characteristic extracellular, capsular, or zoogloeal, matrix. However, matrix formation and its morphological appearance may be controlled nutritionally by the carbon and nitrogen sources, as well as by the carbon to nitrogen ratio, and by the turbulence of the growth medium (Parsons and Dugan, 1971; Unz and Farrah, 1976b). Therefore, the presence or absence of a zoogloeal matrix is not a trait upon which identification can be reliably made. Although the presence of finger-like projections and a zoogloeal matrix is distinctive and a positive indicator, the absence of the zoogloeal matrix does not necessarily indicate that the isolate is not a *Zoogloea* species.

Isolates of *Zoogloea* are Gram-negative, polarly flagellated, rod-shaped bacteria that flocculate and accumulate poly-β-hydroxybutyrate (PHB) or polyalkanoic granules when cultivated in media that are rich in carbon. Cell size ranges from 0.5 to 1.3 μm in diameter and 1.0 to 3.6 μm in length. *Zoogloea* species are oxidative in metabolism and are able to utilize a variety of sugars, sugar alcohols, alcohols, amino acids, and proteins for growth. The inclusion of B vitamins, purines, pyrimidines, and nucleotides in culture media, although not required for growth, decreases the growth lag and increases biomass formation of cultures (Dugan and Lundgren, 1960; Friedman and Dugan, 1968b). The metabolic characteristics of the organisms are not sufficiently distinct to be the basis of identification for the genus *Zoogloea* (Crabtree and McCoy, 1967; Crabtree et al., 1965; Dugan, 1975; Dugan and Lundgren, 1960; Friedman and Dugan, 1968b; Ganapati et al., 1967; Unz, 1974; Unz and Dondero, 1967a, 1967b). However, the overall ability of *Zoogloea* species to oxidatively metabolize many sugars, sugar alcohols, and alcohols, with the formation of esters that often result in the typical fruity odor of esters in culture media, can be of use in restricting the options in identification (Joyce and Dugan, 1972). The strong capability of *Z. ramigera* 115 to form esters from a variety of organic acids and alcohols may be responsible for the addition of pyruvic acid to alcoholic groups on the extracellular zooglocal polymer and may explain why some investigators were unable to observe pyruvate (Friedman et. al., 1969), whereas others observed traces (Norberg, 1984) and others identified a significant amount (Ikeda et al. 1982).

The taxonomy of the genus *Zoogloea* is uncertain with respect to differentiation from other genera within the family Pseudomonadaceae. Also uncertain is whether *Z. filipendula,* a species historically considered as a member of the genus *Zoogloea* validly fits in the genus. The designation of the neotype strain of *Zoogloea ramigera* has also been questionable (Unz, 1984). Some *Zoogloea* isolates re-

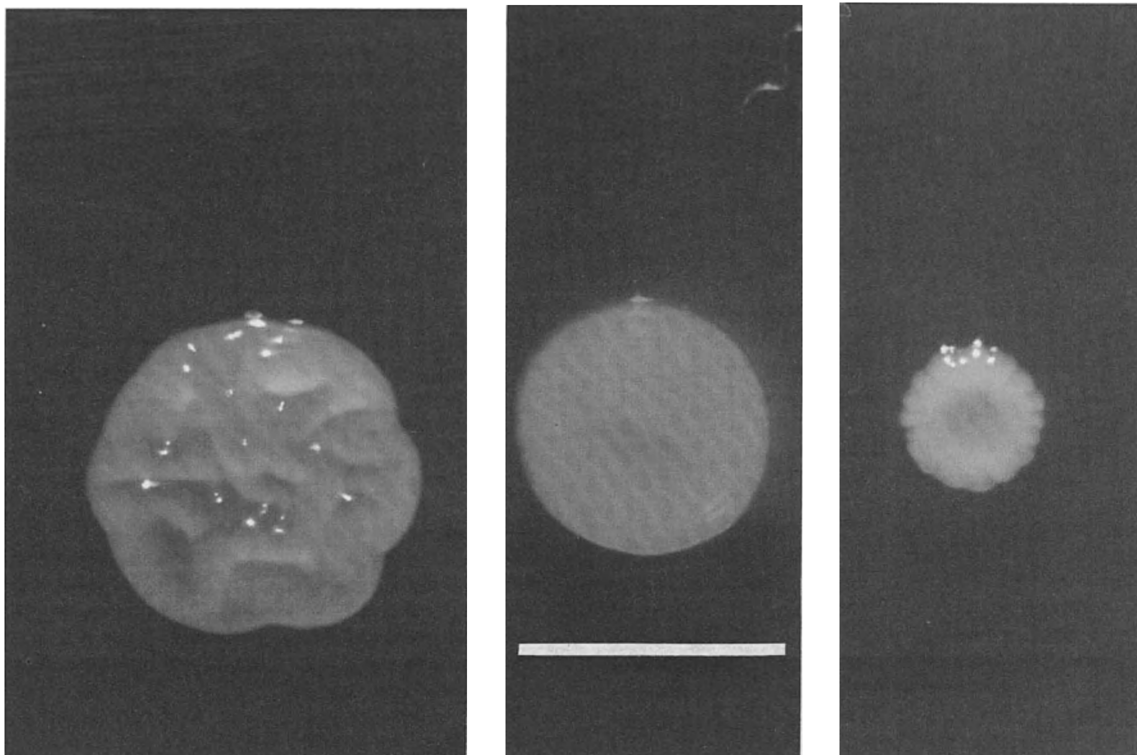

Fig. 8. Colonies of *Zoogloea ramigera* 115 vary in overall appearance and size. Variation is due to culture conditions and age of culture. Bar = 5 mm.

semble species of the genera *Pseudomonas* (particularly *P. denitrificans*) *Gluconobacter*, and *Acetobacter*. *Zoogloea* differ from *Gluconobacter* and *Acetobacter* in the inability to grow at pH 4.5.

Antigenic relationships among some of the *Zoogloea* have been reported (Chorpenning et al., 1978). *Z. ramigera* 115 and I-16-M did not closely relate to *Z. filipendula* P-8-4 on a serologic basis. *Z. ramigera* 115 had greater antigenic relatedness to *Gluconobacter oxydans* subspecies *suboxydans* (ATCC 621) than to either *Z. ramigera* I-16-M or *Z. filipendula* P-8-4. These data indicated that *Z. ramigera* 115 and I-16-M are distinctly different serovars, an observation which is also supported by the difference in composition of their extracellular polysaccharides.

The GC content of the DNA of *Zoogloea* ranged from 60.1 to 64.8 mol% (Pickrum, 1972; Unz, 1984). The data, although not conclusive, reinforce the view that several of the floc-forming isolates are similar organisms that are sufficiently distinctive to allow differentiation from other Gram-negative rods. However, at present there is no definitive description of either the genus *Zoogloea* or its species, other than the gelatinous matrix of finger-like projections, but this matrix is not always present.

Genetics

Because of the potential application of *Zoogloea* in several microbially based technologies, there is a need to isolate and identify genes for polysaccharide and PHB production, determine the biosynthetic pathways, and examine the enzymatic and genetic control mechanisms.

The exopolysaccharides of *Zoogloea* are involved with flocculation (Easson et al., 1987a, 1987b; Friedman et al., 1969) and adsorption of heavy metals (Dugan, 1987; Dugan and Pickrum, 1972; Ikeda et al., 1982). As with other polymers, the structure and physical and chemical properties of polysaccharides define its flocculation and adsorption characteristics. Therefore, manipulation of the structure of the polysaccharide is of importance in developing flocculants and bioadsorbents with desired characteristics that may be applicable to microbially based technologies for waste water treatment or metal reclamation. Genetic control of the biosynthesis of polysaccharides by microorganisms is a means by which the structure of the polymers may be manipulated and production of the polysaccharides increased.

Mutants generated by the insertion of the Tn5 transposon were used to examine the genetics

of polysaccharide production in *Z. ramigera* I-16-M (Easson et al., 1987a, 1987b). There is an inherent instability in the chromosome in which DNA sequences spontaneously delete within the polysaccharide gene region, and this results in the loss of polysaccharide production. The frequency of chromosomal deletions within this gene region was enhanced by the presence of Tn*5* transposon. Polysaccharide production was restored in the mutants by complementation with a gene library constructed with a broad-host-range cosmid vector. The restoration of polysaccharide production in a number of mutants by a single plasmid vector suggests that the genes for polysaccharide production may be clustered on the chromosome.

The genetics involved with PHB synthesis is of interest because of the potential use of PHB and other polydroxyalkanoates in the plastics industry. PHB metabolism in *Z. ramigera* I-16-Mis a cyclic process (Tomita et al., 1983). The biosynthesis of PHB involves the condensation and reduction of acetyl CoA to form D(−)-3-hydroxybutyryl CoA followed by incorporation into PHB granules. Degradation of PHB granules proceeds via the production of D(−)-3-hydroxybutyrate oligomers and hydrolysis and oxidation to acetoacetate. The cycle is completed with the activation of acetoacetate to acetoacetyl CoA.

Studies examining the structure and function of enzymes in PHB synthesis (Davis et al., 1987a, 1987b; Fukui et al., 1976; Nishimura et al., 1978; Saito et al., 1977) and genetic experiments to determine the sequence and location of genes (Peoples et al., 1987) have focused on the enzyme β-ketothiolase, which mediates the first step in the biosynthesis of PHB, the condensation of two acetyl CoA units to form acetoacetyl CoA. A recombinant DNA clone designated LDBK1 expresses the complete thiolase gene sequence in *Escherichia coli* (Peoples et al., 1987). The encoded protein has thiolase enzyme activity and co-migrates with native enzyme in Western blotting and immunodetection experiments. The expression of thiolase-coding sequences is under transcriptional control of the *lac* promoter region contained in the λgtll expression vector that was used to generate the recombinant library of *Z. ramigera* DNA. The structural gene for the thiolase enzyme is 1,173 nucleotides long and codes for a protein of 391 amino acids. The calculated molecular weight and the predicted amino acid sequence of the gene product are in agreement with the molecular weight and amino acid sequence determined experimentally for the native thiolase. The high 6c content of the complete sequence (66.2 mol%) is in agreement with values obtained for the *Z. ramigera* genome (64.5 mol%). The position of the active site peptide, a potential ribosome binding site, and start codons within the DNA sequence have all been identified.

The major DNA-dependent deoxyribonucleic acid polymerase from *Z. ramigera* isolate I-16-Mhas been partially purified and biochemically characterized (Pickrum, 1975). The polymerase activity was eluted as a single peak at 0.1 M potassium phosphate from a DEAE-cellulose column. The partially purified enzyme showed one major, one intermediate, and five minor protein bands after polyacrylamide gel electrophoresis. The DNA polymerase activity was detected in the acrylamide gels by an in situ assay and coincided with the major protein band. Nuclease activity could not be detected in the preparation after electrophoresis.

The DNA polymerase resembles the *Micrococcus luteus* enzyme in many characteristics, and its properties seem to place it as an intermediate between the polymerase I and polymerase II of *Escherichia coli* and *Bacillus subtilus* (Pickrum, 1975). It appears that the enzyme requires all four deoxynucleoside-5′-triphosphates, a divalent cation, 2-mercaptoethanol, and denatured DNA as a template-primer for maximal activity. Dithiothreitol is capable of replacing 2-mercaptoethanol in the reaction. The enzyme replicated, with varying efficiencies, the DNAs isolated from *Bacillus subtilus, Escherichia coli,* calf thymus, salmon, and soft fish roe. Treatment of DNA with pancreatic deoxyribonuclease improved its priming ability, the effectiveness of which depended on the extent of treatment with deoxyribonuclease.

The divalent metal ion requirement of the polymerase could be satisfied with either Mg^{2+} or Mn^{2+}. A slight but observable synthesis was obtained in the presence of 20 mM Co^{2+} or 10 mM Cd^{2+}. The enzyme was unstable in solutions of ionic strength below 0.2 M potassium phosphate or protein concentrations below 1 mg/ml. Enzyme solutions could be stabilized by the addition of glycerol or by increasing the salt or protein concentrations and storing at −20°C.

A synthesis-dependent exonuccease activity, interpreted as a 5′ to 3′ exonuclease, was found, at a low level, with the most purified DNA polymerase preparation; however, this activity was not characterized. Rates were stimulated in the presence of deoxynucleoside triphosphates. The most purified fraction contained no detectable endonuclease activity.

The polymerase enzyme is sensitive to the sulfhydryl-blocking reagents N-ethyl-maleimide, *para*-(chloro)mercuribenzoate, and *para*-

(chloro) mercuriphenylsulfonic acid. Of these, *para*-(chloro) mercuribenzoate is the most effective sulfhydryl reagent.

Applications

Z. ramigera is important in inducing flocculation (Unz, 1974) and reducing biological oxygen demand (BOD) in sewage-waste water treatment (Butterfield, 1935; Joyce and Dugan, 1970). The metal-adsorbing characteristic of *Z. ramigera* has been investigated with application to the treatment of heavy metals and transuranic waste streams (Dugan, 1970, 1987; Dugan and Pickrum, 1973; Friedman and Dugan, 1986a; Kuhn and Pfister, 1989; Norberg and Persson, 1984). Production of PHB or other polyhydroxyalkanoates by *Zoogloea* may be useful for the development of copolymers for commercially useful biodegradable plastics.

Waste Water Treatment

An early and continued use of *Zoogloea* in a treatment process has been in the area of domestic waste water treatment. Although zoogloeal bacteria are not the only microorganisms responsible for a successful waste water treatment process, they are important for the degradation of organic carbon and for the flocculation required for settling. *Zoogloea* bacteria are highly active oxidizers of organic compounds which significantly reduce the biological oxygen demand (BOD) in waste water. Butterfield et al. (1937) reported that 50% of the BOD from sewage could be removed in 5 hours by the aerobic, Gram-negative, floc-forming bacteria indigenous to aerobic waste water system, and 68% could be removed in 3 hr by a natural mixed population of bacteria. *Zoogloea ramigera* converts excess carbon to PHB, extracellular polysaccharide, and esterified organic acids (Joyce and Dugan, 1970). It is the interaction of extracellular microbial polymer by adsorption and bridging that aggregates microorganisms and produces flocs (Busch and Stumm, 1968).

Poly-β-hydroxybutyrate Production

A potential use of *Zoogloea* involves the production of PHB, a highly polymerized lipid reserve material, that is usually synthesized by the bacteria in response to nitrogen limitation in the presence of excess carbon source. The production, enzymology, and genetic control of PHB metabolism in *Zoogloea* is of interest because of the application of PHB and other polyhydroxyalkanoates in the biodegradable plastics industry. PHB produced by some *Alcaligenes* species is currently being marketed as a biodegradable plastic under the trade name Biopol (Holmes, 1985).

Biosorption of Metals

A developing technology is the use of *Zoogloea* to adsorb and concentrate metals and transuranic elements from contaminated waste streams. The significance of extracellular microbial polymers as agents for relatively nonspecific adsorption of dissolved or suspended organic, particulate, and ionic substances has been established with polysaccharides produced by Gram-negative floc-forming bacteria. (Dugan, 1970; Dugan et al., 1971; Friedman and Dugan, 1968a). The adsorptive characteristics of the whole cell or purified polymer can be exploited for commercial waste water treatment to remove heavy metal and transuranic ions and also may be useful in mineral separation processes, where the use of other flocculating agents of biological origin are currently being investigated.

Zoogloea biomass can remove a variety of metallic cations from solution (Dugan and Pickrum, 1973; Friedman and Dugan, 1968a; Norberg and Persson, 1984; Norberg and Rydin, 1984; Sag and Kutsai, 1989a, 1989b). The removal of aluminum, calcium, cobalt, iron, magnesium, manganese, nickel, and silicon from acidic mine water samples demonstrates the effective metal binding characteristic of *Zooglea* and *Zoogloea*-derived polymer that is applicable to the treatment of complex, relatively undefined aqueous metal containing systems (Dugan, 1987; Dugan and Pickrum, 1973). Presumably, microorganisms are partially responsible for the accumulation of metals within the solids generated by sewage treatment and the reduction in metal concentration in effluent supernatant as compared to the metal concentration of the influent and the supernatant from primary settling (Dugan et al., 1971).

Chemically, the purified polysaccharide isolated from *Z. ramigera* 115 interacts with monovalent cations to form soluble salts, binds but does not appear to form cross-linkages with divalent cations, and forms insoluble precipitates with trivalent cations (Ikeda et al., 1982; Stauffer et al., 1980). Successive removal of uranium, copper, and cadmium from solution by *Zoogloea* biomass indicates selective binding among cations in mixed solutions (Norberg and Persson, 1984).

With the potential use of *Zoogloea* for the treatment of metal-bearing waste waters demonstrated, a microbially based technology for

the treatment of these wastes appears to be developing. *Z. ramigera* 115 cells, immobilized in calcium alginate beads, have been used in air-bubbled column reactors to remove mixtures of cadmium, copper, lead, manganese, strontium, and zinc from aqueous solutions (Kuhn and Pfister, 1989, 1990). Although calcium alginate alone sorbed metals, the use of immobilized *Z. ramigera* 115 enhanced metal binding significantly. A batch method was used to study the removal of cadmium, copper, and uranium from solution using biomass of *Z. ramigera* 115 (Norberg and Persson, 1984). A bench-scale continuous process was demonstrated with *Zoogloea* biomass to adsorb copper from solution (Norberg and Rydin, 1984). Affecting adsorption was the concentration of biomass and copper in solution and the operating pH.

Important to the costs of a treatment process, is the ability to recycle biomass for multiple treatment cycles. Acid treatment releases cadmium, copper, and uranium adsorbed to *Zoogloea* biomass, thus enabling multiple cycling for the complete removal of metals from solution (Norberg and Persson, 1984). In experiments with cadmium it was shown that the sorbed cadmium could be eluted by exposing the beads to nutrient solutions whereupon more cadmium could be sorbed (Kuhn and Pfister, 1989, 1990). Alternatively, the release of cadmium from calcium alginate immobilized *Z. ramigera* 115 was achieved by use of a 0.04% aqueous nitrilotriacetate solution (Kuhn and Pfister, 1989, 1990).

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CHAPTER 221

Large Symbiotic Spirochetes: *Clevelandina, Cristispira, Diplocalyx, Hollandina,* and *Pillotina*

LYNN MARGULIS and GREGORY HINKLE

The genera *Cristispira, Clevelandina, Diplocalyx, Hollandina,* and *Pillotina* are morphologically complex, Gram-negative, motile, spirochetes (helical bacteria) in which the flagella are always entirely periplasmic (i.e., located between the inner [plasma] and the outer membrane typical of Gram-negative bacteria) (Fig. 1). (For a general discussion of the morphology of spirochetes, see Chapter 191.) All are obligate symbionts in the digestive system of mollusks or arthropods. These morphologically complex spirochetes have greater than 10 and sometimes as many as 300 flagella inserted at both ends of the cell and overlapping in the middle. If n is the number of flagella at one end of the cell and 2n the number of overlapping flagella in the middle of the cell, then the characteristic array is n:2n:n (e.g., 10:20:10 or 300:600:300). The coated membranes, distinctive cytoplasmic structures (including the sillon, a cell-length invagination or groove of the outer membrane in contact with the inner membrane), and relative proportions that distinguish these genera are depicted in Fig. 2, based on the morphometric analyses summarized in Table 1.

The habitats of these organisms are predictable (e.g., the crystalline style of bivalve mollusks for *Cristispira,* and the intestine of dry wood-eating cockroaches and termites for the others). None has been grown axenically. As molecular biological data are not yet available, species have been determined morphologically. Five species of large, symbiotic spirochetes have been described in the modern bacteriological literature and reverified, revised, or named as: *Clevelandina reticulitermitidis, Cristispira pectinis, Diplocalyx calotermitidis, Hollandina pterotermitidis,* and *Pillotina calotermitidis.* (For genera description, including an explanation of the morphometric analysis of spirochetes, see Bermudes et al., 1988.)

Although often classified on the basis of size and light microscopic morphology in the family. Spirochaetaceae (e.g., Bermudes et al., 1988), these spirochetes are ultrastructurally distinct from all other members of the Spirochaetaceae (see Chapter 191). Therefore, we classify the large symbiotic spirochetes in the family Pillotinaceae. (This family was first suggested by Hollande and Gharagozlou, 1967, who used the incorrect Latin derivative "Pillotaceae.") The spirochetes most similar morphologically to any member of the Pillotinaceae are the tick-borne symbionts, e.g., *Borrelia persica,* (flagella formula 25:50:25) (Karimi et al., 1979); the free-living microbial mat spirochete *Mobilifilum chasei* (10:20:10) (Margulis et al., 1990a); and the pectinolytic rumen spirochete *Treponema saccharophilum* strain PB, which is reported to have approximately 32 flagella (whether 32:64:32 or 16:32:16 is not clear) (Paster and Canale-Parola, 1985). All these spirochetes are significantly smaller than any member of the Pillotinaceae. No free-living *Pillotina*-like spirochete has ever been reported none at least, larger than 0.5 μm in diameter bearing at least 30 flagella.

A summary of the characteristics of the large, symbiotic spirochetes is presented in Table 1. Although only five genera are in the formal taxonomic literature, hundreds of symbiotic spirochetes from a large number of animals have been reported. For a more extensive discourse on *Cristispira* species in a variety of mollusks, see Kuhn (1981) and Breznak (1984a). Large spirochetes in the hindguts of termites and wood-eating cockroaches are more thoroughly described by To et al. (1980).

Cristispira pectinis (Gross) was first described as the trypanosome *Trypanosoma balbiani* (a eukaryote) by Certes (1882). Thought to have a multicellular, chambered body, *Cristispira* was renamed by Gross (1910), the name *Cristispira* being derived from the unusually prominent flagellar bundle or "crest." The modern understanding of the organism comes from Noguchi (1921), who, detecting them in oysters *(Crassostrea),* clams *(Venus),* and mussels *(Modiola),* recognized *Cristispira* as a spirochete bacterium. This identification was confirmed when the crest was shown to be a flagellar bundle by Ryter and Pillot (1965).

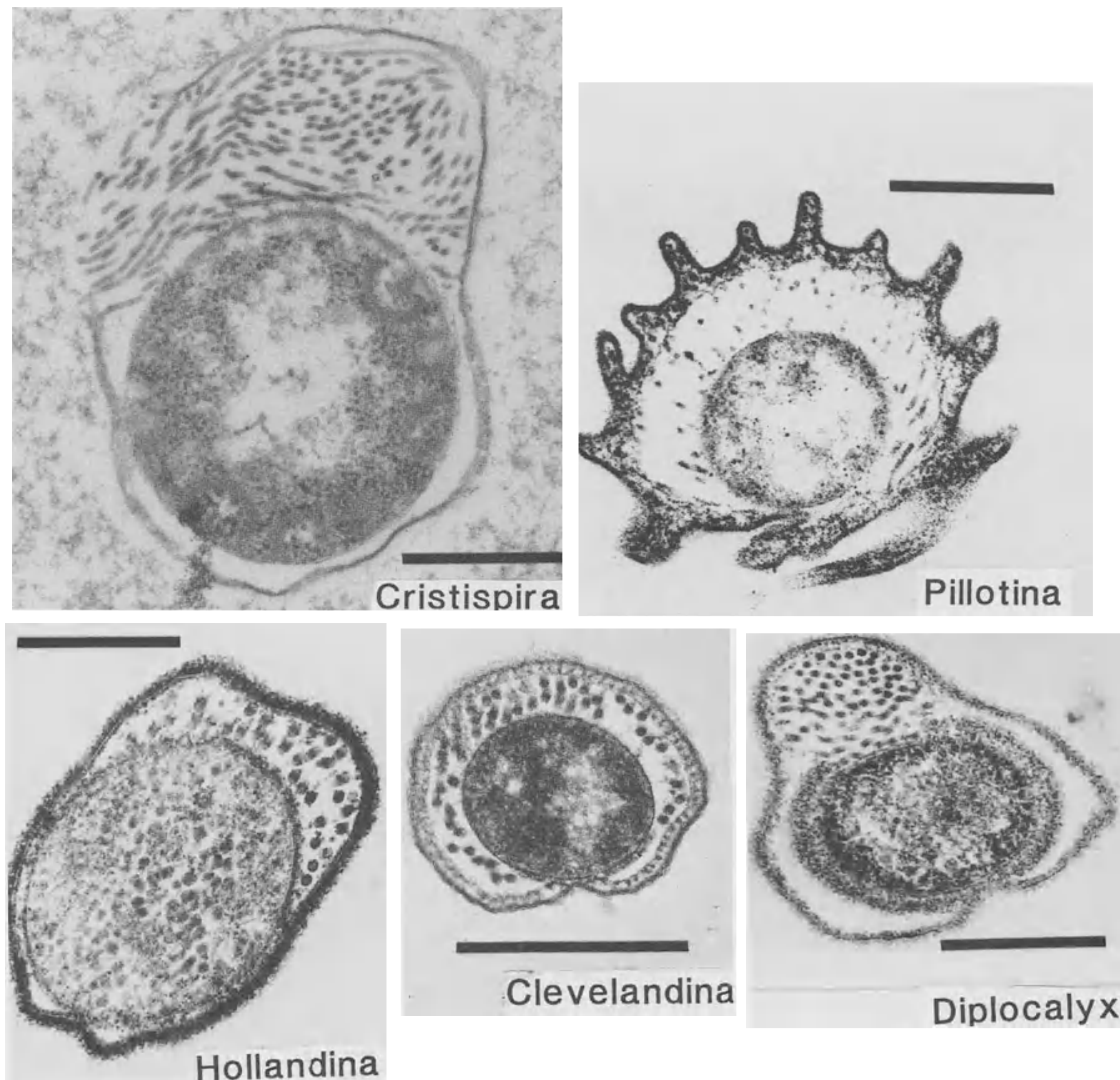
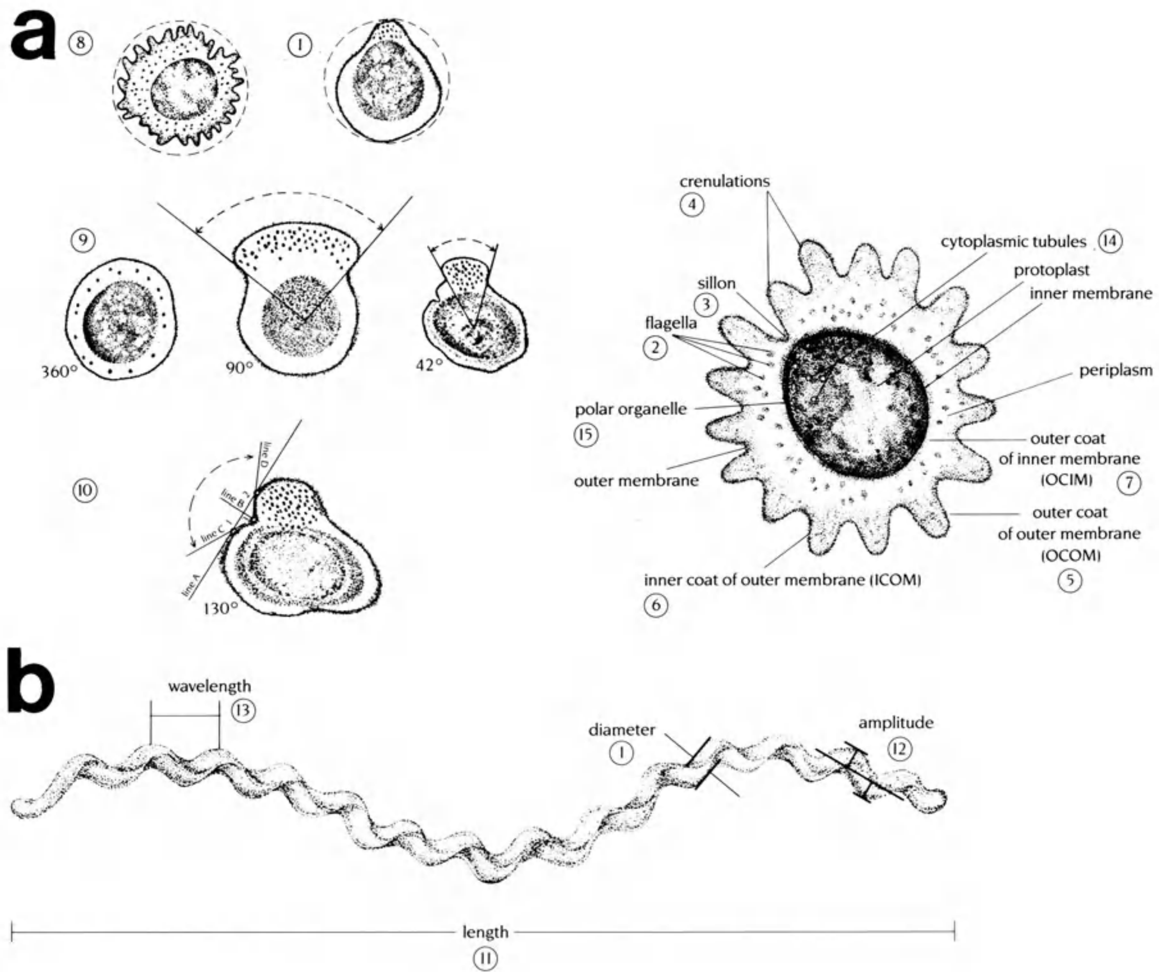


Fig. 1. Large symbiotic spirochetes as shown in transverse electron microscopic sections: *Cristispira* from the East Coast oyster. (Courtesy of J. M. Sieburth.) *Pillotina* from *Reticulitermes flavipes.* (Courtesy of R. Bloodgood.) *Hollandina* from the Sonoran desert termite. (Courtesy of L. P. To.) *Clevelandina* from *Zootermopsis.* (Courtesy of J. Breznak and H. Pankratz.) *Diplocalyx* from the Madeiran termite. Numerous flagella are seen in cross-section in the region between the outer and the plasma membrane. (Bars = 0.5 μm)

Leidy (1850) first described spiral-shaped microbes from termite intestines and called them *Spirillum undula* later renaming them *Vibrio termites* (Leidy, 1881). Dobell (1912) recognized these organisms as spirochetes; he called the larger ones *Treponema termites* and the smaller ones *Treponema minor.* The larger spirochetes were transferred to the genus *Cristipira* by Hollande (1922). They were further described by Damon (1925), Duboscq and Grassé (1929), and Kirby (1941). In an early electron microscopic study, Grimstone (1967) described a large unnamed spirochete from the wood-eating cockroach *Cryptocercus punctulatus.* Electron microscopy and the use of glutaraldehyde as a fixative reinvigorated the study of the termite hindgut microbiota by providing a means of distinguishing morphologically the uncultivable symbiotic bacteria. After the genus *Pillotina* and the family Pillotinaceae were proposed by Hollande and Gharagozlou (1967) for large spirochetes from the Madeiran termite *Incisitermes praecox,* large insect gut spirochetes were informally called "pillotinas." The discovery of a separate spirochete morphotype from the termite *Incisitermes flavicollis* led Gharagozlou (1968) to establish the genus *Diplocalyx.* To et al. (1978) described a third genus, *Hollandina,*



Encircled number	Morphometric criterion
1	Diameter
2	Number of flagella
3	Presence of sillon
4	Presence of crenulations
5	Ratio of thickness of outer coat of outer membrane to outer membrane thickness
6	Ratio of thickness of inner coat of outer membrane to outer membane thickness
7	Ratio of thickness of outer coat of inner membrane to inner membrane thickness
8	Ratio of diameter of protoplasmic cylinder to cell diameter
9	Angle of protoplasmic cylinder subtended by flagella
10	Presence of flagellar bundle (angle of concavity CD <180°
11	Length
12	Amplitude
13	Wavelength
14	Presence of cytoplasmic tubules[a]
15	Presence of polar organelle
16	Presence of rosettes[b]

[a]Cytoplasmic tubules range from 15 to 25 nm in diameter.
[b]The rosettes are not shown in Fig. 2.
Adapted from Bermudes et al. (1988).

Fig. 2. Spirochete structures used in morphometric analyses. (a) Structures discernible by electron microscopy. (b) Structures discernible by light microscopy and a list of the 16 morphometric criteria. Encircled numbers refer to the criteria given in the table in part 2b. (Drawings by C. Lyons.)

Table 1. Criteria used to identify the spirochete genera.

Criterion	*Cristispira*	*Pillotina*	*Hollandina*	*Diplocalyx*	*Clevelandina*
Primary					
Diameter (μm)	0.5–3.0	0.6–1.5	0.4–1.0	0.7–0.9	0.4–0.8
Approximate number of flagella	≥100	30–70	30–60	40–60	30–45
Presence of sillon	−	+	+,−	+	+
Presence of crenulations	−	+	−	−	−
OCOM/OM[a]	ND	0	2.4–8.0	0.6–2.0	0–2.8
ICOM/OM[b]	ND	3.3–11.9	0–1.3	0.9–2.1	2.8–5.0 (chambered)
OCIM/IM[c]	ND	0	1.0–2.5	6.4–7.7	0–3.6
PC/diameter[d]	0.90	0.56–0.67	0.63–0.90	0.47–0.81	0.60–0.81
Angle subtended by flagella (°)	90–160	190–350	105–330	50–100	140–330
Presence of flagellar bundles	+	−	+,−	+	+,−
Auxiliary					
Length (μm)	30–180	ND	ND	ND	ND
Amplitude (μm)	4–6	ND	ND	ND	ND
Wavelength (μm)	10–20	ND	ND	ND	ND
Presence of cytoplasmic tubules	NDT	+	+	+	NDT
Presence of polar organelle	+	+	+	+	NDT
Presence of rosettes	+	−	−	−	−

Symbols: +, present; −, absent; ND, no data; NDT, not detected.
[a]Ratio of thickness of outer coat of outer membrane to outer membrane thickness.
[b]Ratio of thickness of inner coat of outer membrane to outer membrane thickness.
[c]Ratio of thickness of outer coat of inner membrane to inner membrane thickness.
[d]Ratio of diameter of protoplasmic cylinder to cell diameter.
Adapted from Bermudes et al. (1988).

from the hindgut of the Sonoran desert termite *Pterotermes occidentis.* Known informally from the micrographs of D. G. Chase, *Clevelandina* was fixed in the microbiological literature by Bermudes et al. (1988).

Habitat

Cristispira develops especially dense populations in and on the crystalline style of many marine (and a few freshwater) mollusks. Styles are noncellular, gelatinous, cellulase-containing rods that extend into the stomach of many bivalve mollusks. Their presence, appearance, and consistency differ from species to species and even from individual to individual. In actively feeding animals, the style is rotated by epithelial cilia and, much like a pestle, one end is pushed against the stomach wall. The rotation likely aids in the mixing of food particles and digestive enzymes within the mollusk stomach. Some styles dissolve and reform with a tidal rhythm; when the style dissolves, the *Cristispira* disappear. Other smaller bacteria, including *Spirillum ostrea* (Margulis et al., 1991), an unidentified Gram-negative rod, and a smaller spirochete, have also been found in mollusk styles (Kuhn, 1981). Though they do not have a style, gastropods (single-valve mollusks) have been reported to harbor *Cristispira* as well (Morton, 1952; Orton, 1922; Terasaki, 1960). A partial list of the distribution of *Cristispira* in marine mollusks is given in Table 2.

The four other genera of large spirochetes are insect symbionts; they swim freely in the lumen of the distal portion of the intestine, actually an hypertrophied hindgut or paunch. The paunch, which has less oxygen relative to air (Breznak, 1984b), is easily identified and dissected in any of the wood-eating cockroaches or so-called "lower" termites. The geographical distribution of these spirochetes is listed in Table 3. Their abundance is greatest in insects that attack and ingest dry rather than damp or wet wood. As a rule, Pillotinaceae spirochetes are very abundant in both kalotermitid (dry wood) and rhinotermitid (subterranean) termites. In rhinotermitids they tend to be most abundant just posterior of the midgut/anterior hindgut junction. These spirochetes are apparently absent from the damp-wood-eating nasutitermitids and other "higher" termites. In "higher" termites, a bacterial or bacterial/fungal community rather than a protistan/bacterial community has evolved. (Spirochetes 0.5 μm in diameter with approximately 40–50 flagella have been reported in the hindgut of the higher termite *Nasutitermes exitiosus;* Czolij et al., 1985). The presence of Pillotinaceae spirochetes is easily determined by light microscopy on the basis of habitat, size, flexibility, and motility, but genera and species are not identifiable without ultrastructural analysis.

Table 2. Genera of mollusks harboring *Cristispira*.

Marine bivalve genera	
Cardium papillosum	*Penitella ovoidea*
Chama gryphoides	*Pinna nobilis*
Chama pellucida	*Pinna squamosa*
Chama sinistrorsa	*Platyodon cancellatus*
Chione fluctifraga	*Protothaca staminea*
Chione succincta	*Protothaca tenerrima*
Clinocardium nuttallii	*Saxicava arctica*
Crassostrea gigas	*Saxidomus giganteus*
Cryptomya californica	*Saxidomus nuttalli*
Cuminga californica	*Scrobicularia piperata*
Diplodonta orbella	*Siliqua patula*
Entodesma saxicola	*Solen ensis*
Gastrochaena dubia	*Soletellina acuminata*
Gryphaea angulata	*Sphaerium corneum*
Lampsilis anodontoides	*Strophitus* sp.
Lima hians	*Tapes aureus*
Lima inflata	*Tapes decussatus*
Lyonsia pugetensis	*Tapes laeta*
Macoma secta	*Tapes philippinarum*
Mactra sulcataria	*Tapes pullastra*
Modiola barbata	*Taras orbella*
Modiola modiolus	*Tivela stultorum*
Mytilus edulis	*Tresus capax*
Ostrea angulata	*Tresus (Schizothaerus) nuttallii*
Ostrea edulis	*Venerupis japonica*
Ostrea lurida	*Venerupis philippinarum*
Ostrea talienwhaneensis	*Ventricolaria* sp.
Ostrea virginiana	*Venus casta*
Panope generosa	*Venus mercenaria*
Paphia staminea	*Venus verrucosa*
Pecten jacobaeus	*Zirfaea pilsbryi*

From Kuhn (1981).

Morphology

Given ecological information and high resolution ultrastructural analysis, a single transverse thin section suffices to identify one of these large symbiotic spirochetes to genus. Ultrastructural analysis requires morphometrics (Bermudes et al., 1988). Sixteen criteria are useful when sufficient information is available (Fig. 2b). Diameters (criterion 1) in oblong cells are measured at the narrowest and widest points enclosed by the membrane. In crenulated cells, measurements are made at the tips of the crenulations. The number of flagella (criterion 2) is reported as the number inserted at each end. The number of flagella observed ranges from zero (in sections taken beyond the most distal insertion) to twice the number of flagella inserted at one end, where, toward the center, flagella inserted at one end overlap with the flagella from the other end. The sillon or groove (criterion 3) is a contact or pronounced invagination running the length of the cell of the outer membrane toward the inner membrane (Fig. 2). This structure provides a reference point for the description of other structures. Crenulations (criterion 4) are conspicuous folds or ruffles in the outer membrane (Fig. 2); to date they are limited to the genus *Pillotina*. Criteria 5, 6, and 7 deal with the presence of coatings on the inner and outer membranes of the Gram-negative cell walls (i.e., the glycocalyx or sheath). Criterion 8 is the ratio of the diameter of the protoplasmic cylinder to the cell diameter. Criterion 9, the angle subtended by the flagella, is a measurement of the distribution of the flagella in the periplasm. Flagellar bundles (criterion 10) are deemed present if the angle made by lines C and D is less than 180° (Fig. 2a). Criteria 11, 12, and 13 are the length, amplitude, and wavelength of the spirochete (Fig. 2b). Cytoplasmic tubules (criterion 14) are small, hollow structures within the protoplasmic cylinder as observed by negative stain or transmission electron microscopy (Fig. 2 and 6). They vary from 15 to 25 nm in diameter. The polar organelle (synonym, polar membrane; criterion 15) is an electron-dense lamina located on the inside of the inner (plasma) membrane toward the distal ends of the cell (Fig. 2 and 5). Rosettes (criterion 16) are linearly aligned, peripheral structures of the protoplasmic cylinder (Fig. 5); they have only been seen in *Cristispira*.

The largest spirochete in the microbiological literature, *Cristispira* is usually the only spirochete seen in mollusk styles. Healthy termites, however, may contain from one to more than five different morphotypes of pillotina spirochetes difficult to distinguish from each other with a light microscope (Fig. 3, 7, 8, and 9). Length (from 12 to 100 μm), amplitude (from 1.5 to 6.0 μm), and wavelengths (from 6 to 20 μm) can thus only be represented in approximate terms. The combination of large diameter (0.4 to greater than 1.0 μm), great length, and long wavelength distinguish pillotinas from the many other smaller, hindgut spirochetes (Figs. 7 and 8). Idealized drawings based on electron micrographs of transverse sections of all five genera are shown in Fig. 4.

Cristispira

Cristispira cells can be greater than 3 μm in diameter and over 150 μm in length. The enormous number of periplasmic flagella (>100) gives *Cristispira* a figure-eight shape in cross-section, with the flagellar bundle often equal in size to the protoplasmic cylinder (Figs. 1 and 4). In highly motile *Cristispira*, the crest, which is merely the flagellar bundle, is difficult to discern. As the spirochetes lose vitality and speed,

Table 3. Geographic distribution of insect-spirochete symbioses.

Insect family	Insect genus	Location	Spirochete genus
Order Blattaria			
Cryptocercidae (Protoblattidae; wood-eating cockroaches)	*Cryptocercus punctulatus*	United States	*Hollandina*
Order Isoptera			
Hodotermitidae (damp-wood termites)	*Porotermes adamsoni*	Australia	—[a]
Kalotermitidae (dry-wood termites)	*Bifiditermes condonensis*	Australia	—
	Ceratokalotermes apoliator	Australia	—
	Cryptotermes brevis	United States	—
	Cryptotermes cavifrons	United States	—
	Cryptotermes gearyi	Australia	—
	Glyptotermes iridipennis (Kalotermes iridipennis)	Australia	—
	Glyptotermes neotuberculatus	Australia	—
	Kalotermes approximatus[b]	United States	—
	Kalotermes banksiae	Australia	—
	Calotermes flavicollis[b]	France, Spain	*Diplocalyx*
	Kaoltermes jouteli (Neotermes jouteli)	United States	—
	Kalotermes minor (Incisitermes minor)	United States	*Hollandina*
	Kalotermes schwarzi (Incisitermes schwarzi)	United States	*Hollandina* *Pillotina*
	Kalotermes snyderi[b]	United States	*Hollandina*
	Incisitermes milleri	United States	—
	Marginitermes hubbardi (Kalotermes hubbardi)	United States	*Diplocalyx*
	Neotermes insularis	Australia	—
	Paraneotermes simplicicornis	United States	—
	Postelectrotermes praecox (Calotermes praecox)[b]	Madeira, Portugal	*Pillotina*
	Pterotermes occidentis	Mexico, United States	*Hollandina*
Mastotermitidae	*Mastotermes darwiniensis*	Australia	*Hollandina*
Rhinotermitidae (subterranean termites)	*Coptotermes aginaciformis*	Australia	—
	Coptotermes formosanus	Hawaii	*Hollandina*
	Heterotermes aureus	United States	—
	Reticulitermes flavipes	United States	*Clevelandina* *Pillotina*
	Reticulitermes hesperus	United States	*Clevelandina* *Hollandina* *Pillotina*
	Reticulitermes tibialis	United States	*Clevelandina* *Hollandina*

[a]—, There is no ultrastructural information on which identification could be based.
[b]Originally published nomenclature used (*Calotermes* is equivalent to *Kalotermes*).
Adapted from Bermudes et al. (1988).

the crest swells and becomes more obvious. The periplasmic space in *Cristispira* tends to be minimal, but expands to much greater diameter where subtended by the periplasmic flagella.

Rosettes line the periphery of *Cristispira* along its length except where flagella insert (Fig. 5). Although the electron-luminous spheres making up the rosettes generally measure about 100 nm in diameter, the number of component spheres varies from 2 to as many as 9, such that the ultrastructure of the rosettes is not constant. "Chambers" or ovoid inclusions in light micrographs correspond to these rosettes; they have no known function.

The polar organelle, a structure first described in spirilla and subsequently found in other types of flagellated bacteria, is clearly present in *Cristispira* and lies conspicuously beneath the flagellar bundle (Figs. 2 and 5a). The proximity of the polar organelle to flagella insertions suggests a role in motility generation. Nothing is known directly about the reaction of *Cristispira* to gaseous oxygen, though if cytochrome oxidase were present at the polar organelle in

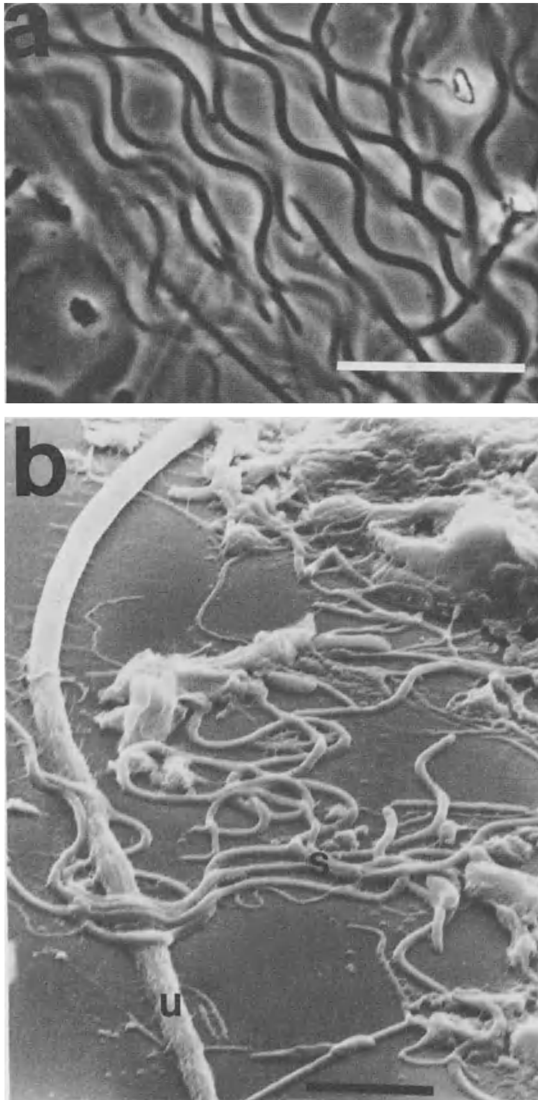


Fig. 3. (a) Live *Hollandina* spirochetes from *Pterotermes occidentis;* light micrograph. (b) *Hollandina pterotermitis* (S) and a huge, unidentified bacterium (U); scanning electron micrograph. (Bar = 5 μm)

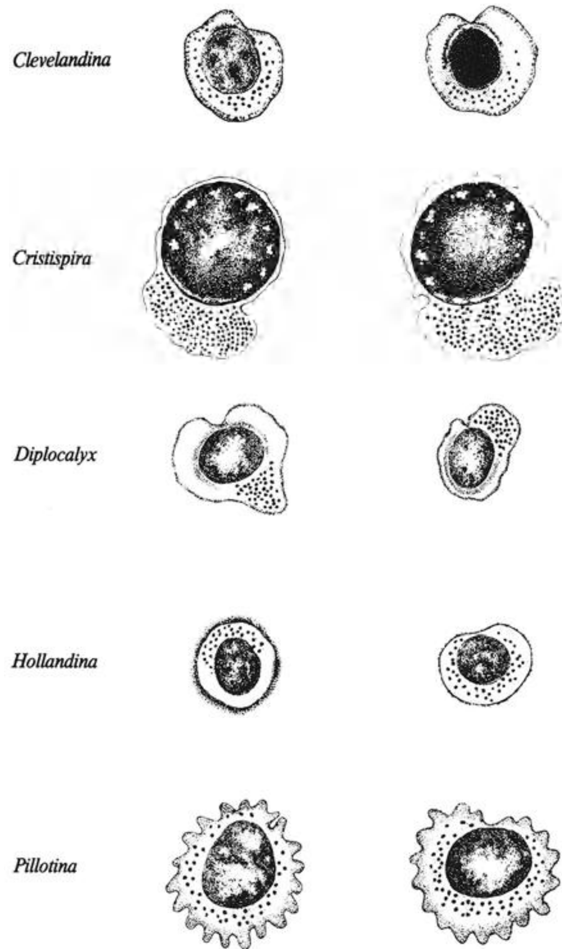


Fig. 4. Idealized drawings of transverse electron microscopic sections showing the range of morphotypes in the five genera of large, symbiotic spirochetes. Not drawn to scale. (Drawings by C. Lyons.)

Cristispira (as it is in *Sphaerotilus natans;* Tauschel, 1985), this would suggest that the metabolism of this spirochete is aerobic or microaerophilic. The type species for the genus is *Cristispira pectinis* (Gross, 1910).

Clevelandina

Clevelandina spirochetes are symbiotic in the paunch of subterranean termites and therefore may be anaerobes. The cells are generally oblong in cross-section, 0.4 to 0.8 μm in diameter and have 30 to 50 periplasmic flagella (Fig. 1, 4, and 9). The angle subtended by the flagella tends to be greater than 180°, and the inner coat (calyx) is correspondingly reduced. The cells are characterized by a thick inner coat (layer) of the outer membrane which tends to be chambered, and an outer coat of the inner membrane which is present except where the circumference is covered by flagella. A sillon, the contact between the inner and outer membrane that extends longitudinally, is present and crenulations are lacking. The type species is *Clevelandina reticulitermitidis* from the California termite *Reticulitermes tibialis* (Bermudes et al., 1988).

Diplocalyx

The *Diplocalyx* spirochetes are symbionts in hindguts of dry wood-eating termites; they are probably either anaerobes or microaerophiles. The cells are helical and 0.7 to 0.9 μm in diameter. In *Diplocalyx* the 40 to 60 periplasmic flagella are tightly bundled between the inner and outer membranes (Figs. 1 and 4). A prom-

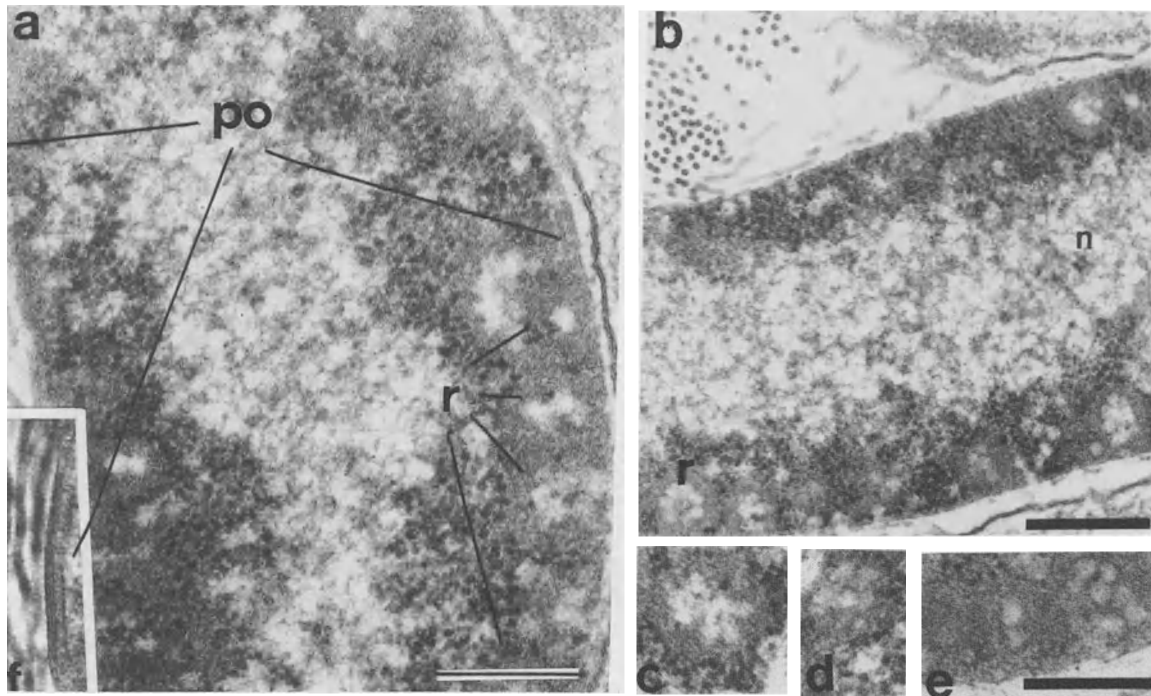


Fig. 5. *Cristispira pectinis* (a) Relationship of rosettes (r) to polar organelles (po); four rosettes and two polar organelles in longitudinal electron microscopic sections. (Bar = 0.2 μm) (b) Peripheral location of rosettes, one with seven-fold symmetry. (Bar = 0.2 μm) (c to e) Rosettes with two to nine spherical components. (Bar = 0.1 μm) (f) Detail of a polar organelle showing quadrilaminar structure. Abbreviations: n, nucleoid. See Margulis et al. (1991) for details.

inent sillon varies in position in different morphotypes relative to the flagellar bundle. A thick outer coat of the inner membrane, forming a calyx (cup), is present except under the flagellar bundle and sillon. Crenulations of the outer membrane are absent. Cytoplasmic tubules, about 21 nm in diameter (Fig. 6b), and polar organelles have been seen in some sections. The type species is *Diplocalyx calotermitidis* from the Mediterranean termite *Incisitermes flavicollis* (Gharagozlou, 1968).

Hollandina

Hollandina spirochetes, found in wood-eating cockroaches and termites, are probably anaerobes or microaerophiles. They are smooth, rounded to oblong, and helical when observed in transverse section; they are 0.4 to 1.0 μm in diameter (Figs. 1 and 4). *Hollandina* spirochetes generally have a thick coating on the outer surface of the outer membrane. Crenulations of the outer membrane are lacking. Cells have from 15 to 70 flagella dispersed in the periplasmic space. A sillon is absent or relatively inconspicuous. Polar organelles and cytoplasmic tubules have been observed in some sections. The type species is *Hollandina pterotermitides* from the Sonoran desert termite *Pterotermes occidentis* (Bermudes et al. 1988).

Pillotina

Pillotina spirochetes are symbionts in the guts of wood-eating cockroaches and termites and are probably anaerobes or microaerophiles. They have helical cells 0.6 to 1.5 μm in diameter with prominent crenulations in the outer membrane (Figs. 1, 4, 6a, 7, 8, and 9). A stellate profile with approximately 30 to 70 flagella distributed throughout the periplasmic space is distinctive for the genus. The inner coat of the outer membrane is consistently prominent; an outer coat of the outer membrane is present in some populations. A deep and narrow crenulation forms the sillon. The type species is *Pillotina calotermitidis* from the Madeiran termite *Incisitermes praecox* (Hollande and Gharagozlou, 1967).

Cytoplasmic Tubules

Hollow tubules, called microtubules by Gharagozlou (1968; Fig. 6a) although they are somewhat smaller in diameter than the standard 25-nm microtubules of eukaryotes (Margulis et al., 1978, 1981; To, 1978), have been seen in the

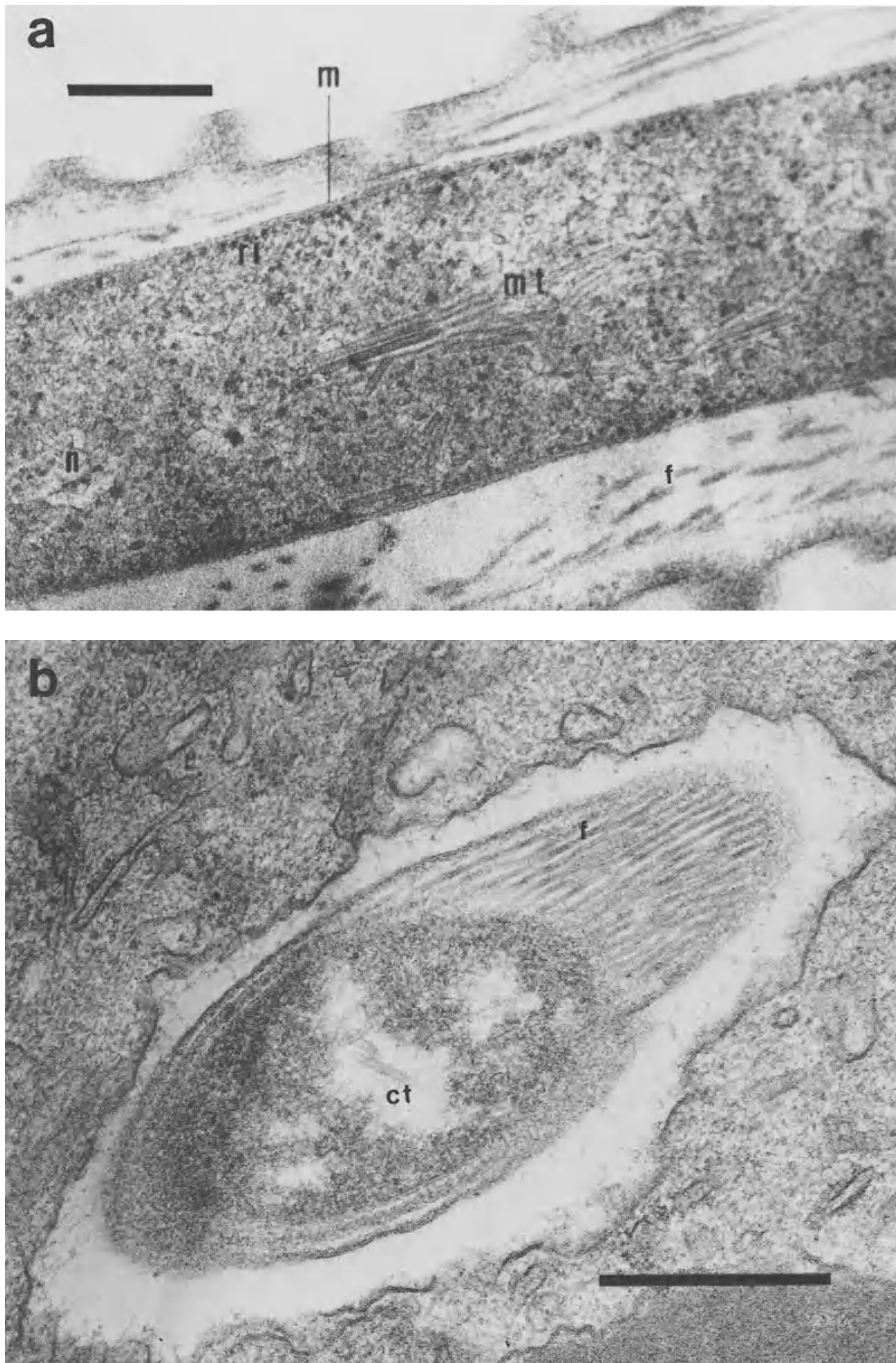


Fig. 6. (a) Cytoplasmic tubules (mt), 24 nm in diameter, in a longitudinal section of the termite *Pillotina calotermitidis* from Madeira. (Bar = 0.5 μm) (b) Cytoplasmic tubules (ct), 21 nm in diameter; and flagella (f), in *Diplocalyx* from *Incisitermes minor* from Newbury Park, California. Abbreviations: m, inner membrane; n, nucleoid; ri, ribosomes. (Bar = 0.5 μm) (Micrograph by D. G. Chase.)

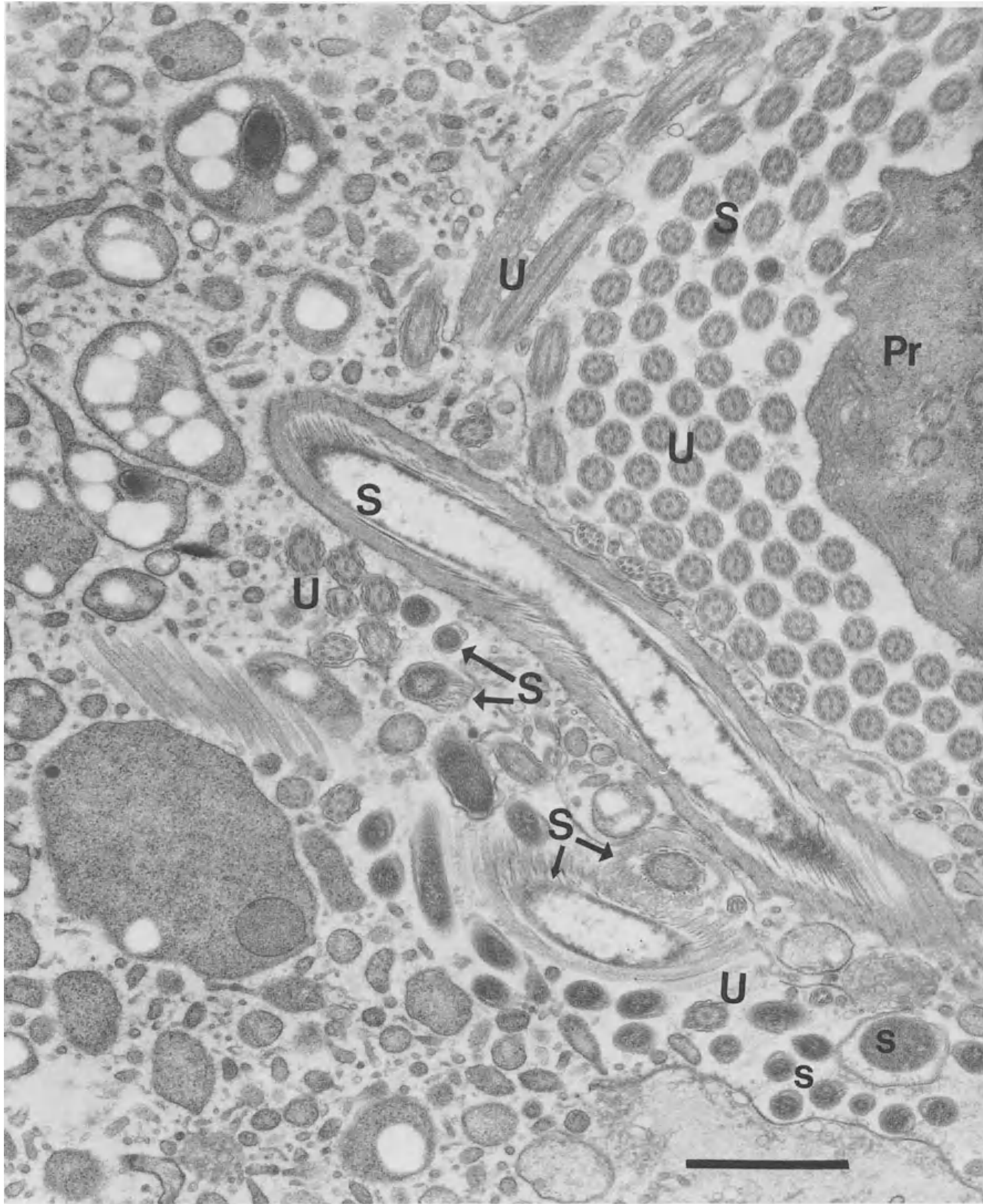


Fig. 7. Densely populated microbial community from the hindgut of the subterranean termite *Reticulitermes hesperus.* An unidentified protist (Pr), undulipodia (U), and unidentified and large spirochetes (S), including *Pillotina* and *Clevelandina,* are seen in this transmission electron micrograph. Bar = 0.5 μm.

protoplasmic cylinders of many termite spirochete genera, including those listed in Table 1. In large spirochetes of the dry-wood-eating termites *Incisitermes schwarzi* and *Pterotermes occidentis,* the presence of a tubulin-like protein was detected by antitubulin immunofluorescence and by co-migration with authentic brain tubulin in acrylamide gel electrophoresis (Bermudes et al., 1987; Margulis et al., 1978). Tubulin proteins, which comprise eukaryotic cilia,

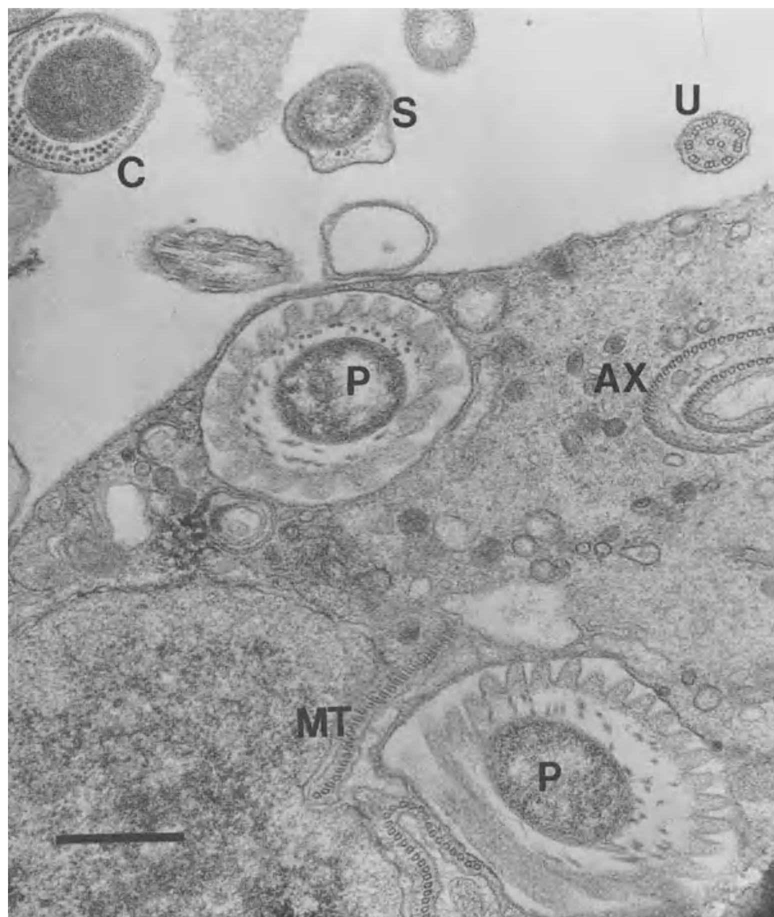


Fig. 8. Two *Pillotina* spirochetes (P) inside an unidentified hindgut protist, associated with host axostyle (AX) and microtubules (MT). Note the *Clevelandina* cell (C) and an unidentified small spirochete (S) at the upper left; U, undulipodium. (Bar = 0.5 μm)

flagella (undulipodia), and the mitotic apparatus, are ubiquitous in eukaryotes. This is the only known evidence of tubulin-like proteins in prokaryotes. An evolutionary relationship between the cytoplasmic tubules of these spirochetes and eukaryotic microtubules has been suggested (Margulis, 1981). Cytoplasmic tubules have not been reported in any *Cristispira* strains.

Distribution of Morphotypes

The geographical distribution of *Cristispira* (Table 2) and of large spirochetes from insect hindguts (Table 3) were summarized earlier. In any given termite, distinctive spirochete morphotypes are consistently and reliably found. Many undescribed spirochete morphotypes, especially smaller ones, are present in termite and cockroach hindguts, often in impressive numbers (Fig. 7). More than 25 species of kalotermitids and rhinotermitids have been examined, and they nearly all harbor some type of pillotina spirochete. Since more than 350 species of drywood termites (Krishna, 1961) and 158 species of subterranean termites are known (Wilson, 1971), and fewer than 30 termite species have been examined with the electron microscope, many more complex spirochetes likely await discovery.

Isolation

Collection and Examination of Symbiotic Spirochetes

Large spirochetes are easily observed live in wet mounts. Styles can be readily dissected from fresh mollusks and crushed with a coverslip for direct observations of *Cristispira.* Intestines are removed with forceps from termites or wood-eating cockroaches. The microorganisms are very densely packed and so are usually diluted with Trager's solution (1934) before observation. They may be maintained for observation for up to 2 hours *(Cristispira)* or 24 hours (some hindgut spirochetes), using petrolatum- or paraffin-sealed microscope slides. The style or gut,

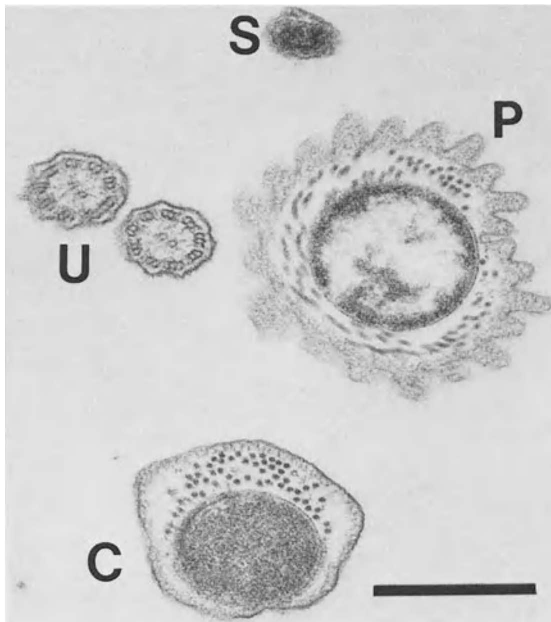


Fig. 9. *Clevelandina* (C), *Pillotina* (P), and an unidentified spirochete (S) coexisting with undulipodia (U) in the hindgut of *Reticulitermes hesperus*. Bar = 0.5 μm.

its microcosm intact, is prepared for electron microscopy by fixation with glutaraldehyde (Margulis et al., 1991; To, 1978) or observed directly.

Collection of *Cristispira*

To observe live *Cristispira*, the host mollusk must be freshly harvested since styles degenerate within a few hours after removal from their natural estuarine habitat. Oysters maintained in the laboratory quickly lose their spirochetes. *Cristispira* tend to be either plentiful or absent in any given style and when present tend to align longitudinally in the style matrix. The environmental conditions indicative of the presence and abundance of *Cristispira* in a mollusk are poorly understood. Mollusks of the same species sharing the same environment will not all contain *Cristispira*. The aeration of the general environment seems to have no correlation with the presence of *Cristispira*. Mollusks in well-aerated surf and mollusks buried deep in black, anaerobic muds both contain *Cristispira*. *Cristispira* can be collected the year round.

Maintenance of Motile *Pillotina* Cells

In spite of many efforts, no medium has yet been devised that permits the in vitro growth of any of these large, symbiotic spirochetes. The following medium, however, did maintain motile termite spirochetes in mixed culture for at least 72 and up to 120 hours:

> Sweet E broth (Holdeman and Moore, 1972) is adjusted to a pH of 6.8, rendered anaerobic by gassing with 80% Ar, 10% CO_2, and 10% H_2, and anaerobically sterilized in Hungate anaerobic tubes containing 1.5% agar. By the syringe method, fetal bovine serum and cocarboxylase are added to a final concentration of 5.0% and 0.05%, respectively. Hindgut contents from a surface-sterilized termite are inoculated into the medium.

The obvious sensitivity of the spirochetes to air bubbles on the slides and in the medium suggests that these organisms are microaerophiles or anaerobes.

Identification

Large symbiotic spirochetes may be identified by light microscopy to at least the family Pillotinaceae and in some cases to genus given identification of their animal hosts. For classification to species, ultrastructural identification is required. The use of molecular sequence analysis, in particular 16S ribosomal RNA sequence comparison, has not been applied to any large symbiotic spirochetes so the phylogenetic relation of these spirochetes with other spirochetes or any bacteria is unknown.

Symbiotic Relationships with Other Organisms

Cristispira

The crystalline styles of oysters digest cellulose in the absence of *Cristispira* symbionts. With respect to cellulose digestion in oysters, styles with spirochetes do not differ from styles without spirochetes (Margulis et al., 1991). Therefore, the role, if any, the spirochete plays in oyster digestion is unknown.

Termite Spirochetes

Termite spirochetes are always found in association with dense populations of hypermastigote and polymastigote protists, as well as with other bacteria. They are present in the hindguts of healthy, dry-wood, and subterranean termite hosts of all insect stages and castes: larvae, pseudergates, alates, and soldiers. The termite communities include some 10^5–10^6 protists per ml of fluid gut: lophomonads, trichonymphids, devescovinids, monocercomonads, and oxymonads. They also regularly contain 10^9–10^{11} bac-

teria per ml of hindgut fluid, including *Arthromitus* (Margulis et al., 1990b) and other Gram-negative rods of many kinds (To and Margulis, 1978; To et al., 1980; Figs. 7, 8, and 9). Live spirochetes are observed in many sorts of relationships with each other and with members of the community: 1) free in the lumen of the intestine (Grimstone, 1967; Margulis et al., 1979; Fig. 3, 7, and 9); 2) attached casually to the surfaces of protists, especially devescovinids and dinenymphids (Bloodgood and Fitzharris, 1976); 3) attached to other bacteria (Margulis and Schaadt, 1976); 4) attached to each other and translating in moving bundles (Margulis and Schaadt, 1976; To et al., 1978); 5) attached to debris and beating in synchrony without translation (Margulis and Schaadt, 1976); 6) attached in regular tufts to the surfaces of mastigotes (these populations of spirochetes may beat in synchrony and thus move their mastigote hosts, thereby forming motility symbioses; Cleveland and Grimstone, 1964; To et al., 1978); or 7) entirely engulfed within the cytoplasm of protists (Margulis et al., 1979, 1981; Fig. 8). Apparently, these spirochetes have sticky surfaces that facilitate attachment to each other and to other microbes. The hindgut spirochetes, along with other members of the hindgut microbial community, are actively transferred to newly hatched larvae and from insect to insect by proctodeal (mouth-to-anus or anus-to-anus) contact. If unprotected or exposed to oxygen, the spirochetes die in a few minutes.

The spirochetes are probably not essential for cellulose digestion because the community and the host can survive their absence, at least for some weeks. The spirochetes appear to depend on the protists in the hindgut community; the spirochetes do not survive more than a few days after the protists are removed by chemical or heat treatment of the termite hosts (Grosovsky and Margulis, 1982). None of the large symbiotic spirochetes seem to have any practical importance.

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CHAPTER 222

The Genera *Succinivibrio* and *Succinimonas*

ROBERT B. HESPELL

Introduction

The rumen is a strictly anaerobic ecosystem inhabited mainly by bacteria and ciliated protozoa, plus smaller numbers of fungi. Although several hundred or more species of bacteria can be found in this ecosystem, only about thirty species are usually found at high enough levels to be considered of ecological significance. *Succinivibrio dextrinosolvens* and *Succinimonas amylolytica* are included in this group of major species, but have not been extensively studied. These two species are usually found in animals fed some grain in their diets. In addition to contributing to ruminal starch digestion, both species produce succinate, a fermentation product, which can be decarboxylated by *Selenomonas ruminantium* to form propionate, a major ruminal volatile fatty acid. The cells of both species stain Gram negatively and are motile by means of monotrichous, polar flagella. However, *S. amylolytica* cells are coccoid whereas cells of *S. dextrinosolvens* are curved rods with tapered ends. At present, these organisms have been isolated mainly from ruminants, but it is reasonable to suspect they may be present in the gastrointestinal tract of other animals.

Habitats

Succinivibrio dextrinosolvens and *Succinimonas amylolytica* are inhabitants of the rumen of cattle and sheep. The numbers of these bacteria vary depending on the diet. *S. amylolytica* is found in the rumen of animals fed mixed diets composed of forages and at least some grain (Bryant, et al., 1958), whereas *S. dextrinosolvens* occurs in high numbers with animals fed high levels of starch or other diets containing large amounts of rapidly fermentable carbohydrates (Bryant and Small, 1956; Wozny et al., 1977). Both species are involved in the digestion of starch and its breakdown products in the rumen. The occurrence of both species in ecosystems other than the rumen has not been well documented. However, there have been at least two reports of *Succinivibrio* species occurring in the blood of humans suffering from bacteremia (Porschen and Chan, 1977; Southern, 1975). In both cases, the patients had gastrointestinal tract disorders and thus, the organisms might have been able to enter the blood as a result of loss of gastrointestinal tract integrity. If so, this suggests that *Succinivibrio* species might be normally present in low numbers in the colon of humans.

Isolation

Selective Enrichment and Isolation

Currently, no procedures have been published regarding selective enrichment or isolation of either *S. dextrinosolvens* or *S. amylolytica.* All strains have been isolated on nonspecific, habitat-simulating media used to enumerate total culturable bacteria from ruminal contents. As with other ruminal bacteria, both species are strict anaerobes. However, three strains of *S. dextrinosolvens* isolated under diverse conditions have been shown to grow on a defined medium (Gomez-Alarcon et al. 1982). This medium lacks the volatile fatty acids, peptides, and a number of other components often used in media to cultivate many species of ruminal bacteria. Thus, it might be possible to use this medium to selectively enrich or isolate *S. dextrinosolvens.*

Cultivation Media

Both species can be grown on a variety of complex, rumen fluid-containing media (Bryant and Burkey, 1953; Bryant and Robinson, 1961) or on more defined media in which the rumen fluid has been replaced by Trypticase, yeast extract, hemin, and volatile fatty acids (Caldwell and Bryant, 1966; Hespell and Bryant, 1981). A typical complex medium for both species and a chemically defined medium for *S. dextrino-*

solvens (adapted from Gomez-Alarcon et al., 1982) are shown in Table 1. Since both species produce succinate as a fermentation product, the use of a bicarbonate-carbonic acid system is needed to provide carbon dioxide and a means of buffering. Ammonium ions serve as a major nitrogen source for both species. *S. amylolytica* has a narrow range of fermentable substrates and only glucose, maltose, dextrin, and starch are known to be used. *S. dextinosolvens* also ferments these substrates plus galactose and xylose, but starch is only partially used and is incompletely hydrolyzed. Neither species ferments amino acids or peptides.

Preservation of Cultures

Both species can be maintained for long times by storage of cultures in liquid nitrogen or ultracold freezers (Hespell and Canale-Parola, 1970). Alternatively, short-term storage is possible by placing glycerol cultures in normal (−20°C) freezers (Teather, 1982).

Table 1. Media for *S. amylolytica* and *S. dextrinosolvens*.[a]

Ingredient	Amount per 100 ml of medium	
	Complex	Defined
Carbohydrate (5%)[b]	5.0 ml	5.0 ml
Trypticase	0.5 g	—
Serine	—	30 mg
Leucine	—	40 mg
Methionine	30 mg	30 mg
Yeast extract	0.2 g	—
p-Aminobenzoic acid	—	10 μg
Ammonium chloride (5.3%)	1.0 ml	1.0 ml
Resazurin (0.1%)	0.1 ml	0.1 ml
IVI VFA solution[c]	3.0 ml	—
L-Cysteine·HCl (2.5%)[b]	1.0 ml	1.0 ml
Mineral A[d]	4.0 ml	4.0 ml
Mineral B[e]	4.0 ml	4.0 ml
Ferrous sulfate	0.3 mg	0.3 mg
Trace minerals[f]	0.5 ml	0.5 ml
Hemin plus 1,4-napthoquinone[g]	1.0 ml	1.0 ml
Sodium carbonate (8%)[h]	5.0 ml	5.0 ml
Distilled water	76.0 ml	79.0 ml

[a]Both media are prepared under carbon dioxide; final pH 6.8. The defined medium is for *S. dextrinosolvens* only.
[b]Autoclaved separately; prepared and stored under a nitrogen gas phase.
[c]Prepared by adding 7 ml acetic, 3 ml propionate, 2 ml butyrate, and 0.6 ml each of isobutyrate, 2-methylbutyrate, and isovalerate to 700 ml of 0.2 M NaOH. Adjust to pH 7.0 with NaOH and to final volume of 1 liter.
[d]Mineral A = 0.5% K_2HPO_4.
[e]Mineral B = 1.0% KH_2PO_4, 1.2% NaCl, 0.58% $NaSO_4$, 0.16% $CaCl_2·2H_2O$, and 0.25% $MgSO_4·7H_2O$.
[f]Trace minerals contained 10 mg each of $ZnSO_4·7H_2O$, H_3BO_3, $NaSeO_3$, $Na_2MoO_4·2H_2O$, $NiCl_2·6H_2O$; 5 mg $CuSO_4·5H_2O$, and 2 mg of $Al(SO_4)_2·12H_2O$ made to 100 mlm with distilled water.
[g]Solution made by dissolving 25 mg of 1,4-napthoquinone in 2 ml of 95% ethanol and combining with 10 mg of hemin dissolved in 50 ml of 0.01 M NaOH and 38 ml distilled water.
[h]Autoclaved separately; prepared, equilibrated, and stored under a carbon dioxide gas phase.

Identification

Succinimonas

Succinimonas form surface colonies that are light tan in color, translucent, smooth, and convex. Even after prolonged incubation, the colonies are only 0.6 to 1.5 mm in diameter. Colonies within agar media are lenticular in shape. Typically, the cell morphology is that of a short, oval rod or coccobacillus (1–1.5 μm by 1.2–3.0 μm long). The cells appear singly or in pairs but can form clumps in older cultures. The cells always stain Gram negatively. In wet-mount preparations, most cells show some degree of translational motility which ceases when oxygen is present. Often cells which have become attached to the glass surface display a rotational movement about one cell end. The flagellar arrangement is polar and monotrichous. Some cells may show granules near the cell periphery. The nature of these granules is unknown but they may be composed of glycogen, which is found in a number of species of ruminal bacteria. In liquid media, growth is even and disperse, but generally dense turbidities do not occur.

The major products formed from glucose are acetic and succinic acids, and the formation of these products requires a large uptake of carbon dioxide. No hydrogen gas, formate, lactate, or ethanol is produced, but trace amounts of acetoin and/or propionate may be formed. No strains are known to hydrolyze gelatin and production of hydrogen sulfide, indole, or catalase has not been observed. Growth occurs at 30–39°C but not at 22°C or 45°C. Strain differences are rather minimal and all strains are considered to be isolates of a single species, *Succinimonas amylolytica* (Bryant, 1984a).

Another major ruminal species found in animals that are fed high-grain diets is *Ruminobacter amylophilus* (formerly *Bacteroides amylophilus*). This organism also forms small, tan colonies on the surface of agar media. However, the cell morphology is distinctly different from *S. amylolytica.* The cells are larger (0.9–1.2 by 1-3 μ long), tend to form pleomorphic, swollen shapes, and most importantly, they are nonmotile (Hamlin and Hungate, 1956). In addition, *R. amylophilus* does not grow on glucose. *R. amylophilus* produces acetate, formate, and succinate as major fermentation products and

trace amounts of lactate and ethanol may also be formed. The results of 16S rRNA cataloging show that *R. amylophilus* is remotely related to the purple sulfur group of bacteria (Stackebrandt and Hippe, 1986), but the phylogenetic status of *S. amylolytica* is unknown.

Succinivibrio

Succinivibrio dextrinosolvens forms light tan, entire, translucent surface colonies that are generally only 1–2 mm in diameter. Occasionally, colonies may be irregular, raised, and yellowish. Subsurface colonies are lenticular and equally small. However, in media having low agar concentrations (0.2 to 0.6%), subsurface colonies take on a diffuse, white, furry appearance. The cells appear singly or in pairs of small, curved rods, usually 0.3–0.5 by 2.0–4.0 μm long with pointed ends. With newly isolated strains, helical or twisted filaments of 2–4 coils composed of two or more cells are common. These filaments often show rotational motility. This helical characteristic varies with strains and can be lost during successive subcultures in the laboratory, resulting in single or pairs of slightly curved rods. Under adverse cultural conditions many strains can form pleomorphic shapes—usually large, swollen rods or spherical bodies. Cells invariably stain Gram negatively and are motile. The motility is translational with a vibrating movement. The flagellation arrangement is polar and monotrichous.

A large production of acetic and succinic acids with an uptake of carbon dioxide is the most common fermentation pattern for *Succinivibrio* strains fermenting glucose (Bryant and Small, 1956; Scardovi, 1963). Small, variable amounts of formate and lactate can be made. Hydrogen gas, propionate, and butyrate are not made. Good growth occurs at incubation temperatures of 30 to 40°C, but no growth occurs at 22°C or 45°C. Biochemical tests for nitrate reduction, catalase, and production of indole, acetoin, or hydrogen sulfide are all negative. Presently, all ruminal strains are considered to constitute a single species, *Succinivibrio dextrinosolvens* (Bryant, 1984b). The succinovibrio-like strains isolated from blood samples (Porschen and Chan, 1977; Southern, 1975) appeared to have lophotrichous flagellation and are probably not true isolates of the this species, but further studies are needed to verify this.

Anaerobiospirillum succiniproducens displays motile, helical rods with cell widths of 0.6–0.8 μm and cell lengths of 3.0–8.0 μm. This organism produces acetate and succinate as major fermentation products. *A. succiniproducens* has been isolated from colonic contents of dogs, but not from the rumen. It is easily differentiated from *S. dextrinosolvens* by having bipolar tufts of about 16 flagella and by its ability to ferment lactose but not galactose or xylose (Davis et al., 1976). The phylogenetic relationships of *Succinivibrio dextrinosolvens, Succinimonas amylolytica,* and *Anaerobiospirillum succiniproducens* to one another and to other higher taxa are not known.

Biochemical and Physiological Properties

The carbohydrate fermentation pathways in *S. dextrinosolvens* have been studied by Scardovi (1963). Enzymatic activities of the Embden-Meyerhof pathway were present in cell-free extracts, but enzymes of the hexose monophosphate pathway were absent. Phosphoenolpyruvate was found to undergo carboxylation to form oxaloacetate as the product, with ADP acting as the phosphate acceptor. Presumably, the oxaloacetate mainly undergoes reduction to form succinate and is partially used in biosynthetic transamination reactions to form amino acids. The pathways for assimilation of ammonia by *S. dextrinosolvens* have been elucidated with strain C18 (Patterson and Hespell, 1985). This strain was shown to possess glutamine synthetase, urease, glutamate dehydrogenase, glutamate-oxaloacetate transaminase, and aspartate synthase. When grown in continuous culture under ammonia limitation, cells contained high levels of glutamine synthetase and urease, but glutamate dehydrogenase activity was low. The opposite pattern is observed with growth in the presence of ample ammonia. The glutamate dehydrogenase was NADPH-linked. The glutamine synthetase was regulated by an adenylation-deadenylation mechanism, allowing for rapid enzyme inactivation when high levels of ammonia are present.

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CHAPTER 223

The Genus *Leptotrichia*

TOR HOFSTAD

The oral cavity of humans is the natural habitat of several different kinds of filamentous organisms, including the Gram-negative anaerobic bacterium *Leptotrichia buccalis.* This organism, which is the only species of the genus *Leptotrichia,* was among the first microorganisms to be recognizably described and drawn in the letters of Antoni van Leeuwenhoek.

The generic name *Leptotrichia* was used by Trevisan (1879) for filamentous organisms found in the human mouth. The species designation *buccalis* had been used several years earlier by Robin (1853), who used the name *Leptothrix buccalis* for filamentous forms that he had observed in wet mounts of tooth scrapings. Wherry and Oliver (1916) were able to cultivate the organism, which they called *Leptothrix innominata* in accordance with Miller (1889), but the first adequate description of *Leptotrichia buccalis* was given by Thjøtta, Hartmann, and Bøe in 1939. These authors, as well as Bøe (1941) and Bøe and Thjøtta (1944), found that the organism had much in common with the fusobacteria and, consequently, should be classified as a Gram-negative anaerobic bacterial species. Later investigators (Davis and Baird-Parker, 1959; Hamilton and Zahler, 1957; Kasai, 1961, 1965) confirmed and extended the cultural and biochemical findings of Thjøtta et al. (1939), but were of the opinion that *L. buccalis* was a Gram-positive organism. However, the ultrastructure of *L. buccalis* is that of a typical Gram-negative bacterium (Hofstad and Selvig, 1969). *Leptotrichia* is now classified as the third genus in the family Bacteroidaceae. Its capacity to produce lactic acid as the only major acid from glucose clearly distinguishes it from *Bacteroides* and *Fusobacterium. L. buccalis* is identical with *L. innominata* of Prévot et al. (1967). The GC content of the DNA is 28–30 mol% (measured by the thermal melting method).

In the past, *L. buccalis* has frequently been confused with *Fusobacterium* species (Hine and Berry, 1937; Kligler, 1915; Morris, 1954; Spaulding and Rettger, 1937a and 1937b). The name *Leptotrichia* has also been used (Bibby and Berry, 1939; Kligler, 1915; Morris, 1954) for the facultative Gram-positive organism termed *L. dentium* by Davis and Baird-Parker (1959) and now classified as *Corynebacterium matruchotii* (Collins and Cummins, 1986). See Chapter 237 for a discussion of *Fusobacterium.*

Habitats

The oral cavity of humans is the habitat of *L. buccalis,* but the organism has also been found in the female genitourinary tract (Bollgren et al., 1979; Evaldson et al., 1980). Also, the organism has been reported in the oral cavity of dogs (Goldstein et al., 1978) and in guinea pigs fed commercial pellets (T. Hofstad, unpublished observations). The principal source of the organism in the oral cavity is the dental plaque, i.e., the bacterial deposit that forms on the tooth surface and at the gingival margin. The concentration of *L. buccalis* in plaque material is uncertain. Slack and Bowden (1965) found the number of *L. buccalis* organisms in 1-day-old experimental plaque to be less than 0.01% of the total viable count; the number increased to 2.3% in 14-day-old plaque. Hillman et al. (1970) have shown that *L. buccalis* adheres to untreated enamel powder and to enamel powder coated with human saliva. However, the occurrence of the organism in the mouth is not solely dependent on tooth eruption since *L. buccalis* can be isolated from the dorsum of the tongue, and, occasionally, from predentate infants (McCarthy et al. 1965).

L. buccalis has infrequently been associated with infection in humans (Duperval et al., 1984; Kohler et al., 1982; Morgenstein et al., 1980; Reig et al., 1985; Thadepalli and Rao, 1979). Being a highly saccharolytic organism, it may participate in the development of tooth decay. The organism has cell wall lipopolysaccharide with the characteristics of an endotoxin (Gustafson et al., 1966). Antibodies reacting with this lipopolysaccharide are present in normal

human sera (Falkler and Hawley, 1976; Mergenhagen et al., 1965). These are IgM antibodies (Hawley and Falkler, 1976) and may be included among the so-called natural antibodies.

Physiology

Leptotrichia buccalis is anaerobic on first isolation although most strains become aerotolerant upon transfer and grow slowly on the surface of solid media in an ordinary CO_2 incubator. The organism grows on ordinary solid media such as Brucella blood agar, and in fluid and semifluid media based on peptone and yeast extract supplemented with a fermentable carbohydrate. Addition of serum to the medium may enhance growth.

L. buccalis is saccharolytic. Several mono- and disaccharides are fermented with the production of lactic acid alone, or accompanied by trace amounts of acetic and succinic acids. The terminal pH is 5.4 or less. Two separate lactate dehydrogenases have been isolated from cell-free extract of *L. buccalis* (Brown and Gross, 1981). Esculin is hydrolyzed. The inability of *L. buccalis* to form ammonia indicates that organic nitrogenous compounds are not metabolized.

L. buccalis is susceptible to penicillins, cephalosporins, tetracyclines, chloramphenicol, clindamycin, and metronidazole, but resistant to aminoglycosides. The minimum inhibitory concentration of erythromycin ranges from 4–16 μg/ml (Duperval et al., 1984; George et al., 1981).

Isolation

Isolation of *L. buccalis* can be performed under nonselective or selective conditions.

Sampling

Leptotrichia buccalis can be isolated from saliva or centrifuged salivary deposits, dental plaque, and the soft tissues of the mouth. It is best isolated from plaque between adjacent teeth (interstitial plaque) and the crevice or pocket that exists between the gingiva and the surface of the tooth. Sampling from the gingival crevice is best performed by the use of sterile filter paper points that are gently inserted into the crevice.

Centrifuged salivary deposits or plaque material can be inoculated onto the medium either directly or after being suspended and diluted in prereduced anaerobically sterilized (PRAS) one-quarter strength Ringers solution (Sutter et al., 1985) or the serum-containing diluent of Bowden and Hardie (1971). The inoculum on the tapering end of the paper point is streaked over a small area of the surface of the solid medium, and further spreading of the deposited material carried out by a sterile wire loop.

Isolation Under Nonselective Conditions

Because of its characteristic colonial morphology (see below), *L. buccalis* can be isolated from mixed cultures on blood agar or the starch-containing medium of Kasai (1961).

The medium of Kasai has the following composition:

Tryptone	20.0 g
Yeast extract	2.0 g
Soluble starch	20.0 g
K_2HPO_2	5.0 g
Sodium chloride	5.0 g
Cysteine hydrochloride	5.0 g
Distilled water	1 liter

The isolation of *L. buccalis* is indirectly favored by starch, which is an unavailable energy source for many of the other organisms present in the inoculated material. It is advisable to prepare 1:10 dilutions of the saliva or plaque samples and spread 0.01–0.05 ml on each freshly prepared plate. Incubation should be carried out at 37°C for 2 or more days in an anaerobic atmosphere containing 5–10% CO_2.

Selective Isolation

Media selective for *Fusobacterium nucleatum* can be used for isolation of *L. buccalis* from mixed cultures. The fusobacterium egg yolk agar (FEA) of Morgenstein et al. (1981) and the Crystal violet erythromycin agar (CVE) of Walker et al. (1979) (see Chapter 237) are most useful. *L. buccalis* grows on the FEA medium with white, raised, and granular colonies which are larger than the convex, round, and entire colonies of *F. nucleatum.* Large, brain-like or rough colonies of *L. buccalis* on the CVE medium are easily distinguished from the smaller and smooth or slightly irregular *F. nucleatum* colonies. However, strains of *L. buccalis,* susceptible to 8 μg/ml, or less, of erythromycin will not grow on the CVE medium.

Axenic Cultivation and Maintenance

L. buccalis can be maintained by weekly serial subculture on blood agar. Viable cells can be stored at −70°C in Greave's solution.

Table 1. Differentiation of *Leptotrichia buccalis* from *Fusobacterium nucleatum* and *Eubacterium saburreum.*

Characteristic	*L. buccalis*	*F. nucleatum*	*E. saburreum*
Microscopic morphology	Gram-negative,[a] 5–15 μm long, thick fusiform bacilli; filaments common	Gram-negative, 5–15 μm long, slender fusiform bacilli; filaments uncommon	Gram-positive,[b] 5–20 μm long bacilli, rounded or blunt ends; bulbous swellings and filaments common
Colonial morphology (blood agar)	Smooth, shiny, rhizoid, or convoluted	Smooth, convex, "flecked" appearance	Rough, rhizoid, adherent; or smooth, rhizoid or convoluted, nonadherent
Terminal pH from glucose	<5.4	>5.8	<5.4
Production of indole	−	+	+
Production of gas	−	−	+
Foul odor	−	+	−
Predominant fatty acids from glucose (GLC)	Lactic	Butyric	Acetic and butyric

[a]Often Gram-positive in young cultures.
[b]Often Gram-negative in old cultures.

Greave's Solution

Bovine serum albumin	50 g
Sodium glutamate	50 g
Glycerol	100 g
Distilled water	100 ml

Mass cultivation is performed in nutrient broth supplemented with yeast extract (0.3%), glucose (0.5%), cysteine HCl (0.1%), and serum (2%). The use of well-filled, narrow-necked bottles or similar containers makes the use of prereduced anaerobically sterilized (PRAS) media unnecessary.

Identification

A reliable identification of *L. buccalis* can be made from cellular and colonial morphology and, in addition, from a few biochemical tests (Table 1).

In Gram-stained specimens, *L. buccalis* is 0.8- to 1.5-μm-wide and 5- to 15 μm-long fusiform rods, commonly occurring in pairs with the adjacent ends flattened.

Filaments are seen in old cultures. The organism is Gram-negative, often with Gram-positive granules, but may be wholly Gram-positive in very young cultures. In young cultures, therefore, the cells of *L. buccalis* are not unlike those of the Gram-positive oral organism *Eubacterium saburreum.* However, *E. saburreum* never has tapered ends.

Young colonies of *L. buccalis* are colorless, smooth, and often shiny, raised and with a filamentous edge; the colonies are described as "medusa-head" colonies. Following prolonged incubation, the filamentous edge may disappear and the surface becomes convoluted, resembling that of a human brain. The colonies are nonadherent to the medium and after 2 days of incubation on blood agar measure 2–3 mm in diameter. Two- to three-day-old colonies on blood agar of *E. saburreum* may be mistaken for *L. buccalis.*

L. buccalis has been identified in plaque material using the fluorescent-antibody technique (Baboolal, 1968).

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CHAPTER 224

The Genus *Francisella*

FRANCIS E. NANO

Introduction

Francisella tularensis was recognized as the causative agent of a febrile illness in three different parts of the world in the early part of the 20th century. McCoy (1911) described a new disease which he found while studying a suspected outbreak of plague in ground squirrels in Tulare County, California. McCoy and Chapin (1912) isolated the etiologic agent of this "plague-like disease" and named the organism *Bacterium tularense* after the county in which it was found. This organism was also recognized as a disease agent of humans at about the same time. Pearse (1911) described what was known as "deer fly fever" in the area around Brigham City, UT, and Wherry and Lamb (1914) identified *B. tularense* as the infectious agent of a diseased meat worker in Cincinnati, OH. Edward Francis carried out an extensive series of experiments on the transmission of this organism and on the pathology of the disease he named *tularemia* (Francis, 1923). Shortly after the description of tularemia in the United States, Ohara (1925) isolated a similar organism (Francis and Moore, 1926) in Japan that caused Yato-byo (hare disease). In 1926, Soviet researchers (Pollitzer, 1967) isolated the same organism from human cases in Siberia. The name of the etiological agent of tularemia has changed over the years from *B. tularense,* to *Pasteurella tularensis,* to what it is called today, *Francisella tularensis,* the latter name in honor of the studies performed with this organism by Francis. In 1950 (Larson et al., 1955), another bacterium was discovered that was eventually placed in the genus *Francisella.* This organism, *F. novicida,* has only been found twice since its original isolation and apparently has a very low infectivity for humans.

F. tularensis is a Gram-negative cocco-bacillus that is 0.2 μm × 0.2–0.7 μm in size; *F. novicida* is larger at 0.7 × 1.7 μm. *F. tularensis* has a loosely associated capsule which is difficult to see with light microscopy and is often stripped away during preparation for electron microscopy. *F. tularensis* cells can appear highly pleomorphic, especially when taken from a stationary growth culture. There are no flagella nor pili associated with this organism but some researchers have observed a long flagellar-like organelle in electron micrographs; it is probable that such structures are electron microscopic artifacts. In the host, *F. tularensis* can be found both intracellularly and extracellularly (see Fig. 1), and there is substantial evidence that *F. tularensis* grows in macrophages.

Initially, researchers from around the world were uncertain of the relationship among the agents causing tularemia-like diseases since the North American cases were clinically much more severe than those found in other parts of the world. After exchanges of convalescent sera and *F. tularensis* strains, it became apparent that the strains from Asia, Europe, and North America were antigenically identical but that the North American strains were considerably more virulent, as demonstrated by both the clinical presentation and the virulence for laboratory animals. Later, it was found that there were two forms of *F. tularensis* in North America: the highly virulent form noted in most clinical descriptions of the disease, and a form that seemed to correspond to the European-Asian isolates.

There are at least three nomenclature systems used to describe the different biovars. Olsufiev et al. (1959) proposed the designation *F. tularensis* var. *tularensis* for the highly virulent North American strain and *F. tularensis* var. *palaearctica* for the less virulent strain found throughout the Northern Hemisphere. More recently Soviet investigators have used *F. tularensis nearctica* Olsufjev and *F. tularensis holarctica* Olsufjev in place of the biovar designations of *tularensis* and *palaearctica,* respectively. Jellison proposed the designations "A" and "B" for the highly virulent and less virulent forms, respectively. I will use these designations since they are simpler and imply nothing about geographical distribution and relatedness, ideas that may change with time. *F.*

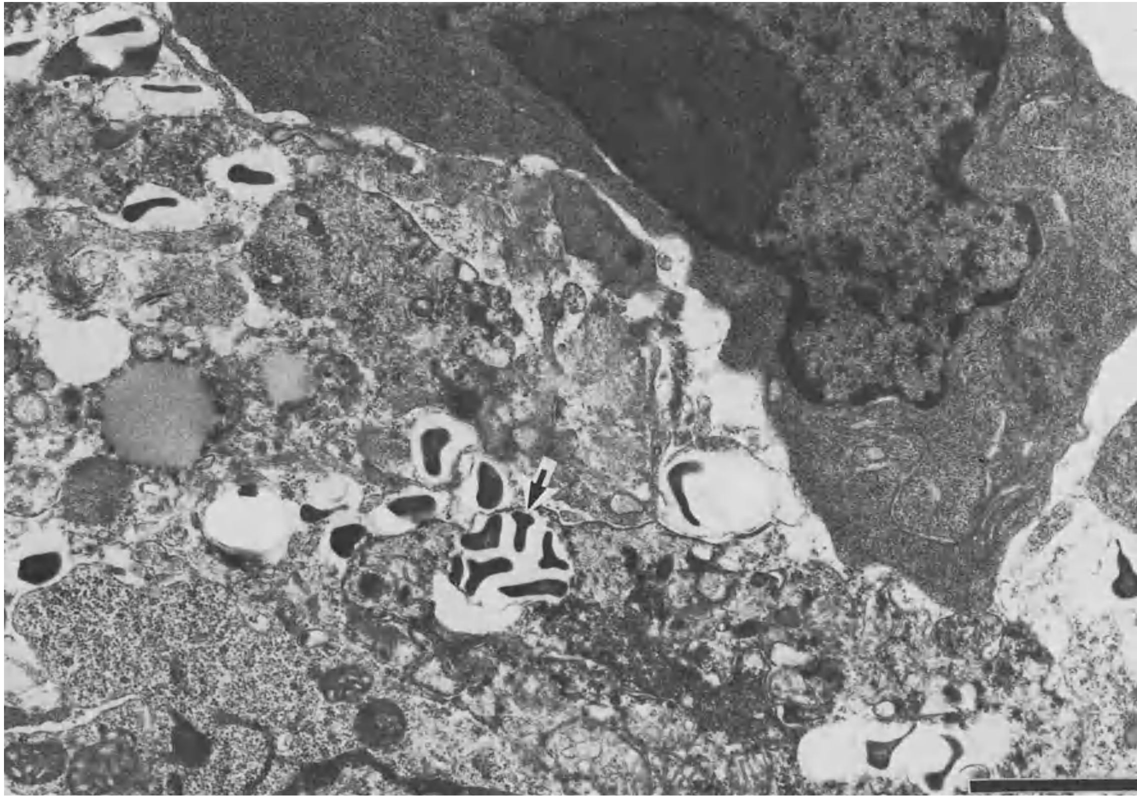

Fig. 1. Intracellular growth of *F. tularensis*. Transmission electron microscope image of a thin-section profile of *F. tularensis*, type A (Schu), in the liver of an RML strain White Swiss mouse. Bacteria are found in both intracellular and extracellular environments. Intracellularly, the organisms appear within endosomes (arrow). Note the pleomorphic forms of *F. tularensis*. Aberrations within the cell, such as mitochondrial swelling, endoplasmic reticular disruption, and lipid droplet formation are indicative of intracellular infection and are considered the result of cytopathic effects. Bar = 1.0 μm. (Courtesy of Stanley F. Hayes.)

novicida has only been isolated from North America.

Distribution and Transmission

F. tularensis has been found on every continent except Australia. Paradoxically, tularemia is not found south of the equator nor in England even though migratory birds have been shown to carry tularemia. It may be that the known foci of tularemia will continue to expand. It was only in 1968 (Young et al., 1969) that Vermont was though to be tularemia-free, only to yield a small epidemic of the disease. Until the 1980s, *F. novicida* had been isolated only once; we now suspect that *F. novicida* is endemic over wide areas of North America (Hollis et al., 1989). Moreover, it is possible that *F. novicida*-like strains are endemic in certain portions of the world, conferring immunity to *F. tularensis* and therefore preventing its establishment. Since *F. novicida* does not infect healthy humans and is not agglutinated by anti-*F. tularensis* antisera, the presence of *F. novicida* may go unnoticed.

Tularemia can be spread in a number of different ways; the prevalent mode of transmission differs from place to place and has been changing since the discovery of tularemia. In North America, during the early part of this century, tularemia was associated with rabbit hunting and the preparation of wild meat; hence, the colloquial names "rabbit fever" and "market men's disease" came about. In the western part of the United States, tularemia is often spread by biting insects and has been referred to as "deer fly fever." With the decline of rabbit hunting, tick bites—and to a lesser extent other insect bites—have become the major mode of transmission. Transmission of the type B form of *F. tularensis* through stream water occasionally causes small outbreaks of tularemia. However, one stream in Montana has been shown to carry *F. tularensis* type B for many years without causing any apparent infections in people using the stream water (Parker et al., 1951). It is likely that many infections with *F. tularensis* type B

occur without being recognized as tularemia and serological evidence suggests that many subclinical or unrecognized cases occur. Although tick-borne tularemia is probably always caused by *F. tularensis* type A and represents "typical" tularemia in North America, the type B forms of *F. tularensis* can also be found in ticks.

In northern Scandinavia and Finland where tularemia is common, tularemia is spread by a number of different routes including insect bites, inhalation of *F. tularensis*-laden agricultural products, and self-inoculation while dressing wild hares. Note that the hare (genus *Lupus*) is susceptible to the type B strain of *F. tularensis,* whereas the rabbit (genus *Sylvilagus*) is resistant to this strain. The similarity of transmission of tularemia by rabbits in North America and hares in Europe and Japan simply reflects close contact of humans with these animals during hunting and subsequent dressing. In Japan, as in Scandinavia, most cases of tularemia are associated with contact with hares.

Most of the world cases of tularemia occur within the Soviet Union, where a large number of hunters and trappers come into contact with infected animals. Also, transmission of *F. tularensis* in water has been responsible for epidemics which involve thousands of people. The reservoir for tularemia in the Soviet Union, as in northern Canada and Scandinavia, is a number of small animals, including rats and lemmings. The public health hazard of tularemia has led to intensive studies of zootic tularemia foci, and large-scale vaccination programs of the Soviet rural populations (Pollitzer, 1967).

Clinical

F. tularensis is an invasive pathogen that is able to cause a wide variety of symptoms which are determined by the route of infection and the immune response of the host. If tularemia is initiated by a self-inflicted cut or an insect bite, an ulcer usually develops at the site of infection, followed by fever, malaise, and swelling of local lymph nodes. This form of tularemia is called ulcero-glandular, and can progress to other forms of disease, depending on the natural immunity of the host and further self-inoculation. In some patients a septicemia can develop which may lead to infection of the lungs and pulmonary involvement; this systemic form of the disease is termed typhoidal tularemia. The typhoidal form of tularemia can also be initiated by ingestion or inhalation (causing pneumonic tularemia) of *F. tularensis.* The ocular-glandular form of tularemia, which is characterized by invasion of the conjunctiva, usually results from individuals unknowingly infecting the eye after touching infected material or an ulcer. However, any site on the body where there is a break in the skin or an exposed mucosal surface can be infected by *F. tularensis;* the terms used to describe the syndromes merely reflect the most common presentations of the disease.

An extensive study of the pathology in tularemia in humans and other animals was carried out by Lillie et al. (1937) and has been extended since then by a number of workers. There is a large variation in the lesions seen in tularemia that vary among the species infected, the virulence of the *F. tularensis* strains, and the route and dose of inoculation. In experimental animals splenomegaly is common and tiny grey focal lesions are seen in both the liver and spleen. In humans, cases of ulcero-glandular forms of tularemia, miliary granulomatous lesions can be found as well as suppurative, ulcerating lymph nodes. The pneumonic form of tularemia is pathologically identical to tuberculosis with caseating granulomatous lesions. An extensive review of tularemia by Bell (1981) includes a section summarizing the pathological lesions seen in human and animal cases.

Streptomycin remains the drug of choice for tularemia despite its toxicity (Anonymous, 1988). Gentamicin, tetracycline, and chloramphenicol have been used clinically and are also effective in treating tularemia. If the infection is not life threatening, tetracycline may be the drug of choice due to its relatively low toxicity. However, as with chloramphenicol, tetracycline treatment often results in relapses. A number of other antibiotics can inhibit *F. tularensis* growth in vitro (Baker et al., 1985) but they have not been tested clinically. Penicillins are not effective in treating tularemia and one study (Baker et al., 1985) showed that of 15 strains tested all had β-lactamase activity.

Identification, Isolation, and Cultivation

Clinically, tularemia can be confused with many other diseases, especially if a cutaneous ulcer is not present. Usually a presumptive diagnosis is made on the basis of a history of a tick bite or contact with diseased rabbits. Conclusive clinical evidence of infection can only be made by a rise in titer of agglutinating antibodies from serum samples taken 10–14 days apart.

F. tularensis is usually suspected when the bacterium is a nonsporeforming, aerobic Gram-negative cocco-bacillus which requires rich media supplemented with cysteine or another reducing agent. Microscopic examination may reveal a tendency to bipolar staining and the presence of a capsule. Definitive identification of *F. tularensis* is accomplished by reaction with antisera, using either agglutination or fluorescent antibody reaction.

Biochemical reactions are not particularly helpful in identifying *F. tularensis* but can be used to a limited extent to separate the biovars of *F. tularensis* and *F. novicida.* The type A biovar is able to ferment glycerol and possesses a citrulline ureidase whereas the type B biovar does not have these characteristics. *F. novicida* can be differentiated by its ability to ferment sucrose. All strains are able to use glucose, mannose, and fructose; *F. novicida* and some strains of *F. tularensis* are unable to ferment maltose.

The most rapid form of laboratory diagnosis can be accomplished by examining the reaction of fluorescence antibody with infected tissue. However, this is not practical for most laboratories which rely on culturing *F. tularensis* and testing for agglutination by specific antisera. Isolation of *F. tularensis* can be done on a number of different types of rich media that are supplemented with cysteine and whole blood. Addition of penicillin (100,000 units/ml) and polymyxin B (100,000 units/ml) will suppress the growth of other bacteria and cycloheximide (0.1 mg/ml) will suppress the growth of fungus. *F. tularensis* appear as grey, viscous colonies surrounded by an area of green discoloration on an agar medium containing blood. When *F. tularensis* needs to be isolated from a dilute source or from a mixture of microorganisms, injection of a mouse can help with the enrichment, although such experiments require Biological Level 3 containment facilities. *F. tularensis* should be easy to isolate from the liver and spleen of moribund animals.

F. tularensis can be cultivated on a number of rich agar media that contain cysteine. In addition, *F. tularensis* can be grown in complex liquid media such as peptone-cysteine broth and trypticase soy broth with cysteine, as well as in defined media. Optimal growth occurs at 37°C with continuous shaking, yielding cultures of up to 4 × 10^{10} cells/ml. *F. novicida* can grow in any of the media used for *F. tularensis.*

Cysteine Heart Agar (Difco) for Growth and Isolation of *F. tularensis*

Beef Heart Infusion	500 gm
Proteose Peptone	10 gm
Sodium Chloride	5 gm
L-cysteine	1 gm
Agar	15 gm
Distilled or deionized water	1000 ml
Final pH 6.8	

After dissolving the medium, autoclave for 15 min at 121°C. Cool to 45–48°C and add 50 ml of defibrinated rabbit or horse blood.

Peptone Cysteine Agar for observing colony morphotypes.

Bacto peptone	20 gm
NaCl	10 gm
Glucose	1 gm
Cysteine-HCl	1 gm
Agar	15 gm

After dissolving all the ingredients (except the agar) adjust the pH to 6.8. Add the agar and autoclave for 15 min at 121°C.

Each lot of media can be tested by growing the avirulent B38 or the live vaccine strain (LVS). The B38 strain normally grows very poorly, so medium that supports its growth should support the growth of virulent strains. *F. tularensis* colonies will reach their maximum size in 3–5 days. *F. novicida* grows considerably faster with colonies appearing within 24 h. *F. novicida* does not require the addition of cysteine but grows faster in its presence.

Francisella strains can be preserved by freezing or lyopholizing log phase cultures that are mixed one- to-one with either skim milk, 2.6% gelatin, or 0.2% agar. Serial passage on solid medium will invariably lead to loss of virulence.

F. tularensis is highly infectious and Biosafety Level 3 facilities should be used when culturing virulent strains or working with infected animals. However, for decades researchers have worked with a number of attenuated *F. tularensis* strains that have proven to be safe; special containment facilities have not been necessary. In particular, the B38 strain and the LVS have been used for antigen preparations and immunological studies, respectively. Personnel who come into contact with highly virulent *F. tularensis* should be vaccinated with the live vaccine strain, which is especially effective in preventing the pneumonic form of tularemia.

Interaction of *F. tularensis* with Its Host

F. tularensis is able to invade and multiply within nonprofessional phagocytes such as L-cells (Merriott et al., 1961) and HeLa cells (Shepard, 1959); the evidence for intracellular growth includes electron microscope observations and intracellular growth in the presence

of streptomycin. The cellular location of *F. tularensis* replication has not been conclusively demonstrated and different authors have suggested that replication takes place inside the cytoplasm (Shepard, 1959) or within a phagolysosome (Lofgren et al., 1988). The significance of in vitro intracellular growth within nonprofessional phagocytes to growth in vivo is unclear. Electron micrographs have demonstrated intracellular growth of *F. tularensis* within liver tissue but the cell type was not identified (J. F. Bell and S. F. Hayes, unpublished observations).

Both in vivo and in vitro experiments reveal that *F. tularensis* grows inside macrophages and monocytes but not within polymorphonuclear lymphocytes (PMN). The growth rate inside macrophages and cultured cell lines correlates with the virulence of the *F. tularensis* strain. *F. tularensis* is not found within PMNs in infected animals presumably due to the antiphagocytic effects of its capsule. In vitro, PMNs cannot phagocytize *F. tularensis* unless immune serum is added. The capsule-minus mutants of the live vaccine strain are readily phagocytized by PMNs whereas the encapsulated strain is not (Sandstrom et al., 1988).

The capsule of *F. tularensis* is clearly a virulence factor. Depending on the parent strain, one can measure one to three logs increase in the LD_{50} in capsule-minus strains (Eigelsbach et al., 1951; Eigelsbach et al., 1952; Hood, 1977; Sandstrom et al., 1988) (much of this information assumes that the "rough" mutants described by different researchers were capsule-minus strains). Hood (1977) was able to dislodge the capsule by incubating *F. tularensis* cells in a solution of 10% saline. Cells treated this way had a large decrease in virulence, but it is impossible to know if this treatment affected other components of the cell besides the capsule. The capsule also seems to protect *F. tularensis* from serum complement; normal serum can kill capsule-minus strains.

Microbiology

Many *F. tularensis* strains generate different colonial morphotypes at a high frequency. Two type A isolates were studied in detail by Eigelsbach et al. (1951) who observed different variants using a dissecting microscope to differentiate the colony types. Wild type *F. tularensis* appear as viscous, rounded colonies; however, when viewed through a dissecting microscope with oblique lighting the colonies appear blue (due to either light interference or selective absorption). The highly virulent Schu strain and the avirulent B38 strain (of biovar type A lineage) both segregate different colony types that had either changes in the blue shade (to "gray" variants) or the shape of the colony. Working with the Schu strain, these workers were able to determine that loss of the blue coloration correlated with the absolute loss of virulence and that some of the colonial shape changes affected virulence to a lesser extent. The European and Asian isolates also segregate gray variants but do not show the extreme variation seen in strain B38. The blue-gray transition is not reversible at detectable frequencies since one blue cell of the Schu strain will kill a mouse whereas $> 10^9$ gray cells will not. Also, the genetic lesions conferring avirulence in strain B38 and the LVS do not revert to wild type at detectable frequencies. Without knowing the molecular basis for the colonial morphotypes, it is difficult to suggest whether the changes reflect point mutations, illegitimate recombination induced by transposons, or the loss of plasmids. Perhaps some of the virulence factors of the North American type A strain of *F. tularensis* are on mobile genetic elements. The plasmid carriage state of most *F. tularensis* strains is unknown; however it is known that the live vaccine strains and *F. novicida* do not have plasmid (F. Nano, unpublished observations).

A large amount of antigenic analysis of *F. tularensis* was done before the 1970s using chemical methods, agglutination reactions, Oucterlony analysis, and delayed and immediate type hypersensitivity reactions. Most of the information from these early studies corresponds well with what has more recently been learned from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots. A capsule covers the *F. tularensis* cell, and antibody against the capsule is agglutinating. The composition of the capsule has not been precisely defined, but the data of Hood (1977) indicates that it was made largely of two saturated fatty acids (16:0 and 1-OH 14:0) and four sugars (mannose, rhamnose, and two unidentified sugars). The data concerning a capsule-minus mutant of the LVS, however, shows no difference in fatty acid composition between the encapsulated strain and the capsule-minus mutant. These two works do not necessarily contradict each other since the "capsule-minus" strain may have a genetic defect that affects assembly of carbohydrate portions of the capsule but not of the lipid portions. *F. novicida* either does not have a capsule or it is different antigenically from that of *F. tularensis,* since no cross agglutination occurs.

The lipid composition of *F. tularensis* is unusual in that it contains hydroxy fatty acids 2-OH-10:0, 3-OH-16:0, and 3-OH-18:0, and fatty acids with long carbon chains (C_{20}–C_{26}). From the published data it is difficult to determine the precise fatty acid composition and the distribution to different cell components. Cumulatively, the data from different publications suggest that the hydroxy fatty acids are not in the inner membrane, but are most likely in the capsule (Anderson and Bhatti, 1986; Hood, 1977; Jantzen et al., 1979).

The capsule blocks the accessibility of outer membrane proteins and lipopolysaccharides to antibody and chemicals, and has thus hindered analysis of the outer membrane. Investigators in Sweden have studied the immunogenic potential of outer membrane components and have described four outer membrane proteins (Sandstrom et al., 1987). Another investigator described a protein, called FopA, and cloned the encoding gene (Nano, 1988). FopA has properties similar to OmpA of *Escherichia coli;* it is an outer membrane protein that appears to have a tight association with LPS. Its mobility in SDS-PAGE is affected by heat, reducing agents, and treatment with organic solvents. FopA is the major protein immunogen in infected humans (Bevanger et al., 1988) and in animals injected with killed cells. The LPS of both *Francisella* species is rough, with no apparent O-side chains visible by silver-staining (F. Nano, unpublished observations).

What are the relationships among the *F. tularensis* strains and *F. novicida* and how is the genus *Francisella* related to other genera of bacteria? Judging by DNA homology, genetic recombination studies and Western immunoblot analysis, the different *F. tularensis* strains and *F. novicida* are closely related. Although attempts have been made to demonstrate a relationship between *F. tularensis* and *Yersinia, Pasteurella,* or *Brucella* species there is no good evidence by modern standards showing a convincing evolutionary relationship. The GC content of the DNA up *Francisella* is 33 to 36 mol%, which is quite different from that of these other bacteria. As 16S RNA sequencing analysis of different bacterial species continues we should learn the correct phylogenetic place of *Francisella.*

Yersinia philomiragia is closely related to *Francisella* species (Hollis et al., 1989), and it has been proposed to encorporate this species into the genus *Francisella.* The origin of the *F. philomiragia* samples that were studied demonstrate that this species is found over wide areas of North America and Europe. Furthermore, the fact that all of the patients (except one) that were the source of the strains had a severe underlying disease or had experienced a near-drowning episode, indicates that these strains have extremely low infectivity for healthy humans. The presence of some of these strains in ocean water suggests that they may be pathogens of fish or marine mammals.

Acknowledgments

I would like to dedicate this review to my predecessors at the Rocky Mountain Laboratories who studied tularemia for many decades (and discovered *F. novicida* and *Y. philomiragia*), especially J. F. Bell, C. R. Owen, and W. L. Jellison.

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CHAPTER 225

The Hemotrophic Bacteria: The Families Bartonellaceae and Anaplasmataceae

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The organisms of the genera included in the families Bartonellaceae and Anaplasmataceae are heterogeneous. It is probable that these organisms were first considered to be related because they all parasitized red blood cells and because they produced diseases characterized by anemia. The additional observations that all of these organisms were best demonstrated by light microscopy of red blood cells after treatment with Romanovsky-type stains, and that they were all quite small, reinforced adherence to the grouping based on the common hemotrophism. The small size and the often intracellular site of growth of the organisms and their dependence for transmission on arthropods also suggested a relationship to the Rickettsiales (Kreier and Ristic, 1972, 1973). The attributes of some members of the two families are summarized in Table 1.

The stroma of erythrocytes is a suitable habitat for a variety of organisms but the erythrocyte membrane may be a barrier to infection. Tedeschi et al. (1978), for example, described growth of corynebacteria in human erythrocytes after experimental introduction despite the normal inability of the organisms to enter red blood cells. It is a fact however that organisms capable of entering and growing in erythrocytes belong to systematically quite diverse groups. Plasmodia, piroplasmids, and endotrypanosomes among the protozoa, as well as the bacteria described in this chapter are all able to enter and grow in erythrocytes. The grouping together, largely based on the common hemotrophism of the organisms treated in this chapter, should thus be considered an interim solution followed for reasons of historical continuity until either confirmed or rejected on the basis of new data.

The organisms of the genera *Bartonella* and *Grahamella* of the family Bartonellaceae are typical bacteria with cell walls morphologically comparable to the cell walls of other Gram-negative bacteria (Krampitz and Kleinschmidt, 1960; Takano-Moron, 1970). Despite the fact that in vivo they often grow intracellularly, they can be cultured without difficulty on nonliving culture media. In culture, organisms of the genus *Bartonella* may be flagellated (Peters and Wigand, 1952) but organisms of the genus *Grahamella* are not (Krampitz and Kleinschmidt, 1960). *Bartonella* and *Grahamella* are thus small, facultatively intracellular Gram-negative bacteria. It is apparent then that these organisms do not have the attributes classically ascribed to the rickettsiae: small, obligately intracellular bacteria which cannot be cultured outside of living host cells (see Chapter 121 and Moulder, 1962).

The organisms of the genera *Anaplasma, Aegyptianella, Haemobartonella,* and *Eperythrozoon* of the family Anaplasmataceae differ from typical bacteria since they lack cell walls and cannot be cultured on nonliving media (Aikawa and Nussenzweig, 1972; Peters et al., 1973; Tanaka et al., 1965). All prokaryotic protists which lack cell walls have some similarities, particularly in morphology (Anderson, 1969; Davis et al., 1973). Prokaryotic organisms lacking cell walls are, despite their morphological similarities, a quite heterogeneous group. Some are mycoplasmas and others may be cell wall-defective bacterial variants (L-phase variants). Some L-phase variants may revert to normal vegetative cells while others are stable. Stable L-phase variants are indistinguishable from mycoplasmas morphologically but nucleic-acid-homology studies show that L-phase bacteria and mycoplasmas are not closely related and that L-phase bacteria are themselves heterogeneous (Davis et al., 1973).

Organisms of the family Anaplasmataceae have not been cultured outside of their hosts. Most mycoplasmas are readily cultured and form characteristic colonies on solid culture media (Hayflick, 1969). L-phase organisms, which often resemble mycoplasmas, in general have the cultural requirements of the bacteria from which they are derived except that the tonicity of the culture medium must be increased to prevent lysis of the cell-wall-less organisms.

Organisms of the various genera of the family Anaplasmataceae are all quite similar morpho-

Table 1. Characteristics of the Bartonellaceae and the Anaplasmataceae.

Characteristic	Family Bartonellaceae		Family Anaplasmataceae			
	Bartonella bacilliformis	*Grahamella*	*Anaplasma*	*Aegyptianella*	*Haemobartonella*	*Eperythrozoon*
Giemsa stain	Intense purple	Intense purple	Intense purple	Intense purple	Intense purple	Pale purple
Gram stain	−	−	−	−	−	−
Morphology:						Easily flattened
Shape (most common)	Rods	Rods	Cocci	Cocci	Cocci	Cocci
Cell walls	+	+	−	−	−	−
Flagella	+	−	−	−	−	−
Motility	+	−	−	−	−	−
Size	0.25–0.5 × 1.0–3.0 μm	0.25 × 0.5–1.0 μm	0.3–0.4 μm	0.3–0.8 μm	0.3–0.5 μm	0.4–1.5 μm
Intracellular location	Intra- and epierythrocytic; in endothelial cells	Intraerythrocytic	Intraerythrocytic inclusions; 0.3–1.0 μm	Intraerythrocytic inclusions; 0.3–3.9 μm	Epierythrocytic; firmly attached in deep grooves	Epierythrocytic; loosely attached in shallow grooves
Multiplication pattern	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission
Growth on culture media	+	+	−	−	−	−
Effect of splenectomy	−	Slight	+	+	+	+
Transmission (cyclic)	Arthropod (Phlebotomus)	Arthropod (fleas)	Arthropod (ticks)	Arthropod (ticks)	Arthropod (lice, fleas)	Arthropod (lice, fleas)
Geographical distribution	Andes, South America	Worldwide	Worldwide	Southern Europe	Worldwide	Worldwide
Host	Humans	Small mammals	Ungulates	Birds	Vertebrates	Vertebrates
Drug sensitivity;						
Arsenic	−	−	−	−	+	+
Sulfanamides	−	ND	−	−	−	−
Penicillin	+	ND	−	−	−	−
Streptomycin	+	ND	−	−	−	−
Tetracycline	+	ND	+	+	+	+
Dithiosemicarbazone	ND	ND	+	+	ND	ND
Clinical disease in intact animals	+	−	+	+	Only *H. felis*	Only *E. suis*

Symbols: +, positive; −, negative; ND, no data.
Data from Weinman, 1944; Peters and Wigand, 1955; Kreier and Ristic, 1968; Ristic and Kreier, 1984a and 1984b; Gothe and Kreier, 1977.

logically and are also morphologically similar to mycoplasmas and L-phase bacteria (Peters et al., 1973; Tanaka et al., 1965). The lack of a cell wall is the major factor in determining the organism's morphology but it may not be assumed that all organisms lacking cell walls are mycoplasmas (Davis et al., 1973).

The Anaplasmataceae occur in the blood plasma, and in or on red cells. Those which enter red cells do so by an endocytic process. Reproduction then occurs by binary fission of the cells that become established in the parasitophorous vacuole (Ristic, 1968; Ristic and Watrach, 1961; Tanaka et al., 1965). The characteristic morphologies of the organisms of the various genera of the Anaplasmataceae seen in stained blood films are determined more by their relationships to the host erythrocytes, grouping of the parasites at the sites of growth, and accessory structures associated with the parasites, than by the characteristics of the organisms themselves (Kreier and Ristic, 1973).

The occurrence of reproduction by binary fission without morphologically specialized infectious forms clearly differentiates the organisms of the family Anaplasmataceae from the chlamydiae, since the chlamydiae are characterized by a unique obligately intracellular growth cycle. This cycle is characterized by the appearance of an infectious elementary body, which is a small, electron-dense form, and a noninfectious initial body which is larger, less electron-dense, and is the vegetative form (Page, 1974).

The cellular nature of the Bartonellaceae and Anaplasmataceae precludes consideration of viral groups for these parasites (Kreier and Ristic, 1973).

As can be seen from the preceding discussion, the Anaplasmataceae and Bartonellaceae do not readily fit into any of the well-established taxons of prokaryotes. The *Bartonella* and *Grahamella* are true, small, hemotrophic bacteria. The Anaplasmataceae are small, cell-wall-less, obligately parasitic, hemotrophic prokaryotes. Information from systematic antigenic analysis and information on nucleic acid homology, which would aid in determining relatedness among these organisms and between them and other organisms, is not available. The taxons into which these organisms are presently placed are based largely upon morphology, host range, tissue and cellular preference, and established tradition (Ristic and Kreier, 1984a, 1984b; Weinman, 1974).

These organisms clearly do not fit comfortably with the rickettsiae as classically defined, nor is there good reason to assume that the Bartonellaceae and Anaplasmataceae are necessarily closely related. To reduce taxonomic confusion, Moulder (1974) redefined the rickettsiae in a very broad fashion, so that it is possible to allow these organisms to remain together in their customary taxonomic position (Ristic and Kreier, 1984a, 1984b; Weinman, 1974). This course of action would appear to be completely justified in light of the meager information available on the relationships among these organisms at the present time.

There are available a number of fairly thorough reviews of the literature on these organisms (Gothe and Kreier, 1977; Kreier and Ristic, 1968; Ristic, 1960, 1968, 1977; Weinman, 1944, 1968; Weinman and Kreier, 1977).

The Bartonellaceae Family

The Genus *Bartonella*

Bartonella bacilliformis is a hemotrophic bacterium which was first isolated from the blood of infected patients by Albert Barton (1909). In humans, it causes a unique clinical syndrome known as bartonellosis or Carrion's disease. Although the disease has been known for centuries in the South American Andes, it first attracted the attention of the world's medical community in 1871 when an epidemic outbreak caused more than 7,000 deaths among workers constructing a railway between Lima and La Oroya in Peru (Weinman, 1944). Further observations concerning the disease were made by the 1913 Harvard Expedition to Peru (Strong et al., 1915). The clinical spectrum of bartonellosis is quite broad and has been the subject of a number of monographs over the past 100 years (Hurtado et al., 1938; Odriozola, 1898; Ricketts, 1949).

Habitats of *Bartonella*

GEOGRAPHICAL DISTRIBUTION. *Bartonella bacilliformis* and the disease it causes are geographically restricted to the western Andes of the South American countries of Peru, Ecuador, and Colombia, where its arthropod vector is found. Even within these locales, it is confined to elevations between 500 and 3,000 meters (Reynafarje, 1972), where temperature and humidity conditions are favorable for survival of the vector. Although organisms resembling *Bartonella* have been described on other continents (Dooley, 1980; Wernsdorfer, 1969; Whitaker et al., 1966), there are no confirmed reports of the occurrence of *B. bacilliformis* outside the Andes region of South America.

AS SYMBIONTS OF ARTHROPODS. *Bartonella bacilliformis* is transmitted through the bite of *Phlebotomus verrucarum* (Hertig, 1942; Shannon, 1929), a hematophagous sandfly with nocturnal feeding habits and a short flight range. Victims are usually infected during the twilight hours, and a single night in an endemic zone is often sufficient to lead to infection (Dooley, 1976). Other species of sandflies have also been implicated as vectors in regions where typical cases of bartonellosis have been described in the absence of *P. verrucarum* (Herrer and Blancas, 1962; Hertig, 1942; Noguchi and Shannon, 1929; Noguchi et al., 1928). The biological cycle of *Bartonella* in the vector is not known.

AS HUMAN PATHOGENS. *Bartonella bacilliformis* is the only species in the genus *Bartonella* (Ristic and Kreier, 1984a). In humans it causes bartonellosis or Carrion's disease, which is typically described as a biphasic disease with two strikingly distinct clinical forms, Oroya fever (a hemolytic disease) and verruga peruana, (a disease characterized by wart-like growths) which usually occur sequentially and are immunologically linked. That both phases are caused by the same organism was proved in 1885 by Daniel Carrion, a Peruvian medical student, who inoculated himself with blood from a verrugous nodule (the wart-like lesion induced by *B. bacilliformis*) and died several weeks later of Oroya fever (Schultz, 1968).

The symptoms of Oroya fever may develop in a patient 1 to 3 weeks following the bite of an infected sandfly. In its most severe form, Oroya fever is an acute, rapidly evolving, febrile anemia. During the anemic period 90 to 100% of a patient's erythrocytes may be parasitized with bacillary forms of *Bartonella* (Fig. 1). The cause of the progressive and severe anemia is a massive destruction of the parasitized red blood cells in the spleen and other organs of the reticuloendothelial system (Reynafarje and Ramos, 1961). The erythrocyte count may fall to less than 1 million cells per mm^3 in a four- to five-day period (Pinkerton, 1963). Without treatment, mortality rates may be as high as 40% (Weinman, 1944). Secondary salmonellosis is a frequent complication and cause of death during the febrile anemic phase of the disease (Cuadra, 1956). A prolonged asymptomatic period may follow the anemic stage. However, in many patients, a chronic eruptive, or verruga peruana, phase develops. The verruga peruana phase is characterized by hemangiomatous skin eruptions, whose characteristic features are endothelial cell proliferation and pronounced vascularization. These lesions vary in size and number and may occur as either miliary, nodular, or mulaire forms. They exhibit a marked tendency to hemorrhage due to their extensive vascularity. Histological studies have revealed the presence of bartonellae and proliferating endothelial cells, and an infiltration of inflammatory cells in the nodules (Arias-Stella et al., 1986; Recavarren and Lumbreras, 1972). Although the role of the bacteria in the development of the verruga stage is unclear, it is possible that they either possess some angiogenic potential as suggested by Garcia et al. (1988), or that they induce inflammatory cells to release angiogenic factors (Arias-Stella et al., 1986), which stimulate endothelial cell proliferation and capillary growth.

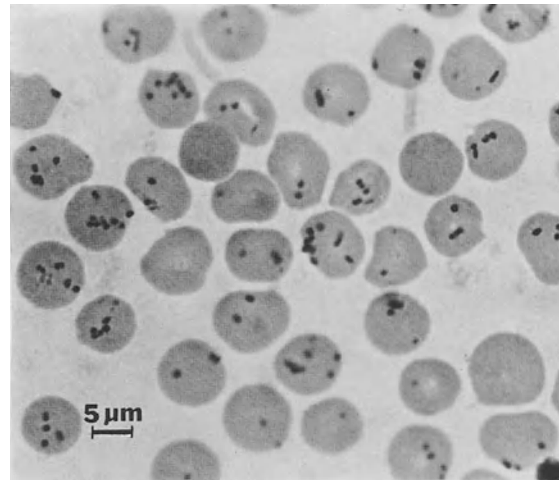

Fig. 1. Wright-stained *Bartonella bacilliformis* in blood of an Oroyo fever-infected human. (From Takano-Moron, 1970, with permission.)

Oroya fever and verruga peruana usually occur sequentially. However, the anemic phase may occur without subsequent development of the eruptive phase and likewise, the eruptive phase may occur without a preceding overt anemic stage (Aldana, 1929; Cuadra, 1957; Hurtado et al., 1938). The fact that the two phases rarely coincide suggests a role for the host's changing immune status.

AS INAPPARENT PARASITES OF HUMANS. Epidemiological studies have shown that in areas endemic for bartonellosis, 5 to 10% of the population with or without a past history of the disease yield blood cultures positive for *B. bacilliformis* (Colichon et al., 1972; Herrer, 1953b; Herrer and Cornejo Ubilluz, 1962; Weinman and Pinkerton, 1937b). Latent infection, during which *Bartonella* can be isolated, may persist in patients for long periods (Ricketts, 1949). These findings strongly support the hypothesis that humans are an important reservoir of *Bar-*

tonella bacilliformis (Weinman and Pinkerton, 1937b). However, other reservoirs may also exist (Herrer, 1953a).

Isolation of *Bartonella*

GENERAL FEATURES. *Bartonella bacilliformis* is a fastidious bacterium whose exact nutritional requirements have not been defined. It can be recovered from a variety of clinical specimens including blood, splenic, lymphoid, and hepatic endothelial cells, and verrugous lesions (Ristic and Kreier, 1984a). Isolation of the organisms is generally easier during the early stages of the disease.

A variety of methods and media for the isolation and cultivation of *Bartonella* have been described (Benson et al., 1986; Geiman, 1941; Jiminez, 1940; Knobloch 1988; Mitchell and Slack, 1966; Noguchi and Battistini, 1926). Although it is an intracellular parasite, it must be considered facultative in this respect since it can be readily grown in culture media provided they are enriched with blood or serum.

The triphasic medium described by Colichon and colleagues (Colichon et al., 1966; Colichon et al., 1971) is particularly useful since it permits good yields of *Bartonella.* The triphasic medium consists of sequential layers of a Bordet blood agar slant, a tryptose agar slant and an overlying layer of tryptose broth prepared according to the following protocol:

Triphasic Medium for Growth of *Bartonella*

Bordet blood agar

Infusion of potato	1 liter
Glycerine	10 ml
Sodium chloride	5.4 g
Proteose peptone	10.0 g
Agar	14.0 g

The components are dissolved by heating and the pH is adjusted to 7.2. After autoclaving (15 min at 15 psi), the medium is cooled to 50°C and 15% sheep blood (v/v)is added. The medium is dispensed into Kolle flasks which are placed in a slanted position until the medium solidifies. When the Bordet blood agar is solidified, add tryptose agar medium whose formula is as follows:

Tryptose agar

Tryptose	15.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Distilled water	1 liter
Agar	13.0 g

The components are dissolved by heating and the pH is adjusted to 7.2. After autoclaving, the medium is allowed to stand until the particulate matter settles. Sheep plasma (15% v/v) is then added to the clear supernatant. The tryptose agar is dispensed as a thin layer over the Bordet blood agar layer and allowed to solidify in a slanted position. Finally, flasks are returned to a vertical position and 30 ml of tryptose broth (same composition as tryptose agar but without the agar and plasma) is added to each. As a sterility check, flasks are incubated for 48 h prior to use.

Culture Media for Preservation and Additional Studies of *Bartonella*

In vitro motility of *Bartonella* is easily observed in cultures maintained in liquid overlays of nutrient medium. Brain heart infusion agar containing 10% human serum (v/v) and 10% human red blood cell lysate (v/v) is prepared as the base medium. Phosphate-buffered saline (PBS), pH 7.4, is used as an overlay. Bartonellae grow readily in the overlay as well as at the agar-overlay interface (Benson et al., 1986). Active growth can be maintained by transfer of aliquots of established cultures to fresh cultures every 48 h. For long-term preservation of *Bartonella,* aliquots of the growth in the overlay can be mixed with dimethylsulfoxide (DMSO) (9:1 v/v) (Lee Ann Benson, personal communication) and stored at −70°C.

A transparent culture medium for culture maintenance and growth studies can also be prepared in test tubes by layering Bordet blood agar in the bottom of 16 × 150 mm test tubes and overlaying with tryptose agar to a depth of 2 cm. The medium is inoculated by puncture. With this system, it is possible not only to demonstrate aerobic and microaerobic growth of *Bartonella,* but also to determine metabolic activity of the organism by adding substrates with appropriate indicators to the tryptose agar.

Technique for Isolation of *Bartonella* from Human Clinical Specimens

Clinical specimens can be processed in several ways for the isolation of *Bartonella.* Cultures can be obtained from a high proportion of infected individuals by use of the following protocol: Ten ml of venous blood is mixed with 0.6 ml of 10% sodium citrate and the mixture centrifuged. The cellular sediment is inoculated into a flask of triphasic culture medium, which is then incubated at 28°C and scored for growth after the fifth day of incubation.

Identification of *Bartonella*

The identification of *Bartonella bacilliformis* depends to a great extent on epidemiological and clinical considerations. The occurrence of appropriate symptoms in an individual who has been in areas of known endemicity is a strong basis for making a presumptive diagnosis of bartonellosis. Identification of *Bartonella* in

blood cultures and blood smears is required for a positive diagnosis of Oroya fever. The identification of *Bartonella* in histologic sections of lesions is used to confirm a clinical diagnosis of verruga peruana (Weinman, 1944).

Morphological and growth characteristics are also considered in the identification of *Bartonella bacilliformis.* The organism is a Gram-negative nonsporeforming coccobacillus which exhibits pleomorphism both in vitro and in vivo. It is most easily observed in Giemsa- or Wright-stained blood smears where it appears as intense purple rods or cocci (see Fig. 1). During the early stages of Oroya fever, the predominant forms are bacilli, 1 to 3 μm in length by 0.2 to 0.3 μm in width. Polar enlargement and polar cytoplasmic granules may be seen. As the patient convalesces or as cultures age, cocci, approximately 0.75 μm in diameter, appear (Peters and Wigand, 1955; Weinman, 1944).

Bartonella have a thin cell wall (Fig. 2) (Cuadra and Takano, 1969; Peters and Wigand, 1955) which, in tissue sections, is seen to enclose a double membrane (Recavarren and Lumbreras, 1972). At least 24 protein antigens, 6 of which are probable cell wall antigens, have been identified by immunoblot and immunoprecipitation procedures (Knobloch, 1988). These cell wall antigens have been used to detect specific antibodies in the sera of bartonellosis patients and may, therefore, have diagnostic value (Knobloch et al., 1985). L-forms of *Bartonella* have been isolated from cultures grown on penicillin-containing medium (Sharp, 1968).

Culture forms of *Bartonella* have flagella at one pole (Fig. 3) (Perez-Alva and Giustini, 1957; Peters and Wigand, 1951). This characteristic serves to differentiate *Bartonella* from *Grahamella* cells, which are non flagellated. Both motile and nonmotile forms may be flagellated. A correlation generally exists between

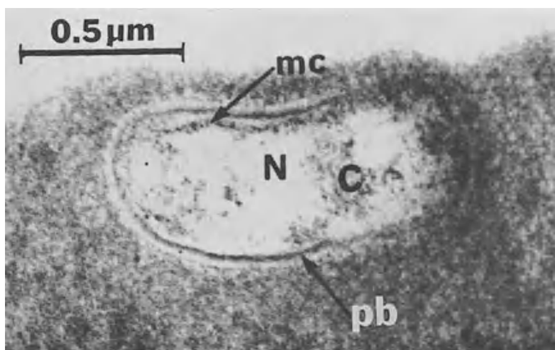

Fig. 2. *Bartonella bacilliformis* in blood cells prepared for electron microscope examination showing cell wall (pb), cell membrane (mc), cytoplasm (C), and nucleoid region (N). (From Takano-Moron, 1970, with permission.)

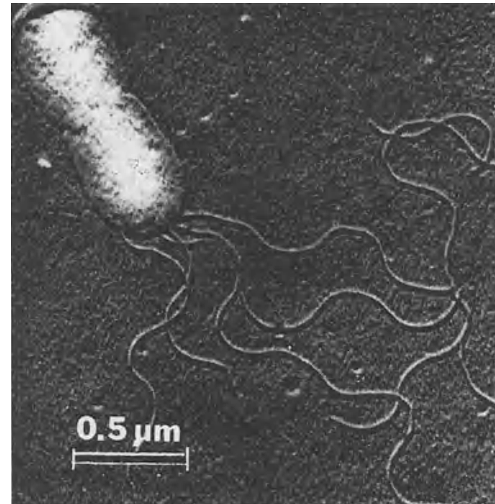

Fig. 3. *Bartonella bacilliformis* from a culture showing flagella and commencement of binary fission. (From Peters and Wigand, 1952, with permission.)

the degree of motility and colony size, with large-colony-size variants exhibiting greater motility (Benson et al., 1986; Walker and Winkler, 1981). *Bartonella* in red blood cell ghosts (G. Mernaugh, personal communication) have been observed to move at a speed of 45 μm per second. This is comparable to or even faster than that reported for most other motile bacteria (Macnab and Koshland, 1972).

The ability to grow in vitro further differentiates *Bartonella* and *Grahamella* from *Haemobartonella* and *Eperythrozoon. Bartonella* grows optimally at a temperature of 28°C and a pH of 7.8 (Noguchi and Battistini, 1926). Colonies are generally visible between the 5th and 8th day of incubation. They are translucent, mucoid, slightly adherent to the agar, and vary in size from minute to 1–2 mm in diameter. Bartonellae are non hemolytic and fail to ferment a variety of common carbohydrates (Noguchi and Battistini, 1926). They are susceptible to the action of penicillin, streptomycin, chloramphenicol, tetracycline, and erythromycin (Aldana et al., 1948; Larrea, 1958; Merino, 1945; Payne and Urteaga, 1951).

Electron microscopic studies have shown that *Bartonella* can adhere to and enter red blood cells (Cuadra and Takano, 1969; Takano-Moron, 1970) where they may be seen within vesicle-like structures. In vitro, the organism can attach to erythrocytes, causing them to spin or "dance" (Cuadra, 1978). Continued interaction between *Bartonella* and the red blood cells leads to extensive deformation of the red cell surface, as pits, trenches, and invaginations develop (Figs. 4 and 5) (Benson et al., 1986; G. Mer-

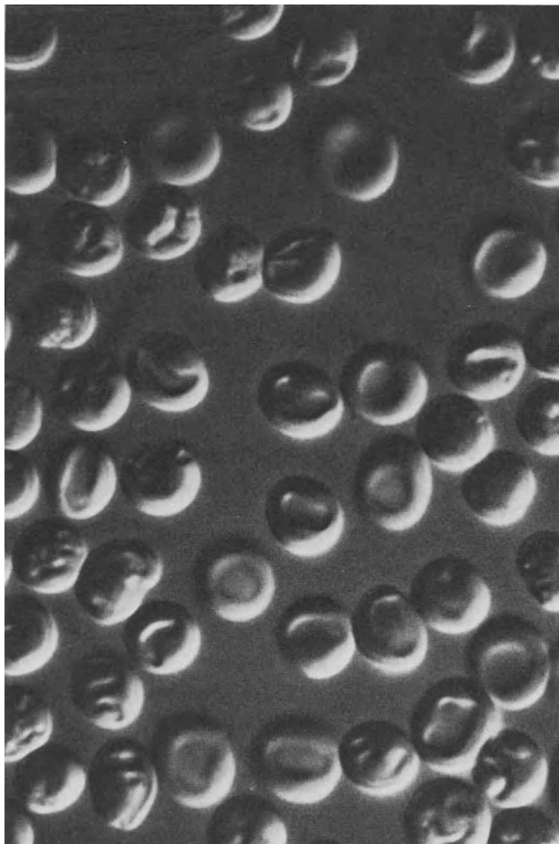

Fig. 4. Deformation of erythrocytes induced by *Bartonella bacilliformis* as seen by scanning electron microscopy. (From Benson et al., 1986, with permission.)

naugh, unpublished observations). The mechanisms by which *Bartonella* adhere to and invade erythrocytes are not known. It has been suggested that specific receptors on the red cell, possibly glycolipid in nature, are involved in the initial recognition step (Walker and Winkler, 1981). The *Bartonella* receptor for binding appears to be located at the polar end opposite the flagellum (Ihler and Tosi, 1987). Furthermore, since individual bacteria do not agglutinate red cells, whereas clusters of bacteria can, it has been proposed that there may be only one binding site per bacterium (Cuadra, 1978). Entry into the red cell most likely involves a process of forced endocytosis since *Bartonella* can be found within vacuoles where their movement is restricted by the confines of the vacuole. Rupture of the vacuoles by hypotonic shock releases the organism into the red cell interior (Benson, et al., 1986; Ihler and Tosi, 1987).

Only the verruga stage of bartonellosis can be experimentally reproduced with regularity in animals; the experimental animal of choice is the rhesus monkey. The symptoms of Oroya fever appear to be unique to humans (Noguchi, 1926; Weinman and Pinkerton, 1937a).

Based on its morphological and biological characteristics, *Bartonella bacilliformis* is considered a true bacterium (Nauck, 1957). It has a cell wall and flagella, divides by binary fission, and can be grown in vitro in culture media. Taxonomically, it is grouped with the rickettsias along with other hemotrophic bacteria (Ristic and Kreier, 1984a).

Bartonella is a somewhat neglected pathogen due to its restricted geographic habitat and the fact that insecticides and antibiotics effectively control its vector and the disease it causes. Nevertheless, it is an interesting organism because it is unique among human pathogens in its parasitic capabilities and in the disease states which it induces.

Grahamella and Its Habitats

GEOGRAPHICAL DISTRIBUTION. The Grahamellae have a world-wide distribution, one consequence of their pattern of cyclic flea-borne transmission. No climatic barriers to their occurrence exist, and a variety ecological niches are used by *Grahamella* when suitable hosts are present. The incidence of *Grahamella* infections in small mammals reflects generation dynamics and the undulating population densities of the respective vertebrate and arthropod host communities. At least outside the tropics, a characteristic rise in incidence of infection usually occurs in the late summer and early fall when the most favorable conditions for transmission occur (Krampitz and Kleinschmidt, 1960). The parasites can be recognized in stained blood films (Fig. 6). The percentage of hosts which appear to be infected in the field usually fluctuates between 0 and 30%, with an average of 10%. This was already known to Graham-Smith (1905), who first observed the parasites in British moles (*Talpa europea*). A "*Drosophila*-like" scale (Chaline, 1977) of both host fertility and parasite generation time favors the development of limited host specificity and of parasite speciation.

AS SYMBIONTS OF ARTHROPODS. Parasitized cells can readily be isolated from arthropods (Fig. 7). The appearance of these cells reminds us of those shown in the photographs by Pinkerton and Weinman (1937) of tissue explants infected with *Bartonella bacilliformis.* There are more or less stable differences between strains of grahamellae from different host species. While only rarely has the experimental transmission from one vertebrate host species to another proved successful (Krampitz and Kleinschmidt, 1960), a species specificity does not exist for arthropods. It has been confirmed

Fig. 5. Deformation of erythrocytes induced by *Bartonella bacilliformis,* as seen by Nomarski microscopy. (Courtesy of G. Mernaugh.)

experimentally that any xenotype of *Grahamella* can use any vector species of the same order (Krampitz and Kleinschmidt, 1960).

For rodent grahamellae, fleas act as arthropod hosts and vectors. This was first proved by Vassiliadis (1935) in Egypt and then confirmed experimentally by Krampitz and Kleinschmidt (1960) and Krampitz (1962) in Germany and Fay and Rausch (1969) in Alaska. It is not known whether other ectoparasites are involved. Adult fleas newly infected with blood discharge large masses of infective parasites a few days after infection (Fig. 8); this discharge persists for several weeks at least. *Grahamella* are ingested by larval fleas from the sheddings of their parents. Infected adults never develop from such infected larvae. No evidence exists for transovarial passage in fleas. It remains to be determined, however, whether grahamellae can penetrate or be transported through the gut wall and invade the fleas' hemocoel. This possibility was taken into consideration by Hertig and Wolback (1924) in their studies on rickettsia-like organisms in insects and by Ito et al., (1975) for *Rickettsia mooseri* but no definite conclusions were reached by those workers. Normally, the fleas' salivary glands and saliva are not involved in transmission (Krampitz, 1962). Since survival outside the host body seems to be limited, direct contamination of the vertebrates' mucous membranes or of small skin wounds by feces appears to be the common mode of infection.

AS PARASITES OF VERTEBRATES. The vertebrate host range (Weinman and Kreier, 1977) is limited mainly to rodents, insectivores, chiroptera, and marsupials. Reported infections in domestic animals, except the dog (Herrer, 1944), have so far not been culturally confirmed. Only a few reports of *Grahamella* in subhuman primates, carnivores, ruminants, reptiles, and fishes exist. Human beings do not seem to be infected. Most of the approximately 40 named species are described only on the basis of their appearance in stained blood films of various host species. For a list of names and citations, see Weinman and Kreier (1977). The last descriptions of that kind were of *G. cuniculi* in Egyptian rabbits (Haiba, 1963) and *G. legeri* in norway rats in Rio de Janeiro (Furlong, 1978).

The stroma of red cells is the exclusive habitat of *Grahamella* in the vertebrate host. Reproduction in this site is by binary fission (Fig. 9). *Grahamella* seen in blood monocytes (Fig. 10) are forms phagocytized after release from lysed erythrocytes. In the arthropod vector, there is obviously an intimate relationship with the cells of the gut wall (see Fig. 7). The common statement that grahamellae are not known to multiply in fixed tissue cells should be restricted to descriptions of its development in the vertebrate host.

Parasites of the genus *Grahamella* seem to be forgotten organisms in the research on blood parasites of animals. We are still missing much basic information about their metabolic and antigenic properties, information available for most of the other parasitic prokaryotes. Only the gross features of their parasitic life can be outlined. Despite their intracellular growth in the erythrocytes of appropriate vertebrates and the intestinal tract of suitable arthropod vectors, the organisms do not cause serious damage to the host cells or produce disease. There may be a mild transitory reticulocytemia in the initial phase of the infection when a large infectious dose is given (see Fig. 6), but a hemolytic crisis, as occurs following infection with some other hemotrophic bacteria that results in a complete breakdown of the erythrocyte population, never takes place.

The method by which the organisms invade the red cells without causing sufficient injury to the membrane to induce pathologic effects is not known. The existence of a host receptor

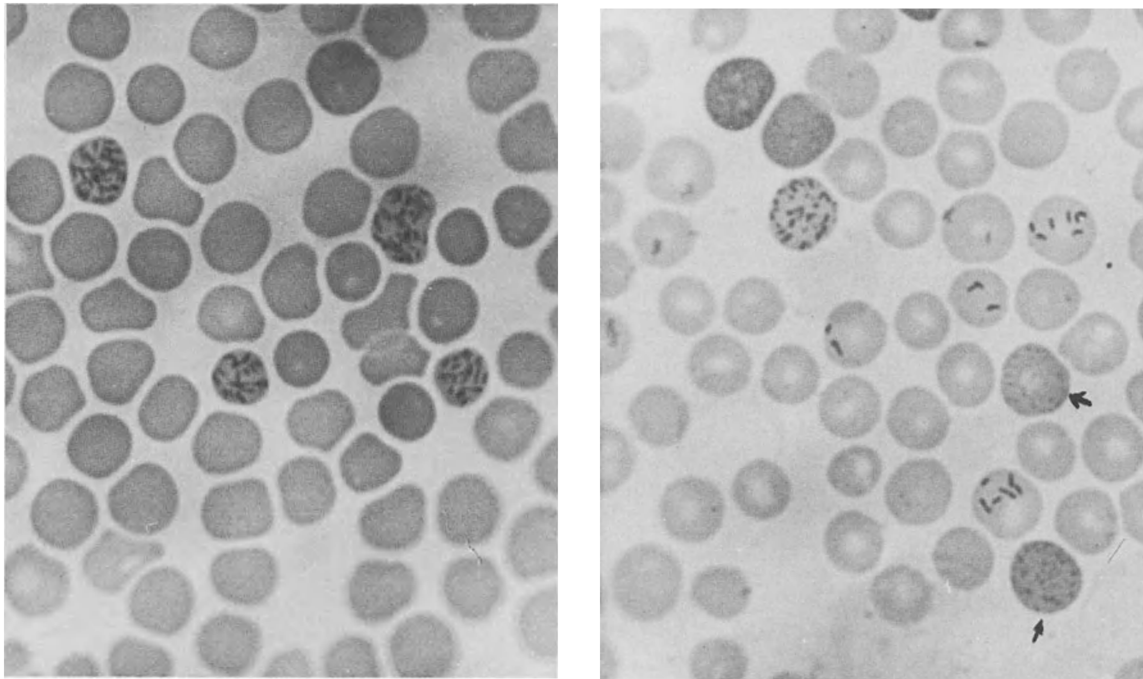

Fig. 6. Development of *Grahamella* sp. in erythrocytes of a European bank vole (*Clethrionomys glareolus*). Left: 3 days after experimental infection; a slight reticulocytosis is evidence of a light anemia. Right: The same infection 6 days after infection; strongly basophilic granulations in young red cells occur frequently in rodent blood and are sometimes misinterpreted as parasites (arrows).

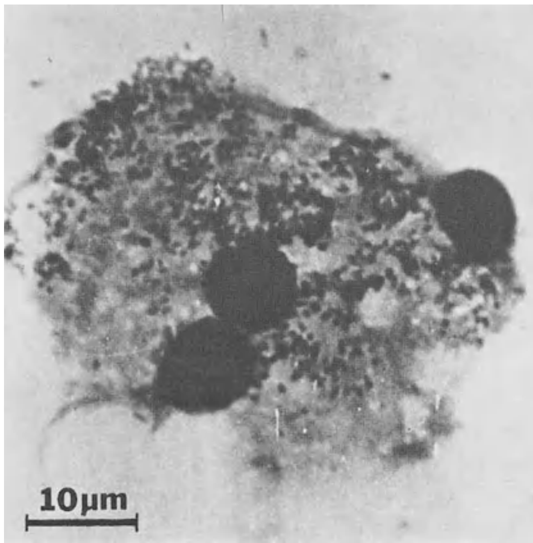

Fig. 7. Isolated midgut cells of the tropical rat flea (*Xenopsylla cheopis*) containing numerous cells of *Grahamella*. Giemsa stain.

mechanism could explain host specificity. The hosts' and parasites' mutual identification methods are unknown. The process of entry into the erythrocyte itself, however, seems different from that of *Bartonella bacilliformis* (Benson et al., 1986; E. Göbel, personal communication). The term "grahamellosis" is more of a construction analogous to the term "bartonellosis" than a term appropriate for characterization of the pathological disorder produced by *Grahamella* in their hosts. The lack of interest in *Grahamella* of the medical and veterinary professions is probably due to their lack of pathogenicity and their failure to infect either humans or domestic animals.

Isolation of *Grahamella*

The difficulty in producing a parasitemia in a susceptible host animal after inoculation of infected donor blood is one of the most puzzling features of the in vivo isolation and maintenance of *Grahamella* strains. The failure is not dose-dependent, nor is it controlled by previous sensitization of the receptor animal in any way. This aspect of *Grahamella* behavior is very different from that of all related intraerythrocytic microorganisms. The reason for this behavior is unknown. However, every *Grahamella* strain can be isolated in a highly virulent condition from the feces of infected fleas (see Fig. 8). The parasites grow easily in vivo when splenectomized, susceptible hosts are inoculated intraperitoneally with the forms from the vector's feces.

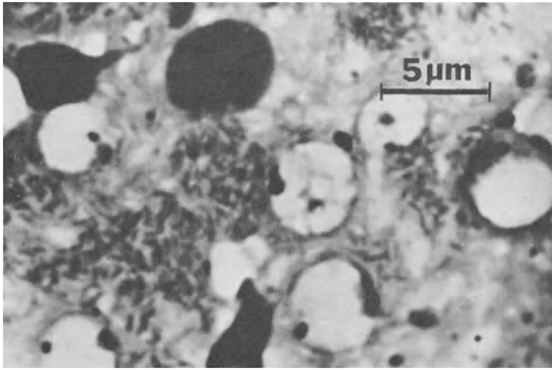

Fig. 8. Infective *Grahamella* in feces of the tropical rat flea (bank vole strain). Giemsa stain.

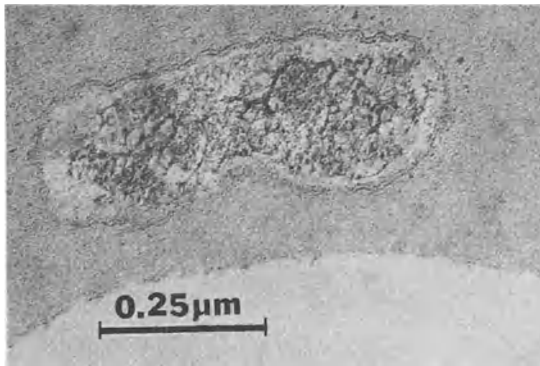

Fig. 9. Binary fission of *Grahamella.* The parasites are within the stroma of the red cell. Ultra-thin section, fixed in glutaraldehyde, embedded in Durcupan ACM (Fluka). (Courtesy of E. Göbel.)

The parasitemia starts 3–6 days after infection with an extensive spread of the organisms all through the bloodstream and with intensive intraglobular reproduction. As many as half the erythrocytes (Fig. 6) may be infected in such animals (Krampitz, 1962). In its density, the early massive blood infection due to *Grahamella* is very "*Bartonella*-like," but only a moderate reactive polychromasia takes place. The parasite does not use reticulocytes or nucleated cells as its microhabitat in the vertebrate at any time. However, in nearly all blood preparations with many *Grahamella*-infected red cells, monocytes can be seen which contain phagocytized and partially disintegrated parasites (Fig. 10). Destruction, not reproduction, occurs in nucleated mammalian blood cells.

The normal aspect of a *Grahamella* infection in its late stages is a concentration of many parasites in only a few host cells which are sometimes difficult to detect. A dense parasitemia guarantees better results if one intends in vitro cultivation or isolation for morphological studies or infection of experimental vectors.

A further remarkable difference between the grahamellae and most other hemotrophic organisms is the failure of splenectomy to accelerate the blood infection unless surgery is carried out immediately after infection, or prior to the infection. Basically, no qualitative differences seem to exist among the numerous xenotypes or species in their host-related properties and behavioral patterns.

In vitro growth of *Grahamella* occurs 2–3 mm below the surface in stab cultures and suggests an oxygen requirement slightly less than that of air (Fig. 11). Only a few semi-defined media have been found to be suitable for growth of *Grahamella,* but little systematic work has been done so far. In view of the organism's proven microaerophilic character, some reservations are appropriate about reports of easy growth on the surface of blood agar and other solid media.

Semisolid media developed for *Leptospira* and *Bartonella* are suitable for *Grahamella.* An important first step in cultivation was the development of the thin serum agar medium by Noguchi and Battistini (1926). Tyzzer (1941) was the first to cultivate *Grahamella* strains from American small mammals in this medium. Krampitz and Kleinschmidt (1960) grew parasites of European origin in rabbit serum with an agar base at pH 7.2. Additional enrichment of the medium with hemoglobin promotes growth.

Medium for Growing *Grahamella*

Saline (0.9%)	800 ml
2% Agar (pH 7.2)	100 ml

Sterilize the agar and distribute 2 ml volumes into small glass tubes. Hold the tubes at 60°C. Add 0.2–0.25 ml sterile (filtered) rabbit serum and 0.1 ml of a concentrated rabbit-hemoglobin solution released from erythrocytes by aqueous lysis. Mix by shaking and autoclave for 30 min at 56°C.

The transfer of cultivated material from tube to tube should be carried out with the platinum loop introduced into the upper part of the medium as for deep stab cultivation. The tubes must be sealed to prevent desiccation. Sand-grain-size colonies appear along the stab 7–10 days after inoculation, most abundantly within a zone situated 3–5 mm below the surface of the medium (Fig. 11); colonies are occasionally also in ring form. The morphology of the colony is greatly influenced by the inoculation technique and the quantity of organisms inoculated. Since no surface growth occurs, it is evident that a reduced amount of oxygen favors growth. An incubation temperature of 27–30°C seems adequate. The white spherical colonies developed

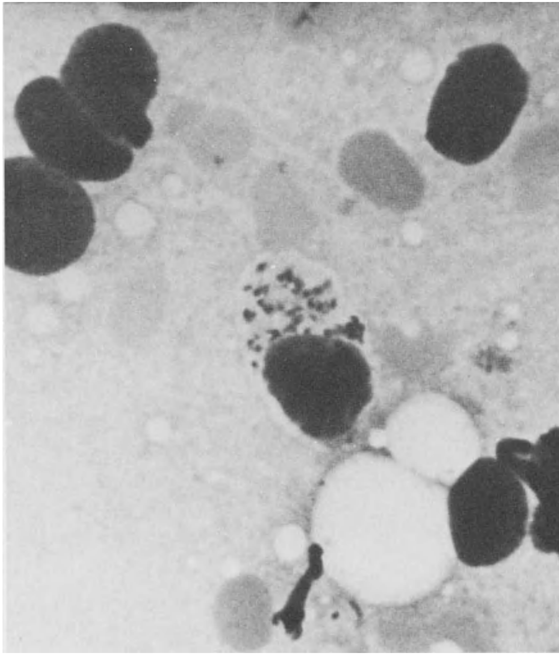

Fig. 10. *Grahamella* sp. in *Clethrionomys glareolus.* Phagocytized and partially destroyed parasites in mononucleated blood cells. Giemsa stain.

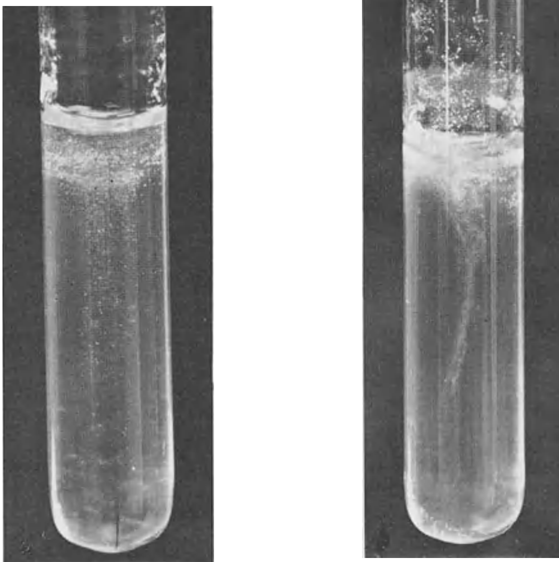

Fig. 11. Deep stab cultures of *Grahamella* in Noguchi's medium. Original isolation from vole blood. (From Krampitz and Kleinschmidt, 1960, with permission.)

on primary inoculation are solid, and they are easy to remove from the medium; colonies produced on subculture are more diffuse and look like snow flakes. Subculture to experimental animals from these cultures may be carried out. Host specificity is never lost in vitro (Krampitz and Kleinschmidt, 1960; Tyzzer, 1941), indicating its genetic basis. Noguchi originally proposed use of rabbit serum in the medium. The addition of hemoglobulin from various sources, even human, promotes in vitro growth of grahamellae of voles and shrews. It is evident, therefore, that host specificity cannot depend on simple nutritional factors.

It is possible to maintain the organisms in the frozen state. All developmental stages except those in the blood may be maintained frozen. The best material for long-term preservation of living strains is the gut content of experimentally infected fleas containing metacyclic grahamellae. The infected gut content must be suspended in the blood of the appropriate host species. Culture material can be preserved in a similar manner, but its infectivity decreases with increasing time of storage. The best methods for preserving grahamellae in the frozen state are those used routinely for preserving blood protozoa (Swoager, 1972). No information is available about cultivation of the organisms in tissue cultures or in hens' eggs, nor have attempts been made to lyophilize the grahamellae, nor has their drug sensitivity been tested.

Identification of *Grahamella*

The easiest characteristic to use for identification of the grahamellae by light and electron microscopy is their appearance inside the erythrocytes. Proper preparation and staining techniques are required and hemolysis of the sample to be analyzed must be prevented. Attempts to concentrate scarce parasitized red cells in thick films have not been successful. In thin blood films stained by the Giemsa method and examined with the aid of a light microscope, grahamellae appear predominately within the erythrocytes as rod-, dumbbell-, or string-shaped purple-red bacteria 1–2 μm long (Fig. 6). Sometimes V- or Y-like figures are found, rarely single cocci, never ring or star forms or chains of several segmenting organisms. Grahamellae are Gram-negative, non acid fast, non flagellated, immobile, and non gas forming. In highly parasitized blood films, groups of extracellular rods can usually be found ("Schwärmende Haufen"; Jettmar, 1932). These are released when injured erythrocytes are broken during film preparation. It is unlikely that free forms can survive and circulate for any significant time. Phagocytes full of *Grahamella* may be observed in stained blood and tissue impression films (Fig. 10). No attempts have been made to determine the organisms' nutritional requirements, metabolic properties, DNA homology, or antigenic structure.

The gross features of the parasites can be established by ultrastructural methods (Krampitz and Kleinschmidt, 1960). Thin sections of the parasitized erythrocytes show *Grahamella* lying within the red cells, always clearly separated from the surface membrane (Fig. 9). They show a cytoplasmic membrane, internal structures, and a trilaminar cell wall characteristic of true bacteria. Reproduction takes place by binary fission. That there is a close ultrastructural relationship between rodent grahamellae and *Bartonella bacilliformis* in human blood is obvious when one compares micrographs of the two organisms (Cuadra and Takano, 1969). The morphological and biological characteristics of the Bartonellaceae are more similar to true bacteria than to the rickettsiae, mycoplasmas, or viruses. The similarities of the characteristics of *Grahamella* and *Bartonella* and their differences from those of the organisms *Anaplasma, Haemobartonella, Aegyptianella,* and *Eperythrozoon* support the legitimacy of the creation of the two families, Bartonellaceae and Anaplasmataceae.

The Family Anaplasmataceae

Eperythrozoon and *Haemobartonella* and Their Habitats

GEOGRAPHICAL DISTRIBUTION. These organisms are obligate parasites, transmitted among vertebrate hosts by a variety of direct and vector-borne means. The distribution of the eperythrozoa and haemobartonellae is therefore, primarily limited only by the distribution of their hosts. In general, the organisms can be assumed to be present within appropriate hosts on all six continents (Gothe and Kreier, 1977; Kreier and Ristic, 1968).

Table 2. Approved *Eperythrozoon* species and principal vertebrate hosts.[a]

Species	Principal vertebrate host
E. coccoides	Albino and wild mice; albino rats; rabbits; hamsters
E. ovis	Domestic sheep, goats, deer, antelope
E. parvum	Domestic pigs
E. suis	Domestic pigs
E. weyoni	Domestic cattle

[a]Based on the *Approved List of Bacterial Names* (1980) or subsequently validly published in the *International Journal of Systematic Bacteriology.* Organisms described as *Eperythrozoon* have been reported from a large number of vertebrate hosts but have not been formally recognized. For a complete list, see Goethe and Kreier (1977).

Table 3. Approved *Haemobartonella* species and principal vertebrate hosts.[a]

Species	Principal vertebrate host
H. muris	Albino and wild mice; albino rats; hamsters
H. canis	Domestic dogs
H. felis	Domestic cats

[a]Based on the *Approved List of Bacterial Names* (1980) or subsequently validly published in the *International Journal of Systematic Bacteriology.* Organisms described as *Haemobartonella* have been reported from a large number of vertebrate hosts but have not been formally recognized. For a complete list, see Goethe and Kreier (1977).

AS SYMBIONTS OF ARTHROPODS. Arthropod-borne transmission between vertebrate hosts is suspected as the primary mode of infection with most species of eperythrozoa and haemobartonellae, and has been proven with several species. Ixodid ticks (for *Haemobartonella canis*), fleas (for *Haemobartonella muris* and *Eperythrozoon coccoides*), and lice (for *Eperythrozoon suis*) are known vectors, while fleas and lice are suspected to be possible vectors for other organisms (Berkenkamp and Wescott, 1988; Crystal, 1958; Nash and Bobade, 1986; Seneviratra et al., 1973). Little information is available on the growth and development of organisms within their arthropod hosts.

AS PARASITES OF VERTEBRATES. The vertebrate host range of both *Eperythrozoon* and *Haemobartonella* is wide and diverse. Although only five species of *Eperythrozoon* (Table 2) are included in the *Approved List of Bacterial Names*, incompletely characterized organisms described as *Eperythrozoon* have been reported from a large number of vertebrate hosts (Kreier and Ristic, 1984). Similarly, while only three species of *Haemobartonella* have been formally recognized (Table 3), there are numerous reports of *Haemobartonella*-like organisms in variety of mammalian and nonmammalian vertebrate hosts (Kreier and Ristic, 1984). Within both genera, a large number of species has been described based primarily on vertebrate host origin. Most are in wild and domestic animals, but there are rare reports of eperythrozoa and *Haemobartonella* in humans (Lwoff and Vaucell, 1930; Kallick et al., 1972; Punaric, 1986). Data supporting speciation is often limited.

In the vertebrate host, *Haemobartonella* infection is limited to the erythrocyte. Eperythrozoa are found either free in the plasma or on the surface of erythrocytes (Kreier and Ristic, 1981). Cells other than erythrocytes (and possibly platelets) do not appear to be infected. Organisms in both genera replicate by binary fission, as seen in Fig. 12 (Tanaka et al., 1965).

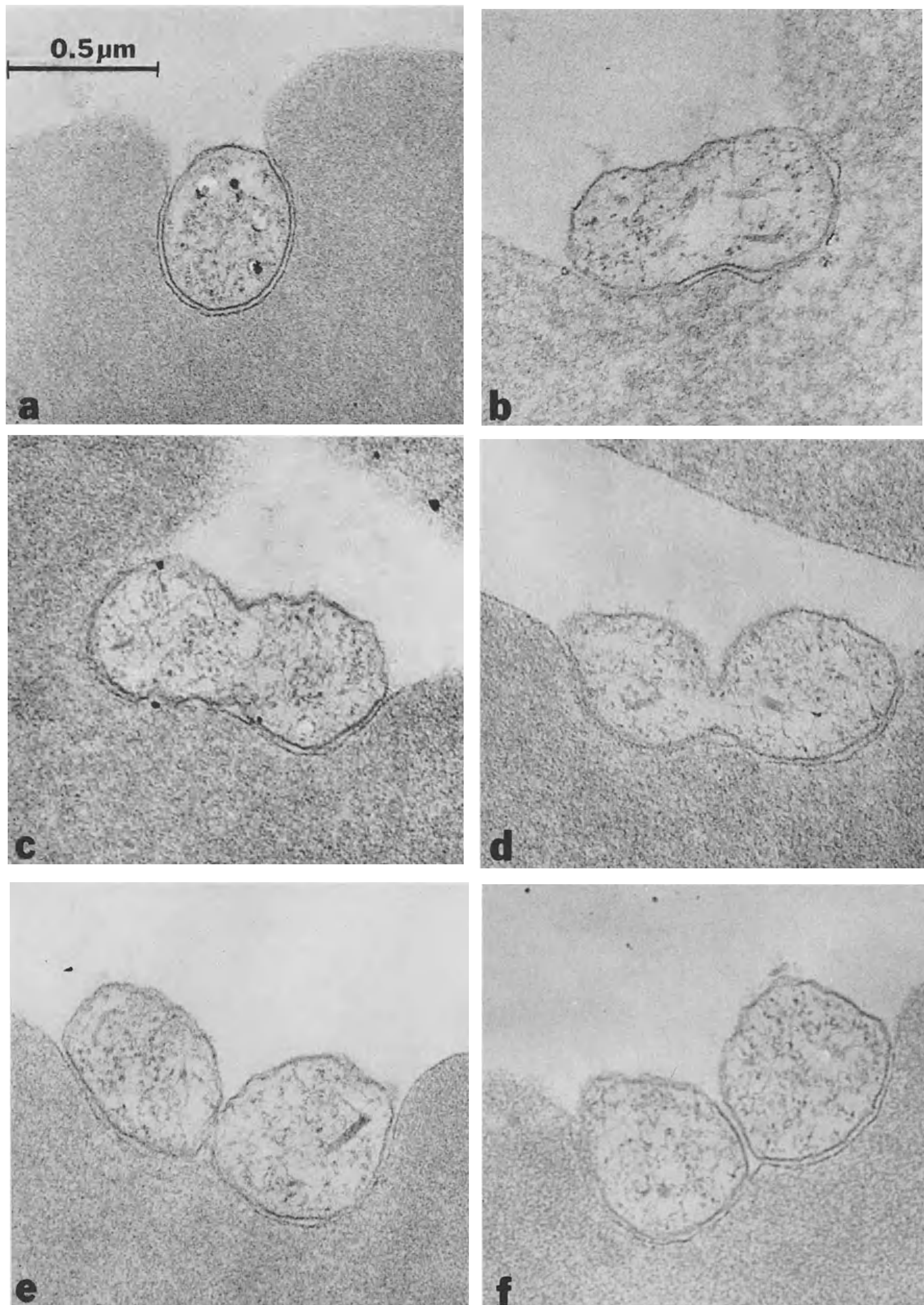

Fig. 12. Transmission electron micrographs of a dividing cell of *Haemobartonella muris.* The coccus first elongates and then constricts in the middle; finally, the two daughter organisms separate. The organisms remain attached to the erythrocytes during reproduction. The erythrocyte membrane appears to remain intact at the site of attachment. (From Tanaka et al., 1965, with permission.)

Organisms do not survive for significant periods outside of their hosts.

Typically, these organisms persist in the blood of immunocompetent hosts without causing significant clinical disease. Serologic assays are available to diagnose persistent infections in animals (Daddow, 1977; Hyde et al., 1973; Smith and Rahn, 1975; Wigand, 1956) Both *Eperythrozoon suis* and *Haemobartonella felis* are pathogenic in healthy, immunocompetent hosts. Acute infection of neonatal pigs with *Eperythrozoon suis* causes severe anemia and frequently high mortality rates (Splitter, 1950). Chronic infection is usually subclinical, but can cause reproductive problems (Blood et al., 1983). *Haemobartonella felis,* the etiological agent of feline infectious anemia, frequently causes anemia in cats immunosuppressed by infection with the feline leukemia virus (Bobade et al., 1988; Cotter et al., 1975). Several reports describe *Haemobartonella* as a cause of anemia in colony-reared nonhuman primates (Adams et al., 1984; Aikawa and Nussenzweig, 1972). *Eperythrozoon ovis* and *E. wenyoni* have been reported to cause anemia in sheep and cattle but are usually not considered pathogenic by themselves (Blood et al., 1983). Immunosuppression of the host by chemotherapy or splenectomy increases the severity of infection with eperythrozoa or haemobartonella (Bellamy et al., 1978; Maede, 1978).

Isolation of *Eperythrozoon* and *Haemobartonella*

No successful cultivation of these organisms has been reported (Gothe and Kreier, 1977). Organisms are isolated by collecting blood from an infected host and maintaining it and the contained organisms in liquid nitrogen (equal volumes of blood and Alsever's solution containing either 10% dimethylsulfoxide or 5% glycerol). Alternatively, because these organisms persist indefinitely in their hosts, a carrier animal can be maintained as a source of the agent. Both methods risk contamination of the organism with infectious agents other than the one desired. Prior to propagation of the organism in a splenectomized host, the recipient animal should be screened for the presence of viruses, other hemotrophic bacteria, and protozoa. Inoculation of organisms from either a frozen blood specimen or carrier animals into the appropriate splenectomized host will result in an infection detectable by microscopic examination of Giemsa-stained blood smears.

In vitro short-term cultivation of hemotrophic rickettsiae is feasible for radiolabeling proteins, carbohydrates, and nucleic acids (Davis et al., 1978; Barbet et al., 1983). The procedure is similar to that used for short-term cultivation of *Anaplasma marginale* and is described later in this Chapter in the section on short-term cultivation of *A. marginale.*

Identification of *Eperythrozoon* and *Haemobartonella*

Microscopic examination of Giemsa- or Wright-stained blood films is used to identify *Eperythrozoon* or *Haemobartonella* organisms during acute stages of infection in the host. Both *Eperythrozoon* and *Haemobartonella* are Gram-negative microbes. They stain poorly with the counter stains used in the Gram-stain procedure. They are non acid fast. Acridine orange may be used as a fluorescent label for organisms in both genera. Morphology and host range are used to classify the organisms into species (Kreier and Ristic, 1984). The morphology of the organisms should be evaluated in animals during the early stages of the infection, prior to development of anemia and reticulocytosis. Host range is helpful in speciation because most *Eperythrozoon* or *Haemobartonella* species usually will grow only in one or, at most, several closely related hosts (Gothe and Kreier, 1977).

Eperythrozoa parasitize the plasma surface of the erythrocyte membrane and appear as rings or cocci 0.5 to 1.0 μm in diameter (Fig. 13). Chains of rod-shaped eperythrozoa may partially or completely circumscribe the erythrocyte. The organisms are clearly separated from the erythrocyte membrane but interact with it in a yet poorly defined manner (Zachary and Basgall, 1985). Eperythrozoa are easily dislodged from the erythrocyte membrane and are frequently found free in the plasma.

Haemobartonellae also parasitize the surfaces of erythrocytes, appearing as 0.3- to 0.5 μm cocci (Fig. 14). Rods are frequently seen, which in reality are composed of pairs and chains of cocci. Organisms appear to bind to the erythrocyte plasma membrane at intermittent "points"; however, the mechanism of binding is unknown (Demaree and Nessmith, 1972; Simpson et al., 1978; Venable and Ewing, 1968). Haemobartonellae are rarely found free in the plasma.

Aegyptianella

Habitats of *Aegyptianella*

GEOGRAPHICAL DISTRIBUTION. Aegyptianellosis of poultry, which is caused by *Aegyptianella pullorum,* occurs enzootically in all the coun-

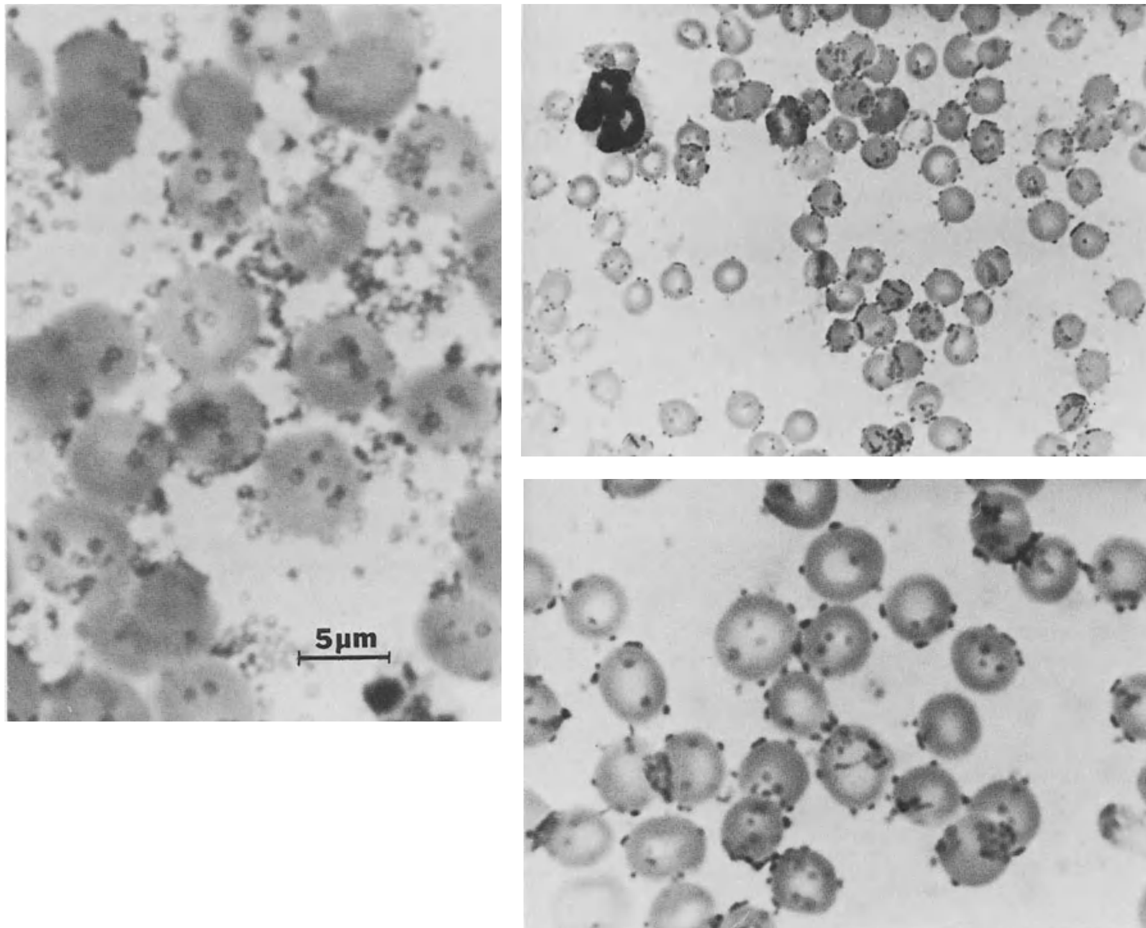

Fig. 13. Photomicrographs of Giemsa-stained thin blood films; blood infected with *Eperythrozoon.* (a) *Eperythrozoon coccoides* may be seen on the erythrocytes and free in the plasma. Organisms appear either as delicate rings on the surface of the erythrocytes and in the plasma or as more deeply staining chains of cocci or rods on the margins of the erythrocytes. (Courtesy of H. J. Baker.) (b) *Eperythrozoon dispar* Bruynoghe & Vassiliadis 1929 in the peripheral blood of a splenectomized, wild-caught European common vole (*Microtus arvalis*) from northern Germany shown at low magnification. Ring forms are not readily visible; (c) ring forms become more apparent at higher magnification.

tries of Africa, the region around the Mediterranean Sea, southern Europe, and southern Asia to Formosa (Gothe, 1971). In these areas, it is of considerable economic significance for the breeding of endemic stock and the maintenance of imported animals. The data concerning the distribution of these parasites are, however, still incomplete and reflect only a fragmentary picture of the actual geographical extension. This is partly because detection of *Aegyptianella* is complicated by the fact that *Borrelia anserina* infections, which are transmitted by the same species of ticks, can mask aegyptianellosis; and *A. pullorum* infections are predominantly latent in older animals.

AS PARASITES OF ARTHROPODS. The complete developmental cycle of *A. pullorum* in *Argas (Persicargas) walkerae* takes approximately 30 days and progresses in three clearly separate phases (Gothe, 1967a, 1971; Gothe and Becht, 1969; Gothe and Koop, 1974). In larval, nymphal, and adult female ticks of this species, it develops and multiplies in epithelial cells of the intestine, then in hemocytes, and finally in the cells of salivary glands. In this latter organ, the infectious forms develop. They are 0.3 to 0.5 µm in diameter, and are roundish anaplasmoidal bodies which are inoculated into the vertebrate with the saliva of the ticks at the next blood meal. Transovarial passage also occurs. *Argas (P.) persicus, Argas (A). reflexus* (Gothe, 1971; Gothe et al., 1981), as well as *Argas (P.) radiatus, Argas (P.) sanchezi* (Gothe and Englert, 1978), and *Argas (A.) africolumbae* (Gothe et al., 1981) can all function as biological vectors. The frequency of infection in ticks can be very high in areas where the parasites occur.

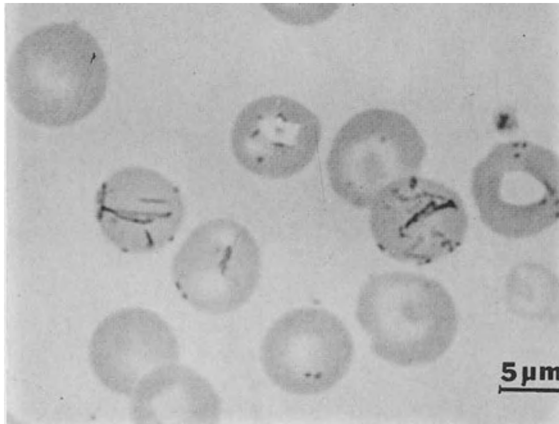

Fig. 14. Photomicrograph of a Giemsa-stained thin blood film from a dog. *Haemobartonella canis* may be seen on the erythrocytes. The organisms appear as solid dots or rods on the surface and margins of the erythrocytes. (From Venable and Ewing, 1968, with permission.)

Gothe and Schrecke (1972a, 1972b) were able to isolate these parasites in 10 out of 11 *Argas walkerae* populations from various parts of the Transvaal. Similarly, all of the 19 wild-derived populations of *Argas (P.) persicus* and the 3 populations of *Argas (A.) africolumbae* collected in chicken coops, dens, and sleeping places from various regions of Burkina Faso transmitted *A. pullorum* to susceptible chickens (Gothe et al., 1981).

AS PARASITES OF VERTEBRATES. In vertebrate hosts, five different species of the genus *Aegyptianella* Carpano, 1928, have been identified and named: *A. pullorum* (Carpano, 1928) from various species of birds; *A. emydis* (Brumpt and Lavier, 1935) from a turtle; *A. moshkovskii* (Schurenkova, 1938) from various species of wild birds; *A. carpani* (Battelli, 1947) from a snake, and *A. ranarum* from various *Rana* spp. (Desser, 1987). Other intraerythrocytic parasites of poikilothermic animals, and also their possible relationship to *Aegyptianella,* were extensively discussed by Pierce and Castleman (1974) as well as Johnston (1975). Gothe (1978) summarized the information on *Aegyptianella-, Anaplasma-,* and *Piroplasma*-like parasites of vertebrates. He described parasites resembling *Aegyptianella* from over 33 species of wild birds and domesticated poultry. The specific identity of these organisms is not clear. Of this genus only *A. pullorum* has been studied sufficiently to provide valid information, thus the characteristics and features of this genus are based exclusively on studies of this species.

A. pullorum, extensively discussed by Gothe (1971), Gothe and Kreier (1977), and Kreier and Gothe (1976), is the agent of an infections but noncontagious infection of birds, which parasitizes only the erythrocytes of the vertebrate host and is transmitted cyclically by argasid ticks.

Vertebrate hosts of *A. pullorum* are chickens, ducks, geese, and quail, as well as ostriches. Concerning guinea fowl, there are contradictory data; doves and turkeys can not be infected (Gothe, 1971). Subinoculations of blood from wild turkeys (*Meleagris gallopavo intermedia*) in Texas, USA, into susceptible domestic broad-breasted white turkeys, however, revealed intraerythrocytic pleomorphic organisms, which were considered on the basis of light and transmission electron microscopic investigations to be *A. pullorum* (Castle and Christensen, 1985). Some wild birds may also be infected at least briefly (Curasson, 1938; Curasson and Andrjesky, 1929; Huchzermeyer, 1969).

In vertebrate hosts, only erythrocytes are host cells. On the basis of electron microscopic studies it is possible to deduce the intraerythrocytic development cycle. The round parasite, 0.3–0.5 μm in diameter, is first seen eperythrocytically situated, surrounded by a double membrane. In the erythrocyte an additional membrane is formed which separates the parasite from the cytoplasm and encloses it in a vacuole in the host cell. In the vacuoles, the parasites grow to 1 μm in size. The process of division starts with invagination of the parasite's double membrane on one or both sides. These invaginations penetrate deeper and deeper, until the organism is cut completely through, and two daughter cells are formed. This process is repeated several times and leads finally to the formation of fully mature marginal bodies containing as many as 26 roundish forms, which each are as little as 0.3–0.5 μm in diameter. The intraerythrocytic development of *A. pullorum* can be completed in 36 h (Gothe, 1967c, 1971). Scanning and transmission electron microscopic studies using ruthenium red as a marker of the red cell plasma-lemma produced evidence of an endocytosis followed by an erythrocytic vesiculation as the possible mode of entrance of *A. pullorum* into erythrocytes. The presence of ruthenium red coating the membrane around the parasitophorous vacuole during the whole invasive process and the complete absence of the stain inside the host cell indicate that the entry of the aegyptianellae is accomplished by invagination of the host cell plasma-lemma and is not preceded nor followed by its breakage. One possible mode of exit of aegyptianellas from parasitized erythrocytes appears to be the invasive mechanism in reverse order, an exocytosis. Generally, however, the affected erythrocytes

are injured by parasitization, resulting in release of the parasites into the plasma following host cell lysis (Gothe and Burkhardt, 1979).

Besides those in the erythrocytes, parasites and fully matured marginal bodies can be observed outside the red blood cells. They occur free in the plasma, and intracellularly in large and small lymphocytes, in neutrophile and eosinophile leukocytes, in monocytes, and in the Kupffer cells of the liver. The proportion of forms outside erythrocytes grows in direct proportion to the extent of the parasitemia. It is probable that most of the parasites in leukocytes have been phagocytized after release from erythocytes. There is probably no exoerythrocytic development parallel to the intraerythrocytic cycle. The plasma forms are transitory phases initiating the infection of new erythrocytes (Gothe, 1969).

The salivary gland forms from the ticks penetrate erythrocytes immediately after injection or at least remain infectious in the blood. Histological investigations of naturally infected chickens have not revealed an exoerythrocytic phase in the development of *A. pullorum* (Gothe, 1967c, 1971).

AS INAPPARENT PARASITES OF BIRDS AND ARTHROPODS. *Aegyptianella pullorum* has a two-host or heteroxenous cycle. The vertebrate hosts function as carriers and thereby as donors of this agent. The argasid species are both biological vectors and an infection reservoir. Parasites existing in both arthropods and vertebrates constitute an important habitat for maintenance of the population in nature.

Isolation of *Aegyptianella*

Attempts to culture *Aegyptianella pullorum* in cell-free media, tissue cultures, or extraerythrocytically in embryonated chicken eggs have all failed. Parasites can only be grown in vivo in their vertebrate and invertebrate hosts. Susceptible birds can be infected very easily by intravenous, subcutaneous, intraperitoneal, or intramuscular injections of parasite-infected blood, as well as through scarification of the skin. The agent can be maintained by serial transfer of infected blood. Chicks infected at 3 weeks have remained carriers for over 1 1/2 years. The duration of the infection in ticks is lifelong and any development stage is infective. The infection has been shown to be present for over 810 days in ticks (Gothe, 1967a, 1971).

Cryopreservation is possible. Any standard procedure for preserving blood protozoa is satisfactory (Swoager, 1972). In liquid nitrogen the maximal storage time determined to date is 6.97 years (Raether and Seidenath, 1977). Freeze-drying of *A. pullorum* is also possible (Bartkowiak et al., 1988). Cryopreservation does not affect the viability of *A. pullorum* or its ability to propagate in the vector tick *Argus Persicargas) walkerae*. The cryopreserved aegyptianellae are capable of transovarial passage (Gothe and Hartmann, 1979).

Identification of *Aegyptianella*

When examined with the aid of a light microscope, *Aegyptianella pullorum* inclusions appear in Giemsa-stained blood films (Fig. 15) in a variety of roundish forms, some ranging up to 0.6 μm in diameter; some as ring forms of 0.8–3.2 μm diameter; and some as half-moon or oblong structures. The roundish structures contain up to 26 small organisms and are approximately 0.3–0.5 μm in diameter. In thin sections examined by electron microscopy (Fig. 16), the parasites appear to be surrounded by a sheath, which consists of two 6- to 8-nm membranes separated by a space of 28 nm. The internal structure or the parasites is made up of electron-dense aggregates of a finely granular material embedded in a less-dense substance. There is no membrane-bound nucleus. The parasites are separated from the host cell plasma by a 6- to 8-nm membrane (Gothe, 1967b, 1971). *Aegyptianella* possess RNA and DNA; the amount of DNA is significantly less than the amount of RNA. The DNA is completely masked by the RNA in conventionally stained preparations (Gothe, 1971). The parasites are susceptible to the action of tetracyclines and certain dithiosemicarbazones (Barrett et al., 1965; Gothe, 1971; Gothe and Kreier, 1977; Gothe and Lämmler, 1970a; Lämmler and Gothe, 1967, 1969). Pleuromutilins also have aegyptianellicidal efficacy (Gothe and Mieth, 1979).

APPLICATIONS. Because of the high cost of experimentation with bovine *Anaplasma*, the biologically and morphologically related *A. pullorum* may be used as an alternative model for quantitative evaluation of the therapeutic and prophylactic effectiveness of drugs (Gothe and Lämmler, 1970b) or in *Anaplasma* vaccine research (Bartkowiak et al., 1988). Investigations on the genetics, immunology, and biochemistry of *Aegyptianella*, however, are still lacking.

Anaplasma and Its Habitats

GEOGRAPHICAL DISTRIBUTION. *Anaplasma marginale*, *Anaplasma centrale*, and *Anaplasma ovis* are obligate intraerythrocytic bacteria of ruminants and are naturally transmitted

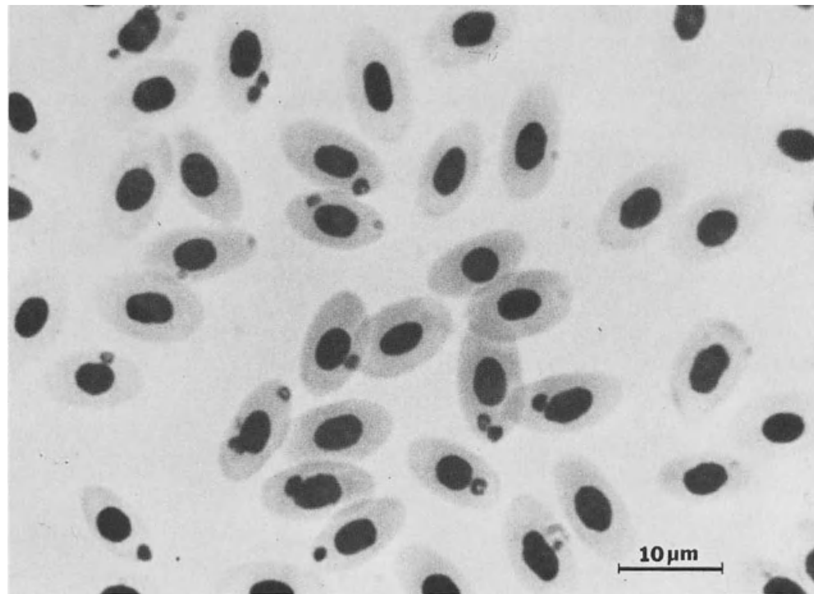

Fig. 15. Photomicrograph of a thin blood film from a chicken with aegyptionellosis stained with Giemsa. A variety of roundish forms, which are the parasitic inclusions, are present in the cytoplasm of the erythrocytes.

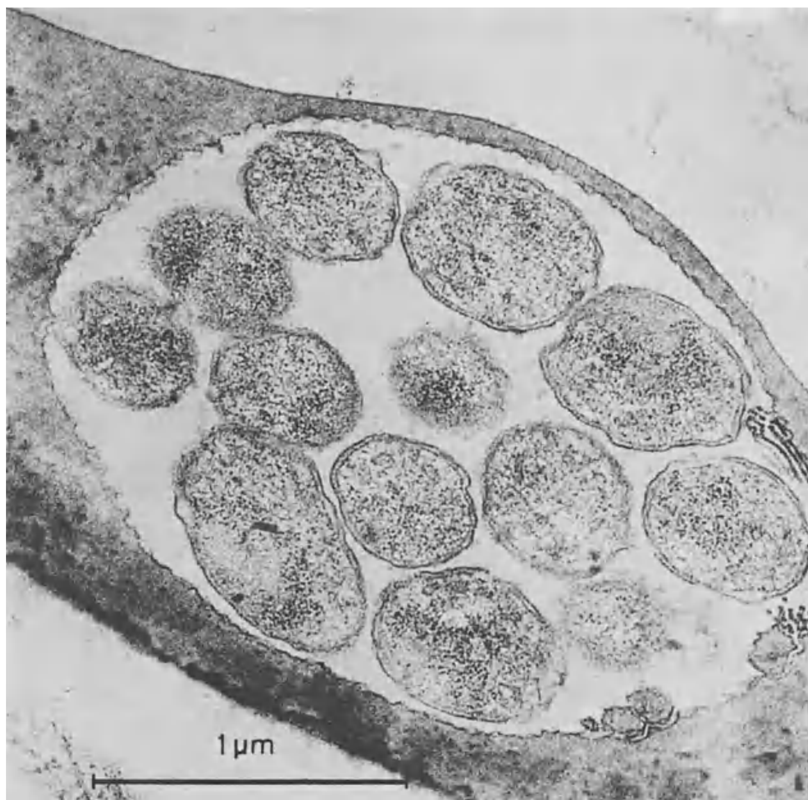

Fig. 16. Electron micrograph of a thin section of an aegyptionella inclusion in a chicken erythrocyte. Inside the inclusion, profiles of parasites can be seen, with one at the upper left probably in the process of division.

biologically and mechanically by a large and diverse group of arthropod vectors. Accordingly, *Anaplasma* infections are widespread in sheep-and cattle-raising areas throughout the tropical, subtropical, and warmer temperate climates, wherever conditions support sufficient populations of the arthropod vectors. Infection prevalence in tropical livestock production regions commonly exceeds 50%, and 90% prevalence of *A. marginale* infection in cattle has been confirmed in tropical South American and Australia (Nicholls et al., 1980; Patarroyo et al., 1978; Rogers and Shiels, 1979). In contrast, prevalence in more temperate areas is fre-

quently less than 50% (Mass et al., 1986). There is little information on prevalence in wild ruminants independent of contract with domestic livestock; certainly, *Anaplasma* infects numerous species of wild ruminants (Kuttler, 1984). The role of wild ruminants as reservoirs of infection for transmission to cattle, or conversely the role of domestic livestock in maintaining a source of infection for spread to wildlife, has not been definitively determined.

Anaplasma marginale is the primary cause of anaplasmosis in cattle and is common on all six continents (Losos, 1986; Theiler, 1910). *A. centrale* is a closely related species, if not the same, which was originally isolated in southern Africa. It is a cause of mild anaplasmosis in cattle (Theiler, 1911). *A. centrale* has been deliberately introduced into Australia, Asia, and South America for use as an agent for immunizing against anaplasmosis by *A. marginale*. *A. ovis* is the etiologic agent of ovine and caprine anaplasmosis. It has a widespread distribution similar to that of *A. marginale* in tropical-to-temperate climates (Bevan, 1912; Lestoquard, 1924).

AS SYMBIONTS OF ARTHROPODS. A diverse group of arthropods may serve as vectors for the transmission of *Anaplasma.* Although 29 species of ticks and numerous hematophagous flies and mosquitos have been shown experimentally to transmit *A. marginale,* the significant natural vectors of *A. marginale* (and probably *A. centrale*) appear to be ticks in the family Ixodidae and flies in the family Tabanidae (Ewing 1981; Yeruham and Braverman, 1981). Ixodid ticks are efficient biological vectors and are believed to be the principal vectors in most regions of the world. The three-host *Dermacentor* ticks have been shown to be important vectors in temperature western North America, while ticks of the one-host *Boophilus* species are highly significant vectors in tropical and subtropical regions worldwide (Callow 1974; Peterson et al., 1977; Potgieter, 1979). Both genera of ticks support transstadial transmission but transovarial transmission has not been reproducibly documented (Connell, 1974; Ewing, 1981; Stich et al., 1989). Within the tick, *A. marginale* undergoes a complex life cycle with development and replication in the midgut epithelial and gut muscle cells (Kocan, 1986). Although *A. marginale* developing within the midgut does become infective to cattle in its latter stages of development, natural transmission likely involves the recently demonstrated infective forms which are found within the salivary glands (Figs. 17 and 18) (Kocan et al., 1988). Ixodid ticks appear to be the predominant natural vectors of *A. ovis.*

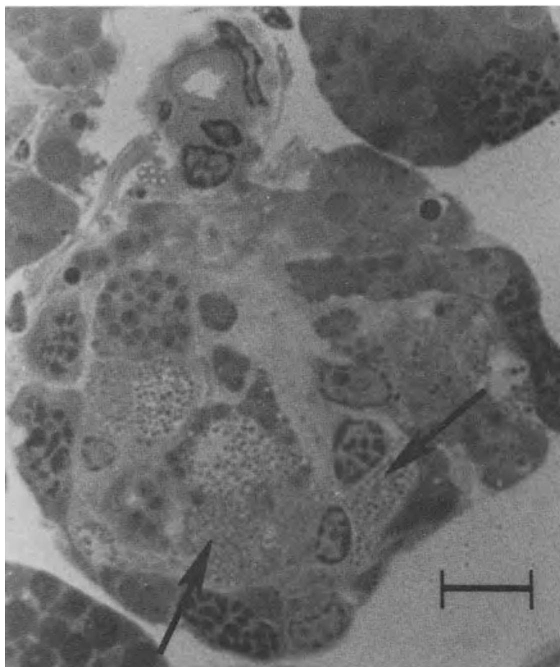

Fig. 17. Photomicrograph of *Anaplasma marginale* colonies (arrow) within salivary glands of infected *Dermacentor variabilis.* Mallory's stain. Bar = 5.0 μm.

Tabanids are efficient mechanical vectors, remaining capable of transmission for up to two hours post-feeding, and have been implicated as the principal vector for *A. marginale* in certain regions within both North America and Africa (Hawkins et al., 1982; Weisenhutter 1975). Certain *A. marginale* isolates, including the Illinois and Florida isolates, have been shown not to infect the predominant North American tick vectors, *Dermacentor andersoni* and *D. variablis,* and may be uniquely dependent on transmission by flies, fomites, or in utero spread (Wickwire et al., 1987).

AS PARASITES OF VERTEBRATES. Only ruminants may be infected with *Anaplasma.* Speciation of *Anaplasma* has been based upon morphologic appearance within erythrocytes (Fig. 19) and pathogenicity in ruminant hosts (Ristic and Kreier, 1984b). *Anaplasma marginale,* the principal cause of bovine anaplasmosis, and *Anaplasma ovis,* the pathogen causing ovine and caprine anaplasmosis, are the only two species included in the *Approved List of Bacterial Names* or in names validly published in the *International Journal of Systematic Bacteriology* (Ristic and Kreier, 1984b). The validity of the speciation of *Anaplasma* into the two species *A. marginale* and *A. ovis* is supported by the lack of cross immunity and differences in sur-

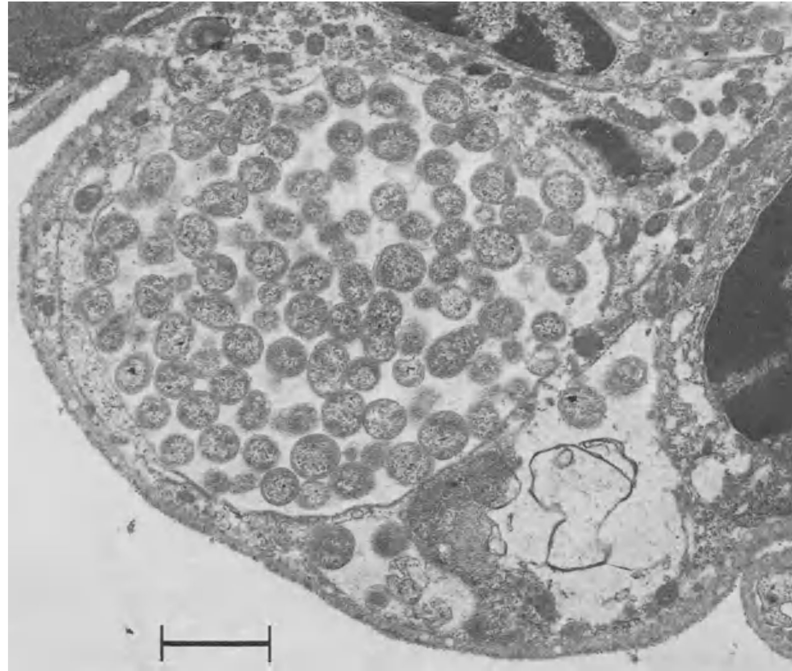

Fig. 18. Transmission electron micrograph of *Anaplasma marginale* within a salivary gland of infected *Dermacentor variabilis*. Bar = 2.0 μm. (Courtesy of K. M. Kocan and D. Stiller.)

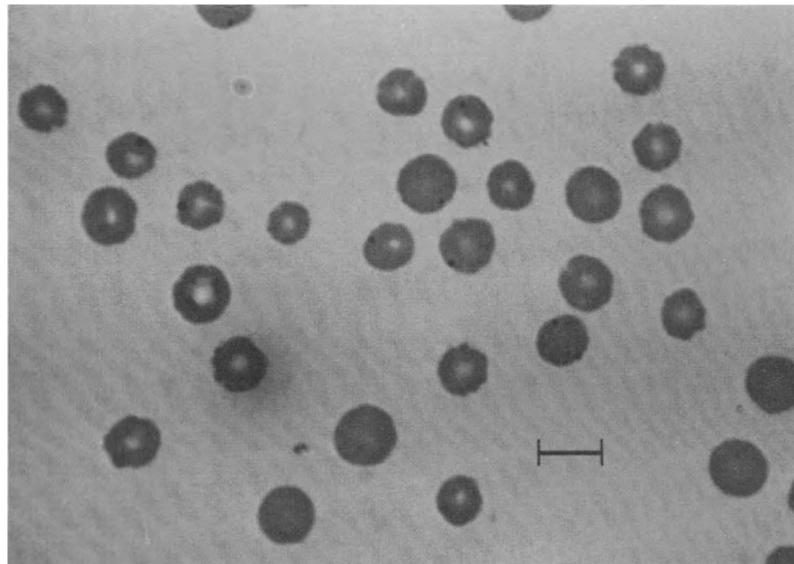

Fig. 19. Photomicrograph of Giemsa-stained *Anaplasma marginale*-infected erythrocytes. The intensely staining inclusion bodies resemble Howell-Jolly bodies in some respects such as size and position in the erythrocyte. They differ from Howell-Jolly bodies, however, in staining characteristics. *Anaplasma* more closely resemble a spot of flat finish paint than one of enamel, i.e., they are not shiny or refractile to light. Bar = 5.0 μm. (Courtesy of K. M. Kocan.)

face antigens of these two organisms (Palmer et al., 1988a; Splitter et al., 1956). Two additional species, *A. caudatum* and *A. centrale,* have been proposed as causes of bovine anaplasmosis (Ristic and Kreier, 1984b; Theiler, 1911) but these names have not been approved. Some authors have not considered the data from antigenic, genetic, and protein structural characterization to be sufficient to warrant differentiation of the proposed *A. caudatum* from *A. marginale* (Barbet et al., 1983; McGuire et al., 1984; Oberle et al., 1988; Palmer et al., 1988a). *A. centrale* was originally proposed as a variant of *A. marginale* by Theiler (1911) based on its more central intraerythrocytic location and on its being less virulent in cattle. Traditionally, *A. centrale* has been considered to be a separate species. While there are demonstrated antigenic and genetic differences between *A. marginale* and *A. centrale,* whether these are great enough to justify speciation is doubtful (Ambrosio and Potgieter, 1987; Kuttler, 1967; Palmer et al., 1988a).

A. marginale and *A. centrale* are defined principally as pathogens of domestic cattle but have been shown experimentally to infect numerous wild ruminant species (Kuttler, 1984). Clearly, certain wild ruminants, such as the black-tailed

deer in western North America, are naturally infected with *A. marginale* and may have a significant epidemiologic role (Osebold et al., 1962). In contrast, natural infection by *Anaplasma marginale* in other animals, such as the giraffe, has not been confirmed (Kuttler, 1984). Domestic sheep can be experimentally infected with *A. marginale* but do not develop clinical disease (Donatien and Lestoquard, 1930). *A. ovis* is principally a parasite of domestic goats and sheep but has been shown experimentally to infect wild ruminants in Africa and North America (Kuttler, 1984). *Anaplasma ovis* can be shown experimentally to infect domestic cattle but in this species it does not cause clinical disease (Kuttler, 1981).

In the vertebrate host, *Anaplasma* infect mature erythrocytes with formation of an erythrocyte-derived vacuole around the organism (Francis et al., 1979). Each organism is 0.55–0.85 μm in diameter and contains dense granular aggregates in an electron-lucid protoplasm, all enclosed in a double membrane 40–50 nm thick (Fig. 20) (Kocan et al., 1978). Within the erythrocyte, the bacteria replicate by binary fission to form up to eight individual organisms within a single vacuole (Fig. 21) (Ristic and Watrach, 1963). *Anaplasma* organisms exit the erythrocyte, using a poorly defined but apparently nonlytic mechanism, and infect additional erythrocytes (Erp and Fahrney, 1975). Following infection of a ruminant host, the number of infected erythrocytes increases with an apparent doubling time of between 24 and 48 hours. The infection becomes patent microscopically two to six weeks post-transmission depending on the number of organisms transmitted and the virulence of the isolate. At peak infection greater than 75% of the erythrocytes may be infected, and a severe anemia develops which persists for one to two weeks. Susceptible animals lose significant weight and may abort if pregnant. A case fatality rate of up to 36% has been documented in acutely ill cattle (Alderink and Dietrich, 1981). *Anaplasma*-infected erythrocytes are rapidly cleared from the blood as immunity develops. Following recovery from acute infection, animals remain infected with a low, microscopically undetectable number of organisms in the blood. The number of infected erythrocytes in this persistent infection, termed the carrier state, has been shown to vary dramatically (from >0.000025% to <0.0025%) both among individual carrier animals and temporally within an individual (Eriks et al., 1989). This variation may significantly influence the efficiency of arthropod transmission from individual animals.

Isolation of *Anaplasma*

Cultivation procedures for *Anaplasma* have not been sufficiently enough established to allow recovery and propagation in vitro. Preservation is currently best achieved by collecting infected erythrocytes and maintaining the organism in the frozen state in dimethylsulfoxide. To each volume of washed, packed erythrocytes to be prepared, an equal volume of 4 M dimethylsulfoxide is added; the mixture is dispensed in convenient volumes and preserved in liquid nitrogen (Love et al., 1976). Infective organisms

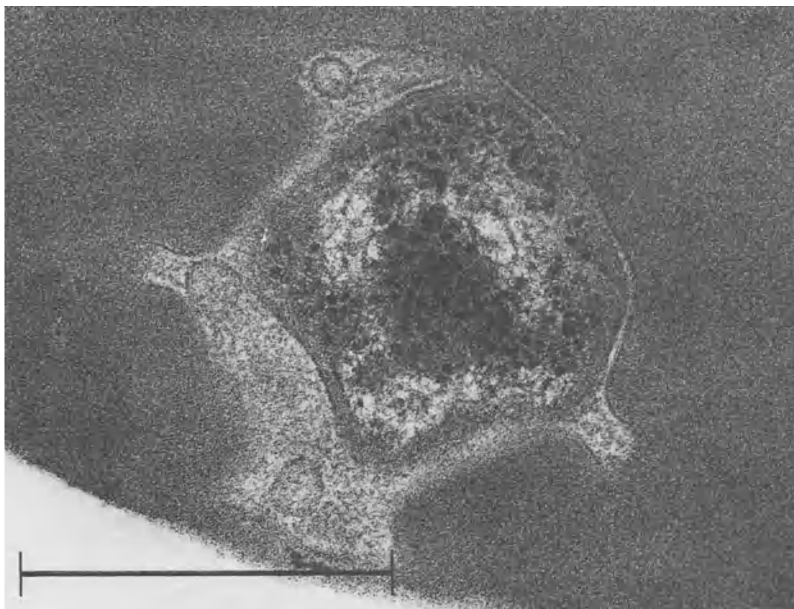

Fig. 20. Transmission electron micrograph of a single *Anaplasma marginale* organism within a vacuole in an intact infected erythrocyte. Bar = 0.5 μm. (From Kocan et al., 1978, with permission.)

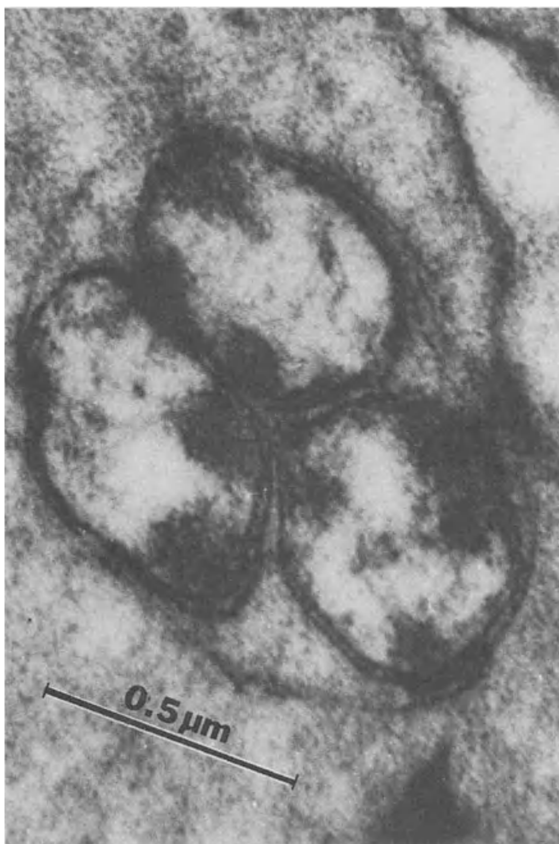

Fig. 21. Three organisms in an inclusion body. (Courtesy of M. Ristic.)

can be separated from the infected erythrocytes using ultrasonic disruption and differential centrifugation. Frozen erythrocytes can be used in this procedure (Palmer and McGuire, 1984). Propagation of the organisms in the cryopreserved isolate is by inoculation of a ruminant host (cattle for *A. centrale* and *A. marginale;* sheep or goats for *A. ovis);* the host subsequently develops an acute infection. Splenectomized animals develop more serious infections with significantly higher numbers of infected erythrocytes than do nonsplenectomized ones. There are no known animal hosts of *Anaplasma* other than domestic and wild ruminants.

In vitro cultivation is currently limited to short-term maintenance within mature erythrocytes. Short-term cultivation has been used to radiolabel *Anaplasma* polypeptides in vitro for structural and antigenic research (Barbet et al., 1983; Palmer et al., 1985—, 1986a, 1986b). Cultivation in nucleated cells has not been reproducibly effective as a source of organisms.

Procedure for the Short-Term Cultivation of *Anaplasma marginale*

The following procedure (Barbet, 1983; Palmer et al., 1985) is used for ^{35}S-methionine labeling of the organism: Blood is collected from splenectomized calves during the ascending parasitemia of animals with acute infection, washed three times in calcium- and magnesium-free Hanks balanced salt solution, and once in Eagle minimal essential medium without methionine; the buffy coat is removed after each centrifugation. The washed, parasitized erythrocytes are added to Eagle minimal essential medium without methionine (10% fetal bovine serum, 2 mM L-glutamine, 100 μg of streptomycin per ml, and 100 units of penicillin per ml) at a ratio of 1 ml packed erythrocytes to 7 ml of medium. The cultures are incubated for 48 hours with ^{35}S-methionine (125 μCi per 5 × 10^8 parasitized erythrocytes) at 37°C in 5% CO_2 in air. Cells are washed four times with Hanks balanced salts solution without calcium or magnesium, and the pelleted erythrocytes are lysed by a 50 mM Tris (pH 8.0) buffer containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-alpha-*p*-tosyl-L-lysyl-chloromethyl ketone (TLCK), 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate (SDS). After centrifugation to pellet the insoluble material, the clean supernatant is collected.

Identification of *Anaplasma*

Microscopic examination of Romanowsky-stained peripheral blood films is used to identify intraerythrocytic *Anaplasma* during acute infection in the ruminant host. *Anaplasma* are Gram-negative microbes that do not stain well with the counter stains used in the Gram stain procedure. Staining with Giemsa or Wright solutions is the most common method of preparation of blood films to be examined for the presence of *Anaplasma.* Infected erythrocytes at a frequency of as low as 0.1% can be detected by this method. What is seen is the round, 0.5- to 1.0-μm inclusion, which stains bluish-purple (see Fig. 19). A frequency of less than 0.1% of infected erythrocytes can be detected by nucleic acid hybridization with defined RNA or DNA probes. It has also been reported that *A. marginale* was detected by nucleic acid hybridization in blood films with as few as 0.000025% infected erythrocytes. *A. ovis* has also been detected by nucleic acid hybridization but sensitivity appears to be lower. *A. ovis* infection was detected in blood films with 0.00035% infected erythrocytes (Goff et al., 1988; Shompole, S. P., 1989; Visser and Ambrosio, 1987). Nucleic acid probes have been used to detect persistently infected carrier animals in field studies (see later).

Differentiation of species is most frequently based on host specificity, which appears to be reliable for the known hosts of *A. marginale* and

A. ovis. Intraerythrocytic location of the organism is the traditional basis for differentiation of *A. centrale* from *A. marginale*. Positive identification of *Anaplasma* can result from nucleic acid hybridization studies using defined nucleic acid probes and from fluorescent antibody staining using monoclonal antibodies (Palmer et al., 1988a; Shompole, S. P., 1989).

Numerous isolates of *A. marginale* which differ one from the other have been described. The isolates differ in antigenic nature, polypeptide composition, and composition of DNA in genes encoding surface proteins (Barbet et al., 1983; Kreier and Ristic, 1963a, 1963b; McGuire et al., 1984; Oberle et al., 1988; Palmer et al., 1988, 1988a). Reactivity in fluorescent-antibody-staining tests with panels of isolate-specific monoclonal antibodies is most commonly used to identify an isolate. Our inability to clone *Anaplasma* and thus to ensure that all organisms in an isolate are derived from a single parent has led to the use of the term "isolate" rather than "strain." Undoubtedly, genetically distinct clonas of *Anaplasma* could be produced, but to date this has not been accomplished.

Anaplasma can be identified within tick tissues by light microscopic examination of tissue sections stained by Mallory's technique and by examination of thin sections with an electron microscope. The morphology of the various developmental stages of *A. marginale* in *Dermacentor* ticks has been well described (Kocan, 1986). The infective stage in the salivary glands is composed of membrane-bound colonies of organisms (see Figs. 17 and 18) (Stiller et al., 1989). Identity of *Anaplasma* in tick tissues can be confirmed using immunofluorescence or nucleic acid hybridization (Friedhoff and Ristic, 1966; Goff et al., 1988).

In one sense *Anaplasma* resembles members of the order Rickettsiales, since it utilizes regulatory elements similar to consensus *Escherichia coli* signals to regulate their genes as do rickettsiae (Anderson et al., 1988; Barbet et al., 1987). There may, however, be differences between *Anaplasma* and the rickettsiae in ribosome-binding sites. The similarity between *Anaplasma* and the rickettsiae in their regulatory signals has allowed expression of *Anaplasma* genes in *E. coli,* using rickettsial promoters. The GC composition of *A. marginale* and *A. centrale* DNA have been reported as respectively, 48.5 and 45.1 mol% GC (Ambrosio and Potgieter, 1987).

Application of Molecular Biology Techniques

Research on the molecular genetics and immunology of *Anaplasma* has been applied in three principal areas: 1) identification of protective immunogens for vaccine development; 2) development of improved serologic diagnostic assays; and 3) development of nucleic acid probes for detection of low numbers of *Anaplasma* and for speciation of *Anaplasma.*

Our inability to cultivate *Anaplasma* has impeded development of effective, widely used vaccines. Immunization at present is only by infection and treatment or by infection with attenuated *Anaplasma* strains (Ristic and Carson, 1977). Identification of protection-inducing surface antigens on *A. marginale* and the cloning and expression of these antigens in recombinant *E. coli* provides a basis for the development of nonliving vaccines (Palmer, 1989). Two surface proteins, major surface protein-1 (MSP-1) and major surface protein-2 (MSP-2), have been shown individually to induce protection in immunized cattle (Palmer et al., 1986a, 1987, 1988). Both surface proteins share common epitopes with *A. centrale* and with various isolates of *A. marginale* from Africa, Asia, North America, and South America (Palmer et al., 1988a). Genes encoding the surface proteins have been expressed in recombinant *E. coli* (Barbet et al., 1987; Palmer, 1989). Although development of *A. ovis* vaccines is also needed, research on development of vaccines for *A. ovis* has not been reported.

Serologic assays with improved sensitivity and specificity for diagnosis of *A. marginale* infected cattle have been developed which utilize individual immunodominant antigens in an enzyme-linked immunosorbent detection system. An 86 KD at *A. marginale* protein is specifically reactive with sera from cattle infected with many different isolates of *A. marginale* regardless of the stage of infection (Palmer et al., 1986b). This assay is more sensitive than are tests using whole organisms as antigens, such as the capillary agglutination test (Ristic, 1962).

The development of nucleic acid probes specific for *A. marginale, A. centrale,* and *A. ovis* has allowed more reliable speciation, and easier identification of persistently infected carrier animals and of *Anaplasma* in tick tissues (Eriks et al., 1989; Goff et al., 1988; Shompole, S. P., 1989; Visser and Ambrosio, 1987).

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CHAPTER 226

Streptobacillus moniliformis

JAMES R. GREENWOOD and SYDNEY M. HARVEY

Introduction

The genus *Streptobacillus* is presently represented by one species, *Streptobacillus moniliformis,* the etiologic agent of rat-bite fever and Haverill fever. *S. moniliformis* has also been known as *Haverhillia multiformis, Actinomyces muris, Actinobacillus muris,* and *Haverhillia moniliformis.* Although the genus *Streptobacillus* was described in the 1920s, taxonomically it continues to be a genus of uncertain affiliation. Previously this genus was placed in the families Parvobacteriaceae (Tribe Haemophileae) and Bacteroidaceae. Since the 8th edition of *Bergy's Manual, Streptobacillus* has been described as a genus of "uncertain affiliation." It was recently suggested (Savage, 1989) that *S. moniliformis* is more similar to some of the Mycoplasmatales. This suggestion is based on the low GC content of the DNA (24–26 mol%), serum or blood requirements for growth, cholesterol incorporation into the cell membrane, animal parasitism, and production of L-phase organisms. Further clarification of the taxonomic position of this genus awaits genetic studies, such as DNA-RNA hybridizations.

Habitats

S. moniliformis is found in the nasopharynx of laboratory and wild rats, with some studies reporting as many as 50–100% of wild rats carrying it as a commensal organism and excreting it in their urine (McHugh et al., 1985). It is reported to have caused tendon sheath infections in turkeys, cervical abscesses in guinea pigs, epizootics in laboratory mice, and lesions in laboratory rats with bronchopneumonia. Humans become involved in the transmission cycle through rat bites or from the bites of squirrels, weasels, and such rat-eating carnivores as dogs, cats, and pigs. Rat bite fever has also been reported following the handling of dead rats. Haverhill fever may result from the ingestion of milk to which rats have had access.

Selective Enrichment

S. moniliformis is both fastidious and slow growing; consequently it is frequently overgrown by normal flora. Because it is an uncommon human pathogen, little work has been done to develop a selective culture medium. Recently, however, colistin nalidixic acid agar (CNA) was used in conjunction with other media to isolate Gram-negative *S. moniliformis* from a 2-month old with a fatal infection following a bite by a wild rat (Sens et al., 1989). CNA is inhibitory for many other Gram-negative bacteria and the use of this medium or a modification combining colistin, nalidixic acid and serum might provide a new approach to isolation of *S. moniliformis* from nonsterile body sites.

Isolation

S. moniliformis growth media should be supplemented with either blood, serum, or ascitic fluid. Because L-phase variants might be present in clinical samples, media formulations must also take the specialized growth requirements of these forms into account. The bacterial phase has been isolated on media with either a meat infusion or tryptose base enriched with 20% horse serum or 15% sterile defibrinated rabbit blood. L-phase variants are more easily observed in culture when grown on the clear, serum-containing agar. Agar plates should be incubated in a humid environment with increased CO_2, such as that obtained in a candle jar, or in a humidified CO_2 incubator. Plates should be incubated at temperatures between 35–37°C. *S. moniliformis* colonies are greyish, round, and have a butyrous consistency. They generally have a discrete edge and reach approximately 1–2 mm after incubation for 3 days. The L-phase colonies exhibit a typical mycoplasma-type "fried egg" appearance and are considerably smaller than bacterial colonies.

When blood cultures are required to diagnose *S. moniliformis* endocarditis, thioglycolate broth appears adequate to support growth. However, it appears important not to use broth that contains sodium polyanethol sulformate, as this has been reported to be inhibiting at levels as low as 0.0125% (Lambe et al., 1973).

Identification

Table 1 lists the salient features of *S. moniliformis*. Because of the fastidious nature of these organisms, the literature frequently reports numerous discrepant biochemical features probably resulting from the use of different basal media. The most comprehensive studies on biochemical reactions of this genus are presently based on the studies of Aluotto et al., (1970) and Cohen et al., (1968).

Perhaps the most reliable and accessible method of testing carbohydrate fermentation is to inoculate a 24-h broth culture of *S. moniliformis* into a cystine trypticase agar (CTA) base that contains one drop of rabbit serum and 1% carbohydrate in final concentration. These tubes are incubated at 35°C and reactions read at 1, 2, 7, and 14 days. Inoculated tubes should be compared to control tubes consisting of CTA with added carbohydrate and serum. For detection of other biochemical features, the work of Cohen et al. (1968) should be consulted. Fatty acid profiles have also been used to rapidly identify *S. moniliformis* during an outbreak of Haverhill fever (Rowbotham, 1983). In this work, strains were grown in serum broth for 24 h, and the cultures were used to prepare fatty acid methyl esters. *S. moniliformis* strains had consistent peaks of palmitic, linoleic, oleic, and stearic acid.

Table 1. Salient features of *Streptobacillus moniliformis*.

Test	Result
Catalase	−
Oxidase	−
Indole	−
Nitrate to nitrate	−
H_2S production	+
Arginine dihydrolase	+
Serum, blood, or ascitic fluid (required for growth)	+
Phenylalanine deaminase	−
Methyl red	−
Voges-Proskauer	−
Esculin in hydrolysis	d[a]
Gas produced from carbohydrate	−
Acid produced from:	
Fructose	+
Glucose	+
Maltose	+
Starch	+
Arabinose	−
Dulcitol	−
Sorbitol	−
Sucrose	−

[a]d = Different biotypes.

The microscopic morphology of *S. moniliformis* may vary depending on the media used, cultural conditions, and age of culture. Generally, cells appear as elongated Gram-negative rods, frequently in chains and filaments with occasional thickenings along the filaments giving rise to a necklace appearance ("moniliformis" means necklace-shaped). *S. moniliformis* is nonencapsulated, nonmotile, and facultatively anaerobic.

Preservation of Cultures

Cultures of *S. moniliformis* can be lyophilized for effective storage. They can also be subcultured in broth, but transfer to fresh broth medium is required as frequently as every 24 h to maintain viability. Refrigerated agar plate cultures can survive up to 15 days.

Applications

Animal research personnel are at risk from developing rat bite fever when working with laboratory rats. From 1958 to 1983, of 13 cases of rat bite fever reported in the United States, six of the cases were associated with bites of laboratory rats (Anderson et al., 1983). This is in keeping with the high rate of colonization by *S. moniliformis* in these animals. Studies on the mechanism of pathogenicity, virulence, and host range have not been reported.

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CHAPTER 227

The Genus *Toxothrix*

PETER HIRSCH

The first description of a bacterium that produced twisted bundles of thin filaments which contained oxidized iron was given by Cholodny (1924). He called this organism *Leptothrix trichogenes,* the filament-producing *Leptothrix.* The flexible bacterium was observed in a water basin next to the river Dnjepr near Kiev, Russia. Its cells (diameter approximately 0.5 μm) were connected to form a trichome of up to 400 μm in length. A peculiar movement was observed: the U-shaped trichome glided with its rounded part forward; both ends of the trichome left bundles of twisted filaments of polymer as parallel "railroad tracks." Occasionally, parts of the bundles appeared to be drawn out in a fan-shaped fashion (Figs. 1a, 2).

Although this first description was very accurate, as we know now, later scientists failed to notice the presence and importance of the flexible, gliding bacterium (trichome); they only observed the often rigid, brittle, iron-encrusted bundles of filaments. Consequently, these filaments were interpreted to be alive, to comprise the organism. It appears strange that the producer of these structures did not attract greater interest.

The explanation for the failure to recognize the true nature of *Toxothrix* came from Krul, Hirsch, and Staley (1970), who employed a partially immersed, phase-contrast microscope to study the formation of such filament bundles in an iron spring in Michigan. The bacterial trichome was observed to consist of up to 40 rods each of 0.5–0.75 × 3–6 μm, with a total length of up to 240 μm. The forward movement of the often U-shaped trichome consisted of symmetrical rotation of both trichome ends, which thereby left a track on their attachment surface that consisted of twisted polymer fibers. Occasionally, the torque on the rounded center part of the U was released by an upward twist followed by a downward "printing" of polymer onto the surface of the glass slide. Later these polymer fibers became encrusted with iron oxide. Attempts to study the flexible filaments in the laboratory resulted in an explosive lysis that occurred within a few minutes after the living

This paper is taken directly from the 1st edition of *The Prokaryotes.*

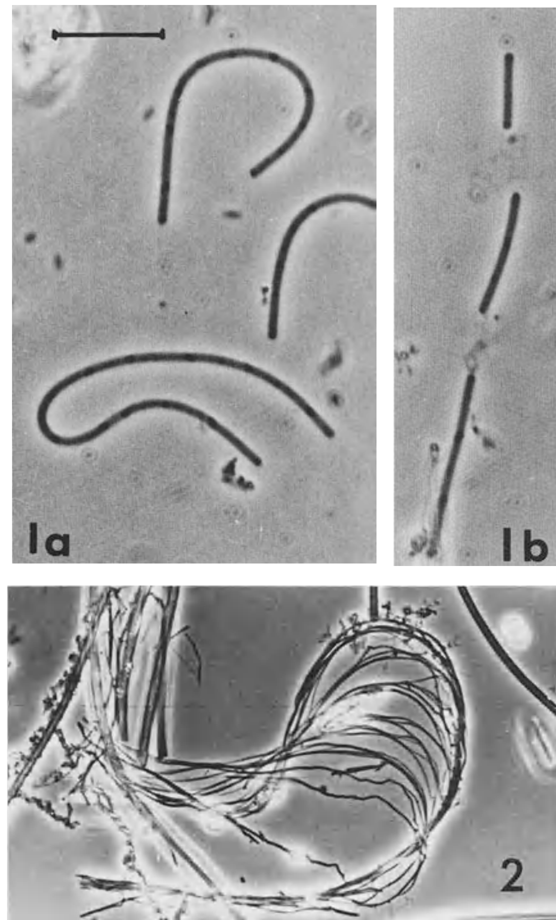

Fig. 1. *Toxothrix trichogenes* growing on a slide submerged in an iron-spring basin. The flexible trichomes with dense granules. (a) Typical U-shape of gliding cells. (b) A trichome has fragmented. Bar = 10 μm.

Fig. 2. *Toxothrix* polymer filaments (twisted bundle, fan-shape) with iron encrustations. There are also sheaths of *Leptothrix* sp. and *Gallionella* bands. Preparation from the same site as in Fig. 1.

preparation had been made (Fig. 1b). Thus the absence of trichomes in samples of many earlier observers could be explained (Krul, Hirsch, and Staley, 1970).

The fact that the bundle of twisted fibers was different from the "true" sheaths produced by other *Leptothrix* spp. caused Molisch (1925) to name this organism *Toxothrix ferruginea* (*toxon,* Greek noun, a bow). Later, Beger and Bringmann (1953) changed the name partially back into *Toxothrix trichogenes,* a more proper name (Hirsch and Zavarzin, 1974).

Habitats

The organism is not at all common in nature, although in specific habitats it has been found with a worldwide distribution. Most observers have seen *Toxothrix trichogenes* in fairly cold iron springs under conditions of reduced oxygen tension and slightly acidic pH (Table 1). The highest water temperature at which *Toxothrix* has ever been reported to grow is 15.5°C; thus this bacterium is obviously a psychrotroph. Concentrations of ferric iron were, when measured, low: 1–2.7 mg/liter Fe^{2+}. Many authors do not mention, in their reports, the occurence of the gliding *Toxothrix* trichomes, and thus the organism itself could have been absent when the more persistent, iron-encrusted filament bundles were seen and the parameters measured.

If one studies the variety of other bacteria present with *Toxothrix trichogenes,* one discovers that *Gallionella ferruginea* usually accompanies it. *Leptothrix* spp., *Siderocapsa* spp. or *Sphaerotilus, Naumanniella* spp., and *Ochrobium tectum* also occur, but if present they appear in smaller numbers. The natural habitat of *Gallionella* seems to be very similar to that of *Toxothrix.* But while the former grows best in nutrient-poor environments, the *Toxothrix* trichomes seem to prefer a higher concentration of organic matter. Also, *Toxothrix* grows best under microaerophilic conditions (Table 1).

Occurrence of *Toxothrix* in lakes or ponds has rarely been reported. Gorlenko, Dubinina, and Kusnezov (1977) found this organism in a 7- to 8-m depth in a Karelian mesotrophic lake and state that, if meromictic lakes were studied, *Toxothrix* could be found in one out of three such lakes.

Enrichment Suggestions

Toxothrix trichogenes has not been obtained in pure culture to date. However, natural *Toxothrix* samples—especially from cold iron springs with low pH—can be kept in the laboratory for several months, provided they contain some sediment with organic matter and are kept cold (5°C) and dark.

Microscopic examination of such stored samples often results in rapid lysis of the trichomes, a behavior also observed in some other gliding bacteria. The reason for the rapid lysis is not entirely clear. It could not be caused by a temperature that is too high, since lysis will occur below temperatures of some natural habitats. Light intensity and lack of oxygen could also be ruled out for the same reasons. *Toxothrix* trichomes may be extremely sensitive to pressure. Therefore, the cover slip of a preparation for microscopy should be carefully supported by small fragments of cover slip glass. It may also be quite harmful to these organisms to be streaked out, for the same reasons.

Future enrichments should be made with natural samples that contain many actively gliding trichomes; the number of twisted rope structures is quite irrelevant. The liquid medium should probably have a pH of 5.5–6.5, a constant iron supply of not more than 1–2 mg/liter Fe^{2+}, and an oxygen concentration of about 1 mg/liter. The incubation should be performed in the dark and at 5–10°C.

Identification

In the absence of iron-encrusted, excreted filament bundles, *Toxothrix trichogenes* could be mistaken for other gliding bacteria such as *Herpetosiphon* spp. The genus *Haliscomenobacter* (= *Streptothrix*), described from polluted environments (see Chapter 201), also shows some morphological similarities to *Toxothrix.* It is quite possible that gliding bacteria without such filamentous sheaths that have been observed in various freshwater, marine, or polluted habitats could have been *Toxothrix* spp. The absence, in *Toxothrix* trichomes, of constrictions at the cross-wall sites is quite characteristic. Their mode of gliding (in the form of a U) separated them from the equally colorless, filamentous, and gliding *Achroonema* spp., which normally remain fairly straight.

Fragmentation of *Toxothrix* trichomes into short rods is more common in some habitats than in others, and indicates the possibility of there being more than one *Toxothrix* species. Even before fragmentation, one recognizes many dense (electron-dense) granules in the *Toxothrix* trichomes. These may be polyphosphate granules (volutin). After fragmentation, each rod-shaped cell usually has two such granules, one at either end.

Table 1. Occurrence and habitats of *Toxothrix trichogenes*.[a]

Location and depth (m)	Time (months)[b]	Temp (°C)	pH	O_2 (mg/liter)	Redox potential (Eh, mV)	Fe^{2+} (mg/liter)	Trichomes	Cell density; other bacteria	Reference
Water basin, Dnjepr, Russia	7–8	·	·	·	·	·	+	Ga, Le	Cholodny, 1924
Iron spring, Braunschweig, Germany (<1)	9	9–11.5	5.5	<1	rH; 26–27	1.8–2.7	?	Ga, Le	Charlet and Schwartz, 1954
Iron spring, Reselithberg, Holstein (0.2)	7	13	6.6	·	·	+	++	Ga, Le, Si	P. Hirsch, unpublished observation
Iron spring, Kokkino Neró, Greece	·	15.5	6.1	0.4–1.2	+210 to +280	·	?	·	R. Schweisfurth, personal communication, 1976
Iron spring basin, Michigan (0–0.4)	1–4	4–9	6.8–6.9	·	·	+	+++	Ga, Le, Sp, Na, etc.	Krul, Hirsch, and Staley, 1970
	8–12	12–4		·	·	+	+++	Ga, Le, Sp	Krul, Hirsch, and Staley, 1970
Iron brook (before swamp), Karelia	·	·	6.25	·	+600	1.0	?	Na, Oc, Ga, Le; 1.3 × 10^3/mol	Dubinina and Derjugina, 1972
Iron brook (behind swamp), Karelia	·	·	5.12	·	+500	1.2	?	Ga, Le; 12.4 × 10^3/ml	Dubinina and Derjugina, 1972
Forest Pond, Michigan (0.6)	2–3	0.5–1.5	7.7	·	·	+	++	Sp, Si	Krul, Hirsch, and Staley, 1970
Lake Putis-Järvi, Karelia (7–8)	7	1	·	<3	·	·	?	5 × 10^3/ml	Gorlenko, Dubinina, and Kusnezov, 1977

[a]Other bacteria: Ga = *Gallionella;* Le = *Leptothrix;* Si = *Siderocapsa;* Sp = *Sphaerotilus;* Oc = *Ochrobium;* Na = *Naumanniella.*
[b]Months in which *Toxothrix* has been observed; 7–8 stands for July-August, etc.

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Fastidious Bacteria of Plant Vascular Tissues and Their Invertebrate Vectors

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Prokaryotes have been recognized as plant pathogens since the pioneering research of Burrill (1881). However, the concept that these pathogens include noncultivable or extremely fastidious prokaryotes developed recently, following the discovery by Doi et al. (1967) and Ishie et al. (1967) that mycoplasma-like organisms are consistently present in the phloem of plants afflicted with various diseases of the yellows type (See Chapter 229). This discovery inspired renewed investigations into the etiology of numerous infectious plant diseases, many of which had been assumed to be of viral etiology. Subsequently, not only were additional mycoplasma-like organisms found associated with plant disease, but several new groups of fastidious prokaryotes were discovered. These organisms include both spiroplasmas (See Chapter 89) and bacteria that are confined to the vascular system of their plant hosts.

The fastidious plant pathogenic bacteria are the subject of this chapter. They are usually limited to either the xylem or phloem of the vascular system, but some inhabit both tissues. They are usually transmitted from plant to plant by insect vectors which also serve as hosts. At first, the bacteria were frequently referred to as "rickettsia-like" because of their fastidious nature, and because their morphology, ultrastructure, and insect transmission resembled those of animal pathogens in the Rickettsiaceae. However, subsequent studies have shown that this resemblance is superficial. Altogether, the fastidious bacteria of plant vascular tissues are phylogenetically diverse and are grouped together largely because of their association with plant disease, their endophytic habitat within plants, and their fastidious nature.

Because these bacteria could not be readily cultured on conventional bacteriological media, their existence in plants was first revealed by light and electron microscopy. Subsequently, the fastidious, xylem-inhabiting bacterium that causes Pierce's disease of grapevines, almond leaf scorch disease, and alfalfa dwarf disease was isolated on media specifically devised for its axenic culture (Davis et al., 1978). Following the development of additional media formulations, most known fastidious, xylem-inhabiting bacteria have been axenically cultured. Furthermore, their role as plant pathogens has been confirmed in most cases.

The fastidious, xylem-inhabiting bacteria include both Gram-positive and Gram-negative organisms. The Gram-positive bacteria include the causal agents of ratoon stunting disease of sugarcane (Davis et al., 1980a) and Bermudagrass stunting diseases (Davis and Augustin, 1984; Davis et al., 1983a). These two plant pathogens were found to be related to some other phytopathogenic coryneform bacteria and were placed together with these pathogens in the newly described genus *Clavibacter* (see Chapter 62) (Davis et al., 1984). Most of the Gram-negative, fastidious, xylem-inhabiting bacteria have been found to be related to one another and have been classified as *Xylella fastidiosa* (Wells et al., 1987). Members of *Xylella* are phylogenetically related to the xanthomonads based on 16s rRNA analysis and belong within the gamma subgroup of the eubacteria. The remaining fastidious, xylem- or phloem-inhabiting bacteria have not been taxonomically classified.

The plant diseases known to be caused, or at least are suspected of being caused, by bacteria classified as *X. fastidiosa* include: Pierce's disease of grapevines (Davis et al., 1978; Goheen et al., 1973; Hopkins and Mollenhauer, 1973), alfalfa dwarf disease (Goheen et al., 1973; Thomson et al., 1978), almond leaf scorch disease (Davis et al., 1980b; Mircetich et al., 1976), phony disease of peach (Davis et al., 1981; Hopkins et al., 1973; Nyland et al., 1973; Wells et al., 1981; Wells et al., 1983), plum leaf scald disease (Davis et al., 1981; French and Kitajima, 1978; Kitajima et al., 1975; Raju et al., 1982; Wells et al., 1981), elm leaf scorch disease (Hearon et al., 1980; Kostka et al., 1984, 1986a); mulberry leaf scorch disease (Kostka et al., 1986b); oak leaf scorch disease (Chang and Walker, 1988; Hearon et al., 1980; Kostka et al.,

1984); periwinkle wilt disease (Davis et al., 1983b; McCoy et al., 1978); Ragweed stunt disease (Timmer et al., 1981); leaf scorch of red maple (Sherald et al., 1987); sycamore leaf scorch disease (Hearon et al., 1980; Sherald et al., 1983); and citrus blight (Feldman et al., 1977; Hopkins, 1982; Hopkins et al., 1978). Diseases associated with other Gram-negative, fastidious, xylem-inhabiting bacteria include: bacterial wilt of Toronto creeping bentgrass (Roberts et al., 1981); Sumatra disease of cloves (Bennett et al., 1987; Hunt et al., 1987); leaf scorch of *Macadamia integrifolia* (Jimenez, 1982); and a dieback of Chinaberry (Yehsiung et al., 1986).

Except for unconfirmed reports, none of the fastidious, phloem-inhabiting bacteria have been isolated in axenic culture, including those which are also capable of inhabiting the xylem. Consequently, little is known about their relationship to one another and to other bacteria. Phloem-inhabiting bacteria have been associated with diseases in citrus (Garnier and Bove, 1977; Garnier et al., 1984a, 1984b; Moll and Martin, 1974), clover (Behncken and Gowanlock, 1976; Benhamou, 1978; Benhamou et al., 1978, 1979; Black, 1944; Grylls, 1954; Liu and Black, 1974; Markham et al., 1975; Windsor and Black, 1973a, 1973b), carrot (Giannoti et al., 1974a), coconut palm (Steiner et al., 1977), grapevine (Rumbos et al., 1977; Ulrychova et al., 1975), hop (Vanek et al., 1976), larch (Nienhaus et al., 1976), onion (Konvicka et al., 1978), *Melaleuca armilaris* (Klein et al., 1979), potato (Klein et al., 1976), *Sida cordifolia* (Hirumi et al., 1974), spinach (Nienhaus and Schmutterer, 1976), sugar beet (Green, 1978; Nienhaus and Schmutterer, 1976; Urbina-Vidal, 1974), wheat (Ploaie, 1973), and willow (Holmes et al., 1972). The bacteria associated with rosette disease of sugar beet were also observed in the parasitic plant, dodder, which was shown to transmit the bacterium to sugar beet (Green, 1978). Double infection of the phloem of dodder with both mycoplasma-like organisms and fastidious bacteria has also been observed (Giannotti et al., 1974b).

Habitats

Plant Xylem Habitat

The xylem is a continuous tissue throughout the plant. It has three primary functions: transport of water and mineral nutrients, storage of nutrients, and structural support (Esau, 1965). Fastidious, xylem-inhabiting bacteria are associated with tracheary elements of two types, tracheids and vessel members, that function primarily in transport. In some hosts, the bacteria are found more frequently in the smaller, thick-walled tracheids than in larger vessels (Hopkins et al., 1973; Kitajima et al., 1975). Occasionally, however, the bacteria are found in the intercellular spaces of the xylem (Goheen et al., 1973). In the process of xylem differentiation, tracheary elements form cell walls with apertures, termed pits. Later, the cells become devoid of cytoplasm, leaving a hollow vessel. All tracheary elements are connected by paired pits which have membranes that separate the adjoining elements. In addition, vessel members are joined end-to-end, forming vessels, and the adjoining ends have open perforation plates. Vessels are not continuous throughout the plant, but are of finite length. The length of vessels is difficult to determine; it varies with plant species and with the time of year the vessels develop. In some species (e.g., *Eucalyptus obliqua*) most vessels are less than 50 cm in length; however, vessels in tree species with ring-porous wood may extend several meters (Esau, 1965; Zimmerman and McDonough, 1978). Sap flows from one vessel to another through pit membranes (Esau, 1965; Zimmerman and McDonough, 1978). Bacteria apparently move from one tracheary element to another by breaching pit membranes, but whether the membranes are enzymatically, or otherwise, dissolved or are forcefully ruptured is unknown. Often, some tracheary elements are packed with bacteria while adjacent elements are not. Pit membranes often appear to prevent lateral spread of the bacteria, but open perforation plates allow relatively unimpeded longitudinal spread within vessels.

Xylem-inhabiting bacteria are confronted with a unique physical and nutritional environment. The ascending flow of xylem sap from the roots fluctuates in response to transpiration of water by the plant; the direction of flow may even reverse under conditions of high atmospheric humidity combined with low soil moisture (Bollard, 1960). Since no evidence of motility has been observed in xylem-inhabiting bacteria, it has been assumed that the flow of xylem sap plays an important role in bacterial dissemination within the plant. However, there is some question as to whether or not vessels might be rendered dysfunctional upon the initial entry of bacteria. Zimmerman and McDonough (1978) suggested that an instantaneous embolism usually results from the invasion or introduction of bacterial pathogens into tracheary elements, thus causing dysfunction. However, Purcell (1989) suggests that such damage to vessels might be minimized by the

highly evolved feeding mechanism of the Homopteran vectors of *X. fastidiosa.* Regardless of the initial consequences of bacterial introduction, colonization of tracheary elements is likely to impede normal xylem function. If infested tracheary elements are dysfunctional, sap from adjacent functional tracheary elements might serve as one source of diffusable nutrients for xylem-inhabiting bacteria. The bacteria might also obtain nutrients from the cytoplasmic remains within the lumen of tracheary elements. Conceivably, sap flow might also be important in removal of bacterial waste products.

The composition and concentration of solutes in xylem sap varies with plant species, location within the plant, time of day, plant age, seasonal cycle, plant nutritional state, and health of the plant (Pate, 1976). The xylem sap provides a qualitatively rich but dilute nutritional environment. Xylem sap usually contains 1–20 mg/ml solids in contrast to phloem sap, which usually contains 50–300 mg/ml solids. These solids contain numerous soluble compounds that might serve as a source of diffusable nutrients for fastidious bacteria. Possible carbon sources for the bacteria include mono- and disaccharides such as glucose, sucrose, and fructose, organic acids such as citrate and succinate, and a number of other organic compounds including amino acids and plant-growth regulators (Andersen et al., 1989; Bollard, 1960; Pate, 1976; Wormall, 1924). Possible nitrogen sources include both organic and inorganic compounds. One organic nitrogenous compound often predominates (Pate, 1976). Amino acids, alkaloids, ureides, and amides are included among the nitrogenous compounds that occur in xylem sap. Organic phosphorous, sulfur-containing compounds, and essential inorganic salts are also present in xylem sap, and vitamins are sometimes present in low concentrations. In view of the low solute concentration of their environment, fastidious xylem-inhabiting bacteria may need special mechanisms for the acquisition of nutrients from sap.

In electron micrographs of ultrathin sections of infected plant material, fastidious, xylem-inhabiting bacteria are sometimes found embedded in an electron-dense matrix that is presumably of plant origin (Huang et al., 1986; Mollenhauer and Hopkins, 1974). Fibrous strands called "osmophilic lines or microfibrils" (Lowe et al., 1976) have frequently been seen connecting these bacteria (French et al., 1977; Lowe et al., 1976; Mollenhauer and Hopkins, 1974; Nyland et al., 1973). Such fibrous stands, discussed in greater detail in this chapter under Identification, resemble the fibrous polysaccharide coats that surround adherent bacteria in diverse habitats (Brooker and Fuller, 1975; Costerton et al., 1978; Latham et al., 1978; McCowan et al., 1978). Costerton et al. (1978) suggest that these are polysaccharide fibers (termed a "glycocalyx") which function in survival and pathogenesis. They suggest that the glycocalyx may confer such advantages to bacteria as: 1) maintaining position in a beneficial environment; 2) adhesion to one another for conservation and concentration of digestive enzymes; 3) provision of a food reservoir that may serve as an ionic net similar to an ion exchange resin; and 4) protection against host defense mechanisms or other stress. If the fibrous stands associated with fastidious, xylem-inhabiting bacteria confer such advantages, this would help to explain how these bacteria survive in the dilute but dynamic environment of the xylem.

With the exception of the fastidious, xylem-inhabiting bacteria associated with some tree diseases (Hearon et al., 1980; Kostka et al., 1984, 1986a, 1986b; Sherald et al., 1983, 1987), the bacteria generally occur in geographical areas with mild winter climates. Purcell (1977, 1979) has proposed that the geographical distribution of some of the diseases may be limited more by cold winters than by the lack of insect vectors or host plants. Exposure of grapevines with Pierce's disease to $-8°C$ to $-12°C$ for various periods of time resulted in remission of symptoms or complete recovery from the disease in some test plants (Purcell, 1977). However, when infected grapevines were exposed to various winter climates, the highest rate of recovery from Pierce's disease was not always associated with colder climates (Purcell, 1980), suggesting that factors other than cold temperatures alone may play an important role in their survival. Perhaps, desiccation of xylem tissues in dormant plants is important in this respect. Another factor might be location of the bacteria within their plant host; longer growing seasons in warmer climates might allow the bacteria the time needed after transmission by insect vectors to systemically spread from leaves and small branches to larger branches, trunks, and roots where there is possibly greater protection from adverse environmental conditions.

Symptoms of diseases in which xylem-inhabiting bacteria are involved include marginal necrosis of leaves, stunting, decline in vigor, and decrease in yield. Such "wilt" symptoms are also produced by many other vascular pathogens of plants, including bacterial pathogens in the genera *Xanthomonas, Pseudomonas, Erwinia, Clavibacter,* and *Curtobacterium* (Nelson and Dickey, 1970; Van Alfen, 1982). Host response varies greatly among plants colonized by fastidious, xylem-inhabiting bacteria. Such

variation may be a function of both the relative disease resistance of different hosts and the distribution of the bacteria within host plants. Peach trees infected with the bacterium that causes phony disease of peach and plum leaf scald disease do not develop marginal necrosis of leaves as symptoms, whereas plums grafted onto peach trees with phony disease develop this symptom (French et al., 1978); leaf marginal necrosis is a characteristic symptom of plum leaf scald disease. Populations of the pathogen are generally much larger in roots than in shoots of both peach and plum; however, greater proportions of the populations are present in plum shoots than in peach shoots (Wells et al., 1980). Interestingly, although wild plum is a symptomless host of the pathogen, it is important in the epidemiology of phony disease of peach. The pathogen apparently is more prevalent in shoots of wild plum than in shoots of peach, as evidenced by graft transmission studies (Hutchins et al., 1953). In grapevines with Pierce's disease, leaf marginal necrosis develops first in older, more mature leaves, and the extent of vascular colonization by the pathogen is directly correlated with development of this symptom (Hopkins and Thompson, 1983). Furthermore, colonization is greater in cultivars which are more susceptible to the disease.

A wide range of plant species can be colonized by fastidious, xylem-inhabiting bacteria. Strains of *X. fastidiosa* causing Pierce's disease of grapevines can colonize members of at least 28 families of monocotyledonous and dicotyledonous plants; however, recognizable disease symptoms are produced in only a few of these (Freitag, 1951; Hopkins and Adlerz, 1988; Raju et al., 1980; Raju et al., 1983). Similarly, strains of *X. fastidiosa* causing phony disease of peach have been found to colonize a wide range of host plants, many of which are symptomless hosts (Wells et al., 1980). Hopkins (1989) speculates that the list of natural hosts of all strains of *X. fastidiosa* is probably limited more by the effort spent looking for other hosts than by the host specificity of the bacterium.

Plant Phloem Habitat

Like the xylem, the phloem is also a continuous tissue throughout vascular plants and comprises another important habitat for fastidious bacteria. It functions primarily in the transport of nutrients synthesized by the plant. The ecological circumstances permitting colonization of the phloem by microorganisms involves a specialized insect fauna of plants that specifically utilizes this tissue. The habitat breadth of the phloem pathogens, therefore, is significantly influenced by the plant-host range of the insect vectors (Whitcomb and Williamson, 1979).

The phloem of plants consists, in part, of sieve tubes that are comprised of sieve cells (or sieve-tube elements) that form a long, pressurized conduit. Continuity of the fluids in the tissue is provided by pores between sieve elements. Although the pores may be less than 1 μm in diameter, they may be as wide as 14 μm, and average more than 2 μm. Thus, prokaryotic organisms can generally pass unrestricted from sieve cell to sieve cell. Electron micrographs of microorganisms jammed into sieve pores are, therefore, probably artifacts resulting from the release of pressure upon sampling of the tissue (McCoy, 1979).

In the phloem of susceptible plants, the microorganisms take advantage of a fluid that is rich in many potential nutrients. These include inorganic cations and anions, organic acids, amino acids, proteins, and carbohydrates. The composition of phloem sap can be favorably compared with the composition of insect hemolymph (Saglio and Whitcomb, 1979). Together, the two fluids comprise an ecological niche that has been filled by several prokaryotic taxa. These include the noncultivable, wall-less prokaryotes that induce proliferation and virescence in plants (see Chapter 229) and the spirophasmas (class Mollicutes; family Spiroplasmataceae; see Chapter 89). The phloem habitat contrasts sharply with that of the xylem. The xylem exudates of sugar beet (*Beta vulgaris,*) contain less than one-tenth the concentration of total solids, sucrose, reducing sugars, and total nitrogen that was found in phloem sap (Fife et al., 1962). Also, nitrogenous compounds were especially low in xylem exudates; physical parameters, such as viscosity, specific gravity, electrical conductivity, pH, and osmotic pressure, also differed in the sap of the two tissues (Bollard, 1960; Fife et al., 1962; Pate, 1976).

Fastidious bacteria of the phloem-insect habitat are small, elongate organisms (1–3 μm × 0.2–0.5 μm). At least 12 disease agents may be included in the group. In certain cases (Küppers et al., 1975; Petzold et al., 1973), the organisms reportedly occur not only in sieve elements, but in other phloem cell types as well. Such observations are not unique to phloem bacteria but have been made for phloem mycoplasma-like organisms as well. Considering that passage through plasmadesmata of the plant cell walls would be necessary for organisms to gain access to cells adjoining the sieve tube elements without disruption of the walls and that cells of developing phloem tissue are difficult to identify, McCoy (1979) concluded that part or all of the

reports of mycoplasma-like organisms in cells other than sieve tube elements can be attributed to misidentification, either of sieve tube elements as parenchyma cells or of membrane-bound vesicles of host origin as mycoplasma-like organisms. Certainly, the difficulties facing mycoplasma-like organisms in such plasmadesmatal passages would be even more insurmountable for the walled (and therefore less plastic) prokaryotes that are discussed in this chapter, unless some possess the ability to actively ramify through plant tissues. Such movement might help to explain reports of fastidious bacteria simultaneously inhabiting the phloem and xylem tissue, and occasionally the parenchyma and meristematic cells (Nienhaus and Sikora, 1979).

In their plant hosts, the phloem-inhabiting bacteria cause diseases characterized by leaf stunting and clubbing. Symptoms are often mild, and spontaneous recovery may be a significant feature (Klein et al., 1976). Other symptoms, such as proliferation of shoots with a resulting witches' broom appearance (Hirumi et al., 1974; Holmes et al., 1972; Maramoroush et al., 1975; Nienhaus et al., 1976; Neinhaus and Schumutterer, 1976) or floral virescences (Black, 1944; Lui and Black, 1974), may be observed in some cases. Symptoms of proliferation and virescence are more characteristic of diseases caused by another group of phloem inhabitants, the mycoplasma-like organisms (see Chapter 229), than those caused by phloem-inhabiting bacteria. At present the physiological basis for the proliferation and virescence syndrome is poorly understood.

Invertebrate Habitat

Despite occasional suggestions that some of the fastidious prokaryotic pathogens of plant vascular tissues are soil borne (Neinhaus et al., 1976; Rumbos et al., 1977), all well-defined vector relationships involve insects. The evolution of insect vector relationships with prokaryotic plant pathogens and the mechanisms involved in insect transmission of these pathogens has been reviewed by Purcell (1982a, 1982b). A large but well-defined group of insect species transmits the xylem-inhabiting bacteria classified as *X. fastidiosa.* Sharpshooter leafhoppers (Homoptera; Cicadellidae; Cicadellinae) and spittlebugs or froghoppers (Homoptera; Cercopidae) are the only known vectors of these pathogens, and both apparently are xylem-feeding insects (Purcell, 1979). The transmission of xylem-inhabiting bacteria by Homopteran insects has recently been reviewed (Purcell, 1989). Little is known about the means of transmission of the fastidious, xylem-inhabiting bacteria that have not been taxonomically classified as *X. fastidiosa.* Machaerotid spittlebugs appear to be principally involved in the transmission of the Sumatra disease bacterium (Eden-Green et al., 1986). Strains of *X. fastidiosa* that cause Pierce's disease are transmitted by at least 26 species of leafhoppers (Frazier, 1966; Hopkins, 1977) and five species of spittlebug (Severin, 1950). Those vectors that have been examined also transmit the pathogen to alfalfa, resulting in the development of alfalfa dwarf disease (Hewitt et al., 1946) and to almond, resulting in the development of almond leaf scorch disease (Auger et al., 1974; Mircetich et al., 1976). Furthermore, the capacity of this pathogen to infect a wide range of plant species, including symptomless as well as symptomatic hosts, was originally determined by means of insect transmissions (Freitag, 1951). This was accomplished even before the nature of the pathogen was known by using susceptible grapevine cultivars as indicator hosts. Transmission of different pathogenic variants among *X. fastidiosa* by the same leafhopper species has been demonstrated on several occasions. For example, *Homalodisca coagulata, Oncometopia undulata,* and *Cuerna costalis* transmit both the Pierce's disease pathogen (Kaloostian et al., 1962) and the phony disease pathogen (Turner and Pollard, 1959), and *Oncometopia nigricans* transmits the pathogens causing Pierce's disease (Hopkins, 1977) and the periwinkle wilt disease (McCoy et al., 1978). Thus, it appears that insect-vector transmission of *X. fastidiosa* is not governed by strict vector-pathogen specificities. In this respect, the vector relationship may mirror the pattern of wide host preference of the vectors themselves. Host preference of vectors, in turn, may be governed largely by the amino acid composition, especially amides, of xylem sap (R. F. Mizell, personal communication).

Although other sap-feeding, suctorial insects have been observed to probe the xylem, only those adapted for prolonged ingestion of xylem sap are efficient vectors of *X. fastidiosa* (Purcell, 1989). A large cibarial pump and pump musculature permits these insects to ingest copious amounts of xylem sap, and a highly developed filter chamber in their digestive tract facilitiates the efficient absorption of nutrients from the sap (Andersen et al., 1989). The mechanism of transmission of *X. fastidiosa* has been extensively studied in only a few vector species. Transmission appears to be noncirculative but persistant over the adult lifetime. There is no evidence that *X. fastidiosa* is pathogenic to its insect vectors. Evidence supporting the noncirculative transmission of *X. fastidiosa* includes:

1) the ability of some vectors to very quickly acquire and transmit the bacterium (Purcell and Finlay, 1979); 2) the lack of transovarial passage of the bacterium from one generation of insect to the next (Severin, 1949); 3) the failure of vector insects to transmit following experimental injection of the bacterium into their body cavity (McCoy et al., 1978; Purcell et al., 1979); and 4) the lack of transtadial transmission, in other words, the failure of previously transmitting insects to transmit after molting (Purcell and Finlay, 1979). This last feature directly points to the involvement of the foregut in transmission, because the lining of the foregut is lost during molting.

Microscopic examinations have revealed extensive colonization of the foregut of leafhopper vectors by *X. fastidiosa* (Fig. 1) (Purcell et al., 1979; Brlansky et al., 1983). Mats of the bacterium have been observed lining the entrance to the cibarial pump chamber and the chamber itself. Colonization of the precibarium has also been noted. Purcell (1989) suggests that from a functional standpoint, the precibarium is most likely the site from which bacteria are egested into plants by these vectors. One interesting feature of insect colonization by *X. fastidiosa* is the polar attachment of the bacteria to the lining of the foregut. This manner of attachment leds one to suspect that the fibrous strands seen extending from the polar ends of *X. fastidiosa* in electron micrographs might be directly responsible for attachment. Because of the high velocity of xylem sap passing through narrow portions of the foregut, such attachment may be essential for insect colonization (Purcell et al., 1979) and, ultimately, survival of *X. fastidiosa.*

In many respects, knowledge of insects as habitats for phloem-specialized bacteria is scanty. Most of these agents are known only as suspected plant pathogens; although they are assumed to be insect borne, in most cases no vector is known. Vertical transmission (transmission from one generation to the next) has been demonstrated in the leafhoppers (Homoptera: Cicadellidae) that carry two of these agents (Black, 1944; Grylls, 1954); the rates were as high as 99%. The demonstration that the clover club leaf pathogen was transmitted vertically through multiple generations (Black, 1948, 1950) was the first demonstration that a microorganism could multiply in both animal and plant reservoirs in the course of a complex biological cycle. In contrast, little is known of the vector-pathogen relationships in the case of the leafhopper vectors of other clover diseases or the psyllid (Homoptera; Psyllidae) vectors of citrus disease (Capoor et al., 1974; Catling and Atkinson, 1974; McClean and Oberholzen, 1965). In the latter case, there is some evidence that the organisms multiply in the vector hemolymph (Moll and Martin, 1973). This claim offered hope for eventual cultivation of the agents, since other agents that multiply extracellularly in arthropods, such as *Rochalimaea quintana* (Vinson, 1966) and spiroplasmas (Whitcomb and Williamson, 1979) have proven to be cultivable. Recent reports on isolation of the citrus greening disease agent (Garnett, 1985) would seem to support this contention if they can be substantiated; however, it should also be noted that with few possible exceptions, the phloem borne bacteria and mycoplasmalike organisms remain noncultivable despite their ability to exist extracellularly in insects.

Isolation

Selection of Host Material

Plant hosts are frequently used as a source for isolation of fastidious, xylem-limited bacteria. Isolation from insects is also possible but is more difficult because they normally harbor contaminating microorganisms which can not be eliminated by surface sterilization. However, the efficiency of isolation can be improved by using only the heads of surface-sterilized insects (Purcell et al., 1979), thus avoiding microflora associated with other body parts. Factors that govern the choice of plant material used for isolation include: 91) distribution of the organism within the plant; 92) avoidance of potential contaminants; and 93) ease of bacterial extraction from tissue. It is advisable to avoid necrotic tissues and, when possible, older tissues that harbor more contaminants. Generally, the bacteria are isolated more easily from more mature tissues of the current year's growth. Plant parts with mild symptoms, or that are adjoining symptomatic parts, are preferable. In this respect, petioles of leaves exhibiting marginal necrosis symptoms have frequently been used.

Surface Sterilization

Selective media are not available for isolation of fastidious, xylem-inhabiting bacteria; consequently, surface sterilization of host material is necessary to avoid contamination by other microorganisms. Surface sterilization of plant material is effective largely because the bacteria are systemically distributed within and limited to the xylem where few, if any, other microorganisms exist. Aqueous solutions of sodium hypochlorite may be used for surface sterilization of plant parts (Davis et al., 1978). The plant material is immersed in a 0.5% or 1.0% hypo-

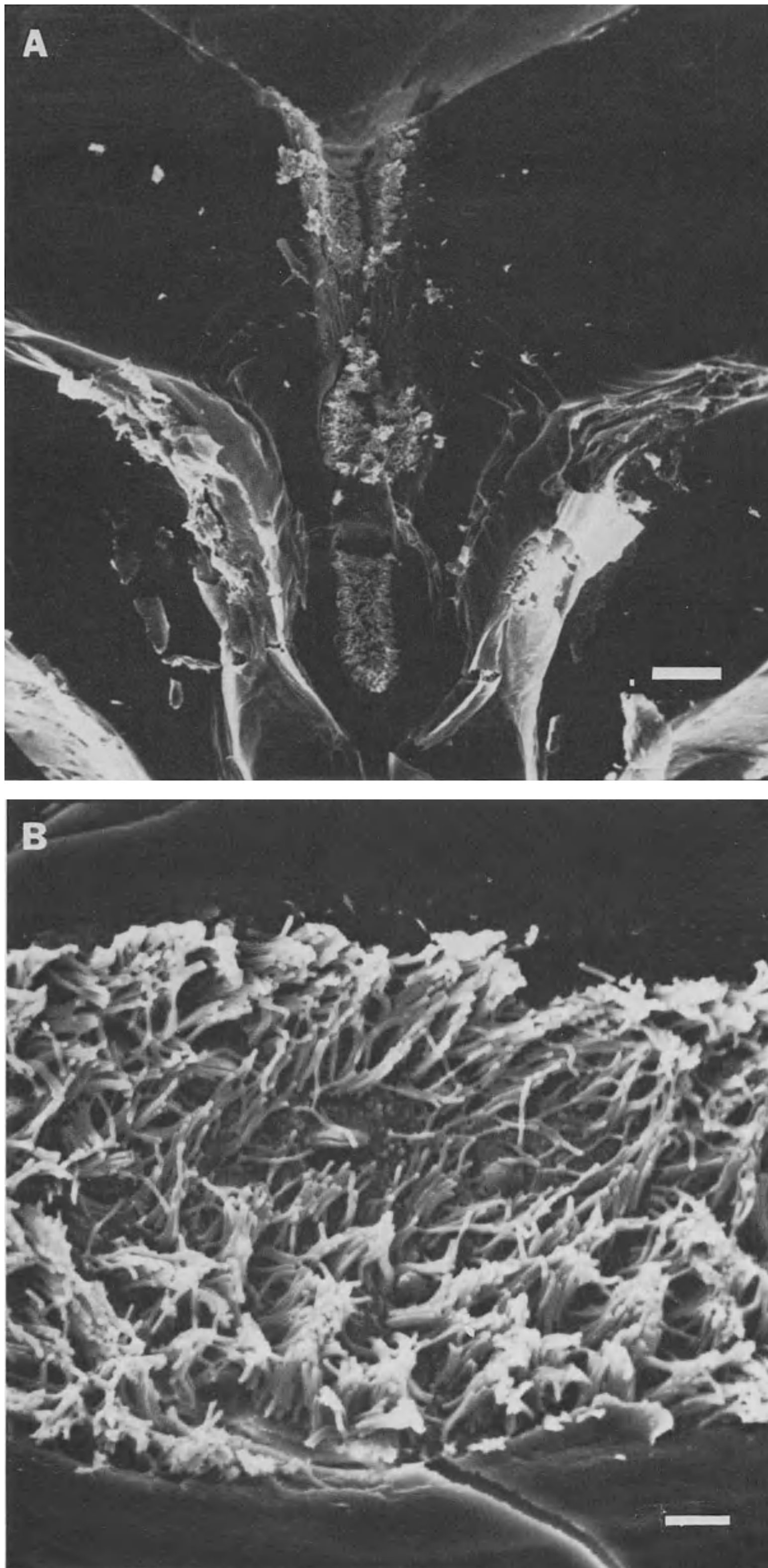

Fig. 1. Scanning electron micrograph of the precibarium of the leafhopper vector *Homalodisca coagulata* colonized by *Xylella fastidiosa.* (A) Epipharynx of the precibarium; floor of the cibarial pump visible at top and precibarial valve visible toward bottom. Bar = 25 μm. (B) Enlargement of anterior (distal) precibarium colonized by a dense mat of *X. fastidiosa.* Bar = 5 μm. (Courtesy of R. H. Brlansky.)

chlorite solution for 2–5 minutes and rinsed 2–3 times with sterile water to remove residual hypochlorite. It is helpful to first dip the plant material into 95% ethanol before treatment with sodium hypochlorite solution. In addition to being a disinfectant, ethanol also acts as a wet-

ting agent to facilitate surface sterilization. Unwanted portions of the plant material, such as cut ends that may have absorbed the hypochlorite solution, are then aseptically excised and discarded.

Inoculum Preparation for Isolation

Several different procedures have been used to extract fastidious, xylem-inhabiting bacteria from surface-sterilized plant parts. Extracts are often prepared by expressing sap from stems, rhizomes, petioles, or other plant parts with the aid of sterile forceps, pliers, or a hand vice. The sap is then collected with a pipette or blotted directly onto agar-solidified media. To obtain extracts, some plant parts may be ground in several volumes of buffer, such as 0.01 M phosphate buffer, pH 6.9, using a sterile mortar and pestle or a mechanical homogenizer. The liquid is then decanted from the plant debris and used as inoculum (Davis et al., 1981). A similar procedure has also been used to isolate the bacteria from their insect vectors (Davis, unpublished observations). Centrifugation or vacuum infiltration may be used to obtain extracts directly from sections of roots and stems. Such procedures are advantageous because extracts often contain relatively high numbers of xylem-inhabiting bacteria and are free of most plant debris. Although it has not been a widely recognized problem, plant tissue extracts may inhibit bacterial growth. Such inhibition has been found for the Sumatra disease bacterium when grown in the presence of tissue from clove trees (Bennett et al., 1987). When centrifugation is used, the plant material is aseptically placed in a conical centrifuge tube such that it does not contact the sap after extraction. Otherwise, the sap will often be drawn back into the plant material by capillary action. Centrifugation at 1000–4000 × times *g* for 1–5 minutes is routinely used to obtain sap containing *Clavibacter. xyli* subsp. *xyli* from sugarcane stalk sections (Davis et al., 1980a; Davis and Dean, 1984), and grapevine sap containing the Pierce's disease bacterium has been obtained in a similar manner from leaf petioles using a microcentrifuge (Davis et al., 1978). In the vacuum infiltration procedure, sections of stems or roots are attached to a vacuum flask via rubber couplings (Davis et al., 1980a; French et al., 1977; Teakle et al., 1973); cells of the pathogen are then flushed out by pulling water or buffer through the xylem with vacuum pressure.

Inoculation of Culture Media

Several methods of media inoculation have been successfully used to isolate fastidious, xylem-inhabiting bacteria. Direct inoculation of a medium with plant extracts is often the most expedient method and may be done simply by streaking extracts onto medium or by repetitive blotting of expressed sap from the cut surface of the plant material onto the medium. Serial dilutions of extracts may be used as inoculum resulting in the elimination of less numerous microbial contaminants by dilution. Furthermore, plating a series of dilutions enables enumeration of single colonies for quantitative studies. Spot inoculations with small volumes of inoculum, usually 10 μl, may prove useful. Spot inoculations allow different inocula or several dilutions of the same inoculum to be placed on the same plate of agar medium, thereby, conserving medium and labor. Also, because only a small portion of the surface of the medium is inoculated, locating and counting the small colonies commonly developed by fastidious, xylem-inhabiting bacteria may be easier.

Culture Media

The requirements for culture media vary among the different species and strains of xylem-inhabiting bacteria. For *X. fastidiosa,* this variation is usually much less among strains of specific pathogens grouped together within the species. The bacteria causing Sumatra disease of cloves (Bennett et al., 1987), bacterial wilt of Toronto creeping bentgrass (Roberts et al., 1981), and some strains of *X. fastidiosa* (Kostka et al., 1986b; Fry et al., 1988) will grow on full-strength or diluted nutrient agar. In general, however, isolation and continuous cultivation of the xylem-inhabiting bacteria is better on media specifically formulated for these organisms. Following the initial isolation of the Pierce's disease bacterium (Davis et al., 1978), the PD2 medium was developed for culturing this bacterium (Davis et al., 1980c). Two completely autoclavable derivations of the PD2 medium were also developed by replacing bovine serum albumin with either potato starch (2 g/l; PD3 medium) or activated charcoal (0.5 g/l; PD4 medium). Growth of the Pierce's disease bacterium may be slightly better on the PD2 medium than on the autoclavable formulations.

PD2 Medium for Xylem-Inhabiting Bacteria

Pancreatic digest of casein (Tryptone [Difco] or Trypticase peptone [BBL]	4 g
Papaic digest of soy meal (Soytone [Difco] or Phytone peptone [BBL]	2 g
Trisodium citrate	1 g
Disodium succinate	1 g
Hemin chloride stock (0.1% bovine hemin chloride dissolved in 0.05 N NaOH)	10 ml
$MgSO_4 \cdot 7H_2O$	1 g

K_2HPO_4	1.5 g
KH_2PO_4	1 g
Agar	15 g
Distilled water	1 liter
BSA stock (20% bovine serum albumin fraction five [sigma Chemical Co.] in water)	10 ml

All ingredients except the BSA stock are autoclaved in one liter of distilled or deionized water. After autoclaving, the medium is cooled to 50°C, and the filter-sterilized BSA stock is aseptically added. The final pH of the medium should be approximately 6.9 without adjustment.

The more fastidious strains of *X. fastidiosa* will not grow on PD2 medium. Several media formulations have been developed which will support growth of at least some if not all of these strains in culture (Davis et al., 1980a; Davis et al., 1981; Chang and Walker, 1988; Wells et al., 1981). The PW medium (Davis et al., 1981) is perhaps the most widely used of these formulations and will support the isolation and continued cultivation of even the most fastidious strains of *X. fastidiosa,* such as those that cause both phony disease of peach and plum leaf scald disease. The SC medium (Davis et al., 1980a) was developed for cultivation of *C. xyli.* subsp. *xyli* and *C. xyli.* subsp. *cynodontis,* which will not grow on PD2 or PW media, and will also support growth of less fastidious strains of *X. fastidiosa.* Consequently, it may be advantageous to employ several media when attempting to isolate a xylem-inhabiting bacterium whose identity is unknown. Generally, cycloheximide can be added to the PD2, PW, or SC media to inhibit fungal contamination without affecting bacterial growth; it is filter-sterilized and added to the medium at a final concentration of 50 mg/l.

PW Medium for Isolating More Fastidious Strains of *X. fastidiosa*

Pancreatic digest of casein (Tryptone [Difco] or Trypticase peptone [BBL]	1 g
Papaic digest of soy meal (Soytone [Difco] or Phytone peptone [BBL]	4 g
Hemin chloride stock (0.1% bovine hemin chloride dissolved in 0.05 N NaOH)	10 ml
$MgSO_4 \cdot 7H_2O$	0.4 g
K_2HPO_4	1 g
KH_2PO_4	1.2 g
Agar	12 g
Phenol red stock (0.2% phenol red in water)	10 ml
Distilled Water	1 liter
BSA stock (20% bovine serum albumin fraction five in water)	30 ml
Glutamine (8% glutamine in water)	50 ml

All ingredients except the BSA and glutamine stocks are autoclaved in one liter of distilled or deionized water. After autoclaving, the medium is cooled to 50°C, and the filter-sterilized BSA and glutamine stocks are aseptically added. The final pH of the medium should be approximately 6.6 without adjustment.

SC Medium for Isolating Xylem-Inhabiting Bacteria

Papaic digest of soy meal (Soytone [Difco] or Phytone peptone [BBL]	8 g
Hemin chloride stock (0.1% bovine hemin chloride dissolved in 0.05 N NaOH)	15 ml
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	0.5 g
KH_2PO_4	1.5 g
Corn meal agar (BBL)	17 g
Distilled Water	1 liter
Glucose (50% glucose in water)	1 ml
Cysteine (free base; 10% cysteine in water)	10 ml
BSA stock (20% bovine serum albumin fraction five in water)	10 ml

All ingredients except the glucose, cysteine, and BSA stocks are autoclaved in one liter of distilled or deionized water. After autoclaving, the medium is cooled to 50°C, and the filter-sterilized glucose, cysteine, and BSA stocks are aseptically added. The pH should be 6.6–6.7 without adjustment.

Several broth formulations have been used for cultivation of fastidious, xylem-inhabiting bacteria. The PD2 and PW media can be prepared as broth media by omitting the agar. The elm leaf scorch bacterium (*X. fastidiosa),* which has been difficult to isolate on agar solidified media, can be isolated in S8 broth (SC medium without cornmeal agar) inoculated with infected wood chips (Kostka et al., 1981). However, once isolated in broth culture, this bacterium will grow when transferred onto semi-solid PD2, SC, or PW media.

Cultivation

All of the fastidious, xylem-inhabiting bacteria are obligate aerobes and grow best in culture at 27–29°C. One to three weeks of incubation are usually required to produce visible growth in primary cultures. Since most contaminating bacteria grow much faster than this, plates can be screened early for such contaminants. Because of this relatively long incubation period, precautions against excessive desiccation of the medium are often necessary.

Preservation of Cultures

Cultures of *X. fastidiosa* lose viabity rapidly when incubated past log-phase growth, and viability has not been successfully prolonged by refrigeration. Viability can be maintained at room temperature (25°C) for 1 to 2 months when cells are suspended in 0.01 M phosphate

buffer, pH 7.2, or 10% skim milk in water and for at least 4 months at −40°C in 10% skim milk in water or 15% glycerol in PW broth medium (Jimenez and Davis, unpublished observations). Long-term preservation of *X. fastidiosa* is possible with preparations that have been freeze-dried in 10% skim milk and stored at −20°C (Davis, unpublished observations). Long-term preservation of the bacteria on silica gel in a freezer has also been recommended (Hopkins, 1988).

Identification

Xylem-Inhabiting Bacteria

CELL MORPHOLOGY AND ANATOMY The Gram-negative, fastidious, xylem-inhabiting bacteria are all nonmotile, aflagellate rods usually measuring 0.25 μ to 0.5 μm in width and 1 μm to 4 μm in length (Chen et al., 1982; Hopkins, 1977, 1989). Constriction furrows in the cell wall have been interpreted as evidence that the bacteria reproduce by binary fission (Hopkins, 1977; Lowe et al., 1976; Mollenhauer and Hopkins, 1974). In culture, elongated forms of these bacteria, which measure several times the normal cell length in the host plant, are sometimes found (Davis et al., 1978; Davis et al., 1981; Wells et al., 1981). Fewer elongated cells were seen on media that support more rapid growth of the bacteria, suggesting that cell elongation without division occurs in response to a sub-optimal nutritional environment (Davis et al., 1978). The association of filamentous forms with smooth colonies and shorter rods with rough colonies was also reported (Wells et al., 1987). The cell walls of the bacteria have a typical Gram-negative type ultrastructure with outer and inner trilaminar unit membranes enclosing a periplasmic space (Fig. 2) (Bennett et al., 1987; Brlansky et al., 1983; Lowe et al., 1976; Mollenhauer and Hopkins, 1974). A peptidoglycan or "R-layer" has often been observed in the periplasmic space. Although many Gram-negative bacteria have outer cell wall membranes which have periodic enfoldings, the enfoldings of *X. fastidiosa* are often more exaggerated. The outer membranes have been described as rippled, ridged, corrugated, and furrowed, and the enfoldings tend to be perpendicular to the long axis of the cells. Within the plant, host, the cell topography may vary within individual tracheary elements, ranging from greatly enfolded to smooth (Mollenhauer and Hopkins, 1974). This variation may be a result of cell deterioration due to aging (Mollenhauer and Hopkins, 1974) or defense mechanisms of the host (Huang et al., 1986).

The cytoplasm of fastidious, xylem-inhabiting bacteria resembles that of other prokaryotes in its content of ribosomes, nuclear regions with DNA-like strands, and osmophilic granules (Bennett et al., 1987; Lowe et al., 1976; Mollenhauer and Hopkins, 1974; Nyland et al., 1973). Membranous inclusion bodies, which appear to be associated with the cytoplasmic membrane, were seen in situ in the Pierce's disease bacterium (Davis, 1978; Lowe et al., 1976). Occasionally, an electron-lucent zone, which may be an artifact of fixation or a capsule, has been observed surrounding individual bacteria in plants (Davis, 1978; Hopkins, 1977; Lowe et al., 1976; Mollenhauer and Hopkins, 1974).

In the tracheary elements of plants, fastidious, xylem-inhabiting bacteria often form aggregates that appear to be held together by extracellular material produced by the bacteria (Mollenhauer and Hopkins, 1974). This material has been termed fibrous forms (Mollenhauer and Hopkins, 1974), "osmophilic lines" or microfibrils (Lowe et al., 1976), and electron-dense strands (French et al., 1977; Nyland et al., 1973). Often these strands are more abundant at the ends of the bacteria (Fig. 2) (French et al., 1977; French and Kitajima, 1978; Lowe et al., 1976). Negatively stained preparations of the phony disease bacterium revealed filaments (French et al., 1977), which might be analogous to either the acid polysaccharide coat (Costerton et al., 1978) or to fimbriae (Ottow, 1975) observed in other bacteria. Other filaments, possibly composed of subunits, were observed either attached to the cell wall or in the vicinity of cells (Lowe et al., 1976; Nyland et al., 1973). Hopkins (1977) considered these to be analogous to either fimbriae or possibly to sex pili in other bacteria. These filaments were 28–30 nm in diameter and of undetermined length; their width was approximately three times the usual width of fimbriae. Similar appendages have been observed attached to the cell wall of a bacterial symbiont in the pharyngeal diverticulum of the olive fly (Poinar et al., 1975). The extracellular materials of *X. fastidiosa* may also be associated with virulence within the plant host in addition to their possible role in the attachment of cells to the lining of the foregut of its insect vectors as discussed previously in the section on invertebrate habitat. Cells of these bacteria tend to agglutinate when suspensions are made from recently isolated cultures, and Hopkins (1989) has observed that a correlation exists between the loss in culture of the ability to agglutinate and of virulence.

CULTURE CHARACTERISTICS. Fastidious, xylem-inhabiting bacteria usually produce colonies on semi-solid media which are circular with entire

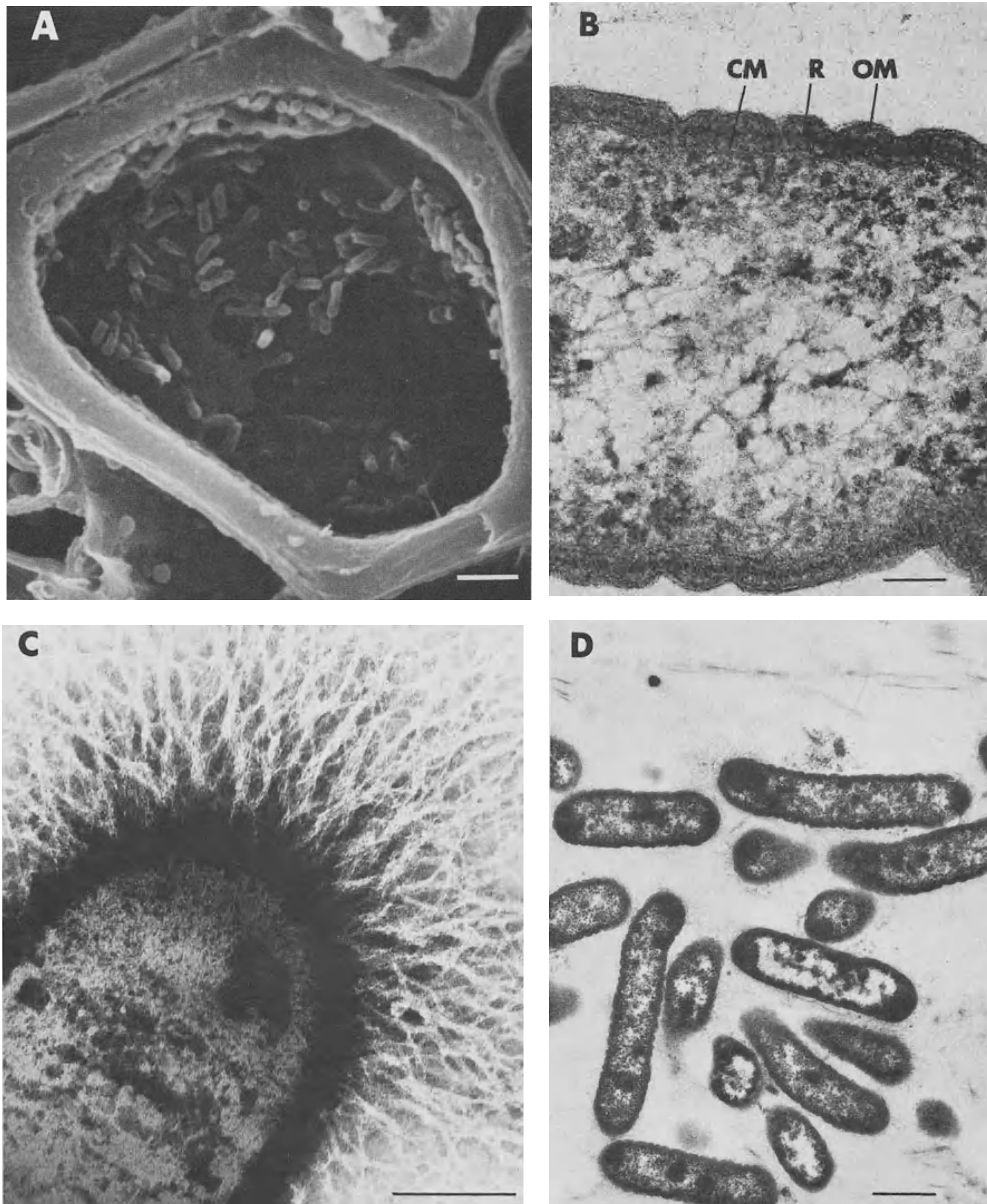

Fig. 2. Xylem-limited bacteria of the species *Xylella fastidiosa.* (A) Scanning electron photomicrograph of a freeze-fractured tracheary element of grapevine with Pierce's disease, showing the causal bacterium in the lumen. Bar = 2.5 μm (Courtesy of D. G. Garrott and M. J. Davis.) (B) Pierce's disease bacterium in grapevine showing rippled cell wall with cytoplasmic membrane (CM), R-layer (R), and outer membrane (OM). Bar = 50 nm. (Courtesy of H. H. Mollenhauer and D. L. Hopkins.) (C) Negatively stained bacterium causing plum leaf scald, showing prominent "fimbriae" extending from the cell wall at a longitudinal end of the bacterium. Bar = 0.2 μm. (From French and Kitajima, 1978.) (D) Bacterium with rippled cell wall associated with elm leaf scorch disease in tracheary element of elm. Bar = 0.5 μm (Courtesy of J. Sherald and S. Hurtt.)

margins and convex, but sometimes colonies are produced that are also circular with undulate margins and have an umbonate to flat appearance. With the phony disease of peach and plum leaf scald bacteria, the convex colonies have a smooth surface, whereas the umbonate to flat colonies have a rough surface, and both colony types can appear in cultures of the same strain (Davis et al., 1981). Colonies of freshly isolated strains of the Pierce's disease bacterium are often very viscid, becoming almost granular with age, and do not disperse easily in water or broth medium. Colonies are usually clear to opalescent white and without pigmentation. Generally, the slower growing pathogens, such as the phony disease bacterium, produce colonies in 2 to 3 weeks that reach diameters from 0.1 to 0.7 mm. The faster growing pathogens, such as the Pierce's disease bacterium and Sumatra disease bacterium, produce colonies in 1 to 2 weeks that reach a diameter from 0.5 to 5 mm. In broth cultures, doubling times ranging from 9 hours to 2.3 days have been reported for different strains of *X. fastidiosa* (Davis, 1978; Wells et al., 1987).

SEROLOGY. Serological techniques, such as gel double diffusion, immunofluoresence, and enzyme-linked immunosorbent assay (ELISA), presently provide the most practical means of differentiating *X. fastidiosa* from other bacteria. Both monoclonal (Wells et al., 1987) and polyclonal (Davis et al., 1979; French et al., 1978; Davis et al., 1981; Davis et al., 1983b; Lee et al., 1978; Nome et al., 1980; Raju et al., 1978) antibodies have been used. All strains of *X. fastidiosa* appear to be antigenically related, but quantitative differences exist among strains as measured by ELISA (Davis et al., 1983b; Nome et al., 1980; Raju et al., 1982; Wells et al., 1983). However, serological means to distinguish among different pathogens within *X. fastidiosa* have not been found, and preliminary reports suggest that alternative means, such as analysis of protein profiles from cell envelopes (Chang and Schaad, 1982) or cloned DNA probes (Jimenez and Davis, 1987), may prove more useful in this respect.

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS. All fastidious, xylem-inhabiting bacteria are oxidase negative, catalase positive, and obligately aerobic. Strains of *X. fastidiosa* hydrolyze gelatin, and utilize hippurate but do not produce indole, H_2S, β-galactosidase, lipase, amylase, coagulase, or phosphatase (Wells et al., 1987). Generally, the optimum temperature for growth in culture is 26–28°C, and the optimum pH is 6.5–6.9. Growth of strains of *X. fastidiosa* and the Sumatra disease bacterium in culture is usually accompanied by increased alkalinity of the medium (Bennett et al., 1987; Davis et al., 1980c; Wells et al., 1987). In minimal medium, strains causing Pierce's disease utilize organic acids, such as succinate and citrate, but not carbohydrates, such as glucose and sucrose (Davis, 1983), and similar results were obtained in more complex media (Davis et al., 1980c). However, in modified PW medium, growth of a number of strains of *X. fastidiosa,* representing different pathogenic types, was stimulated by glucose and several other carbohydrates and organic acids but inhibited by citrate and succinate (Wells et al., 1987). This discrepancy may be due to strain differences or inadequacy of the methods used to test utilization of organic acids and carbohydrates. Interestingly, the predominant organic compounds in xylem fluid of four of the plant host species of *X. fastidiosa* include citrate and succinate but not carbohydrates (Andersen et al., 1989). Completely defined media would be useful to examine the nutritional requirements of fastidious, xylem-inhabiting bacteria but have not been developed. Alternatively, Chang (1988) has examined the enzyme activities of two strains of the Pierce's disease bacterium and found no indication that these bacteria have a glycolytic pathway. The strains derived energy through the Krebs cycle and pentose phosphate shunt. The Krebs cycle enzymes found to be active were citrate synthetase, aconitase, fumarase, succinyl-CoA synthetase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase. Phosphohexose isomerase and glucose-6-phosphate dehydrogenase, but not lactate dehydrogenase, were also detected. These results support the earlier contention that organic acids are the primary source of energy for *X. fastidiosa.*

A high degree of DNA homology (75–100%) exists between pathologically different strains of *X. fastidiosa,* suggesting that the strains are indistinguishable at the species level (Kamper et al., 1985; Wells et al., 1987). Less than 5% homology was found between these strains and other strains representing different genera containing phytopathogenic bacteria and other Gram-negative bacteria. The content of guanine plus cytosine (GC) in various strains of *X. fastidiosa* ranged from 49.5 to 53.1 mol%, and the genome size ranged from 1.27 to 1.6×10^9 daltons. Similarity and signature analysis of 16S rRNA sequences indicated that strains of *X. fastidiosa* were most closely related to xanthomonads; the highest similarity being with a strain of *Xanthomonas maltophilia* (Wells et al., 1987).

Analyses of the composition of total cellular fatty acids of strains of *X. fastidiosa* indicate uniformity within the species (Wells and Raju, 1984; Wells et al., 1987). The major components were $C_{16:0}$ (30%), $C_{16:1}$ (26.7%), $C_{17:0}$ (11.6%), and $C_{15:0}$ (8%) fatty acids. The profile was unique when compared to those of other taxa. The percentage of saturated, odd-numbered carbon straight chains was higher than in other Gram-negative, phytopathogenic bacteria, and cyclopropane acids were entirely absent.

PATHOGENICITY. Host colonization and pathogenicity in plant diseases caused by fastidious, xylem-inhabiting bacteria has recently been reviewed (Davis, 1989; Hopkins, 1989; Purcell, 1989). In many situations, pathogenicity tests are impractical for routine identification of these bacteria, because of the long incubation period required for symptom development in plants, usually 2–6 months. Furthermore, loss of virulence in culture may complicate matters (Hopkins, 1984). Also, the pathogenicity of some fastidious, xylem-inhabiting bacteria has not been conclusively established.

Often, plants can be successfully inoculated with fastidious, xylem-inhabiting bacteria from culture; however, at least in one case, attempts to use insect vectors to transmit the bacteria from culture to plants has met with very limited success (Davis et al., 1978). Various modifications of two techniques, the vacuum infiltration technique (Davis et al., 1978; Davis et al., 1980b; Sherald et al., 1983) and the needle puncture technique (Davis et al., 1980b, Hopkins, 1980), have frequently been used for inoculation of plants. Inoculation of roots wounded to expose the xylem has also been reported to result in a low frequency of infection (Davis et al., 1980b). The prerequisite for successful inoculation regardless of the technique used appears to be the direct introduction of inoculum into the xylem which is necessary because the bacteria apparently do not have an active mechanism for ingress (or egress) through other tissues.

Phloem-Inhabiting Bacteria

Lafleche and Bove (1970) were the first to recognize a group of fastidious, phloem-inhabiting prokaryotes, whose limiting structure was more complex than that of the wall-less prokaryotes. Their studies, discussed also by Saglio et al. (1972), clearly showed that the limiting structure of the suspected causal agent of citrus greening disease consists of a double membrane and, therefore, differs fundamentally in its structure from that of the phloem-inhabiting, wall-less agent which has since been named *Spiroplasma citri* (Saglio et al., 1973) and shown to incite citrus stubborn disease (Markham et al., 1974).

Although there have been reports of the in vitro cultivation of the fastidious, phloem-inhabiting bacteria associated with plant disease, these reports have not been independently confirmed. They include the cultivation in chick embryos of a bacterium from yellows-diseased grapevine (Nienhaus et al., 1978) and the cultivation in axenic culture of the bacterium associated with citrus greening disease (Garnett, 1985). With these possible exceptions, failures in cultivation have prohibited the development of a clear taxonomic concept. However, the organisms appear to have several common structural features, which may indicate a common phylogeny and eventual recognition as a taxon. The organisms are elongate, 1.3 × 0.2–0.5 μm, with the usual ribosomal and diffuse nuclear regions characteristic of prokaryotic cells (Fig. 3). They are bounded by a "wavy" structure that consists, in part, of two single membranes (about 8 nm wide). Presence of cell wall constituents is suggested by the total width of the limiting structure (20–30 nm) and by the susceptibility of the organisms to penicillin derivitives. It is generally agreed that these phloem organisms are similar to Gram-negative bacteria, although their dimensions are certainly at the lower limit of the size range. Of special significance is the apparent absence of an R-layer in the periplasmic space of the cell wall (Moll and Martin, 1974), which is characteristically present in the Gram-negative cell wall (Costerton et al., 1974). Although the organisms have been referred to as "rickettsia-like" by many authors, there is no direct analogy to members of the Rickettsiaceae; for this and other reasons, Moll and Martin (1974) preferred to refer to the organisms as "bacteria-like," a term that has gained some acceptance (Klein et al., 1976). Some of the organisms are so small that they are visible by phase-contract microscopy, but not by conventional brightfield microscopy (Liu and Black, 1974). Under phase-contrast microscopy, the organisms exhibit an undulatory motility in 30% glycerol that ceases upon the addition of mercuric chloride or potassium penicillin G (Lui and Black, 1974). Thus sensitivity to the inhibition by penicillin of cell wall biosynthesis has been shown both in vivo and in vitro (Windsor and Black, 1973a; Ulrychova et al., 1975). The in vivo sensitivity of the organisms to tetracycline derivatives has also been established (Schwarz and Van Vuuren, 1971; Schwarz et al., 1974; Su and Chang, 1976).

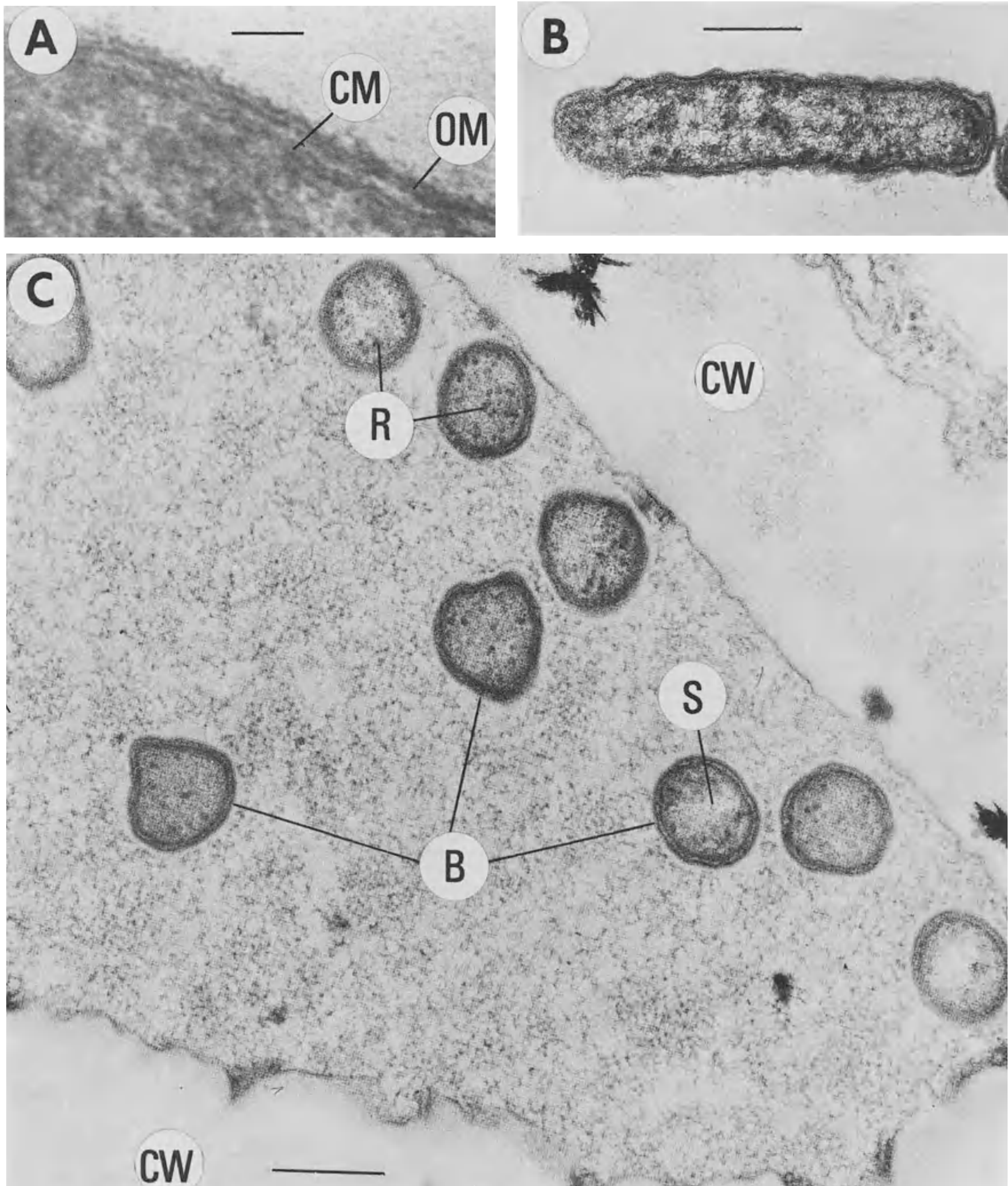

Fig. 3. Fastidious phloem bacteria. (A) Detail from thin section of cell of citrus greening bacterium. Cytoplasmic membrane (CM) and outer membrane (OM) are present. Bar = 40 nm. (Courtesy of J. M. Bove.) (B) Thin section of bacterium from phloem of white clover (*Trifolium repens*). Cells are elongate with wavy contour. Bar = 0.2 μm. (Courtesy of P. G. Markham.) (C) Cells of clover club leaf bacterium (B) in transverse section of phloem cell of periwinkle (*Catharanthus roseus*) shoot. The cells contain ribosome-like particles (R) and fine strands (S) representing DNA. CW, cell wall. Bar = 0.2 μm. (From Windsor and Black, 1973b.)

Lui and Black (1974) were able to partially purify the clover club leaf organism, prepare antiserum that agglutinated organisms to a titer of 1/1024, and stain the organisms specifically in leafhopper tissue using the fluorescent antibody test. More recently, monoclonal antibodies have been produced for detection of the citrus greening bacterium (Garnier et al., 1987). Serological techniques may eventually be used to demonstrate relationships even in the ab-

sence of successful cultivation of the fastidious, phloem-inhabiting bacteria.

Applications

Although the economic importance of fastidious bacteria of plant vascular tissues is presently related directly to their role as plant pathogens, ironically, some may eventually prove useful in the control of plant disease and pestilence. This point is exemplified by the recent effort to control lepidopterous plant pests through genetic enhancement of *C. xyli* subsp. *cynodontis* (Kostka et al., 1988). By inserting the gene for production of the δ-endotoxin from *Bacillus thuringensis* var. *kurstaki* into the genome of *C. xyli* subsp. *cynodontis,* it is hoped that the bacterium will effect a degree of pest control by systemically colonizing plants and at the same time producing sufficient amounts of the toxin. This concept for an endophytic delivery system relies on the relatively wide host range of this fastidious, xylem-inhabiting bacteria and the negligible amount of damage due to its presence in target hosts. A further advantage of using *C. xyli* subsp. *cynodontis* is the lack of insect vectors which lessens the risk of environmental release of the recombinant organism. Other fastidious, xylem-inhabiting bacteria with insect vectors might make more suitable delivery systems in some situations, if factors governing virulence and vector transmission can be understood and manipulated when necessary.

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Mycoplasma-Like Organisms—Plant and Invertebrate Pathogens

BRUCE C. KIRKPATRICK

Mycoplasma-like organisms (MLOs) are wall-less prokaryotes that cause disease in many higher plants and in some cases in the insects that transmit them. Although MLOs morphologically resemble culturable members of the class Mollicutes and are susceptible to the same antibiotics, the inability to continuously culture these organisms in vitro has prevented their definitive classification as Mollicutes (Razin and Freundt, 1984). However, recent sequence analysis of MLO 16S ribosomal RNA (rRNA) has clearly established that these pathogens form a unique cluster of organisms that are phylogenetically related to Gram-positive bacteria and to culturable mollicutes. These results, and rapid advancements in MLO taxonomy made possible by serological and nucleic acid hybridization analyses, suggest molecular analyses will generate the most important criteria for classifying currently nonculturable wall-less prokaryotes.

The primary subject of this chapter is plant pathogenic MLOs; other plant-associated mycoplasmas are only briefly discussed. Emphasis is placed on the advancements that have been made in MLO detection, taxonomy, and phylogeny.

Biological and Biophysical Characteristics of MLOs

Since their discovery by Doi et al. (1967), MLOs have been shown to be associated with more than 200 diseases of higher plants (McCoy et al., 1989). MLOs were originally thought to be large, unstable viruses (Black, 1954; reviewed by Whitcomb and Black, 1982). Electron microscopic observations of plants infected with "yellows agents" revealed the presence of pleomorphic, wall-less prokaryotes in the phloem sieve tube elements of infected plants. These pathogens ranged in size from 50 nm, theoretically nonviable "elementary bodies" (Maniloff and Morowitz, 1972; McCoy, 1979), to filaments that were several microns long (McBeath et al., 1972). Because these long, thin filaments appear to be small spherical cells when sectioned transversely, the true morphology of an MLO cell is best revealed by constructing three dimensional models from serial sections (Waters and Hunt, 1980; Florance and Cameron, 1978). Such analyses show that at least some MLOs are highly pleomorphic, multi-branched, filamentous organisms, whose filaments are often aligned parallel to the length of the sieve tube element. Numerous ribosomes and occasional fibers, which are thought to be DNA, are usually observed within sections of viable MLOs (Fig. 1 and 2). These structures are useful morphological markers for differentiating MLOs from host vesicles that are often present in plant phloem. Although similar morphological characters are observed in MLO-infected insect vectors (Fig. 2), the true morphology of MLOs in their insect vectors has not

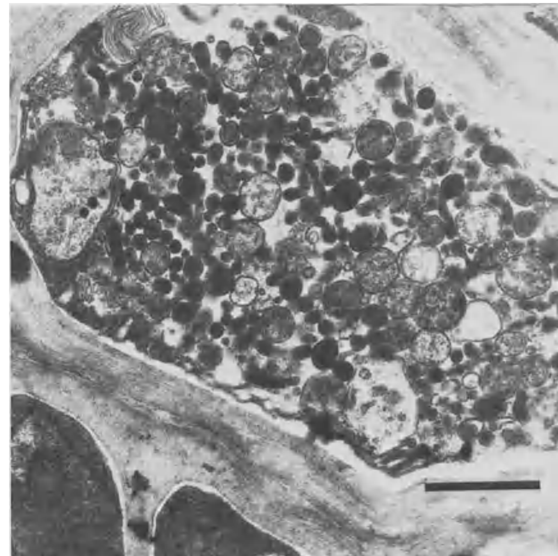

Fig. 1. Electron micrograph of X-disease mycoplasma-like organisms (X-MLOs) in a sieve-tube element of sweet cherry (*Prunus avium*). Bar = 1 μm.

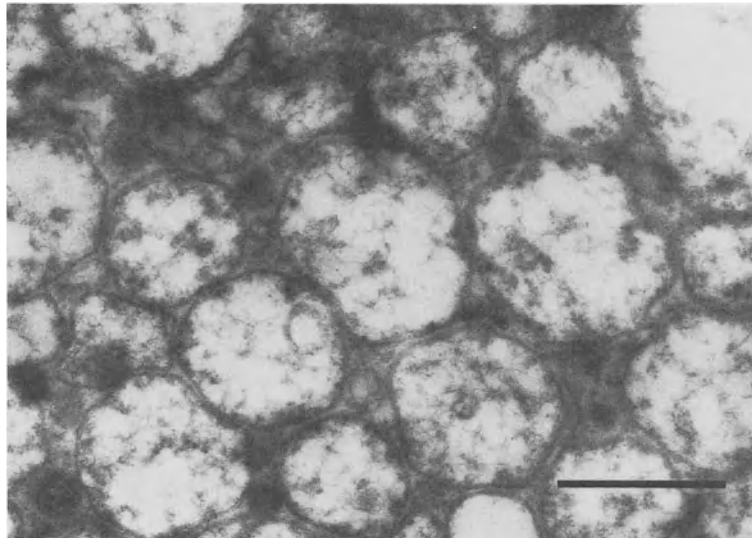

Fig. 2. Electron micrograph of X-MLOs in the brain of an infected *Collandonus montanus* leafhopper. Note absence of a cell wall. Bar = 0.5 μm.

been rigorously established by serial thin sections.

MLOs are susceptible to antibiotics, such as tetracyclines, that inhibit prokaryotic metabolic functions. Because MLOs are wall-less, they are resistant to antibiotics such as penicillin that block cell wall synthesis (Ishiie et al., 1967; Davis et al., 1968). When MLO-infected plants are injected with tetracyline antibiotics, or given foliar application or root drenches of this antibiotic there is a temporary remission of plant disease symptoms and a concurrent reduction in the number of MLOs within the plant. Tetracycline antibiotics have thus served as a basis of therapy and prophylactic treatment for some tree crops, such as coconuts (McCoy et al., 1976), pears (Nyland and Moller, 1973), peaches, and cherries (Nyland, 1971; Rosenberger and Jones, 1977). Unfortunately, the therapeutic benefits of tetracycline are only temporary and symptoms recur unless plants are regularly treated.

MLOs systemically infect plants by passing through phloem sieve plate pores. Their small size and lack of a rigid cell wall allow MLOs, like culturable mycoplasmas, to pass through filters (0.45 μm) that normally exclude most bacteria. The ability of MLOs to pass through filters has also been exploited for separating MLOs from host contaminants (Kirkpatrick et al., 1987; Davis et al., 1988). In addition, extracts containing infectious MLOs are usually filtered prior to injection into healthy insect vectors in order to exclude potentially deleterious bacteria (Gold and Sylvester, 1982; Markham, 1982b).

Habitats

Plant Pathogenic MLOs

Plant pathogenic MLOs are obligate, intracellular parasites that are primarily restricted to phloem sieve tubes. Although there were several early reports of MLOs residing within phloem parenchyma and cell types other than phloem, McCoy (1979) dismissed these as either misidentifications of immature phloem as parenchyma or misidentifications of plant vesicles as MLOs. However, the hypothesis that MLOs reside only within phloem sieve elements remains controversial. For example, two recent reports provide substantial evidence that MLOs can be found, at least in some plants, in cells other than phloem sieve elements. Siller et al. (1987) provided very thorough and convincing histological evidence that MLOs can be found in the parenchyma cells of a dodder species, *Cuscuta odorata.* Their numerous micrographs clearly documented the presence of MLOs in thin-walled, nucleated cells that contained mitochondria, dictyosomes and other plant organelles not normally found in mature sieve tubes. Sears and Klomparens (1989) examined MLO-infected *Oenothera* growing as differentiated plantlets in tissue culture and described MLOs in nucleated cells that contained plastids and mitochondria. Based on the presence of these organelles and other morphological criteria, they classified these MLO-infected cells as phloem parenchyma. Although it should be noted that neither the MLO-infected *Cuscuta* nor the tissue-cultured *Oenothera* represent "typical" MLO plant hosts, evidence presented

in these and other reports (Esau et al., 1976) suggests that MLOs are not restricted only to phloem tissues in all infected plants. One major question raised by these reports is how the MLOs were able to enter the parenchyma cells because parenchyma plasmodesmata are thought to be too small (less than 50 nm) to permit the passage of viable MLOs. The dilemma posed by MLO movement through parenchyma plasmodesmata is further supported by observations that culturable mycoplasmas cannot pass through 0.1 μm filters.

MLOs can be classified into three main groups on the basis of plant disease symptoms (Markham, 1982a; Kirkpatrick, 1989): 1) The "decline agents" produce a general stunting, foliar chlorosis, limb or shoot dieback, root necrosis, and a reduction in leaf and flower size; however, flower color and morphology is normal. 2) "Proliferation agents" may produce some of the same symptoms as the decline agents, but this group also causes proliferative growth of leaves and shoots whose internodes are shortened in at least some of their plant hosts. These infected plants have a bunchy growth habit, producing symptoms that have been described as "witches' brooms." 3) Most "virescence agents" also cause leaf chlorosis and leaf, shoot, and flower proliferation. In addition, flower morphology and pigmentation are dramatically altered. Plant tissues that would normally become petals and sepals instead develop into leaf-like structures (phyllody) and normally pigmented flowers become green (virescence).

Symptoms produced by virescence MLOs strongly suggest that these pathogens alter the normal balance of plant growth regulators in infected plants. Several studies have shown altered levels of phytohormone activity in virescence-infected plants (Davey et al., 1981; Gaborjanyi and Sziraki, 1978). However, it is not known whether the MLOs synthesize plant growth regulators, like some other phytopathogenic prokaryotes (Panopolous and Peet, 1985; Morris, 1986) or if the MLOs interfere with the normal balance of endogenous plant hormones. Perhaps the best example of an MLO that appears to produce a plant growth regulator is the beet leafhopper transmitted virescence agent (BLTVA-MLO) (Golino et al., 1987). Several plants that normally require short photoperiods or cold temperatures to induce flowering will flower under noninductive conditions when infected with the BLTVA-MLO. Exogenous applications of gibberellic acid (GA) also induce similar flowering responses in these plants. Inhibitors that block the action of GA prevent the premature flowering of BLTVA-MLO-infected plants, suggesting either that the BLTVA-MLO may produce a compound(s) with GA-like activity or that this pathogen alters normal endogenous GA levels in the plant (Golino et al., 1988).

MLOs appear to systemically colonize plant hosts in one of two ways, depending on the pathogen and possibly the plant species. One colonization pattern is typified by the X-disease MLO (X-MLO), which first multiplies to high cell densities in the roots of experimental plant hosts such as celery *(Apium graveolens)* and periwinkle *(Catharanthus roseus)*. As the roots become necrotic, the numbers of X-MLO cells in the leaves increase and typical foliar symptoms develop. Celery or periwinkle infected with virulent X-MLO isolates usually collapse one or two months after inoculation, at which time virtually nothing remains of the root system (Kirkpatrick, 1986, 1989). Extensive root necrosis is also observed in several other MLO "decline diseases" such as pear decline (Schaper and Seemüller, 1982), apple proliferation (Seemüller et al., 1984a, 1984b), and elm yellows (Braun and Sinclair, 1976). Most of the decline agents of perennial woody plants recolonize the above-ground portions of their hosts from the roots each spring (Seemüller et al., 1984a 1984b). Decline-type MLOs also differentially colonize the above ground portions of a plant host. For example, in the spring the X-MLO initially multiplies to higher densities in the fruit peduncles than in the leaves of sweet cherry *(Prunus avium)* trees (Kirkpatrick, 1986). X-MLO densities are highest in the peduncles when the fruit is fully mature, are low in the leaves until after the fruit ripens and senesces, and they continue to increase in the leaves until late in the fall. The ability of healthy insect vectors to acquire X-MLO from infected cherry trees also correlates with this seasonal change of MLO densities in the leaves (Suslow and Purcell, 1982; Kirkpatrick et al., 1987).

In contrast to the decline agents, the "virescence" MLOs, such as the aster yellows (AY) pathogens, usually cause little root necrosis, and infected plants may live as long as healthy ones. Furthermore, the colonization pattern of periwinkle by the severe strain of western aster yellows MLO (SAY-MLO) was quite different from the X-MLO (Kuske, 1989; Kuske and Kirkpatrick, 1990b). After a SAY-MLO-infected scion was grafted onto a healthy plant, the pathogen colonized and multiplied to high numbers in the immediately adjacent shoot, rather than in the roots. The SAY-MLO continued to preferentially multiply in the shoots, and pathogen numbers remained uniform and comparatively low in the roots.

MLOs Associated with Insect Vectors

Despite numerous attempts, it has not been possible to mechanically infect healthy plants with infectious MLO extracts. Although the reasons for this failure are uncertain, the rapid accumulation of callose and P-protein that occurs when a phloem sieve tube is injured may play a significant role (McCoy, 1979). Because of the cost and logistical difficulties involved with maintaining colonies of healthy and MLO-infectious insect vectors, the development of methods to mechanically inoculate MLOs into plants would greatly facilitate research on this group of recalcitrant plant pathogens.

Although MLOs can be transmitted from infected to healthy plants by scion and root grafts, most natural transmission occurs via phloem-feeding insects, especially leafhoppers (Cicadellidae). A few species of planthoppers (Fulgoridae) and psylla (Psyllidae) also transmit MLOs (Tsai, 1979). The specificity of MLO/vector relationships has been historically important in identifying and differentiating MLOs. However, there are some insect vectors, such as the leafhopper *Fieberiella florii,* that can efficiently transmit several distinctly different types of MLOs (Severin, 1946; Wolfe et al., 1951). The utility of vector transmission characteristics in classifying MLOs is therefore limited.

All MLOs circulate through and multiply in their insect vectors before being transmitted to a healthy plant. The transmission latent period within a vector is typically 2 to 8 weeks (Tsai, 1979; Purcell, 1982). After the MLO is acquired from the phloem of infected plants, it penetrates and multiplies in the vector's gut epthelial cells. The MLO then passes through the gut epithelial cells, enters the insect's hemocoel, and presumably multiplies in the hemolymph. Depending on the particular MLO, it may also invade adipose tissues, nerve ganglia, the brain, and other organs of the insect vector (Fig. 2) (Whitcomb et al., 1967, 1968a, 1968c; Nasu et al., 1970; Sinha and Chiykowski, 1967). However, the MLO can be transmitted to a healthy plant only if it can penetrate, multiply in, and exit from the insect's salivary gland. The existence of gut and salivary gland "barriers" has been experimentally substantiated. One can bypass the "gut barrier" and greatly shorten the normal latent period within the vector by injecting MLO-infectious insect extracts into healthy vectors using fine needles (Whitcomb et al., 1966; Markham, 1982b). If infectious MLO extracts are injected into a nonvector, the MLOs can multiply in the nonvector's hemolymph and possibly other organs, but the pathogen will not enter the salivary gland nor will it be transmitted to healthy plants (Purcell et al., 1981). Similarly, some MLOs can multiply in a nonvector's gut cells, but in such cases MLOs are not found in the hemolymph or salivary glands and the insects do not transmit the pathogen to healthy plants (Sinha and Chiykowski, 1967). Thus, there are a number of tissue-specific "barriers" within a potential insect vector that an MLO must cross before it can be transmitted to a plant host. Furthermore, once inoculated into a potential plant host, the MLO may or may not multiply; the factors determining plant host susceptibility are currently unknown.

It has been clearly established that some MLOs are pathogenic to their insect vectors. Jensen (1959) reported that *Colladonus montanus* leafhoppers infected with the peach yellow leafroll strain of X-MLO lived approximately half as long as uninfected leafhoppers. Light microscopy revealed numerous pathological lesions in the brain, thoracic ganglia, salivary gland, and adipose tissues of X-MLO-infectious *C. montanus* leafhoppers (Whitcomb et al., 1967, 1968a, 1968c). Electron microscopic examination of infectious *C. montanus* leafhoppers showed that these same tissues contained large numbers of X-MLOs (Nasu et al., 1970), thus suggesting that MLO-induced pathologies were responsible for increased vector mortality. Jensen (1971) also established that X-MLO-infected *C. montanus* produced fewer offspring than did healthy leafhoppers. Similar increases in mortality were reported for six leafhopper species which transmit the maize bushy stunt MLO (MBS-MLO) (Madden and Nault, 1983; Nault et al., 1984), although no deleterious effects were observed on two other MBS-MLO vector species. In summary, it appears that some decline agents (X-MLO) and proliferation agents (MBS-MLO) negatively affect their insect vectors. Interestingly, there does not appear to be any significant deleterious effect of the AY-MLO, a virescence agent, on the longevity or fecundity of its insect vectors (Severin, 1946); however, some cytological abnormalities were observed in AY-MLO-infected *Macrosteles severini* (Hemmati and McLean, 1980; Hemmati, 1977).

At least two plant pathogenic mycoplasmas can change the host plant preference of a nonvector leafhopper. *Dalbulus maidis,* a leafhopper that can transmit both the MBS-MLO and *Spiroplasma kunkelii,* will normally feed and reproduce only on maize *(Zea mays)* and closely related species such as teosinte *(Zea mays mexicana)* (Nault, 1985). When experimentally confined to other plant hosts the leafhoppers quickly die. However, *D. maidis* placed on any of several plant species infected with the AY-

MLO (Maramorosch, 1958; Purcell, 1988) or *Spiroplasma citri* (Purcell, 1988) subsequently live for prolonged periods on nonhost plants. *D. maidis* never transmitted either AY-MLO or *S. citri* to plants.

Nonphytopathogenic MLOs in Insects

Nonculturable, walled and wall-less prokaryotes have been observed in many insect orders that feed on plant vascular fluids, especially the Homopterans, several families of which transmit plant pathogenic mycoplasmas (Buchner, 1965; Houk and Griffiths, 1980). Extensive electron microscopic examination of *Colladonus montanus,* a vector of X- and AY-MLOs, and *Macrosteles severini,* a vector of the AY-MLO, documented the presence of wall-less prokaryotes in these healthy leafhoppers (Fig. 3) Nasu et al. (1970) reported that the extracellular MLOs were normally associated with the midgut epithelial cells of healthy *C. montanus.* However, if an extract containing these wall-less symbionts from healthy *C. montanus* was injected into healthy *C. montanus* leafhoppers, the gut-associated symbionts rapidly multiplied to high cell densities in the fat bodies, malpighian tubules, and even the salivary glands of injected leafhoppers. But, the gut-associated organisms were apparently never transmitted to plants. Similar nonculturable mycoplasmas were observed in the midgut epithelial cells of *M. severini* (Hemmati, 1977). Unlike some of the plant pathogenic MLOs, the wall-less organisms associated with *M. severini* did not appear to produce pathology in the cells that they colonized.

Given the prevalence of obligate insect symbionts and the intimate relationship the plant pathogenic MLOs have with their insect vectors, it may be possible that plant pathogenic MLOs evolved from an insect symbiont(s) or a common progenitor mollicute. The ability to isolate and characterize evolutionarily conserved markers, such as the 16S rRNA, may provide direct genetic evidence concerning the possible relationship between insect symbionts and plant pathogenic mollicutes.

Mollicutes Isolated from Plant Surfaces

A number of culturable, nonhelical and helical mollicutes have been isolated from plant surfaces, especially flowers (reviewed by Tully, 1989; Hackett and Clark, 1989; see also Chapter 89). Because no organisms were isolated from flowers caged to exclude insects, it is assumed that these mollicutes were deposited in the floral nectaries by visiting insects. Whether these wall-less prokaryotes are truly epiphytes is uncertain because it has not been established that they multiply on plant surfaces.

A spiroplasma, later designated *Spiroplasma floricola* (Davis et al., 1981), and some nonhelical mycoplasmas were isolated from non-surface-sterilized flowers of the tulip tree, *Liriodendron tulipifera* (Davis, 1978). Although the nonhelical isolates obtained in this study were not further characterized, other workers have isolated both mycoplasmas and acholeplasmas from insect-visited plants. A serologically unique acholeplasma, *Acholeplasma florum,* was isolated from the surface of several tropical flowers growing in South Florida (McCoy et al., 1984). Several sterol-requiring mycoplasmas, which were serologically unrelated to the 80 recognized *Mycoplasma* species, were isolated from *Melaleuca quinquenervia* flowers. One of the isolates was characterized, and it has been proposed that this organism be named *Mycoplasma melaleucae* (Tully et al., 1990). Even though their A·T rich genome should be sus-

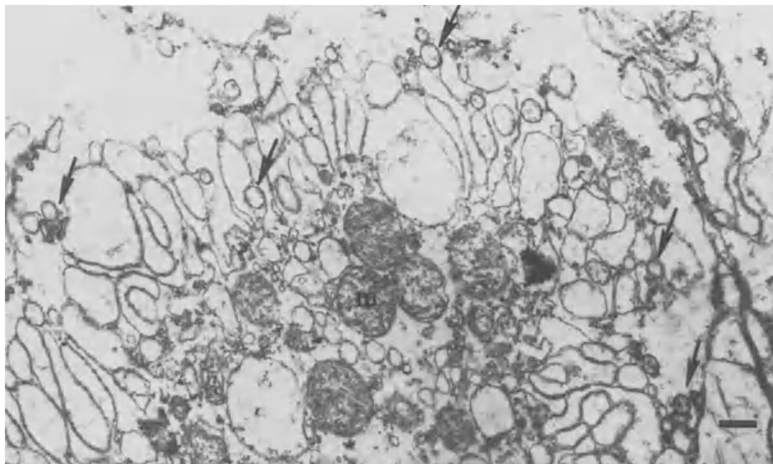

Fig. 3. Wall-less prokaryotes (arrows) associated with a midgut cell in a healthy *Macrosteles severini* leafhopper. m, mitochondrion. Bar = 0.5 μm. (Courtesy of K. Hemmati).

ceptible to damage by ultraviolet-induced thymine dimerization, it appears that some *Mycoplasma* species can survive on leaf surfaces. For example, a serologically distinct organism, whose proposed name is *M. lactucae,* was isolated from nonsterilized lettuce leaves (Somerson et al., 1982; Rose et al., 1990). These studies suggest that a number of other mycoplasmas will likely be isolated from plant surfaces that are visited by insects or other animals. Future studies directed towards understanding the effects of these organisms on the microbial ecology of the phyllosphere and the relationship they have with their insect hosts should be most interesting.

Cultivation of MLOs

As previously stated, there have been no confirmed reports of continuous in vitro cultivation of nonhelical, plant pathogenic mollicutes. Some success has been achieved in maintaining the infectivity of insect-derived MLO extracts for several days (Nasu et al., 1974b; Smith et al., 1981). The infectivity of X-MLOs residing within dissected salivary glands was maintained for 120 days in insect tissue culture medium; however, there was no evidence that the MLOs within the cultured salivary glands multiplied (Sugiura et al. 1984).

Several very fastidious spiroplasmas were recently cultured in vitro by growing the spiroplasmas in media conditioned by monolayers of cultured insect cells (Hackett and Lynn, 1985; Hackett et al., 1986). Although similar attempts to infect monolayers of cultured leafhopper epithelial cells with the X-MLO were unsuccessful (Richardson and Jensen, 1971), the particular system used to culture the fastidious spiroplasmas has not yet been applied to MLO culture.

Some of the virescence MLOs have been successfully maintained for long periods of time in plantlets but not undifferentiated callus grown in tissue culture. Jacoli (1974, 1978) maintained an isolate of eastern aster yellows MLO (EAY-MLO) in carrot explants growing in plant tissue medium containing kinetin and auxin. In contrast to the numerous roots produced by healthy carrot tissues, the EAY-MLO-infected explants produced numerous shoots, but no roots. Similarly, Sears et al. (1989) were able to maintain AY-MLO-infected *Oenothera* explants in plant tissue culture. Like the MLO-infected carrots, these explants also produced numerous shoots but no roots. In both cases the MLOs grew to very high cell densities, offering a uniform and continuous supply of MLO-infected tissues which can be easily manipulated in the laboratory.

Microscopic Identification of MLOs

Electron microscopy (EM) provided the first evidence that plant yellows diseases were caused by wall-less prokaryotes rather than by viruses (Doi et al., 1967). Transmission EM is still a valuable tool for confirming the presence of MLOs in a particular plant or insect host. The reader is referred to several excellent reviews concerning the specifics of sample preparation and potential interpretation problems inherent in EM studies (Waters, 1982; Markham, 1988; Chen et al., 1989; Norris and McCoy, 1983). Because MLOs are pleomorphic organisms, neither electron nor light microscopy can be directly used to morphologically distinguish one MLO from another (McCoy, 1979). However, EM may be useful for differentiating MLOs from spiroplasmas, if the latter organisms exhibit their typical helical morphology in the tissues being examined.

Several staining procedures have been used to visualize MLOs by light microscopy (reviewed by Waters, 1982). Perhaps the most widely used light microscopic technique involves staining MLO DNA with DAPI (4′-6-diamidino-2-phenylindole), a DNA-binding fluorochrome, and examining stained tissues with an epifluorescence microscope. Although this method is considerably faster than examining tissues by EM, it requires some experience to differentiate the fluorescence of DNA-containing organelles such as mitochondria from MLOs. DAPI visualization of MLOs is most efficacious for examining high titer herbaceous plant hosts; however, if the appropriate controls are used, this technique can also be used to detect MLOs in woody plants (Seemüller, 1976; Douglas, 1986; Schaper and Converse, 1985), in which they are usually present in low numbers.

Purification of MLOs from Infected Hosts

Considerable progress has been made recently in the development of protocols to purify MLOs from infected plant and insect hosts (reviewed by Kirkpatrick, 1989). Although MLO-enriched fractions can now be routinely obtained from both infected plants and insects, much of the early work on purifying MLOs was done with infected insects. Infected insects, rather than plants, were used in these early studies because the MLO numbers were much higher in insects than in plants. The efficacy of a particular purification protocol was bioassayed by injecting

the preparation into healthy vectors and observing whether the injected insects transmitted the pathogen to test plants. Extracts derived from plants were never as infections as extracts derived from insects, possibly because of lower pathogen numbers and inhibitors present in plant extracts (Liao and Chen, 1980). Early infectivity assays helped establish the optimum buffers, pH, and osmolarity that best maintained MLO infectivity (Lee and Chiykowski, 1963; Whitcomb et al., 1968b; Smith et al., 1981). Viability of the pathogen was achieved using buffers between pH 6.5 to 8.0 containing divalent cations and glycine, and whose osmolarity was 300 to 900 mOsmol.

Most MLO purification protocols are very similar to those that are used to isolate mitochondria from plant or insect hosts (reviewed by Kirkpatrick, 1989). In such procedures, the tissue is disrupted and the MLOs are separated from host debris and large organelles by differential centrifugation. MLOs are then pelleted by centrifugation at approximately 20,000 × *g* for 20 minutes. In some cases these simple extracts have been used as immunogens for the production of MLO-specific polyclonal or monoclonal antibodies (Caudwell et al., 1982; Clark et al., 1983, 1989). However, polyclonal antisera produced against partially purified MLOs usually requires extensive cross-absorption with healthy plant extracts to reduce cross reactions with plant antigens. In other protocols, MLO-enriched extracts were further purified by repeated differential centrifugation or fractionation on sucrose (Whitcomb et al., 1968b; Nasu et al., 1974a) or Percoll[R] density gradients (Jiang and Chen, 1987; Davis et al., 1988). Other modifications of this basic isolation technique include the use of plant-cell-wall degrading enzymes to enhance the recovery of intact MLOs (Lee and Davis, 1983, 1988) and the removal of host contaminants from MLO-enriched extracts using antibodies directed against healthy host antigens (Kirkpatrick, 1986 and unpublished observations). High quality MLO preparations were also obtained by affinity chromatography using MLO-specific monoclonal antibodies (Jiang et al., 1988). However, even MLOs purified by affinity chromatography still contain some residual host contaminants which may or may not interfere with MLO-specific analyses.

Serological Detection and Antigenic Properties of MLOs

Historically, the taxonomy of mycoplasmas has relied heavily on the serological differentiation of these cultivable but morphologically indistinguishable organisms. It is now clear that like the classical mycoplasmas, MLOs can also be differentiated on the basis of their antigenic properties. The results of the following MLO serological studies are summarized in Table 1.

Several polyclonal (Pab) and monoclonal (Mab) antisera have been prepared against plant pathogenic MLOs isolated from plants. Sinha and coworkers used an elaborate purification procedure to obtain immunogens from plants infected with Canadian asters yellows (CAY), Canadian clover phyllody (CCP) and Canadian isolates of eastern X-disease (EX) MLOs (Sinha, 1974, 1979; Sinha and Chyikowski, 1984). Pabs produced against these immunogens were used in enzyme-linked immunosorbent assays (ELISA) and immunospecific electron microscopy to detect these MLOs in plants. In their studies, the CAY-MLO and the CCP-MLO were serologically indistinguishable and neither was serologically related to the EX-MLO (Sinha and Benhamou, 1983). In contrast, Clark et al. (1983) produced Pab against European clover phyllody (ECP) MLO and reported no cross reactivity between this antiserum and an isolate of European AY (EuAY-MLO). Because very high ELISA readings were obtained when strawberry plants infected with green petal disease were tested using the ECP-MLO antiserum, these two diseases are probably caused by similar, if not identical, MLOs. The apparent antigenic similarity between these two MLOs also agrees with previous insect vector transmission studies (Frazier and Posnette, 1957). Hobbs et al. (1987) produced Pab against the peanut witches' broom (PWB) MLO using an extraction protocol very similar to Clark's. No serological cross reactions were observed with the other MLOs that were examined. Similar procedures were used to prepare immunogens and Pab against four virescence MLOs (eastern and western AY, potato witches' broom and clover phyllody) (da Rocha et al., 1986). When used in an indirect immunofluorescence labelling procedure, all four antisera detected MLOs in infected periwinkle tissues and all of the antisera cross-reacted with all four MLOs.

Caudwell et al. (1982) were the first to produce MLO-specific Pab using leafhopper-derived immunogens. MLO-specific antibodies were obtained when extracts of *Euscelidius variegatus* leafhoppers infected with the MLO that causes grapevine flavescence doree (FD-MLO) were injected into rabbits. FD-MLO-specific antibodies were also produced against FD-MLO immunogens prepared from infected *Vicia faba* plants. Antisera produced against the leafhopper immunogen were most effective in detecting the pathogen in plants, and antibodies

Table 1. Serological relationships of MLOs.

MLO-specific antibody	Organisms tested											Reference
	WX	EX	WB	EY	WAY	BLTVA	*S.c.*	*S.k.*				
WX (Pab)	+	+	+	$+^{w}$	–	–	–	–				Kirkpatrick et al., 1984, 1988b
	CAY	CCP	EX	CYE	*S.c.*							
CAY (Pab)	+	+	–	–	–							Sinha and Benhamou, 1983
	BLTVA	IV	WAY	WX	*S.c.*							
BLTVA (Pab)	+	+	–	–	–							Golino et al., 1989
	EX	CAY	CCP	CYE								
EX (Pab)	+	–	–	–								Sinha and Chiykowski, 1984
	ECP	SGP	EuAY	*S.c.*								
ECP (Pab)	+	+	–	–								Clark et al., 1983
	PeWB	ELL	VWB	DWB								
PeWB (Pab)	+	–	–	–								Hobbs et al., 1987
	EX	WX	EY	CAY	CCP	WAY	LP	MBS	PaWB	*S.c.*	*S.k.*	
Ex (Mab)	+	+	–	–	–	–	–	–	–	–	–	Jiang et al., 1989
	EAY1	WAY	AsY	EY	PaWB	PWB	PR	MBS	LWB			
EAY1 (Mab)	+	–	–	–	–	–	–	–	–			Lin and Chen, 1985
	MBS	EAY1	CCP	EY	PaWB	PWB	EX	*S.c.*	*S.k.*			
MBS (Mab)	+	–	–	–	–	–	–	–	–			Chen and Jiang, 1988
	S	EuAY	ECP	CC	AP	WBL	*S.c.*	*S.k.*				
S (Mab)	+	–	–	–	–	–	–	–				Garnier et al., 1990
	EuAY	PY	ECP	ABB	*S.c.*							
EuAY (Mab)	+	+	–	–	–							Clark et al., 1989

Pab, polyclonal antibody; Mab, monoclonal antibody; +, positive reaction in enzyme-linked immunosorbent assay (ELISA); $+^{w}$, weakly positive by ELISA; –, no reaction by ELISA; WX, western X-disease; EX, eastern X-disease; WB, walnut bunch; EY, elm yellows; WAY, western aster yellows; BLTVA, beet leafhopper-transmitted virescence agent; *S.c., Spiroplasma citri; S.k., Spiroplasma kunkelii;* CAY, Canadian aster yellows; CCP, Canadian clover phyllody; CYE, clover yellow edge; IV, Illinois virescence; ECP, European clover phyllody; SGP, strawberry green petal; EuAY, European aster yellows; PeWB, peanut witches' broom (India); ELL, eggplant little leaf (India); VWB, vinca witches' broom (India); DWB, *Datura* witches' broom; LP, little peach (S. Carolina); MBS, maize bushy stunt; PaWB, Paulowina witches' broom; EAY1, eastern aster yellows (New Jersey); AsY, ash yellows; PWB, potato witches' broom; PR, peanut rosette; LWB, loofah witches' broom; S, stolbur (Europe); CC, cabbage chloranty; AP, apple proliferation; WBL, witches' broom of lime; PY, Primula yellows; ABB, Australian tomato big bud.

against the plant-derived immunogen were most useful for detecting FD-MLO in leafhoppers. Antigenic relationships between the FD-MLO and other MLOs were not reported. Specific Pab were also obtained from rabbits injected with immunogens prepared from western X (WX) MLO-infected *C. montanus* leafhoppers (Kirkpatrick and Garrott, 1984). No antigenic relationship was observed between the WX- and the AY- or BLTVA-MLOs, *S. citri,* or *S. kunkelii.* However, strong antigenic similarity was found between the western and eastern strains of X-disease and the walnut bunch (WB) MLO. Weaker, but positive, ELISA readings were obtained with periwinkle plants infected with the elm yellows (EY) pathogen. Western blot analyses of infected plants showed that the major WX-MLO antigen was a 29 kDa protein which was also present in the WB- and EY-MLOs (Kirkpatrick et al., 1988b). Immunogens derived from BLTVA-MLO-infected *Circulifer tenellus* leafhoppers were used to elicit Pab against this MLO. No antigenic cross reactions were obtained between the BLTVA- and the WX- or AY-MLOs or *S. citri* (Golino et al., 1989).

Although MLO-specific Pab are comparatively easy to prepare and can potentially recognize several MLO antigens, most have relatively high background reactions with healthy host antigens. Healthy host background reactions are virtually eliminated in ELISA and immunofluorescence analyses using MLO-specific monoclonal antibodies (Mabs). Lin and Chen (1985) were the first to produce Mabs against MLOs. Mabs produced against a New Jersey strain of eastern aster yellows (EAYI-MLO) provided sensitive and specific detection of this pathogen in plants. These Mabs did not react with antigens in seven other MLO-infected plants or the two plant pathogenic spiroplasmas that were tested. Unexpectedly, EAYI-MLO Mabs did not react with other geographic isolates of AY-MLOs. These results were the first to suggest that although Mabs provide very sensitive and specific detection of a particular MLO isolate, they may be of limited utility for defining larger taxonomic groups of MLOs.

Mabs produced against the maize bushy stunt MLO (MBS-MLO) also provided sensitive detection of that MLO in infected maize by ELISA and immunofluorescence. No reactions were obtained between MBS-MLO Mabs and seven other MLOs, *S. citri,* or *S. kunkelii* (Chen and Jiang, 1988). Mabs have also been prepared against the eastern (Jiang et al., 1989) and the western (M. F. Clark, personal communication) X-disease MLOs. EX-MLO Mabs reacted with both EX- and WX-MLO antigens but no reactions occurred with plants infected with several virescence MLOs or plant pathogenic spiroplasma species. In extensive comparisons between WX-MLO Pabs and Clark's WX-MLO Mabs, both serological reagents tested positively with the same field and laboratory samples. Healthy host background reactions of the Mabs were lower than those obtained with Pabs (B. C. Kirkpatrick and M. F. Clark, unpublished observations). Pabs were produced against European aster yellows (EuAY-MLO) and Mabs were produced against the virescence MLO causing primula yellows (PY-MLO) (Clark et al., 1989). Strong heterologous ELISA reactions were obtained between PY- and EuAY-MLO antisera, suggesting that these two MLOs are antigenically similar. No reaction was obtained when either EuAY- or PY-MLO antisera were tested against European clover phyllody (ECP) MLO-infected plants, results that confirmed earlier observations (Clark et al, 1983). Similar results were obtained by Garnier et al. (1990) who produced Mabs against another ECP-MLO isolate and the stolbur MLO (S-MLO). No antigenic cross reactions were obtained when ECP-MLO Mabs were tested against another isolate of EuAY-MLO, providing additional evidence that the ECP- and EuAY-MLO are antigenically distinct. S-MLO Mabs detected this pathogen in naturally infected tomato plants, whether or not the plants showed virescence symptoms. S-MLO Mabs were highly specific for this pathogen; no cross reactions were observed with plants infected with five other MLOs or with *S. citri* or *S. kunkelii.*

In addition to characterizing the antigenic properties of MLOs these serological diagnostic reagents have been valuable tools in identifying alternate plant hosts and insect vectors of these MLOs (B. C. Kirkpatrick, unpublished observations).

Genetic Characterization of MLOs

Isolation and Composition of MLO DNA

Although antigenic properties have been the primary basis for differentiating species of mollicutes, DNA-DNA and DNA-rRNA hybridization analyses are also used to establish taxonomic relationships among other prokaryotes. Until recently it was not possible to obtain MLO DNA that was free of host nucleic acids so that direct comparisons between MLO genomes using DNA-DNA hybridization analyses were not possible. Recently, two groups have reported the purification of MLO DNA by

equilibrium centrifugation in cesium chloride/bisbenzimide gradients (Sears et al., 1989; Kollar and Seemüller, 1989; Kollar et al., 1990). Bisbenzimide preferentially binds to A·T rich DNA and, depending upon the base composition of a particular plant host, it can be used to separate MLO DNA from plant DNA. A unique band of A·T rich DNA was isolated from tissue-cultured *Oenothera* explants infected with a virescence MLO (O-MLO) (Sears et al., 1989). No hybridization occurred between this DNA and chloroplast- or mitochondria-specific hybridization probes, suggesting that this DNA band was primarily MLO DNA. The GC content of the O-MLO DNA was 29.5 mol%, a value similar to that of most culturable mycoplasmas (Stanbridge, 1979; Bové, 1984; Razin, 1985). In another study, repeated equilibrium centrifugations in cesium chloride/bisbenzimide gradients were used to purify five MLO DNAs from infected plants (Kollar and Seemüller, 1989). The GC contents of the MLO DNAs ranged from 23.0 mol% for an isolate of EuAY-MLO to 26.2 mol% for an MLO causing virescence in *Diplotaxis.* Although the presence of contaminating host DNA in these preparations was not determined, several MLO-specific fragments were cloned from one of these preparations (Kollar et al., 1990). As yet there have been no reports on the homology between various MLOs using these gradient-purified DNAs.

Taxonomic Relationships Determined by Cloned MLO DNA

Cloned fragments of the MLO genome have also provided considerable insight into grouping and differentiating MLOs. The results of comparative hybridization studies using cloned, MLO-specific DNA probes is discussed below and summarized in Table 2.

Twenty-four unique, cloned fragments of the WX-MLO chromosome were identified on the basis of hybridization with ^{32}P-labelled DNA from X-diseased but not healthy plants and insects (Kirkpatrick et al., 1987). These WX-MLO-specific fragments were used as hybridization probes to investigate the genetic relatedness of the WX-MLO with other MLOs and culturable mollicutes. No hybridization occurred between any of the WX-MLO cloned probes and DNAs extracted from seven *Mycoplasma* species or 16 strains of plant pathogenic and other spiroplasmas. In contrast, most of the WX-MLO cloned probes hybridized with DNAs extracted from plants infected with several decline agents, such as eastern X-disease, walnut and pecan bunch, and peach yellows MLOs. Only two of the WX-MLO genomic probes hybridized with DNA from several strains of western aster yellows MLO (Kirkpatrick et al., 1988b, 1990). In similar studies, several chromosomal fragments of the apple proliferation MLO (AP-MLO), a European tree decline agent, were recently cloned and characterized (Bonnet et al., 1990). Two of the AP-MLO clones that were characterized hybridized with the decline MLOs that cause apricot chlorotic leafroll and "dormancy breaking disease" of plum. Neither AP-MLO DNA probe hybridized with DNA from plants infected with EuAY- or S-MLO. These results indicate that the X-MLO and several other decline type MLOs form a cluster of genetically related organisms which are distinct from the virescence agents. The decline MLOs are even less closely related to culturable *Mycoplasma* and *Spiroplasma* spp. than they are to the virescence MLOs.

Similar hybridization analyses, using cloned fragments of an eastern strain of aster yellows (EAY-MLO), established the existence of a cluster of genetically related virescence agents (Lee and Davis, 1988). Although hybridization occurred between cloned fragments of the EAY-MLO and DNA from some decline MLOs under low stringency conditions, ^{32}P-labelled EAY-MLO RNA probes hybridized only with three other virescence MLOs under more stringent hybridization and wash conditions. Similar results were obtained using two cloned chromosomal fragments from the severe strain of western aster yellows (SAY-MLO) (Kuske et al., 1991a). Positive hybridization was obtained with DNA from 14 virescence isolates from North and South America and Europe; no hybridization occurred with eight decline agents from North America, Europe, and Thailand. In addition, it was possible to subdivide those virescence isolates that hybridized with the SAY-MLO chromosomal probes on the basis of similarities in restriction fragment length polymorphisms (RFLPs). Hybridization analyses also established that some virescence agents are genetically distinct from the "typical" AY-MLO strains that are transmitted by *Macrosteles severini.* For example, no hybridization occurred between cloned SAY-MLO probes and DNA from Canadian isolates of aster yellows, clover proliferation or potato witches' broom, nor DNA from sesame phyllody MLO-infected plants from Thailand (Kuske et al., 1991a). Neither the EAY- (Lee and Davis, 1988) nor the SAY-MLO (Kuske et al., 1991a) DNA probes hybridized with DNA from plants infected with the beet leafhopper transmitted virescence agent (BLTVA), a virescence MLO that is transmitted by *Circulifer tenellus,* a leafhopper that does not transmit the AY-MLO.

Table 2. Genetic relationships of MLOs.[a]

Origin of MLO DNA probes	DNAs tested																Reference
	EX	WB	PB	PY	EY	PR	WAY	O	BLTVA	AP	LY	WL	MBS	*S.c*	*S.k.*	*Myco*[b]	
WX	++	++	++	++	+	+	+	–	–	–	–	–	–	–	–	–	Kirkpatrick et al., 1988b
	ACL	PDB	EuAY	ECP	S	C	WX	*S.c.*									
AP	++	++	–	–	–	–	–	–									Bonnet et al., 1990
	EAY1	O	DV	RV	SY	BLTVA	CAY	CCPr	PWB	WX	EY	AP	LY	MBS	*S.c.*	*S.k.*	
WAY	++	++	++	++	++	–	–	–	–	–	–	–	–	–	–	–	Kuske et al., 1991a.
	WAY	BB	WX	EX	EY	BLTVA	*S.c.*										
EAY2[c]	++	++	–	–	–	–	–										Lee and Davis, 1988
	EAY3	WX	EX	*S.c.*													
O	++	–	–	–													Sears et al., 1989

+, At least one, but not most MLO probes hybridized; ++, Most or all MLO probes hybridized; –, No hybridization occurred; WX, western X-disease; EX, eastern X-disease; WB, walnut bunch; PB, pecan bunch; PY, peach yellows; PR, peach rosette; EY, elm yellows; WAY, western aster yellows; O, *Oenothera* MLO; BLTVA, beet leafhopper-transmitted virescence agent; AP, apple proliferation; LY, coconut lethal yellows; WL, sugarcane white leaf; MBS, maize bushy stunt; *S.c., Spiroplasma citri; S.k., Spiroplasma kunkelii; Myco,* 7 *Mycoplasma* sp.; ACL, apricot chlorotic leafroll; PDB, plum dormancy breaking; EuAY, European aster yellows; ECP, European clover phyllody; S, stolbur; C, chloranty; EAY1, eastern aster yellows from New Jersey; DV, *Diplotaxis* virescence (Spain); RV, rape virescence (France); SY, stolbur (Yugoslavia); CAY, Canadian aster yellows; CCPr, Canadian clover proliferation; PWB, Canadian potato witches' broom; EAY2, eastern aster yellows from Maryland; BB, tomato big bud from Arkansas; EAY3, eastern aster yellows from Wisconsin.

[a]As determined by hybridization analyses using cloned fragments of the MLO chromosome as probes.

[b]Seven *Mycoplasma* species were tested.

[c]Results of RNA riboprobes.

Extrachromosomal MLO DNA

Extrachromosomal DNA has been found in many of the MLOs that cause virescence in herbaceous hosts. Davis et al. (1988) were the first to clone MLO extrachromosomal DNA from a Florida isolate of the maize bushy stunt MLO (MBS-MLO). Four MBS-MLO-specific recombinant clones hybridized with several low-molecular-weight, extrachromosomal DNAs in MBS-MLO-infected corn and *D. maidis* leafhoppers. Unexpectedly, no hybridization occurred between any of the four extrachromosomal DNA probes and DNA from a Texas isolate of MBS-MLO, indicating the Texas isolate had no extrachromosomal DNA. Nevertheless, hybridization occurred between Florida MBS-MLO chromosomal DNA probes and chromosomal DNA of the Texas isolate. Symptoms produced by the Texas MBS-MLO were very similar to symptoms produced by the Florida isolates, so the function of plasmid-encoded genes in the MBS-MLO, as well as all of the other MLO plasmids identified thus far, are unknown.

Supercoiled, extrachromosomal DNAs present in three strains of western aster yellows were isolated on cesium chloride/ethidium bromide gradients (Kuske and Kirkpatrick, 1990a; Kuske, 1989). Although virus-like particles (VLPs) have been occasionally associated with some MLOs (Allen, 1972; Giannotti et al., 1973; Gourret et al., 1973), no VLPs were observed in these strains of western aster yellows infected plants (Hemmati and McLean, 1980; B. C. Kirkpatrick, unpublished observations). For this reason, these extrachromosomal DNAs are most likely MLO plasmids and not DNAs from encapsidated viruses. Four plasmids, ranging in size from 7.4 to 1.7 kbp, were isolated from the severe strain of western aster yellows (SAY-MLO). Similar, but not identical, plasmids were found in the dwarf and Tulelake strains (Freitag, 1964) of western aster yellows. The native SAY-MLO plasmids were labelled and used as probes in Southern blots of decline and virescence MLO DNA. Most of the virescence agents that were examined contained plasmid DNAs which shared some homology with SAY-MLO plasmids (Kuske et al., 1991b).

Unexpectedly, SAY-MLO plasmid DNA hybridized with MBS-MLO extra-chromosomal DNA. In reciprocal experiments, a recombinant plasmid containing a fragment of MBS-MLO extrachromosomal DNA hybridized with all of the native western AY-MLO plasmids (Kuske et al., 1988, 1991b). These results are significant because both the plant host range and the insect vectors of the AY- and MBS-MLO are presently mutually exclusive (Nault, 1980, 1985), which suggests these ecologically distinct MLOs either evolved from similar ancestors or that they occupied common habitats, or niches, at some time in the past. Both native and recombinant SAY-MLO plasmids hybridized with numerous virescence isolates from North America and Europe; however, the number and size of the plasmids in these virescence agents varied considerably (Kuske et al., 1991).

Although no hybridization occurred between SAY-MLO plasmids and DNA isolated from BLTVA-MLO-infected plants, we have identified and cloned portions of several plasmids that are present in the BLTVA-MLO (Shaw and Kirkpatrick, 1990). Thus it appears there are at least two major subgroups of the virescence agents whose chromosomal and extrachromosmal DNAs, as well as their antigenic properties, are distinct.

No extrachromosomal DNAs have been found in any of the tree decline agents examined thus far either by Southern blot analysis using AY-MLO plasmids as probe or MLO DNA electrophoresced in ethidium-stained gels (Kuske, 1989). These data provide additional evidence that the virescence and decline MLOs represent distinctly different groups of pathogens.

The identification of plasmid DNA in the virescence MLOs may provide a means for introducing foreign DNA into MLOs. It is theoretically possible to identify specific MLO genes by transposon mutagenesis using engineered MLO plasmids. For example, a Gram-positive transposon, Tn*916*, has been introduced into *Mycoplasma* and *Acholeplasma* species (Dybvig and Cassell, 1987; Dybvig and Alderete, 1988; Mahairas and Minion, 1989). In addition, it may be possible to characterize plasmid-encoded genes if MLO plasmids could be introduced into a genetically compatible, culturable prokaryote. Phylogenetic analyses suggest that *Acholeplasma* species, and perhaps even *Bacillus subtilis,* might be amenable to transformation with MLO plasmid DNA.

Phylogenetic Relationships Between MLOs and Other Prokaryotes

Nucleotide sequences of evolutionarily conserved genes, such as the 5S and 16S rRNA, have been extensively used to determine the phylogenetic relationships of the class Mollicutes (Woese et al., 1980, 1985; Rogers et al., 1985; Weisberg et al., 1989) and other prokaryotes (Woese, 1987). Because it is not necessary

to culture an organism in order to clone and sequence its rRNA genes, it is possible to determine the phylogenetic relationships of MLOs using this evolutionarily conserved marker. Early comparisons, using 16S rRNA oligonucleotide catalog sequences, indicated the culturable mollicutes were derived from a Gram-positive clostridial ancestor from which *Bacillus* and *Lactobacillus* species also evolved (Woese et al., 1980). Full-length 16S rRNA sequences provide the most comprehensive data for phylogenetic comparisons and more than 50 full-length 16S rRNA sequences of wall-less prokaryotes have now been determined, nearly all of these obtained by Weisburg and colleagues (1989).

Full-length 16S rRNA sequences have been determined for three MLOs to date. Lim et al. (1989) cloned and sequenced the 16S rRNA gene of an MLO (O-MLO) that was obtained from field-infected *Oenothera* plants in Michigan. The O-MLO is thought be a strain of the eastern aster yellows MLO because it can be transmitted by *Macrosteles severini* leafhoppers. However, the exact identity of this MLO is uncertain because symptoms produced by the O-MLO in vector-inoculated test plants are not typical of eastern AY-MLO (Sears and Klomparens, 1989). Full-length sequence comparisons of the O-MLO and other culturable prokaryotes established that the O-MLO was most closely related to *Mycoplasma capricolum* (80% homology), followed by *Bacillus subtilis,* thus establishing that MLOs are evolutionarily related to Gram-positive rather than Gram-negative organisms. Comparisons of the O-MLO 16S sequence with 16S rRNA oligonucleotide catalog sequences of selected mycoplasmas and clostridial bacteria suggested that the O-MLO was more closely related to *Acholeplasma laidlawii* than to other *Mycoplasma* or *Spiroplasma* species.

The full-length 16S rRNA sequence of another virescence agent, the SAY-MLO, has been determined (Kuske and Kirkpatrick, 1989; Kuske, 1989). The sequence of the SAY-MLO 16S structural gene was 99.2% homologous to the O-MLO, indicating that these two virescence agents are very closely related phylogenetically. Analysis of oligonucleotide rRNA catalog sequences also supported the conclusion that the SAY-MLO was closely related to *A. laidlawii.*

The 16S rRNA sequence of a decline type MLO, the WX-MLO, was also recently determined (Kirkpatrick and Fraser, 1989). The sequence of the WX-MLO 16S gene was 78% homologous to both *M. capricolum* and *B. subtilis* and only 72% homologous with *E. coli,* providing evidence that decline MLOs are also evolutionarily related to Gram-positive organisms. The WX-MLO was 89% homologous to both the SAY- and the O-MLOs. Although the 89% homology between the WX-MLO and the two virescence MLOs is higher than WX-MLO homologies with other prokaryotes, this relatively low value indicates that decline and virescence MLOs are distinctly different groups of MLOs, a conclusion supported by the results of serological and DNA hybridization analyses.

The 16S rRNA sequence of the O-, SAY-, and X-MLOs were recently compared with those or other mollicutes and Gram-positive prokaryotes (C. R. Woese, personal communication). The three MLOs were more closely related to each other than to any other prokaryotes. The MLOs formed a phylogenetically distinct cluster within the "anaeroplasma group" which contains all the *Anaeroplasma* species that were examined, as well as *Acholeplasma laidlawii* and *A. modicum* (Weisburg et al., 1989). In addition to finally establishing that MLOs are truly members of the class Mollicutes, this information may also provide valuable clues for those who are attempting to culture these obligate plant pathogens.

Several interesting structural features were also identified in the MLO rRNA operons. A $tRNA^{ile}$ gene was found in the spacer region between 16S and 23S rRNA genes of the X-, SAY- and O-MLOs. However, at least one significant difference was found between the two virescence agents. A $tRNA^{tyr}$ gene was found just upstream from the 16S rRNA structural gene in the SAY-MLO (Kuske, 1989) but not in the O-MLO (P. -O. Lim, personal communication). No tRNAs were found within a 1.2 kb region upstream from the WX-MLO 16S rRNA gene. Two putative rRNA operon promoters were identified upstream from the SAY-MLO 16S rRNA gene. These were similar to rRNA promoters identified in culturable mollicutes and *Bacillus subtilis* (Kuske, 1989). Similarly, two putative promoters, that were very similar to the rRNA operon promoter of *B. subtilis,* were identified upstream from the WX-MLO 16S gene (Kirkpatrick and Fraser, 1988a).

Further evidence that MLOs are related to Gram-positive organisms was provided by the sequence of the RNase III cleavage site in the stem region formed from sequences flanking the SAY-MLO 16S rRNA structural gene. The SAY-MLO cleavage site sequence was highly homologous to culturable *Mycoplasma* species and *B. subtilis* but not to *E. coli.* A Gram-positive RNase III cleavage site whose sequence was nearly identical to the SAY-MLO site, was identified in the WX-MLO.

The MLO rRNA sequences also provided a sensitive method for detecting very low numbers of MLOs using techniques originally developed for culturable mycoplasmas (Göbel et al., 1987). Computer-assisted sequence comparisons of the WX-MLO 16S rRNAs with the 16S rRNA sequences of other prokaryotes and plant organelles identified several oligonucleotide sequences that were MLO-specific. ^{32}P-labelled oligonucleotide probes provided 10 to 20 times more sensitive detection of the X-MLO than similarly labelled fragments of the X-MLO chromosome (Kirkpatrick and Fraser, 1989).

Conclusion

In the short period of time since the first edition of this Handbook was published, there have been several significant advances in our ability to detect and differentiate plant pathogenic MLOs. The development of sensitive, MLO-specific serological and nucleic acid hybridization assays has greatly facilitated both basic and applied studies on plant pathogenic MLOs and the diseases they cause. Genetic analyses using cloned fragments of MLO DNA, together with comparisons of MLO 16S rRNA sequences, have clearly established that the MLOs represent a diverse but distinct taxon of presently nonculturable mollicutes. The ability to isolate and characterize MLO DNA, RNA, and antigenic proteins has obviated, at least to some extent, the requirement to culture these pathogens in order to characterize some of their basic biological properties. Future classification and differentiation of wall-less prokaryotes will be based on serological, genetic, phylogenetic, and other biological data. If the criteria established for naming and classifying wall-less prokaryotes can be revised, more formal taxonomic designations may eventually be proposed for plant pathogenic MLOs.

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The L-Forms of Bacteria

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Introduction

It is clearly established that many or possibly all bacteria can produce L-forms as a result of the complete or partial inhibition of cell wall synthesis. The resultant wall-deficient organisms (L-forms) lack the classical bacterial structure; in culture they manifest a distinctly variant type of independent growth. Under certain conditions, L-forms have the potential to revert to the parent bacterial form.

The modern microbiologist can profit from a knowledge of the bacterial L-forms. Bacteria are exposed to traumatic influences exerted by environmental changes, host-dependent mechanisms, and biologic interdependency with other microorganisms. In this context, evolutionary trends and pathologic conditions may be the result of genetic modifications of bacteria mediated by changes in their cell wall.

In recent years, the L-forms have come to be recognized as invaluable tools in new and innovative areas of microbial research. With the recent advances in molecular biology, the L-forms are being used to study the biosynthesis of bacterial membranes, ribosomal functions, the replication of DNA, plasmid-related effects, mechanisms of antibiotic activities on bacteria and their membranes, and many other aspects of bacterial physiology at the genetic level. The study of these complex and divergent disciplines can be expected to yield valuable information as to the role of L-forms in the biology of bacteria and as to their participation and significance in infectious disease processes. For a recent review, see Madoff (1986).

The discovery of the L-forms over 50 years ago ushered in a new era in the understanding of the bacterial cell. The name L-forms (L for Lister Institute) was first proposed by Klieneberger (1935); she discovered colonies resembling mycoplasma in cultures of *Streptobacillus moniliformis* isolated from the rat, and named them L_1. Dienes (1939) studied the spontaneous development of the L_1 from *Streptobacillus* and its reversion to the bacillary form. Dienes thereby established the bacterial derivation of the L-forms. Within a few years, the spontaneous development of L-form colonies was observed in cultures of *Bacteroides, Haemophilus, Escherichia,* and *Neisseria.* For an early review, see Dienes and Weinberger (1951).

The discovery that L-forms could be induced by exposure of bacteria to penicillin (Pierce, 1942) was an important advance. Other antibiotics were found to produce a similar result by interfering with biosynthesis of cell wall. Exposure of bacteria to bacteriostatic chemicals, to phage, and to antibodies also resulted in L-transformation. In 1954, Sharp recognized that organisms lacking a rigid cell wall needed osmotic protection and he induced L-forms from group A streptococci on media containing high salt concentrations. The current belief is that conversion to L-forms may be a universal property of bacteria provided that suitable conditions for induction and growth can be determined. L-forms have been derived from bacteria of many genera (Hijmans and Clasener, 1971) as shown in Table 1.

Terminology and Biological Characteristics

An L-form is a cell-wall-deficient organism derived or induced from a bacterium following suppression of the synthesis of its rigid cell wall.

Table 1. Some bacterial genera from which L-forms have been derived.

Agrobacterium	*Erysipelothrix*	*Salmonella*
Bacillus	*Escherichia*	*Sarcina*
Bacteroides	*Flavobacterium*	*Serratia*
Bartonella	*Haemophilus*	*Shigella*
Bordetella	*Listeria*	*Staphylococcus*
Brucella	*Neisseria*	*Streptobacillus*
Clostridium	*Proteus*	*Streptococcus*
Corynebacterium	*Pseudomonas*	*Vibrio*

In culture the classical bacterial forms are replaced by soft spherical granular forms and large bodies that penetrate the agar gel and produce colonies that resemble those of the mycoplasma. Other characteristics similar to those of the mycoplasma, such as fragility, pleomorphism, and filterability, may be related to the absence of rigid wall (Dienes, 1968). These are the fundamental characteristics of the L-forms and represent the criteria by which they are recognized. Definite identification can be made microscopically by means of agar blocks stained in situ by the Dienes technique (Madoff, 1960). The terms "L-phase" and "L-phase variants" are synonymous and have been used interchangeably with the term L-forms.

Protoplasts and spheroplasts are not the same as L-forms. Rather, they are spherical structures that originate from bacteria following partial (spheroplasts) or complete (protoplasts) removal of the cell wall by enzymatic digestion in a hyperosmolar environment (Brenner et al., 1958). A major difference from the L-forms is that protoplasts and spheroplasts are unable to replicate as such. However, they may be capable of producing L-forms when transferred to solid media of the proper osmolarity. Thus, "spheroplast L-forms" and "protoplast L-forms" are descriptive for L-forms that differ in properties relating to the presence or absence of cell wall components. (Ghuysen et al., 1986).

Atypical, aberrant, transitional, or variant bacterial forms are bacteria with altered cell walls that are occasionally recovered from clinical specimens (McGee et al., 1971). These forms have unstable morphology and may require osmotic protection for isolation and growth. However, they tend to regain normal growth characteristics upon subculture without producing L-type growth. Most clinical isolates of wall-defective bacterial forms probably fall within this group (Wittler, 1968).

The ability of "unstable" L-forms to revert to their parent bacteria is an important property. When this ability is lost, the cultures are considered to be "stable." Reversion is unpredictable, however, and has been shown to occur in apparently stable L-forms. The term "stabilized" would, therefore, be more appropriate. Bacteria recovered from the L-forms most often retain the essential characteristics of the parent strains. However, certain revertant bacteria may show profound alterations that indicate that changes can occur at some time during conversion, propagation, or reversion of the L-forms (Dienes, 1970; Pachas and Madoff, 1978; Owens and Nickerson, 1989).

Given the currently available methodology, a description of the L-forms only in terms of morphology and ultrastructure is insufficient. Future investigators should include other pertinent data in the description of the organisms, such as origin of the parent bacterium, method of L-transformation, requirements for osmotic protection, tendency for reversion, extent of the wall defectiveness, and, if possible, some comparative information on the physiological and biochemical properties of the parent, the derived L-forms, and the revertant bacteria. In this manner, the nomenclature will be clarified and the essential information regarding the L-cultures will be made available. For detailed studies of the physiology and biochemistry of the bacterial L-forms in comparison with mycoplasma, see Smith (1971, 1978).

The production of L-forms may reflect genetic differences among the bacteria in a population. In one well-known experiment with *Streptococcus*, only 1 colony out of 20 was shown to produce progeny capable of L-transformation (Hijmans and Dienes, 1955). Biochemical studies support the hypothesis of genetic determinants in the transformation to the stable L-form state. Stable L-forms of streptococci were shown to be defective in important stages of cell wall biosynthesis. Altered constituents of cytoplasmic membrane (Cohen and Panos, 1966) and the lack of certain cell wall components (King et al., 1970) have also been demonstrated. Gregory and Gooder (1977) noted the loss of enzyme function is *S. faecium* L-forms at the membrane stage of peptidoglycan synthesis. Pachas and Shor (1977) suggested that penicillin had a mutagenic effect, based on structural and functional changes in *Proteus* L-forms. Differences in antibiotic sensitivity patterns seen in L-forms of various species are also suggestive of genetic modifications (Schmitt-Slomska and Roux, 1977). Stable L-forms of a phytopathogenic bacterium, *Erwinia*, have been produced by ultraviolet irradiation with apparent mutagenic effect (Cabezas de Herrera and Garcia Jurado, 1977).

There is a paucity of studies at the molecular level. An important contribution in this area was made by Hoyer and King (1969), who demonstrated the loss of a portion of the chromosomal DNA in a stable L-form of *Streptococcus faecalis*. Wyrick et al. (1973) used DNA transformation to transfer the stable L-form state to intact cells of *Bacillus subtilis*. Further studies by molecular biologists should do much to elucidate the nature of the L-forms and to explore their role as a factor of genetic change in bacteria. (For review and discussion, see Gilpin and Young, 1986.)

Induction of L-forms: General Principles

The techniques used for the induction of L-forms have been described in several diverse publications (Dienes and Weinberger, 1951; Gooder and Maxted, 1961; Guze, 1968; Hijmans et al., 1969; Lawson, 1982; Madoff, 1986; Madoff et al., 1967; Sharp, 1954). The general principles of induction and maintenance will be reviewed here and detailed directions for particular strains will be given.

The conversion of a bacterium to the L-form depends on the species, the strain of the species, and the experimental conditions. In general, induction is accomplished by exposing bacteria to penicillin or other β-lactam antibiotics on a suitable medium. Important aspects of the medium are its physical and chemical properties, which include the consistency of the agar gel, the presence of animal serum, and, especially, the osmolarity (Dienes and Sharp, 1956).

In general, the L-forms prefer a fairly soft medium with the addition of 10–20% of serum. Many bacteria, notably the Gram-negative *Proteus, Salmonella,* and *Shigella* species, can be converted to L-forms on media containing the normal concentration of sodium chloride (0.5%). Others require a hypersomolar environment for stabilization. These include the Gram-positive bacteria (e.g., *Streptococcus, Staphylococcus, Corynebacterium, and Listeria*) and some Gram-negative species (e.g., *Neisseria, Serratia, and Pseudomonas*). Sodium chloride (1–5%) is the commonly used osmotic stabilizer. Other neutral salts (e.g., potassium chloride, magnesium chloride, and phosphates) have also been used. Sucrose, in concentrations ranging from 5–20%, plus Mg^{2+} can be more effective for osmotic stabilization in some strains. The combination of sodium chloride and sucrose in varying concentrations has been successful in some experiments. Polyvinylpyrrolidone (PVP) has also been found to support L-form growth in several bacterial species (Lawson and Douglas, 1973; Wyrick and Gooder, 1977).

Often, even a slight modification in the concentration of inducing agent or osmotic stabilizer may make a decisive change in the success of an experiment. Anaerobiosis may be necessary for L-transformation for some species; for others it is helpful for primary induction. The L-cultures can later be adapted to aerobic growth. Certain strains of bacteria appear to be impervious to all known methods of induction.

The penicillins, with their action on the peptidoglycan cross-linkages of bacterial cell wall, are the most effective inducing agents. Other antibiotics that act on the cell wall (cycloserine, ristocetin, bacitracin, vancomycin, and the cephalosporins) have also been shown to produce L-forms. High concentrations of certain amino acids, notably glycine, methionine, and phenylalanine, have been shown to produce L-forms from some bacteria. Inorganic salts such as mercury, lithium, and cadmium have also been used to produce L-type growth.

L-forms can be induced by certain enzymes, particularly lysozyme, that digest the peptidoglycan of the cell wall of bacteria. The bacteria are grown in osmotically protective media and are treated with the enzyme. Protoplasts are released which then produce L-forms when transferred to appropriate hyperosmolar solid media (King and Gooder, 1970; Madoff et al., 1967) (see Fig. 1). In 1961, Gooder and Maxted produced protoplasts of group A streptococci using a phage-associated muralytic enzyme found in a group C phage lysate. After inoculation on appropriate agar media, a high yield of L-forms developed. This method has been successfully applied to many other strains of *Streptococcus* (Hryniewicz, 1977).

For the induction of L-forms with penicillin, a culture is grown in broth medium for several hours. A small aliquot of broth culture is then spread over the surface of an appropriate agar medium and allowed to dry. A penicillin gradient is established in the plate by means of antibiotic discs saturated with varying concentrations, or the penicillin is introduced into small troughs cut in the agar. Other inducing agents (antibiotics, lysozyme, and amino acids) may also be tested in this manner. The concentration of the inducing agent may be a critical factor; later, the appropriate concentration can be incorporated into the medium for maintenance of the L-cultures. The use of pour plates may be effective; the inoculated agar medium is poured into a plate containing a thin layer of solid medium with similar nutrient and penicillin concentrations.

The experimental bacterial culture should be tested on a variety of media, both with and without osmotic stabilizers. L-forms have been obtained on routine culture media (e.g., horse blood agar), even from strains that would seem to require high salt concentration. L-forms induced on media of high osmolarity can sometimes be adapted to media of normal osmolarity. Adaption may represent either selection or mutation of viable organisms (Hijmans and Clasener, 1971; Leon and Panos, 1976; Panos, 1986).

L-forms of some organisms (e.g., *Proteus* and *Bacillus* species) may be formed several hours after exposure of the bacteria to penicillin

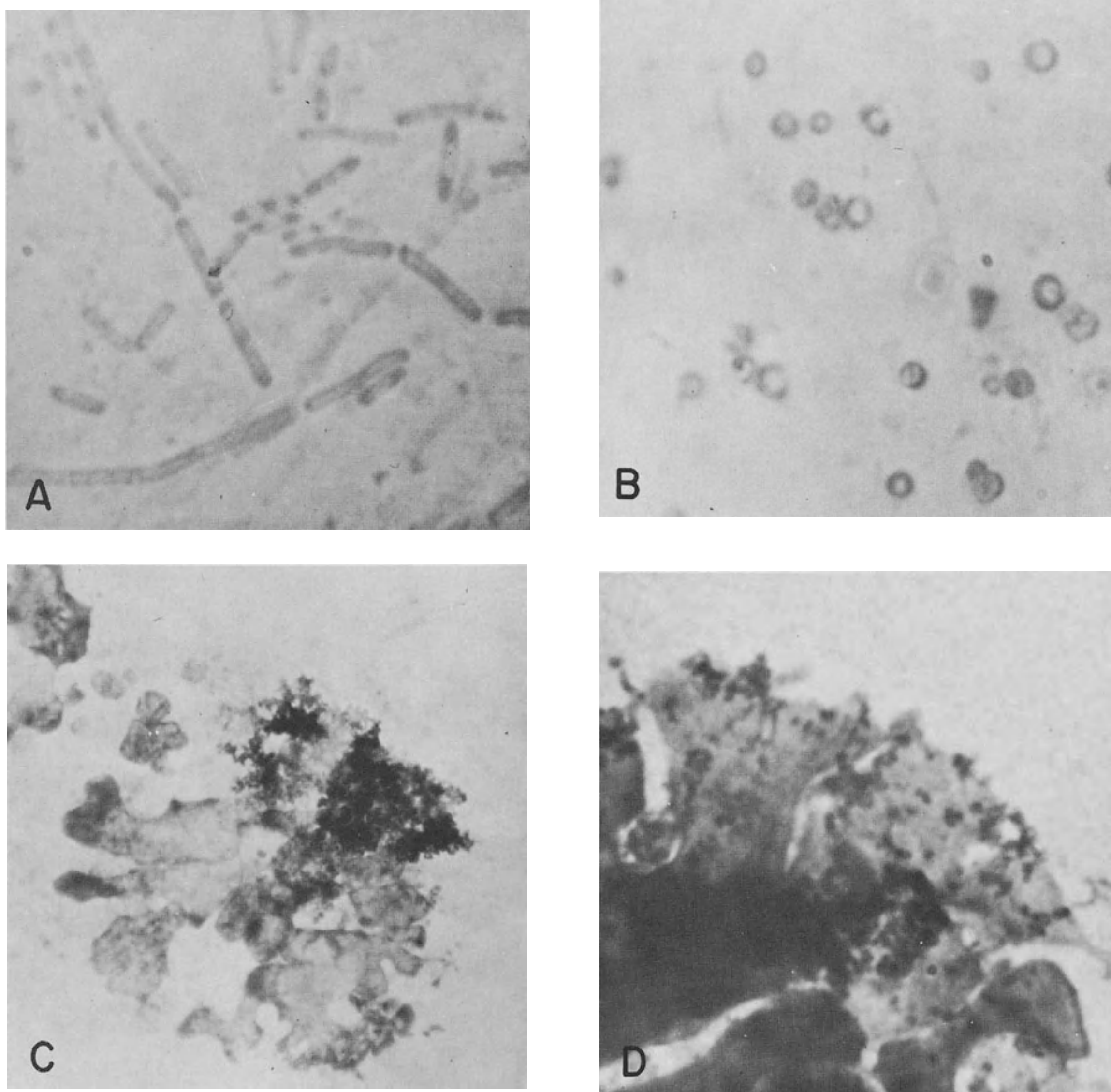


Fig. 1. Induction of L-forms of *Bacillus* species by means of lysozyme treatment. Photomicrographs made from Dienes-stained preparations. ×2,250. (A) Organisms grown 2 h in broth medium. (B) Following incubation for 2 h, in the presence of lysozyme, protoplasts are formed which are then transferred to hypertonic L-form agar medium. (C) Protoplasts enlarge to large body forms with granular elements developing into L-forms colony. (D) Detail of periphery of mature L-colony.

whereas others (e.g., *Streptococcus, Staphylococcus,* and *Listeria*) may take several days or weeks to develop. Successful transfer of the L-culture depends on the viability of the organisms; some require frequent transfers and others remain viable for extended periods.

Subcultures are made from agar to agar by the "push-block" technique, in the manner used for mycoplasma. A block of agar bearing L-form growth is cut from the plate and pushed gently along the surface of the fresh medium. The agar block should be left on the plate because growth in the transplant may occur only under the block. Growth in broth is also initiated with agar blocks, but in contrast to mycoplasmas, is often difficult to obtain. A long period of adaptation to growth in broth may be necessary to obtain an adequate concentration of viable organisms. This difficulty has hampered the study of many important biological properties of the bacterial L-forms. An alternative method of inducing broth cultures is by the use of a diphasic medium. The appropriate broth is added after the production of L-form growth on agar slants in tubes of flasks. After further incubation, the L-colonies may be dislodged by shaking or by the use of a Vortex mixer, and serial subcultures in broth are continued.

Growth in chemically defined media is not easily obtained; only a few species of L-forms have been adapted to grow in such media (van Boven et al., 1967; Gilpin et al., 1973; Bacigalupi and Lawson, 1973). Periodic transfer of broth cultures to agar are always necessary to determine viability as well as stability of the L-cultures.

Reversion and Identification of Revertants

Transfer of freshly isolated L-colonies in the absence of inducing agent will often result in reversion to the parent bacterium. For some species, when the cultures have become stabilized reversion can no longer be effected under any known circumstances. Special techniques have been devised to induce reversion of L-forms. Thus, reversion of *Bacillus subtilis* has been achieved by changes in the physical environment, e.g., by increasing the agar concentration, by the use of 15–35% gelatin in the medium, or by the application of membrane filters (Landman et al., 1968). Similar techniques using gelatin media were employed successfully with *Streptococcus* L-forms (King and Gooder, 1970; Wyrick and Gooder, 1977). In rare instances, reversion of L-forms has been influenced by the presence of factors produced by other bacteria or fungi. Landman and Halle (1963) induced reversion of *B. subtilis* L-forms by using *B. subtilis* cell wall material as a primer for wall regeneration. In L-forms of *H. influenzae* growth and reversion were found to be enhanced by a low-molecular-weight peptide produced by a strain of *Neisseria perflava* (Madoff, 1977, 1979).

The identification of an L-form culture from an unknown source depends, to a large extent, on its ability to achieve reversion. In cases where the L-form is no longer revertible, DNA-base composition and DNA hybridization may provide information as to its origin. Serological testing by agglutination, complement fixation, and fluorescent antibody methods may be useful for identifying unknown L-forms (Feinman et al., 1973; Lynn and Haller, 1968). Modified biochemical tests, useful for bacteria and their derived L-forms, can aid in characterizing and identifying stable, nonreverting L-forms (Cohen et al., 1968). Comparison of membrane proteins by polyacrylamide gel electrophoresis appears to offer a reliable method for comparing L-forms and bacteria (King et al., 1969; Gilpin and Young, 1986). Further studies are needed to detect key antigenic and metabolic markers for the L-forms.

Induction of L-Forms: Selective Procedures

Variations in requirements for nutrients, osmotic protection, inducing agents, and gel consistency make it necessary to tailor the techniques to the experimental organism. The following media and methods have proved successful with certain strains of selected species:

Gram-Negative Species (Dienes and Weinberger, 1951; Madoff and Pachas, 1970)

For induction and maintenance of L-forms of Gram-negative species (*Proteus, Salmonella, and Shigella),* a simple medium consisting of trypticase soy agar (BBL) or brain heart infusion (Difco), containing 10% horse serum will support L-form growth. Yeast extract (5%) may be added. Penicillin concentrations vary according to the strain. Anaerobic incubation may be useful. Other Gram-negative species may require a hyperosmolar environment. As examples, *Escherichia coli, Serratia marcescens,* and *Klebsiella* and *Pseudomonas* species have been converted to L-forms and maintained only in the presence of high salt or sucrose concentrations (Guze et al., 1976; Lederberg and St. Clair, 1958).

High-Salt-Requiring L-Forms (Marston, 1968; Sharp, 1954; Madoff, 1970)

For the induction and propagation of L-forms requiring high-salt concentration (*Streptococcus, Staphylococcus, Corynebacterium,* and *Listeria,* an all-purpose L-form medium is prepared in the author's laboratory as follows:

To 300 ml of distilled water is added 12 g of trypticase soy agar (BBL). The solution is thoroughly mixed and autoclaved without previous heating. The agar is cooled to 56°C (in a water bath) and the following sterile components are added in the order listed (it is advisable to warm the solutions slightly and to mix well after each addition): 30 ml of a 30% solution of NaCl, 30 ml of inactivated horse serum (56°C for 30 min), and 15 ml of yeast extract. The inducing agent may be incorporated into the medium or added, as noted above. The medium is poured into sterile petri dishes; plates are used after standing overnight at room temperature.

This medium is also useful for maintaining stock L-form cultures which normally do not require increased salt concentration e.g., (*Proteus* and *Salmonella.,*).) For maintenance, penicillin is added to the medium in concentration of 500–1,000 units per milliliter.

Liquid media that can be used are Todd-Hewitt or Mycoplasma Broth Base (BBL) containing similar concentrations of horse serum, yeast extract, NaCl, and the appropriate antibiotic. Growth may be enhanced initially by the addition of 0.1% agar.

Sucrose in varying concentrations has been used most successfully as an osmotic stabilizer with L-forms of numerous bacterial species (*Listeria,* Brem and Eveland, 1968; *Neisseria,* Roberts, 1966; *Streptococcus,* Madoff, 1970). Madoff and Dienes (1958) induced growth of

L-forms of pneumococci in media containing 10–20% sucrose, 0.01% $MgSO_4 \cdot 7H_2O$ and 10% horse serum. With the pneumococcus, the critical factor was the appearance of L-forms only in a narrow range of low penicillin concentrations (0.1–0.3 units per milliliter).

Streptococcus by Lysozyme (Gooder, 1968; Wyrick and Gooder, 1977)

Protoplasts were prepared in the following manner: Cultures of *Streptococcus faecium* strain F 24 were grown overnight (stationary phase) in trypticase soy broth, harvested, and washed three times in distilled water. Sucrose (0.6 M) or polyethylene glycol (Carbowax 4000, Union Carbide Co.) 8% (w/v) was used as osmotic stabilizer in 0.01 tris-chloride buffer, pH 7.1. A mixture of $2 \times 10_9$ streptococcal colony-forming units (CFU) and 200 μg lysozyme (Nutritional Biochemicals Corp.) per milliliter was incubated at 37°C for 2 h. Tenfold serial dilutions of the protoplast suspension were then plated onto the surface of tryptone soy agar (Oxoid) containing 0.43 M NH_4Cl, 0.5% (w/v) additional glucose, and 2% inactivated horse serum. Incubation of the plates was at 37°C. L-colonies could be counted in 2–5 days.

Broth medium for *S. faecium* L-forms consisted of Albimi brucella broth (Gibco Diagnostics) prepared in 0.43 M NH_4Cl with 0.5% glucose added. Reversion medium consisted of tryptone soy broth prepared in 0.6 M sucrose and 35% gelatin (Difco) as the solidifying agent. Plates were incubated at 25°C.

Neisseria (Lawson, 1986; Roberts, 1966; Roberts and Wittler, 1966)

L-forms of *Neisseria meningitidis* were produced with penicillin using the gradient plate technique. The medium consisted of brain heart infusion (BHI; Difco) of pH 7.2–7.4 containing 1.2% agar, 10% sucrose, 0.5% yeast extract, and 10% inactivated horse serum. L-forms obtained were serially propagated on media containing benzylpenicillin (1,000 units per milliliter). Incubation of plates was at 37°C in CO_2 (candle jar). L-forms of *N. meningitidis* have also been produced by methicillin, ampicillin, cycloserine, cephalothin, ristocetin, bacitracin, and vancomycin.

L-forms of *N. gonorrhoeae* were induced and propagated under similar conditions with the exception that 100 units penicillin per milliliter was required. Growth of L-forms in broth was obtained by the use of diphasic media. Agar slanted in flasks was inoculated and then overlayered with BHI broth of similar composition but containing 0.01% agar. Frequent serial transfers were required before heavy L-form growth was obtained in broth lacking traces of agar.

Lawson and Bacigalupi (1977) produced L-forms of *N. gonorrhoeae* using penicillin and L-form media supplemented with 7.0% PVP as osmotic stabilizer. The PVP was subjected to extensive dialysis before use. Induction frequencies were higher than those obtained in the presence of sucrose. Two other antibiotics which inhibit cell wall synthesis, cephalothin and D-cycloserine, also permitted L-transformation; transformation did not occur with vancomycin, bacitracin, or novobiocin. The L-forms of *Neisseria* species were reviewed by Lawson (1986).

Identification of L-Forms

Transformation of bacteria to L-forms may occur in periods ranging from several hours to several days. Cultures must be examined frequently. In a penicillin gradient plate, L-colonies may be detected by the naked eye or with a hand lens in the zone of penicillin inhibition. Microscopic examination under oil immersion is necessary for the identification of L-form colonies. The Dienes technique of stained agar preparation is recommended (Madoff, 1960). The method is simple and equally applicable to mycoplasma; it is indispensable for distinguishing L-colonies and viable organisms from tiny bacterial colonies and from artifacts.

The Dienes-staining solution is made by dissolving 2.5 g of methylene blue, 1.25 g of Azur II, 10 g of maltose, 0.25 g of NA_2CO_3, and 0.2 g of benzoic acid in 100 ml of distilled water. A thin film of stain is painted on a coverslip by means of a cotton applicator and allowed to dry. Many coverslips can be prepared at a time and stored for future use. The coverslip is cut into convenient squares with a diamond pencil. A block of agar bearing suspected colonies is cut from the plate and placed face up on a glass microscope slide, and the stained coverslip is placed face down on the agar. The preparation is then ready for microscopic observation.

Microscopically, L-colonies and mycoplasma colonies stain a deep blue at the center and pale blue at the periphery. Nonviable organisms and artifacts either do not stain or they assume a pinkish to violet hue. Viable bacterial colonies also take the Dienes stain. Decolorization of the stain may occur in bacterial colonies, as in large L-colonies, if the preparation is not examined at once. Contrary to other reports, decolorization of the Dienes stain is not a criterion for distinguishing between mycoplasma and L-form colonies. Identification is based rather on the distinctive morphologic characteristics of the organisms.

Morphology and Ultrastructure

The use of stained agar preparations and oil immersion magnification permits the observation of the transformation from bacteria to L-forms to be followed in serially examined

blocks of agar. The initial change is the enlargement of the bacterial cell to produce a "large body." In some instances, large bodies are formed during division when swelling occurs within segmenting cells. The large bodies appear to form a connecting link between the growth of bacteria and the transformation of L-forms (Dienes, 1968). The large body stage may be followed by fragmentation of these forms with release of typical bacterial forms. Alternatively, granular elements of varying size develop within or at the periphery of the large bodies; these soon penetrate the agar and multiply, forming an L-colony. The L-colony may grow to resemble the mycoplasma, with dense central growth into the agar surrounded by a periphery of surface growth ("fried egg" appearance), as shown in Fig. 2A; or the colony may become grossly irregular in shape and contour (Fig. 2B). L-colonies are usually larger and of coarser appearance than those of the mycoplasma. The distinctive morphology of the L-form colonies results from the growth into the agar of coarse granular elements, the presence of large bodies, and the complete absence of any residual bacterial forms (Dienes, 1939, 1968, 1970).

Young L-colonies of some bacterial species, notably, *Haemophilus influenzae, Corynebacterium* species, various streptococci, and *Streptobacillus moniliformis,* can be very small, irregular, and consist of very small granular forms. Therefore they can be difficult to distinguish from mycoplasma colonies purely by morphology. A problem can arise if such tiny colonies are produced on penicillin-containing media inoculated primarily for the isolation of mycoplasma from clinical material. In such cases, the final identification of the L-forms is by proof of derivation from the bacterium by their reversion to the parent, or by serological or biochemical means. This may present considerable difficulty in some instances.

Two morphologic types of L-colonies have been described, the A-and B-type L-forms (Fig. 3). Partial loss of cell wall by the bacteria usually produces the highly revertible B-type L-forms (Fig. 3, middle), whereas the complete loss of cell wall yields the stable or difficult-to-revert A-type L-forms (Fig. 3, bottom). Gram-positive organisms such as streptococci, staphylococci, and corynebacteria tend to produce A-type colonies almost exclusively.

Enterobacteriaceae and other Gram-negative species may produce B-type L-forms predominantly, but can also produce the stable A-type. In *Proteus,* for example, L-colonies growing on agar medium are distinguished by their small size, fine granularity, and scarcity of large bodies. With electron microscopic examination, they are seen to be devoid of cell wall (Dienes and Bullivant, 1968). The pleomorphic elements are bound by a single unit membrane and they are filled with ribosomes and DNA material. Their resemblance to mycoplasma is notable. Mesosomal bodies are absent. By light microscopy, the B-type colonies appear to be composed almost entirely of large bodies. When examined with the electron microscope, L-organisms of the B-type show the presence of cell wall components apparently similar to the wall of the parent bacteria, but lacking its rigidity.

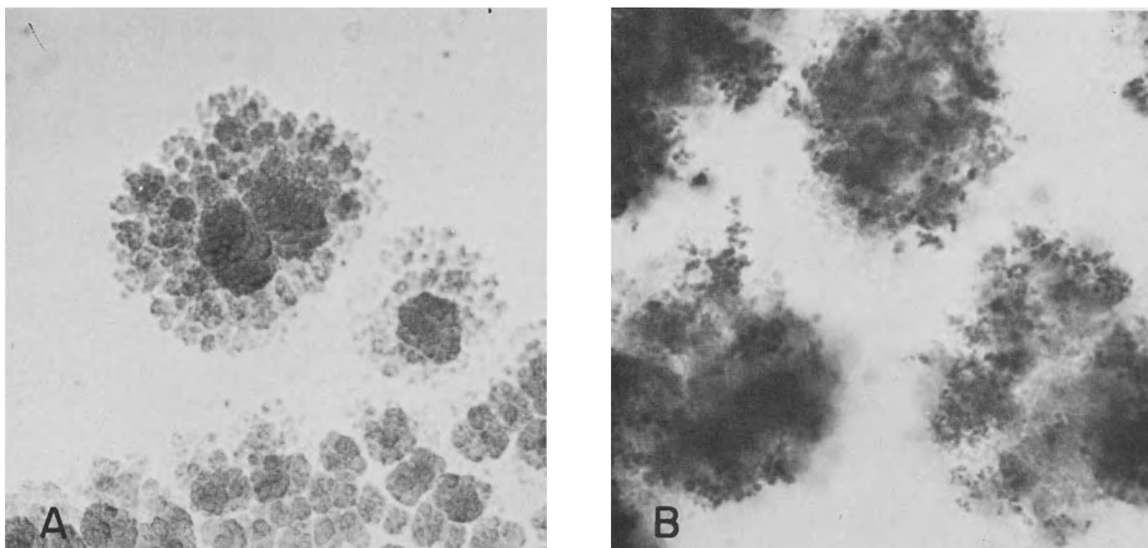
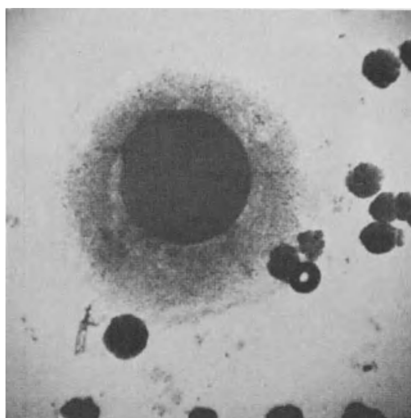
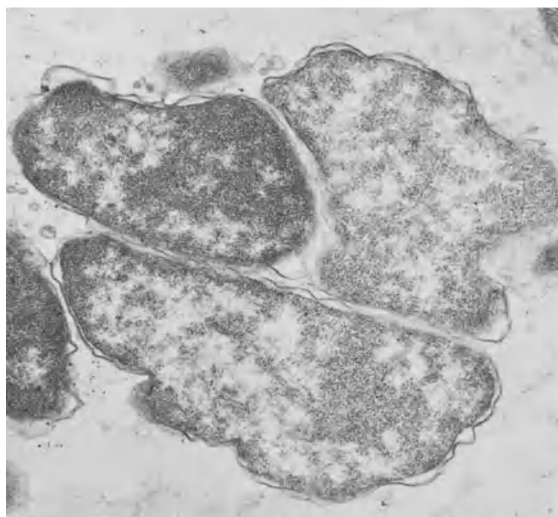


Fig. 2. (A) L-forms of *Neisseria gonorrhoeae.* ×65. (B) L-forms of *Streptococcus pyogenes.* ×110.





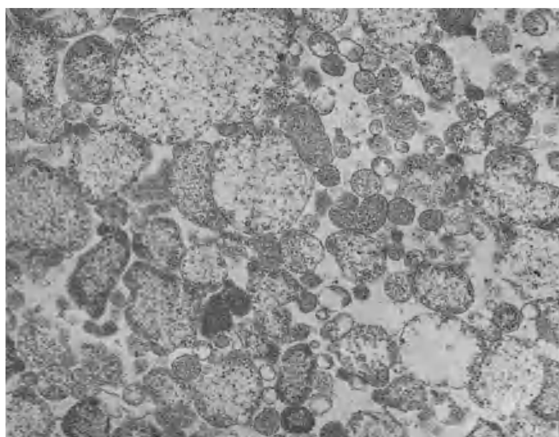


Fig. 3. L-forms of *Proteus*. In the electron micrographs, the presence of cell wall constituents is clearly visible in the B-type (middle photograph). In the A-type (bottom photograph) there is only the cytoplasmic membrane. Note the size difference between the colonies of A- and B-type L-forms (top photograph). ×65. [Reproduced by permission of the American Society for Microbiology (Pachas and Madoff, 1978).]

The presence of altered mucopeptide layers in revertible B-type L-forms of *Proteus* has also been noted (Cole, 1971).

Similarly, in *Proteus mirabilis,* exposure of the bacteria to β-lactam antibiotics has led to the production of the stable "protoplast L-forms" and the unstable "spheroplast L-forms," the latter retaining portions of defective cell wall and the former having the plasma membrane as the sole integument. With a long period of subcultivation of the spheroplast L-forms in the presence of benzylpenicillin, spheroplast L-forms can be detected that are stable and have lost the ability to revert (Martin et al. 1977). Concomitantly, these forms show resistance to the action of benzylpenicillin and mecillinam combined. Thus, as emphasized by Dienes (1970), the presence or absence of cell wall constituents does not appear to be the decisive factor in the ability of an L-form to revert to the parent form. At the present time, the mechanism by which these "mutants" are formed remains unexplained. (For a comprehensive review of B-lactam induced *Proteus* L-forms, see Ghuysen et al., 1986.)

Conflicting morphological descriptions are also given for other L-forms, particularly of the Gram-negative species. In *Escherichia coli, Neisseria meningitidis,* and *Brucella abortus,* L-forms have been shown to possess a layer of damaged cell wall external to the cytoplasmic membrane. In PVP-stabilized L-forms of *Neisseria gonorrhoeae* (Lawson and Bacigalupi, 1977) and in L-forms of *Haemophilus influenzae* (Madoff, 1977), only the unit cytoplasmic membrane was visible. In studies of thin section of *Bacillus licheniformis, Bacillus subtilis,* and *Salmonella paratyphi,* no trace of cell wall could be seen and the organisms were bounded only by cytoplasmic membrane (Wyrick and Rogers, 1973).

D. Albrewczynski and J. W. Lawson (unpublished observations) have isolated from gelatin a low-molecular-weight (<1,000 MW) peptide which, on addition to a culture of stable L-forms of *S. faecalis* strain F24L, induces near-complete conversion of the culture to large bodies. This peptide was obtained after 4 days of dialysis of 60% gelatin. The dialysate was then filtered through a 1,000 MW cutoff filter using an Amicon ultrafiltration apparatus. Upon freezing the filtrate, biologically active crystals precipitated. The crystals were recovered by filtration and solubilized in L-broth. The induced large bodies attained diameters of more than 20 μm and upon rupture-released viable units. Imidazole (30 μm), a cyclic AMP (cAMP) phosphodiesterase stimulator, induced similar changes in the L-form cultures. On the other

hand, the anti-pyretic analgesic drug indomethacin, a cAMP phosphodiesterase inhibitor, inhibited large body formation in the presence of the gelatin factor but not imidazole. The level of cAMP in L-form broth cultures and cultures supplemented with indomethacin rose steadily during exponential growth and then fell rapidly. The cAMP levels in the gelatin factor and imidazole supplemented cultures remained low until the stationary phase and then rose slowly. Electron microscopy revealed that large bodies in imidazole supplemented cultures appeared devoid of internal membranes. In contrast, large bodies from cultures containing the gelatin factor were highly vacuolated and contained viable granules.

By electron microscopy, stable L-forms of a strain of *Escherichia coli* show the presence of laminate structures and microtubules (Eda et al., 1976) resembling similar configurations previously observed in *Streptococcus faecalis* (Cohen et al., 1968) and in *Pseudomonas* species. Microtubule-like cores have also been observed in thin sections of L-forms of *Nocardia* (Beaman, 1986). The nature and function of these structures have not been established.

Replication and Reversion

As seen with transmission and scanning electron microscopy, L-forms of diverse bacteria species are surprisingly similar in their morphology and reproductive processes. Although organisms as small as 200 to 300 nm have been seen, the size of the smallest units which are viable and capable of reproduction remains unclear. Like those of the mycoplasma, the mechanisms of replication appear to vary from simple binary or asymmetric fission to budding or segmentation of small, dense bodies from large, spherical, or filamentous forms in sequential growth. In *Proteus* L-forms, sequential enlargement of protoplasmic bodies appears to be followed by release of new viable granules (Dienes and Bullivant, 1968; Cole, 1971). Thin sections of L-form colonies of *Haemophilus influenzae* grown within agar reveal small, dense bodies seen as condensations at the periphery of large cells apparently capable of detachment as budding forms (Madoff, 1977).

Among the Gram-positive bacteria, growth and reversion of L-forms have been studied in *Bacillus subtilis* (Landman and Forman, 1969; Landman et al., 1968), *Bacillus licheniformis*, (Elliott et al., 1975), *Streptococcus pyogenes* (Cole, 1970), *S. faecalis* (Green et al., 1974b), and *S. faecium* (King and Gooder, 1970; Wyrick and Gooder, 1977). Of particular interest in these reports are the studies that describe the sequential stages of reproduction and the reversion of L-forms to bacteria.

Green et al. (1974b) have proposed a reproductive cycle for L-forms of *S. faecalis*. Small, dense, nonvesiculated L-form bodies are thought to divide by simple binary fission and budding and develop within vesicles of mature "mother" forms; under certain conditions, these dense forms may undergo transition and revert to the bacteria. Reversion may be accompanied by the formation of mesosome-like structures in a manner similar to that seen in *B. subtilis* (Landman et al., 1968). In a study of *S. faecium* with ferritin labeling, reversion appears to arise through excretion of cell wall material around the unstable L-forms. Pieces of wall associated with cell membrane form a scaffolding to create the intact (complete) bacterial cell wall (Wyrick and Gooder, 1977).

Antibiotic Sensitivities of L-forms and Parent Bacteria

The susceptibilities of L-forms to antimicrobial agents have been reviewed in several publications (Hijmans, et al., 1969; Schmitt-Slomska and Roux, 1977. For a recent review, see Schmitt-Slomska, 1986). Although the subject is too extensive to treat here, a few general statements can be made.

L-forms are not susceptible to the β-lactams or to other drugs that inhibit the synthesis of the bacterial cell wall. In recent years, the interaction of β-lactams with bacteria has been the focal point of extensive investigations. Such studies show the existence of multiple, functionally different penicillin targets: pencillin-binding proteins (PBPs), penicillin-sensitive enzymes, and murein-hydrolases have been described. The modes of action of other inhibitors of peptidoglycan synthesis (e.g., bacitracin, ristocetin, and D-cycloserine) are poorly understood. Neither has shown any effect on the growth of L-forms.

Polymixin B increases the permeability of the cell membrane through affinity for the phospholipid of the membrane. It has been shown to exert a greater effect on L-forms than on the parent bacteria. L-forms derived from polymyxin-resistant Gram-negative bacteria, *N. gonorrhoeae, Brucella melitensis*, and *Proteus* species are as resistant to polymyxin as the parent bacteria.

The broad-spectrum antibiotics exert their primary influence by inhibition of nucleic acid functions and protein synthesis. They have, in

general, a greater effect on the L-forms than on the parent bacteria. Greater susceptibility has been shown by the L-forms of *S. faecalis* to the tetracyclines and to the aminoglycoside antibiotics, kanamycin, streptomycin, and vancomycin (Montgomerie et al., 1966). *Staphylococcus* L-forms have also shown greater susceptibility to the tetracyclines. In *N. meningitidis* and *N. gonorrhoeae,* both parent bacteria and derived L-forms showed similar susceptibilities to tetracylcine, novobiocin, erythromycin, and sulfadiazine (Roberts, 1966; Roberts and Wittler, 1966). More recent studies with *Neisseria* L-forms (Lawson and Bacagalupi, 1977) showed considerably greater susceptibility of the L-forms than the parent to the same antibiotics. The authors explain the divergent results as being due to the complete absence of cell wall constituents in the PVP-stabilized forms of *Neisseria* as opposed to those induced on sucrose-containing media.

Studies with the L-forms of *Proteus* species (Ghuysen et al., 1986) and with *Streptococcus pyogenes* (Leon and Panos, 1988) have shown changes in numbers and relative concentrations of penicillin-binding proteins with the loss of normal cell synthesis.

In summary, the L-forms share with their parent bacteria, to a greater or lesser degree, susceptibility to antibiotics other than those that act on bacterial cell wall. Agents that exert their influence on cytoplasmic membrane tend to have a greater inhibitory effect on the wall-defective forms. Recent studies confirm the unpredictability of the degree of susceptibility of the L-forms in comparison with the parent bacteria, and, in particular, with bacterial revertants from L-forms. Alterations in biochemical or genetic properties of the cytoplasmic membranes following prolonged cultivation in the L-form may be important factors. Should the L-form be found to play a significant role in infectious disease, these considerations could be important (Schmitt-Slomska, 1986).

Occurrence and Pathogenicity

The controversial aspects of L-forms as pathogens have been discussed in several publications (Clasener, 1972; Hijmans et al., 1969; Kagan et al., 1977; Pachas and Madoff, 1978; Roux, 1977; Wittler, 1968). For a thorough review, see Pachas (1986).

L-forms of the following species have been found to be adaptable to growth in tissue cultures of diverse origin: *Brucella abortus* in hamster kidney cells (Hatten and Sulkin, 1968); *Streptococcus faecalis* in human embryonic kidney cells (Green et al., 1974a); and *S. pyogenes* in human heart cells (Leon and Panos, 1976). In the last example, a marked cytotoxic effect was found with a L-strain adapted to physiologically isotonic environment (Panos, 1986).

Early experiments with L-forms in animals failed to show any pathogenicity unless it was caused by the revertant bacteria, as in *Streptobacillus* and *Listeria,* or unless toxins were produced, as in *Clostridium* and in *Vibrio* species. *Staphylococcus* L-forms failed also to colonize experimental endocarditis in rabbits (Linneman et al., 1973). The persistence of L-forms of *Streptococcus* has been shown in mouse experiments, but pathogenic effects have not been noted (Schmitt-Slomska et al., 1967). L-forms of *Streptococcus pyogenes,* which had been adapted to grow in isotonic conditions, survived in immunosuppressed mice (Panos, 1986) suggesting that the minimal response of the mouse (host) may play a role in suppressing the L-forms in vivo.

Some recent studies in vivo suggest a pathogenic role for the L-forms. Khostikian et al. (1987) have shown that the intraperitoneal inoculation of stable L-forms of group B streptococci into animals led to a progressive inflammatory, necrotic and sclerotic degeneration of the hypothalmus, hypophesis, and adrenal glands. Persistence of the organisms appeared to result in hormonal disturbances. Owens (1987) demonstrated that L-forms of *Staphylococcus aureus* could be isolated from bovine mastitis lesions following penicillin treatment. After cessation of therapy, the cows that harbored L-forms yielded the parent organisms. This observation may perhaps account for the poor response of staphylococcal bovine mastitis to treatment with antibiotics.

The L-forms of *Nocardia* have clearly shown their pathogenic potential. L-forms of actinomycetes, *Nocardia, Actinomyces,* and *Actinomadura* have been isolated from infected tissues of humans and animals (Beaman, 1982, 1985, 1986; Buchanan et al., 1982). The possible relationship between these L-forms and clinical manifestations have been studied. It was shown that the immunologically intact host induced the formation of L-forms (probably by activated macrophages) (Bourgeois and Beaman, 1974), which persisted within the tissues for long periods. In contrast, L-forms were not isolated from T-cell deficient hosts (Beaman, 1980; Beaman and Scates, 1981). In experimental murine infections with *Nocardia caviae* (Beaman, 1980; Beaman and Scates, 1981), it was shown that L-forms could grow in tissues to form granules characteristic of those found in mycetomas. Frequently, the L-forms reverted to the parental

cell so that both cell types could be recovered from these granules; however, some remained composed entirely of bacterial L-forms. It was shown that in vitro induced L-forms of *N. caviae* injected into mice induced mycetomatous disease. Furthermore, the L-forms from these lesions could be isolated in vitro, and transmitted to new animals, with the resultant formation of new lesions, thus, fulfilling "Koch's postulates" for the pathogenicity of *Nocardia* L-forms (Beaman, 1980, 1982, 1985; Beaman and Scates, 1981).

Summary

The pathogenicity of the bacterial L-forms remains unclear. Certain factors in persisting and recurrent infections may predispose to the production of wall-defective organisms. Factors such as the dissolution of bacterial cell wall by immune mechanisms, by cellular enzymes, or by antibiotics may create favorable conditions for the establishment of chronic infections by wall-defective organisms.

To establish a definitive role for the L-forms in disease, a conscientious search will have to begin with careful observations in the laboratory. In searching for L-forms in human infection, however, it is important to keep in mind the potential for misinterpreting bacteria that show atypical or aberrant morphology. In the last decade, progress on the clinical significance of the L-forms has produced little new information. On the other hand, strong new data such as the production of chronic or latent infection in animals by bacterial L-forms offer interesting possibilities for future studies, particularly as they may relate to similar conditions in humans.

Clinical studies have reported the recovery of L-forms and other wall-defective organisms from blood, body fluids, and tissues of animals and humans. However, their relation to pathogenic states are at present unclear. Studies by Domingue et al. (1977) describe the recovery of variant wall-defective bacterial forms from the blood of normal as well as diseased humans. Likewise, the role of L-forms of *Mycobacteria* in tuberculous infection or in resistant states merits further attention (Mattman, 1968; Khomeno et al., 1980). A comprehensive survey of the L-forms and bacterial variants in clinical disease has been presented by Pachas (1986).

The bacterial L-forms deserve investigations with new experimental models, isolation methods, and culture techniques. For example, provocative observations have been made on the stimulation of growth of L-forms by certain bacteria (satellite effect) (Bouvet and Acar, 1977; Madoff, 1977, 1979; Falcon et al., 1989). Studies showing the enhanced survival of L-forms of *Streptococcus* in immunosuppressed mice (Panos, 1986) and the production of mycetomatous disease in animals with L-forms of *Nocardia* (Beaman, 1986) represent new and promising avenues of approach.

The L-forms of bacteria deserve to be studied for their intrinsic scientific value. That the bacteria can, under certain conditions, enter into a wall-less state, that they can survive and reproduce themselves in this wall-deficient state, that they can ultimately revert to their bacterial form—or lose the ability to revert—are remarkable phenomena. These phenomena cannot be insignificant. Further studies with regard to their structure, biochemical function, and genetic composition should elucidate the role of the L-forms in the biology of bacteria and their potential role in clinical disease.

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CHAPTER 231

The Genus *Gallionella*

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The bacteria of the genus *Gallionella* belong to the so-called "iron bacteria," which achieved their theoretical importance after Winogradsky (1888, 1922) postulated his conception of chemolithotrophy for these organisms. These bacteria also have practical significance since they clog drains, water pipes, and wells with deposits of iron oxide compounds. In connection with freshwater supply problems and the rapid growth of agriculture, hydrotechnicians and land-reclamation (underground drainage) specialists have become increasingly interested in bacterial iron oxidation as a common source of interference with wells and drainage systems (Ford, 1978; Hanert, 1974b; Khrutskaya, 1970; Martin et al., 1978). Iron bacteria are also interesting for ecological and biogeochemical reasons. Like algae, iron bacteria may develop in their natural habitats in such masses that the idea of their participation in the sedimentary formation of iron ore is plausible, as was recognized in the first description of the iron bacterium *Gallionella ferruginea* (Ehrenberg, 1836).

Iron bacteria are reputed to be difficult to work with. The general opinion of microbiologists is that it is difficult to cultivate these organisms in the laboratory and that there are difficulties in isolating and identifying them. Therefore, it is one of the main purposes of this contribution to show that this poor reputation is unjustified. It will be demonstrated that cultivation and handling of the treated bacteria are in no way more difficult than for other microorganisms, if attention is paid to only a few fundamental peculiarities, such as "sessility" and "gradient growth." Disregard of these factors may be one of the most common practical reasons for failure to locate these organisms in natural habitats, for failure to cultivate them successfully, for misinterpretation of presumed developmental stages, and for discouragement which can often be observed when students are starting in this field. Therefore, the following text is narrowly restricted to methods and practical advice—without any theoretical discussion on the controversial life cycle, taxonomic position, and physiology, which are described in the literature cited.

This paper is taken directly from the 1st edition of *The Prokaryotes*.

Habitats

Chemistry of the Habitats

Gallionella ferruginea characteristically occurs most abundantly in very pure, iron-bearing waters that contain only traces of organic material. Habitats with the best prospects for a successful search are ferruginous mineral springs, water works, wells, and drainages, especially underground drainages in regions near the groundwater table. Swamp ditches and lakes are unusual habitats that contain *Gallionella* in large quantities only at places where pure, ferrous waters infiltrate. Occurrence is not restricted to fresh waters or to low-temperature habitats, as commonly assumed; *Gallionella* has been convincingly found in salt water, marine bays, and thermal springs with temperatures up to 47°C (Hanert, 1973b, 1981a; Sharpley, 1961; Volkova, 1939; Vouk, 1960).

Common to all of the various *Gallionella* localities investigated are sharply limited physicochemical conditions characterized by a low redox potential in an Eh range of +200 to +320 mV (rH_2 values generally vary from 19 to 21) and a slightly acidic environment caused by the solution of considerable amounts of CO_2. Adding the data determined by Volkova (1939) in slightly alkaline mineral waters, the pH environmental limits range from 6.0 to 7.6, which excludes growth in acid habitats. Occurrence of *Gallionella* in acid mine waters as described by Walsh and Mitchell (1969, 1970) may be a result of confusing *Gallionella* with *Metallogenium* and/or nonbiological structures.

These Eh and pH limits characterize *Gallionella* as a very good example of a gradient

organism that develops under neither strongly reducing conditions nor in a highly oxidizing zone, but in a level between the two extremes with redox conditions about 200–300 mV lower than typical surface waters. This region in which *Gallionella* grows characterizes the lowest zone of Eh-pH environmental limits of iron bacteria established by Baas-Becking et al. (1956), in which there are usually only low concentrations of oxygen.

Comparison with geochemical-stability field data for ferrous iron, published by Garrels and Christ (1965) and Hem (1972), shows that the *Gallionella* Eh-pH milieu, in all the cases that have been measured, lies significantly within the zone in which ferrous ions are stable. This stability appears to be the essential factor in the environmental conditions for the existence of *Gallionella,* much more important than the factors of temperature and oxygen content to which *Gallionella* manifests greater adaptability than generally thought. The marked psychrophily and the microaerophily generally ascribed to *Gallionella* seem to be only secondary effects that depend on the fact that bivalent iron is most stable under these conditions (Hanert, 1975). Thus, it is easy to understand why *Gallionella* also occurs in O_2-saturated waters, for example, in aerated, iron-removal treatment plants in water works or in thermal springs, when the only essential condition, stability of bivalent iron in the presence of oxygen, is fulfilled. These observations do not invalidate the most common values of physicochemical factors measured in *Gallionella* habitats, which are 0.1–1 mg/liter O_2, 8–16°C, 5–25 mg/liter Fe(II), around 20 or more mg/liter CO_2, and a very slight content of organic material, not above 12 mg/liter $KMnO_4$ (Hanert, 1975).

Growth Measurement and Analysis of Iron-Oxidation Structures by In Situ Exposure

The red-brown deposits at *Gallionella* sites consist primarily of the typical spiral bands of dead matter excreted from the terminal *Gallionella* cells. These deposits may be extremely pure. However, their *Gallionella*-nature may be very difficult to recognize when the deposits have been altered by additional chemical iron-oxidation processes. False conclusions may then be drawn about the nature of the iron-oxide precipitation. A direct measurement of *Gallionella* growth is indispensable for analyzing such structures, as well as for determining whether or not *Gallionella* is actually growing on the site.

There are three proven methods of in situ exposure for light microscopy and for transmission and scanning electron microscopy: (1) The exposure of cover slips or slides for several hours or days, singly or in multiple vertical alignment, in plastic clamp fittings. This method, a modification of the on-growth method of Naumann (1919) and Cholodny (1924) allows the localization of the zones in which *Gallionella* develops in still waters, the densitometric and photometric registration of the total iron oxidation (Hanert, 1981a) and, by determining stalk production, the quantification of the momentary *Gallionella* development (Hanert, 1973a). (2) The exposure of Formvar-coated, platinum or gold, electron-microscope grids (copper grids are inappropriate due to the frequent presence of H_2S). With these procedures, the fine structure of the bands, as well as of the terminal *Gallionella* cells on the sessilely excreted intact stalks, may be observed. The latter is not included when suspension preparations of the stalk fragments are used (Hanert, 1970; Hirsch and Pankratz, 1970). (3) Exposure of round glass slides for scanning electron microscopy and electron probe microanalysis (FeKα). This procedure is especially important because it allows tiny globular particles (0.04–0.3 μm in diameter) to be chemically and physically analyzed (Hanert, 1981a). Until now, they were thought to be mycoplasma-like developmental stages and buds of *Gallionella,* but they have been shown to be pure Fe(III)-oxide particles.

The advantages of these three procedures, which all make use of the sessile way of life, are obvious when one considers that the failure to find the characteristic apical cells in natural habitats (van Iterson, 1958) was the starting point for the studies that aimed at proving the mycoplasma-like and *Metallogenium*-like viable nature of *Gallionella* stalks (Balashova, 1967b, 1969, 1974; van Iterson, 1958; Zavarzin, 1961). The three procedures described above may also be applied to pure cultures of *Gallionella.*

Isolation

Physiological Basis for Isolation

Although chemolithotrophy has not as yet been generally recognized for *Gallionella,* it must be emphasized that all of the successful cultures were able to develop only in mineral media with iron(II) as energy source. This is true for Lieske's first cultures (1911) containing metallic iron as well as for the excellent culturing procedures of Kucera and Wolfe (1957) that use ferrous sulfide as a source of reduced iron. Culture procedures that used organic media con-

taining serum, as suggested by Balashova (1969), have remained unsuccessful. Classic *Gallionella* organisms with stalks and apical cells as well as developmental stages producing such forms have not been obtained. It thus seems evident that *Gallionella* is truly a chemolithotrophic bacterium and that ferrous-containing mineral media are best for cultivation and isolation.

Isolation Principles and Procedures

Two isolation procedures have proven useful. Both use mineral Wolfe's FeS medium (see below) as a selective isolation medium for *Gallionella,* in which neither sheathed nor capsulated iron bacteria will grow.

One procedure is a test-tube dilution method in which a final concentration of one colony per culture is reached. The ability to stick to the vessel walls is used to separate *Gallionella* from the contaminants (Engel and Hanert, 1967; Hanert, 1968; Hanert 1981b).

Isolation of *Gallionella* by Dilution and Serial Transfer (Engel and Hanert, 1967; Hanert, 1968)

One *Gallionella* colony is withdrawn with a capillary pipette from the wall of a test tube containing an enrichment culture, suspended homogeneously in 10 ml of Wolfe's medium without FeS, diluted to 10^{-5} (maximally 10^{-6}, one colony of 1 mm in diameter consists of about 100,000 apical cells), and then inoculated into test tubes with Wolfe's FeS medium (30–50 parallel cultures starting with a 10^{-4} dilution).

After 1 week of culture, a one-colony culture is treated, in essentially the same way as above, to obtain a pure culture. The colony, which is attached to the wall of the test tube, is carefully rinsed using a pipette with approximately 200 ml of fresh, sterile medium without FeS, after first disposing of the FeS sediment (1 ml) and the mineral medium (9 ml). Then the colony is transferred five times into test tubes containing 10 ml of sterile medium (without FeS) and washed with gentle shaking to rid the colony of clinging contaminations. The washed colony is then suspended into sterile medium (10 ml) and again inoculated into test tubes containing Wolfe's FeS medium at dilutions of maximally 10^{-6} (again 30–50 parallel cultures). Five to ten serial transfers are necessary to achieve pure culture in this manner. Although this procedure requires up to 10 weeks, it is a very certain method of continually reducing the number of contaminants and obtaining a pure culture (Hanert, 1975).

The second isolation technique requires much less time and leads, according to the authors, to a pure culture in 7 out of 10 cases. It is based on the observation that some strains of *Gallionella,* in contrast to most contaminants, is resistant to 0.5% formalin.

Isolation of *Gallionella* by Formalin Resistance (Nunley and Krieg, 1968)

Five-tenths milliliter of formalin (40% formaldehyde solution) was added to a dilution bottle of Wolfe's medium containing 10 ml of ferrous sulfide agar and 100 ml of fluid medium. Samples of *Gallionella* from its natural source were centrifuged at 3000 × *g* for 3 minutes. One to five milliliters of sediment was transferred to the Wolfe's medium with formalin. The medium was incubated at 25°C for 1–2 days. One-milliliter aliquots of the culture were transferred to fresh Wolfe's medium without formalin. Cultures were incubated at 25°C for 2–3 weeks. Tests for culture purity were performed using the third serial transfer after the formalin treatment.

Purity is checked by use of a variety of heterotrophic and autotrophic test media; for example, yeast extract bouillon, nutrient agar, *Nitrosomonas* medium, *Ferrobacillus* medium, and *Thiobacillus* agar. The length of observation is up to 4 weeks.

Micro- and Macrocultures and Axenic Maintenance

There are no principal difficulties in cultivating *Gallionella* in containers ranging from small chambers to 12-liter fermentors. The difficulties of culture that have been reported in almost all publications on *Gallionella* generally are due to overlooking interactions in the medium among the following factors: phosphate, ferrous and ferric iron, oxygen, pH, and redox potential. The most important growth-inhibiting chemical processes in the medium are: (1) iron phosphate precipitation caused by too high a concentration of phosphate; (2) chemical autoxidation of ferrous iron caused by too high a concentration of oxygen or by the redox potential being too high; and (3) the pH becoming more acidic when elevated O_2 concentration causes hydrolysis of ferric iron. All three processes eventually reduce the Fe(II) content of the mineral medium of the FeS cultures below the lower limit of 5 ppm. In order to retain the required Fe(II) concentration, it is absolutely necessary to have the following initial conditions in the medium: oxygen content 1 ± 0.2% (0.42 ppm), pH 6.0 (values from Co_2 bubbling, border values between 5.5 and 6.3), Eh +330 mV to +350 mV (Eh border values from −55 to +395 mV), phosphate (as K_2HPO_4) 0.05 g/liter. These conditions were established in optimum growth zones of 3-liter Erlenmeyer flasks and confirmed in test tube cultures (Hanert, 1975). They lead, following interactions between mineral medium and FeS-sediment, to an iron(II) concentration of 10–20 ppm which guarantees good growth of *Gallionella.* The conditions of gassing must be varied according to the volume in each vessel, so that

common standardized instructions may not be given for all sizes of flasks. Identical for all cultures, though, is the production and storage of ferrous sulfide as well as the production of modified mineral Wolfe's medium. During the first process, essential mistakes may be made that inhibit the growth of *Gallionella.*

Preparation and Storage of Ferrous Sulfide

For preparation of ferrous sulfide, two precipitation reactions with equal molar quantities are possible. FeS can be produced either by reacting 78 g ferrous ammonium sulfate [$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$] with 44 g sodium sulfide ($Na_2S \cdot 9H_2O$) or 140 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) with 120 g sodium sulfide in deionized water (50°C; ferrous salts are added in solid form while stirring). The resulting FeS precipitate must be washed extensively using deionized water (decanting of the supernatant and its replacement with deionized water at 50°C), removing Na^+, NH_4^+, and, above all, S^{2-} ions until the precipitate reacts neutrally (pH measurement in FeS, not in the supernatant).

Washing of FeS while continually checking pH is indispensable, since hydrolysis of any residual sulfide ions ($S^{2-} + H_2O \rightarrow HS^- + OH^-$) raises the pH to 7.7 directly over the FeS sediment. The elevated pH prevents bivalent iron from dissolving and *Gallionella* development does not begin (Gebauer, 1978). Because of this indirect effect of sulfide on the development of *Gallionella,* sodium sulfide, used to manufacture FeS, is added in slightly less than the equimolar amount. The separation of the adsorbed sulfide ions from the FeS precipitate during the washing procedure is a slow process and takes approximately 5 days (5–9 washings at intervals of at least 4 h). Delayed FeS sedimentation caused by formation of FeS-hydrosol can easily be eliminated by including a few drops of a saturated $FeCl_3$ solution or by once washing with tap water. After this cautious washing, FeS precipitate can be stored without oxidation in glass-stoppered bottles that are completely filled.

Modified Mineral Wolfe's Medium

The modified mineral medium contains per liter distilled water: 1 g NH_4Cl, 0.2 g $MgSO_4 \cdot H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, and only 0.05 g $K_2HPO_4 \cdot 3H_2O$ (one-tenth of the original concentration).

With the strongly reduced concentration of phosphate, it is no longer necessary to separately autoclave the individual components (no earth alkali phosphates are formed during autoclaving). Low phosphate concentration also prevents iron phosphate precipitate in the culture medium. Such precipitation would result in a total growth inhibition of *Gallionella* due to lack of dissolved iron(II).

Microculture Slide Chambers for In Vivo Studies on Cell Division, Cell Rotation, and Stalk Twisting

In constructing a chamber, one cover glass (20 × 50 mm) is mounted on each slide (26 × 76 mm). The cover glass is maintained at 0.05 or 0.1 mm above the slide by initially inserting a 0.05- or 0.1-mm flat bar and then sealing the two sides with sealing wax. The bar is removed, the third side (bottom of the chamber) is then sealed, and the slide is held erect for filling. First, a tiny drop of FeS is inserted (by capillary pipette) and then mineral medium is added so that the chamber is two-thirds full, whereby the final ratio of FeS:medium is 1:10. The chamber is then inoculated and the fourth side is sealed. Microaerobic conditions are produced by replacing the air with CO_2 or with a mixture of 94% N_2–5% CO_2–1% O_2. *Gallionella* growth (stalk production) starts immediately after the chamber is closed (Hanert, 1974a).

Procedure for Maintenance of Axenic Cultures of *Gallionella.*

The following procedure has proven useful in maintaining pure cultures of *Gallionella.*

Thirty-four test tube cultures are set up with the dilutions 10^{-1} (4 cultures; inoculum 150,000–300,000 cells), 10^{-2} (5 cultures), 10^{-3} (15 cultures), and 10^{-4} (10 cultures; inoculum 150–300 cells). For this purpose, the cotton-plugged test tubes are aseptically filled with 9 ml autoclaved mineral medium. Then, 22.8 cm^3 of cotton-filtered CO_2 is bubbled through each tube using a 1-ml pipette (length of bubbling approximately 5 s; the pH of the medium is now 4.5–4.8). One milliliter of sterile FeS precipitate is then slowly added with a pipette.

Six hours after the addition of FeS, the inoculation follows subsequent to dissolving 5–10 ppm bicarbonatic Fe(II) in the medium; meanwhile, the tubes are stored in jars at 17°C under an atmosphere of 94% N_2–5% CO_2–1% O_2. In preparing the initial suspension for inoculation (10^0 dilution), two colonies with a diameter of 2 mm (or one colony with a diameter of 4 mm) are suspended in 10 ml of sterile medium using a vortex mixer. Dilution series and inoculation are repeated as described above. The cultures are then incubated in preserving jars at 17°C in an atmosphere of 94% N_2–5% CO_2–1% O_2. This gas mixture can also be used for bubbling through the cultures (135 cm^3 for each test tube). The best *Gallionella* growth is usually at a dilution of 10^{-3} and is macroscopically visible after 3–5 days. Enrichment cultures may be obtained in the same manner, using a drop of natural *Gallionella* sediment for inoculation. Cultures are transferred every 4 weeks.

Preservation of *Gallionella* culture material in viable form for at least 13 weeks has been reported by Nunley and Krieg (1968) using the following procedure.

Culture Preservation by Freezing (Nunley and Krieg, 1968)

The organisms were centrifuged at 3000 × *g* for 3 minutes, resuspended in the fluid portion of fresh Wolfe's medium containing 15% glycerol, and stored in 1-ml quantities in a low-temperature cabinet at −80°C. Sur-

vival was tested by transfer to Wolfe's medium at weekly intervals; in every case, the organisms were viable, forming a distinct mat of growth on the submerged ferrous sulfide agar after 7–10 days.

Batch Cultures in Erlenmeyer Flasks for Physicochemical Studies in the Optimal *Gallionella* Growth Zone

In order to obtain the described physicochemical growth conditions, FeS (100 ml of FeS precipitate or 100 ml of 1.5% FeS agar) is added to the sterile culture medium (2 liters) in 2-liter Erlenmeyer flasks (silicon stoppers with glass tubes for bubbling), and then bubbled with 94% N_2–5% CO_2–1% O_2 up to pH 6 (gassing takes approximately 90 min). Then, pH, O_2, and Eh values are checked electrometrically and the medium is additionally gassed with N_2 and CO_2 to adjust to the required conditions (see above). The inoculation is with a suspension containing 10–15 *Gallionella* colonies (2 mm in diameter) into 10 ml of mineral medium. *Gallionella* growth becomes macroscopically visible after a lag phase of 4–6 days, during which the redox potential of the culture medium is lowered 300–400 mV by the bacteria (Hanert, 1975).

Fermentor Cultures in Mineral Ferrous Bicarbonate Medium

A clear $Fe(HCO_3)_2$ medium is made by adding 200 ml of FeS precipitate to 10 liters of modified Wolfe's medium, and CO_2 is bubbled through for approximately 24 h until a Fe(II) concentration of 50 ppm (pH 4.6) is reached. After FeS sedimentation, 4.5 liters of the supernatant, combined with 4.5 liters of modified Wolfe's medium [without Fe(II)], is filtered into the autoclaved fermentor through a membrane-filter apparatus containing coarse prefilter (10-μm pore size) followed by a Sartorius membrane filter (0.2-μm pore size) using a gas mixture of 94% N_2–5% CO_2–1% O_2 under 2 atm pressure. This filtration results in 9 liters of culture volume with 25 ppm ferrous iron. For measurement, control, and eventual readjustment of the physicochemical conditions with N_2 or CO_2 gas, the solution is left in the fermentor vessel for 24 h. The conditions are so reductive that no autoxidation of the ferrous iron occurs (culture temperature, 17°C). Inoculation is with 10–15 colonies (4 mm in diameter), suspended in 10 ml of medium. In contrast to normal fermentation cultures, the medium should be only moderately stirred (25 rpm) and gassed (1 bubble/2 s).

Gallionella growth becomes macroscopically visible after 2 days (white flakes on the walls of the vessel; after 5 days, the entire Fe(II) in the culture medium has been oxidized. *Gallionella* grows on all the inner surfaces of the culture vessel without gradient formation. This procedure seems suitable for the development of *Gallionella* fermentor cultures with a continuous flow of culture solution, during which it is particularly important to prevent contamination of the culture (changing of redox potential during *Gallionella* growth; Hanert, 1975).

Identification

The characteristic, spirally twisted, stalk structure formed by rotation of the apical cell makes *Gallionella* very easy to identify. It is uncertain, though, whether the genus consists of one or more species. This question has repeatedly been discussed at length (Beger and Bringmann, 1953; Pringsheim, 1949; van Iterson, 1958; among others). These authors have pointed out—and rightly so—that in almost all cases the differentiation into species has been based only on differences visible under light microscopy in the bands of *Gallionella* collected in nature.

Studies on quantification of the iron content of the bands of pure cultures of *Gallionella ferruginea* (Hanert, 1975) have shown that, due to additional chemical-physical iron oxidation on the surface of the bands, these surfaces may vary greatly in morphology. Six stalk types have been differentiated. Thus, the differentiation into species on the basis of light-microscopic band differences is definitely unreliable.

The genus is presently differentiated into two species, *Gallionella ferruginea* (stalk consists of 40 filaments or more) and *Gallionella filamenta* (stalk consists of only 3–8 filaments), based on the electron-microscopic band differentiation of Balashova (1967a, 1968). But this differentiation is only a morphological definition in another dimension; it does not exclude the possibility that "filamenta" is only an underfed "ferruginea." It seems conceivable that the presumption of Pringsheim (1949), which we favor, is accurate—the genus may consist of only a single species *(Gallionella ferruginea)* and all the other described species are only ecological growth forms of this one. The proof of this presumption by the results of additional physiological investigations would show that this bacterium—unique in its physiology and stalk excretion—is not nearly as complex as *Gallionella* literature may give the impression.

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CHAPTER 232

The Genus *Nevskia*

PETER HIRSCH

The Russian microbiologist A. Famintzin (1892) discovered a rod-shaped bacterium that was characterized by the formation of a laterally excreted slime stalk. The organism was often slightly bent; during division, both daughter cells continued to laterally produce the slime, which therefore became dichotomously branched. Groups of cells were thus kept together by a disk-shaped or spherical structure consisting of the polymer (Fig. 1). Famintzin (1892) named this morphologically unique organism *Nevskia ramosa.*

Famintzin observed this bacterium on the water surface of aquaria in his laboratory, but he was unable to obtain a culture of it. The bacterium, together with its polymer stalk, vaguely resembled aggregates of *Pasteuria ramosa* Metchnikoff, a bacterial parasite on (in) *Daphnia,* which had just been described. *P. ramosa* had rosettes of drop-shaped cells and was thought to divide longitudinally. But Famintzin correctly recognized and described the nature of the center of *Nevskia* aggregates, a polymer soluble in 1% KOH.

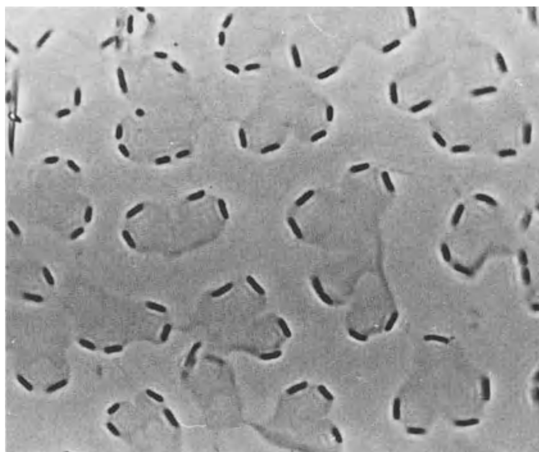

Fig. 1. *Nevskia* sp. cell families surrounding the central polymer. From a pond water enrichment culture with 0.1% Na lactate. × 1,280.

This paper is taken directly from the 1st edition of *The Prokaryotes.*

Additional observations made by Famintzin (1892) must be considered significant for the recognition of *Nevskia* spp. He described *N. ramosa* to contain highly refractile spherical bodies of variable diameter. In 35% ethanol, these globules swelled; in 70% ethanol, they completely dissolved. Famintzin thought that the globules consisted of "ethereal oils." Occasionally, he also observed that individual cells of a group actively left the central polymer to disappear in the water. Although motility was not observed as such, he inferred that such cells had become motile.

It is surprising that, with its rather peculiar properties, *Nevskia* was not seen very often later on. Henrici and Johnson (1935), in their now-famous paper on the stalked bacteria, mention finding *Nevskia* on the surface of a water sample taken from a greenhouse lily pond "to which a little sodium sulphide had been added to encourage a growth of *Beggiatoa.*" They were able to transfer *Nevskia* to new water samples containing Na_2S, but pure culturing was not attempted.

Babenzien (1965) reported seeing *Nevskia* spp. on the water surface of a swamp ditch; surface-contact preparations that had been transferred to sterilized pond water (pH 5.9, with 0.1% Na lactate) yielded pure cultures of slime-producing bacteria, but the lateral slime stalk was only seen occasionally, in older cultures. Additional information on *Nevskia* was published later (Babenzien, 1966, 1967; Babenzien and Schwartz, 1970).

H. Hippe (personal communication, 1964) found *Nevskia* on a greenhouse pool. From his samples, the present author successfully isolated neuston bacteria of the *Nevskia* shape and size, capable of slime production. But lateral polymer stalks could not be found. Zimmermann (1975) showed a scanning electron micrograph of a Nuclepore-filter preparation with *Nevskia* sp. His sample came from the brackish water of the Bight of Kiel (Baltic Sea). The di-

chotomously branched, lateral slime stalks are clearly recognizable.

Habitats

Most findings of *Nevskia*-like bacteria have been reported from the water surface, usually from fresh water. In fact, *Nevskia* appears to be a typical neuston microorganism, according to Babenzien, (1965, 1966, 1967) and to Babenzien and Schwartz (1970). Table 1 summarizes observations of various authors on the occurrence of *Nevskia* spp. The lengths of lateral polymer stalks evidently varied considerably, as did the cell sizes. It is quite probable that identification solely on the basis of lateral slime production and intracellular granules may not have been sufficient and, thus, various different bacteria could have been observed.

Nevskia-like bacteria are widely distributed in shallow, aquatic habitats; they have never been reported from soils. Their occurrence in brooks, ponds, and even lakes is shown in Table 2. From these data, we can see that bacteria with lateral polymer stalks can be found during most of the year. There does not seem to be a distinct temperature dependency and, in all but one case, the organisms were found in or near the (aerobic) water surface. *Nevskia*-like bacteria do not show preference for a specific type of aquatic environment as long as they can live at the surface and are not overgrown by other organisms.

Isolation

Henrici and Johnson (1935) obtained their *Nevskia* enrichments by adding "a little sodium sulphide" to a jar of water taken from a greenhouse lily pond. Continued growth was observed when the *Nevskia*-containing scum was serially transferred to sterilized, Na_2S-containing water from the same lily pond. Babenzien (1965) could not confirm beneficial effects of a sulfide addition.

Babenzien (1965) collected his *Nevskia* sp. by touching the swamp brook-water surface with a glass slide, by washing the film off the slide with sterile water from the same site (pH 4.8), and by repeatedly transferring to new, sterile water. Growth of families occurred within 4 weeks. Addition of C or N sources other than 0.1% Na lactate to this water resulted in overgrowth of the *Nevskia* by the other microorganisms. Finally, a pure culture was obtained using water from a dystrophic pond (pH 5.9), 0.1% lactate, and agar. The organisms grew only on the water's surface, and lateral polymer stalks developed only in cultures after 2 weeks of incubation at 20–28°C.

The enrichment technique employed by H. Hippe (personal communication, 1964) resembled that of Henrici and Johnson (1935) somewhat. A water sample from a greenhouse pool was enriched with water lily leaves, then filled to the top, closed, and incubated in darkness and warmth for several months. When this sample was exposed to light and air, a surface pel-

Table 1. Occurrence and habitats of *Nevskia* and similar bacteria as reported in the literature.

Location	Depth	Remarks: Habitat	Remarks: *Nevskia* cells	Reference
Aquarium in laboratory, St. Petersburg	Surface		Size: 2–6 × 12 μm; globules of ethereal oils; lateral stalks	Famintzin (1892)
Greenhouse lily pond	Surface	Surface scum	Size: 1 × 3–4 μm; each cell with 3–4 "vacuoles," considered to be sulfur	Henrici and Johnson (1935)
Greenhouse pool	Surface	Sample with water lily leaves, kept warm	Cells small; lateral stalks; cells motile	H. Hippe (personal communication, 1964)
Swamp ditch	Surface	Water, pH 4.8; in surface pellicle	Size: 0.7 × 1.8–2.7 μm; stalks 3 μm; cells with 1–3 flagella and 1–6 storage bodies (not S°)	Babenzien (1965)
Little Lake, Wisconsin	8 m	Anaerobic, 4°C	Cells short rods with gas vesicles; stalks long and slender	D. E. Caldwell (personal communication, 1975)
Kiel Bight (Baltic Sea)	?	Brackish water	Cells small, with lateral polymer stalks	Zimmermann (1975)

Table 2. Occurrence of *Nevskia*-like bacteria in various aquatic habitats and some data on the habitats and organisms.[a]

		Nevskia habitat			Cell properties				
Location	Time	Depth	Temp.	O_2	Size (μm)	Granules	Flagella	Stalks	Remarks
Lake Lansing (Michigan)	7 Dec. 1968	15 cm	1°C	(+)	0.5–0.7 × 1.2–2.0	–	–	+	Lake, ice-covered; in enrichments
Lake Plußsee (Holstein)	3 Oct. 1975	Surface	15.5°C	+	0.5–0.6 × 0.9–1.7	+	–	+	In enrichments after 30–40 days
Knaack Lake (Wisconsin)	14 May 1975	17.5 m	5°C	–	0.4–0.8 × 1.0–1.5	–	–	+ Long	Cells with gas vesicles; in natural samples
Gull Lake (Michigan)	15 Sept. 1969	Moist beach sand	ND	+	0.4–0.7 × 1.7–3.3	–	–	+ Short	Enrichment with *Elodea* and tap water; 8 months
Forest Pond (Michigan)	21 July 1968	40 cm	22°C	(+)	0.6–0.8 × 0.7–1.0	–	+ (1)	+ Branched	On EM grid submerged 5 days
Occom Pond (New Hampshire)	10 July 1964	Surface	20°C	+	0.7–0.8 × 2.0–5.0	+	–	+	In enrichments, pH 5.9, with 0.1% Na lactate
Iron Spring Brook (Connecticut)	30 March 1967	Surface	15°C	+	0.6–0.8 ×1.7–5.8	+	–	+	In enrichments at 5°C/dark
Humus Brook (Connecticut)	26 July 1967	Surface	22°C	+	0.7–0.9 × 2.5–5.0	+	–	+ Short	In enrichments at room temp.
Aquarium water (laboratory)	10 Feb. 1968	Surface	21°C	+	0.7–0.9 × 2.0–4.8	–	–	+	
Warburg water bath (laboratory)	25 Apr. 1968	Surface	22°C	+	0.7–0.8 × 2.1–5.0	+	–	+ Very short	

[a]ND, not determined; +, present; (+), low oxygen concentration; –, absent. P. Hirsch, unpublished observations.

licle that contained 90% *Nevskia* developed within a month. Transfers into sterile water with or without 0.1% Na acetate resulted in some growth, but *Nevskia* developed better without acetate. These enrichments were inoculated by the present author into pond water, whose pH had been adjusted to 5.9 with H_3PO_4 and to which 0.1% of Na lactate had been added after sterilization. Excellent development of *Nevskia* occurred within 7 days of dark incubation at 22°C. *Nevskia* likewise grew well under these conditions when the inoculum came from Occom Pond, a eutrophic pond in Hanover, New Hampshire. Isolation of these organisms was accomplished by streaking onto the same medium solidified with 2% Noble agar (Difco). The pure cultures obtained, however, produced polymer all around the cells rather than just laterally.

Enrichment of *Nevskia* from Lake Water

Enrichment from lake surface water was done repeatedly by using the following procedure: 100 ml of surface-water samples (0–20 cm) are poured into 500-ml Erlenmeyer flasks and covered with a loose cotton plug. To some flasks, 0.005% (wt/vol) of either peptone (Difco) or yeast extract (Difco), or 1 ml of vitamin solution no. 6 (Van Ert and Staley, 1971) are added. Incubation is in the dark and at room temperature (20–22°C). *Nevskia* families will occur on the surface within 30–40 days. The enrichments will have to be monitored often, since a *Nevskia* cell bloom develops rapidly and lasts only for a few days; lysis will then cause a rapid drop of cell numbers. Although the polymer structures remain for a long time, the only remnants of *Nevskia* cells after lysis are dense, dark granules located at the former site of the cell. Addition of 0.005% (wt/vol) peptone (Difco) usually results in the greatest numbers of *Nevskia* families.

Identification

Nevskia ramosa Famintzin 1892 has not been obtained in pure culture. Key characteristics, such as slime formation and the presence of fragile, reserve-material globules, are also found in other rod-shaped bacteria that are slightly bent *(Brachyarcus thiophilus, Microcyclus aquaticus)*. However, *lateral* slime formation has not been found too often. *"Bacterium pediculatum,"* described by Koch and Hosaeus (1894) as a contaminant of sugar factories, showed with its long, branched stalks a certain morphological similarity to *Nevskia* spp. Henrici and Johnson (1935) regarded this organism as a species of *Nevskia*. *Siderophacus corneolus*

(Dorff) Beger 1949, 12 (=*Gallionella corneola* Dorff 1934, 26) may also be related to *Nevskia* spp. But its longer and less-branched stalks, as well as the deposition of ferric oxide hydrate on the stalks, distinguish it from *Nevskia* spp.

The type species, *Nevskia ramosa,* was described by Famintzin (1892) as being 2–6 × 12 μm in size ("the average length of the cell measures 0.012 mm, in the mature state it is 2–6 times longer than wide"). One wonders if these measurements could have been correct. Although within the bacterial range, Henrici and Johnson (1935) pointed out that all other *Nevskia*-like bacteria seen to date were smaller. The average sizes were: 0.4–0.8 × 1.0–3.3 μm (from lakes); 0.7 × 1.8–2.7 μm (only from swamp ditch); and 0.6–1.0 × 1.7–5.8 μm (from ponds, brooks, aquaria, etc.). Therefore, more than one species of the *Nevskia* genus might exist. Famintzin (1892) mentioned that he had seen a form identical to *N. ramosa,* but of much smaller dimensions. Babenzien and Hirsch (1974) list but one species.

Apart from size, there were remarkable differences with respect to stalk formation. Stalk formation depends on the original source as well as on growth conditions and cell age (Babenzien, 1965). Slow growth under oligotrophic conditions and greater cell age favor lateral stalk formation; faster growth and younger cell age result in less polymer formation, equally produced over the whole cell surface.

The physiology of *Nevskia* spp. is still nearly unknown. From the data in Tables 1 and 2, as well as from the work of Babenzien (1965, 1966, 1967), we can deduce the following properties: *Nevskia* spp. should be chemoorganotrophic and oligocarbophilic, with an ability to utilize low concentrations of lactate and perhaps acetate. They are aerobic, nonphotosynthetic, occasionally motile (1–3 flagella), and capable of cell lysis and storage of alcohol-soluble reserves. They should be mesophilic and may be rather UV resistant (since they are found in the neuston). Nevskiae evidently prefer slightly acid environments (pH < 6.0). Growth is probably not vitamin dependent. There may be forms that are slightly halotolerant.

The organisms found by D. E. Caldwell and this author in Little Lake and Knaack Lake (Wisconsin) contained gas vesicles. Their cells (short rods to almost cocci) sat on long, slender stalks with only few branches. They came from anaerobic, cold strata of these two meromictic lakes. All of these properties render them distinctly different from the other *Nevskia* spp.

Acknowledgments

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CHAPTER 233

The Genera *Caulococcus* and *Kusnezovia*

JEAN M. SCHMIDT and GEORGI A. ZAVARZIN

Perfil'ev and Gabe (1965) observed bacteria of the genera *Caulococcus* and *Kusnezovia,* along with *Metallogenium* and *Siderococcus,* as manganese- and, to a lesser extent, iron-depositing or -oxidizing organisms of mud and sandy deposits. The use of capillary devices that permit microscopic observation (peloscopes) has permitted some observations of structure and colonial morphologies. Their morphologies suggest that these organisms might be related to *Acholeplasma, Metallogenium,* or *Siderococcus,* but lack of axenic cultures has prevented thorough study of their physiology and composition; understanding of the nature of these rather rare bacteria is quite meager.

Habitats

Caulococcus has been found in samples of mud and sandy deposits as observed with microcapillary techniques (Kutuzova et al., 1972; Perfil'ev and Gabe, 1961; Perfil'ev et al., 1965). It was originally found in Lake Khepo-Yarvi (Karelian Isthmus), "especially in ore deposits," and was "frequently observed in the upper layers of bottom mud deposits, above the reducing horizon," or in the bottom water (periphyton) over the mud (fine mineral or sandy) surface (Perfil'ev et al., 1965).

Kusnezovia was observed, using the peloscope technique, only "in mud samples from Lake Ukshezero, Karelian ASSR" (Perfil'ev et al., 1965), and in secondary profiles in zones of manganese oxidation of mud samples stored in the laboratory.

As described by Perfil'ev and Gabe (1965), in capillary peloscope samples from many ore-bearing lakes, for all those "with a black-orange microzone, the blackish-brown horizon of the microzone in the peloscope canals showed a mass-development of *Metallogenium,* rarely *Caulococcus,* and sometimes *Kusnezovia,* the biogenic deposits of which consist mostly of manganese oxides."

Isolation

Neither *Caulococcus* nor *Kusnezovia* has been isolated in pure culture. Distinctive morphologies, particularly those of microcolonies using microcapillary techniques, have been used to define these two groups (Perfil'ev et al., 1965).

Organisms could be maintained in the isolated samples of mud (Perfil'ev et al., 1965): a sample of water (approximately ⅓) and mud (⅔) from the natural habitat was mixed thoroughly and incubated in a 200-ml beaker at room temperature for months. Peloscopes were placed across the upper 3 cm of the mud. Development of manganese- and iron-depositing organisms was observed to occur in sharp, horizontal microzones (a few millimeters in width) in the upper layers of mud following the reduction of metal oxides in the bottom layers.

Identification

In the absence of axenic cultures and information on cultural traits, colonial morphology is used, along with the characteristic manganese deposition, for tentative recognition of these kinds of bacteria. To observe cell morphology, Perfil'ev and Gabe (1965) dissolved manganese deposits with 0.2–1.0% oxalic acid, washed them with distilled water, and stained them with a carbol solution of gentian violet. All steps were monitored with the light microscope. The bacteria appeared as minute bodies without sharp contours, and appeared to reproduce by budding. They were embedded in a matrix or connected with thin filaments. For identification, the original illustrations of Perfil'ev et al. (1965) should be consulted. Some other characteristics are given in Table 1.

Colonial morphology is variable. Three sorts of microcolony morphologies have been de-

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Table 1. Characteristics of *Caulococcus* and *Kusnezovia* spp.

Trait	*Caulococcus manganifer*	*Kusnezovia polymorpha*
Coccoid cell diameter (μm)	0.5	0.5–1.5
Connecting filament diameter (μm)	0.1	0.1–0.2
Mode of reproduction	Budding (?)	Budding (?)
Motility	Present	Not observed
Gram reaction	Not reported	Not reported
Zoogleal accumulations	Present	Not observed
Oxygen relationship	Microaerophilic	Microaerophilic (presumed)
Occurrence	Rare, in iron-manganese ore from several lakes (Karelian Isthmus, ASSR)	Extremely rare, found in only one lake (Ukshezero, Karelia)

scribed for *Caulococcus manganifer.* The most commonly observed microcolonies occur as dense, irregular clusters, the surfaces of which appear ribbed. The central portion of the microcolony is more heavily mineralized than the periphery, due to manganese deposition. The mineralized colonies are blackish brown in color. Occasionally, colonies have radial arms (termed "radial lobate" colonies); the rarest colonial form in *Caulococcus* microaccretions is the trichospherical colony with very fine, radiating processes.

Kusnezovia polymorpha microcolonies (polymorphous cenobia) also occur in a variety of forms: filamentous sprouts, open lily-of-the-valley leaves, or goblets with a toothed margin, with the wider portion of the microcolony pointing downward in the peloscope canals. Candelabra-like and clavate, filamentous cenobia structures attached to the peloscope wall, with an appearance analogous to *Cladonia* (a bryophyte), have also been attributed to *Kusnezovia.* Removal of manganese from these deposits revealed the cocci, connected by thread (Perfil'ev et al., 1965).

These organisms will require further attention in order to distinguish them from other quite similar manganese depositers, such as *Metallogenium.* In fact, we strongly suspect that these genera, particularly *Caulococcus,* are merely growth forms of *Metallogenium* in a certain habitat. The description of *Siderococcus* (this handbook, Chapter 236) should be consulted, since these iron-depositing organisms are very probably members of the same group, although they are not presently classified as such.

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The Genus *Brachyarcus*

PETER HIRSCH

Light-microscopic investigation of the microorganisms in the hypolimnion of many lakes, shows the presence of a highly diverse population of bacteria. Here, most of the recognizable forms have never been cultured and many of them have not even been described morphologically. Among these bacteria are rod-shaped organisms that are bent in an arc by asymmetric, polar cell growth and that frequently assume the shape of a pretzel. Cell division in the center and separation result in two arcs that face each other. Additional cell divisions and the presence of masses of polymer lead to even numbers of arcs facing each other in a symmetric, mirror-image way (Fig. 1).

Phase-contrast light microscopy, using slides coated with water agar to improve resolution, reveals the presence of gas vesicles in most of these organisms. The wide distribution and often the high cell numbers of such arc-shaped bacteria warrants attempts to cultivate and study them, but such attempts have been unsuccessful. Skuja (1964) found similar bacteria in a Lappish lake, and he described them as *Brachyarcus thiophilus*. The species name was used to suggest a possible sulfur metabolism, since the cells occasionally possessed intracellular structures resembling sulfur globules.

Since *Brachyarcus* spp. have never been cultivated, not even in enrichments, their possible identification presents some problems. In fact, a rodlike, arc-shaped cell shape is quite common among other bacteria. But the cell arrangement after division, the presence of gas vesicles, the absence of photosynthetic pigments, and the frequent observation of these bacteria among other "thiophilic" organisms may indeed allow a recognition sufficient for a descriptive treatment here, as has been done in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Hirsch, 1974).

Habitats

The original observations were made by Skuja (1964) on a hypolimnion water sample collected April, 1953, at a depth of 12–13.5 m in Lake

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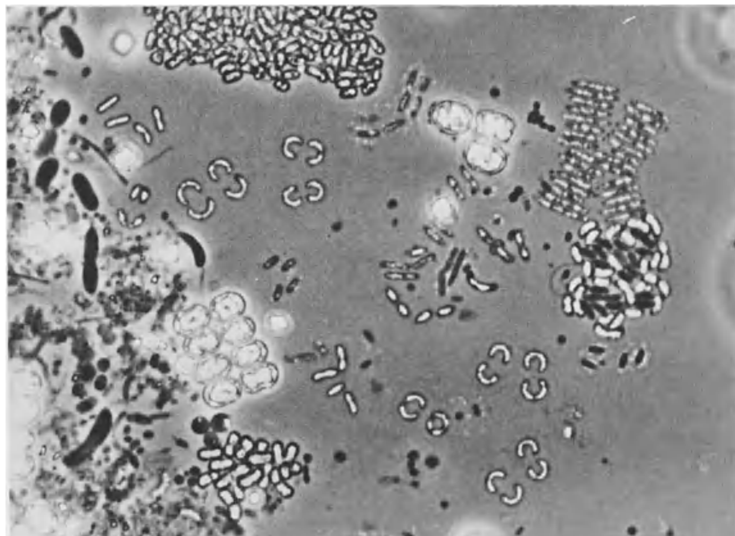

Fig. 1. Arc-shaped cell families of a *Brachyarcus* sp. from Wintergreen Lake (Michigan), collected from 4 m. Magnification × 1,280.

Vuolep Njakajaure, Swedish Lappland (408 m above sea level). The water sample had a temperature of 2°C; the lake was ice-covered. Together with *Brachyarcus thiophilus,* large numbers of other "thiobiontic" bacteria were found, many of which contained sulfur storage globules but none of which has ever been cultured (for nomenclature, see Skuja, 1964), e.g., *Macromonas bipunctata, M. mobilis, Gigantomonas cucullata, G. capitata, Thiovulumspora, Hyalobotrys hypolimnicus.* Also present in this sample were some photosynthetic bacteria (*Thiocystis violacea, Pelochromatium roseum* consortium) and sulfate reducers *(Microspira desulfuricans).*

Similar crescent-shaped organisms have been found by other authors (Table 1) in various lakes or ponds. Bacteria of such cell arrangement and/or shape were also found in soil (Nikitin, 1971, 1973; *"Renobacter vacuolatum"*) or even in reindeer rumen (Tarakanov, 1972). The latter bacteria were 0.4–0.5 × 1.1–1.2 μm and occurred in the above-described cell-plate arrangements. Their shape, however, was more that of a vibrio than that of an arc.

Isolation

Although isolation has not yet been successful, the data on occurrence in Table 1 allow us to speculate on possible enrichment techniques. In Wintergreen Lake, temperatures in the *Brachyarcus* layers during the time of observation varied between 11° and 20°C, with the maximum cell density of *Brachyarcus* spp. at 13°C (depth, 5 m). In the other sites—as far as data are available—temperatures ranged from 5° to 12.5°C. These observations indicate that *Brachyarcus* is psychrophilic.

In all cases observed, the organism occurred under anaerobic conditions. A pH of 7.5 and a phosphate concentration below 2.56 mg/liter would be additional parameters. No information is available on the carbon nutrition, although one could guess that *Brachyarcus* (being nonphotosynthetic) would have a chemoorganotrophic metabolism. Their presence in strata that contain methane (Wintergreen Lake) or reducible sulfate (Lake Vuolep Njakajaure) points to preference for a low redox potential in the environment. Storage of intracellular sulfur

Table 1. Occurrence of *Brachyarcus*-like bacteria.

Location	Date	Depth (m)	Temperature (°C)	Remarks	Reference
Lake Vuolep, Njakajaure (Lappland)	April 1953	12–13.5	2		Skuja, 1964
Burke Lake (Michigan)	23 Aug. 1972	9	6	Anaerobic; sulfide +	Caldwell and Tiedje, 1975
Cassidy Lake (Michigan)	1 Aug. 1968	7	8.5	Anaerobic; sulfide +	P. Hirsch, unpubl.
	11 Aug. 1970	7	7.6	Anaerobic; sulfide +; pH, 7.12	P. Hirsch, unpubl.
Knaack Lake (Wisconsin)	14 May 1975	17.5	5	Anaerobic	P. Hirsch, unpubl.
Wintergreen Lake (Michigan)	23 July 1970	4	16.5	O_2, 0.22 mg/liter; pH, 7.74	P. Hirsch, unpubl.
	22 July 1975	3.5–6.0	20–11	Anaerobic; pH, 7.8–7.3; DOC, 9–7 mg/liter; POC, 2–5 mg/liter; methane, 60–320 μM/liter × 10^{-3}; PO_4^{3-}; 0.23–2.56 mg/liter; max. distribution at 5 m (= 13°C)	P. Hirsch, unpubl. (M. Klug, R. Wetzel, unpubl.)
Lake Blunkersee (Holstein)	16 Sept. 1975	8	12.5	Anaerobic; sulfide +	P. Hirsch, unpubl.
Lake Plußsee (Holstein)	17 June 1967	27	5.5	Anaerobic	P. Hirsch, unpubl.
Forest Pond, Augusta (Michigan)	15 Oct. 1968	0.25–0.51	15.5	Anaerobic; sulfide (+)	P. Hirsch, unpubl.
	17 Nov. 1968	0.55–0.65	6	Anaerobic; sulfide +	P. Hirsch, unpubl.
Arco Lake (Minnesota)	Sept. 1972	6.5–9		Anaerobic ?	Walsby, 1974
	May, June 1973	6.5–9			Walsby, 1974

globules indicates participation in the sulfate reduction or sulfide oxidation processes.

Identification

Brachyarcus Skuja 1964, 19, is a genus of colorless rod-shaped bacteria of approximately 1.0 × 1.5–2.5 μm that usually contain few to many gas vesicles (Fig. 1). Occasionally, globules resembling sulfur were observed by Skuja (1964), but these have not been reported by other authors nor have they been seen by this author. The cells are bent like a bow or arc, almost to a circle or even a pretzel shape. Upon division, a 3-shape is initially assumed until two semicircles lie opposite each other, embedded in a thick polymer capsule with diffuse edges (Fig. 1) Families of two to 16 synchronously dividing cells are common; they often form cell plates, which may be free floating or grow attached to a surface. Three-dimensional, free-floating families of polymer-embedded cells of up to 100-μm family size or larger have been observed. The type species is *Brachyarcus thiophilus* Skuja 1964, 20, so named for the "thiophilic" environment in which it has been found.

Identification is still largely on the basis of morphology. Cells or cell aggregates of similar shape are formed by the purple bacterium, *Rhodocyclus* (Rhodospirillaceae), or by the green bacteria, *Chlorobium vibrioforme* and *Pelodictyon luteolum* (Gorlenko, Dubinina, and Kuznetsov, 1977; Pfennig, 1978). The former two bacteria lack gas vesicles while *P. luteolum* does contain them. Since *Brachyarcus* has been described (Skuja, 1964) and has subsequently been found to be colorless (see Table 1), differentiation from these photosynthetic bacteria should be possible.

Morphological similarity with a colorless, gas-vacuolated soil bacterium called *Renobacter vacuolatum* (Nikitin, 1971, 448; 1973) also holds true for the size (0.7–1.0 × 1.6–1.8 μ). However, this bacterium appears to grow out, before cell division, to an S-shape rather than a 3-shape or pretzel. Similarity also exists with *Microcyclus* spp. Electron micrographs of the nonvacuolated *Microcyclus marinus* (Raj, 1977) or the gas-vacuolated strains of *M. aquaticus* (Van Ert and Staley, 1971) show cells that appear to be more vibrioid; in pure culture, the characteristic orderly arrangement in symmetric cell plates, as seen in *Brachyarcus* (in nature), is not seen in the genus *Microcyclus*. Furthermore, both of the *M. aquaticus* strains that were studied in greater detail had temperature optima of 37° and 30°C, respectively, and neither strain grew anaerobically. *Brachyarcus thiophilus* can be expected to be an anaerobic psychrophile.

Acknowledgments

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CHAPTER 235

The Genus *Pelosigma*

PETER HIRSCH

The Danish scientist E. Warming observed, during a walk along Kallebo Beach in 1874, a reddish discoloration of mud and decaying algae. His microscopic investigation of a sample started the now-famous study of "bacteria living on Denmark's coasts" (Warming, 1875). One of the organisms he found in "stinking mud at the height of decay" at several locations was a highly unusual, flat and striated, motile "bacterium" (which he called *Spiromonas cohnii*) that resembled a flagellate *Spiromonas* previously described from Switzerland. Although Warming himself was not quite sure of what he saw, his drawings are remarkably accurate illustrations of bacteria that can still be found in hypolimnia and on anaerobic sediment surfaces.

About 40 years later, these organisms were observed again and described as the bacterial genus *Pelosigma* (Lauterborn, 1913, 1915). Lauterborn clearly stated that a bacterium could not belong to a flagellate genus, but otherwise his observations confirmed the findings of Warming. Another 40 years later, a study on Swedish lakes revealed *Pelosigma* organisms in many different locations (Skuja, 1956). But now the old theory that this organism was a flagellate—at least in part—was revived. Skuja was convinced that he had found another example of a flagellate/bacterium symbiosis, and he produced detailed drawings of various stages of such consortia. Nevertheless, the genus name *Pelosigma* was retained for the consortium for both the flagellate and the bacterium organisms.

Detailed microscopic investigations of aquatic microorganisms and the study of several lakes and ponds in the United States and northern Germany revealed the presence of *Pelosigma* spp. in many locations, often in large numbers (P. Hirsch, unpublished observation). These studies allowed a preliminary description and interpretation for the eight edition of *Bergey's Manual of Determinative Bacteriology* (Hirsch, 1974). Since then, many additional samples have been found to contain *Pelosigma* spp., and evidence is mounting that these are, indeed, bacteria living in sigmoid, bundle-shaped aggregates just as originally drawn by Warming (1875) and described by Lauterborn (1913, 1915). Unfortunately, all attempts to cultivate these organisms so far have failed. Suggestions for enrichment cultures can be made, however, on the basis of the observed occurrence.

This paper is taken directly from the 1st edition of *The Prokaryotes*.

Habitats

The original description (Warming, 1875) does not contain many details on the natural habitat. It is not even clear whether *Spiromonas (= Pelosigma) cohnii* was found in mud of salt water, brackish water, or fresh water. However, the location "Limfjord" suggests brackish water, and the purpose of the article by Warming was to describe bacteria living at the coasts. Warming listed four locations for *S. cohnii* and stressed its occurrence in mud that was most active in the decaying process. He stated also that *S. cohnii,* like other bacteria discussed later in his paper, did not contain granules such as those of the organism now known as *Chromatium okenii.* His description of these granules makes it quite likely that he meant sulfur globules. Although obviously an anaerobe, *S. cohnii* thus did live in a sulfide-rich environment but did not store elementary sulfur.

The habitat of *Pelosigma (=Spiromonas) palustre* (Lauterborn, 1913) was likewise poorly described: fresh water; in the decaying sediment especially in ponds with characean algae.

The observations of Skuja (1956) could be added to the habitat description, if one accepts the interpretation that his *Pelosigma* organisms indeed were identical with those seen by Warming and Lauterborn. Skuja found *Pelosigma* spp. in Swedish lakes (Hönsan, Lushavet, Munkbosjön, Tjärnatjärn), usually at depths ranging from 10 to 14 m. Although he did not describe these environments chemically or

physically, some information can be obtained from the list of accompanying bacteria. Skuja mentioned *Peloploca* spp. *(P. pulchra, P. fibrata, P. taeniata), Macromonas mobilis, Achroonema* sp., *Pelonema tenue, Achromatium oxaliferum, Gigantomonas cucullata, Ochrobium tectum, Beggiatoa arachnoidea, Leukobium maior, Pelochromatium roseum,* and *Thiocystis violace* (nomenclature sensu Skuja, 1956). These bacteria are generally known to be anaerobic or microaerophilic, hypolimnetic organisms, many of which are found in sulfide-rich environments. Thus, we can expect the presence of low sulfide concentrations in an otherwise mostly anaerobic and dark environment.

More information comes from recent observations on *Pelosigma* habitats (Table 1; P. Hirsch, unpublished). The presence of *Pelosigma* spp. appears to be correlated with anaerobic conditions and, generally, temperatures below 11°C. Like *Brachyarcus* spp., often found in the same location, *Pelosigma* spp. can be expected to be psychrophilic. It should be pointed out, however, that *Pelosigma* spp. have been found occasionally at higher temperatures.

The presence, in Wintergreen Lake (Michigan), of sulfide together with measurable concentrations of dissolved methane during *Pelosigma* occurrence points to a need of these organisms for a low redox potential.

Isolation

From the data given by Warming (1875), Lauterborn (1915), Skuja (1956), and in Table 1, an enrichment procedure could be conceived. It would consist of inoculating an anaerobic medium of pH 7.5 that contains organic matter (such as organic acids, amino acids, etc.), a mixed vitamin solution and, perhaps, a low concentration of sulfide (10^{-4} M or less). The sample for inoculation should be drawn anaerobically, and it should be kept at temperatures below 10°C. Incubation would be in the dark at 5–10°C.

Identification

All *Pelosigma* spp. observed were more or less flat, band-shaped aggregates of slender, long, and S-shaped bacterial rods. The aggregates appear to be formed preferentially by four cells or by multiples of four. At least one end of the aggregates appears to be more pointed. If a flagellar structure is present, it is located at the more pointed end. The overall length of the aggregates (9–25[30] μm) is not the length of the individual, component rod, since uneven elongation and cross-division occur. The maximum width of aggregates is 5–11 μm and depends on the number of rods in the aggregate (Fig. 1).

At present, there are two species recognized (Hirsch, 1974): *Pelosigma cohnii* (Warming) Lauterborn 1913, 100 (synonym: *Spiromonas cohnii* Warming 1875, 370) and *P. palustre* Lauterborn 1915, 418. Both species show great morphological similarity but differ in size. Aggregates of *P. cohnii* are 1.2–4 μm wide and 9–20 μm long, while those of *P. palustre* are 8–10 μm wide and 20–25 μm long. The aggregates of *P. cohnii* are usually pointed at both ends (and

Table 1. Occurrence and morphological characteristics of *Pelosigma* spp. and some data on their environment.

Site	Time	Depth (m)	Length of aggregate (μm)	Width of aggregate (μm)	No. cells per aggregate	Width of cells (μm)	Flagella (no.)	Remarks
Lake Plußsee (Holstein)	June 1967	27	20.3	ND[a]	4 + 4	ND	1	T = 5.5°C; $O_2(-)$
		29	19.0	ND	4 + 3	0.31	0	T = 5°C; $O_2(-)$
		30	19.0	ND	12;6;8;4	ND	0	T = 5°C; $O_2(-)$; very numerous
		31	19.0	ND	4 + 2	ND	0	T = 5°C; $O_2(-)$
Little Lake (Wisconsin)	May 1975	12	19.5	ND	3 + 4	ND	1	T = 4°C; $O_2(-)$; actively motile
Wintergreen Lake (Michigan)	Aug. 1970	4.5	29.7	10.2	Numerous	0.23	0	$O_2(-)$
		4.5	19.5	5.1	4 + 4 (+4)	ND	0	$O_2(-)$
	July 1975	4.0	18.7	ND	4 + 4	ND	0	T = 19°C; $O_2(-)$; pH = 7.65; S^{2-}+; CH_4+
		5.5	19.3	ND	4 + 4 + 4	ND	0	T = 10.5°C; $O_2(-)$; pH = 7.25; S^{2-}+; CH_4+
		6.0	19.5	ND	4 + 4	ND	0	T = 10°C; $O_2(-)$; S^{2-}+; CH_4+
Forest Pond (Michigan)	July 1968	0.7	19.6	10.1	3 + 8	ND	0	T = 17.5°C; $O_2(-)$; $S^{2-}(+)$

[a]Not determined.
Data from P. Hirsch, unpublished observations.

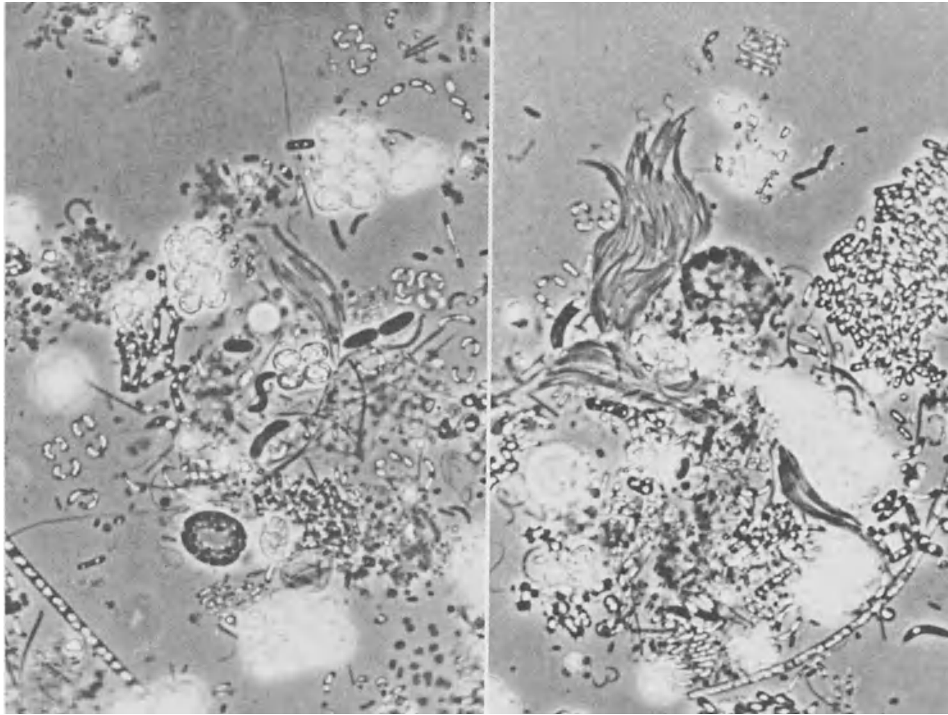

Fig. 1. A mixed bacterial flora with *Pelosigma palustre* cell aggregates from Wintergreen Lake (Michigan), collected from 4.5 m on 5 August 1970. × 1,280.

often flagellated on both ends), they are actively motile, and the individual cells measure approximately 0.4 μm in diameter (from the drawings of Warming, 1875). The aggregates of *P. palustre* normally show only one pointed end; they are much less actively motile, and flagella have not been seen. They occur in freshwater habitats.

The *Pelosigma* aggregates found by Skuja (1956) were thought to surround a colorless flagellate which multiplied by longitudinal fission. One to eight (usually four) colorless, S-shaped rods were kept by the flagellate "subpellicularly" with the ends sometimes sticking out. The aggregate (consortium) of bacteria and flagellate were described as *"Pelosigma cohnii"*; the overall sizes were 1–7 × 11–27 μm. The bacterial component was 0.3–0.4 × 7–15 (20) μm in size, colorless, without any granules, and sometimes yellowish pigmented. Cross-division was not synchronous with the longitudinal fission of the monad. The consortium did not have two flagella; the aggregates seen by Warming were likewise not supposed to have front and rear flagella—despite the fact that Warming (1875) mentions these and has actually drawn three double-flagellated aggregates (Table VII, Fig. 4a, 4c by Warming). The consortium was thought to be "oligothiophilic" and possibly involved in the decomposition of cellulose and/or hemicelluloses.

The aggregates described in Table 1 (Fig. 1) were, in general, 19.5 μm long and either 5–6 or 10–11 μm wide; thus, they resembled *P. palustre.* One sample (Wintergreen Lake, 4.5 m, August 1970) had much larger aggregates with thinner cells. None of the numerous aggregates seen contained anything that could have resembled a flagellate. Only two cases of flagellation and motility were seen; in both cases, the flagellum had the appearance of bacterial flagella, with a permanent, constant wavelength. Therefore, the author believes that such visible flagella constitute tufts of several prokaryotic flagella, attached polarly to each individual bacterium and combined to form the visible structure. Another argument against a eukaryotic flagellum would be the absence of two flagella from the "dividing" cell pole (as would be formed during longitudinal fission of a eukaryotic flagellate). Instead, the *one* "flagellum" (tuft) is located on the nondividing cell pole. Occasional bipolar flagellation (as has been observed by Warming) would be expected to occur in polarly flagellated bacteria. The transparent area in between the aggregate's bacteria is probably filled with an extracellular, common polymer. It is also possible that there exist, in nature, *Pelosigma*-like consortia of S-shaped bacteria

with colorless flagellates. Only observation of laboratory cultures and directly embedded and sectioned natural material could solve this problem. For the time being, it may be advisable to restrict the name *Pelosigma* to the bacteria that are agreed upon by all observers.

Acknowledgments

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CHAPTER 236

The Genus *Siderocapsa* (and Other Iron- or Manganese-Oxidizing Eubacteria)

HANS H. HANERT

This chapter discusses those species of *Siderocapsa* and members of the family Siderocapsaceae (Pribram, 1929) whose morphology, ecology, and to some extent, culture physiology, have been so exactly described that, in spite of disagreement concerning their taxonomic position, there can be no doubt of their existence and great ecological significance. The discussion concentrates on such existing and ecologically significant representatives of this family in order to preserve the practical orientation of this Handbook. Information on many genera and species whose position is uncertain has been drawn together in *Bergey's Manual of Determinative Bacteriology,* eighth edition (Buchanan and Gibbons, 1974; contributor G. A. Zavarzin) and seventh edition (Breed et al., 1957; contributor R. S. Breed in cooperation with H. Beger) and by Dorff (1934). A comparison of the genera and species treated in these references provides a bird's-eye view of this entire group of organisms.

The family Siderocapsaceae is defined as unicellular, nonfilamentous or nonstalkforming, iron and/or manganese bacteria that are able to deposit these metal oxides under natural conditions, on or in capsules or on extracellular mucoid material excreted by these bacteria. This definition is taxonomically rather imperfect, but for practical purposes it is quite adequate. That the validity of this family, in particular of the species of the genus *Siderocapsa,* has often been questioned is to be ascribed exclusively to the present taxonomic inadequacy of the definition. Such questions have been raised most recently by Dubinina and Zhdanov (1975); but a final clarification of the taxonomic question will not be possible until the complete developmental cycle of these bacteria in their natural habitat has been clearly described. Culture experiments are doubtless very helpful here, but they cannot provide a final clarification of the taxonomic question as long as the natural development of the organisms remains unknown.

Merely because there have been controversial questions of taxonomy it does not follow that these bacteria do not exist. Their existence and great ecological significance have become firmly established in the last decades through the discovery of their mass development in the hypolimnion of many lakes.

This paper is taken directly from the 1st edition of *The Prokaryotes.*

Habitats

Occurrence and Natural Mass Development

Siderocapsaceae are widely distributed in nature (see Table 1). They are found in all habitats which contain iron and manganese. They were first found in meadow and swamp ditches and mesosaprobic flowing streams, as well as in stagnant waters (Dorff, 1934; Drake, 1965; Galinsky and Hanert, 1979a; Hardman and Henrici, 1939; Molisch, 1910; Naumann, 1921). The organisms occur in these habitats as epiphytes on submerged water plants or on growths of exposed slides, or they are free-living and neustonic in metallic, glossy, iron oxide films on the water. A second important source of these organisms is deep wells, pipes, and waterworks, as described by Beger (1949) and by Hässelbarth and Lüdemann (1967). Greatest development of Siderocapsaceae has been found in the hypolimnion of lakes, often near growths of *Metallogenium.* At present, five species of the genus *Siderocapsa* (see Table 1) have been shown to be planktonic, not sessile, and so are not susceptible to slide growth (Kalbe, 1965; Redinger, 1931; Skuja, 1948, 1956; Wawrik, 1956).

The occurrence of Siderocapsaceae is not limited to typical freshwater habitats. As the investigations by Ten (1967, 1968, 1969) have shown, the family Siderocapsaceae *(Naumanniella polymorpha)* occurs in brown forest soils and iron-manganese crusts on soil-forming rocks as well. A marine *Siderocapsa* has been found in the iron bay of Palaea Kameni in Santorini, Greece (Hanert, 1981).

Table 1. Spectrum of Siderocapsaceae habitats and environmental parameters.

Habitats	Organisms	Environment	Physicochemical basic conditions inferred from natural occurrence or culture experiments
Iron-rich meadow and swamp ditches, flat pools (often stagnant) and mesosaprobic flowing streams	*Siderocapsa*		
	S. treubii	Typically epiphytic (attached to submerged roots and leaves of water plants; also ongrowth on slides; mesosaprobic waters)	Aerobic, organotrophic, pH 6.2–8.7
	S. major	Neustonic and epiphytic	As above
	S. monoica	Epiphytic	Aerobic tending to microaerobic, organotrophic
	Siderocystis		
	S. vulgaris	Ongrowth in the upper water layer	Aerobic, organotrophic, pH 6–7
	S. confervarum	Forming large iron aggregates on algae	Aerobic, organotrophic, pH 6–7
Wells, pipes, and waterworks	*Siderocapsa*		
	S. botryoides		
	Naumanniella		
	N. pygmaea	All found in cool waters of pipes and deep wells by Beger (1949)	Microaerobic, psychrophilic, organotrophic
	N. elliptica		
	N. minor		
	N. catenata		
	Siderobacter		
	S. gracilis		
	S. brevis		
	S. latus		
Lakes (planktonic forms)	*Siderocapsa*		
	S. anulata	Epilimnetic (water surface up to 6 m)	Aerobic, pH around 8.0, organotrophic
	S. coronata	Generally neustic, mass development at time of vernal thaw, also found at depths of 17–20 m	Aerobic to microaerobic, organotrophic
	S. arlbergensis	Epilimnetic in alpine pools with mass development at time of thaw	Aerobic, organotrophic, ferrous iron up to 4 ppm, pH 4.5–8.5
	S. eusphaera	Generally hypolimnetic in larger meso- and eutrophic lakes, at depths of 6–30 m, mass development	Microaerobic to aerobic, organotrophic
	S. geminata	Generally hypolimnetic, epilimnetic occurrence during vernal and autumnal circulation, at depths of 0–26 m, mass development in lakes	Microaerobic to aerobic, organotrophic
	Metallogenium		
	M. personatum	Hypolimnetic with mass development in the chemocline zone, where O_2 disappears, at an iron and manganese content of 1–5 ppm	Microaerobic, organotrophic
Soils, bottom deposits of water bodies, mud, iron manganese crusts on soil-forming rocks	*Siderocapsa* sp. (Ten, 1967)	Different soil types	Aerobic to microaerobic, organotrophic

(*continued*)

Table 1. *Continued*

Organisms	Environment	Physicochemical basic conditions inferred from natural occurrence or culture experiments
Naumanniella		
N. polymorpha	Iron-manganese crusts and brown forest soils	Aerobic to microaerobic, possibly mixotrophic by manganese oxidation
Siderococcus		
S. limoniticus	Mud horizons and bottom deposits of fresh waters	Microaerobic, neutral pH

In all habitats, the most characteristic feature of *Siderocapsa* is its capacity to form large masses of iron and/or manganese oxide. This mass development of Siderocapsaceae, like that of the classical stalk- and threadforming iron bacteria, is often visible to the naked eye. Such readily visible deposits usually develop during vernal and autumnal circulation or during vernal thaw in alpine lakes and pools; deposits have been reported from all planktonic lake Siderocapsaceae, as well as from some epiphytic *Siderocapsa* species (Sokolova, 1959). A yearly cycle of Siderocapsaceae development with vernal and autumnal maxima in swamp ditches has

Table 2. Procedures for Siderocapsaceae enrichment in natural waters and synthetic media.

Procedure	Procedure suitability and incubation time
A. Procedures using natural waters:	Procedure suitability and incubation time:
1. Maintenance of sample flasks (totally or 2/3 filled) at 4°C and room temperature without any stirring. Flask size: up to 1 liter. Modifications: addition of 1 mg $MnCO_3$/liter or 1 mg $FeCO_3$/liter.	These procedures with natural waters seem suitable for the majority of all known Siderocapsaceae
2. Test tubes filled with sample water underlayed by 1 ml natural sediment. Modification as above.	
3. 30- to 40-liter aquarium cultures adding 0.07 g yeast extract/liter and 1 mg $FeCO_3$/liter or 1 mg $MnCO_3$/liter (divided in 5 portions added in intervals of 2 days).	Incubation time: up to 3 weeks
B. Procedures with synthetic liquid media (frequently used with 0.1% agar):	
1. Yeast extract-$MnSO_4$ medium; Pringsheim (1949)—medium in modification of Tyler and Marshall (1967) and application according to Dubinina and Zhdanov (1975). Composition (%): yeast extract, 0.005; $MnSO_4$, 0.002; distilled water, 1 liter—in place of manganese, to the bottom of the test tubes iron oxalate or ferrous sulfide were added.	
2. Peptone-$MnCO_3$ medium; manganese-peptone media have been frequently used, see Schweisfurth, 1972. Composition according to Schmidt (1976) with addition of glucose (%): peptone, 0.00002; $MnCO_3$, 0.0002; glucose, 0.00002; distilled water, 1 liter.	Growth usually within 2–3 days
3. Yeast and beef extract–manganese–iron medium (according to Mulder and van Veen, 1963). Composition (%): $MnCO_3$, 0.2; $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.015; Difco yeast extract, 0.0075; beef extract, 0.1; cyanocobalamin, 0.005 mg/liter, Na-citrate-2-hydrate, 0.015; distilled water, 1 liter.	
4. Starch-$MnCO_3$ medium (Zavarzin, 1964—medium modified according to Dubinina, 1970). Composition (%): starch, 0.1; $MnCO_3$, 0.5 (freshly prepared); distilled water, 1 liter.	
C. Procedures with solid media for isolation: Media as B 1–4 with 1–1.5% agar in petri dishes.	Growth within 2 days; iron and/or manganese oxidation within 7 days

been demonstrated and quantified for *Siderocystis vulgaris* (Galinsky and Hanert, 1979b). Detailed measurements which give some impression of Siderocapsaceae mass development in nature were taken by Dubinina, Gorlenko, and Suleimanov (1973) in the meromictic lake Gek-Gel', using the membrane-filter method (filters stained with erythrosine and treated with potassium ferricyanide and hydrochloric acid). These determinations counted up to 60,000 cells/ml at a depth of 27–29.5 m in the zone of massive development of *Siderocapsa eusphaera* (distinct narrow zone below *Metallogenium* growth zone), and also demonstrated the gradient growth of this organism (see also Sorokin, 1968).

Aspects of Siderocapsaceae Physiology Deduced from Environmental Variables

The old idea that Siderocapsaceae are aerobic iron bacteria arose from their initial discovery in epiphytic, neustonic, and surface film habitats. More recent discoveries of Siderocapsaceae in deep wells, and especially in the hypolimnion of lakes, have forced the revision of this idea. *Siderocapsa treubii, Siderocapsa major,* and *Siderocapsa monoica* (which are probably only growth forms of one species—Drake, 1965; Hardman and Henrici, 1939) as well as *Siderocystis vulgaris, Siderocystis confervarum, Naumanniella neustonica,* and possibly *Siderocapsa anulata* (see Table 1) appear to be the only markedly aerobic species. All the other Siderocapsaceae (*Siderococcus limoniticus, Naumanniella polymorpha,* and the lake *Siderocapsa* species) tend to be microaerophilic. The oxygen content of the mass growth zone in lakes is at the level where oxygen disappears (narrow water layer at the chemocline: $O_2 < 1$ ppm, $rH_2 < 19$, Fe^{2+} 1–2 ppm, Mn^{2+} 1–5 ppm; Dubinina, Gorlenko, and Suleimanov, 1973). These natural mass growth conditions established for *Siderocapsa eusphaera* are very similar to those for *Siderocapsa geminata* (Schmidt, 1976), which also grows under extremely microaerobic conditions (see later, Fig. 2a-f). Generalizing, and including the macroscopic observations concerning the onset of a mass development of lake *Siderocapsa* described by the first observers (see Table 1), it seems that optimal Siderocapsaceae growth takes place at the beginning of a change from extremely reduced to oxidized conditions in a neutral-to-light alkaline environment.

Table 3. Enriched or presumably isolated Siderocapsaceae.

Organism	Enrichment or isolation procedure[a]
Siderocapsa	
S. treubii	Enriched: method A3 (Moldau-water resp. tap water + 0.1% $MnCl_2$ as epiphytic growth; Molisch, 1910).
	Enriched: method A3 (swamp-ditch water + 0.007% yeast extract + 0.0001% $FeCO_3$; growing as neustic form in the water surface layer).
S. eusphaera	Enriched: method B1; presumably isolated: on medium B1 with 1% agar by Dubinina and Zhdanov (1975).
S. geminata	Enriched: method A1; presumably isolated: on media B1-4 by Schmidt (1976).
Naumanniella	
N. neustonica	Enriched: method A3 (swamp-ditch water + 0.007% yeast extract + 0.0001% $FeCO_2$; growing as neustic form in the water surface layer).
N. polymorpha	Presumably isolated: on Beijerinck's medium.
	(K_2HPO_4, 0.05%; NH_4Cl, 0.05%; $MnCO_3$ or $Mn(HCO_3)_2$, 1%; agar, 2%); further on Aristovskaya's (1965) medium (ulminofulvate complex) and on iron ammonium salt of citric acid by Ten (1968, 1969).
Ferribacterium	
F. duplex	Enriched: modified method A2 underlayed with peat and iron bars, Brussoff (1916); presumably isolated: in iron ammonium citrate medium, Brussoff (1916).
Siderocystis	
S. vulgaris	Enriched: method A1 in 3-liter Erlenmeyer flasks 3/4 filled with swamp-ditch water; growing as rust-spot ongrowth on the flask walls at room temperature, H. H. Hanert (unpublished observations).
S. confervarum	Enriched: method A1 in 100-ml flasks filled to the top with swamp-ditch water and some algae threads, cultivated at room temperature and near a window (daylight).
Siderococcus	
S. limoniticus	Enriched: method A2, coupled with slide and capillary ongrowth, Dorff, 1934; Zavarzin, 1972; Kutuzova, 1974.
S. communis	Enriched: in ferrous ammonium citrate, Dorff, 1934.

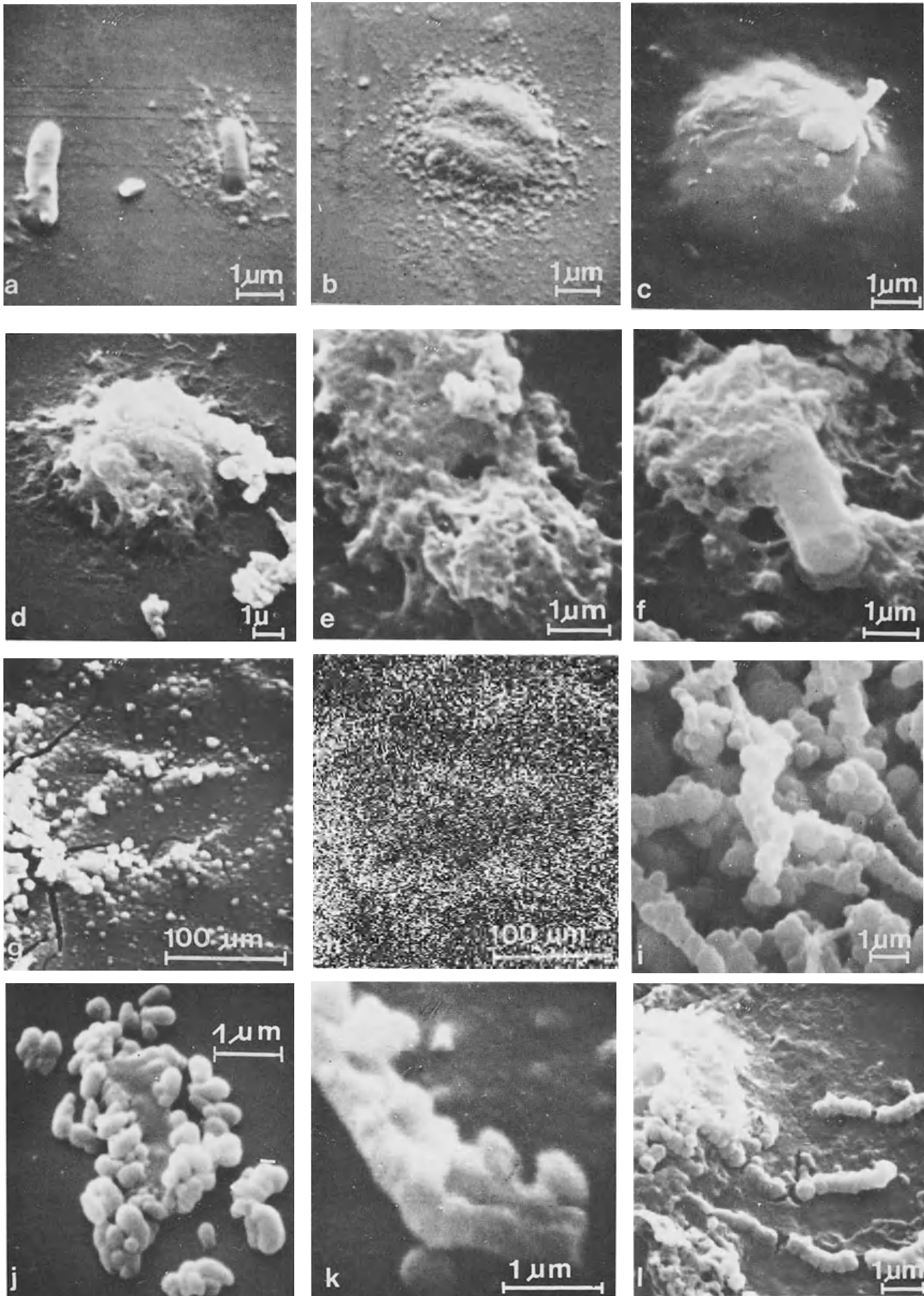
[a]See Table 2 for methods.

Table 4. Procedures necessary for identification of Siderocapsaceae.

Procedure	Methods for realization
1. Exact light-microscopic visualization of the bacterial cell and surrounding iron capsule	a. Phase-contrast observation: Yellowish iron capsule with light-refrangible central bacterial cell. b. Direct microscopic control of capsular ferric iron dissolution by adding 5% HCl to the edge of the cover glass (suspension preparations), immediately followed by carbolfuchsin staining of the central cell under microscope. c. Carbolfuchsin–Prussian blue double-staining (air-dried preparations): Prussian blue solution (70 ml 0.1% HCl + 30 drops 2% $K_4[Fe(CN)_6]\cdot 3H_2O$): 10 min (cuvette); washing with water (cuvette); 10 min into nonheated Ziehl-Neelsen carbolfuchsin (cuvette). d. Benzidine staining for manganese reaction: Material on slides or filters is first wetted with benzene, and 2 or 3 drops of 0.5% benzidine hydrochloride in 50% acetic acid are applied to the wet surface. After 1–2 s, the benzidine is removed with filter paper (Dubinina, Gorlenko, and Suleimanov, 1973). e. Fluorescence staining of the bacterial cell: air-dried preparations covered with a filtered FITC solution for 5 min (0.1 N Na_2CO_3 + 0.1 N $NaHCO_3$ in 3:2 proportion + 3.5 mg FITC per 10 ml)—washing with the same buffer. Very suitable for visualization of bacterial cells in rust-spot-forming bacteria.
2. Scanning electron microscopy and electron probe microanalysis	a. Stereoscan: Necessary for detection of the slimy nature of the capsule as well as the globular ferrihydrite particles in rust spots. Preparation in the usual way, placing a drop of material directly onto the specimen stub or using ongrowth method (specimens metal-coated). Figs. 1 (without 1h), 2a–d, and 2h–k. b. Electron-probe microanalysis for proof concerning iron or manganese incorporation of the capsules using X-ray analysis (area- or point-scanning). Figs. 1h, 2e, f, and 1.
3. Transmission electron microscopy	Realization in the usual way using formvar-coated grids (ongrowth method for sessile Siderocapsaceae; see also Kutuzova, 1974, 1975; Kutuzova, Gabe, and Kravkina, 1972); cell observation is better when capsular iron or manganese is removed by dissolving.
4. Direct microscopic observation of developmental cell cycle in micro-chambers or reverse light microscope	The possibility of pleomorphic development of Siderocapsaceae members makes it necessary to compare cell development in chambers filled with habitat water or culture media for identifying growing cells with the natural originals. This should be possible by directly proving stalk excretion from the apical cell in the way done for *Gallionella* (Hanert, 1974) or by using the reverse microscope (Schmidt, 1976, cell cycle of *Siderocapsa geminata;* see also Dubinina and Zhdanov, 1975, cell cycle of *Arthrobacter siderocapsulatus*).

A more essential difference between classical stalk- or threadforming bacteria and capsular iron bacteria appears to be the latter's obvious growth dependence on organic material. It appears to be no accident that Siderocapsaceae have not yet been found in iron springs or mineral water, very poor in organic substances (with the exception of certain results concerning very unsure forms obtained by Beger, 1949). From this standpoint, classifying Siderocapsaceae as a chemolithotrophic bacterial group (Buchanan and Gibbons, 1974) appears less justified and a physiologically indeterminate classification would correspond better to the facts

Fig. 1. Identification and differentiation of Siderocapsaceae cells, capsules, and ferrihydrite particles. (a–c) *Siderocapsa treubii,* in situ growth on slide in aquarium-natural water culture: (a) single cells at the initial phase of capsule formation; (b) two cells coated with slime and iron particles; (c) slime capsule completely coating the cell. (d–f) *Siderocapsa* sp. from the Siderocapsaceae zone in the marine iron bay of Palaea Kameni, in situ growth on slide, Hanert, 1981: (d) thread-slime capsule; (e) capsule with two holes; (f) cell half-coated with thread-slime capsular material. (g–i) Globular, chain-forming ferrihydrite particles on the slime surface of the rust-spot bacteria (formed in the marine bay): (g and i) ferrihydrite particles; (h) FeKα picture to (g). (j) Single cell of *Siderocystis vulgaris* surrounded by ferrihydrite particles. (k) Single cell of the marine rust-spot-former surrounded by ferrihydrite particles. (l) Slime capsule of the marine *Siderocapsa* sp. coated with ferrihydrite particles.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and would avoid invalid inferences. Until now, only *Naumanniella polymorpha* cultivated by Ten (1968, 1969) in Beijerinck's $MnCO_3$ medium appeared to have a certain probable capacity for mixotrophic growth by manganese oxidation.

For the other capsular iron or manganese bacteria, the natural conditions of the habitat suggest that the highest probability is organotrophic nutrition, possibly of the sort first postulated by Aschan (1907) (utilizing the organic portion of iron humates or other iron organic substances and precipitating ferric iron in or on the capsules).

Isolation

Enrichment

Table 2 lists suitable enrichment procedures for Siderocapsaceae. In particular, the procedures with natural water are extraordinarily simple, straightforward, and characteristic for the speedy enrichment of iron bacteria in general. In habitat-water samples maintained in cold storage or at room temperature without any stirring, a rapid growth of iron bacteria begins within a few days (often overnight). The growth can be recognized mostly by the formation of yellow to black-brown (iron and/or manganese oxides) flocs which sediment slowly. The Siderocapsaceae in general are no exception; nor, for example, is *Siderocapsa geminata,* observed in the hypolimnion of the "Plußsee" (Plön, Germany) and investigated by Schmidt (1976), among others. Our own samples from the same site behaved in the same way, and led within a week to an intensive development of *Siderocapsa geminata* in the sample flasks.

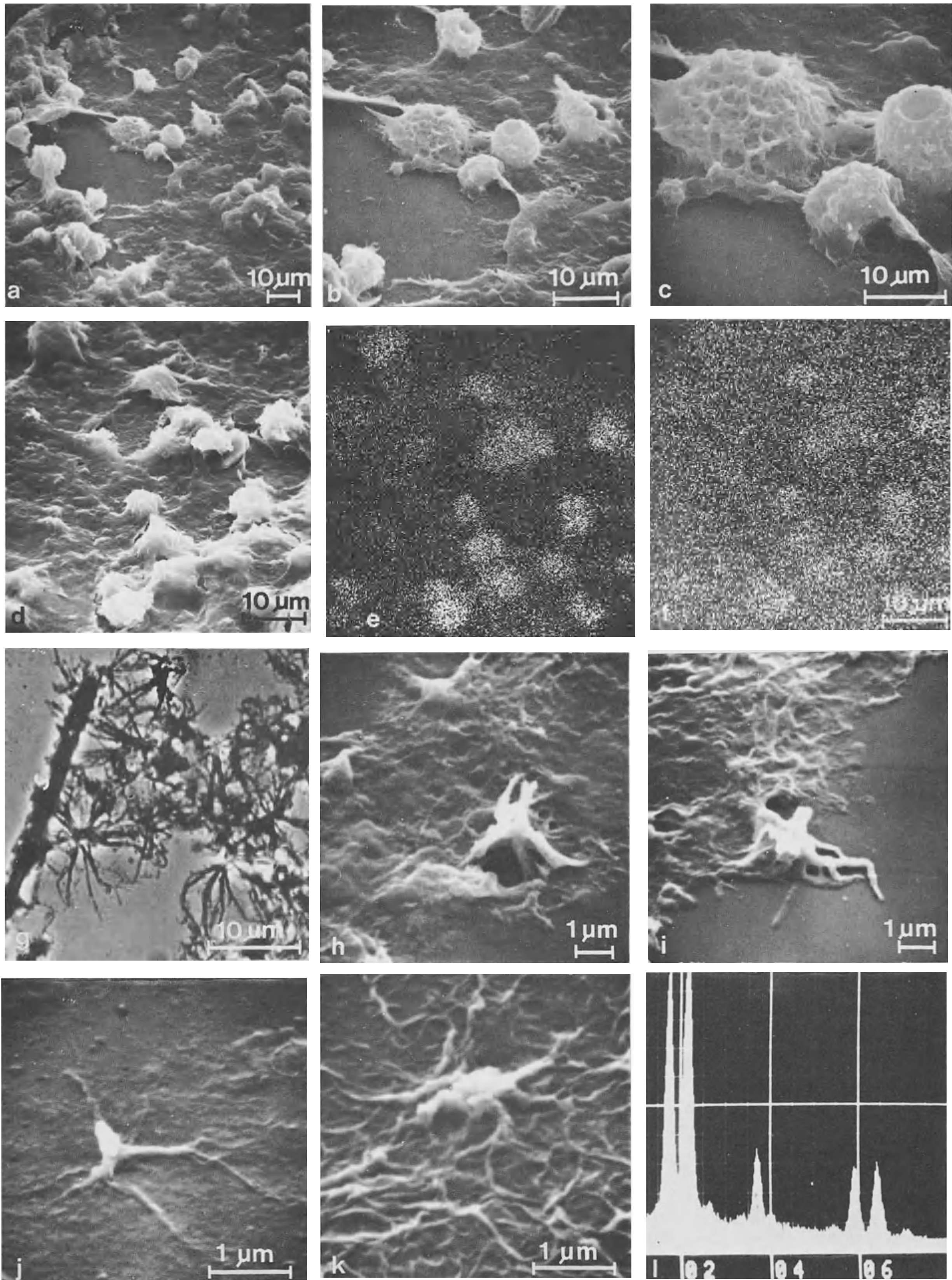
A second suitable enrichment method involves the use of 30- to 40-liter aquaria cultures with habitat water after the addition of 0.007% yeast extract and 0.0001% $FeCO_3$ or $MnCO_3$. At first, within 2 days after the culture start and before the addition of yeast extract and $FeCO_3$, thread-forming bacteria develop. Then, 7 days after the addition of $FeCO_3$ and yeast extract and the start of desulfurication, an intensive development of Siderocapsaceae takes place at the end of the desulfurication phase. The Siderocapsaceae form an ochreous layer, 2–3 mm thick, on the water surface. This method makes enrichment possible by more closely simulating natural conditions, particularly with *Siderocapsa treubii* and *Naumanniella neustonica,* which both form dense nests of bacterial cells. The first enrichment of a *Siderocapsa* strain, as carried out by the discoverer of this organism group, is fundamentally similar; *Siderocapsa treubii* was cultivated as an epiphytic growth on water plants in aquaria with the addition of 0.1% $MnCl_2$ (Molisch, 1910). Test tubes with sample water underlayed with natural sample mud were successfully used for the enrichment of *Siderococcus limoniticus,* and showed growth 3–4 days after the beginning of the culture (Dorff, 1934). The enrichment of *Siderocystis confervarum,* an organism which grows in direct contact with filamentous algae, also uses natural sample water.

In Table 2, section B, four synthetic media are presented which have proven to be particularly suitable for cultivating Siderocapsaceae. Yeast extract is particularly suitable as an organic carbon and nitrogen substrate, as is peptone and beef extract in weak concentration; iron and manganese are best given as carbonate or sulfate salts. On the basis of the presently available facts, in particular the culture experiments by Dubinina and Zhdanov (1975) and Schmidt (1976), as well as indications from the older literature (summarized by Schweisfurth, 1972), Siderocapsaceae can be regarded as organisms that grow well in weak concentrations of organic media, when cultivated in a neutral environment under aerobic to microaerobic conditions. Growth without oxygen appears to be impossible. Table 3 provides a bird's-eye view of all Siderocapsaceae that have been enriched so far.

Isolation

The isolation of Siderocapsaceae is much more a problem of identification than the application of particular isolation techniques. As Dubinina and her co-workers indicated for *Siderocapsa eusphaera* (1973, 1975), Schmidt for *Siderocapsa geminata* (1976), and Ten for *Nauman-*

Fig. 2. Iron/manganese capsules and slimes of *Siderocapsa geminata* and *Metallogenium personatum,* (a–f) *Siderocapsa geminata,* flocs grown in Plußsee sample flasks, three-quarters filled at a depth of 26 m in the natural habitat and maintained in cold storage at 4°C, where the *S. geminata* flocs formed in clear, natural habitat water: (a–d) capsules closed or with a hole; (e) MnKα; (f) FeKα picture to (d), proving simultaneous presence of manganese and iron, the latter at a much lower level. (g–l) *Metallogenium personatum,* flocs grown in habitat water-sample flasks from 4°C at a depth of 27 m, Plußsee: (g) light microscopic; (h–k) young *Metallogenium* organisms and thread-slime structure; (l) simultaneous manganese (MnKα, 5894keV) and iron (FeKα, 6398keV) evidence in the center of *Metallogenium* (k).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niella polymorpha (1968, 1969), pure cultures of these organisms could be conventionally produced without difficulty in liquid and solid media, using serial solution or plating techniques, respectively, (Table 3).

The main problem in isolating Siderocapsaceae is the question of relating the isolated organism with the Siderocapsaceae organism of the inoculated material from the natural environment. The isolated strains of *Naumanniella polymorpha, Siderocapsa eusphaera,* and *Siderocapsa geminata* show—as described by the authors—an extreme polymorphism and a quite complex cycle of development, whereas it has not yet been possible to demonstrate corresponding changes directly in the natural environment immediately from an individual object. This is one of the reasons why the task of identifying Siderocapsaceae has such crucial importance at the moment, and why isolation work cannot lead to really secure results so long as the individual developmental cycle remains unexplained.

Identification

Initial Identification

Siderocapsaceae are defined purely morphologically, without physiological features and the morphological definition provided by the people who first described them refers to their morphology in their natural environment or in habitat water cultures. This fact is the starting point from which all identification work has to proceed. The second important point to make is that the first morphological descriptions of the organisms corresponded to the then present state of microscopic technology; the descriptions used only light-microscopic photographs or drawings and the natural ultramorphology and ultrastructure remained unknown. *Siderocapsa anulata* is the only Siderocapsaceae which, at the time of its discovery, was described by means of an electron microscope (Kalbe et al., 1965) so that a clear picture of its morphology was produced which was completely adequate for purposes of identification.

The description which defines the morphology of the Siderocapsaceae contains three components: the bacterial cell, the capsule or excreted gelatinous material, and iron and/or manganese compounds deposited in or on the excreted material. The capacity to form capsule-like material has been recently shown for *Siderococcus limoniticus* (Kutuzova, 1974), which had previously been regarded as a non-capsule-forming Siderocapsaceae, so that this component appears to be common to all Siderocapsaceae. For the identification of Siderocapsaceae, the analysis of natural ultramorphology of these three components is of crucial importance, and thus, in virtue of new ideas concerning the pleomorphic development of these organisms, the analysis of the individual natural development must be added as a further new component.

Identification by Analysis of the Natural Ultramorphology and Development

The methods that are indispensable for a clear identification, which must be carried out with all Siderocapsaceae whose existence has been made certain by repeated finds, and ecological descriptions are summarized in Table 4. They relate to the visualization of the bacterial cell, the differentiation of cell and capsule or slime material, the ultramorphology of the capsular material, the specific evidence for the presence of iron and manganese, the ultramorphology of the iron deposits, and the analysis of the individual development under natural conditions.

LIGHT MICROSCOPY, STAINING, AND DIFFERENTIATION OF THE BACTERIAL CELL. In phase contrast microscopy, the central cells can only be distinguished from the surrounding capsule which contains metal oxide by their differing light reflection. This appearance can also be created by the shape of the capsule, and cell and capsule staining are indispensable. Carbolfuchsin and fluorescence staining have been found suitable for cell staining. Erythrosine staining has been applied just as successfully by other authors. In combination with the Prussian blue reaction for iron and the benzidine hydrochloride reaction for manganese, a differentiating double staining of the cell and metal-containing capsule is possible. Fluorescein isothiocyanate (FITC) fluorescence staining is especially suitable for the visualization and differentiation of the bacterial cells from iron/manganese oxides in rust-spot-forming bacteria, since the inorganic deposits are not stained (Hanert, 1981). A specific staining of the capsular polysaccharides has not yet been carried out, so that the capsule until now has only been made recognizable by staining the iron or manganese which it contains.

ULTRAMORPHOLOGY OF THE CAPSULE AND EXCRETED GELATINOUS MATERIAL. In all the Siderocapsaceae material which has been sampled in the natural environment or in habitat water cultures, the capsules showed a very characteristic slime-thread ultramorphology and struc-

ture, which can be recognized best with scanning electron microscopy. The capsules are slimy but, nevertheless, form casings which the bacteria vacate, leaving a noticeable hole when they depart. The bacteria produce this gelatinous thread slime, cover themselves with it—at this stage the bacteria cannot be recognized with a scanning electron microscope—and break through the capsule after a certain time. Even though this process cannot be observed in vivo, the electron microscope pictures allow scarcely any other inference. (See Fig. 1a-c, *Siderocapsa treubii;* Fig. 1d-f, *Siderocapsa* sp.; Fig. 2a-d, *Siderocapsa geminata* from flocs.) On the basis of this characteristic ultramorphology, it seems reasonable to think that the slime capsule might be a very characteristic feature of genuine Siderocapsaceae. It should be noted that the gelatinous slime of the Siderocapsaceae also resembles ultramorphologically those thread slimes which we have observed without the formation of capsules in *Metallogenium* habitat water cultures (Fig. 2g-k, *Metallogenium* flocs).

ULTRAMORPHOLOGY OF DEPOSITED IRON PARTICLES AND ELEMENT-SPECIFIC, X-RAY DETERMINATION OF THE PRESENCE OF IRON/MANGANESE IN THE CAPSULES IN COMBINATION WITH POSTULATED DEVELOPMENTAL STAGES. There has been intensive discussion concerning the existence of tiny mycoplasma-like developmental and budding stages in certain iron and manganese bacteria, for example, in *Gallionella* (Balashova, 1969), *Metallogenium* (Dubinina, 1970), and also in Siderocapsaceae (*Siderococcus limoniticus,* Kutuzova, 1974; Kutuzova, et al., 1974; *Naumanniella polymorpha,* Ten, 1968, 1969; *Siderocapsa geminata,* Schmidt, 1976). Evidence which has previously been presented in favor of this hypothesis has largely been pictures obtained by transmission electron microscopy which, however, show only tiny dense particles.

Relevant here are observations on rust-spot-forming bacteria (rust spots in the Palaea Kameni Bay, Hanert, 1981; *Siderocystis vulgaris* rust spots, Galinsky and Hanert, 1979a). The

Table 5. Siderocapsaceae which have clearly been shown to exist by repeated finds.

Organisms	Morphological features and characteristic ecology
Siderocystis	
S. confervarum	Large ovoid iron precipitations on *Conferva*-algae; iron-containing swamp ditches.
S. vulgaris	Forming rust spots up to 1 mm in diameter on exposed glass slides (Fig. 1j); iron-containing swamp ditches.
Naumanniella	Slim rods with a thin, sharply limited iron capsule in the form of a torus (marginal thickening); single and in short chains.
N. neustonica	Rod-shaped cells, including the torus, 1.8–3.3 by 4.9–10 μm; surface of iron-bearing well and swamp water.
N. polymorpha	Ellipsoid rods of 0.7–1.0 × 1.0–2.0 μm that form coccoid cells by budding and fission; thin capsules; manganese- not ferrous iron-oxidizing; colony-forming in manganese carbonate or manganese acetate agar; brown forest soils and rock crusts.
Siderococcus limoniticus	Cocci (0.2–0.5 μm in diameter) without capsules (recently, capsule-like formations in which iron-oxide deposition takes place have been found by Kutuzova, 1974); in mud horizons and zone growth in culture.
Siderocapsa	Coccus resp. ovoid or short rods, single or in groups in a very thick, slimy iron capsule.
S. treubii	(Including the intermediary form of *S. major* and *S. monoica* found by Hardman and Henrici, 1939 and Drake, 1965.) Coccoid cells of 0.6–1.6 μm in diameter and small rods 0.4–1.0 × 2 μm; single or in groups; most frequently of 6–8 up to 30 cells and more in mucoid capsules averaging about 2 μm by 4.5 μm with round, elliptical, or irregular outer edge surrounded by thick, rust-brown deposits of iron (light microscopic photographs by Hardman and Henrici, 1939).
S. anulata	Single coccus (0.2–0.5 μm in diameter) surrounded by sharply limited ferric iron ring (1.2–1.9 μm), excellent electron microscopic photographs by Kalbe, Keil, and Theile, 1965; planktonic and epilimnetic.
S. coronata	Two to eight cocci (maximal 1.2 μm) in one capsule (up to 24 μm in diameter); aggregating to coenobia of up to 90 capsules and a length of 400 μm (described and pictured by drawings; Redinger, 1931); in alpine lakes.
S. arlbergensis	Cocci (0.4–1.0 μm) normally in pairs in capsules (diameter 6–15 μm); coenobia similar to *S. coronata,* but single capsules not fusing; light-microscopic photographs by Wawrik, 1956; planktonic and neustic in alpine pools.
S. eusphaera	Coccoid to ovoid (1–2 μm) cells up to 60 in large capsules (regular spheres up to 50 μm in diameter); capsules occur singly (drawings by Skuja, 1948); planktonic and hypolimnetic in large lakes.
S. geminata	Ovoid cells (0.5 × 0.8 μm) usually in pairs in round capsules (7–11 μm in diameter); capsules occur singly (drawings by Skuja, 1956); planktonic and hypolimnetic in lakes.

iron oxides in these rust spots consist of tiny, spherical ferric iron particles which are often chainlike in form; these particles correspond to the iron bodies which have been analyzed as ferrihydrite by Tschukrov (1974). These particles consist exclusively of iron, which can easily be demonstrated by X-ray analysis (point-scanning) and dissolving in acids.

Another useful aid in structural analysis in this field is area-scanning electron microscopy in combination with element-specific scanning. The existence of both iron and manganese incorporation in the capsules can be indicated simultaneously (see Fig. 2d-f for *Siderocapsa geminata* and Fig. 2l for *Metallogenium*).

The methodological possibilities for analyzing natural individual development in vivo are represented in Table 4.

In summary, it should be emphasized that an adequate number of identification and culture methods are available for the investigation of Siderocapsaceae, which can be applied to those genera and species (Table 5) whose existence has been made certain. One of these forms is *Siderocystis confervarum,* which forms large, round, iron oxide aggregates on algae, and can be found in profusion in iron-bearing swamp ditches in the vicinity of Braunschweig. The application of the methods described here to this bacterium has resulted in the surprising discovery that this organism lives not only on the surface of the algae but also within it. This bacterium might have a lytic function for this algae, similar to those lytic bacteria which colonize on the hyphae of fungi in natural habitats (e.g., *Phytophthora cinnamomi;* Nesbitt, Malajczuk, and Glenn, 1978).

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CHAPTER 237

The Genus *Fusobacterium*

TOR HOFSTAD

The genus *Fusobacterium* includes several species of obligately anaerobic, nonsporeforming, motile or nonmotile, Gram-negative rods. Some are slender, spindle-shaped bacilli, others are pleomorphic rods with parallel sides and rounded ends. Their habitat is the mucous membranes of humans and animals.

During the last decade of the nineteenth century, several authors, among them Miller (1898), Plaut (1894), and Vincent (1896, 1899, 1904), observed spindle-shaped, or fusiform, bacilli in material from both the diseased and the healthy human mouth. Veillon and Zuber (1998), Lewkowicz (1901), and Ellermann (1904) were the first to cultivate fusiform bacilli. Loeffler, in 1884, observed pleomorphic rods in diphtheritic lesions of calves and doves. The same organism, identifiable with the *F. necrophorum* of today, was cultured by Bang (1890–1891) from necrotic lesions of a number of domestic animals, and by Schmorl (1891) from an epizootic in rabbits.

The more pleomorphic fusobacteria without tapering ends have been described under different generic names. Examples are *Bacteroides, Sphaerophorus, Bacterium, Necrobacterium, Pseudobacterium, Bacillus, Actinomyces, Corynebacterium, Ristella,* and *Zuberella.*

The family name Bacteroidaceae was first used by Pribram (1929) for strictly anaerobic rods. Ten years earlier, Castellani and Chalmers (1919) proposed that the genus *Bacteroides* should only contain obligately anaerobic bacilli that did not form spores. Eggerth and Gagnon (1933) and Weiss and Rettger (1937) excluded the Gram-positive rods from the genus. The generic name *Fusobacterium* was proposed by Knorr (1923) for obligately Gram-negative bacilli that were fusiform. Prévot (1938), who argued that the generic name *Fusobacterium* (and also the names Bacteroidaceae and *Bacteroides*) was invalid, used the term *Sphaerophorus* for the nonmotile, pleomorphic fusobacteria, and the term *Fusiformis* for the fusobacteria that had tapered ends. The seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957) divided the family Bacteroidaceae into three genera: *Bacteroides,* defined as rods with rounded ends; *Fusobacterium,* defined as rods with tapering ends; and *Sphaerophorus,* defined as rods with rounded ends that showed a market pleomorphism and where filaments were common.

Cell morphology therefore, had so far been the main criterion for the classification of the nonsporeforming, anaerobic rods. Physiological studies (Beerens et al., 1962; Werner, 1972a; Werner et al., 1971) and studies of DNA base ratios (Sebald, 1962) showed that there was insufficient evidence to separate the genera *Fusobacterium* and *Sphaerophorus.* In the eight edition of *Bergey's Manual* (Moore and Holdeman, 1974a) the genus *Fusobacterium* was restricted to anaerobic, nonsporeforming, Gram-negative rods which form butyric acid as a major endproduct from peptone or glucose (without isobutyric and isovaleric acids). Further, *Leptotrichia* (see Chapter 223) was reestablished as a genus for the saccharolytic fusiform bacilli producing lactic acid as the only major fermentation product.

Up to now, 12 human species of *Fusobacterium* have been adequately described (Table 1). The GC content of these species ranges from 26 to 34 mol%. In addition, *F. prausnitzii,* which has a GC content of 52–57 mol%, has been included in the genus. The species isolated most frequently from humans and animals are *F. nucleatum* and *F. necrophorum,* respectively. *F. simiae* is a new species which has been isolated from the mouth of the stumptailed macaque (*Macaca arctoides*) (Slots and Potts, 1982).

Habitats

As Normal Microbiota of Humans and Animals

All *Fusobacterium* species are parasites of humans and animals. Anaerobic Gram-negative rods with the same morphological and bio-

Table 1. The main human sources of *Fusobacterium* isolates.

Species	Normal flora		Clinical specimens
	Mouth	Gastrointestinal tract	
F. nucleatum	+		+
F. necrophorum		+	+
F. alocis	+		
F. gonidiaformans		+	+
F. mortiferum		+	+
F. naviforme	+		+
F. necrogenes		+	
F. periodonticum	+		
F. prausnitzii		+	
F. russii		+	
F. sulci	+		
F. ulcerans			+
F. varium		+	+

Adapted from Moore et al. (1984), Slots et al. (1983), Cato et al. (1985), and Adriaans and Shah (1988).

chemical properties as *Fusobacterium* species have also been isolated from the hindgut of the cockroach *Eublaberus posticus* (Foglesong et al., 1984) and the gastrointestinal tract of the grass carp *Ctenopharyngodon idella* (Trust et al., 1979).

The main human habitats of the different *Fu-sobacterium* species are listed in Table 1.

F. nucleatum is a constant member of the oral microflora of adults, but has also been isolated from the oral cavity of predentate children (Hurst, 1957; McCarthy et al., 1965). The principal habitat of *F. alocis* and *F. sulci* is the human gingival sulcus. The incidence of *F. periodonticum* in the human oral cavity is unknown. Occasionally, *F. naviforme* has been found in the mouth or the upper respiratory tract (Holdeman et al., 1977). The isolation of *F. necrophorum* from pleuropulmonary infections suggests that this organism is also able to live as a parasite on the mucous membranes of the oral cavity and upper respiratory tract of humans. The number of fusobacteria per milliliter of saliva has been estimated to be 5.6×10^4 (Richardson and Jones, 1958). In different surveys, fusobacteria have been found to make up from 0.4 to 7% of the cultivable dental plaque flora (Hardie and Bowden, 1974). There are, however, great individual variations. In patients with different forms of periodontitis, *F. nucleatum* is a predominant member of the subgingival plaque flora (Williams et al., 1976; Moore et al., 1982, 1983). Hadi and Russel (1969) reported a mean viable count of *F. nucleatum* per gram wet weight of gingival plaque material from patients with advanced chronic periodontal disease and acute ulcerative gingivitis of 3.3×10^7 and 9.3×10^7, respectively. In subjects with healthy gingivae, the corresponding figure was 5.7×10^6.

Fusobacterium makes up a small part of the fecal microflora of man, with individual variation ranging from about 7% to less than 1% of the cultivable fecalflora (Finegold et al., 1974; Finegold et al., 1975; Holdeman et al., 1976; Moore and Holdeman, 1974b; van Houte and Gibbons, 1966). The most prevalent species seem to be *F. prausnitzii, F. russii,* and *F. mortiferum.* Both the number of fusobacteria in feces and the relative frequency of the different species are influenced by the diet (Finegold et al., 1974; Maier et al., 1974; Peach et al., 1974). Thus, Japanese on a traditional diet rich in carbohydrate have a relatively high number of *F. necrophorum* in feces (Ohtani, 1970a; Ueno et al., 1974).

The occurrence of fusobacteria on the mucous membranes of the genitourinary tract is virtually unknown. Fusobacteria were not present in the normal microflora of the cervix of 30 healthy females examined by Gorbach et al. (1973), and Hite et al. (1947) found no fusobacteria in the healthy vaginas of pregnant women. Fusobacteria, particularly *F. necrophorum,* were, however, present in the vagina of pregnant women with trichomoniasis and in the postpartum uterus of such women. Spaulding and Rettger (1937) found *F. nucleatum* in the normal vagina but not in the vagina of pregnant women. Davis and Pilot (1922) and Brams et al. (1923) isolated fusiform bacilli (and spirochetes) from the clitoris region in females, and from preputial secretions of 50 out of 100 men.

The habitat of *F. ulcerans* is unknown. The organism has been isolated from tropical ulcers (Adriaans and Drasar, 1987) and from a few specimens of mud (Adriaans and Shah, 1988).

Tropical ulcer is a form of skin ulceration predominantly affecting children and is most commonly in the tropics (Robinson and Hay, 1985)

F. necrophorum is a normal inhabitant of the alimentary tract of cattle, horses, sheep, and pigs. Fuller and Lev (1964), in a study of the Gram-negative bacteria of the pig alimentary tract, found *F. necrophorum* to be present from the age of 43 days. Aalbæk (1972) isolated the organism from the colon of pigs in numbers up to 10^3 per gram of wet material, but in considerably higher number in the ileus, cecum, and colon of pigs with experimental enteritis. *F. necrophorum* has also been found in infections and in feces of other animals, such as mules (Nolechek, 1918), goats (Jensen, 1913), reindeer (Horne, 1898–1899), antelope (Mettam and Carmichael, 1933), macropods (Bang, 1890–1891; Samuel, 1983), wildebeest (Grainer, 1983), dogs (Jensen, 1913), rabbits (Cameron and Williams, 1926; Schmorl, 1891), rats (Lewis and Rettger, 1940), chickens (Jensen, 1913), and apes (Dack et al., 1935; Dack et al., 1937). It has also been reported in buffaloes, cats, guinea pigs, mice, snakes, tortoises, and fowl (Simon and Stovell, 1969; Weinberg et al., 1937).

Less is known about the presence in animals of the other *Fusobacterium* species. Fusiform bacilli, probably *F. nucleatum,* have been isolated from the alimentary tract of pigs (Aalbæk, 1977) and mice (Syed, 1972), and from the oral cavity and the throat of monkeys (Krygier et al., 1973; Pratt, 1927; Slanetz and Rettger, 1933), dogs (Slanetz and Rettger, 1933), cats (Prèvot et al., 1951), rabbits (Pratt, 1927; Slanetz and Rettger, 1933), and guinea pigs (Pratt, 1927; Spaulding and Rettger, 1937). Terada et al. (1976) isolated *F. necrogenes* and *F. mortiferum* from pig feces, and *F. perfoetens* has been found in piglet feces (van Assche and Wilssens, 1977). *F. necrogenes* is a member of the cecal flora of poultry (Holdeman et al., 1977). *F. russii* is a member of the normal oral flora of cats (Love et al., 1987) and has been mentioned as being part of the normal microflora of mice and pigs, as well as of the rumen flora of cattle (Smith, 1975).

As Pathogens of Humans and Animals

Next to members of the "*Bacteroides fragilis* group" and the black-pigmented bacteroides, *F. nucleatum* is the Gram-negative anaerobic organism most often encountered in human infections. Also, *F. necrophorum* is clearly pathogenic in humans. Before the advent of antibiotics and other antimicrobial drugs, this organism was frequently isolated from suppurative infections of the oral cavity and the upper respiratory tract, and from pleuropulmonary infections. Reviewing the literature concerning anaerobic pleuropulmonary infections, Finegold (1977) found that *F. necrophorum* accounted for 24% of all anaerobic bacteria isolated from 358 cases. Today the organism is less commonly isolated from human infections. *F. nucleatum* is usually found associated with other anaerobic and/or facultative organisms. On a percentage basis, *F. nucleatum* and *F. necrophorum* have been isolated in pure culture from pyogenic infections more frequently than have other anaerobic bacteria (Beerens and Tahon-Castel, 1965; Werner and Pulverer, 1971; Bartlett et al., 1974). However, when in mixture with other organisms the recovery of fastidious strains of *F. nucleatum* and *F. necrophorum* may fail. Other species of *Fusobacterium* are occasionally isolated from clinical specimens, and nearly always in mixed culture.

Pathogenic fusobacteria are in particular isolated from inflammatory processes accompanied by necrosis and ulceration. They are most frequently found in head and neck infections, pleuropulmonary infections, abscesses of the brain and the liver, and in infections following human and animal bites. *Fusobacterium* species are also recovered from subcutaneous and soft-tissue abscesses, obstetrical and gynecological infections, and abdominal abscesses. Because of variations in nomenclature and anaerobic culture technique, the incidence of *Fusobacterium* infections is difficult to determine from the literature. In a series of 15,844 clinical specimens submitted over 12 years (1973–1985) to the microbiological laboratories in two military hospitals, *Fusobacterium* species accounted for 4% of all anaerobic isolates (Brook, 1988). *F. nucleatum* was the most common *Fusobacterium* species (47% of all *Fusobacterium* species). The incidence of fusobacteria was somewhat higher in a 10-year series of anaerobic isolates from hospital patients (Sutter et al., 1985). *F. nucleatum* and *F. necrophorum* may also be encountered in blood cultures, particularly when the upper respiratory and the female genital tract are the portals of entry (Felner and Dowell, 1971; Henry et al. 1983). *Fusobacterium* species may be a cause of chorioamnionitis (Altshuler and Hyde, 1985). *F. nucleatum* is invariably present in Plaut-Vincent's angina (fusospirochetal angina) and in acute necrotizing ulcerative gingivitis.

F. necrophorum is the cause of human necrobacillosis. This is a rare, but life-threatening septicemia which predominantly affects healthy young adults. The infection is characterized by sore throat, followed by rigor and the formation

of abscesses, usually involving the lung (Lemierre, 1936; Moore-Gillon et al., 1984).

F. necrophorum is an animal pathogen that is frequently isolated from necrotic and gangrenous lesions in cattle, sheep, and pigs, and less frequently from other animals. Carnivorous animals appear to be resistant. The most common manifestations of diseases associated with *F. necrophorum* are liver abscess (hepatic necrobacillosis) and footrot.

Liver abscesses are especially encountered in heavily fattened cattle. Ninety % or more of such abscesses contain *F. necrophorum* as the only organism or in combination with other organisms (Hussein and Shigidi, 1974; Kanoe et al., 1976; Newsom, 1938; Simon and Stovell, 1971; Berg and Scanlan, 1982). The disease is associated with inflammation of the forestomach, presumably caused by irritating substances produced by fermentation of the high-caloric feed or by foreign bodies (Jensen and Mackay, 1965). *F. necrophorum* present in the stomach contents is thought to gain entry to the vascular system through the injured mucosa. Liver abscesses have thus been produced experimentally in cattle and sheep by intraportal injections of viable cells of a bovine isolate of *F. necrophorum* (Jensen, et al., 1954). Hepatic necrobacillosis in feedlot cattle may present an economic problem in meat-producing countries (Langworth, 1977; Panel report, 1973). Footrot is frequently encountered in sheep and cattle. The disease affects the epidermal tissues of the interdigital skin and hoof and leads to separation of the hoof from the soft tissues. Lameness and morbidity from infection lead to a reduction in productivity and economic losses. Footrot in sheep is caused by *Bacteroides nodosus* in combination with *Fusobacterium necrophorum*. *B. nodosus* is the principal causative agent transmitting the disease from one animal to another (Beveridge, 1941), whereas *F. necrophorum* is essential for the later inflammatory destruction of tissue (Egerton et al. 1969; Roberts and Egerton, 1969). Injury to the foot and damp soil are predisposing factors (Graham and Egerton, 1968). Effective protection against ovine footrot is achieved through immunization with killed, fimbriated *B. nodosus* cells (Egerton and Roberts, 1971; Stewart et al., 1982).

The primary cause of epizootic footrot in cattle has not been found. *F. necrophorum* is present in the lesions as a concurrent pathogen or a secondary invader. Typical lesion have ben produced in cattle by the intradigital or intradermal inoculation of *F. necrophorum* alone or in combination with black-pigmented bacteroides (Berg and Loan, 1975; Clark et al., 1985).

Another disease associated with *F. necrophorum* is calf "diphtheria," which is necrotic laryngitis that occurs in calves up to 2 years of age. *F. necrophorum* is also involved in several other suppurative or gangrenous processes in domestic animals, such as interdigital dermatitis and heel abscess in sheep (Parsonson, et al., 1967; Roberts et al., 1968), neonatal bacteremias in calves and lambs, necrotic enteritis of pigs, necrotic rhinitis of growing pigs, and oral infections in several animals.

Necrobacillosis have long been known as a serious cause of death of macropods, mainly in zoological collections, but also in wild habitats. The body sites most commonly affected are the face, the stomach wall, and the hind limbs. There is good evidence that *F. necrophorum* is the main etiological agent of the disease, but other organisms, especially *Bacteroides* species, are often also present in the lesions in high numbers (Oliphant et al., 1984; Samuel, 1983). *Fusobacterium* species are the predominant species of the mixed flora in soft tissue infections of cats caused by contamination from the oral flora (Love et al., 1980).

Virulence Determinants

The natural infections have verified the infectivity and invasiveness in animals of *F. necrophorum* and in man of *F. nucleatum* and *F. necrophorum*. Experimental investigations in animals have shown that synergistic mechanisms may be of importance in the pathogenesis of mixed infections involving fusobacteria (Brook and Walker, 1986; Hamp and Mergenhagen, 1963; Hill et al., 1974; Kaufman et al., 1972; Onderdonk et al., 1976; Roberts, 1967a, b).

Our current knowledge of virulence determinants is cursory. Fimbriation has been seen in *F. necrophorum* (Shinjo and Kiyoyma, 1986), but not in *F. nucleatum* or other *Fusobacterium* species. Encapsulation has been observed in *F. nucleatum* and *F. necrophorum* (Brook, 1986; Emery 1988).

F. necrophorum isolates from cattle and sheep have been assigned to biovars A and B, and to an intermediate type, AB, on the basis of cultural characteristics (Fievez, 1963). A and AB biovars are frequently isolated from lesions, are highly virulent in mice, hemolytic, and produce a hemagglutinin. B biovars are of low virulence in mice and nonhemolytic. The agglutinin is associated with the cell wall, has a subunit molecular weight of 19,000, and is heat labile (Nagai et al. 1984). Certain strains of *F. necrophorum* produce a leukocidin which is also destructive for erythrocytes and a variety of cul-

tivated cells (Coyle-Dennis and Lauerman, 1978; Fales et al., 1977; Garcia et al., 1975a; Ishii et al., 1988; Roberts, 1967a, b; Scanlan et al., 1982). The toxin is possibly a phospholipase (Abe et al., 1979). High amounts of leukocidin is produced by A and AB biovars of *F. necrophorum,* indicating a correlation between toxin production and virulence. (Coyle-Dennis and Lauerman, 1979; Emery and Vaughan, 1986; Scanlan et al. 1982).

A hemagglutinin is also produced by *F. nucleatum* (Dehazya and Coles, 1980; Falkler and Hawley, 1977). *F. nucleatum* and *F. necrophorum,* as well as other *Fusobacterium* species, posses a cell wall lipopolysaccharide with the characteristics of an endotoxin (Garcia et al., 1975; Hofstad and Kristoffersen, 1971; Sveen et al., 1977; Warner et al., 1975).

Physiology

Fusobacterium species are not particularly demanding with regard to a low oxidation-reduction potential. The maximum E_h value permitting growth varies, depending on the species, the size of inoculum, and the medium. Stock strains of *F. nucleatum* were able to grow in an oxygen tension of up to 6% (Loesche, 1969). Fusobacteria are, however, fairly readily killed by exposure to air. This is possibly due to their susceptibility to hydrogen peroxide and is especially noticeable when thioglycolate or cysteine HC1 is incorporated into media that are exposed to air before inoculation and incubation.

Fusobacteria grow readily on ordinary solid media, such as Brucella blood agar and brain heart infusion agar, and in fluid media with a base of peptone and yeast extract. Proteose peptone, tryptone, and trypticase have better growth-promoting effects than casamino acids (Gharbia and Shah, 1988a).

Energy is obtained from peptides and amino acids, which are fermented to give a mixture of butyric and acetic acids. Peptides represent the most important energy source (Gharbia et al., 1989). Lysine is likely catabolized by the 3-keto, 5-aminohexanoate pathway (Barker et al., 1982). Glutamate may be degraded through different pathways (Gharbia, 1987). *F. necrophorum* has an absolute need for protein-containing polypeptides (Wahren and Holme, 1973). Most *Fusobacterium* species convert threonine to proprionate. Lactate is converted to proprionate by *F. necrophorum.*

All the *Fusobacterium* species are either nonfermentative or only weakly fermentative. All species examined are able to utilize glucose, which is incorporated into cellular components (Robrish et al., 1987; Gharbia and Shah, 1988a). The accumulation of glucose is dependent upon energy supplied by fermentation of amino acids (Robrish et al., 1987). A low terminal pH (seldom lower than pH 5.5) in glucose-containing media indicate that, in at least a few species, some glucose is fermented.

Glutamate dehydrogenase is produced by all *Fusobacterium* species. The activity at different pH values and the electrophoretic mobility of the enzyme differ between species or groups of species (Gharbia and Shah, 1988b). Possibly all fusobacteria produce deoxyribonuclease (Porschen and Sonntag, 1974). *F. necrophorum* is able to deconjugate bile salts (Shimada et al., 1969).

The fusobacteria are susceptible to many of the commonly used antibiotics, including penicillins and cephalosporins. They are, however, resistant or relatively resistant to vancomycin, neomycin, and erythromycin. *F. nucleatum* may produce beta-lactamase. *F. varium* and *F. mortiferum* are resistant to rifampicin (rifampin). The growth of these two organisms is not inhibited by bile, to which other species of *Fusobacterium* are susceptible. Along with several other Gram-negative bacteria, the fusobacteria will grow in the presence of low concentrations of various dyes.

Isolation

Sampling

Fusobacterium nucleatum is best isolated from saliva or centrifuged salivary deposits, or from the crevice or pocket that exists between the gingiva and the tooth surface. Sampling from the crevice area is performed by the use of sterile filter paper points (absorbent dental points), which are gently inserted into the crevice. Saliva or salivary deposits may be inoculated into the medium either directly or after being resuspended in a reducing diluent, such as the serum-containing diluent of Bowden and Hardie (1971) or in prereduced anaerobically sterilized (PRAS) one-fourth-strength Ringer solution (Sutter et al., 1985). The inoculated tapering end of the paper point is streaked on a small area of the solid medium, and further spreading of the deposited material is carried out by a wire loop. Because of their presence in small numbers, isolation of other *Fusobacterium* species from their natural habitat in man can be difficult. Detailed directions for collection, transport, and processing of fecal specimens have been given by Sutter et al. (1985). It is essential

that the specimens are thoroughly homogenized and adequately diluted in a reducing diluent before inoculation. This applies also to the isolation of *F. necrophorum* from the intestinal tract of animals.

Isolation Under Nonselective Conditions

When present in clinical specimens, *Fusobacterium nucleatum* and *F. necrophorum* and the less commonly isolated *Fusobacterium* species are usually recovered on solid nonselective media. If the colonies are carefully inspected, the Gram stain is properly used and if subculturing is performed promptly, isolation is usually straight forward.

Nonselective isolation of fusobacteria from their natural habitats on the mucous membranes in man and animals is laborious and time-consuming. Such isolation attempts should be avoided in those instances where isolation on selective media is possible. However, isolation under nonselective conditions seems to be the most reliable method for examination and quantitation of viable cells in normal flora specimens. For this purpose the roll tube method (Holdeman et al., 1977; Moore, 1966) or the use of a glove box (Aranki et al., 1969) is to be recommended. By inoculating roll tubes with 1 ml each of 10^8, 10^9, and 10^{10} dilutions of homogenized feces, bacterial species—including fusobacteria—present in numbers as low as 3×10^{10} per gram of fecal dry matter (0.06% of the fecal bacterial population), were counted (Moore and Holdeman, 1974).

Selective Isolation

Media formulations have been developed and evaluated for selective isolation of fusobacteria from human (Baird-Parker, 1957; Morgenstein et al., 1981; Ohtani, 1970b; Omata and Disraely, 1956; Sutter et al., 1971; Walker et al., 1979) and animal (Fales and Teresa, 1972a) sources.

The following medium of Morgenstein et al., (1981) is recommended for the isolation of *Fusobacterium* species from their natural habitats and from clinical specimens.

Fusobacterium Egg Yolk Agar (FEA) Medium
(Morgenstein et al., 1981; Sutter et al., 1985)

Brucella agar base	37 g
Disodium phosphate	5 g
Monopotassium phosphate	1 g
Magnesium sulfate	0.1 g
Hemin solution (5 mg/ml)	1 ml
Polysorbate 80	1 ml
Neomycin solution (100 mg/ml)	1 ml
Distilled water	1,000 ml
Vancomycin solution (7.5 mg/ml)	0.67 ml
Josamycin (Yamanouchi Pharmaceuticals)	3 mg
Egg yolk emulsion (Difco)	50 ml
(or Egg yolk emulsion) (Oxoid)	74 ml

The vancomycin, the josamycin and the egg yolk emulsion are added to the medium after adjustment of pH to 7.6, dissolving by boiling, autoclaving at 121°C for 15 min, and cooling to 50°C.

The FEA medium is selective because of its content of antibiotics. Addition of egg yolk makes it differential for *F. necrophorum,* which is lipase positive. All species of *Fusobacterium* grow on the medium with only minimal inhibition. The typical colonial morphology is translucent to white, convex, round, and entire colonies. *Leptotrichia* grows with white, raised, and granular colonies, and *Veillonella* forms small and translucent colonies. The growth of a majority of facultative Gram-negative organisms is inhibited. Gram-positive organisms do not grow on the medium.

The CVE medium of Walker et al. (1979) may be used in addition to the FEA medium for isolation of *F. nucleatum* from the oral cavity.

Crystal Violet Erythromycin Agar (CVE) Medium
(Walker et al., 1979)

Trypticase	10 g
Yeast extract	5 g
Sodium chloride	5 g
Glucose	2 g
Tryptophane	0.2 g
Agar	15 g
Crystal violet	5 mg
Distilled water	1,000 ml
Sterile defibrinated sheep blood	50 ml
Erythromycin (dissolved in small volume of 95% ethanol)	4 mg

The blood and the erythromycin are added to the medium after adjustment of the pH to 7.0–7.2, autoclaving at 121°C for 20 min, and cooling to 50°C.

The selectivity of the CVE medium depends on its content of erythromycin and crystal violet. Strains of *F. nucleatum* exhibit one of two different colony types on CVE. Some strains form transparent, smooth, round, blue colonies having an entire edge with a darker blue center. Other strains form transparent, rounded or irregular, blue colonies with a speckled internal appearance. Several species of Gram-positive and Gram-negative organisms grow on the medium, but with a colonial morphology distinguishable from those of *F. nucleatum.*

The medium of Sutter et al. (1971) is selective for *F. varium* and *F. mortiferum* and can be used for isolation of these species from feces or from other sources. The selectivity of this medium

depends on the addition of rifampin to standard blood agar.

Rifampin Blood Agar (Sutter et al. 1971; Sutter et al., 1985)

A total of 50 μg/ml of rifampin is added to Brucella blood agar just before the plates are poured. Rifampin stock solution: 0.1 g of rifampin is dissolved in 20 ml of absolute ethyl alcohol, and 80 ml of sterile distilled water is added. This gives a final concentration of 1,000 μg/ml. The solution can be stored at 4°C for up to 2 months.

F. varium and *F. mortiferum* grow freely on this medium, while the growth of *Bacteroides* and most other organisms present in human feces in high numbers is inhibited.

A medium selective for the isolation of *F. necrophorum* from bovine liver abscesses was reported by Fales and Teresa (1972a). The medium is based on the trypticase and egg yolk medium of McClung and Toabe (1947) and contains crystal violet and phenethyl alcohol as selective agents.

Isolation Medium for *Fusobacterium necrophorum* (Fales and Teresa, 1972a)

To 415.0 ml of distilled water add the following:

Trypticase	16.0 g
Biosate	4.0 g
Thiotone	2.0 g
Glucose	0.5 g
$MgSO_4$ (5% solution)	0.1 ml
Na_2HPO_4	2.5 g
Agar	8.3 g

Adjust to pH 7.3.

After autoclaving, the basal medium is cooled to 50°C, and 1.35 ml (0.27% vol/vol) of phenethyl alcohol is added. One egg yolk mixed with an equal volume of a 0.9% sterile saline solution (total volume, approximately 45.0 ml) and blended with the basal medium, and then 11.5 mg of crystal violet dissolved in 25.0 ml of sterile distilled water is added. Finally, the volume is adjusted to 500 ml with sterile distilled water.

Small colonies of *Proteus* species appearing on the medium are easily distinguished from the larger colonies (1.5–1.7 mm in diameter after 48 h of incubation) of *F. necrophorum.*

As previously mentioned, the various selective media designed for isolation of *Fusobacterium* species allow other organisms to grow to a varying extent. In order to gain experience with these media it is important, therefore, to check the different colony types by Gram-staining.

Axenic Cultivation and Maintenance

Fusobacterium strains can be maintained by weekly serial subcultures on blood agar. Viable cells can be stored at −70°C in Greave's solution. Stock cultures can also be prepared in skim milk (Sutter et al., 1985).

Greave's Solution

Bovine serum albumin	50 g
Sodium glutamate	50 g
Glycerol	100 g
Distilled water	1,000 ml

Batch cultivation is best performed in a nutrient broth with a tryptone base, which is supplemented with yeast extract (0.3%), glucose (0.25%), and cysteine HCl (0.1%), or in the selective media bases. If narrow-necked, well-filled containers are used, PRAS media are usually not necessary.

F. necrophorum has been grown in continuous culture with glucose as the growth-limiting factor (Wahren et al., 1971). Maximal cell yields (3.5 mg/ml dry weight) were achieved at dilution rates between 0.19 h^{-1} and 0.40 h^{-1}, at a pH of 6.8, and at temperatures of 33–36°C.

Identification

All *Fusobacterium* species are susceptible to colistin and kanamycin, resistant to vancomycin, and produce butyric acid without isobutyric or isovaleric acids. In the clinical diagnostic laboratory identification is based on both cell and colony morphology and on biochemical properties. Chemotaxonomic methods, such as DNA-DNA hybridization, lipid analysis, electrophoretic mobility of glutamate dehydrogenase, outer membrane protein patterns, and peptidoglycan composition, are useful for taxonomical purposes.

Morphology

Fusobacterium nucleatum has a characteristic cell morphology that makes presumptive identification relatively easy. The cells are Gram-negative, slender, spindle-shaped bacilli with sharply pointed ends, often appearing in pairs and end-to-end. Most cells are 5–10 μm long, but both shorter and longer rod forms may be seen. *F. periodonticum* has a similar cell morphology. This organism has, however, not been isolated from clinical specimens. The fusiform cells of *Capnocytophaga* species are generally smaller than those of *F. nucleatum.* Those of *Leptotrichia buccalis* are thicker and usually larger (distinguishing characters are given in Table 1 of Chapter 223). The cells of *F. necrophorum* are pleomorphic, often curved, and may have spherical enlargements. Free coccoid bod-

Table 2. Differential characteristics of *Fusobacterium* species encountered in clinical specimens.

Characteristic	*F. nucleatum*	*F. necrophorum*	*F. gonidiaformans*	*F. naviforme*	*F. mortiferum*	*F. russi*	*F. varium*
Growth in 20% bile	−	−	−	−	+	−	+
Production of							
Gas from PYG[a]	−	+	+	−	+	+	+
Indole	+	+	+	+	−	−	+
Lipase	−	+	−	−	−	−	−
Hydrolysis of esculin	−	−	−	−	+	−	−
Propionate from							
Lactate	−	+	−	−	−	−	−
Threonine	+	+	+	−	+	−	+
Fatty acids from PYG	Acetic, propionic, butyric; sometimes formic, lactic, succinic	Acetic, propionic, butyric; sometimes lactic, succinic	Acetic, propionic, butyric; sometimes formic, lactic, succinic	Acetic, butyric, lactic; sometimes formic, propionic, succinic	Acetic, propionic, butyric; sometimes formic, lactic, valeric, succinic	Acetic, butyric, lactic; sometimes formic	Acetic, propionic, butyric, lactic; sometimes succinic

+, positive reaction for majority of strains; −, negative reaction for majority of strains.
[a]PYG, peptone-yeast extract-glucose broth.

ies and, especially, filaments are common. *F. naviforme* strains may have boat-shaped cells. Gonidial forms may be seen in old cultures of *F. gonidiaformans*. The other *Fusobacterium* species have no distinctive cellular morphology.

Colonies of *F. nucleatum* on blood agar are low convex, glistening, and slightly irregular in form. Those of *F. necrophorum* are circular, rough, and often β-hemolytic. The other *Fusobacterium* species form smooth, small- to-punctiform colonies on blood agar after incubation for two days.

Biochemical Properties

Differential characteristics of the most common *Fusobacterium* species are shown in Table 2. In addition, *F. periodonticum* hydrolyzes hippurate; nitrate is reduced by *F. ulcerans;* the pH may be slightly lowered in cultures of *Fusobacterium* species grown in carbohydrate-containing media, but also in cultures grown without added carbohydrate. This makes the interpretation of fermentation reactions difficult.

Commercial multitest systems for identification of bacteria are generally associated with a percentage of misidentifications, which may be either system or laboratory dependent. Experience is limited with respect to identifications of *Fusobacterium* species.

DNA probes for identification of *Fusobacterium* species are not available. The fluorescent antibody technique has been used for identification of fusobacteria in clinical specimens from man (Griffin, 1970; Stauffer et al., 1975) and for *F. necrophorum* in bovine liver abscesses (Fales and Teresa, 1972b). Simon (1975) has described a hemagglutination inhibition test for rapid identification of *F. necrophorum.*

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