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Advances in
MICROBIAL PHYSIOLOGY

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OBITUARY

Anthony H. Rose

Tony Rose, who was born in Birmingham on 18 July 1930, died suddenly at a squash club in Bath on 10 June 1993, just a few months before his planned retirement at the end of 1993.

Tony's formal training was completed at Birmingham University where he was awarded a First Class honours degree in Applied Biochemistry (1950) and a PhD in 1954. The title of his thesis was *Growth of a yeast and a lactic-acid bacterium in mixed culture*. Following Birmingham, Tony worked at the Institute of Microbiology at Rutgers University, USA (1954–1955), then the Microbiology Division of Biosciences, National Research Council of Canada (1957–1958), and he spent two years as an Education Officer in the Flying Training Command of the Royal Air Force.

On his return from Canada, Tony held teaching appointments at several institutions, Heriot-Watt College (1958–1961), King's College, University of Durham (1961–1963), University of Newcastle-upon-Tyne (1963–1965). In 1968 he was appointed to the newly created Chair of Microbiology at

the University of Bath, an institution that had only recently evolved out of the Bristol College of Advanced Technology. Over the next quarter of a century, Tony unleashed his boundless energy on four main tasks: teaching and research, publishing, administration and enjoyment!

Many generations of undergraduates will no doubt entertain memories of Tony the lecturer, whose clear exposition of microbial physiology was achieved without recourse to notes. If a student failed to grasp some cogent point of a lecture, he or she had access to a classic textbook, *Chemical Microbiology* which Tony published (Academic Press) in 1965 with subsequent editions in 1968 and 1971. A great number of postgraduate students and post docs will recall also their efforts in the Zymology laboratory, the lunch-time meetings at which their work was discussed and the many rehearsals of papers that they were to present to audiences outside the University. Their efforts, principally on the physiology of yeasts, resulted in the publication of upwards of 100 papers in learned journals. This flow of primary papers was complemented by the publication of more than 60 review articles, again with aspects of yeast physiology being key topics.

The publishing facet of Tony's endeavours began during his time at Heriot-Watt with the publication (1961) of *Industrial Microbiology* (Butterworths, London). Over the next 30 years, he edited, and often contributed to, 20 books which included an eight-volume series published by Academic Press on *Economic Microbiology* (1977–1983), a three-volume series on *The Yeasts* in 1969 and a six-volume series on the same theme (1987–1994). *The Yeasts* volumes, of which J.S. Harrison was co-editor, have become accepted universally as standard books.

Of course many will be convinced that Tony's main contribution to the promulgation of microbiology to a wide audience was achieved through *Advances in Microbial Physiology*, the first volume of which appeared in April 1967. Over the next 26 years, 36 volumes of this series have been published. Over this time span Tony collaborated with several editors – John Wilkinson, Gareth Morris and Dave Tempest – but since 1991 he worked alone on what must have been a demanding task.

As the Professor of Microbiology in the School of Biological Sciences at Bath University, Tony had from time to time to acquiesce to suggestions from the Vice Chancellor that the time had come for the School to have a new head. Tony undertook this onerous chore between 1973 and 1976 and again between 1981 and 1984. The zenith of his University administration came in 1988–1989 when he held a post that would be called Dean in most other British universities. Outside of the University, Tony played a leading role as its first Secretary General (1974–1980) in establishing the Federation of European Microbiological Societies.

Tony's interest in *Saccharomyces cerevisiae* was far from being a detached, academic pursuit. Over the years he acquired an encyclopaedic knowledge of alcoholic beverages in general and wines in particular. Indeed his knowledge of wine led him to become a widely respected taster and selector of wines for the Senior Common Room at the University of Bath and other bodies.

The moving ball proved to have a life long fascination for Tony. Throughout his career he was a regular spectator at soccer matches and a dyed-in-the-wool supporter of West Bromwich Albion FC. Additionally he played squash with an enthusiasm and fervour that matched his academic endeavours.

At this time many microbiologists worldwide will be saddened by the untimely death of a colleague who did so much to promote microbiology and their thoughts will undoubtedly encompass compassion for his wife, Jane, and their three children.

R. G. BOARD
SCHOOL OF BIOLOGICAL SCIENCES
UNIVERSITY OF BATH

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I. Introduction

There has been no attempt to produce a comprehensive review of peptide transport by micro-organisms for over ten years. Prior to that, several reviews presented a balanced description of transport and utilization of peptides by micro-organisms. From this nutritional standpoint, the essential, complementary role played by intracellular peptidases was also considered. In addition, attention was devoted to the particular opportunities conferred by peptide uptake compared with amino-acid transport, and to the advantages in possession of both types of systems. Overall, these reviews presented a largely phenomenological description of a developing research area (Payne, 1975, 1976, 1980d; Payne and Gilvarg, 1978; Matthews and Payne, 1980).

The present review is restricted to consideration of the process of peptide transport in micro-organisms. Reflecting the main thrust of the intervening period, it concentrates on a molecular approach to the subject. Thus, it attempts to provide an integrated view of advances in understanding of the structures of transport components, their molecular mechanisms, synthesis and assembly, energetics and regulation. Application of this fundamental knowledge to exploitation of peptide permeases in the design of peptide-based antimicrobial compounds is also considered. Only when felt important for a balanced discussion is material covered in earlier reviews presented here. However, problems in adopting this approach need to be recognized. For, as G. K. Chesterton might have said, the disadvantage of not knowing the past is that you cannot fully understand the present.

II. Occurrence of Peptide-Transport Systems in Nature

When this aspect was addressed in the most recent review on peptide transport (Matthews and Payne, 1980), the authors felt the need to persuade their readers of the special benefits that can accrue to organisms from possession of peptide-transport systems. At that time, there were still some workers who found it difficult to appreciate a need for mediated

peptide transport, when amino-acid transport systems were so well recognized; although, we hasten to add, such individuals were to be found in areas associated mostly with medicine and mammalian physiology rather than amongst microbial physiologists. However, even if some observers of Nature had been slow to appreciate the benefits of peptide transport, Nature itself had long recognized its virtues and, by 1980, peptide-transport systems were known to occur not only amongst prokaryotic and eukaryotic micro-organisms but also in a range of animal cells and in higher plants. Today, there is no longer the need to proclaim the advantages of mediated peptide transport, for the ensuing decade has seen an explosion in studies related to this topic. These studies have revealed, across a spectrum of biological species, extensive conservation of structural and functional features, within and between peptide systems and related transporters and channels. If a plea be needed today, it is that workers pursuing studies on this topic in various species (micro-organisms, animals and plants) make themselves better aware of each other's literature; all too often results are presented seemingly in ignorance of other publications that have addressed identical questions in analogous systems.

In line with the above view, brief reference will be made here to peptide transport in non-microbial systems. The most extensive studies have been performed on the brush-border membrane of the intestine (reviewed in Matthews and Payne, 1980; Matthews, 1985). In a standard reference to this area, completed just before his death, Matthews (1991) presented in book form an integrated view of the role of peptide transport in protein absorption by mammals. In this book, the following conclusions were presented. In the intestinal mucosal cell, transport of di- and tripeptides is carrier mediated through a common pathway (Addison *et al.*, 1975a, b). There is no firm evidence that higher peptides can be absorbed, although peptides of varied composition and structure are substrates. Uptake is coupled directly to a proton gradient that is generated by a Na^+/H^+ antiporter, which helps to explain the common observation that peptide uptake shows a dependence on sodium ions. Matthews (1991) states that: "While it is plain that the hypothesis that the brush border membrane contains only one transport system for all di- and tripeptides is no longer tenable, ... I think that any temptation to premature classification should be resisted in the present state of knowledge. At this stage it seems virtually impossible to construct any coherent transport scheme . . . ." Notwithstanding these reservations, further clarification is important, not least because other studies have demonstrated that the intestinal peptide-carrier system(s) is also used by a variety of compounds, many of which are clinically important; for example, inhibitors of the aspartic-protease renin (Kramer *et al.*, 1990) and of the angiotensin-converting enzyme, such

as captopril and enalopril, also β-lactam antibiotics with a free α-amino group, such as cefatrizine, cephalexin and amoxycillin, and dipeptidyl derivatives of α-methyldopa (Bai *et al.*, 1991; Kramer *et al.*, 1992). It is clear that, in the rational design of orally-effective drugs (Section XI), characterization of the intestinal peptide carrier will play a crucial role. To this end, a protein of M_r 127 k, identified following photo-affinity-labelling by β-lactam analogues, has been purified and functionally reconstituted into liposomes (Kramer *et al.*, 1992). In complementary studies, uptake of the β-lactam cephalexin by a dipeptide carrier has also been demonstrated in a human intestinal cell culture (Dantzig and Bergin, 1990). Extensive studies into the renal handling of peptides have also led to the identification of a transport system(s) in the kidney (Tiruppathi *et al.*, 1991), which shares many characteristics with the intestinal system(s) (Matthews, 1991).

Another area of great current interest is transport of amino acids and peptides in the central nervous system (Rakic *et al.*, 1988; Banks *et al.*, 1991). Application of concepts developed in other systems to the mediated bidirectional passage of natural neurotransmitters and opiates, together with other peptido-compounds, across the blood–brain barrier, should prove especially rewarding (Banks and Kastin, 1990, 1992).

In summary, therefore, peptide permeation mechanisms are demonstrably of considerable importance in several mammalian organs, and they also function in red-blood cells and a number of other cells and tissues (Matthews, 1991). Comparative aspects of this topic are considered further in Section XII.

In higher plants, peptide transport has been reported to occur in several tissues and species; the early literature was reviewed by Higgins and Payne (1982). However, the only well-characterized example of active peptide transport is in seeds. During seed germination, nitrogen reserves, mainly present as storage proteins in the endosperm, are hydrolysed and transferred to the growing embryo for early synthesis of shoots and roots. In monocotyledons, this transfer occurs across the scutellum, a specialized absorptive tissue abutting the endosperm, which shows many features in common with the intestinal mucosa. A scutellar peptide-transport system has been extensively characterized in terms of substrate specificity, kinetic parameters, energy coupling, and development and regulation, by Sopanen and his co-workers (Sopanen, 1984; Sopanen and Vaisanen, 1985; Salmenkallio and Sopanen, 1989) and by Payne and his colleagues (Higgins and Payne, 1982; Walker-Smith and Payne, 1985; Payne and Walker-Smith 1987). Recently, protein components of the peptide carrier have been isolated, following photo-affinity labelling with a dipeptide analogue (Hardy and Payne, 1991). An integrated review of amino-acid and peptide transport in higher plants has recently appeared (Hardy and Payne, 1992).

Thus, it can be concluded that micro-organisms are not the only species that have evolved to benefit from peptide permeases. Although it may seem obvious that enteric micro-organisms such as *Escherichia coli* will have systems that allow them to capitalize on their ecological niche of the peptide-rich lumen of the gut, it is an example from the extremes of microbial physiology that provides perhaps the final word on the exceptional value of peptide utilization. A hyperthermophilic, sulphur archaebacterium, *Hyperthermus butylicus*, which grows in sea water at up to 108°C, ferments peptides as carbon and energy sources (Zillig *et al.*, 1990). Thus, the evolutionary antiquity of peptide uptake would appear to be established. At present, the molecular basis of transport in this bacterium is not known but, in another species of archaebacteria, evidence has been presented for the presence of a system belonging to the large family of transporters of which microbial peptide permeases are members (Miyauchi *et al.*, 1992) (Sections VI and XII).

III. Mechanisms of Peptide Transport

Earlier reviews considered several hypothetical schemes for peptide transport in a variety of membrane systems. Ones specifically relevant to micro-organisms were discussed in Payne and Gilvarg (1978) and Payne (1980a). Several schemes envisaged the involvement of specific membrane peptidases. These were considered to bind peptides on the outside of the cytoplasmic membrane and, either independently (by a group-translocation mechanism) or through further involvement of associated carriers, to deliver the constituent amino-acid residues in free form into the cell. Today such schemes seem largely redundant. There appear to be no aspects of peptide transport that are not explained most simply through operation of energized transport systems that bind peptides and translocate them intact into the cell, where they subsequently undergo hydrolysis. Thus, of the previous conceptual models, the simplest appears to be the one that occurs.

What is surprising in reconsidering these earlier models is their paucity of molecular detail. It is in this regard that major advances have been made in the last decade. The multiprotein components of several peptide permeases have been sequenced and their primary role in the transport complex determined. Information on their topology within and across the membrane, and their spatial relationship with one another has been obtained. Conserved elements and 'loops', 'spanners' and 'motifs', which are proposed to play specific roles in recognition, binding, information transfer, translocation and energy coupling, have been identified. It is now

possible to construct models with a large measure of true molecular detail, and this aspect is considered further in Section VI. However, in principle, it still remains a possibility that peptide transport could occur through a simpler protein system energized by an ion gradient (analogous to the *E. coli lac* permease); time will tell.

Further progress requires multidisciplinary inputs from molecular biologists, biochemists, biophysicists and others in order to refine further current models and to obtain a clearer description of the molecular events occurring during membrane transport. Here, as in a number of other current areas of research in physics, chemistry and biology, the trend is towards the emergence of unifying principles. Thus, there is increasing debate as to whether distinctions between transporters and channels are not more semantic than mechanistic. We can pose the question: Do the two represent specifically evolved members of a (anticipated) continuum of membrane transport (and information transfer) systems, that share fundamental structural and functional principles? This topic is addressed again in Section XII.

IV. Influence of the Microbial Cell Envelope on Peptide Uptake

Microbial transport systems involved in uptake of exogenous peptides (and other nutrients) are located in the cytoplasmic membrane. In virtually all micro-organisms this membrane is surrounded on its outer surface by a complex cell envelope or wall structure that is important for protection from surface-active agents and in maintaining cell structure. The nature of the cell wall varies from species to species, but when present it can act as a permeability barrier to potential nutrients.

A. GRAM-NEGATIVE BACTERIA

The cell wall of Gram-negative bacteria is composed of an outer membrane and a peptidoglycan layer. The outer membrane is a bilayer, containing in the outer monolayer a unique lipopolysaccharide component that affords protection against detergents, hydrolytic enzymes and certain antibiotics. The structure and function of the outer membrane have been reviewed (Lugtenberg and Van Alphen, 1983; Nikaido and Vaara, 1985; Nakae, 1986). Although this membrane fulfils an important role in exclusion of certain molecules, it must at the same time allow passage of nutrient molecules and of waste products.

1. Outer-membrane Proteins (Porins)

In 1968, Payne and Gilvarg (1968b) demonstrated that the cell wall in *E. coli* acts as a molecular sieve for peptides. By studying growth of amino-acid auxotrophs on members of several series of homologous peptides, a size limit of about 650 Da (typically a penta- or hexapeptide) was found, above which growth on a peptide could not occur. This exclusion limit was shown to be dependent on the overall volume (Stokes radius) of the peptide, rather than the number of amino-acid residues. Subsequent work confirmed a similar exclusion limit for *Salmonella typhimurium* of about 600 Da for saccharides and polyethyleneglycols (Nakae and Nikaido, 1975; Decad and Nikaido, 1976). The molecular sieving property of the cell wall was subsequently found to reside in the outer membrane, and to result from the presence of water-filled diffusion channels, formed by proteins known as porins. These outer-membrane proteins (Omp) are described in detail in the reviews already referred to and also in Benz (1988) and Nikaido (1992).

The OmpC and OmpF proteins are the main porins of *E. coli* and form channels of 1.1 and 1.2 nm diameter, respectively. Their expression is differentially regulated by osmolarity (Stock *et al.*, 1989; Mizuno and Mizushima, 1990) and temperature; thus, OmpF is preferentially synthesized under conditions of low osmolarity and low temperature. These two proteins possess significant amino acid sequence homology, both with each other and with PhoE (Mizuno *et al.*, 1983), a porin whose expression is derepressed under conditions of phosphate starvation. Many porins, including those already referred to, form stable trimers (Nakae *et al.*, 1979) with each monomer possessing a single pore. It has been found that outer-membrane lipopolysaccharide is required for trimerization of OmpF porin *in vitro* (Sen and Nikaido, 1991). It has been suggested for some porins that the three pores of a trimer merge on the inner side of the outer membrane to form a single channel (Engel *et al.*, 1985; Weiss *et al.*, 1991). However, recent work has shown that this is not so for OmpF (Cowan *et al.*, 1992). Porins OmpF and OmpC produce relatively non-specific diffusion channels, although the size, charge and hydrophobicity of a molecule can affect its diffusion rate through them. These channels have been shown to be weakly cation-selective, whereas the PhoE porin produces an anion-selective channel, hence its role in phosphate transport (Benz *et al.*, 1985). Crystal structures have recently been obtained for OmpF and PhoE (Cowan *et al.*, 1992). These proteins are unusual membrane proteins since they are predominantly composed of β-sheets. They consist of a 16-stranded antiparallel β-barrel, containing a pore. One of the loops connecting the strands folds into the pore and forms a constriction zone within it. This

zone is important in determining the size-exclusion limit and ion-selectivity of the pore. Indeed, site-directed mutagenesis of one of the residues in this loop, substituting residue Lys-125 with Glu, converts PhoE to a cation-selective channel (Bauer *et al.*, 1989).

The role of porins in peptide uptake by *E. coli* has been studied by Andrews and Short (1985) and by Alves *et al.* (1985), using various porin-deficient mutants. Andrews and Short (1985) studied the effect of *ompf* and *ompc* mutations on the sensitivity to toxic tripeptides, and also the ability of peptides to act as the sole source of a required amino acid. Strains with mutations in *ompf ompc* were unable to grow on any peptide tested, and were fully resistant to all toxic tripeptides tested, implicating these porins in permeation of peptides across the outer membrane with no evidence for a peptide-specific porin. Some differential specificity for tripeptides was shown, whereby some tripeptides used both OmpF and OmpC and others used only OmpF. Alves *et al.* (1985) measured kinetic parameters for peptide uptake in mutants deficient in OmpF and OmpC, by using continuous peptide-transport assays (Section V). These studies indicated the greater importance of OmpF for peptide uptake. Mutational loss of OmpA protein was also found to produce a significant decrease in outer-membrane permeability to peptides. Subsequently, OmpA has also been shown to form pores. It has recently been purified and reconstituted in proteoliposomes (Sugawara and Nikaido, 1992). The pore formed by OmpA is of a similar size to those of OmpF and OmpC, although diffusion of L-arabinose through it is about 100-fold slower than in these porins. No evidence was obtained that OmpA forms an amino acid- or peptide-selective pore.

Porins exclude some important compounds from the cell, which necessitates synthesis of alternative substrate-specific channels, e.g. *E. coli* LamB for maltose and maltodextrins, and Tsx for nucleosides. The outer membrane of enteric bacteria also contains energy-dependent transport systems for vitamin B_{12} and iron–siderophore complexes. These systems require a functional TonB protein, a cytoplasmic-membrane protein that couples energy to uptake across the outer membrane.

Porins have been identified in many other Gram-negative bacteria (Nakae, 1986; Jeanteur *et al.*, 1991). Of particular interest are the porins of *Pseudomonas aeruginosa*, since this species possesses considerable intrinsic resistance to many antibacterial compounds (Section VII.B). The main, non-specific porin of this species appears to be OprF (Nikaido *et al.*, 1991), which is functional as a monomer, and has amino-acid sequence homology with the OmpA protein from *E. coli*. In common with OmpA, OprF plays a role in maintenance of cell structure by virtue of cross-linking to the peptidoglycan. Porin OprF forms a channel of about 2 nm diameter

that allows diffusion of saccharides with molecular weights of up to 3000 (Hancock *et al.*, 1990). Although the pore of this protein is about twice the diameter of the OmpF and OmpC porins in *E. coli*, diffusion of solutes through it proceeds at a rate about 100-fold slower (Nikaido, 1992). Suggested explanations for this discrepancy are that either only a small fraction of the proteins form open channels or that there is high friction between solutes and the pore (Nikaido *et al.*, 1991). Recent studies using intact cells have sought to explain the anomaly (Bellido *et al.*, 1992). *Pseudomonas aeruginosa* also possesses several substrate-specific porins including OprB (glucose), OprP (phosphate) and OprO (polyphosphate and tripolyphosphate (Hancock *et al.*, 1990, 1992). Of particular relevance here is the OprD protein, originally identified through its role in uptake of the β-lactam antibiotic imipenem. Uptake of this compound was found to be competitive with basic amino acids and lysine-containing dipeptides (Trias and Nikaido, 1990). Tri- and tetrapeptides also inhibited imipenem uptake but only when they possessed a carboxy-terminal lysyl residue. Porin OprD forms channels by trimeric aggregation; within each monomer two domains have been identified, one showing channel-forming activity and the other acting as a gate or cap to control access to the pore (Yoshihara and Nakae, 1992).

2. *The Periplasm*

Between the outer membrane and the cytoplasmic membrane is a region known as the periplasm. This includes the peptidoglycan layer, which is covalently linked to the outer membrane through interactions with OmpA protein and lipoprotein (Braun, 1975). Within the periplasm there is a collection of proteins important to many transport systems, including peptide permeases (Section VI). Relatively little is known about the structure of the periplasm. It has been estimated to account for 20–40% of the total cell volume in *E. coli* (Stock *et al.*, 1977), although there has been some debate over the size of this compartment (Ferguson, 1990). Van Wielink and Duine (1990) proposed a width of 50 nm, whereas Graham *et al.* (1991a) measured it as being 10–25 nm. It seems likely that different bacterial species may have different sized periplasms, which may relate to their reliance on periplasmic activities, e.g. periplasmic redox carriers. It has been proposed that the size of the periplasm may also vary with the metabolic activity of the cell (Graham *et al.* 1991a). It is necessarily iso-osmolar with the cytoplasm, and this parameter appears to be regulated by synthesis of membrane-derived oligosaccharides consisting of 8–10 glucose residues (Kennedy, 1982). These molecules were found to regulate porin activity measured by patch-clamping of reconstituted outer

membranes such that, in low osmolarity medium, synthesis of the oligosaccharides is increased leading to a closing of porin channels (Delcour *et al.*, 1992).

From early electron microscope work, peptidoglycan was assumed to form a discrete layer in the periplasmic space. The use of more sophisticated techniques led to the proposal that the polymer may actually fill the entire periplasm (Hobot *et al.*, 1984; Labischinski *et al.*, 1991). Cross-linking of peptidoglycan was proposed to be greater near the outer membrane, decreasing possibly to zero near the cytoplasmic membrane. This model yields a periplasm consisting of a viscous gel 13 nm thick with a high water content and large pores. This theory is consistent with the observations of Brass *et al.* (1986), who measured the mobility of fluorescently-labelled periplasmic proteins. Lateral diffusion of these proteins was about 1000-fold slower than that in aqueous solution. Not all species appear to possess a periplasmic gel; for example, *Caulobacter crescentus* has a peptidoglycan layer about 5 nm wide, compared with the periplasmic gel in *E. coli* about 14 nm wide (Graham *et al.*, 1991b). Other work has indicated that the periplasm may be compartmentalized as a result of the tight contact between the outer membrane and the cytoplasmic membrane at regions known as periseptal annuli (Foley *et al.*, 1989). The exact relevance of periplasmic structure to solute uptake in general, and to peptide transport in particular, is not known.

B. GRAM-POSITIVE BACTERIA

The cell wall of Gram-positive bacteria is considerably simpler than that in Gram-negative bacteria. In the former bacteria, the cytoplasmic membrane is overlaid by polymers consisting of peptidoglycan and teichoic or teichuronic acids. Further layers of either polysaccharide or protein may also be present, depending on the species and strain. Gram-positive bacteria lack a periplasm, although the structures described above may produce an extracellular matrix serving some of the functions of this Gram-negative component. This view is supported by the finding of solute-binding proteins attached to the cytoplasmic membrane by lipoprotein modification, which appear to be functionally equivalent to periplasmic binding proteins in Gram-negative bacteria (Sections VI and VII).

C. YEASTS

Yeasts possess a thick and complex cell-wall structure, which is broadly analogous to that in Gram-negative bacteria. Glucans and chitin-based polymers occur in place of a peptidoglycan. Between the wall and the

cytoplasmic membrane is a region known as the periplasmic space. As in Gram-negative bacteria, in *Saccharomyces cerevisiae* this contains binding proteins for solute-transport systems, which can be released by osmotic shock (Section VIII. B). It is presently unclear to what extent the cell wall in *Sacch. cerevisiae* may act as a permeability barrier and a molecular sieve to peptides and other nutrients (Denobel *et al.*, 1990).

V. Methods for Studying Peptide Transport and Utilization

A. INTRODUCTION

There have been particular problems associated with attempts to measure peptide transport in micro-organisms because, unlike most other transport substrates, very few radioactively-labelled peptides have been produced commercially, so that conventional transport assays could not routinely be performed. Consequently, a variety of alternative methods have been devised, and their applicability, advantages and disadvantages will now be considered. Some aspects of certain of these methods have been reviewed previously (Payne, 1980a; Higgins and Gibson, 1986).

B. INDIRECT METHODS USING AMINO-ACID AUXOTROPHS

Most of these methods involve some technique for monitoring growth response. Peptides of appropriate amino-acid composition can generally satisfy the nutrient requirements of amino-acid auxotrophs. If an auxotroph has intracellular peptidase activity but lacks extracellular activity, then intact peptide uptake can occur and this can be monitored by studying the auxotrophic growth response to various peptides containing the required amino acid(s). Clearly, assessment of transport is only indirect, for growth response depends on a series of steps, of which transport is the first but may not be rate-limiting. Growth is usually assessed by measuring the increase in culture density. The method has been used extensively, especially in early studies, with various species of bacteria and fungi (Payne, 1976, 1980a; Payne and Gilvarg, 1971, 1978; Becker and Naider, 1980). It can provide no detailed kinetic data, simply indicating whether or not a peptide is absorbed. However, some indication of relative transport abilities can be inferred from the competitive abilities of other peptides to inhibit growth on nutrient peptides. A limitation is that uptake can only be studied under growth conditions, precluding any study of effects of pH value, temperature or energy coupling. The procedure is

relatively insensitive, requiring millimolar concentrations of substrate, and may take up to 24 hours to complete.

Certain of these limitations have been overcome in two related procedures. In the first, instead of measuring peptide-dependent growth over many generations, synthesis of a specific inducible enzyme is measured (Bell *et al.*, 1977; Payne, 1980a). The method is sensitive and quick, requiring only a few minutes to monitor β-galactosidase production, but it still requires auxotrophs and incubation conditions that facilitate growth (protein synthesis). This approach has been adapted as an extremely sensitive and specific assay for nutritionally available amino acids (Tuffnell and Payne, 1985; Payne, 1989). In the second method, transport is measured from incorporation of a radioactively-labelled amino acid into protein. Using a double auxotroph, one of the required amino acids is supplied radioactively-labelled and the other in peptide form, the latter being made rate limiting for protein synthesis (Payne and Bell, 1977b; Payne, 1977). Results based upon these procedures were more informative but in broad agreement with those from growth assays.

C. USE OF RADIOACTIVELY-LABELLED PEPTIDES

A number of workers have used radioactively-labelled peptides, in conventional filtration assays, to determine kinetic parameters of transport in both bacteria and fungi (early studies are reviewed in Payne and Gilvarg, 1971; and Payne, 1980a). However, in most studies, measurements were made in ignorance of the fact that transported peptides are very rapidly hydrolysed by cytoplasmic peptidases, and released amino acids (and on occasions peptide fragments (Payne, 1980a) may undergo immediate exodus concomitant with continuing uptake of the peptide substrate (Payne and Bell, 1977a,c; 1979). In consequence, only net influx will be measured, leading to serious underestimates of the rate and extent of peptide transport (Payne and Nisbet, 1980a,b). Exodus has been observed in many bacteria, e.g. *E. coli, Staphylococcus aureus, Streptococcus faecalis* and others; in fungi similar effects occur, although exodus is mainly in the form of deaminated amino acids (Perry and Abraham, 1979; Nisbet and Payne, 1980; Shallow *et al.*, 1991). The problem may be exacerbated by catabolism and decarboxylation reactions, all of which can be significant even during the short time periods used in these uptake assays. Ironically, therefore, the unavailability of radioactively-labelled peptides has not been a great problem, for conclusions based on their use would in most instances have proved to be erroneous (Payne, 1980a).

D. USE OF FLUORESCENCE TECHNIQUES

Two different procedures, based upon production of peptides fluorescently labelled with either dansyl chloride or fluorescamine, offer the best approach for determining uptake parameters. In the first procedure, micro-organisms are incubated with one or more peptides and, periodically, samples are filtered to remove cells. Peptides in the filtrate are dansylated, separated by thin-layer chromatography and their amounts assessed from the intensities of their fluorescent spots. Thus, the transport rate for any peptide or analogue can be monitored by following its removal from the incubation medium (Payne and Bell, 1979; Nisbet and Payne, 1979a). In a complementary manner, accumulation of non-hydrolysable peptides in the intracellular pool can similarly be measured. Application of this method first provided evidence for exodus of cleaved amino acids. There are several advantages in using this method. Detection is very sensitive so that uptake at low concentrations (μM) over short periods (several minutes) can readily be performed. Competition and differential uptake rates can be studied using peptide mixtures, as can the influence of amino acids or other compounds in the incubation suspension (Payne and Bell, 1977c, 1979). Over the years, the method has been successfully applied to a range of bacteria and fungi and also to measuring transport into plants (Hardy and Payne, 1992). Dansyl chloride is superior to fluorescamine as a labelling reagent in this type of procedure (Hulen and LeGoffic, 1987). As micro-organisms do not need to be growing, they can be incubated under various conditions allowing study of the effects of pH value, temperature and metabolic inhibitors. When comparison was made between transport rates in *E. coli* during exponential growth, and harvested cells in suspension, the same rates were found (Payne and Bell, 1979). Interestingly, in this study, it was found that in typical growth assays all of the peptide became absorbed by the cells within the first few hours, after which growth was maintained by re-absorption of the effluxed amino acids.

The rapid reaction of fluorescamine with primary amino groups on peptides allowed development of a sensitive solution assay similar to the dansyl-chloride procedure for monitoring removal of peptides from incubation mixtures as a result of microbial transport (Nisbet and Payne, 1979b). This is the most sensitive means of measuring peptide transport and has proved applicable to all organisms tested. Its advantages over use of radioactively-labelled substrates have been repeatedly demonstrated (Nisbet and Payne, 1979b, 1980; Payne and Nisbet, 1980a,b; Payne *et al.*, 1982; Shallow *et al.*, 1991). Subsequently, an automated fluorescence technique that allowed peptide transport to be measured continuously was

developed (Payne and Nisbet, 1981; Payne, 1983; McCarthy *et al.*, 1985c). Such a system allows precise measurement of transport kinetic parameters. Uptake can be monitored as it is taking place and also interactively so that, for example, the effects on transport of adding energy uncouplers can be studied (Payne and Nisbet, 1981; Nisbet and Payne, 1982). The continuous system has been linked to a microcomputer for storage and processing of transport data and, in this mode, provides an unrivalled facility for studying transport (Payne, 1983, 1986; Alves *et al.*, 1985).

E. OTHER METHODS

A spectrophotometric method, using synthetic peptide substrates that contain glycine residues α-substituted with thiophenol, has provided useful information on peptide permease affinities in several organisms (Perry and Gilvarg, 1984; Kingsbury, 1990). Transport and peptidase action results in intracellular release of thiophenol, which rapidly exits from the cell. Measurement of released thiophenol using Ellman's reagent gives an indication of transport rate, and the effects of competitor peptides on this release allow an estimate of their relative affinities. Application of the procedure is limited by the restricted range of α-substituted peptides available and their relatively high affinities compared with competitor substrates. In a novel, but indirect, approach to measuring peptide transport and metabolism, a biosensor technique was described, consisting of microbial cells on an oxygen electrode, in which changes in respiration rate were monitored following addition of peptides (Riedel *et al.*, 1989). However, these procedures offer no advantages over the fluorescence techniques already described.

VI. Peptide Permeases of *Escherichia coli* and *Salmonella typhimurium*

A. INTRODUCTION

Current understanding of the physiological importance, structures and properties of bacterial peptide permeases derives largely from studies carried out with *Escherichia coli* and *Salmonella typhimurium*. In *E. coli*, detailed characterization of the specificities of peptide transport, and isolation of transport mutants, revealed the existence of three peptide permeases. These are the oligopeptide-, dipeptide- and tripeptide-permeases. A combination of biochemical and molecular genetical studies has shown that similar permeases occur in both species. Overall, these studies have facilitated characterization of analogous systems in other micro-organisms

(Sections VII and VIII). Additionally, molecular characterization of the oligopeptide permease in particular has contributed to identification of a group of membrane-transport systems, which are widely distributed in Nature and which share fundamental structural and mechanistic principles (Section XII).

B. THE OLIGOPEPTIDE PERMEASE

1. Introduction

The occurrence of separate systems for uptake of di- and oligopeptides was originally shown by differences in their mutual competition in transport assays. The existence of the oligopeptide permease (Opp) in *E. coli* was demonstrated by isolation of mutants, selected as resistant to the toxic peptide triornithine, in which transport of tripeptides and higher oligomers was largely lost, while dipeptide uptake was only slightly affected (Payne, 1968). Subsequently, an analogous system was described in *S. typhimurium* and several other species. Early studies have been reviewed (Payne, 1980a; Matthews and Payne, 1980).

2. Substrate Specificities of the Oligopeptide Permease

Substrate specificities and kinetic parameters for the Opp have been determined using a variety of transport assays (Section V). The system has a high affinity for substrates, with a typical K_t value of 0.1–2 μM, and is of high throughput with a typical V_{max} value of 2–20 nmol min^{-1} (mg protein)$^{-1}$. Early conclusions (reviewed in Payne and Gilvarg, 1978; and Payne, 1980a) have been endorsed and extended by later studies. Much of the information on this topic has derived from studies aimed at exploiting this system for uptake of peptide-carrier prodrugs (Section XI). Oligopeptides, up to and including hexapeptides, can be transported. This upper limit is controlled by the outer membrane porins (Section IV; Alves *et al.*, 1985). It follows that the actual limit for the permease cannot be determined by measuring uptake of exogenous peptides, whether or not the Opp is potentially able to handle larger peptides. However, evidence for this possibility has been obtained (Section VI.B4). For efficient transport, oligopeptides must possess a positively-charged, primary or secondary *N*-terminal α-amino group. Any variation from this requirement, e.g. *N*-acyl or *N*-dialkyl substitutions, effectively prevents transport. Various studies testify to the critical importance of this structural feature for substrate recognition (Payne, 1980a, 1986). In spite of this, a few instances have been observed in which α-*N*-acyl oligopeptides show biological

properties that appear to require their transport. Examples are utilization of α-*N*-acetyltrilysine (Losick and Gilvarg, 1966), toxicity of α-*N*-t-butyloxycarbonylaminoxy peptides (Payne *et al.*, 1984) and utilization of α-*N*-acetyltrialanine as the sole nitrogen source; in every case, these biological effects were lost in *opp* mutants (J. W. Payne, unpublished results). Assuming that all observations of this type have a common cause, it was suggested (Payne, 1980a) that this might arise from operation of a dipeptidylcarboxypeptidase (dcp) activity reported to be present in the bacterial periplasm (Deutch and Soffer, 1978). However, further studies (J. W. Payne, unpublished observation), with dcp-negative mutants, has shown this not to be true and, in consequence, it appears that limited uptake of such α-*N*-acyl oligopeptides involving the Opp may occur. Other recognition features have also been studied (Payne, 1980a; Section XI). The *C*-terminal α-carboxyl group is desirable but is not essential, and it may be removed or modified in various ways without loss of transport. The system shows stereochemical specificity, with preference for all L-residues but with some tolerance of D-residues within the chain. The nature of side-chain residues influences individual kinetic parameters, but the system is characterized by its ability to handle an enormous range of natural and modified residues. Substrates require normal α-peptide bonds for effective transport; methylation of the peptide-bond nitrogen impairs, but does not prevent, transport. Somewhat surprisingly, the peptide backbone can be extended, as in aminoxy and aza derivatives, with very little effect on transport rates (Section XI). The system is also able to absorb peptide components derived from cell-wall peptidoglycan; these compounds have structural features that differ significantly from those already described, and their transport raises queries about the basis of the molecular recognition process in this system (Goodell and Higgins, 1987) (Section VI, p. 26). In other studies, claims have been made for involvement of the Opp in uptake of polyamines and aminoglycoside antibiotics (Kashiwagi *et al.*, 1992). Clearly, before the determinants of molecular recognition can be fully appreciated, direct studies upon the Opp substrate-binding component(s) are needed (Section VI.B4).

3. The Oligopeptide Permease is a Periplasmic Binding Protein-dependent System

Active transport systems in Gram-negative bacteria can be functionally separated into two classes, based upon their sensitivity to a cold osmotic-shock procedure (Neu and Heppel, 1965). After this treatment, the activity of so-called shock-sensitive systems is severely decreased, whereas that of shock-insensitive systems is largely unaffected. This osmotic-shock

treatment causes selective release of periplasmic proteins, and it is their loss that impairs transport activity. Amongst these periplasmic proteins is a class of solute-binding proteins that are essential to functioning of shock-sensitive systems, hence, their alternative name of binding protein-dependent transport systems (Ames, 1986). In contrast, the shock-insensitive systems are composed of a single membrane-spanning protein; the lactose permease of *E. coli* is the best studied example of this class (Hengge and Boos, 1983). A further mechanistic distinction between the two classes is that transport involving the shock-insensitive systems is energized chemiosmotically, using an ion gradient in either an antiport or symport mechanism, whereas the binding protein-dependent systems are linked directly to phosphate-bond energy.

Early studies indicated that, in *E. coli*, peptide transport was susceptible to osmotic shock, was largely independent of the proton-motive force but was inhibitable by agents such as arsenate, implying energization through ATP (Cowell, 1974; Payne and Bell, 1979, and references cited in Payne, 1980b). All of these studies pointed to a binding-protein dependence. The finding that the oligopeptide permease is encoded by an operon of several genes, similar to that described previously for other shock-sensitive systems, provided strong endorsement for this view (Higgins and Hardie, 1983). However, early studies failed to detect peptide-binding activity among material released by osmotic shock (Cowell, 1974; Payne, 1980a), probably because of the unusually low affinity of the glycine-containing peptides used and the degradative effect of peptidase activity in the shockates. Subsequently, using different radioactively-labelled oligopeptides, binding-protein activity was readily detected (Guyer *et al.*, 1985; Tyreman *et al.*, 1992). In *S. typhimurium*, identification of an abundant periplasmic protein, that was absent from a number of *opp* mutants, provided the first direct evidence for an oligopeptide-binding protein (Higgins and Hardie, 1983), although its reported M_r value of about 52 k proved erroneously low. In *E. coli*, an equivalent periplasmic protein with an M_r value of about 60 k was described (Guyer *et al.*, 1985).

4. The Oligopeptide Binding Protein

The oligopeptide-binding protein (OppA) is the product of the first gene (*opp*A) of the *opp* operon (Higgins and Hardie, 1983; Hiles *et al.*, 1987a,b). The nucleotide sequences of the *opp*A genes from *E. coli* (Kashiwagi *et al.*, 1990) and *S. typhimurium* (Hiles and Higgins, 1986) have been reported, and are 75% homologous. These genes encode polypeptides of 543 and 542 amino-acid residues, respectively, which include amino-terminal signal peptides that determine their periplasmic location.

The OppA protein is one of the most abundant periplasmic proteins, typically representing 5–8% of the total (Higgins and Hardie, 1983; Guyer *et al.*, 1985; Tyreman *et al.*, 1992). The protein has been purified from *S. typhimurium* (Hiles and Higgins, 1986) and from *E. coli* (Guyer *et al.*, 1985; Tyreman, 1990; Kashiwagi *et al.*, 1990).

The binding specificities of the protein from *E. coli* were first studied using equilibrium-dialysis assays, in which the ability of various compounds to inhibit binding of Ala-Phe[^{3}H]Gly to purified OppA was measured (Guyer *et al.*, 1986). As expected, amino acids showed no competition. The tripeptide Ala_3 was, however, an excellent competitor, although α-*N*-acetylation abolished its competitive ability and esterification severely decreased it. Tripeptides DDD-Ala_3 and LDL-Ala_3 were also unable to inhibit binding of Ala-Phe[^{3}H]Gly. The specificity towards peptide length was studied using alanine homopeptides. The dipeptide Ala_2 was not an inhibitor, whereas Ala_3 and Ala_4 both showed good and similar competitive abilities, while Ala_5 was a relatively poor inhibitor. Side-chain specificity was studied using a range of homo-tripeptides as competitors. In general, peptides comprising amino acids with ionic side chains (e.g. Lys_3) showed no competition, whereas peptides composed of either polar or hydrophobic side-chain residues were good competitors. Peptide-binding to OppA was also assessed using fluorescence emission spectroscopy, involving excitation at 290 nm and tryptophan-fluorescence emission at 349 nm. This technique has been used to study ligand binding to a variety of periplasmic-binding proteins (Furlong, 1987). However, neither Ala_3 nor Ala-Phe-Gly caused a change in the fluorescence emission spectrum, although both had been shown to bind well by equilibrium dialysis. In contrast, Lys_3 caused an increase in fluorescence and a shift to longer wavelength, although this peptide failed to act as an effective competitive inhibitor in the equilibrium-dialysis assay. Given the apparent contradiction in these results, it was proposed that OppA possesses two (or more) separate substrate-binding sites that distinguish peptides on the basis of their side-chain residues. Others have also advanced the view that two or more separate binding sites may exist (Goodell and Higgins, 1987), based on the finding that the structurally anomalous cell-wall peptides are accumulated by the Opp (and see p. 79), and also on the observation that *opp*A mutants have been isolated that are resistant to triornithine but which retain the ability to transport Pro-Gly-Gly and other peptides known to use the Opp (Higgins *et al.*, 1983).

Further information relevant to this topic has come from an extensive study of the substrate-binding specificity of the OppA from *E. coli* using a filter-binding assay (Tyreman, 1990; Tyreman *et al.*, 1992). In this assay, binding of a radioactively-labelled peptide substrate is measured as an

OppA–ligand complex after its precipitation by ammonium sulphate on a membrane filter; the relative affinities of other peptides are determined from their competitive abilities to prevent binding of the radioactively-labelled substrate. Comparative studies were also performed using equilibrium dialysis. Several substrates were used in these studies, ranging from the high-affinity [^{14}C]Ala_3 to low-affinity, high specific activity ligands, such as [^{125}I]Tyr-Gly-Gly. The results broadly endorsed and extended those of Guyer *et al.* (1986). Most importantly, they confirmed that the detailed substrate specificities and binding affinities determined for transport by the Opp are mirrored exactly in the binding properties of OppA. This finding highlights the utility of the binding assays in design of peptide carriers for prodrug delivery (Section XI). This conclusion also has important implications for any model of peptide translocation by a binding protein-dependent system (Section VI.B6), and would seem to limit any substrate-recognition features that might be present in a membrane pore to being a subset of those of OppA, and to having a markedly lower affinity.

Using the filter-binding assay, it was found that, in contrast to the observation of Guyer *et al.* (1986), Lys_3 and Orn_3 could be effective competitors of binding of other tripeptides. Such tripeptides, such as [^{125}I]Tyr-Gly-Gly, could lack any charged side chains and be shown to share a common binding site with Ala_3 and Ala-Phe-Gly; it was only necessary that they be of low affinity. Thus, the simplest interpretation of these results is that a single binding site is used (or conceivably, overlapping sites) by all of these peptides. If, indeed, only a single binding site exists, comment is needed on the views already reported for the existence of two (or more) separate binding sites. Firstly, the poor affinities of peptides with charged residues presumably precludes their potential competitive ability being detected by use of the less discriminating equilibrium-dialysis technique. Secondly, there is the apparent anomaly of the fluorescence-emission results. The indole nucleus of a tryptophan residue is a sensitive intrinsic fluorophore, and its emission spectrum reflects the polarity of its surrounding environment. However, in a protein, the fluorescence spectrum observed represents the summation of signals that result from all of the tryptophan residues present. But, since any substrate-induced change will occur predominantly near the binding site, the micro-environments of tryptophan residues located away from the binding site are unlikely to be altered. Evidence for this situation has been obtained by tryptophan-residue substitution in the maltose-binding protein (Martineau *et al.*, 1990). Thus, the binding site could have a tryptophan residue near it that would have the polarity of its micro-environment, and hence its intrinsic fluorescence, altered by encroachment of the charged

side-chains of Lys_3, but would be unaffected by smaller, neutral residues on other substrates. With small peptides, lacking an extended secondary structure, one domain of the binding protein might bind the peptide backbone predominantly, while another domain might interact with side chains on the substrate. Thus, the fluorescence results of Guyer *et al.* (1986) can be explained without the need to invoke two binding sites. A final point is the mutational loss of triornithine sensitivity with retention of growth using proline-containing peptides in certain *opp*A mutants. Here a missense mutation at a common substrate-binding site in OppA could detrimentally affect its binding capacity, with the result that uptake of a peptide of normally low affinity, such as tri-ornithine, is decreased to a level that prevents expression of its inhibitory effect. At the same time, uptake of a higher-affinity substrate, such as Pro-Gly-Gly, although decreased could still satisfy auxotrophic growth requirements for proline. This type of transport event was discussed by Payne (1980a). Thus, a definitive conclusion on the existence of one or more (possibly overlapping) binding sites in OppA must await results of X-ray crystallographic studies.

In an attempt to identify residues at the binding site of OppA, the protein from *E. coli* has been derivatized with a radioactively-labelled peptide analogue carrying a photolabile azido residue (Hardy and Payne, 1991). The photoaffinity probe was shown to bind competitively with Ala_3 and thus, after selective proteolysis of the derivatized protein and sequencing of fragments that carry the labelled ligand, information on residues around the binding site should be forthcoming (N. Marshall, M. W. Smith, and J. W. Payne, unpublished results, and see p. 79).

Use of the filter-binding assay also provided the novel observation that large peptides, containing up to 16 residues, bound competitively to the same site (or overlapping sites) as all other tested small peptides. This conclusion was endorsed by the finding that binding of [^{125}I]angiotensin (10-mer) was competitively inhibited by LLL-Ala_3 but not by DDD-Ala_3. Based on these observations, it is possible that OppA might also be involved in uptake of polypeptides originating within the cell envelope, possibly resulting from protein turnover or from degradation of cleaved signal peptides (Tyreman *et al.*, 1992).

The OppA protein is the largest of about two dozen periplasmic-binding proteins identified in these Gram-negative bacteria. These proteins have been the subject of intensive study, and their properties have been extensively reviewed (Ames, 1986, 1988; Furlong, 1987; Adams and Oxender, 1989; Higgins *et al.*, 1990; Quiocho, 1990, 1992). Most have an M_r value of about 30 k, with the smallest being the vitamin B_{12}-binding protein from *E. coli* with an M_r value of 22 k. They are unusually stable,

being particularly resistant to heat and to proteases. They often have broad pH optima of binding, and all bind their substrates with high affinity, with typical k_m = 0.1–1 μM (Furlong, 1987). This property often results in binding proteins retaining some bound substrate molecules during purification (Boos *et al.*, 1972; Richarme and Kepes, 1974). An explanation for co-purification of protein with ligand, based on a phenomenon known as the retention effect, has been proposed (Silhavy *et al.*, 1975; Amanuma *et al.*, 1976). Ligand-free proteins can be obtained by reversible denaturation with guanidine-hydrochloride, followed by dialysis against a large volume of ligand-free buffer (Miller *et al.*, 1983). Additionally, we have found that, if purified OppA is applied to a reverse-phase chromatography column (Pharmacia ProRPC) equilibrated with 0.1% (v/v) trifluoroacetic acid, the protein is recovered as a sharp, ligand-free peak eluting at 40% acetonitrile (Tyreman, 1990).

Many of the binding proteins have been crystallized and their tertiary structures determined using X-ray diffraction. This work has revealed strong structural similarities within this group of proteins (Quiocho, 1990, 1992). Thus, although in general binding proteins share very little sequence similarity, they do have very similar overall conformations. To date, all have been shown to be elongated ellipsoidal molecules with dimensions of about 3.5 nm × 4.0 nm × 7.0 nm. They consist of two globular domains, the lobes being connected by two or three short polypeptide segments that form a hinge between the domains. The substrate-binding site is located in a prominent cleft between the two domains. Interestingly, the mammalian iron-binding protein, lactoferrin, has a similar structure to that of the bacterial binding proteins (Baker *et al.*, 1987; Anderson *et al.*, 1990). It consists of two lobes, each possessing a single binding site for iron. Each of the lobes is composed of two domains that are almost superimposable on the structure of the sulphate-binding protein from *S. typhimurium*. Despite this remarkable structural similarity, the two proteins share no sequence homology (less than 10%).

Three different conformational forms of the binding proteins have been identified from X-ray structural studies, two of which are considered to result from substrate-induced conformational changes. Thus, in the native state, the protein exists in an open conformation. When this binds a ligand molecule, the initial binding stage involves contact between the substrate and only one domain of the protein, and yields an 'open liganded' conformation. Subsequent closure of the two domains around the substrate produces a 'closed liganded' conformation. In this state, the substrate binds to both domains and is inaccessible to external solvent. Surprisingly, substrate binding is mediated mainly by hydrogen bonds and Van der Waals forces, irrespective of the nature of the substrate. This aspect is

considered important for the function of binding proteins and is discussed by Quiocho (1990).

Evidence for conformational changes has been obtained experimentally for many binding proteins, using a variety of techniques (Furlong, 1987). The most commonly used method is fluorescence-emission spectroscopy, as discussed previously. For OppA, novel observations on its ability to undergo substrate-induced conformational changes have come from use of isoelectric focussing (Tyreman, 1990; Tyreman *et al.*, 1992). The p*I* value of the protein from *E. coli* has been reported to be 5.95 (Guyer *et al.*, 1986). However, in our laboratory, the purified protein runs as three distinct bands on IEF gels, with p*I* values of 6.20, 6.26 and 6.55 respectively; each of these species yields the same single band on a sodium dodecylsulphate–polyacrylamide gel and has an identical *N*-terminal sequence. The apparent variation in OppA is also seen during its purification. Thus, when it is isolated on ion-exchange columns (Pharmacia FPLC MonoQ and MonoS columns), it elutes as several poorly-resolved peaks, each of which converts to a single, sharp peak when again separated on a reverse-phase column (see above). This form of the protein yields a single band on IEF with a p*I* value of 6.20, and which shows maximal binding activity and univalent substrate stoicheiometry in the filter-binding assay already described. This species, which we consider to be the unliganded form, can be converted to either of the other forms with different p*I* values by addition of different peptide substrates. For example, binding of Ala_3 yields the form with a p*I* value of 6.26, whereas binding of LysAlaAla yields the p*I* 6.55 form. Using a variety of charged and uncharged substrates, no correlation was found between the p*I* value of the liganded form and the net charge of the peptide. Thus, the three forms are considered to be different conformers, representing the native (p*I* 6.20) and two liganded forms (p*I* 6.26 and 6.55). It is not yet clear which structural features of a peptide determine which liganded form is adopted, or whether the forms may be related to the 'open-' or 'closed-liganded' conformations observed from X-ray studies. The galactose-binding protein has been reported to undergo a change in electrophoretic mobility on substrate binding (Boos and Gordon, 1971; Boos *et al.*, 1972; Mowbray and Petsko, 1982), but only one liganded form was detected. Whether there is any possible biological relevance for the two liganded forms of OppA is presently unclear. It is tempting to speculate, however, that their binding to membrane proteins may induce formation of slightly different translocation pores, each designed to accommodate distinguishable peptide substrates (Section VI.B6).

The OppA protein from *S. typhimurium* has been crystallized (Tolley *et al.*, 1988), although no structural data are available. This protein is

presumably very similar structurally to OppA from *E. coli*, and antibodies raised against the former protein cross-react with the latter, and vice versa (Hiles and Higgins, 1986). In our laboratory (C. Schuster and J. W. Payne, unpublished results), it has been found that antibodies raised against OppA from *E. coli* cross react with similar sized proteins from a range of Gram-negative and Gram-positive bacteria. When detailed structural information is available for OppA, it will be interesting to see how closely it resembles the consensus structure for binding proteins, given that it is about twice the size of all other characterized proteins. Such information will also be invaluable for aiding synthesis of novel antibacterial smugglins, designed to exploit peptide permeases in gaining access to intracellular targets (Section XI, p. 63).

5. *Membrane Proteins of the Oligopeptide Permease*

The *opp* operon of *S. typhimurium* has been cloned by Higgins and his co-workers and found to occupy about 5 kb of DNA (Hiles *et al.*, 1987a,b). This region was found to be a hot spot for IS1 and IS5 insertions, a feature that may contribute to the relatively high mutation rate of *opp* and adjacent genes (Jackson and Yanofsky, 1973; Payne, 1980a; Higgins *et al.*, 1983) (Section XI, p. 64). Sequencing of the *opp* genes revealed five open reading frames, rather than the four detected in complementation studies (Hogarth and Higgins, 1983; Hiles *et al.*, 1987b). These genes are transcribed in the order *opp* ABCDF and all are essential for peptide transport controlled by *opp*.

The *oppB* and *oppC* genes encode polypeptides of 33.4 kDa and 33.1 kDa, respectively, that have 21% identity (Hiles *et al.*, 1987b). Fusions of LacZ were made to these genes and the hybrid OppB/C-LacZ polypeptides were found to be associated with the cytoplasmic membrane. This location is consistent with the hydrophobicity plots for these proteins, which indicate that each possesses multiple membrane-spanning segments. The membrane topology of these two proteins has been studied using β-lactamase fusions to the *opp B/C* genes (Pearce *et al.*, 1992). Trypsin accessibility studies helped to identify sections of the polypeptides that were located in the periplasm or cytoplasm. Both OppB and OppC have six transmembrane segments, connected by hydrophilic loops, and with both the *N*- and *C*-termini located in the cytoplasm. Their overall topology accords with the two-times-six transmembrane helix paradigm for active transporters (Maloney, 1990). A similar study has been made of the topology of membrane-bound components of the histidine permease; comparisons with similar systems indicated conservation of structural features, and a generalized model for prokaryotic and eukaryotic systems was proposed (Kerppola and Ames, 1992).

The *oppD* and *oppF* genes encode polypeptides of 36.9 kDa and 37.1 kDa, respectively, that show extensive amino-acid sequence homology (Hiles *et al.*, 1987b). Both are relatively hydrophilic proteins but, despite this, LacZ–OppD fusion proteins were found to be associated with the cytoplasmic membrane. More precise information on the subcellular location of the OppF protein has come from overproducing the cloned gene product and detecting its presence in cellular fractions using peptide-specific antibodies (Gallagher *et al.*, 1989). These studies, together with protease accessibility data, revealed the protein to be associated in a tight, non-ionic interaction with the inner face of the cytoplasmic membrane. Furthermore, this location was independent of the presence of other Opp proteins. The OppD and OppF proteins each contain two regions of sequence characteristic of nucleotide-binding proteins; this structural motif consists of two highly conserved blocks of sequence that form a nucleotide-binding fold (Walker *et al.*, 1982; Higgins *et al.*, 1985). The OppD protein shares about 30% sequence identity with HisP and MalK, components of the histidine permease of *S. typhimurium* and the maltose permease of *E. coli*, respectively (Gilson *et al.*, 1982; Higgins *et al.*, 1985).

Studies on HisP indicate that its properties are intermediate between those of integral and peripheral membrane proteins, and it has been suggested that it is deeply embedded in the membrane and may extend through to the periplasmic surface (Kerppola *et al.*, 1991). It is possible that the cytoplasmic location determined for OppF may be artefactual, resulting from its overproduction in mutants lacking other membrane proteins of the oligopeptide permease.

Each of these proteins possesses the nucleotide-binding motif, although their sequence homology extends beyond this feature, implying a closer evolutionary relationship than just a nucleotide-binding domain. The function of the proposed nucleotide-binding site of these proteins has been confirmed by their ability to bind ATP and/or ATP analogues (Hobson *et al.*, 1984; Higgins *et al.*, 1985; Dean *et al.*, 1989). These observations lent support to the suggestions from earlier studies that energy coupling to shock-sensitive transport systems (including Opp) probably involved high-energy phosphate bonds, most probably ATP (reviewed in Payne, 1980b). In recent years, reconstitution from purified components of the histidine and maltose permeases in proteoliposomes has confirmed that ATP is the primary energy source (Ames *et al.*, 1989; Bishop *et al.*, 1989; Prossnitz *et al.*, 1989; Ames and Joshi, 1990; Dean *et al.*, 1990; Higgins, 1990). Moreover, ATP-hydrolysing activity by the MalK protein has been demonstrated (Walter *et al.*, 1992). However, the stoicheiometry of molecules of ATP hydrolysed for each molecule transported has yet to be determined. It should not necessarily be inferred that this stoicheiometry is two, simply

because these transporters possess two nucleotide-binding proteins; in the analogous cystic fibrosis chloride channel (Section XII) it has been proposed that ATP binding may also serve a regulatory role (Quinton and Reddy, 1992).

6. *Mechanism of Peptide Transport by the Oligopeptide Permease*

The oligopeptide permease, together with the much studied histidine and maltose permeases, belongs to a widely distributed family of membrane-transport systems (Section XII). Knowledge gained from studies of these bacterial permeases has contributed significantly to development of models for the molecular mechanisms of these systems. A model for the Opp is shown in Fig. 1. The occurrence of permease proteins in the form of a

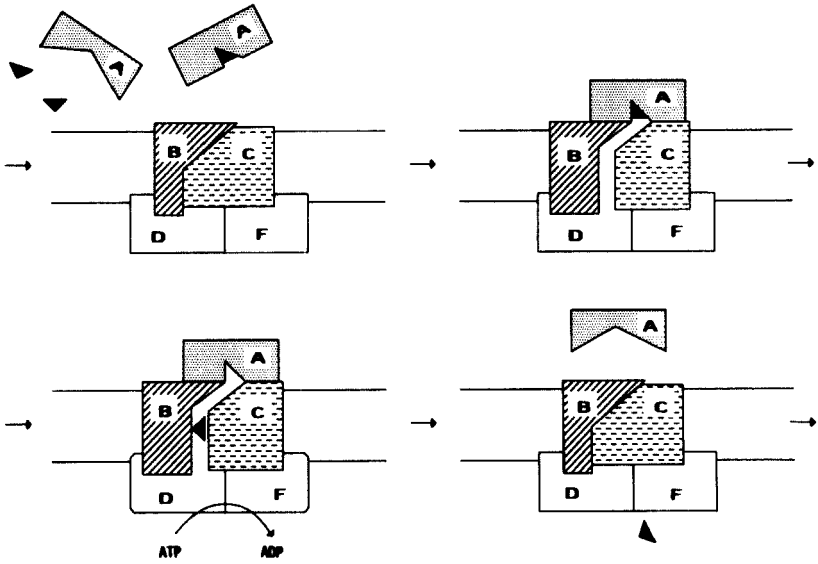

FIG. 1. A model for peptide transport by the oligopeptide permease. The speculative sequence of events is initiated (top left) by a peptide substrate (▲) interacting with the periplasmic oligopeptide-binding protein OppA(A); binding of the peptide ligand leads to a conformational change in OppA. This change facilitates ligand–complex binding to one or more of the membrane-bound proteins OppB and OppC (B,C) (top right); this binding in turn causes a conformational change that creates a transmembrane pore. Peptide translocation through the pore is triggered by this conformational re-arrangement and may be linked to ATP hydrolysis by one or more of the peripheral proteins OppD, OppF (D,F) (bottom left). Some specific interactions of the substrate with residues in the membrane pore may occur. Ligand release into the cytoplasm is followed by a return to the initial state of the permease (bottom right), either spontaneously or by energy-dependent conformational re-arrangement.

topographically-defined complex has been shown from cross-linking and reconstitution studies for the histidine, maltose and galactose permeases (Kerppola *et al.*, 1991; Davidson and Nikaido, 1991; Richarme *et al.*, 1992). Recently, assembly of the maltose permease has been shown to occur in a sequential manner (Traxler and Beckwith, 1992). The integral membrane components, MalF and MalG, are independently inserted into the membrane. Their folding and lateral diffusion follows, before they assemble together and then with MalK to form the full complex. But, unlike the oligopeptide permease, which has OppD and OppF, the histidine and maltose permeases have two copies of HisP and MalK, respectively, the nucleotide-binding component that is presumed to function as a dimer.

A unified model for the mechanism of transport by these binding protein-dependent systems is as follows. The initial, specificity-determining recognition step involves substrate binding to the cognate binding protein. This leads to a ligand-induced conformational change(s) in the binding protein (OppA), endowing it with a recognition site of increased affinity for one or both of the membrane proteins OppB and OppC. Evidence for an interaction between the binding protein and the membrane complex has been provided for the histidine permease (Prossnitz *et al.*, 1988). This interaction is, in turn, considered to trigger a sequence of conformational changes in the membrane proteins that drive the translocation event. The precise order and interdependence of these changes is not clear. Structural change is translated into information for signalling hydrolysis of ATP by OppD and/or OppF. A pore region, possibly with some substrate-recognition features, is created within the transmembrane domains of the integral membrane proteins. Dissociation of substrate from the binding protein, possibly proceeding through an intermediate 'open-liganded' form, may possibly be co-ordinated with creation of the translocation pore. The presence of ligand-recognition features (of low affinity), vectorially aligned within the pore would facilitate forward translocation of the substrate. Release of ADP would contribute to a reverse sequence of conformational changes, with dissociation of the binding protein restoring the permease machinery. The binding proteins are clearly an important component in conferring specificity and high affinity on these systems, although mutants lacking binding proteins for the maltose and histidine permeases have been isolated and were shown to retain ability to transport, albeit with much lower affinity (Shuman, 1982; Treptow and Shuman, 1985; Speiser and Ames, 1991). These strains had mutations in either the integral membrane proteins or the nucleotide-binding proteins which allowed hydrolysis of ATP in the absence of the signal(s) normally initiated by binding of the liganded protein. Indeed, the mutant permeases

hydrolysed ATP continuously at about five times the rate of the wild type (Petronilli and Ames, 1991; Davidson *et al.*, 1992). The mutants appeared to retain some substrate specificity, implying that the membrane-bound complex possesses some ligand-recognition capacity.

The above model for transport is highly speculative and many elements require clarification. Indeed, a fundamental reappraisal of all such models (and see Section XII) may be required following recent evidence on the topography of the membrane complex of the histidine permease of *S. typhimurium* (Baichwal *et al.*, 1993). It appears that the conserved ATP-binding component, HisP, is also exposed to the periplasmic (external) surface, requiring that it be a membrane-spanning protein rather than a peripheral protein confined to the cytoplasmic (inner) surface.

7. *Regulation of the* opp *Operon*

The *opp* operon is transcribed from a single promoter, upstream of the *oppA* gene. In common with other analogous permeases, the binding protein (OppA) is, however, present in a considerable excess over the membrane proteins. For example, in *E. coli* grown on maltose, it is estimated there are about 30 000 molecules of maltose-binding protein in each cell (Dietzel *et al.*, 1978), but only 100–1000 copies of the integral membrane protein MalF (Shuman *et al.*, 1980). The differential expression of genes for the binding protein and other membrane proteins has been proposed to result from an intercistronic region that could potentially form a stable stem–loop structure which might either act as a transcription terminator or serve to stabilize upstream mRNA (Higgins *et al.*, 1982). However, attempts to find experimental evidence for this have been unsuccessful, leading to the suggestion that differential gene expression may result from the presence of ribosome-binding sites with different affinities for mRNA (Hiles *et al.*, 1987a).

In studies from various laboratories, mainly using *E. coli*, no evidence was found that cells grown under a variety of nutrient limitations (e.g. of carbon or nitrogen-containing nutrients) differed in their uptake as regulated by *opp*, and it was concluded that transport was expressed constitutively at a high level (reviewed in Payne, 1980c). Exposing *E. coli* to mixtures of amino acids and/or peptides in growth media showed no short-term effects on peptide uptake mediated by Opp that could be interpreted as regulation (Payne and Bell, 1977a,c). Similarly, the level of OppA was also unchanged under these various nutrient differences (Higgins and Hardie, 1983). However, the possibility that an integrated regulatory network might exist, linking peptide and amino-acid transport to the amino-acid pool, was considered previously (Payne, 1980c).

The possibility of transcriptional regulation of *opp* has been studied in *S. typhimurium* (Jamieson and Higgins, 1984) and *E. coli* (Andrews and Short, 1986) using *lac* fusions. The studies led to different conclusions. In *S. typhimurium*, transcription of *opp* was reported to be unaffected by changes in carbon and nitrogen source, aeration, or the presence of amino acids or peptides. In contrast, transcription in *E. coli* was reported to be increased by anaerobic conditions, and by incorporation of leucine or alanine to the medium. Growth in the presence of leucine was also found to increase the amount of OppA found in the periplasm, and to enhance somewhat transport and utilization of some peptides (Andrews *et al.*, 1986; J. W. Payne, unpublished results), as well as increasing the sensitivity of cells to toxic tripeptides (Tyreman, 1990). Whether there is any connection between these findings and the observation that addition of putrescine to a polyamine-requiring mutant of *E. coli* stimulates synthesis of OppA is not known at present (Kashiwagi *et al.*, 1990). Seeking to explain the discrepancy between their results with *E. coli* and those of Jamieson and Higgins (1984), Andrews and Short (1986) introduced their *lac–opp* fusions on an episome into both species. They found the same expression in both cases, indicating that the same controls operated. Subsequent work has shown that the leucine effect is mediated by the leucine-responsive regulatory protein (Lrp), originally designated OppI (Austin *et al.*, 1989). This protein is a DNA-binding protein that regulates synthesis of at least 30 other proteins, considered to be members of a global response regulon in *E. coli* (Ernsting *et al.*, 1992). The control exerted by Lrp can be either positive or negative, and may be independent of leucine (Newman *et al.*, 1992). Deletion of the *Lrp* gene caused increased expression of the *opp* operon (Austin *et al.*, 1989) indicating that Lrp is a negative regulator of *opp* transcription. The Lrp regulon has been proposed to distinguish between 'feast and famine' through its ability to decrease expression of transport and degradative genes and to increase expression of biosynthetic genes (Newman *et al.*, 1992). The utility of such a process in regulating uptake and utilization of peptides was considered earlier (Payne, 1980c).

In our laboratory, we observed that synthesis of OppA is also regulated by availability of phosphate (Smith and Payne, 1992). In *E. coli*, phosphate limitation leads to derepression of synthesis of several proteins important for uptake and utilization of available sources of phosphorus; amongst these are the periplasmic alkaline phosphatase and phosphate-binding protein. This positive regulation is mediated by the PhoB/PhoR two-component regulatory system, known as the Pho regulon (Wanner, 1987; Torriani, 1990). However, these same conditions for limiting availability of phosphate lead to a decrease in synthesis of OppA (and several other periplasmic proteins), although pre-existing protein is stable for up to

7 hours. The possibility that this may arise from negative regulation mediated by the Pho regulon, acting to repress *opp* transcription, is supported by identification of a putative PhoB binding site at the −35 region of the *oppA* gene (Smith and Payne, 1992).

C. THE DIPEPTIDE PERMEASE

1. Introduction

It is not straightforward to attempt to characterize transport of dipeptides, because they can be handled by several permeases. However, the presence of a specific dipeptide permease (Dpp) in *E. coli* became clear as a result of work on mutants that were defective in the Opp (Payne, 1968). Early investigations of this system, and its counterpart in other species, have been reviewed (Payne and Gilvarg, 1978; Payne, 1980a).

2. Substrate Specificities of the Dipeptide Permease

Studies similar to those used to characterize the Opp (Section VI. B2) have shown the Dpp to have analogous structural specificities, and to transport substrates with comparable kinetics. However, in one respect the specificity of the Dpp appears stricter than the Opp, in that loss or derivatization (amidation, esterification) of the *C*-terminal α-carboxyl on a dipeptide dramatically decreases its uptake capacity (Payne and Gilvarg, 1968a; Payne, 1973; Hirshfield and Price, 1975). Dipeptides with modified peptide bonds and extended backbones are well transported and, interestingly, in these modified structures D-residues are better tolerated than usual (Morley *et al.*, 1983a,b; Payne *et al.*, 1984). Although the system handles varied dipeptides, their uptake is more influenced by side-chain modifications than is found with the Opp (Alves and Payne, 1980; Perry and Gilvarg, 1984). With the ability to measure transport directly (Section V), and the availability of well characterized *dpp* mutants, it was found, somewhat surprisingly, that the Dpp has a limited ability to transport tripeptides also (Payne, 1983; Higgins and Gibson, 1986). The specificity of the Dpp is therefore complementary to the Opp, which has high affinity for tripeptides and limited ability to transport dipeptides.

3. The Dipeptide Permease is a Periplasmic Binding Protein-dependent System

In *E. coli*, uptake of Gly-Gly was shown to be severely inhibited by osmotic shock (Cowell, 1974), implying that dipeptide uptake occurs by a

binding protein-dependent system (Section VI.B3). However, in early studies, no dipeptide-binding activity was detected in shock fluids, probably because the affinity of the glycine peptide was too low and it was hydrolysed (Cowell, 1974; results are cited in Payne, 1980b). Studies showing that dipeptide transport was coupled to phosphate-bond energy (arsenate-sensitive) in *E. coli* supported the idea of a shock-sensitive system (Payne and Bell, 1979; Payne, 1983). Subsequently, this was confirmed when a dipeptide-binding protein (DppA), which was identified from *E. coli* and *S. typhimurium*, was missing in a number of *dpp* mutants (Manson *et al.*, 1986).

One reason for the problems encountered in characterizing the dipeptide permease and identifying its cognate binding protein has been the difficulty in isolating *dpp* mutants. This arises because dipeptides can also enter through Opp. In most instances, therefore, *dpp* mutants have been isolated in strains that are already defective in *opp* (usually selected as resistant to triornithine) (Payne, 1980a). Using this strategy, *dpp* mutants have been isolated using as a selection procedure resistance to the toxic peptides bacilysin and bialaphos (Section XI) (Manson *et al.*, 1986; Abouhamad *et al.*, 1991). However, it is possible to obtain *dpp* mutants directly in various ways (Payne, 1983; and J. W. Payne, unpublished results), for example by selecting for resistance to the toxic analogues lysyl-2-aminopropionic acid (lysyl-aminoxyalanine) or alanylaminopimelic acid (Section XI), both of which show high specificity for Dpp in *E. coli.* This selection procedure can be greatly enhanced if accompanied by certain physiological constraints. For example, use of a lysine auxotroph in which a high concentration of trilysine is used; the tripeptide acts as a competitor for uptake of the toxic dipeptides through the Opp, and ensures that the oligopeptide permease is retained as being the sole uptake route for the required amino acid. Alternatively, cells are grown under conditions of nitrogen limitation using α-*N*-acetyltrialanine as the sole nitrogen source, accumulation of which is through the Opp (Section VI.B2). In all instances, mutants can be mapped to the *dpp* locus and a proportion are defective in synthesis of the dipeptide-binding protein.

4. The Dipeptide Binding Protein, DppA

The gene, *dppA*, encoding the dipeptide binding protein in *E. coli*, has been cloned and sequenced by two groups (Olson *et al.*, 1991; Abouhamad *et al.*, 1991). Olson and his coworkers noted that, quite uniquely, *E. coli* strain JM101, grown in minimal medium, produces a protein of about 50 kDa that represents about 90% of the total periplasmic proteins. Genetic mapping showed the locus for the gene to be at 80 min. Based on

the *N*-terminal sequence of the purified protein, oligonucleotides were synthesized, and used to probe a library of lambda clones from the 78–80 min region. This approach facilitated cloning of the corresponding gene, with an open reading frame encoding a polypeptide of 535 amino-acid residues containing a 28 amino acid-residue signal peptide. This protein has 26.5% amino acid sequence identity with OppA from *S. typhimurium*. The nucleotide sequence reported by Abouhamad *et al.* (1991) was identical to that found by Olson *et al.* (1991); the gene was localized to 79.2 min. Similarities in the sequences of DppA and OppA are also reflected in their structures, for polyclonal antibodies raised against either of the proteins from *E. coli* cross-react with the other (C. Schuster and J. W. Payne, unpublished results).

The protein DppA is amongst the most abundantly produced periplasmic proteins under typical growth conditions, although it is synthesized in smaller amounts than OppA. The dipeptide-binding protein from *E. coli* has been purified to homogeneity (Olson *et al.*, 1991; Tyreman, 1990; Tyreman *et al.*, 1992). When purified by ion-exchange chromatography on Pharmacia FPLC, the protein runs as three species on isoelectric focusing (IEF) gels with p*I* values of 5.9, 6.0 and 6.1, respectively. However, when subjected to Edman degradation, these samples gave only a single protein sequence corresponding to the first 33 residues predicted from the nucleotide sequence (Smith, 1992). When this mixed DppA was run on SDS–PAGE, only one band was obtained and, similarly, when it was applied to a reverse phase column (Pharmacia ProRPC), it yielded a single species with a p*I* value of 6.1 on IEF. This species, which we consider to be the unliganded form, can be converted to the p*I* 5.9 and 6.0 forms by addition of dipeptide substrates; although many dipeptides yield the latter form, we have only found Ala-Phe to convert the protein to the form of p*I* 5.9. Thus, DppA shows properties exactly analogous to OppA in its ability to exist in different ligand-induced conformational forms (see Section VI.B4).

As well as monitoring dipeptide binding from mobility shifts on IEF gels, the substrate specificities of DppA have also been determined using radioactively-labelled peptides in the filter-binding assay, and also from fluorescence emission changes (Section VI.B4) (Tyreman, 1990; Smith, 1992; Blank, 1987). Results from the filter-binding assay showed the structural specificities found for transport through the Dpp to be paralleled by the binding to DppA. The protein has a much lower affinity for tripeptides than for dipeptides. The binding affinities of a range of dipeptides paralleled those reported for transport (Perry and Gilvarg, 1984). The protein binds Ala-[^{14}C]Phe with a 1:1 stoicheiometry. Ligand binding was also demonstrated from a decrease in fluorescence emission. Although use of this technique provided relevant information, for example, LL-Ala_2

caused a shift but DD-Ala_2 did not, there was no correlation between the extent of the fluorescence shift and the relative affinities of the peptides derived from the filter-binding assay. The likely dependence of any fluorescence shift on the nature of substrate side chains has already been considered for OppA (Section VI.B4).

In *E. coli*, DppA has also been shown to be the primary receptor for peptide chemotaxis (Manson *et al.*, 1986). The secondary receptor for this response in *E. coli* is the Tap signal transducer. Mutants possessing DppA but otherwise defective in dipeptide transport (*dpp* mutants) could still carry out chemotaxis. This indicates that, as with analogous systems, transport is not necessary for peptide chemotaxis (Bourret *et al.*, 1991). Whether this distinction might involve differential binding to transport and chemotaxis receptors by different conformational forms of DppA, observed following peptide binding, is not known. In the chemotaxis system, understanding the molecular basis of transmembrane signalling induced by attachment of a liganded-binding protein has been advanced by the finding that the information-transfer effect can be mimicked by single-residue mutations in the periplasmic ligand-binding domain of the membrane-bound chemoreceptor (Yaghmai and Hazelbauer, 1992).

Cloning studies with *dppA* indicate that there are other genes downstream of *dppA* that are required for transport but not for chemotaxis. The gene immediately 3′ to *dppA* has homology with *oppB* and presumably codes for one of the integral membrane proteins of the dipeptide permease (Abouhamad *et al.*, 1991). It seems likely that the *dpp* operon will closely resemble *opp* and that the molecular mechanism of transport by their gene products will also be analogous (Section VI.B5,6).

5. *Regulation of the Dipeptide Permease*

This aspect has not been studied very intensively, although it is to be expected that it will be subject to similar controls as *opp*. As a general observation, DppA expression seems somewhat variable amongst different strains of *E. coli* and *S. typhimurium* but the reasons for this are obscure. Expression of *dpp* has been studied in *E. coli* by monitoring DppA levels under different growth conditions, and in *S. typhimurium* using *dpp-lacZ* fusions. In the latter case, production of β-galactosidase (and thus, by presumption, *dpp* expression) was unaffected by growth in media containing glycerol or glucose, addition of casamino acids or peptides, the level of nitrogenous nutrients or anaerobic growth. In *E. coli*, the level of DppA was unaffected by growth in media containing glycerol or glucose, or addition of casamino acids, or Pro-Leu. It was concluded that *dpp* expression is probably constitutive. However, as described for OppA

(Section VI.B7), there is a decrease in DppA synthesis under conditions of phosphate limitation. As for *opp*, the regulatory region of *dpp* contains a sequence similar to the consensus binding site for PhoB, raising the possibility of repression being mediated by the Pho regulon (Smith and Payne, 1992).

The reason for the exceptionally high production of DppA in *E. coli* strain JM101 grown in minimal glucose and its decrease by addition of casamino acids is not known (Olson *et al.*, 1991). The control appears to operate at the level of transcription. The nucleotide sequences reported for the gene isolated from this strain, and from another non-overproducing strain, were identical, so the basis for the differential expression must either be encoded in the sequence upstream of those determined or it is a separate strain-specific effect. However, it was noted that, in *E. coli* strain W3110, which produces DppA at a normal level, its synthesis was also repressed by casamino acids (Olson *et al.*, 1991). Clearly, further work is needed to reconcile the different reports and to provide a detailed explanation of regulation in this system.

D. THE TRIPEPTIDE PERMEASE

The existence of an additional system in *E. coli* able to transport tripeptides was speculated upon when *opp* mutants were first isolated (Payne, 1968). Its presence was confirmed when well-characterized *opp* mutants were shown to be able to grow on a restricted range of tripeptides (Barak and Gilvarg, 1975; Naider and Becker, 1975).

This system showed a similar substrate specificity to Opp with respect to its need for a positively-charged amino terminus and its ability to transport tripeptides without a free *C*-terminal carboxyl group (Naider and Becker, 1975) (Section VI,B2). Subsequent work, using *opp dpp* mutant strains, has shown that it can transport with varied ability a wide variety of tripeptides and, less effectively, dipeptides (Alves and Payne, 1980). It is clear that it is unable to transport tetra- or higher peptides, hence it was termed the tripeptide permease (Tpp) (Payne, 1983 and J. W. Payne, unpublished results; Naider and Becker, 1975). This permease possesses greater affinity for peptides composed of hydrophobic amino acids, particularly with *N*-terminal Val or Met residues (Payne, 1983); a similar situation appears to be true for *S. typhimurium* (Gibson *et al.*, 1984). Mutations in *tpp* can be isolated through selection of resistance to alafosfalin (Section XI), a dipeptide which is taken up predominantly through this system (Payne, 1983; Gibson *et al.*, 1984). Mutations leading to alafosfalin resistance in *E. coli* have been mapped to 86 min, and the *tppB* locus to 27 min in *S. typhimurium* (Payne, 1983; Gibson *et al.*, 1984).

In addition, a locus designated *oppE* that appeared to encode a system with a similar specificity to the Tpp has been localized to 98.5 min in *E. coli* (Andrews and Short, 1985, 1986; Andrews *et al.*, 1986). Further studies are needed to clarify this matter.

The *tppB* locus in *S. typhimurium* has been cloned and, although the nucleotide sequence was not determined, it was considered from the size of the cloned DNA to consist of more than one gene (Gibson *et al.*, 1984). Few studies have been performed to determine the class of permease to which Tpp belongs. It has been reported to be sensitive to arsenate and thus a possible binding-protein dependent system (Payne, 1983). However, analysis of the shock fluid from a variety of *tpp* mutants has failed to reveal a putative Tpp-binding protein (Smith, 1992). In the shock fluid of an *oppA dppA* mutant, binding activity for Val-[^{125}I]Tyr-Val and Leu-Leu[^{125}I]Tyr has been detected, but this could not be related to one specific protein (Smith, 1992).

Regulation of transcription was investigated using *lac* fusions to the *tppB* locus in *S. typhimurium* (Jamieson and Higgins, 1984, 1986). Expression was unaffected by the nature of the carbon or nitrogen source, whereas addition of leucine or alanine, or anaerobic growth, caused a tenfold increase in transcription. The anaerobic induction is repressed by nitrate, which implies that it is the redox potential that is sensed rather than the concentration of oxygen. Interestingly, a peptidase (PepT) with related substrate specificity to the Tpp is also induced by anaerobic growth (Jamieson and Higgins, 1984). A further locus, originally called *tppA*, acts as a positive regulator; this was shown to correspond to the *ompB* locus, comprising *ompR* and *envZ* genes (Gibson *et al.*, 1987; Stock *et al.*, 1989). When grown anaerobically, but not aerobically, *tppB* expression is dependent on medium osmolarity (Ni Bhriain *et al.*, 1989). An effect on DNA supercoiling has been implicated in regulation of gene expression (Higgins *et al.*, 1988a; Dorman *et al.*, 1988). Little is known about regulation of *tpp* in *E. coli*, although it also appears to be controlled by *ompR/envZ* (Gibson *et al.*, 1987). It is surprising that the *opp* in *E. coli* and *tpp* in *S. typhimurium* have been reported to be regulated similarly (by leucine and anaerobiosis). Although Tpp is generally considered to play a minor role, it could become more important for certain species in the anaerobic environment of the mammalian gut.

VII. Peptide Transport in other Bacterial Species

A. INTRODUCTION

Few bacterial species other than *E. coli* and *S. typhimurium* have been studied in detail. Nevertheless, conclusions based on a variety of studies

testify to the occurrence and importance of peptide transport in a wide range of bacteria. Subsequent studies have endorsed this conclusion and have increasingly highlighted similarities between the systems present in various species. Many fastidious organisms utilize peptides as sources of essential amino acids and this has proved a useful means by which to study transport; commonly, peptides prove to be nutritionally more effective than corresponding mixtures of amino acids. Useful general information on peptide uptake has also come from screening bacteria for sensitivity to a range of peptide-carrier prodrugs (smugglins) (see for example Atherton *et al.*, 1982 and Section XI). Inhibition requires that smugglins be accumulated intact and hydrolysed by intracellular enzymes; thus, sensitivity provides prima facie evidence for peptide transport. The various species studied have considerable differences in their physiology and cell structure and, to facilitate discussion of their transport characteristics, it is useful to divide them into groups that take account of these differences.

B. *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is a clinically important pathogen, one resistant to many antimicrobial agents mainly because of its unusual and complex cell-envelope structure. Some confusion has surrounded studies of peptide transport by this bacterium because of reports of membrane-bound peptidases that may function in peptide utilization (Haas *et al.*, 1981; Hulen and LeGoffic, 1988). Conclusions from early auxotrophic-growth studies indicating physiologically important peptide transport (reviewed in Payne 1980a) have been confirmed by direct studies of transport (Alves, 1984; Hulen and LeGoffic, 1987; J. W. Payne and R. A. Alves, unpublished results). In our laboratory, using radioactively-labelled peptides and fluorescence assays, we have demonstrated the presence of two systems with properties analogous to the Dpp and Opp of *E. coli*. Intact accumulation of a non-hydrolysable peptide, namely Gly-Sar, was demonstrated. Compared with Dpp in *E. coli*, this dipeptide system showed a similar affinity (K_t = 19 μM for Gly-Phe), but an uptake rate (V_{max} = 0.4 nmol Gly-Phe min^{-1} (mg protein)$^{-1}$) about two orders of magnitude lower. Analogous conclusions were reached for uptake by the oligopeptide system; kinetic values were K_t = 18 μM for Ala_3, and V_{max} = 0.7 nmol Ala_3 min^{-1} (mg protein)$^{-1}$. Both systems showed stereospecificity. Various oligopeptides, up to and including hexa-alanine, compete with trialanine for transport. No evidence for efflux of peptide-derived amino acids was found, perhaps not surprising in view of the slow uptake rates, added to which extracellular peptide hydrolysis was not detectable. The fact that arsenate inhibited uptake by both systems is compatible with their

energization being linked to ATP directly. It is to be expected that, at a molecular level, they will resemble model systems in *E. coli* (Section VI), and we have demonstrated cross-reactivity of antibodies raised against OppA from *E. coli* with proteins present in shock fluids from *Ps. aeruginosa*. It is not clear to what extent slow peptide (and amino-acid) transport is a characteristic of the respective permeases, or reflects a rate-limiting penetration of outer membrane porins. Interestingly, the D2 porin contains binding sites for basic amino acids and basic peptides (Trias and Nikaido, 1990) (Section IV.A). Whether analogous porins for anionic species may recognize negatively-charged peptides remains to be determined (Siehnel *et al.*, 1992).

C. RUMEN MICRO-ORGANISMS

Rumen micro-organisms comprise an important and heterogeneous group. They play an essential role in breakdown of ingested feedstuffs, degradation products from which become available to the small intestine for absorption. However, in attempts to calculate the ruminant's requirements for dietary protein, it has become recognized that degradation of proteins in the rumen can be wasteful, much being lost as ammonia. Consequently, much effort is being directed at characterizing the system with the aim of maximizing protein utilization. Studies with the mixed microbial population present in rumen fluid have led to the conclusion that peptide uptake can be the rate-limiting step in ruminal proteolysis (Chen *et al.*, 1987). Various species of protozoa, anaerobic fungi and bacteria are involved in this proteolysis, although bacteria are most important in peptide hydrolysis (Wallace *et al.*, 1990). These bacteria are strict anaerobes. The most important organism involved in peptide breakdown is the Gram-negative *Bacteroides ruminicola* (Wallace and McKain, 1991). Oligopeptide hydrolysis appears to be initiated mainly through action of an intracellular dipeptidyl aminopeptidase activity. It seems likely from the limited studies described that peptide transport resembles that found for other Gram-negative bacteria. The anaerobe *Streptococcus bovis* is another important proteolytic bacterium in the rumen, and good evidence for its ability actively to accumulate peptides has been provided (Westlake and Mackie, 1990). To date, however, most transport studies with these species may have been compromised by the occurrence of secreted and cell-bound peptidases. The recent studies already cited recognize these pitfalls, and clear characterization can be expected now that pure cultures are being used in transport studies.

D. LACTIC-ACID BACTERIA

Lactic-acid bacteria are a further group of proteolytic, fastidious organisms that have been studied extensively, mainly because of their commercial importance in casein breakdown in cheese production. For effective utilization of proteins, lactic-acid bacteria possess extracellular, cell wall-bound proteinases and peptidases that act in concert to provide the cell with essential and growth-stimulating amino acids and peptides. Early studies with lactic-acid bacteria indicated that specific peptide transport occurred, and that peptides were often nutritionally superior to amino acids (Law, 1978; Rice *et al.*, 1978); conclusions from early investigations have been reviewed by Payne, (1980a) and VanBoven and Konings (1986). However, confusion has surrounded descriptions of the physiological implications of peptide transport in lactic-acid bacteria because of uncertainties in classification and nomenclature of the species. Recently, a new genus *Lactococcus* has been proposed to aid classification (Schleifer and Kilpper-Balz, 1987). Currently, five species in this genus are recognized as forming a single DNA homology group of which *Lactococcus lactis* is the most studied. However, three strains can be distinguished on the basis of phenotypic differences, namely subspp. *lactis, cremoris* and *hordniae.* Members of the genus *Lactococcus* are Gram-positive, non-motile, facultative anaerobic cocci, with complex nutritional requirements. *Steptococcus lactis* is phylogenetically distinct from lactococci and constitutes a new genus. Taxonomic studies show that the genus *Lactococcus* is not closely related to streptococci and enterococci. A useful network showing phylogenetic relationships between these species and other Gram-positive organisms has been described (Smid, 1991).

In *L. lactis*, dipeptide uptake, intracellular cleavage and consequent amino-acid efflux have been described (VanBoven and Konings, 1988) that resemble the behaviour of *E. coli* (Section V). For growth of *L. lactis* on casein, uptake of dipeptides is essential (Smid *et al.*, 1989a; Smid and Konings, 1990). Studies on the energetics of dipeptide transport in *L. lactis* have led to conflicting conclusions. Initially a phosphate bond-driven system, regulated by internal pH value, was proposed (VanBoven and Konings, 1987) but, in later studies using membrane vesicles, it was proposed that transport was coupled directly to the proton-motive force (pmf) (Smid *et al.*, 1989b). Further studies of dipeptide uptake by *L. lactis* has characterized a system of broad specificity that has a limited capacity to transport tripeptides also, as found for the Dpp in *E. coli* (Section VI.C2); an alanyl-β-chloroalanine-resistant mutant was defective in this dipeptide transport system (Smid *et al.*, 1989a; Smid, 1991). This mutant proved useful in characterizing a further system(s) for transporting di-, tri-

and oligopeptides (Smid, 1991) that shows similarities to the Opp in *E. coli*. Based on the observation that valinomycin and nigericin inhibited oligopeptide uptake, it was suggested that this system also might be driven by a pmf, although a possible role for other energy sources was not excluded (see also p. 79).

E. GRAM-POSITIVE BACTERIA

The role of peptides in the physiology of a range of other Gram-positive organisms has been described, and the transport systems involved described at various levels of molecular detail. In *Staphylococcus aureus*, the main peptide-transport system recognizes both di- and tripeptides (Perry, 1981). In *Enterococcus* (formerly *Streptococcus*) *faecalis*, two typical peptide permeases exist (Nisbet and Payne, 1982). One is a high-rate system used by dipeptides, and to a lesser extent tripeptides; the other is a low-rate oligopeptide system. Exodus of amino-acids can occur after peptide uptake and cleavage. The specificities of these systems show broad similarities with the Dpp and Opp in *E. coli*, although uptake rates are markedly faster in *Enterococcus faecalis*. Transport of peptides is stopped immediately on addition of a protonophore such as carbonylcyanide chlorophenylhydrazone (CCCP) that collapses the pmf (Payne and Nisbet, 1981). However, the fact that this treatment simultaneously causes exodus of intracellular substrates cautions against any premature conclusion on the possible role of the pmf in energy coupling to peptide transport. Uniquely amongst peptide-transport systems, a further system occurs in *Enterococcus faecalis* with specificity apparently restricted to di- and tripeptides with *N*-terminal glutamyl or aspartyl residues (Payne *et al.*, 1982). Peptides containing anionic residues at other positions are transported by other peptide permeases. The significance of this specialized system awaits further study. *Bacillus subtilis* is able to transport and to utilize peptides, and this has been made the basis for the design of a biosensor device, which include the bacteria on an oxygen electrode, for selective determination of peptides (Riedel *et al.*, 1989).

The results already described for Gram-positive bacteria provide clear evidence for the presence of various peptide permeases, and reveal strong similarities in their complementary specificities to the better characterized systems in Gram-negative bacteria. However, given the marked differences in cell-envelope structures of the two types of bacteria it might not have been anticipated that they would have peptide permeases with such similar molecular architectures. Nevertheless, evidence for high-affinity, binding protein-dependent transport systems in Gram-positive bacteria and in *Mycoplasma hyorhinis* has recently been provided. Dudler

et al. (1988) presented DNA sequence data indicating that *Mycoplasma hyorhinis* possesses a transport system similar to the traffic ATPases of Gram-negative bacteria (Section XII, p. 66). A protein (p37) was identified that appeared to be the analogue of binding proteins in Gram-negative bacteria. This result was surprising given that mycoplasmas lack a cell wall and a periplasmic space and, based on their rRNA sequences, seem to be related to Gram-positive bacteria. However, a significant difference to the binding proteins in Gram-negative bacteria was noted, for the predicted amino acid sequence of p37 comprised not only a typical signal sequence to allow its translocation across the cytoplasmic membrane but, distinctively, it possessed an *N*-terminal sequence for the mature protein that matched the *N*-terminal consensus sequence of secreted bacterial lipoproteins. In such lipoproteins, the *N*-terminal cysteine is modified to carry a glyceride moiety that anchors them to the membrane. The gene for p37 was part of an operon coding for two additional proteins, namely p29 and p69; p29 is homologous to the ATP-binding subunits, and p69 has similarity to the integral membrane proteins of periplasmic binding-protein systems (see Section VI.B5).

In a linked publication, Gilson *et al.* (1988), presented evidence that the Gram-positive *Streptococcus pneumoniae* also possessed extracytoplasmic, binding lipoprotein-dependent transport systems. Extensive homology was found between two proteins from *Strep. pneumoniae*, namely MalX and AmiA, and periplasmic-binding proteins, MalE and OppA, which are involved in transport of maltose and oligopeptides, respectively, in Gram-negative bacteria. In a later publication (Alloing *et al.*, 1990), the complete nucleotide sequence of the *ami* operon of *Strep. pneumoniae* was described. Overall, the operon is strikingly similar to the *opp* locus of *S. typhimurium*. It encodes six proteins, with AmiA being the first gene product. Extensive homologies exist between the respective genes for the hydrophobic membrane components and the (presumed) peripheral ATP-binding proteins. The Ami system, is, however, dispensable for cell viability. Nevertheless, *ami* mutant strains show various pleiotropic effects, including enhanced resistance to aminopterin and methotrexate. Although the mechanism of this resistance is unclear, *amiA* mutations do not confer full resistance, which led the authors to speculate that facilitated diffusion of these drugs might occur without involvement of AmiA. This model may find relevance in the mechanism of the multiple-drug resistance protein (Section XII). The AmiB protein, which has no counterpart in the operon in Gram-negative bacteria, is homologous to ArsC, a cytosolic modifier subunit of the anion pump encoded by the arsenate-resistance operon of the R-factor R773 from *E. coli*. Its possible functional relevance to peptide uptake in *Strep. pneumoniae* remains to be determined. The Ami system is likely to play

an important nutritional role since *Strep. pneumoniae* is auxotrophic for several amino acids, and peptides could satisfy these requirements. However, no studies have yet been carried out on the physiological role of peptide uptake in this organism.

In oral streptococci, the organism implicated as of greatest significance in the causation of dental caries is *Strep. mutans*. This bacterium has recently been shown to possess a lipoprotein-dependent periplasmic-type of transport system for sugars, termed *msm* (Russell *et al.*, 1992). The *msm* operon shows considerable similarities in organization to those systems in Gram-negative bacteria and to the Ami locus of *Strep. pneumoniae*. It seems reasonable to anticipate that analogous peptide permeases will be found in *Strep. mutans*, for peptides are common constituents of its physiological niche, and peptide utilization has been reported for *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, which are also important pathogens in human periodontal disease (Gharbia and Shah, 1991; Bakken *et al.*, 1989).

F. SPORE-FORMING BACTERIA

A similar approach, based on cloning and sequence studies, has led to the identification in *Bacillus subtilis* of separate transport systems for oligopeptides (Perego *et al.*, 1991; Rudner *et al.*, 1991) and dipeptides (Mathiopoulos *et al.*, 1991; Slack *et al.*, 1991). Many mutations have been characterized in *B. subtilis* that cause defects in sporulation; one of these, *spoOK*, is defective in the first step in sporulation. This mutation was shown to reside in an operon of five genes, structurally identical to the *opp* locus of Gram-negative bacteria (Perego *et al.*, 1991; Rudner *et al.*, 1991) (Section VI). The deduced amino acid sequences of the five open reading frames were very similar to the OppA, B, C, D and F proteins of *S. typhimurium*. The binding protein corresponding to OppA was least conserved, especially at its amino terminus. The ATP-binding proteins (SpoOKD and SpoOKF) were highly conserved, but the hydrophobic membrane components (SpoOKB and SpoOKC) slightly less so. Studies on the sensitivity of *B. subtilis* to the toxic dipeptide bialaphos (Section XI) confirmed that the *spoOK* gene products function in peptide transport. Mutations in *SpoOKA*, *SpoOKB*, *SpoOKC* or *SpoOKD* all affected sporulation, and all conferred resistance to bialaphos by preventing its uptake. Surprisingly, however, mutants with insertions into *SpoOKF* remained sensitive to bialaphos; in contrast, in *S. typhimurium*, both *SpoOKD* and *SpoOKF* are required for peptide transport (Hiles *et al.*, 1987a). Mutations in *SpoOKF* were also without effect upon sporulation. The presumed binding protein, SpoOKA, has a cleavable *N*-terminal signal sequence to allow its translocation that

is characteristic of lipoproteins with a *N*-terminal glyceryl-cysteinyl membrane anchor, as found in *Strep. pneumoniae*. This protein was partly associated with the cell during the exponential phase of growth, although the nature of the association was not clarified, but it became increasingly released into the medium as the cells entered the stationary phase of growth. In these studies, it was speculated that the SpoOK system may serve to accumulate extracellular peptide factors, perhaps from turnover of peptidoglycan, that play a signalling role in initiation of sporulation. The sporulation defect was considered to result from failure to transport these peptides. However, an alternative transmembrane-signalling model could be envisaged in which binding of the SpoOKA–peptide factor complex could be sensed intracellularly, precluding the need for the peptide factor itself to be transported.

In a related study, analysis of the sequence of the *dciA* operon, transcripts from which accumulate rapidly immediately after induction of sporulation, showed that it encoded five proteins organized in a similar manner to that of binding protein-dependent systems in Gram-negative bacteria (Mathiopoulous *et al.*, 1991). However, *dciA* differed in one unique respect in that the putative binding protein was coded by the fifth rather than the first gene. This protein, DciAE, was similar to DppA and OppA of *S. typhimurium* and *E. coli*, and to SpoOKA of *B. subtilis*. A mutation in *dciAE* abolished the ability of a proline-requiring auxotroph to grow on the dipeptide Pro-Gly as sole source of proline, providing evidence for the products of this operon constituting a dipeptide permease. The amino-terminal region of DciAE has a predicted signal peptide and a terminal sequence typical of lipoproteins in Gram-positive bacteria. The *dciA* operon was shown to be under several transcriptional controls (Slack *et al.*, 1991). In vegetative cells, transcription is at a very low level, but this is induced rapidly by several conditions that cause cells to enter the stationary phase of growth and to initiate sporulation. The protein AbrB, a known regulator of early sporulation genes, is a repressor of *dciA* transcription. Expression of *dciA* in growing cells is also repressed independently by glucose and by a mixture of amino acids; neither of these effects is mediated by AbrB.

G. GENERAL COMPARISONS

Recently, therefore, Gram-positive bacteria have been shown to possess peptide permeases analogous to those already well characterized in Gram-negative bacteria. The finding that Gram-positive species have analogues of periplasmic-binding proteins that are anchored, rather than freely diffusible, may not be too surprising. Within the gel-like periplasm of a

Gram-negative bacterium, protein diffusion may be limited and mostly lateral (Brass *et al.*, 1986) (Section IV). Therefore, in a Gram-positive bacterium, restricting the mobility of an anchored binding protein to two-dimensional diffusion in the extracellular monolayer of the cytoplasmic membrane may not be very different. The interesting finding had been made earlier that the maltose-binding protein in a mutant of *E. coli*, in which the signal peptide was not cleaved and the protein remained attached to the outer face of the membrane, could still function in transport (Fikes and Bassford, 1987). And, recently, a natural example has been described that combines the two distinctive features of binding proteins from Gram-negative and Gram-positive bacteria. *Haemophilus influenzae* is a Gram-negative bacterium, but in many respects it resembles *Strep. pneumoniae*. It has complex growth requirements, amongst which is a dependence on an exogenous source of haem for aerobic growth. A protein has been identified from *H. influenzae* that binds haem *in vitro*. The nucleotide sequence of the gene for this protein (HbpA) revealed the protein sequence to be 53% identical to the dipeptide-binding protein DppA from *E. coli* (Hanson *et al.*, 1992). But sequence data and biochemical evidence show HbpA to be a lipid-anchored binding protein. Although the full functional and mechanistic role of HbpA awaits clarification, it represents an intriguing hybrid structure and perhaps we should now anticipate further examples being found. It remains only for lipoprotein-binding proteins from Gram-positive bacteria to be shown to perform a functional role in the free diffusible form they adopt as cells progress into the stationary phase of growth, for the circle to be closed fully!

VIII. Peptide Transport in Fungi

A. INTRODUCTION

Early studies on transport and utilization of peptides in yeasts and other fungi have been reviewed (Becker and Naider, 1980; Wolfinbarger, 1980; Matthews and Payne, 1980). Since then, detailed investigations have been restricted to *Saccharomyces cerevisiae* and *Candida albicans*, and these will be considered separately.

B. *SACCHAROMYCES CEREVISIAE*

In *Sacch. cerevisiae*, studies on growth and response of amino-acid auxotrophs showed that a variety of di- and tripeptides were taken up, and results of competition studies indicated that they shared a common system

that was distinct from amino-acid permeases (Marder *et al.*, 1977; Becker and Naider, 1977, 1980). A mutant of a leucine-lysine auxotroph, isolated on the basis of its resistance to L-ethionylalanine, was cross resistant to Leu-Leu-L-ethionine, did not utilize di- and tripeptides, although it contained peptidases able to cleave them, and had lost the capacity to accumulate radioactivity from [^{14}C]Gly-Leu. The characteristics of this peptide-transport mutant, and of a spontaneous revertant, indicated that di- and oligopeptides shared a common system (Marder *et al.*, 1978). A similar conclusion was reached for a bacilysin-resistant, peptide transport-deficient mutant of a wild-type strain Σ1278b (Nisbet and Payne, 1979a,b), and for a nikkomycin Z-resistant transport mutant (Moneton *et al.*, 1986a). This system has been termed the general peptide permease (Gpp) (Nisbet and Payne, 1979a). Confirmation of these earlier conclusions, and preliminary genetic studies indicating that Gpp involves at least three components of which one may be regulatory, have appeared (Island *et al.*, 1991).

Structural specificities and kinetic parameters of Gpp have been determined for several strains, using a variety of assays based on growth, fluorescence or radioactively-labelled peptides (Section V). Peptide transport and hydrolysis were shown to be mechanistically separate by showing intact accumulation of non-hydrolysable sarcosine peptides (Nisbet and Payne 1979b; Moneton *et al.*, 1986a) and by demonstration of the intracellular locations of peptidases involved in substrate hydrolysis (Parker *et al.*, 1980). Conclusions regarding the influence of peptide size on uptake are conflicting. Growth studies with various auxotrophs showed that certain strains could utilize tetra- and pentapeptides whereas others could not (Becker and Naider, 1980); uptake of peptides larger than tripeptides was undetectable in direct transport assays (Nisbet and Payne, 1979a,b). A study (Naider *et al.*, 1980) apparently showing competitive inhibition of trimethionine transport by a soluble, macromolecular peptide (Met_3-polyethyleneglycol, M_r value of 5000) is difficult to interpret in relation to the complex cell envelope structure of *Sacch. cerevisiae*, and its general exclusion of compounds with M_r values greater than about 700. The observation may have relevance to the interaction of yeast α-mating factor (a dodecapeptide) with its receptor in the plasma membrane.

Somewhat varied conclusions have been reached regarding the influence of side chains on transport. However, these differences probably reflect assay artefacts, for example peptidase-dependence of growth tests, rather than significant inter-strain variations. Systematic studies measuring transport directly are the most reliable (Nisbet and Payne, 1979b). Overall, Gpp prefers bulky substrates with hydrophobic side chains, and discriminates against negatively-charged amino-acid residues. The system shows

high stereospecificity for L-residues, since transport of LD, DL, LLD, LDL and DDD peptides of alanine was not detectable (Nisbet and Payne, 1979b). Various peptides containing D-residues were poor competitors for uptake of [^{14}C]Met_3 (Marder *et al.*, 1977; Becker and Naider, 1977). In contrast to studies of other peptide-transport systems (Matthews and Payne, 1980) in which a free α-*N*-terminal amino group is required, auxotrophic growth tests indicated that α-*N*-acetylated peptides could be utilized (Becker and Naider, 1977), although not by all strains (Marder *et al.*, 1977). The situation still remains to be clarified. Clearly, substitution of the *C*-terminal α-carboxyl group, for example by esterification, has little effect on uptake and utilization (Marder *et al.*, 1977; Becker and Naider, 1977). Uptake of sarcosine-containing di- and tripeptides establishes that the system can handle peptides with a methylated peptide-bond nitrogen atom (Nisbet and Payne, 1979a).

Kinetic parameters for peptide transport have been determined from fluorescence assays and using radioactively-labelled peptides (Becker and Naider, 1977; Nisbet and Payne, 1979b; Payne and Nisbet, 1980a; Payne *et al.*, 1982; Moneton *et al.*, 1986a). The system has a pH optimum of about 5.5. Initial rates for all typical peptides were about 2–5 nmol (mg dry wt)$^{-1}$ min^{-1} and K_m values were typically about 10^{-5}M.

In a preliminary attempt to identify protein components of the peptide carrier, the system was photo-inactivated with two different substrates (Becker *et al.*, 1982). Substrates Leu-Leu-4-azido-2-nitrophenylalanine and (surprisingly) Leu-*p*-nitroanilide were shown to be competitive inhibitors of peptide transport and, upon photolysis, to give specific, irreversible inactivation of peptide uptake. Unexpectedly, the amino acid *p*-nitroanilide appeared to show greater affinity for the peptide carrier than did the peptide analogue. The authors concluded that a component(s) of the peptide-transport system was irreversibly modified by photolysis, but no further reports on its characterization have appeared.

Thus, at present, no good biochemical information exists on the number or nature of the protein components of Gpp; although, it should be recalled that genetic evidence points to at least three components being involved (Island *et al.*, 1991). Preliminary attempts (Shallow, 1987) to look for a putative binding protein, analogous to that implicated in leucine transport (Wainer *et al.*, 1988) proved inconclusive. The availability of antibodies against peptide-binding-proteins from *E. coli* may help to resolve this question (C. F. Schuster and J. W. Payne, unpublished results). Clearly, there is much need for a combined biochemical and molecular genetical approach to clarify the situation. Such information may find application to strain selection for commercially-used yeast species, where utilization of peptide nutrients may play an important role in cell physiology and product yield.

Sacch. cerevisiae has been shown to possess all of the enzymes of the γ-glutamyl cycle, and speculations have arisen that this cycle might be involved in transport of amino acids and peptides (Mooz and Wigglesworth, 1976; Jaspers and Penninckx, 1981). However, Robins and Davies (1981) reported that turnover of glutathione through the cycle was too slow for its stoicheiometric involvement in transport. In addition, activity of the key enzyme γ-glutamyltransferase was not changed between a wild-type strain and derived *gap* and *gpp* mutants, and selective inhibition of this membrane-bound enzyme also failed to inhibit transport (Payne and Payne, 1984). It was concluded that the γ-glutamyl cycle is not involved in transport of peptides, amino acids or γ-glutamylamino acids, although it was suggested that it could play a coordinating role in transmembrane regulation by signalling the presence of external nutrients by delivery of low levels of γ-glutamyl derivatives into the cell cytoplasm.

Energization of peptide transport is linked to a proton-motive force across the plasma membrane. *Sacch. cerevisiae* produces ATP by fermentation and by oxidative phosphorylation. A specific plasma-membrane ATPase acts as a proton pump; the enzyme is sensitive to N,N^1-dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (DES). Pre-incubation with the metabolic inhibitors (azide, dinitrophenol or cyanide) blocks transport (Becker and Naider, 1977) as also does arsenate, DES and the protonophore CCCP (Payne and Nisbet, 1981). Furthermore, with cells actively accumulating peptide, addition of DES or CCCP stopped uptake almost immediately (Payne and Nisbet, 1981). Similar results were obtained for amino acid substrates of the general amino acid permease (Gap). It appears, therefore, that peptide and amino-acid transport are likely to be energized in the same manner. Few systematic studies have been carried out into possible regulation of peptide transport. Initial uptake rates are enhanced about tenfold in cells grown on poor nitrogen sources such as proline, rather than on glutamate or ammonium ions (Becker and Naider, 1977; Nisbet and Payne 1979a; Moneton *et al.*, 1986b). This places peptide transport in the general category of systems that are subject to repression by nitrogen sources that support a high growth rate (Wiame *et al.*, 1985). In addition, low concentrations of a variety of amino acids induce large increases in dipeptide uptake. For example, in defined media containing allantoin as the sole nitrogen source, tryptophan (15 mM) increased uptake 25-fold within 30 min; the increase is blocked by cycloheximide, implying that *de novo* protein synthesis is required (Island *et al.*, 1987, 1991). The molecular mechanism of the effect is unknown, but it appears to differ from analogous effects reported for *Candida albicans* (Payne *et al.*, 1991).

C. *CANDIDA ALBICANS*

In the dimorphic, opportunistic fungal pathogen *Candida albicans*, peptide transport and utilization have been studied in some detail. A main aim has been to devise anticandidal agents based on the smugglin principle (Naider and Becker, 1987) (Section XI). The early literature contains conflicting information on the number and specificities of its peptide permeases (Lichliter *et al.*, 1976; Logan *et al.*, 1979; Davies, 1980; Becker and Naider, 1980). This arose for a variety of reasons, including considerable inter-strain variation, the influence of different nitrogen sources on permease expression, and problems inherent in determining transport properties from assays based on growth response and substrate competition and using radioactively-labelled substrates. The influence of these various aspects has been clarified in later studies and general principles have emerged.

Attempts to characterize the number of peptide permeases have mainly involved mutant selection. Characterization of mutants isolated as resistant to various toxic peptides has shown the high frequency with which various transport mutants can arise and also revert. A number of these mutants show transport properties characteristic of other so-called wild types, illustrative of the variation in peptide sensitivities and transport capacities observed with different strains, which appear to be a general finding with *C. albicans* (Payne and Shallow, 1985). The results indicate that several peptide permeases are present. A mutant isolated as being resistant to the toxic dipeptide nucleoside nikkomycin Z (Section XI) lost most of its capacity to take up dipeptides but apparently simultaneously increased its ability to transport tripeptides (Yadan *et al.*, 1984). A mutant, isolated by a similar selection procedure from a different strain was unable to transport dipeptides, while oligopeptide uptake was unaffected (McCarthy *et al.*, 1985a,b). Spontaneous mutants selected for resistance to one of the toxic peptide analogues bacilysin, polyoxin (Section XI) and nikkomycin showed cross resistance to the other analogues and associated defects in dipeptide transport, whilst retaining normal oligopeptide uptake (Payne and Shallow, 1985; Mehta *et al.*, 1984). A revertant of a bacilysin-resistant mutant, selected for its ability to utilize Ala-Ala as sole nitrogen source, regained wild-type dipeptide uptake ability and analogue sensitivity. In addition, a mutant resistant to the toxic tripeptide *m*-fluorophenylalanyl-Ala-Ala showed normal dipeptide transport but had lost the ability to accumulate oligopeptides (Payne and Shallow, 1985). Finally, characterization of mutants resistant to dipeptides containing the toxic N^3-4-methoxyfumaroyl-L-2,3-diamino-propanoic acid residue (Section XI) lends further support for the multiplicity of peptide permeases (Milewski *et al.*, 1988). The unifying conclusion from these studies is that two peptide permeases operate in *C. albicans*. These are a dipeptide permease, which

may have very limited ability to transport tripeptides, and an oligopeptide permease that handles tri- and higher peptides. This conclusion is endorsed from studies involving photo-affinity inhibition of tripeptide transport (Sarthou *et al.*, 1983).

Problems in obtaining detailed information on structural specificities and kinetic parameters from growth studies and use of radioactively-labelled peptides have also been noted with *C. albicans* (McCarthy *et al.*, 1985c; Payne and Shallow, 1985). But more reliable information has come from use of fluorescence and colorimetric assays and well-controlled experiments using radioactive substrates (McCarthy *et al.*, 1985b,c; Payne and Shallow, 1985; Milewski *et al.*, 1988; Shallow *et al.*, 1991). Most studies have been carried out using wild-type strains in which both di- and oligopeptide permeases are functional and, except for one study that looked at mycelial cells (Milewski *et al.*, 1988), transport has been measured in the unicellular yeast form. With respect to size, oligopeptides containing up to six residues are transported at comparable rates (McCarthy *et al.*, 1985b,c; Payne and Shallow, 1985; Milewski *et al.*, 1988; Shallow *et al.*, 1991). Whether this cut off operates at the level of permease or more generally at the cell-envelope has not been clarified (Section IV.C).

Peptides containing a range of normal amino-acid residues and analogues can be transported, but the permeases function best with substrates containing alanyl residues or more bulky hydrophobic residues, whereas the presence of acidic or basic side-chain groups restricts uptake (McCarthy *et al.*, 1985c; Milewski *et al.*, 1988; Shallow *et al.*, 1991). Although all studies indicate a preference for peptides with free α-*N*-termini, some studies, mainly involving competition, indicate that α-*N*-acetyl peptides can bind and be transported to a limited extent (Logan *et al.*, 1979; Chmara *et al.*, 1982; McCarthy *et al.*, 1985b; Milewski *et al.*, 1988). The *C*-terminal carboxyl group may be replaced by other acidic groups, such as phosphonate, sulphonate or tetrazole, without abolition of uptake (Davies, 1980) but some reports indicate amidation or esterification may decrease transport (Lichliter *et al.*, 1976; Logan *et al.*, 1979; McCarthy *et al.*, 1985b). Direct-transport assays show a strict stereospecific requirement for L-residues (Milewski *et al.*, 1988; Shallow *et al.*, 1991).

In the yeast form of *C. albicans*, the optimum pH value for peptide uptake is about pH 4.5 (Logan *et al.*, 1979; Davies, 1980; Payne and Shallow, 1985), whereas for the mycelial form it has been reported as pH 6.8 (Milewski *et al.*, 1988). Kinetic parameters determined from various transport assays are typically V_{max} 1–10 nmol (mg dry wt)$^{-1}$ min^{-1} (Payne and Shallow, 1985; Milewski *et al.*, 1988; Shallow *et al.*, 1991; McCarthy *et al.*, 1985b,c); values are about two times higher for mycelial cells, with the

oligopeptide permease being enhanced relative to the dipeptide permease (Milewski *et al.*, 1988); K_m values are about 2 μM (Logan *et al.*, 1979; McCarthy *et al.*, 1985b,c; Yadan *et al.*, 1984).

Attempts to identify permease components have been limited and only preliminary in nature. Photo-affinity labelling of the tripeptide permease (Sarthou *et al.*, 1983; Milewski *et al.*, 1991a) has not yet led to isolation of derivatized components. Attempts to label permease components with protein reagents showed that little selectivity was possible (Shallow *et al.*, 1991).

Similarly, only preliminary attempts have been made to characterize mechanisms by which peptide transport is energized. Demonstration of intact accumulation of various peptides resistant to intracellular peptidases confirms that an active transport process operates (Davies, 1980; Shallow *et al.*, 1991). *Candida albicans* can use stored energy for transport quite effectively, for cells incubated for up to 30 min without an energy source show no deterioration in transport, unlike *Sacch. cerevisiae* which requires an added energy supply (Shallow *et al.*, 1991). A variety of studies indicate both di- and oligopeptide permeases require maintenance of a proton-motive force and dependence on a plasma-membrane ATPase (sensitivity to DCCD and DES) (Logan *et al.*, 1979; Davies, 1980; Shallow *et al.*, 1991).

Regulation of permease activity operates at several levels. A mechanism for ammonium (nitrogen) repression inhibits a range of transport systems, including peptide permeases (Becker and Naider, 1980; Wiame *et al.*, 1985). A second level of regulation operates when certain amino acids act as the sole nitrogen source, which leads to a co-ordinate derepression (activation) of a range of permeases, again including peptide permeases (Logan *et al.*, 1979; Mehta *et al.*, 1984; Payne and Shallow, 1985; Milewski *et al.*, 1988; Payne *et al.*, 1991). With both ammonium ion- and amino acid-grown cells there is an approximate inverse relationship between growth rate and permease activity. Peptide substrates also control specifically the activity of their own permeases (Payne *et al.*, 1991). The presence of peptides in the growth medium increases peptide transport about fivefold over that observed in amino acid-grown cells; addition of peptides (but not amino acids) overrides ammonium repression. The increase and decrease in peptide-permease activity observed, respectively, with step-up (from ammonium and amino acids to peptides as nitrogen sources) and step down (from peptides to amino acids as nitrogen sources) both occur rapidly (about 20 min at 28°C) and do not require *de novo* protein synthesis. It is possible that the process operates by endocytosis involving a cycle of insertion and retrieval of preformed permease components, similar to that described for the α-mating pheromone receptor (Riezman *et al.*, 1986; Payne *et al.*, 1991).

IX. Energetics of Peptide Transport

This topic has already been referred to a number of times in this review, but there are benefits in reconsidering it in an integrated manner and in discussing some additional features.

It is now known that ATP energizes binding protein-dependent permeases (Ames and Joshi, 1990). Thus, Opp and Dpp in *E. coli* and *S. typhimurium* (Sections VI.B5 and C3) are energized in this way. Other species possessing similarly organized peptide-transport operons have permeases which are presumably similarly energized (Section VII). However, even with final confirmation of what had been anticipated for over a decade, much remains to be established. No good picture exists as to how or at what stage ATP hydrolysis contributes to the translocation process. The stoicheiometry of energy coupling has yet to be clarified. Answers to these questions will no doubt emerge. But will all microbial peptide permeases be found to conform to this model? There is evidence that peptide transport in certain bacteria, such as certain enterococci and lactobacilli show a requirement for a proton gradient (Section VII.D and E). A similar conclusion has been reached for yeast peptide permeases (Section VIII.B and C). Further studies are needed to clarify this situation. It should be noted that peptide transport systems in the mammalian gut (Matthews, 1991) and in barley embryos (Hardy and Payne, 1992) appear to be energized by coupling to a proton gradient (Section II). Using glucose as an energy source to generate a proton gradient, a stoicheiometry of two protons for each peptide was derived for uptake into *Enterococcus faecalis* (Payne, 1983).

Peptides are endowed with unique properties in relation to the energetics of substrate translocation. First is their structural variability, which allows neutral, positively- or negatively-charged substrates to be handled by a single system. Second is their oligomeric nature. Some implications of these features were considered earlier (Payne, 1980b). A transporter that has the potential ability to handle Ala_4, Lys_4 and Glu_4 is an interesting system from an energy-coupling point of view. Furthermore, if the periplasmic His permease has a stoicheiometry of one (or two) ATP molecules for His transport, then an oligopeptide permease that operates with the same stoicheiometry for transport of His_5 is certainly an energetically superior system. Following peptide uptake and intracellular cleavage, there is the possibility of additional energy gains (inherent in the chemi-osmotic nature of the released residues) resulting from efflux of the released amino acids down an (electro)chemical gradient (Section V.C and D). The various hypothetical ways in which this efflux might lead to generation of energy gradients and fuel counterflux were considered by

Payne (1980b). Energy recycling by solute efflux has been described in other systems (Brink *et al.*, 1985). Studies into these peptide-specific aspects of transport energetics should prove interesting.

X. Regulation of Peptide Permeases

When this topic was last reviewed (Payne, 1980c), little or no evidence existed for any regulation of peptide-transport systems. It still remains a fact that, under typical laboratory conditions, high expression of peptide transport activity is the norm for bacteria, but it is now clear that a variety of controls do operate in many different micro-organisms, and these have been described in previous sections (Sections VI.B7 and C5; VII; VIII). Many of the observations made accord with earlier assumptions of a need for integration between peptide transport and maintenance of amino acid-pool levels (Payne, 1980c). Findings on the influence of individual amino acids and of protein hydrolysates on expression of *opp* and *dpp* in *E. coli* can be viewed in this light (Section VI.B7 and C5), as can the relationship with induction of sporulation (Section VII.F). However, the effects of anaerobiosis and phosphate limitation were unanticipated (Section VI.B7 and C5). In yeasts, hierarchical controls involving nitrogen availability, amino acid availability and, uniquely to date, peptide substrate induction have been described (Section VIII.C). All of these observed effects need to be interpreted in relation to the particular niches which these organisms occupy, in which feast and famine conditions and prolonged periods of specific nutrient limitation will operate.

Although many instances have now been noted in which environmental switches lead to changes in expression and activity of peptide permeases, in no micro-organism has the underlying molecular mechanism been adequately described. How membrane regulation is integrated into general cell physiology may prove to be the most interesting aspect of this whole area. Controls that operate at the level of DNA and mRNA are being uncovered, but it will be more challenging to identify information encoded in permease-protein domains that can interact with the cytoplasm. In studies that employ directed mutagenesis to unravel the molecular mechanisms of solute translocation and energization, the possible effects on permease regulation should not be ignored.

XI. Exploitation of Peptide Permeases: Peptide-Carrier Prodrugs

A. INTRODUCTION

There is now an extensive literature concerning the use of peptide-transport systems for uptake of antimicrobial agents. There are many

examples, both synthetic and naturally occurring, of peptide-carrier prodrugs active against bacteria and/or fungi. However, fundamental aspects of this topic still require clarification before the commercial potential of this approach can be fully exploited by the pharmaceutical industry.

For some considerable time before the existence of microbial peptide permeases was established, it was appreciated that peptides could be either nutritionally more active or more inhibitory than a combination of their free component amino acids (early studies were reviewed in Payne, 1975, 1976). Early work in the 1950s and 1960s, showing that peptides containing inhibitory amino-acid mimetics, such as ethionine, norleucine or β-2-thienylalanine, could often possess enhanced antibacterial activities (reviewed in Ringrose, 1980; and Payne, 1976), were not always correctly interpreted. However, subsequent appreciation of the widespread occurrence of microbial peptide permeases and their rather broad structural specificities (Section VI) soon suggested the possibility that peptides containing modified amino acid-residues or synthetic analogues might also be generally recognized as substrates. Moreover, this provided the means to transport otherwise impermeant moieties, if these were attached to, or incorporated into, a peptide structure without compromising the substrate specificities of the permeases (Payne, 1972). It was but a short step to conclude that, if the impermeant moiety were potentially toxic, it provided an opportunity for development of a broad range of peptide-carrier prodrugs.

Different groups have coined various terms to describe peptide-mediated accumulation of impermeant residues, including illicit transport (Ames *et al.*, 1973), warhead delivery (Ringrose, 1980) and portage transport (Gilvarg, 1981). The peptide-carrier complexes are known as smugglins (Payne, 1976). The concept of illicit transport was experimentally endorsed at an early stage with peptides containing the impermeant residues histidinol phosphate and homoserine phosphate (Ames *et al.*, 1973; Fickel and Gilvarg, 1973). To function as prodrugs, an essential further step is for the smugglin to undergo intracellular cleavage (most commonly by peptidase action) in order to release its component amino-acid mimetic or warhead. The inhibitory compound, being unable to cross the membrane except as a smugglin component, cannot undergo exodus once released and accumulates to a high concentration intracellularly. This overall process is illustrated in Fig. 2.

To consider this topic in more detail, it is convenient to distinguish between examples of natural and synthetic smugglins, and to consider antibacterial and antifungal agents separately. Several reviews relevant to this topic have appeared (Ringrose, 1980, 1983a,b, 1985; Gilvarg, 1981; Diddens *et al.*, 1979; Payne, 1986; Thomas, 1986; Naider and Becker, 1987; Vining, 1990; Tyreman *et al.*, 1992; Gadebusch *et al.*, 1992; Bugg and Walsh, 1992).

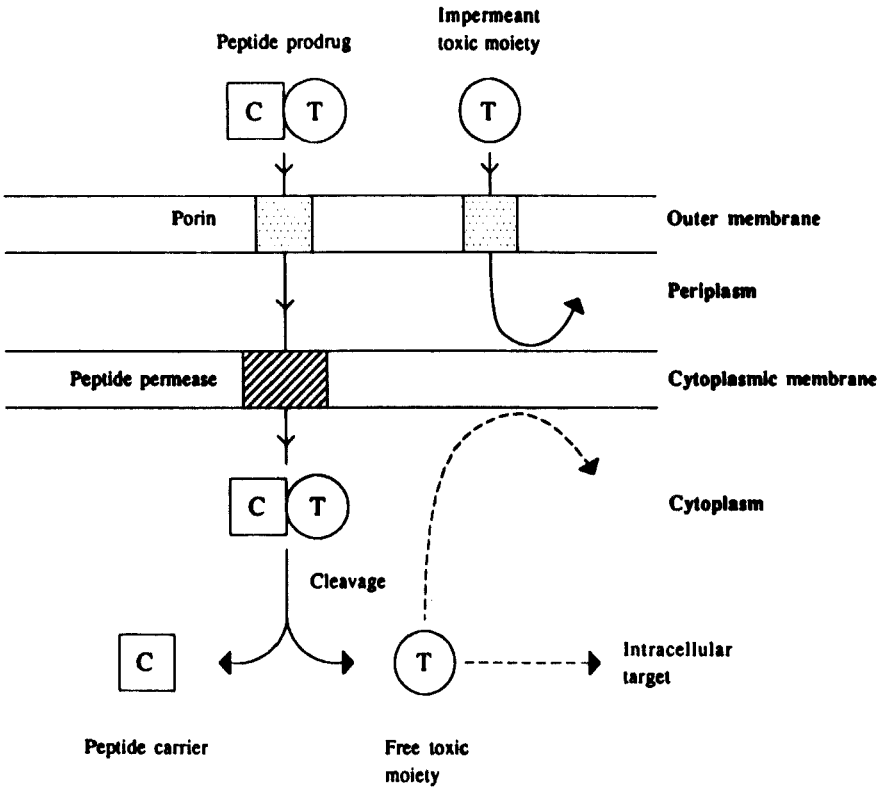

FIG. 2. Exploitation of peptide permeases by peptide-carrier prodrugs. The inhibitory moiety (T) is impermeant in its free form, but can be actively accumulated through a peptide permease(s) when linked to a peptide carrier (C). Following intracellular release, T can reach a high cytoplasmic concentration, enhancing its activity against its specific target.

B. NATURAL SMUGGLINS

1. Introduction

It was some years after the concepts of illicit transport and smugglins had been proposed before it was appreciated that Nature had long before acted upon the ideas. Thus, a range of natural peptide smugglins is now recognized, and no doubt further compounds will be found to function in this manner. Certainly, current appreciation that the activities of such compounds will be antagonized by the peptide components typically found in media used for antibiotic testing should facilitate their identification in

the future. To date, the compounds characterized appear to have been designed as "weapons of chemical warfare", to deliver toxic compounds into competitor micro-organisms, or to invade host systems. Here, it is convenient to consider antibacterial and antifungal compounds separately.

2. *Antibacterial Compounds*

Amongst antibacterial compounds, alanine is commonly found as the carrier function in natural smugglins, both dipeptides and oligopeptides. One of the first examples of a naturally occurring peptide-carrier prodrug to be identified was the dipeptide bacilysin (tetaine, bacillin), produced by *Bacillus subtilis* (Kenig and Abraham, 1976). It comprises an alanyl residue and the *C*-terminal amino acid mimetic, L-β-(2,3-epoxycyclohexanono-4)-alanine known as anticapsin. Anticapsin itself is not antibacterial but, as the dipeptide derivative, it inhibits both Gram-positive and Gram-negative bacteria, as well as *Candida albicans* but not *Sacch. cerevisiae* (Kenig and Abraham, 1976; Chmara *et al.*, 1980, 1981, 1982; Payne and Shallow, 1985). In all organisms, it is transported predominantly through the dipeptide permease; to date, mutants isolated as resistant have been permease-defective rather than peptidase-deficient. After transport, it is hydrolysed by intracellular peptidases to yield anticapsin, which acts as a glutamine analogue to inhibit glucosamine synthetase. In consequence, peptidoglycan synthesis is inhibited in bacteria, and mannoprotein and chitin synthesis in fungi (Chmara *et al.*, 1980; Chmara and Zahner, 1984; Chmara, 1985). Synthetic analogues of bacilysin were also prepared that provided detailed information on the role of the different structural elements in transport, hydrolysis and enzyme inhibition (Chmara *et al.*, 1982). Interestingly, the analogue L-alanyl-L-β-(2,3-epoxycyclohexyl-4)-alanine is as effective as bacilysin, although it lacks the ring keto group. A natural dipeptide related to bacilysin is lindenbein, (N^3-fumaramoyl-L-2,3-diaminopropanoyl-L-alanine) in which the warhead is the *N*-terminal residue (Ringrose, 1980).

A variety of natural inhibitors are based on phosphorus-containing amino-acid analogues (Uri *et al.*, 1988). In the tripeptide phosphinothricyl-Ala-Ala (bialaphos), the toxic amino-acid mimetic phosphinothricin (L-2-amino-4-methylphosphinobutyric acid; PPT) is a δ-phosphinate analogue of glutamic acid and is a potent inhibitor of glutamine synthetase. The intact peptide is inactive against the isolated enzyme, whereas the mimetic is only active against whole cells, as the peptide derivative. Bialaphos shows antibacterial and herbicidal activity (Diddens *et al.*, 1976, 1979; Kumada *et al.*, 1991); related antibiotics, such as phosalacine, have been described (Omura *et al.*, 1984). Bialaphos is produced by *Streptomyces*

hygroscopicus; its biosynthetic pathway has been established and the gene cluster cloned and characterized (Murakami *et al.*, 1986; Kumada *et al.*, 1991). Although bialaphos is the main product, it is produced as one of a number of products that each contain PPT, including PPT-Gly-Ala, PPT-Ala-Gly, PPT-Ala-α-aminobutyric acid, PPT-Ala-Val, PPT-Ala-Ser; in addition, bialaphos itself can be modified to PPT-Ala-Ala-PPT and PPT-Ala-Ala-PPT-Ala-Ala (Kumada *et al.*, 1991). Interestingly, resistance of the host to bialaphos is conferred in part by producing the *N*-acetylated form (Kumada *et al.*, 1991). A related tripeptide antibiotic is L-(N^5-phosphono)methionine-*S*-sulphoximinyl-Ala-Ala which, together with the synthetic analogue L-methionine-*S*-dioxydyl-Ala-Ala, was shown to have a similar mode of action and spectrum of activity to bialaphos (Diddens *et al.*, 1976, 1979). Another antimicrobial compound, Gly-Leu-2-amino-2-propenyl-phosphonate, has a similar broad spectrum (Hunt and Elzey, 1988). It is noteworthy that many naturally occurring antibiotics of this type carry glutamine mimetics and inhibit glutamine synthesis or utilization in one way or another, which renders them unsuitable for clinical use. Related examples are the tripeptides duazomycin B and alazopeptin that both carry two residues of the mimetic 6-diazo-5-oxo-L-norleucine.

Phaseolotoxin is a phytotoxic agent produced by *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo blight in beans (Mitchell and Bieleski, 1977). The structure originally proposed for the major component was (*N*-phosphosulphamyl)ornithylalanylhomoarginine; a minor component occurs in which a serine residue replaces one of alanine (Mitchell *et al.*, 1981). However, a revised structure has been proposed for the phosphorus-containing moiety, showing it to be a phosphotriamide (Moore *et al.*, 1984). This toxic mimetic inhibits ornithine carbamoyltransferase. It also inhibits growth of various bacteria but, when incorporated into the tripeptide, it is over 1000-fold more inhibitory, being transported through the oligopeptide permease (Staskawicz and Panopoulos, 1980; Mitchell *et al.*, 1981). The genes involved in phaseolotoxin synthesis have been cloned and shown to be clustered (Peet *et al.*, 1986).

A wide range of other natural smugglins composed of toxic amino-acid mimetics and varied carrier residues have been described (see, for example, reviews by Ringrose, 1980, 1983b, 1985). A particularly interesting class are those that contain residues with β-lactam rings, e.g. the phytotoxin tabtoxin (Ringrose, 1980). Conventional β-lactam antibiotics act against enzymes of peptidoglycan synthesis located in the cell envelope. Thus, smugglins that contain β-lactam structures might be expected to inhibit alternative or additional targets located intracellularly. One such class are the clavams which possess an oxygen atom instead of a sulphur atom at position 1 and are active against bacteria and fungi (Rohl *et al.*,

1987). Towards *E. coli* the toxicity of hydroxyethylclavam was enhanced about 500-fold by incorporation into the peptide-carrier form known as valclavam. This peptide enters through both the dipeptide and oligopeptide permeases, and mutants defective in one or other systems retain sensitivity, a feature important for design of efficacious smugglins (Section XI.D). Valclavam contains a bond between an α-amino-acid residue and one of α-hydroxy-β-amino acid, which extends further the range of structural analogues recognized by these permeases (Section VI.B2). The antibiotic TA-243, produced by *Streptomyces griseofuscus*, is actively transported into *Staphylococcus aureus* by a peptide-transport system and hydrolysed intracellularly by aminopeptidases to yield amino-oxysuccinic acid; the primary intracellular target of the toxic agent is thought to be alanine racemase (Kamogashira and Takegata, 1988).

3. Antifungal Compounds

Amongst the antifungal smugglins characterized to date, a few, such as bacilysin and valclavam, also show antibacterial activity, but most carry warheads targeted against enzymes absent from bacteria. In this category, the best characterized are the polyoxins and nikkomycins, which are related peptide-nucleoside complexes. The nucleoside component acts as a substrate analogue to inhibit chitin synthase. In consequence, they are active against a range of phytopathogenic fungi, yeasts and filamentous fungi, zoopathogenic fungi including *C. albicans*, diatoms and insects (Becker and Naider, 1980; Becker *et al.*, 1982, Mehta *et al.*, 1984; Payne and Shallow, 1985; Gooday *et al.*, 1985, Furter and Rast, 1985). Their dependence for this activity upon peptide uptake has been demonstrated in some cases (Yadan *et al.*, 1984; Payne and Shallow, 1985; McCarthy *et al.*, 1985a) (Section VIII.C). The polyoxins are produced by *Streptomyces* sp. in a range of dipeptidyl and tripeptidyl forms containing a variety of amino-acid carrier functions (Borman *et al.*, 1985). Information on these natural structural variants, and knowledge of the substrate specificities of the peptide permeases in *C. albicans*, have provided the basis for design and synthesis of polyoxin and nikkomycin analogues (Section XI.C3).

C. SYNTHETIC SMUGGLINS

1. Introduction

It was Gilvarg and Ames and their colleagues who were the first to capitalize on knowledge concerning peptide permeases in bacteria to

produce synthetic smugglins (Fickel and Gilvarg, 1973; Ames *et al.*, 1973), but these were not designed as antimicrobial agents. Commercial interest in the concept concerns the design of novel vehicles for delivering antimicrobial agents (and potentially prodrugs designed against mammalian targets), and we concentrate on this aspect here. Antibacterial and antifungal agents will be considered separately. These studies, often by workers in the pharmaceutical industry, have contributed extensively to characterizing the specificities of microbial peptide permeases.

2. *Antibacterial Compounds*

One of the first examples of an antibacterial agent designed to exploit a peptide carrier for transport of a toxic amino-acid mimetic was Gly-Gly-*N*-δ-(phosphonoacetyl)-L-ornithine, containing the phosphonic analogue of the transition state of the reaction catalysed by ornithine carbamoyltransferase (Penninckx and Gigot, 1979). However, the most extensive studies have been those on synthetic phosphonopeptides by the Roche group (Hassall, 1983; Kafarski and Lejczak, 1991; Ringrose, 1985). The best studied example is the dipeptide alafosfalin (alaphosphin, Ro-03-7008), in which the toxic alanine mimetic L-1-aminoethylphosphonic acid (Ala(P)) is linked to an *N*-terminal alanyl residue (Allen *et al.*, 1978). The mimetic itself is not able to penetrate cells and, in consequence, following transport in peptide form it can be accumulated to concentrations 1000-fold in excess of that of the smugglin outside (Allen *et al.*, 1979a; Atherton *et al.*, 1983). Concomitant exodus of amino acid-carrier residues occurs similarly to that observed with common peptides (Payne and Bell, 1977a,b; Perry and Abraham, 1979; Payne and Nisbet, 1980a; Section V.C. and D). Alafosfalin showed broad-spectrum antibacterial activity against Gram-negative and Gram-positive bacteria, which primarily involved inhibition of alanine racemases and thus of peptidoglycan synthesis (Allen *et al.*, 1979a; Atherton *et al.*, 1979a,b). In wide-ranging studies, many di- and oligophosphonopeptides were tested, varying in chain length, stereochemistry and carrier residues; differences in their antibacterial activities *in vitro* were related to the specificities of the peptide permeases and intracellular peptidases of various species (Atherton *et al.*, 1979a,b, 1980, 1983). Much of the rationale for design of the initial structures was based on known specificities of the Dpp and Opp from *E. coli*, (Hassall, 1983, 1985). Ironically, alafosfalin proved not to use these permeases to any great extent, (Atherton *et al.*, 1983), instead being taken up predominantly via Tpp (Payne, 1983). However, depending upon the nature of the peptide-carrier moiety, all three permeases can be involved in transport, either singly or in conjunction (Payne, 1986; Atherton *et al.*, 1983). Selection of

mutants resistant to phosphonopeptides generally yields permease-deficient strains (Atherton *et al.*, 1980). Alafosfalin can be used to select *tpp* mutants (Payne, 1983, 1986; Payne *et al.*, 1984) (Section VI.D). Other analogues can be used to select mutants in the other permeases. Evidence for a specific anionic peptide permease in *Streptococcus faecalis* has come from isolation of mutants resistant to analogues containing Asp or Glu carrier-moieties (Payne *et al.*, 1982). Resistance acquired by loss of peptidase activity has also been reported (Nisbet and Payne, 1982). Although not proven, this was likely to arise from defective aminopeptidase activity, for phosphonopeptides seem not to be susceptible to cleavage by carboxypeptidases, at least in *E. coli* (Atherton *et al.*, 1983). Alafosfalin, and certain other analogues underwent extensive testing *in vivo*, including detailed studies on oral absorption, and pharmacokinetics (Allen *et al.*, 1979b; Allen and Lees, 1980). In many of these aspects, alafosfalin closely resembled the β-lactam antibiotic cephalexin, with which it showed good synergy both *in vitro* and *in vivo* (Allen and Lees, 1980; Maruyama *et al.*, 1979; Atherton *et al.*, 1980, 1981). Synergistic activity was also found between Nva-Ala(P) and nocardin A (Angehrn *et al.*, 1984). In further studies, synthesis and structure–activity relationships were described for phosphonopeptides based on aminomethylphosphonic acid (Atherton *et al.*, 1982, 1986). This analogue behaved as a mimetic of both D- and L-alanine and inhibited not only alanine racemase but also D-Ala-D-Ala synthetase and UDP-*N*-acetylmuramyl-L-alanine synthetase; in general, however, smugglins based on this mimetic were less inhibitory than ones containing Ala(P). Phosphonate analogues of other amino acids were generally inactive (Atherton *et al.*, 1986). Phosphono-oligopeptides, such as Ala-Ala-Ala(P), showed a much broader antibacterial spectrum *in vitro* than their phosphonodipeptide homologue, but this was not generally expressed *in vivo*, probably due in part to rapid hydrolysis to the corresponding dipeptide and also to decreased absorption. Compounds designed to be resistant to host peptidohydrolases, such as Sar-L-Nva-L-Nva-Ala(P), showed a greater potency and a broader antibacterial spectrum *in vivo*, although they were generally absorbed less well than the corresponding dipeptide (Atherton *et al.*, 1986).

Overall, the Roche work has contributed to a definition of bacterial peptide permease specificities in general, particularly for many clinically important bacterial species, and it has greatly extended the smugglin concept in the pharmaceutical arena. The extensive studies *in vivo* showed that development of transport resistance was not a problem for any of the tested analogues (Ringrose, 1985) and that compounds could be selected that showed considerable therapeutic potential for use either alone or in combination. Regrettably, problems with long-term Ala(P) toxicity

prevented their implementation and have caused the studies to be discontinued. However, the fact that none of these compounds proved commercially successful should in no way lead to the conclusion that the concept cannot be successfully exploited.

Related work on phosphonopeptides has been carried out in other laboratories. Studies on the influence of mimetic structure on the stability towards mammalian aminopeptidases were performed by Lejczak *et al.* (1985) for several diastereomeric phosphonodipeptides with *C*-terminal Ala(P), Val(P) and aminopropane phosphonate residues. In general, they were all hydrolysed extremely slowly (compared with the carboxyl homologues), and some were weak inhibitors. In addition, the antibacterial activity of a series of phosphonodipeptides containing a variety of *C*-terminal aminoalkylphosphonic acids was described (Lejczak *et al.*, 1986). Compounds based on 1-amino-1-methylethanephosphonic acid (MeAla(P)) proved most active, although only in the form of smugglins. Other compounds, namely AlaMeAla(P) and LeuMeAla(P), were only slightly less inhibitory than alafosfalin, presumably through inhibition of alanine racemase in each case (Zboinska *et al.*, 1990). Uptake rates matched their relative inhibitory activities and, significantly, all were equally active against wild-type and oligopeptide-permease mutants, indicating that these methyl derivatives may also use the tripeptide permease (Tpp) predominantly, as shown earlier for alafosfalin (Payne, 1983). Other derivatives such as (β-chloro-α-aminoethyl)phosphonic acid have been shown to be inhibitors of alanine racemase and of D-Ala:D-Ala ligase, but to possess poor antibacterial activity (VoQuang *et al.*, 1986); it will be of some interest to test the antibacterial efficacy of suitable peptide-carrier derivatives. Other potential peptide inhibitors of these enzymes are generally inactive because the presence of D-residues precludes adequate transport by permeases (Neuhaus *et al.*, 1977; Neuhaus and Hammes, 1981; Cheung *et al.*, 1983). The biological activity of aminophosphonic acids and their derivatives has recently been reviewed (Kafarski and Lejczak, 1991). Other changes to the *C*-terminal carboxylic group have been attempted, including phosphinate, sulphonate, tetrazole, sulphonamide and hydroxamate substitutions (Ringrose, 1980). Some peptides incorporating *C*-terminal aminotetrazole mimetics have shown limited antibacterial activity (Ringrose, 1980), whereas others, as well as corresponding hydroxamic acid derivatives, were not inhibitory although well transported (Morley *et al.*, 1983a,b).

Several other classes of novel smugglins have been synthesized, and the characteristics of the most interesting of these are now discussed. Dipeptides containing a residue of either of the amino-acid mimetics β-chloroalanine or L-propargylglycine (an acetylenic amino acid, (2-amino-

4-pentynoate; Lpp) which are mechanism-based inhibitors of alanine racemase and cystathionine γ-synthase, respectively, showed good antibacterial activity *in vitro* (Cheung *et al.*, 1983). However, inhibitory activity was increased by up to 4000-fold by incorporation of the mimetics into dipeptides. Smugglins containing only one residue of either mimetic showed a restricted range of antibacterial activity, but dipeptides containing two such residues (β-Cl-L-Ala-β-Cl-L-Ala or LppGly-LppGly) were broad-spectrum antibacterials. The reasons for this are not clear but it presumably reflects interdependence of permeases and peptidases for smugglin action. A double-warhead smugglin (β-Cl-L-Ala-LppGly) gave apparent synergy of antibacterial action for its constituents. The relative activities of compounds endorsed the stereochemical specificities of bacterial peptide permeases (Cheung *et al.*, 1983). Further studies confirmed that uptake of β-Cl-L-Ala-β-Cl-L-Ala occurred mainly through Dpp in various Gram-positive and Gram-negative bacteria and, following peptidase activation, the β-chloroalanine inhibited alanine racemase (Cheung *et al.*, 1986; Boisvert *et al.*, 1986). In an intriguing extension of this work, periplasmic β-lactamases were co-opted to deliver the above inhibitory dipeptide specifically to the vicinity of the dipeptide permease by cleaving "pre-pro-drugs" that were of the general form of cephalosporin C_{10}-dipeptide esters (Mobashery *et al.*, 1986; Mobashery and Johnston, 1987). Antibacterial activity based on inhibition of alanine racemase by β-chloroalanine was demonstrated with these compounds also. The antibacterial spectrum of several 3-halovinylglycines, which act as suicide substrates of alanine racemase, was also shown to be dramatically enhanced when incorporated into dipeptides, according to the smugglin principle (Patchett *et al.*, 1988).

A novel class of smugglin, in which a relatively impermeant molecule was attached to the side-chain of an amino-acid residue of the peptide carrier, was described by Gilvarg and his co-workers (Boehm *et al.*, 1983). The thiol compounds 2-mercaptopyridine and 4-[*N*-(2-mercaptoethyl]-aminopyridine-2,6-dicarboxylic acid were transported into *E. coli* as mixed disulphides with a cysteine residue of a di- or tripeptide. Transport was shown to occur via Dpp and Opp, with intracellular release occurring as a result of (non-enzymic) disulphide exchange within the thiol-rich cytoplasm; once cleaved, the latter thiol underwent exodus as a relatively lipophilic uncharged metal chelate (Perry and Gilvarg, 1983). The compounds exemplified a new type of smugglin, although they were not themselves effective antibacterial compounds. A further example of a novel prodrug that is dependent on disulphide exchange for activation acts on lipopolysaccharide synthesis (Norbeck *et al.*, 1989). A more general synthetic approach that involved linkage to the side-chain residues of the peptide carrier was described for a range of di- and oligopeptides that

contained nucleophilic moieties attached to the α-carbon of a glycine residue (Kingsbury *et al.*, 1984a). Following uptake through Dpp and Opp in *E. coli* (and other micro-organisms) and peptidase action to liberate its free amino group, the α-substituted glycine is rendered unstable (whilst being stable in peptide linkage) and decomposes, releasing its nucleophilic moiety. Smugglins containing glycine residues α-substituted with phenol, thiophenol or aniline exemplified the broad applicability of this approach (Kingsbury *et al.*, 1984a). Several of these compounds were antibacterial, being at least two orders of magnitude more effective when combined in peptide form. However, peptides containing the pyrimidine analogue 5-fluorouracil were particularly effective antimicrobial agents (Kingsbury *et al.*, 1984b).

A truly rational class of antibacterial compounds, designed specifically to inhibit Gram-negative bacteria by acting on lipopolysaccharide biosynthesis, involved linkage of analogues of β-ketodeoxyoctonate (KDO) (an inhibitor of CMP–KDO synthetase) to various small peptides (Hammond *et al.*, 1987; Goldman *et al.*, 1987; Claesson *et al.*, 1987). Active accumulation of the compounds mainly through Opp yielded activity with a broad antibacterial spectrum. (Hammond *et al.*, 1987; Goldman *et al.*, 1987; Smith and Payne, 1990).

The lysine biosynthetic pathway as a target for synthetic antibacterial compounds has been discussed by various authors (Girodeau *et al.*, 1986). Peptides containing residues of 2-aminopimelic acid that inhibit diaminopimelic acid biosynthesis and, thus, peptidoglycan synthesis, have been described (Berges *et al.*, 1986; Leroux *et al.*, 1991). Other inhibitory analogues of diaminopimelic acid may offer potential for further smugglin developments (Lam *et al.*, 1988).

In the examples already described, mimetics were attached to a peptide carrier at either the *N*- or *C*-termini or to side-chain residues. Other studies have explored the effects of modifications to the peptide backbone (Morley *et al.*. 1983a,b; Payne *et al.*, 1984; Smith and Payne, 1990). Thus, in a series of di- and trialanine analogues, the usual peptide linkage (–CO–NH–) was reversed (–NH–CO–) (retro), extended as in –CO–NH–NH (hydrazino) or –CO–NH–O (aminoxy), or an α-carbon was replaced by a nitrogen (α-aza) (Morley *et al.*, 1983a,b). The aminoxy and hydrazino compounds were inhibitory towards Gram-negative and Gram-positive bacteria, but the other analogues were inactive; in general, activities correlated with ease of uptake. Following uptake, the toxic mimetics were rapidly released intracellularly, but only the normal carrier residues could undergo exodus. A more detailed study was made of a collection of aminoxy peptides (containing L- or D-aminoxy propionic acid; L- or D-OAla) (Payne *et al.*, 1984). Synthetic analogues with a D-OAla residue

proved more antibacterial than the corresponding L-OAla homologues. Detailed studies showed that smugglins with L- or D-OAla residues were transported by all three peptide permeases in *E. coli* and were cleaved by a range of peptidases, although earlier studies indicated they were relatively resistant to cleavage by certain mammalian peptidohydrolases. The facile ability to transport these peptides containing D-residues contrasts sharply to the normal situation, in which D-stereochemical configurations severely restrict uptake (Section VI.B2 and C2). This feature warrants further exploration in synthetic smugglin design. Results with these compounds have contributed towards defining structural features that are acceptable to bacterial peptide permeases, but the intrinsic toxicity of these particular mimetics precluded any therapeutic applications. Interestingly, a natural smugglin containing amino-oxysuccinic is known (Kamogashira and Takegata 1988).

Finally, in addition to incorporating residues of toxic amino-acid mimetics into designed smugglins, there have been many attempts to enhance the potency and spectrum of known antimicrobial agents by attaching peptide carriers (Ringrose, 1985), including chloramphenicol (Ringrose and Lambert, 1973), nalidixic acid (Kametani *et al.*, 1981), isonicotinic acid hydrazide (Bruckner *et al.*, 1983) and erthromycin (LeMahieu *et al.*, 1982). However, none of these compounds showed increased potency, in some cases because the resulting structures vitiated permease specificities, in others it was probably because their overall size precluded penetration through outer-membrane porins (Section IV).

3. *Antifungal Compounds*

Although there are broad similarities between bacterial and fungal peptide permeases, there do seem to be differences that may be exploited in antifungal smugglin design. Many reports have appeared on synthetic antifungal smugglins, although the variety of toxic mimetics used is rather narrow. In most instances, the aim has been to design compounds inhibitory to *C. albicans* (Naider and Becker, 1987). Early studies, in which the toxic agent 5-fluorocytosine was linked to various peptides, showed that design of anticandidal smugglins was possible but that the nature of the mimetic linkage was critical (Becker and Naider, 1980). Subsequent studies led to compounds with more effective anticandidal activity (Ti *et al.*, 1980). A collection of analogues of Arg-D-allo-Thr-Phe, a naturally occurring peptide with antifungal activity, were active against *C. albicans* (Meyer-Glauner *et al.*, 1982). A series of di- and tripeptides containing D- or L-*m*-fluorophenylalanine was synthesized, and those containing the latter mimetic showed anticandidal activity (Kingsbury *et al.*, 1983).

Peptides containing 5-fluorouracil as an α-substituted glycine residue (as discussed previously) were also actively accumulated by *C. albicans*, causing antifungal effects (Kingsbury *et al.*, 1984b; Kingsbury, 1990).

Synthetic peptides containing residues of various glutamine analogues based on N^3(X)-L-2,3-diaminopropanoic acid have been described, in which X is fumaroyl (Andruszkiewicz *et al.*, 1984; Milewski *et al.*, 1983), 4-methoxyfumaroyl (MF) (Andruszkiewicz *et al.*, 1987; Milewski *et al.*, 1991a) or iodoacetyl (Andruszkiewicz *et al.*, 1990a,b). The mimetic acts against glucosamine-6-phosphate synthase. Based on the wide range of compounds available, the anticandidal efficacy was shown to be dependent mainly on the rate of transport and to a lesser degree on the rate of peptidase activation (Milewski *et al.*, 1991a,b). The Nva-MF compound proved to be an effective antifungal agent in the mouse model of systemic candidosis.

The naturally occurring peptidyl nucleotides, the polyoxins (Isono and Suzuki, 1979) and nikkomycins (Muller *et al.*, 1981; Borman *et al.*, 1985), are antifungal components acting on chitin synthase. Much effort has gone into synthesizing analogues in attempts to produce improved anticandidal agents. Some di- and tri-peptidyl derivatives retained high activity against the isolated target enzyme (Shenbagamurthi *et al.*, 1983; Naider *et al.*, 1983) but susceptibility to peptidase action decreased their effectiveness against *C. albicans in vitro*. Attempts to minimize this problem by insertion of peptidase-resistant bonds markedly decreased inhibitory activity against chitin synthetase (Shenbagamurthi *et al.*, 1986). More successfully, a range of polyoxin analogues containing residues of α-amino fatty acids showed good enzyme inhibition, good affinity for the peptide permeases, increased stability against intracellular peptidase action and, in consequence, showed acceptable anticandidal activities (Khare *et al.*, 1988). Further effective analogues have been described (Krainer *et al.*, 1991). Evidence of synergism between natural nikkomycins and azole antifungals (Milewski *et al.*, 1991b; Hector and Scheller, 1992) raises the possibility that even more effective combinations may be achieved with synthetic analogues.

D. PROSPECTS FOR RATIONAL DESIGN OF SYNTHETIC SMUGGLINS

The fact that Nature has capitalized so extensively on the smugglin principle, to produce a wide range of peptide-based antibiotics, is the best possible endorsement of the concept. Certainly, there appears no alternative drug-delivery mechanism for which this claim can be made. The various examples of synthetic smugglins described in this review indicate that the scientific community is also recognizing this fact. A framework for

rational design of smugglins of chemotherapeutic utility is clearly in place. However, before it will be possible to propose optimal peptide carriers for diverse applications in prodrug development, further information is needed concerning microbial physiology, added to which a similar situation exists for other applications of peptide prodrugs (Ringrose, 1983a,b; Thomas, 1986). In acquiring this information, as already illustrated with current smugglins, characterizing the responses to new derivatives will itself contribute to this process. Clearly, the pharmacokinetic and pharmacodynamic characteristics of individual smugglins will contribute significantly to their effectiveness, but these factors will not be considered in the present context.

Questions of microbial physiology that need further clarification for problem pathogenic organisms, are as follows: (1) How many different peptide permeases occur, and what are their substrate specificities? (2) How are the permeases regulated and to what extent are they expressed *in vivo*? (3) What are the most likely mechanisms of resistance to peptide prodrugs? Some aspects of these topics have already been discussed (Payne, 1986; Tyreman *et al.*, 1992).

Considerable progress has been made with respect to the first of these questions, and for several important classes of microbial pathogens their complement of peptide permeases has been established (Section VI, VII). Given this background, and by application of procedures used successfully in these studies such as selection of mutants resistant to toxic peptides and characterization of their cross-resistance patterns, it should in principle be relatively straightforward to define the number of permeases for other organisms. However, problems can be envisaged with some species, including high levels of resistance to available toxic peptides, so that additional approaches will be needed. In one such approach, antibodies raised against binding proteins from *E. coli* have been used to probe periplasmic/membrane/envelope fractions from other species to look for the presence of cross-reacting proteins (C. Schuster and J. W. Payne, unpublished results). Similarly, cloned peptide-permease genes can be used to probe genomic material from heterologous organisms for evidence of hybridizing species. With this background information, transport specificities for particular permeases can be determined using either direct transport assays and/or ligand-binding procedures that are now well established (Section V). A collection of peptides encompassing systematic structural variations is needed as substrates in such studies. To extract structural information from these results, applicable to generic carrier design, it is helpful to use computer-based molecular modelling, together with precise knowledge on specific peptide conformations obtained from techniques such as X-ray crystallography and n.m.r. The ability to define

preferred structural features should facilitate the design of peptide carriers applicable to broad-spectrum prodrugs or for targeting against a particular problem organism.

With regard to regulation of microbial peptide permeases, accumulated evidence indicates that a high constitutive level of expression is the normal situation, and this is to be expected given the high nutrient value of peptides, their energy efficiency of accumulation (Section X), and their continual presence in most environments. However, more recent studies have indicated that a variety of regulatory mechanisms do operate to increase or to decrease these levels (Sections VI and X). Nevertheless, it seems a fair assumption from measurements of permease expression and knowledge of these regulatory controls, together with proof of the efficacy of various smugglins *in vivo*, that peptide-permease activity will be present at levels sufficient for successful exploitation. However, in a hypothetical situation, if it were suspected that a species such as *E. coli* was growing *in vivo* under conditions in which phosphate-limitation prevailed, leading to decreased *opp* and *dpp* expression (Section VI), cognisance could be taken of this to exploit more fully uptake through Tpp (assuming it is not regulated in a similar manner!).

Whenever antibiotics are used, attempts to circumvent development of resistance to smugglins is a paramount consideration. It is our view that, in evaluating the potential of a new compound, this feature should be weighed relatively more importantly against MIC values than is currently prevalent. With smugglins, resistance can arise in several ways. Firstly, the warhead target may mutate to resistance; this feature is common to all antimicrobial agents and its likelihood will vary with each situation. It is difficult to predict and will not be considered here. Specifically for smugglins, in common with other forms of prodrug, an essential activation mechanism (commonly peptidase activity) may be lost. Micro-organisms generally possess many intracellular peptidohydrolases with overlapping specificities; therefore, mutation at the activation step may not normally be a problem, for inactivation of several peptidases may be required, which in itself may be compromising for growth. In addition, use of smugglins in combinative therapy (see below) would also be expected to minimize this problem. Nevertheless, it is a potential difficulty, and knowledge of the range and specificities of enzymes recruited for prodrug activation would be helpful. Finally, resistance may arise from mutational inactivation of permease function. Generally, this potential problem will be minimized by the fact that several permeases, with some overlap in specificities, co-exist. However, the potential effect of this feature is highlighted by the unusually high frequency at which *opp* mutations occur in *E. coli* (Section VI). It is a teleological debate whether the *opp* locus occurs in a mutational hotspot

merely by chance, or whether it is a favoured locus to provide a balanced population able to capitalize on benefits from absorbing peptide nutrients and recycling cell-envelope degradation products, whilst on the other hand having an in-built resistance mechanism to toxic natural peptides. There is little definitive evidence on the frequency of permease mutation in other bacteria.

In principle, there are various ways in which the design and use of smugglins might be tailored to overcome these resistance mechanisms. In addressing this aim it is advisable to take note of the way that Nature has tackled similar problems. Firstly, smugglins could carry two or more different warheads, which have unrelated targets or are aimed at different enzymes within a common pathway. Increased inhibition and/or possible synergy could result from this approach. Phaseolotoxin is an excellent natural example of this concept. A few synthetic examples exist, some from an early period (Hill and Dunn, 1969; Ringrose, 1980), others more purposefully designed (Cheung *et al.*, 1983; Leroux *et al.*, 1991). This principle has also been implemented with other antibacterial compounds (Beskid *et al.*, 1990). A second approach is to use a carrier peptide that effectively allows a particular smugglin to use more than one permease. Several natural examples have been described (Diddens *et al.*, 1979; Ringrose, 1980; Rohl *et al.*, 1987) but, in the limited number of cases for which information is available, synthetic smugglins enter predominantly through one permease. It may prove difficult to realize this design objective in most cases. Alternatively, a smugglin mixture could be used, linking a single warhead with two or more carrier moieties that facilitates complementary transport through several permeases. This approach certainly finds endorsement in Nature. Thus, various peptide antibiotics are produced as isomeric mixtures, incorporating various amino-acid residues and of variable size; examples are bialaphos (Kumada *et al.*, 1991), phaseolotoxin (Mitchell *et al.*, 1981), polyoxins and nikkomycins (Borman *et al.*, 1985). It would seem that the involvement of such unusually non-specific enzymes for synthesis of these compounds is purposeful, for such isomeric mixtures presumably provide beneficial variation in delivery routes and activation mechanisms, as well as varied resistance to enzymic inactivation. These compounds seem to exemplify the advantages of combinative therapy against different microbial populations.

The idea of using smugglins in combinative therapy probably provides the best solution to potential resistance. It also offers the most effective way to capitalize on the resource of novel agents possessing toxicity towards intracellular targets; used alone such compounds may not have a sufficient antimicrobial spectrum, but mixtures may display synergy. Examples already exist, for example between peptide prodrugs and amino

acids (Diddens *et al.*, 1976, 1979) or between various antibacterial smugglins and β-lactams (Cheung *et al.*, 1983; Atherton *et al.*, 1981, 1982; Maruyama *et al.*, 1979; Hassall, 1983; Smith and Payne, 1990) or other antibiotics (Angehrn *et al.*, 1984; Smith and Payne, 1990). Similar effects have been observed with anticandidal nikkomycins and azoles (Hector and Scheller, 1992; Milewski *et al.*, 1991b).

Using the information and principles already discussed, innovations in the design and application of novel synthetic smugglins can be expected. However, development of a chemotherapeutically useful agent will require attention to factors additional to those of microbial physiology considered here. For example, good oral absorption and resistance to degradative enzymes in the host will be important; it is here, perhaps, that non-peptide carrier mimetics will find a role (Freidinger, 1989).

XII. The ATP-Binding Cassette Superfamily of Membrane Transporters

The oligopeptide permease of Gram-negative bacteria belongs to a class of bacterial periplasmic transport systems comprising a soluble substrate-binding receptor and a membrane-bound complex of from two to four proteins. These proteins include one or more that binds ATP and is involved in energizing transport. In recognition of these features, these systems have been termed traffic ATPases (Ames *et al.*, 1990). Interestingly, over the last few years, membrane-bound proteins have been identified in various eukaryotes that share a high level of homology to the conserved components of the bacterial periplasmic permeases. Each system has two hydrophobic protein components (analogous to OppB and OppC) and two ATP-binding components (corresponding to OppD and OppF). These four components can be present either as a single polypeptide or as between two and four polypeptides that constitute a complex (Higgins *et al.*, 1990; Ames *et al.*, 1990). Many, but not necessarily all, of these proteins appear to function in transport processes (Higgins *et al.*, 1988b). In an overly contrived manner, characteristic of the acronym-blighted culture (ABC) of the 1990s, they have been named ABC (ATP-binding cassette) proteins (Hyde *et al.*, 1990). Currently, there is no evidence that the systems present in mammals, plants and insects involve binding proteins (Juranka *et al.*, 1989; Ames *et al.*, 1990; Higgins *et al.*, 1990). Nevertheless, their overall similarities encourage transfer of models and concepts developed from the bacterial systems to eukaryotic examples. This is particularly exciting because, amongst mammalian systems, are ones of much medical significance, such as the multidrug resistance protein and the cystic fibrosis gene product.

Resistance of tumour cells to a variety of structurally-unrelated cytotoxic agents arises from increased expression of the multidrug-resistance protein (Endicott and Ling, 1989; Pastan and Gottesman, 1991). This protein, also known as P-glycoprotein, is a 170 kDa membrane component that mediates efflux of chemotherapeutic agents in the above case but, presumably, in the normal state it functions to protect cells against toxic compounds generated from the diet. It has also been reported that it (and perhaps related proteins) can transport peptides; this led to the speculation that the multidrug-resistance protein may also play a role similar to that of two other family members, namely the bacterial haemolysin transport protein and the yeast STE6 protein which secretes α-factor mating pheromone (Sharma *et al.*, 1992). And, one might ask, perhaps it could play a role in peptide transport of a similar nature to that considered in this review? Recently, the multidrug-resistance pump has been partially purified and reconstituted into an artificial membrane; this system carried out ATP hydrolysis that was stimulated by drugs known to be substrates of the pump (Ambudkar *et al.*, 1992). Analogues of this protein have been identified in organisms as diverse as the protozoan *Plasmodium falciparum*, in which it mediates chloroquine resistance (Foote *et al.*, 1989), and the plant *Arabidopsis thaliana* (Dudler and Hertig, 1992).

Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein. This protein is also a member of the ABC superfamily that functions as a regulated chloride channel (Anderson *et al.*, 1991a,b). It contains, uniquely at present, in addition to the family characteristics of 12 membrane-spanning domains and two nucleotide-binding domains, a large highly-charged domain located in the cytoplasm that contains numerous phosphorylation consensus sites. This extra component is involved in regulation, a process which is complicated and presently ill understood, with channel opening requiring both phosphorylation and binding of ATP. Recent studies indicate that control of chloride conductance is mediated by ATP through non-hydrolytic binding (Quinton and Reddy, 1992). In elegant studies by Ames and her colleagues, detailed structure–function analysis of the membrane components of the His permease has been compared with cystic fibrosis mutations and with features in other family members (Shyamala *et al.*, 1991; Ames and LeCar, 1992; Kerppola and Ames, 1992). From these studies, a functional model for membrane-spanning regions of conserved components has been proposed which provides an alternative to an earlier model (Hyde *et al.*, 1990). An exciting finding, particularly relevant to this discussion, is that of genes with homology to ABC transporters in the mammalian class II major histocompatibility complex (Monaco *et al.*, 1990; Deverson *et al.*, 1990; Trowsdale *et al.*, 1990; Spies *et al.*, 1990). The

putative transport complex consists of two subunits, each possessing one hydrophobic and one hydrophilic (ABC) component. Studies indicate that such pumps function to deliver peptides to antigen-presenting molecules. Recent results are discussed, and further references are given in Parham (1990, 1991, 1992) and Robertson (1991). See also p. 79. No doubt future studies on microbial peptide permeases will provide additional insights into the nature of members of the ABC superfamily, and vice versa.

XIII. Conclusions and Prospects

There is now an extensive literature relevant to peptide transport by micro-organisms. The ripples from the very earliest studies on this subject now extend very far, and touch upon topics as diverse as intestinal and renal peptide transport, protein absorption and synthetic peptide diets, neuropeptides in the central nervous system and their passage across the blood brain barrier, the early nitrogen nutrition of germinating seeds, rational design of peptide-based antimicrobial agents and herbicides, cystic fibrosis, multiple drug resistance in cancer chemotherapy, and the major histocompatibility complex and antigen presentation. Some of these topics have been discussed in this review. Only a few could have been foreseen by those who were involved in the earliest studies on peptide transport by micro-organisms. In 1965, could Charles Gilvarg have anticipated, "O what a tangled web we weave, when first we find this permease?" In 1993, the ramifications of the topic look more like a contribution to the current debate on chaos theory.

Recent developments imply that the future will bring exciting advances in these interrelated areas. One wonders how the genealogy of the ABC superfamily will look by the time the final returns are in from the Human Genome Project? The availability of that information, combined with the application of recombinant DNA technologies seem to offer the potential for an evolutionary leap. It would appear that man could become an agent for purposeful evolution rather than being part of one driven by the crude forces of natural selection. Teilhard de Chardin, in *The Phenomenon of Man*, had a relevant thought: "If progress is a myth, that is to say: 'What's the good of it all?,' Our efforts will flag. With that the whole of evolution will come to a halt—because we are evolution."

On a more down to earth note, any review necessarily looks back, but we hope that this one will have helped some readers to bridge an information gap to the present and for others it will have provided a stimulus for future studies (or at least, it may prevent their efforts from flagging). In conclusion, we wish to repeat the sentiments expressed in an

earlier review on peptide transport written for this series (Payne, 1976): "It is hoped that studies on . . . microbial peptide permeases . . . may be useful not only in their immediate context, but that they may also act as a model system to provide information relevant to . . . interactions in more complex biological systems."

REFERENCES

Abouhamad, W. N., Manson, M. D., Gibson, M. M. and Higgins, C. F. (1991). *Molecular Microbiology* **5**, 1035.

Adams, M. D. and Oxender, D. L. (1989). *Journal of Biological Chemistry* **264**, 15 739.

Addison, J. M., Burston, D., Dalrymple, J. A., Matthews, D. M., Payne, J. W., Sleisenger, M. H. and Wilkinson, S. (1975a). *Clinical Science and Molecular Medicine* **49**, 313.

Addison, J. M., Burston, D., Payne, J. W., Wilkinson, S. and Matthews, D. M. (1975b). *Clinical Science and Molecular Medicine* **49**, 305.

Allen, J. G. and Lees, L. J. (1980). *Antimicrobial Agents and Chemotherapy* **17**, 973.

Allen, J. G., Atherton, F. R., Hall, M. J., Hassall, C. H., Holmes, S. W., Lambert, R. W., Nisbet, L. J. and Ringrose, P. S. (1978). *Nature* **272**, 56.

Allen, J. G., Atherton, F. R., Hall, M. J., Hassall, C. H., Holmes, S. W., Lambert, R. W., Nisbet, L. J. and Ringrose, P. S. (1979a). *Antimicrobial Agents and Chemotherapy* **15**, 684.

Allen, J. G., Havas, L., Leicht, E., Lenox-Smith, I. and Nisbet, L. J. (1979b). *Antimicrobial Agents and Chemotherapy* **16**, 306.

Alloing, G., Trombe, M.C. and Claverys, J. P. (1990). *Molecular Microbiology* **4**, 633.

Alves, R. A. (1984). Ph.D. Thesis, University of Durham.

Alves, R. A. and Payne, J. W. (1980). *Biochemical Society Transactions* **8**, 704.

Alves, R. A., Gleaves, J. T. and Payne, J. W. (1985). *FEMS Microbiology Letters* **27**, 333.

Amanuma, H., Itoh, J. and Anraku, Y. (1976). *Journal of Biochemistry* **79**, 1167.

Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M. and Pastan, I. (1992). *Proceedings of the National Academy of Sciences of the United States of America* **89**, 8472.

Ames, B. N., Ames, G. F. L., Young, J. D., Isuchiya, D. and Lecocq, J. (1973). *Proceedings of the National Academy of Sciences of the United States of America* **70**, 456.

Ames, G. F. L. (1986). *Annual Review of Biochemistry* **55**, 397.

Ames, G. F. L. (1988). *Journal of Bioenergetics and Biomembranes* **20**, 1.

Ames, G. F. L. and Joshi, A. K. (1990). *Journal of Bacteriology* **172**, 4133.

Ames, G. F. L. and LeCar, H. (1992). *The FASEB Journal* **6**, 2660.

Ames, G. F. L., Mimura, C. S. and Shyamala, V. K. (1990). *FEMS Microbiology Reviews* **75**, 429.

Ames, G. F. L., Nikaido, K., Groarke, J. and Pitithory, J. (1989). *Journal of Biological Chemistry* **264**, 3998.

Anderson, B. F., Baker, H. N., Norris, G. E., Rumball, S. V. and Baker, E. N. (1990). *Nature* **344**, 784.

Anderson, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E. and Welsh, M. J. (1991a). *Cell* **67**, 775.

Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A.E. and Welsh, M. J. (1991b). *Science* **253**, 202.

Andrews, J. C. and Short, S. A. (1985). *Journal of Bacteriology* **161**, 484.

Andrews, J. C. and Short, S. A. (1986). *Journal of Bacteriology* **165**, 434.

Andrews, J. C., Blevins, T. C. and Short, S. A. (1986). *Journal of Bacteriology* **165**, 428.

Andruszkiewicz, R., Chmara, H. and Borowski, E. (1984). *Journal of Antibiotics* **37**, 1479.

Andruszkiewicz, R., Chmara, H., Milewski, S. and Borowski, E. (1987). *Journal of Medicinal Chemistry* **30**, 1715.

Andruszkiewicz, R., Milewski, S., Zieniawa, T. and Borowski, E. (1990a). *Journal of Medicinal Chemistry* **33**, 132.

Andruszkiewicz, R., Chmara, H., Milewski, S., Zieniawa, T. and Borowski, E. (1990b). *Journal of Medicinal Chemistry* **33**, 2755.
Angehrn, P., Hall, M. J., Lloyd, W. J. and Westmacott, D. (1984). *Antimicrobial Agents and Chemotherapy* **25**, 607.
Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W. and Ringrose, P. S. (1979a). *Antimicrobial Agents and Chemotherapy* **15**, 677.
Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W., Lloyd, W. J. and Ringrose, P. S. (1979b). *Antimicrobial Agents and Chemotherapy* **15**, 696.
Atherton, F. R., Hall, M. J., Hassall, C. H., Holmes, S. W., Lambert, R. W., Lloyd, W. J. and Ringrose, P. J. (1980). *Antimicrobial Agents and Chemotherapy* **18**, 897.
Atherton, F. R., Hall, M. J., Hassall, C. H., Holmes, S. W., Lambert, R. W., Lloyd, W. J., Nisbet, L. J., Ringrose, P. S. and Westmacott, D. (1981). *Antimicrobial Agents and Chemotherapy* **20**, 470.
Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W., Lloyd, W. J., Ringrose, P. S. and Westmacott, D. (1982). *Antimicrobial Agents and Chemotherapy* **22**, 571.
Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W., Lloyd, W. J., Lord, A., Ringrose, P. S. and Westmacott, D. (1983). *Antimicrobial Agents and Chemotherapy* **24**, 522.
Atherton, F. R., Hassall, C. H. and Lambert, R. W. (1986). *Journal of Medicinal Chemistry* **29**, 29.
Austin, E. A., Andrews, J. C. and Short, S. A. (1989). *Molecular Genetics of Bacteria and Phages* p153, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Bai, P. F., Subramanian, P., Mosberg, H. I. and Amidon, G. L. (1991). *Pharmaceutical Research* **8**, 593.
Baichwal, V., Liu, D. and Ames, G. F. L. (1993). *Proceedings of the National Academy of Sciences of the United States of America* **90**, 620.
Baker, E. N., Rumball, S. V. and Anderson, B. F. (1987). *Trends in Biochemical Sciences* **12**, 350.
Bakken, V., Hogh, B. T. and Jensen, H. B. (1989). *Scandinavian Journal of Dental Research* **97**, 43.
Banks, W. A. and Kastin, A. J. (1990). *American Journal of Physiology* **259**, 1.
Banks, W. A. and Kastin, A. J. (1992). *Progress in Brain Research* **91**, 139.
Banks, W. A., Kastin, A. J. and Barrera, C. M. (1991). *Pharmaceutical Research* **8**, 1345.
Barak, Z. and Gilvarg, C. (1975). *Journal of Bacteriology* **122**, 1200.
Bauer, K., Struyve, M., Bosch, D., Benz, R. and Tommassen, J. (1989). *Journal of Biological Chemistry* **264**, 16 393.
Becker, J. M. and Naider, F. (1977). *Archives of Biochemistry and Biophysics* **178**, 245.
Becker, J. M. and Naider, F. (1980). *Journal of Bacteriology* **143**, 1066.
Becker, J. M., Dunsmore, K. P., Steinfeld, A. S. and Naider, F. (1982). *Biochemistry* **21**, 5967.
Bell, G., Payne, G. M. and Payne, J. W. (1977). *Journal of General Microbiology* **98**, 485.
Bellido, F., Martin, N. L., Siehnel, R. J. and Hancock, R. E. W. (1992). *Journal of Bacteriology* **174**, 5196.
Benz, R. (1988). *Annual Review of Microbiology* **42**, 359.
Benz, R., Schmid, A. and Hancock, R. E. W. (1985). *Journal of Bacteriology* **162**, 722.
Berges, D. A., DeWolf, W. E., Dunn, G. L., Grappel, S. F., Newman, D. J., Taggart, J. J. and Gilvarg, C. (1986). *Journal of Medicinal Chemistry* **29**, 89.
Beskid, G., Siebelist, J., McGarry, C. M., Cleeland, R., Chan, K. K. and Keith, D. D. (1990). *Chemotherapy* **36**, 109.
Bishop, L., Agbayani, R., Ambudkar, S. V., Maloney, P. C. and Ames, G. F. L. (1989). *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6953.
Blank, V. (1987). Diploma Thesis, University of Konstanz, Germany.
Boehm, J. C., Kingsbury, W. D., Perry, D. and Gilvarg, C. (1983). *Journal of Biological Chemistry* **258**, 14 850.
Boos, W. and Gordon, A. S. (1971). *Journal of Biological Chemistry* **246**, 621.

Boos, W., Gordon, A. S., Hall, R. E. and Price, H. D. (1972). *Journal of Biological Chemistry* **247**, 917.
Boisvert, W., Cheung, K. S., Lerner, S. A. and Johnston, M. (1986). *Journal of Biological Chemistry* **261**, 7871.
Borman, C., Huhn, W., Zahner, H., Rathmann, R., Hahn, H. and Konig, W. A. (1985). *Journal of Antibiotics* **38**, 9.
Bourret, R. B., Borkovich, K. A. and Simon, M. I. (1991). *Annual Review of Biochemistry* **60**, 401.
Brass, J. M., Higgins, C. F., Foley, M., Rugman, P. A., Birmingham, J. and Garland, P. B. (1986). *Journal of Bacteriology* **165**, 787.
Braun, V. (1975). *Biochimica et Biophysica Acta* **415**, 335.
Brink, B. T., Otto, R., Hansen, U. P. and Konings, W. N. (1985). *Journal of Bacteriology* **162**, 383.
Bruckner, H., Jung, G., Werner, R. G. and Appel, K. R. (1983). *Arzneimittel-Forschung, Analen der Chemie und Pharmacie* **33**, 1630.
Bugg, T. D. H. and Walsh, C. T. (1992). *Natural Product Reports* **9**, 199.
Chen, G., Russell, J. B. and Sniffen, C. J. (1987) *Journal of Dairy Science* **70**, 1211.
Cheung, K. S., Wasserman, S. A., Dudek, E., Lerner, S. A. and Johnston, M. (1983). *Journal of Medicinal Chemistry* **26**, 1733.
Cheung, K. S., Boisvert, W., Lerner, S. A. and Johnston, M. (1986). *Journal of Medicinal Chemistry* **29**, 2060.
Chmara, H. (1985). *Journal of General Microbiology* **131**, 265.
Chmara, H. and Zahner, H. (1984). *Biochimica et Biophysica Acta* **787**, 45.
Chmara, H., Smulkowski, M. and Borowski, E. (1980). *Drugs and Experimental Clinical Research* **6**, 7.
Chmara, H., Woynarowskq, B. and Borowski, E. (1981). *Journal of Antibiotics* **34**, 1608.
Chmara, H., Milewski, S., Dzieduszycka, M., Smulkowski, M., Sawlewicz, P. and Borowski, E. (1982). *Drugs and Experimental Clinical Research* **8**, 11.
Claesson, A., Jansson, A. M., Pring, B. G., Hammond, S. M. and Ekstrom, B. (1987). *Journal of Medicinal Chemistry* **30**, 2309.
Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N. and Rosenbusch, J. P. (1992). *Nature* **358**, 727.
Cowell, J. L. (1974). *Journal of Bacteriology* **120**, 139.
Dantzig, A. H. and Bergin, L. (1990). *Biochimica et Biophysica Acta* **1027**, 211.
Davidson, A. L. and Nikaido, H. (1991). *Journal of Biological Chemistry* **266**, 8946.
Davidson, A. L., Shuman, H. A. and Nikaido, H. (1992). *Proceedings of the National Academy of Sciences of the United States of America* **89**, 2360.
Davies, M. B. (1980). *Journal of General Microbiology* **121**, 181.
Dean, D. A., Davidson, A. L. and Nikaido, H. (1989). *Proceedings of the National Academy of Sciences of the United States of America* **86**, 9134.
Dean, D. A., Davidson, A. L. and Nikaido, H. (1990). *Research in Microbiology* **141**, 348.
Decad, G. M. and Nikaido, H. (1976). *Journal of Bacteriology* **128**, 325.
Delcour, A. H., Adler, J., Kung, C. and Martinac, B. (1992). *FEBS Letters* **304**, 216.
Denobel, J. G., Klis, F. M., Munnik, T., Priem, J. and Vandenende, H. (1990). *Yeast* **6**, 483.
Deutch, C. E. and Soffer, R. L. (1978). *Proceedings of the National Academy of Sciences of the United States of America* **75**, 5998.
Deverson, E. V., Gow, I. R., Coadwell, W. J., Monaco, J. J., Butcher, G. W. and Howard, J. C. (1990). *Nature* **348**, 738.
Diddens, H., Zahner, H., Kraas, E., Gohring, W. and Jung, G. (1976). *European Journal of Biochemistry* **66**, 11.
Diddens, H., Dorgerloh, M. and Zahner, H. (1979). *Journal of Antibiotics* **32**, 87.
Dietzel, I., Kolb, V. and Boos, W. (1978). *Archives of Microbiology* **118**, 207.
Dorman, C. J., Barr, G. C., Ni Bhriain, N. and Higgins, C. F. (1988). *Journal of Bacteriology* **170**, 2816.
Dudler, R. and Hertig, G. (1992). *Journal of Biological Chemistry* **267**, 5882.

Dudler, R., Schmidhauser, C., Parish, R. W., Wettenhall, R. E. H. and Schmidt, T. (1988). *The EMBO Journal* **7**, 3963.
Endicott, J. A. and Ling, V. (1989). *Annual Review of Biochemistry* **58**, 137.
Engel, A., Massalski, A., Schindler, H., Dorset, D. L. and Rosenbusch, J. P. (1985). *Nature* **317**, 643.
Ernsting, B. R., Atkinson, M. R., Ninfa, A. J. and Matthews, R. G. (1992). *Journal of Bacteriology* **174**, 1109.
Ferguson, S. J. (1990). *Trends in Biochemical Sciences* **15**, 377.
Fickel, T. E. and Gilvarg, C. (1973). *Nature (New Biology)* **241**, 161.
Fikes, J. D. and Bassford, P. J. (1987). *Journal of Bacteriology* **169**, 2352.
Foley, M., Brass, J. M., Birmingham, J., Cook, W. R., Garland, P. B., Higgins, C. F. and Rothfield, L. I. (1989). *Molecular Microbiology* **3**, 1329.
Foote, S. J., Thompson, J. K., Cowman, A. F. and Kemp, D. J. (1989). *Cell* **57**, 921.
Friedinger, R. M. (1989). *Trends in Pharmacological Sciences* **10**, 270.
Furlong, C. E. (1987). *In "Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology" (F. C. Neidhardt, ed.), pp. 768–796. American Society for Microbiology, Washington DC.
Furter, R. and Rast, D. M. (1985). *FEMS Microbiology Letters* **28**, 205.
Gadebusch, H. H., Stapley, E. O. and Zimmerman, S. B. (1992). *Critical Reviews in Biotechnology* **12**, 225.
Gallagher, M. P., Pearce, S. R. and Higgins, C. F. (1989). *European Journal of Biochemistry* **180**, 133.
Gharbia, S. E. and Shah, H. N. (1991). *Current Microbiology* **22**, 159.
Gibson, M. M., Ellis, E. M., Graeme-Cook, K. A. and Higgins, C. F. (1987). *Molecular and General Genetics* **207**, 120.
Gibson, M. M., Price, M. and Higgins, C. F. (1984). *Journal of Bacteriology* **160**, 122.
Gilson, E., Alloing, G., Schmidt, T., Claverys, J. P., Dudler, R. and Hofnung, M. (1988). *The EMBO Journal* **1**, 3971.
Gilson, E., Higgins, C. F., Hofnung, M., Ames, G. F. L. and Nikaido, H. (1982). *Journal of Biological Chemistry* **257**, 9915.
Gilvarg, C. (1981). *In* "The Future of Antibiotherapy and Antibiotic Research" (L. Ninet, P. E. Bost, D. H. Bouanchand and J. Florent, eds), pp. 351–361. Academic Press, New York.
Girodeau, J. M., Agouridas, C., Masson, M., Pineau, R. and LeGoffic, F. (1986). *Journal of Medicinal Chemistry* **29**, 1023.
Goldman, R., Kohlbrenner, W., Lartey, P. A. and Pernet, A. (1987). *Nature* **329**, 162.
Gooday, G. W., Woodman, J., Casson, E. A. and Browne, C. A. (1985). *FEMS Microbiology Letters* **28**, 335.
Goodell, E. W. and Higgins, C. F. (1987). *Journal of Bacteriology* **169**, 3861.
Graham, L. L., Beveridge, T. J. and Nanninga, N. (1991a). *Trends in Biochemical Sciences* **16**, 328.
Graham, L. L., Harris, R., Villiger, W. and Beveridge, T. J. (1991b). *Journal of Bacteriology* **173**, 1623.
Guyer, C. A., Morgan, D. G., Osheroff, N. and Staros, J. V. (1985). *Journal of Biological Chemistry*, **260**, 10812.
Guyer, C. A., Morgan, D. G. and Staros, J. V. (1986). *Journal of Bacteriology* **168**, 775.
Haas, M. W., Becker, J. M. and Miller, R. V. (1981). *Biochimica et Biophysica Acta* **643**, 256.
Hammond, S. M., Claesson, A., Jansson, A. M., Larsson, L. G., Pring, B. G., Town, C. M. and Ekstrom, B. (1987). *Nature* **327**, 730.
Hancock, R. E. W., Siehnel, R. J. and Martin, N. L. (1990). *Molecular Microbiology* **4**, 1069.
Hancock, R. E. W., Egli, C., Benz, R. and Siehnel, R. J. (1992). *Journal of Bacteriology* **174**, 471.
Hanson, M. S., Slaughter, C. and Hansen, E. J. (1992). *Infection and Immunity* **60**, 2257.
Hardy, D. J. and Payne, J. W. (1991). *Planta* **186**, 44.

Hardy, D. J. and Payne, J. W. (1992). *Plant Physiology. Life Science Advances* **11**, 59.
Hassall, C. H. (1983). "Antibiotics VI. Modes and Mechanisms of Microbial Growth Inhibitors" (F. E. Hahn, ed.), pp. 1–11. Springer Verlag, Berlin.
Hassall, C. H. (1985). *Antimicrobial Agents and Chemotherapy* **12**, 193.
Hector, R. F. and Scheller, K. (1992). *Antimicrobial Agents and Chemotherapy* **38**, 1284.
Hengge, R. and Boos, W. (1983). *Biochimica et Biophysica Acta* **737**, 443.
Higgins, C. F. (1990). *Research in Microbiology* **141**, 353.
Higgins, C. F., Ames, G. F. L., Barnes, W. M., Clement, J. M. and Hofnung, M. (1982). *Nature* **298**, 760.
Higgins, C. F., Dorman, C. J., Stirling, D. A., Wadell, L., Booth, I. R., May, G. and Brener, E. (1988a). *Cell* **52**, 569.
Higgins, C. F., Gallagher, M. P., Mimmack, M. L. and Pearce, S. R. (1988b). *Bioessays* **8**, 111.
Higgins, C. F. and Gibson, M. M. (1986). *Methods in Enzymology* **125**, 365.
Higgins, C. F. and Hardie, M. M. (1983). *Journal of Bacteriology* **153**, 1434.
Higgins, C. F., Hardie, M. M., Jamieson, D. J. and Powell, L. M. (1983). *Journal of Bacteriology*, **153**, 830.
Higgins, C. F., Hiles, I. D., Whalley, K., and Jamieson, D. J. (1985). *The EMBO Journal* **4**, 1033.
Higgins, C. F., Hyde, S. C., Mimmack, M. L., Gileadi, U., Gill, D. R. and Gallagher, M. P. (1990). *Journal of Bioenergetics and Biomembranes* **22**, 571.
Higgins, C. F. and Payne, J. W. (1982). *Encyclopaedia of Plant Physiology* **14A**, 438.
Hiles, I. D. and Higgins, C. F. (1986). *European Journal of Biochemistry* **158**, 561.
Hiles, I. D., Gallagher, M. P., Jamieson, D. J. and Higgins, C. F. (1987a). *Journal of Molecular Biology* **195**, 125.
Hiles, I. D., Powell, L. M. and Higgins, C. F. (1987b). *Molecular and General Genetics* **206**, 101.
Hill, J. T. and Dunn, F. W. (1969). *Journal of Medicinal Chemistry* **12**, 737.
Hirshfield, I. N. and Price, M. B. (1975). *Journal of Bacteriology* **122**, 966.
Hobot, J. A., Carlemalm, E., Villiger, W. and Kellenberger, E. (1984). *Journal of Bacteriology* **160**, 143.
Hobson, A. C., Weatherwax, R. and Ames, G. F. L. (1984). *Proceedings of the National Academy of Sciences of the United States of America* **81**, 7333.
Hogarth, B. G. and Higgins, C. F. (1983). *Journal of Bacteriology* **153**, 1548.
Hulen, C. and LeGoffic, F. (1987). *FEMS Microbiology Letters* **40**, 103.
Hulen, C. and LeGoffic, F. (1988). *FEMS Microbiology Letters* **49**, 167.
Hunt, A. H. and Elzey, T. K. (1988). *Journal of Antibiotics* **37**, 802.
Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E. and Higgins, C. F. (1990). *Nature* **345**, 362.
Island, M. D., Naider, F. and Becker, J. M. (1987). *Journal of Bacteriology* **169**, 2132.
Island, M. D., Perry, J. R., Naider, F. and Becker, J. M. (1991). *Current Genetics* **20**, 457.
Isono, K. and Suzuki, S. (1979). *Heterocycles* **13**, 333.
Jackson, E. N. and Yanofsky, C. (1973). *Journal of Bacteriology* **116**, 33.
Jamieson, D. J. and Higgins, C. F. (1984). *Journal of Bacteriology* **160**, 131.
Jamieson, D. J. and Higgins, C. F. (1986). *Journal of Bacteriology* **168**, 389.
Jaspers, C. and Penninckx, M. (1981). *FEBS Letters* **132**, 41.
Jeanteur, D., Lakey, J. H. and Pattus, F. (1991). *Molecular Microbiology* **5**, 2153.
Juranka, P. F., Zastawny, R. L. and Ling, V. (1989). *FASEB Journal* **3**, 2583.
Kafarski, P. and Lejczak, B. (1991). *Phosphorus, Sulphur and Silicon and their Related Elements* **63**, 193.
Kametani, T., Kigasawra, K., Hiragi, M. and Yambe, S. (1981). *Heterocycles* **16**, 1205.
Kamogashira, T. and Takegata, S. (1988). *Journal of Antibiotics (Tokyo)* **41**, 803.
Kashiwagi, K., Miyaji, A., Ikeda, S., Tobe, T., Sasakawa, C. and Igarashi, K. (1992). *Journal of Bacteriology* **174**, 4331.
Kashiwagi, K., Yamaguchi, Y., Sakai, Y., Kobayashi, H. and Igarashi, K. (1990). *Journal of Biological Chemistry* **265**, 8387.

Kenig, M. and Abraham, E. P. (1976). *Journal of General Microbiology* **94**, 37.
Kennedy, E. P. (1982). *Proceedings of the National Academy of Sciences of the United States of America* **79**, 1092.
Kerppola, R. E. and Ames, G. F. L. (1992). *Journal of Biological Chemistry* **267**, 2329.
Kerppola, R. E., Shyamala, V. K., Klebba, P. and Ames, G. F. L. (1991). *Journal of Biological Chemistry* **266**, 9857.
Khare, R. K., Becker, J. M. and Naider, F. (1988). *Journal of Medicinal Chemistry* **31**, 650.
Kingsbury, W. D. (1990). *Pharmacology and Therapeutics* **90**, 187.
Kingsbury, W. D., Boehm, J. C., Mehta, R. J. and Grappel, J. F. (1983). *Journal of Medicinal Chemistry* **26**, 1725.
Kingsbury, W. D., Boehm, J. C., Perry, D. and Gilvarg, C. (1984a). *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4573.
Kingsbury, W. D., Boehm, J. C., Mehta, R. J., Grappel, S. F. and Gilvarg, C. (1984b). *Journal of Medicinal Chemistry* **27**, 1447.
Krainer, E., Becker, J. M. and Naider, F. (1991). *Journal of Medicinal Chemistry* **34**, 174.
Kramer, W., Girbig, F., Gutjahr, U., Kleemann, H. W., Leipe, I., Urbach, H. and Wagner, A. (1990). *Biochimica et Biophysica Acta* **1027**, 25.
Kramer, W., Girbig, F., Gutjahr, U., Kowaleski, S., Adam, F. and Schiebler, W. (1992). *European Journal of Biochemistry* **204**, 923.
Kumada, Y., Imai, S. and Nagaoka, K. (1991). *Journal of Antibiotics* (Tokyo) **44**, 1006.
Labischinski, H., Goodell, E. W., Goodell, A. and Hochberg, M. L. (1991). *Journal of Bacteriology* **173**, 751.
Lam, L. K. P., Arnold, L. D., Kalantar, T. H., Kelland, J. G., Lanebell, P. M., Polcic, M. M., Pickard, M. A. and Vederas, J. C. (1988). *Journal of Biological Chemistry* **263**, 11 814.
Law, B. A. (1978). *Journal of General Microbiology* **105**, 113.
Lejczak, B., Kafarski, P. and Mastalerz, P. (1985). *European Journal of Medicinal Chemistry* **20**, 378.
Lejczak, B., Kafaraski, P., Sztajer, H. and Mastalerz, P. (1986). *Journal of Medicinal Chemistry* **29**, 2212.
LeMahieu, R. A., Pruess, D. and Carson, M. (1982). *Journal of Antibiotics* (Tokyo) **35**, 1063.
Leroux, P., Blanot, D., Menginlecreulx, D. and Vanheijenoort, J. (1991). *International Journal of Peptide and Protein Research* **37**, 103.
Lichliter, W. D., Naider, F. and Becker, J. M. (1976). *Antimicrobial Agents and Chemotherapy* **10**, 483.
Logan, D. A., Becker, J. M. and Naider, F. (1979). *Journal of General Microbiology* **114**, 179.
Losick, R. and Gilvarg, C. (1966). *Journal of Biological Chemistry* **241**, 2340.
Lugtenberg, B. and Van Alphen, L. (1983). *Biochimica et Biophysica Acta* **737**, 51.
Maloney, P. C. (1990). *Research in Microbiology* **141**, 374.
Manson, M. D., Blank, V., Brade, G. and Higgins, C. F. (1986). *Nature* **321**, 253.
Marder, R., Becker, J. M. and Naider, F. (1977). *Journal of Bacteriology* **131**, 906.
Marder, R., Rose, B., Becker, J. M. and Naider, F. (1978). *Journal of Bacteriology* **36**, 1174.
Martineau, P., Szmelcman, S., Spurlino, J. C., Quiocho, F. A. and Hofnung, M. (1990). *Journal of Molecular Biology* **214**, 337.
Maruyama, H. B., Arisawa, M. and Sawada, T. (1979). *Antimicrobial Agents and Chemotherapy* **16**, 444.
Mathiopoulos, C., Mueller, J. P., Slack, F. J., Murphy, C. G., Patankar, A., Bukinsoglu, G. and Sonenshein, A. L. (1991). *Molecular Microbiology* **5**, 1903.
Matthews, D. M. and Payne, J. W. (1980). *Current Topics in Membranes and Transport* **14**, 331.
Matthews, D. M. (1985). *Physiological Reviews* **55**, 537.
Matthews, D. M. (1991). *In* "Protein Absorption". pp. 235–320. Wiley-Liss, New York.
McCarthy, P. J., Troke, P. F. and Gull, K. (1985a). *Journal of General Microbiology* **131**, 775.

McCarthy, P. J., Nisbet, L. J., Boehm, J. C. and Kingsbury, W. D. (1985b). *Journal of Bacteriology* **162**, 1024.
McCarthy, P. J., Newman, D. J., Nisbet, L. J. and Kingsbury, W. D. (1985c). *Antimicrobial Agents and Chemotherapy* **28**, 494.
Mehta, R. J., Kingsbury, W. D., Valenta, J. and Actor, P. (1984). *Antimicrobial Agents and Chemotherapy* **25**, 373.
Meyer-Glauner, W., Bernard, E., Armstrong, D. and Merrifield, B. (1982). *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale A* **252**, 274.
Milewski, S., Chmara, H. and Borowski, E. (1983). *Archives of Microbiology* **135**, 130.
Milewski, S., Andruszkiewicz, R. and Borowski, E. (1988). *FEMS Microbiology Letters* **50**, 73.
Milewski, S., Andruszkiewicz, R., Kasprzak, L., Mazerski, J. Mignini, F. and Borowski, E. (1991a). *Antimicrobial Agents and Chemotherapy* **35**, 36.
Milewski, S., Mignini, F. and Borowski, E. (1991b). *Journal of General Microbiology* **137**, 2155.
Miller III, D. M., Olson, J. S., Pflugrath, J. W. and Quiocho, F. A. (1983). *Journal of Biological Chemistry* **258**, 13 665.
Mitchell, R. E. and Bieleski, R. L. (1977). *Plant Physiology* **60**, 723.
Mitchell, R. E., Johnston, J. S. and Ferguson, A. R. (1981). *Physiological Plant Pathology* **19**, 227.
Miyauchi, S., Komatsubara, M. and Kamo, N. (1992). *Biochimica et Biophysica Acta* **1110**, 144.
Mizuno, T., Chou, M.-Y. and Inouye, M. (1983). *Journal of Biological Chemistry* **258**, 6932.
Mizuno, T. and Mizushima, S. (1990). *Molecular Microbiology* **4**, 1077.
Mobashery, S. and Johnston, M. (1987). *Biochemistry* **26**, 5878.
Mobashery, S., Lerner, S. A. and Johnston, M. (1986). *Journal of the American Chemical Society* **108**, 1685.
Monaco, J. J., Cho, S. and Attaya, M. (1990). *Science* **250**, 1723.
Moneton, P., Sarthou, P. and LeGoffic, F. (1986a). *Journal of General Microbiology* **132**, 2147.
Moneton, P., Sarthou, P. and LeGoffic, F. (1986b). *FEMS Microbiology Letters* **36**, 95.
Moore, R. E., Niemczura, W. P., Kwok, O. C. H. and Patil, S. S. (1984). *Tetrahedron Letters* **25**, 3931.
Mooz, E. D. and Wigglesworth, L. (1976). *Biochemical and Biophysical Research Communications* **68**, 1066.
Morley, J. S., Hennessey, T. D. and Payne, J. W. (1983a). *Biochemical Society Transactions* **11**, 798.
Morley, J. S., Payne, J. W. and Hennessey, T. D. (1983b). *Journal of General Microbiology* **129**, 3701.
Mowbray, S. L. and Petsko, G. A. (1982). *Journal of Molecular Biology* **160**, 545.
Murakami, T., Anzai, H., Imai, S., Satoh, A., Nagaoka, K. and Thompson C. J. (1986). *Molecular and General Genetics* **205**, 42.
Muller, H., Furter, R., Zahner, H. and Rast, D. M. (1981). *Archives of Microbiology* **130**, 195.
Naider, F. and Becker, J. M. (1975). *Journal of Bacteriology* **122**, 1208.
Naider, F. and Becker, J. M. (1987). *Current Topics in Medical Mycology* **2**, 170.
Naider, F., Khan, S. A., Parker, D. D. and Becker, J. M. (1980). *Biochemical and Biophysical Research Communications* **95**, 1187.
Naider, F., Shenbagamurthi, P., Steinfeld, A. S., Smith, H. A., Boney, C. and Becker, J. M. (1983). *Antimicrobial Agents and Chemotherapy* **24**, 787.
Nakae, T. (1986). *Critical Reviews in Microbiology* **13**, 1.
Nakae, T., Ishii, J. and Tokunaga, M. (1979). *Journal of Biological Chemistry* **254**, 1457.
Nakae, T. and Nikaido, H. (1975). *Journal of Biological Chemistry* **250**, 7359.
Neu, H. C. and Heppel, L. A. (1965). *Journal of Biological Chemistry* **240**, 3685.
Neuhaus, F. C., Goyer, S. and Newhaus, D. W. (1977). *Antimicrobial Agents and Chemotherapy* **11**, 638.

Neuhaus, F. C. and Hammes, W. P. (1981). *Pharmacology and Therapeutics* **14**, 265.
Newman, E. B., D'Ari, R. and Lin, R. T. (1992). *Cell* **68**, 617.
Ni Bhriain, N. N., Dorman, C. J. and Higgins, C. F. (1989). *Molecular Microbiology* **3**, 933.
Nikaido, H. (1992). *Molecular Microbiology* **6**, 435.
Nikaido, H. and Vaara, M. (1985). *Microbiological Reviews* **49**, 1.
Nikaido, H., Nikaido, K. and Harayama, S. (1991). *Journal of Biological Chemistry* **266**, 770.
Nisbet, T. M. and Payne, J. W. (1979a). *Journal of General Microbiology* **115**, 127.
Nisbet, T. M. and Payne, J. W. (1979b). *FEMS Microbiology Letters* **6**, 193.
Nisbet, T. M. and Payne, J. W. (1980). *Biochemical Society Transactions* **8**, 705.
Nisbet, T. M. and Payne, J. W. (1982). *Journal of General Microbiology* **128**, 1357.
Norbeck, D. W., Rosenbrook, W., Kramer, J. B., Grampornik, D. J. and Lartey, P. A. (1989). *Journal of Medicinal Chemistry* **32**, 625.
Olson, E. R., Dunyak, D. S., Jurss, L. N. and Poorman, R. A. (1991). *Journal of Bacteriology* **173**, 234.
Omura, S., Hinotozawa, K., Imamura, N. and Murata, M. (1984). *Journal of Antibiotics* (Tokyo) **37**, 939.
Parham, P. (1990). *Nature* **348**, 674.
Parham, P. (1991). *Nature* **351**, 271.
Parham, P. (1992). *Nature* **357**, 193.
Parker, D. D., Naider, F. and Becker, J. M. (1980). *Journal of Bacteriology* **143**, 1066.
Pastan, I. and Gottesman, M. M. (1991). *Annual Review of Medicine* **42**, 277.
Patchett, A. A., Taub, D., Weissberger, B., Valiant, M. E., Gadebusch, H., Thornberry, N. A. and Bull, H. G. (1988). *Antimicrobial Agents and Chemotherapy* **32**, 319.
Payne, G. M. and Payne, J. W. (1984). *Biochemical Journal* **218**, 147.
Payne, J. W. (1968). *Journal of Biological Chemistry* **243**, 3395.
Payne, J. W. (1972). *CIBA Foundation Symposium* 4, pp. 17–32. Associated Scientific Publishers, Amsterdam.
Payne, J. W. (1973). *Biochimica et Biophysica Acta* **298**, 469.
Payne, J. W. (1975). *In* "Peptide Transport in Protein Nutrition" (D. M. Matthews and J. W. Payne, eds), pp. 283–364. North Holland, Amsterdam.
Payne, J. W. (1976). *Advances in Microbial Physiology* **13**, 55.
Payne, J. W. (1977). *CIBA Foundation Symposium* **50**, 305.
Payne, J. W. (1980a). *In* "Microorganisms and Nitrogen Sources" (J. W. Payne, ed.), pp. 211–257. John Wiley & Sons, Chichester and New York.
Payne, J. W. (1980b). *In* "Microorganisms and Nitrogen Sources" (J. W. Payne, ed.), pp. 359–381. John Wiley & Sons, Chichester and New York.
Payne, J. W. (1980c). *In* "Microorganisms and Nitrogen Sources" (J. W. Payne, ed.), pp. 335–356. John Wiley & Sons, Chichester and New York.
Payne, J. W. (ed.) (1980d). "Microorganisms and Nitrogen Sources: Transport and Utilisation of Amino Acids, Peptides, Proteins and Related Substrates." John Wiley & Sons, Chichester, New York.
Payne, J. W. (1983). *Biochemical Society Transactions* **11**, 794.
Payne, J. W. (1986). *Drugs in Experimental and Clinical Research* **12**, 585.
Payne, J. W. (1989). *In* "*In vitro* Techniques in Research" (J. W. Payne, ed.), pp. 145–159. Open University Press, Milton Keynes.
Payne, J. W., Barrett-Bee, K. J. and Shallow, D. A. (1991). *FEMS Microbiology Letters* **79**, 15.
Payne, J. W. and Bell, G. (1977a). *FEMS Microbiology Letters* **2**, 259.
Payne, J. W. and Bell, G. (1977b). *FEMS Microbiology Letters* **1**, 91.
Payne, J. W. and Bell, G. (1977c). *FEMS Microbiology Letters* **2**, 301.
Payne, J. W. and Bell, G. (1979). *Journal of Bacteriology* **137**, 447.
Payne, J. W. and Gilvarg, C. (1968a). *Journal of Biological Chemistry* **243**, 335.
Payne, J. W. and Gilvarg, C. (1968b). *Journal of Biological Chemistry* **243**, 6291.
Payne, J. W. and Gilvarg, C. (1971). *Advances in Enzymology and Related Areas of Molecular Biology* **35**, 187.

Payne, J. W. and Gilvarg, C. (1978). *In* "Bacterial Transport" (B. P. Rosen, ed.), pp. 325–383. Marcel Dekker, New York.
Payne, J. W., Morley, J. S., Armitage, P. and Payne, G. M. (1984). *Journal of General Microbiology* **130**, 2253.
Payne, J. W. and Nisbet, T. M. (1980a). *FEBS Letters* **119**, 73.
Payne, J. W. and Nisbet, T. M. (1980b). *Biochemical Society Transactions* **8**, 683.
Payne, J. W. and Nisbet, T. M. (1981). *Journal of Applied Biochemistry* **3**, 447.
Payne, J. W., Payne, G. M. and Nisbet, T. M. (1982). *FEMS Microbiology Letters* **14**, 123.
Payne, J. W. and Shallow, D. A. (1985). *FEMS Microbiology Letters* **28**, 55.
Payne, J. W. and Walker-Smith, D. J. (1987). *Planta* **170**, 263.
Pearce, S. R., Mimmack, M. L., Gallagher, M. P., Gileadi, U., Hyde, S. C. and Higgins, C. F. (1992). *Molecular Microbiology* **6**, 47.
Peet, R. C., Lindgreen, P. B., Willis, D. K. and Panopoulos, N. J. (1986). *Journal of Bacteriology* **166**, 1096.
Penninckx, M. and Gigot, D. (1979). *Journal of Biological Chemistry* **254**, 6392.
Perego, M., Higgins, C. F., Pearce, S. R., Gallagher, M. P. and Hoch, J. A. (1991). *Molecular Microbiology* **5**, 173.
Perry, D. (1981). *Journal of General Microbiology* **124**, 425.
Perry, D. and Abraham, E. P. (1979). *Journal of General Microbiology* **115**, 213.
Perry, D. and Gilvarg, C. (1983). *Journal of Biological Chemistry* **258**, 14 856.
Perry, D. and Gilvarg, C. (1984). *Journal of Bacteriology* **160**, 943.
Petronilli, V. and Ames, G. F. L. (1991). *Journal of Biological Chemistry* **266**, 16 293.
Prossnitz, E., Gee, A. and Ames, G. F. L. (1989). *Journal of Biological Chemistry* **264**, 5006.
Prossnitz, E., Nikaido, K., Ulbrich, S. J. and Ames, G. F. L. (1988). *Journal of Biological Chemistry* **267**, 17 917.
Quinton, P. M. and Reddy, M. M. (1992). *Nature* **360**, 79.
Quiocho, F. A. (1990). *Philosophical Transactions of the Royal Society of London, Series B* **326**, 342.
Quiocho, F. A. (1992). *Current Opinion in Structural Biology*, **1**, 922.
Rakic, L., Begley, D. J., Davson, H. and Zlokoviv, B. V. (eds) (1988). "Peptide and Amino Acid Transport Mechanisms in the Central Nervous System." Stockton Press, New York.
Rice, G. H., Stewart, F. H. C., Hillier, A. J. and Jago, G. R. (1978). *Journal of Dairy Research* **45**, 93.
Richarme, G., El Yaagoubi, H. and Kohiyama, M. (1992). *Biochimica et Biophysica Acta* **1104**, 201.
Richarme, G. and Kepes, A. (1974). *European Journal of Biochemistry* **45**, 127.
Riedel, K., Renneberg, R. and Scheller, F. (1989). *Bioelectrochemistry and Bioenergetics* **22**, 113.
Riezman, H., Chratchko, Y. and Dulic, V. (1986). *Trends in Biochemical Sciences* **11**, 325.
Ringrose, P. S. (1980). *In* "Microorganisms and Nitrogen Sources" (J. W. Payne, ed.), pp. 641–692. John Wiley & Sons, Chichester and New York.
Ringrose, P. S. (1983a). *Biochemical Society Transactions* **11**, 804.
Ringrose, P. S. (1983b). *Medical Microbiology* **3**, 179.
Ringrose, P. S. (1985). *In* "Scientific Basis of Antimicrobial Chemotherapy" (D. Greenwood and F. O'Grady, eds), pp. 219–266. Cambridge University Press, Cambridge.
Ringrose, P. S. and Lambert, R. W. (1973). *Biochimica et Biphysica Acta* **299**, 374.
Robertson, M. (1991). *Nature* **353**, 300.
Robins, R. J. and Davies, D. D. (1981). *Biochemical Journal* **194**, 63.
Rohl, F., Rabenhorst, J. and Zahner, H. (1987). *Archives of Microbiology* **147**, 315.
Rudner, D. Z., LeDeaux, J. R., Ireton, K. and Grossman, A. D. (1991). *Journal of Bacteriology* **173**, 1388.
Russell, R. R. B., Opoku-Aduse, J., Sutcliffe, I. C., Tao, L. and Ferretti, J. J. (1992). *Journal of Biological Chemistry* **267**, 4631.
Salmenkallio, M. and Sopanen, T. (1989). *Plant Physiology* **89**, 1285.
Sarthou, P., Gonneau, M. and LeGoffic, F. (1983). *Biochemical and Biophysical Research Communications* **110**, 884.

Schleifer, K. H. and Kilpper-Balz, R. (1987). *Systematic Applied Microbiology* **10**, 1.
Sen, K. and Nikaido, H. (1991). *Journal of Bacteriology* **173**, 926.
Shallow, D. A. (1987). Ph.D. Thesis, University of Durham.
Shallow, D. A., Barrett-Bee, K. J. and Payne, J. W. (1991). *FEMS Microbiology Letters* **79**, 9.
Sharma, R. C., Inoue, S., Roitelman, J., Schimke, R. T. and Simon, R. D. (1992). *Journal of Biological Chemistry* **267**, 5731.
Shenbagamurthi, P., Smith, H. A., Becker, J. M., Steinfeld, A. S. and Naider, F. (1983). *Journal of Medicinal Chemistry* **20**, 1518.
Shenbagamurthi, P., Smith, H. A., Becker, J. M. and Naider, F. (1986). *Journal of Medicinal Chemistry* **29**, 802.
Shuman, H. A. (1982). *Journal of Biological Chemistry* **257**, 5455.
Shuman, H. A., Silhavy, T. J. and Beckwith, J. R. (1980). *Journal of Biological Chemistry* **255**, 168.
Shyamala, V. K., Baichwal, C., Beall, E. and Ames, G. F. L. (1991). *Journal of Biological Chemistry* **266**, 18 714.
Siehnel, R. J., Egli, C. and Hancock, R. E. W. (1992). *Molecular Microbiology* **6**, 2319.
Silhavy, T. J., Szmelcman, S., Boos, W. and Schwartz, M. (1975). *Proceedings of the National Academy of Sciences of the United States of America* **72**, 2120.
Slack, F. J., Mueller, J. P., Strauch, M. A., Mathiopoulos, C. and Sonenshein, A. L. (1991). *Molecular Microbiology* **5**, 1915.
Smid, E. (1991). Ph.D. Thesis, University of Groningen.
Smid, E. J. and Konings, W. N. (1990). *Journal of Bacteriology* **172**, 5286.
Smid, E. J., Plapp, R. and Konings, W. N. (1989a). *Journal of Bacteriology* **171**, 6135.
Smid, E. J., Driessen, A. J. M. and Konings, W. N. (1989b). *Journal of Bacteriology* **171**, 292.
Smith, M. W. (1992). Ph.D. Thesis, University of Wales, Bangor.
Smith, M. W. and Payne, J. W. (1990). *FEMS Microbiology Letters* **70**, 311.
Smith, M. W. and Payne, J. W. (1992). *FEMS Microbiology Letters* **100**, 183.
Sopanen, T. (1984). *In* "Membrane Transport in Plants" (W. J. Cram, ed.), pp. 365. John Wiley & Sons, Chichester.
Sopanen, T. and Vaisanen, E. (1985). *Plant Physiology* **78**, 684.
Speiser, D. M. and Ames, G. F. L. (1991). *Journal of Bacteriology* **173**, 1444.
Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D. and De Mars, R. (1990). *Nature* **348**, 744.
Staskawicz, B. J. and Panopoulos, N. J. (1980). *Journal of Bacteriology* **142**, 474.
Stock, J. B., Ninfa, A. J. and Stock, A. M. (1989). *Microbiological Reviews* **53**, 450.
Stock, J. B., Rauch, B. and Roseman, S. (1977). *Journal of Biological Chemistry* **252**, 7850.
Sugawara, E. and Nikaido, H. (1992). *Journal of Biological Chemistry* **267**, 2507.
Thomas, W. A. (1986). *Biochemical Society Transactions* **14**, 383.
Ti, J. S., Steinfeld, A. S., Naider, F., Gulumoglu, A., Lewis, S. V. and Becker, J. M. (1980). *Journal of Medicinal Chemistry* **23**, 913.
Tiruppathi, C., Ganapathy, V. and Leibach, F. H. (1991). *Biochimica et Biophysica Acta* **1069**, 14.
Tolley, S. P., Derewenda, Z., Hyde, S. C., Higgins, C. F. and Wilkinson, A. J. (1988). *Journal of Molecular Biology* **204**, 493.
Torriani, A. (1990). *Bioessays* **12**, 371.
Traxler, B. and Beckwith, J. (1992). *Proceedings of the National Academy of Sciences of the United States of America* **89**, 10 852.
Treptow, N. A. and Shuman, H. A. (1985). *Journal of Bacteriology* **163**, 654.
Trias, J. and Nikaido, H. (1990). *Journal of Biological Chemistry* **265**, 15 680.
Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A. and Kelly, A. (1990). *Nature* **348**, 741.
Tuffnell, J. M. and Payne, J. W. (1985). *Journal of Applied Bacteriology* **58**, 333.
Tyreman, D. R. (1990). Ph.D. Thesis, University of Wales, Bangor.

Tyreman, D. R., Smith, M. W., Payne, G. M. and Payne, J. W. (1992). *In* "Molecular Aspects of Chemotherapy" (D. Shugar, W. Rode and E. Borowski, eds), pp. 127–142. Springer Verlag, Berlin.
Uri, J. V., Bendas, C. M. and Burdash, N. (1988). *Acta Microbiologica Hungarica* **35**, 221.
VanBoven, A. and Konings, W. N. (1986). *Netherlands Milk Dairy Journal* **40**, 117.
VanBoven, A. and Konings, W. N. (1987). *Applied and Environmental Microbiology* **53**, 2897.
VanBoven, A. and Konings, W. N. (1988). *Biochimie* **70**, 535.
Van Wielink, J. E. and Duine, J. A. (1990). *Trends in Biochemical Sciences* **15**, 136.
Vining, L. C. (1990). *Annual Review of Microbiology* **44**, 395.
VoQuang, Y., Carniato, D., VoQuang, L., Lacoste, A. M., Neuzil, E. and LeGoffic, F. (1986). *Journal of Medicinal Chemistry* **29**, 148.
Wainer, S., Boveris, A. and Ramos, E. H. (1988). *Archives of Biochemistry and Biophysics* **262**, 481.
Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982). *The EMBO Journal* **1**, 945.
Walker-Smith, D. J. and Payne, J. W. (1985). *Planta* **164**, 550.
Wallace, R. J. and McKain, N. (1991). *Journal of General Microbiology* **137**, 2259.
Wallace, R. J., McKain, N. and Newbold, C. J. (1990). *Journal of Science of Food and Agriculture* **50**, 191.
Walter, C., Höner, Z. U., Bentrup, K. and Schneider, E. (1992). *Journal of Biological Chemistry* **267**, 8863.
Wanner, B. L. (1987). *In* "*Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology" (F. C. Neidhardt, ed.), pp. 1326–1333. American Society for Microbiology, Washington DC.
Weiss, M. S., Kreusch, A., Schiltz, E., Nestel, U., Welte, W., Weckesser, J. and Schulz, G. E. (1991). *FEBS Letters* **280**, 379.
Westlake, K. and Mackie, R. I. (1990). *Applied Microbiology and Biotechnology* **34**, 97.
Wiame, J-M., Grenson, M. and Arst, H. N. (1985). *Advances in Microbial Physiology* **26**, 2.
Wolfingbarger, L. (1980). *In* "Microorganisms and Nitrogen Sources" (J. W. Payne, ed.), pp. 281–301. John Wiley & Sons, Chichester.
Yaghmai, R. and Hazelbauer, G. L. (1992). *Proceedings of the National Academy of Sciences of the United States of America* **89**, 7890.
Yadan, J-C., Gonneau, M., Sarthou, P. and LeGoffic, F. (1984). *Journal of Bacteriology* **160**, 884.
Yoshihara, E. and Nakae, T. (1992). *FEBS Letters* **306**, 5.
Zboinska, E., Sztajer, H., Lejczak, B. and Kafarski, P. (1990). *FEMS Microbiology Letters* **70**, 23.
Zillig, W., Holz, I., Janekovic, D., Klenk, H. P., Imsel, E., Trent, J., Wunderl, S., Forjaz, V. H., Coutinho, R. and Ferreira, T. (1990). *Journal of Bacteriology* **172**, 3959.

Note Added in Proof

Binding proteins of Gram-negative bacteria (p. 16) and homologous lipoproteins from Gram-positive bacteria (p. 38) have been classified into eight clusters based on homology of their amino acid sequences (Tam and Saier, 1993). In this scheme, the peptide binding proteins form a separate cluster, together, surprisingly, with a nickel-binding protein (Navarro *et al.*, 1993). A further binding protein, part of the Sap system in *S. typhimurium*, required for virulence and for resistance to the antimicrobial polypeptides melittin and protamine, has also been assigned to this cluster

(Parra-Lopez *et al.*, 1993). It has been suggested that recycling of peptidoglycan-derived peptides (p. 18) may involve another transport system in addition to Opp (Park, 1993). These two papers raise the issue of the number of peptide transport systems present in bacteria such as *E. coli*, and the structural specificities needed to define a system as a peptide permease. A preliminary report on the crystallization of DppA (p. 30) has appeared (Dunten *et al.*, 1993). The Dpp (p. 29) has been shown to transport the peptide analogue 5-aminolevulinic acid (Elliott, 1993; Verkamp *et al.*, 1993); in filter-binding assays (p. 18) the affinity of this compound is about 500-fold lower than AlaAla (N. Marshall and J. W. Payne, unpublished results). The substrate-binding site fragment of OppA labelled with the photoaffinity probe (p. 20) is AspIleIleValAsnLys (residues 300–305); the two Cys residues of OppA and the four of DppA have been shown to be present as disulphide bridges (N. Marshall and J. W. Payne, unpublished results). In *L. lactis* (p. 37), di- and tripeptides are taken up by a proton-motive force driven system distinct from an ATP-energized system for oligopeptides (Kunji *et al.*, 1993). The substrate specificity and ATP-dependence of the peptide transporter involved in antigen translocation into the endoplasmic reticulum (p. 68) has been further characterized (Androlewicz *et al.*, 1993; Shepherd *et al.*, 1993).

Androlewicz, M. J., Anderson, K. S. and Cresswell, P. (1993). *Proceedings of the National Academy of Sciences of the United States of America* **90**, 9130.

Dunten, P. W., Harris, J. H., Feitz, V. and Mowbray, S. L. (1993). *Journal of Molecular Biology* **231**, 145.

Elliott, T. (1993). *Journal of Bacteriology* **175**, 325.

Kunji, E. R. S., Smid, E. J., Plapp, R., Poolman, B. and Konings, W. N. (1993). *Journal of Bacteriology* **175**, 2052.

Navarro, C., Wu, L. F. and Mandrandberthelot, M. A. (1993). *Molecular Microbiology* **9**, 1181.

Park, J. T. (1993). *Journal of Bacteriology* **175**, 7.

Parra-Lopez, C., Baer, M. T. and Groisman, E. A. (1993). *The EMBO Journal* **12**, 4053.

Shepherd, J. C., Schumacher, T. N. M., Ashtonrickardt, P. G., Imaeda, S., Ploegh, H. L., Janeway, C. A. and Tonegawa, S. (1993). *Cell* **74**, 577.

Tam, R. and Saier, M. H. (1993). *Microbiological Reviews* **57**, 320.

Verkamp, E., Backman, V. M., Bjornsson, J. M., Soll, D. and Eggertsson, G. (1993). *Journal of Bacteriology* **175**, 1452.

Dinucleoside Oligophosphates in Micro-organisms

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I. Introduction

The dinucleoside oligophosphates to be discussed in this review are made up of two nucleosides joined in a 5′-5′ linkage by a chain of two to six phosphodiester linkages (Fig. 1). They appear in the literature under various names. Thus, the most popular dinucleoside oligophosphate, Ap_4A (Fig. 1), is named either diadenosine 5′,5‴-P^1,P^4-tetraphosphate or bis(5′-adenosyl) tetraphosphate, or even adenosine(5′)tetraphospho(5′)adenosine.

The first biological occurrence of dinucleoside oligophosphates was demonstrated in the early 1960s, when Finamore and Warner observed that encysted dehydrated embryos of the brine shrimp *Artemia salina* contained large amounts of Gp_4G and Gp_3G (Finamore and Warner, 1963; Warner and Finamore, 1965a). A few years later, Zamecnik and his coworkers showed that lysyl-tRNA synthetase from *Escherichia coli* was capable of synthesizing Ap_4A and other adenylylated dinucleoside oligophosphates (Zamecnik *et al.*, 1966, 1967; Randerath *et al.*, 1966; Zamecnik and Stephenson, 1968, 1969). However, it was only in the late 1970s and in the early 1980s that reports appeared which triggered rapid progress in establishing possible functions for dinucleoside oligophosphates. In 1976, Rapaport and Zamecnik reported that the Ap_4A concentration in mammalian cells correlated with the index of proliferative activity (Rapaport and Zamecnik, 1976). From this result and other observations, Ap_4A was suggested to be a positive pleiotropic activator involved in control of the cell cycle. In 1983, Ames and his coworkers showed that several oxidizing compounds produced a very strong accumulation of dinucleoside oligo-

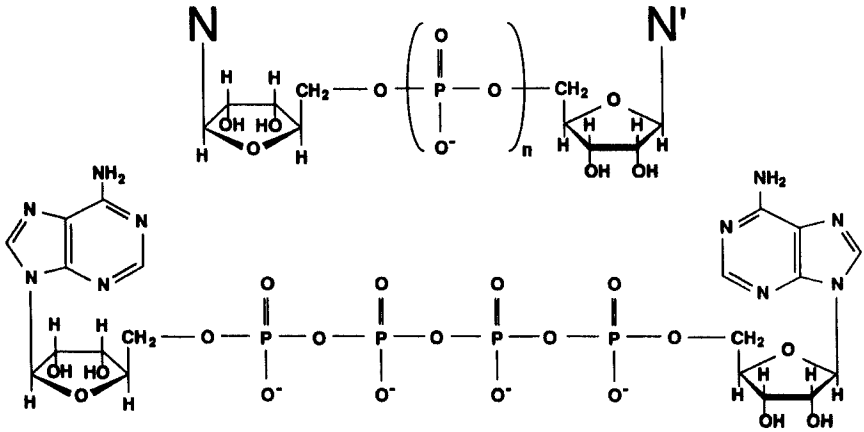

FIG. 1. General structure of (a) a dinucleoside oligophosphate (Np_nN') in which N and N′ indicate nucleoside residues and (b) structure of Ap_4A (N, N′ = A; n = 4).

phosphates in cells of prokaryotes (Lee *et al.*, 1983a,b). This led to a second hypothesis for the physiological role of dinucleoside oligophosphates, giving to Ap_4A the role of an alarm signalling to the cell at the onset of an oxidative stress. Later, observations, such as the ubiquitous character of these nucleotides and the effect of Ap_4A *in vitro* on the activity of various enzymes, reinforced the idea that dinucleoside oligophosphates were potentially important regulatory molecules.

At the present time, several enzymes capable of synthesizing or degrading dinucleoside oligophosphates have been identified, giving credit to the idea that these compounds result from specific metabolic pathways. However, the exact biological role of these nucleotides remains to be determined.

In this review, we summarize the work performed on dinucleoside oligophosphates in micro-organisms and, in particular, emphasize the construction of mutant strains affected in their dinucleoside oligophosphate concentrations. These strains are precious tools to experiment directly on possible functions of these nucleotides. For a more comprehensive view on all aspects of dinucleoside oligophosphate biology, the reader is referred to previous reviews (Silverman and Atherly, 1979; Zamecnik, 1983; Grummt, 1983, 1988; Baril *et al.*, 1985; Bambara *et al.*, 1985; Andersson, 1989) and to contributions to a recent monograph (McLennan and Zamecnik, 1992; Holler, 1992; Garrison and Barnes, 1992; Plateau and Blanquet, 1992; Guranowski and Sillero, 1992; Kitzler *et al.*, 1992; Remy, 1992; Günther Sillero and Cameselle, 1992; Ogilvie, 1992; Warner, 1992; Blackburn *et al.*, 1992).

II. Occurrence of Dinucleoside Oligophosphates in Micro-organisms and Accumulation During Stresses

Dinucleoside oligophosphates have been discovered in all micro-organisms in which they have been searched for. They have been found in *E. coli* (Plesner *et al.*, 1979; Lee *et al.*, 1983b; Plateau *et al.*, 1987a,b; VanBogelen *et al.*, 1987; Coste *et al.*, 1987), *Salmonella typhimurium* (Lee *et al.*, 1983a,b; Bochner *et al.*, 1984; Kramer *et al.*, 1988), *Bacillus brevis* (Federn and Ristow, 1986), *Clostridium acetobutylicum* (Balodimos *et al.*, 1988), *Halobacterium halobium* (Grummt, 1988), *Synechococcus* sp. (Pálfi *et al.*, 1991), *Dictyostelium discoideum* (Ogilvie and Jakob, 1983), *Physarum polycephalum* (Weinmann-Dorsch *et al.*, 1984b; Garrison and Barnes, 1984; Garrison *et al.*, 1986, 1989) and *Saccharomyces cerevisiae* (Garrison and Barnes, 1984; Baltzinger *et al.*, 1986; Coste *et al.*, 1987). In exponentially growing cells, Ap_4A concentration usually ranges between 0.1 and 3 μM (Garrison and Barnes, 1992). Other dinucleoside oligophosphates

occurring in living cells include Ap_4G, Ap_4C, Ap_4U, Ap_3A, Ap_3G, Ap_3C, Ap_3U, Ap_3Gp_2 and the six possible non-adenylylated Bp_4B' nucleotides (B, B' = C, G or U).

In 1983, Ames and his coworkers reported that the concentration of dinucleoside oligophosphates markedly increased when cells of *S. typhimurium* were exposed to the bacteriostatic quinone, ACDQ (6-amino-7-chloro-5,8-dioxoquinoline) (Lee *et al.*, 1983a). This important observation led to a systematic search for the physiological conditions capable of inducing an increase in dinucleoside oligophosphate concentration. The main results obtained using micro-organisms are summarized below.

A. *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

In *S. typhimurium* and *E. coli*, the factors known to trigger dinucleoside oligophosphate accumulation are temperature upshift, UV irradiation and the addition of ethanol or of oxidizing compounds such as hydrogen peroxide, cadmium chloride, diamide, 1-chloro 2,4-dinitrobenzene, *N*-ethylmaleimide, iodacetamide, t-butylhydroperoxide and a variety of quinones (Lee *et al.*, 1983a,b; Bochner *et al.*, 1984; VanBogelen *et al.*, 1987; Plateau *et al.*, 1987a,c). The common feature shared by all of these stresses could be their oxidizing character. Ultraviolet irradiation produces free radicals (Kramer and Ames, 1987), while temperature or ethanol may increase superoxide and hydrogen peroxide side-products from electron transport by damaging cellular membranes (Lee *et al.*, 1983b). In contrast, dinucleoside oligophosphate concentrations could never be modified upon blocking DNA, RNA or protein syntheses, or during starvation of sources of carbon, nitrogen or phosphate (Bochner *et al.*, 1984).

Various dinucleoside oligophosphates were observed to accumulate in response to heat or oxidative shocks; they included Ap_4N (N = A, C, G or U), Ap_3N, Bp_4B' and Ap_3Gp_2. Their individual intracellular concentrations could be increased more than 100-fold. Interestingly, the relative rates of accumulation of the various dinucleoside oligophosphates depend on the applied stimulus. *N*-Ethylmaleimide increases only Ap_3A concentration, while t-butylhydroperoxide or UV irradiation mainly increase concentrations of Ap_3Gp_2 (Bochner *et al.*, 1984; Kramer *et al.*, 1988). The concentrations of the precursor nucleotides are likely to play a role in these variations. Thus, Ap_3Gp_2 is elevated in concentration during mild heat shock (from 28 to 42°C), along with the concentration of its putative precursor nucleotide ppGpp, while it does not accumulate during more severe heat shock (from 28 to 50°C) which do not stimulate ppGpp synthesis (Lee *et al.*, 1983b).

B. *CLOSTRIDIUM ACETOBUTYLICUM*

In response to hyperthermia, *Cl. acetobutylicum* accumulates Ap_4A and Ap_4G to high levels (Balodimos *et al.*, 1988). These nucleotides increase also in this anaerobic bacterium when cells switch from the acidogenic phase of growth to the phase of production of solvents (butanol, acetone, ethanol). Noteworthy, when cells progress from the acid to the solvent phase, accumulation of Ap_4N in response to heat stress becomes higher, suggesting a synergy between hyperthermia and ethanol in increasing Ap_4N production (Balodimos *et al.*, 1988). During the solventogenic phase, most of the Ap_4N nucleotides become extracellular, maybe as a result of the chaotropic effect of alcohols and solvents rendering cell membranes permeable to small molecules (Balodimos *et al.*, 1988; Hutkins and Kashket, 1986; Terracciano *et al.*, 1987).

C. *SACCHAROMYCES CEREVISIAE*

In *Sacch. cerevisiae*, concentrations of the four Ap_4N rise from 0.2–0.55 μM to 34–270 μM in 180 min after a temperature shift from 30 to 46°C, while concentrations of the non-adenylylated Bp_4B' increase from 0.03–0.2 μM to 0.28–4.75 μM (Coste *et al.*, 1987). To calculate intracellular concentrations in these experiments, intracellular volumes were estimated using a cell-sizing apparatus (Plateau *et al.*, 1987b). Accumulation of Ap_4N and Bp_4B' was also observed after addition of 500 μM cadmium chloride to a yeast culture (Coste *et al.*, 1987). The effect of hyperthermia on Ap_3A variation is less clear. Denisenko (1984) reported an increase in concentration of this nucleotide after a temperature upshift, but this effect could not be confirmed by Baltzinger *et al.* (1986).

D. *PHYSARUM POLYCEPHALUM*

Concentrations of Ap_4A and Ap_4G transiently increase by a factor of three to seven in cells of *P. polycephalum* after treatment with 0.1 mM dinitrophenol (Garrison *et al.*, 1986). At such a concentration, dinitrophenol is an effective uncoupler of oxidative phosphorylation in mitochondria from *P. polycephalum* (Holmes and Stewart, 1979), and may have caused an oxidative stress, because uncoupling of respiration is likely to increase production of hydrogen peroxide (Garrison *et al.*, 1986). However, the subsequent observation that oxidants, including hydrogen peroxide, cadmium chloride or paraquat, only slightly modified Ap_4A concentration renders this mechanism questionable (Garrison *et al.*, 1989). In addition, Ap_4A concentrations increase three- to seven-fold in *P. polycephalum*

grown anaerobically (Garrison *et al.*, 1989). Anoxia is expected to acidify the cytoplasm through an increase in the concentrations of acids produced by glycolysis, while pH value may be an important factor in regulation of Ap_4A concentration since synthesis and degradation of this nucleotide are accompanied by consumption or release of protons. In this context, it is noticeable that dinitrophenol is a proton ionophore capable of transferring protons from the medium (pH 4.6) to the cytoplasm (Garrison *et al.*, 1989). The effect of anoxia and dinitrophenol might be, in fact, related to a downshift in the cell pH value.

III. Biosynthesis of Dinucleoside Oligophosphates

To elucidate mechanisms through which dinucleoside oligophosphate concentrations vary in a cell, it is important to identify which enzymes are responsible for synthesis and catabolism of these nucleotides. As shown in the following sections, the list of enzymes possibly involved in metabolism of dinucleoside oligophosphates is already quite large, in particular with regard to degrading enzymes. However, while a few of the reported catabolic enzymes appear to have a very specific function, enzymes involved in producing dinucleoside oligophosphates are systematically involved in another cellular function.

A. *IN VITRO* SYNTHESIS OF ADENYLYLATED DINUCLEOSIDE TETRA- OR TRIPHOSPHATES BY AMINOACYL-tRNA SYNTHETASES

The first enzymic synthesis of a dinucleoside oligophosphate was observed in 1966, when Zamecnik and his coworkers incubated lysyl-tRNA synthetase from *E. coli* in the presence of ATP, magnesium ions and lysine, and discovered synthesis of Ap_4A (Zamecnik *et al.*, 1966; Randerath *et al.*, 1966). Aminoacyl-tRNA synthetases catalyse covalent attachment of each amino acid to the cognate tRNA(s). In a first step, the amino acid reacts with ATP to produce pyrophosphate and an aminoacyl-adenylate molecule which remains bound to the synthetase:

$$\text{ATP} + \text{amino acid} + \text{enzyme} \leftrightarrow \text{aminoacyl}\sim\text{AMP:enzyme} + \text{pyrophosphate}$$

In a second step, tRNA reacts with the aminoacyl-adenylate to give aminoacyl-tRNA and AMP:

$$\text{aminoacyl}\sim\text{AMP:enzyme} + \text{tRNA} \leftrightarrow \text{aminoacyl}\sim\text{tRNA} + \text{AMP} + \text{enzyme}$$

Each reaction is reversible. In particular, a pyrophosphate molecule can react with the aminoacyl-adenylate to regenerate ATP and the free amino acid. This reaction is not fully specific for pyrophosphate, for an ATP molecule can also attack the aminoacyl-adenylate. This leads to slow formation of an Ap_4A molecule (Zamecnik *et al.*, 1966):

aminoacyl~AMP:enzyme + ATP ↔ Ap_4A + amino acid + enzyme

Several properties of Ap_4A synthesis by aminoacyl-tRNA synthetases result from this mechanism. The first is requirement of aminoacyl-adenylate synthesis. Hence, Ap_4A synthesis cannot occur in the absence of the cognate amino acid (Zamecnik *et al.*, 1966, 1967; Plateau *et al.*, 1981b; Goerlich *et al.*, 1982; Nakajima *et al.*, 1989). The second is that because Ap_4A synthesis from ATP and an amino acid generates pyrophosphate which is a competitor of ATP in attack on the aminoacyl-adenylate, the presence of pyrophosphatase shifts the equilibrium of the reaction towards Ap_4A synthesis (Plateau *et al.*, 1981b; Goerlich *et al.*, 1982; Nakajima *et al.*, 1989). Finally, in agreement with the involvement of aminoacyl-adenylate as an intermediate, Ap_4A synthesis proceeds with retention of configuration at the α-phosphate position of ATP (Harnett *et al.*, 1985; Lowe, 1991).

Nearly all aminoacyl-tRNA synthetases are capable of synthesizing Ap_4A, whatever their origin (Table 1). At the present time, the sole aminoacyl-tRNA synthetases from micro-organisms found not to be able to catalyse Ap_4A synthesis are arginyl- and glutaminyl-tRNA synthetases from *E. coli* and arginyl-tRNA synthetase from *Sacch. cerevisiae*. They correspond to those peculiar synthetases which do not succeed in catalysing an isotopic ATP-[^{32}P]pyrophosphate exchange in the absence of the cognate tRNA.

Numerous other compounds with a free diphosphoryl terminus can react with aminoacyl-adenylate. Thus, various XTP (X = A, C, G, U, dA, dG or dT) and XDP (X = A, C, G, U, dA or dT) molecules having a 5′-tri- or diphosphate group produce an Ap_4X or an Ap_3X molecule, respectively (Zamecnik *et al.*, 1966, 1967; Randerath *et al.*, 1966; Zamecnik and Stephenson, 1968, 1969; Zamecnik, 1969, 1983; Plateau *et al.*, 1981a; Plateau and Blanquet, 1982; Jakubowski, 1983; Traut, 1987). Other nucleotides (ppppA, ppGpp, pppGpp, ATPαS, pCH_2ppA), 5′-diphosphorylated RNAs and non-nucleotidic compounds (inorganic tripolyphosphate, thiamine pyrophosphate) can also substitute for pyrophosphate in the back reaction of aminoacyl-adenylate synthesis (Zamecnik and Stephenson, 1968, 1969; Rapaport *et al.*, 1975; Led *et al.*, 1983; Nakajima *et al.*, 1989; Lazewska and Guranowski, 1990). Reactivities towards these substrates of

TABLE 1. *In vitro* Ap_4A synthesis by aminoacyl-tRNA synthetases from various microorganisms

Amino acid	Organism	Ap_4A synthesis?	Stimulation by zinc ions?	Reference
Class I aminoacyl-tRNA synthetases				
Cys	*Escherichia coli*	yes	no	Blanquet *et al.* (1983)
Ile	*Escherichia coli*	yes	no	Goerlich *et al.* (1982), Blanquet *et al.* (1983)
Leu	*Bacillus stearothermophilus*	yes	no	Kitabatake *et al.* (1987), Nakajima *et al.* (1989)
	Saccharomyces cerevisiae	yes	nd[a]	Goerlich *et al.* (1982)
Met	*Escherichia coli*	yes	no	Blanquet *et al.* (1983)
Trp	*Escherichia coli*	yes	no	Blanquet *et al.* (1983)
	Bacillus stearothermophilus	yes	no	Traut (1987)
Tyr	*Escherichia coli*	yes	no	Goerlich *et al.* (1982), Blanquet *et al.* (1983)
Val	*Escherichia coli*	yes	no	Lazewska and Guranowski, (1990)
	Saccharomyces cerevisiae	yes	no	Goerlich *et al.* (1982)
Arg	*Escherichia coli*	no	no	Zamecnik *et al.* (1966)
	Saccharomyces cerevisiae	no	no	Goerlich *et al.* (1982)
Gln	*Escherichia coli*	no	no	Traut (1987)
Class II aminoacyl-tRNA synthetases				
Ala	*Escherichia coli*	yes	yes	Blanquet *et al.* (1983), Traut (1987), Biryukov *et al.* (1990)
Lys	*Escherichia coli*	yes	yes	Zamecnik *et al.* (1966), Plateau and Blanquet (1982), Goerlich *et al.* (1982), Biryukov *et al.* (1990)
	Saccharomyces cerevisiae	yes	yes	Blanquet *et al.* (1983)
Phe	*Escherichia coli*	yes	yes	Plateau *et al.* (1981b), Goerlich *et al.* (1982), Biryukov *et al.* (1990)
	Methanosarcina barkeri	yes	yes	Rauhut *et al.* (1985)
	Physarum polycephalum	yes	yes	Goerlich *et al.* (1982)
	Saccharomyces cerevisiae	yes	yes	Plateau *et al.* (1981b), Goerlich *et al.* (1982), Guédon *et al.* (1987)
	Saccharomyces cerevisiae mitochondria	yes	yes	Rauhut *et al.* (1985)
	Thermus thermophilus	yes	yes	Biryukov *et al.* (1991)
Pro	*Escherichia coli*	yes	yes	Blanquet *et al.* (1983)
Asp	*Saccharomyces cerevisiae*	yes	nd[a]	Goerlich *et al.* (1982)
Gly	*Escherichia coli*	yes	no	Led *et al.* (1983)
His	*Escherichia coli*	yes	no	Goerlich *et al.* (1982), Blanquet *et al.* (1983)
	Bacillus stearothermophilus	yes	no	Traut (1987)
Ser	*Saccharomyces cerevisiae*	yes	no	Goerlich *et al.* (1982)

[a] nd indicates that the response was not determined.

each aminoacyl-adenylate bound to the corresponding aminoacyl-tRNA synthetase are variable. For instance, lysyl-tRNA synthetase from *E. coli* catalyses production of the four possible Ap_4N at comparable rates (Plateau and Blanquet, 1982), while alanyl-tRNA synthetase from the same bacterium produces Ap_4U much more slowly than Ap_4A, Ap_4C or Ap_4G (Traut, 1987).

In 1981, our laboratory showed that zinc ions could strongly accelerate production of dinucleoside oligophosphates by several aminoacyl-tRNA synthetases (Plateau *et al.*, 1981a,b; Plateau and Blanquet, 1982). Micromolar concentrations of this ion inhibit the tRNA aminoacylation by lysyl-, phenylalanyl-, alanyl- and prolyl-tRNA synthetases from *E. coli* and by lysyl- and phenylalanyl-tRNA synthetases from *Sacch. cerevisiae* (Plateau *et al.*, 1981b; Blanquet *et al.*, 1983). At the same time, zinc ions stimulate 25- to 200-fold rates of Ap_4A synthesis by these enzymes. These ions also stimulate Ap_4N and Ap_3N syntheses from the various NTP and NDP precursor nucleotides (Plateau and Blanquet, 1982). In contrast to the aminoacyl-tRNA synthetases already referred to, enzymes specific for leucine, valine or histidine remain insensitive to addition of zinc ions, whatever their origin (Table 1). From the presence or absence of conserved motifs in their primary and tertiary structures, the family of aminoacyl-tRNA synthetases was recently partitioned in two classes (Eriani *et al.*, 1990). It may be significant that all of the synthetases known so far to be sensitive to the presence of zinc ions belong to the class II.

The action of zinc ions on the phenylalanyl-tRNA synthetase from *E. coli* was studied in detail (Mayaux and Blanquet, 1981; Goerlich and Holler, 1984). This enzyme binds eight zinc ions to each $\alpha_2\beta_2$ enzyme, with an apparent dissociation constant of 1.25 μM (pH 8.0, 4°C, 150 mM KCl, 7 mM $MgCl_2$). Binding of zinc ions is paralleled by inhibition of both tRNA aminoacylation and the ATP-pyrophosphate exchange reaction, and by a strong stimulation of Ap_4A synthesis. At higher concentrations of the ions (> 40 μM), inhibition of Ap_4A synthesis occurs, which may be attributed to an artefactual zinc-induced aggregation of the protein (Goerlich and Holler, 1984).

B. *IN VIVO* SYNTHESIS OF Ap_4N BY AMINOACYL-tRNA SYNTHETASES

The capacity of aminoacyl-tRNA synthetases to synthesize Ap_4N *in vitro* did not prove that these enzymes significantly contributed to Ap_4N synthesis *in vivo*. To solve this question, *E. coli* strains overexpressing either lysyl-, methionyl-, phenylalanyl- or valyl-tRNA synthetase were obtained by transforming strain JM101TR (Hirel *et al.*, 1988) with a multicopy plasmid harbouring the gene for the corresponding synthetase

(Brevet *et al.*, 1989). In every strain studied, overproduction of the considered aminoacyl-tRNA synthetase (20- to 80-fold) resulted in an increase in the cellular Ap_4N concentration (3- to 14-fold). The observed increases in Ap_4N were likely to be directly caused by the overproduced aminoacyl-tRNA synthetase for the following reasons: (i) overproduction of an inactive mutant aminoacyl-tRNA synthetase does not increase Ap_4N; (ii) as expected from the specificity of aminoacyl-tRNA synthetases, concentrations of the non-adenylylated Bp_4B' nucleotides are not changed by aminoacyl-tRNA synthetase overproduction; and (iii) concentrations of cellular Ap_4N hydrolase are not modified in extracts of the overproducing strains (Brevet *et al.*, 1989).

As already stated, the rate of Ap_4N synthesis by lysyl-tRNA synthetase is strongly enhanced in the presence of zinc ions, while that by valyl-tRNA synthetase is indifferent to the ion. However, when overproduced at similar concentrations, the two aminoacyl-tRNA synthetases sustain somewhat identical increases in cellular Ap_4N concentration (Brevet *et al.*, 1989). Therefore, it may be concluded that the zinc ion did not interfere with the *in vivo* Ap_4A synthetase activity of lysyl-tRNA synthetase under the growth conditions used.

In spite of the large concentrations of Ap_4N they contain, strains overproducing aminoacyl-tRNA synthetases still respond by hyperthermia and continue to accumulate the latter nucleotides after a temperature upshift (Brevet *et al.*, 1989). Concentration of Ap_4N in overproducing cells, from before to after the temperature transition, is multiplied by a factor roughly identical to that measured in control cells that do not overproduce. Thus, in a strain overproducing lysyl-tRNA synthetase, Ap_4N concentration increases from 47 to 2000 μM after an upshift of 60 min from 37 to 48°C. In a strain overproducing valyl-tRNA synthetase, it increases from 28 to 1350 μM. In both examples, the Ap_4N concentration is increased by a factor of about 50. This factor is identical to that measured in the host strain where Ap_4N vary in concentration from 3.4 to 190 μM (Brevet *et al.*, 1989).

In *E. coli*, lysyl-tRNA synthetase is exceptional since it occurs as two species encoded by two distinct genes, namely *lysS* and *lysU* (Hirshfield *et al.*, 1981; Lévêque *et al.*, 1990b). One of these genes, *lysU*, usually almost silent, is highly expressed in response to various conditions including hyperthermia (Neidhardt and VanBogelen, 1981). Therefore, it was tempting to imagine that this gene played a special role in accumulation of Ap_4N following heat shock. However, a Δ*lysU* mutant strain of *E. coli* was recently constructed by inserting a kanamycin gene cassette in the chromosomal *lysU* gene (Lévêque *et al.*, 1991). In this strain, accumulation of Ap_4N after a temperature upshift from 30 to 48°C remains almost identical

to accumulation in the control *lysU*$^+$ strain (J. Chen, P. Plateau and S. Blanquet, unpublished results). Therefore, the heat-induced increase in Ap_4N concentration cannot be related to the heat-induced increase in *lysU* expression. Rather, raising of Ap_4N concentration seems to be the result of mobilization of several, if not all, aminoacyl-tRNA synthetases.

C. SYNTHESIS OF DINUCLEOSIDE OLIGOPHOSPHATES BY ALTERNATIVE ENZYMES

In 1974, Warner and his coworkers reported synthesis of Gp_4G and Gp_3G by a GTP:GTP guanylyltransferase from *Artemia salina* yolk platelets (Warner *et al.*, 1974; Warner and Huang, 1974). This enzyme catalyses the two following reactions:

$$2\ GTP \leftrightarrow Gp_4G + pyrophosphate$$

$$GDP + Gp_4G \leftrightarrow Gp_3G + GTP$$

The occurrence of such an enzymic activity was searched for in other organisms by measuring production of [^{32}P]GTP from [^{32}P]pyrophosphate and Gp_4G. A high activity was found in extracts from either *E. coli* or various yeasts (*Sacch. cerevisiae*, *Sacch. uvarum*, *Hansenula subpelliculosa*, *Rhodotorula mucilaginosa*). After partial purification of this activity, it was shown that the enzymes from *E. coli* and yeast also catalysed conversion of GTP into Gp_4G (Warner, 1992). However, characterization of these enzymes was not pursued.

The enzyme detected in *Sacch. cerevisiae* may be the guanylyltransferase responsible for mRNA capping. This enzyme forms a covalent enzyme–GMP complex during the course of the capping reaction:

$$E + GTP \leftrightarrow E\text{–}GMP + pyrophosphate$$

Reaction of NDP and NTP with the enzyme–GMP complex leads to formation of Gp_3N and Gp_4N molecules, respectively (Wang and Shatkin, 1984).

The mechanisms of dinucleoside oligophosphate production by aminoacyl-tRNA synthetases or guanylyltransferases are rather similar. Both are able to transfer a nucleotide monophosphate moiety accompanied by release of a pyrophosphate ion. In principle, as underlined by Guranowski *et al.* (1990), any enzyme catalysing such a transfer of nucleoside monophosphates is a candidate for producing dinucleoside oligophosphates under appropriate experimental conditions. This was exemplified with the firefly luciferase from *Photinus pyralis* which can synthesize Ap_4A, Ap_5A, Ap_4G or Gp_4G at the expense of luciferyl-adenylate or luciferyl-guanylate intermediates (Guranowski *et al.*, 1990; Sillero *et al.*, 1991).

IV. Catabolism of Dinucleoside Oligophosphates *in vitro*

Enzymes identified for their ability to degrade dinucleoside oligophosphates *in vitro* will now be described. This classification is somewhat arbitrary because we include Ap_4A phosphorylases which, under appropriate conditions, synthesize dinucleoside oligophosphates as well. In addition, as already stated, Ap_4N synthesis catalysed by aminoacyl-tRNA synthetases is reversible. Consequently, Ap_4N can easily be degraded *in vitro* by aminoacyl-tRNA synthetase in the presence of the amino acid and of a high concentration of pyrophosphate (Goerlich *et al.*, 1982), while Ap_4N can be used instead of ATP as an energy source for aminoacylation of the tRNA (Zamecnik *et al.*, 1966).

Many types of enzyme catabolizing dinucleoside oligiphosphates have been described, depending on the organism from which they were characterized. Non-specific phosphodiesterases or nucleotidases occur in various eukaryotic and prokaryotic organisms. They hydrolyse Ap_4A into AMP and ATP (Jakubowski and Guranowski, 1983; Bartkiewicz *et al.*, 1984; Cameselle *et al.*, 1984; Robinson and Barnes, 1986; Lüthje and Ogilvie, 1987; Ogilvie *et al.*, 1989; Ruiz *et al.*, 1989; Rodriguez-Pascual *et al.*, 1992; Guranowski and Sillero, 1992 and references therein). In several bacteria and in *Physarum polycephalum*, a specific Ap_4A hydrolase converts a molecule of Ap_4A into two molecules of ADP (Barnes and Culver, 1982; Guranowski *et al.*, 1983; Plateau *et al.*, 1985). In crude extracts of *Sacch. cerevisiae* and of the protozoan *Euglena gracilis*, Ap_4A can be phosphorolytically cleaved into ADP and ATP (Guranowski and Blanquet, 1985; Guranowski *et al.*, 1988b; Wasternack *et al.*, 1989). Enzymes which specifically hydrolyse dinucleoside triphosphates were detected in both prokaryotic and eukaryotic cells (Sillero *et al.*, 1977; Jakubowski and Guranowski, 1983; Costas *et al.*, 1984; Hurtado *et al.*, 1987; Brevet *et al.*, 1991; Prescott *et al.*, 1992). The 3′-phosphokinase of *Streptomyces morookaensis* converts Ap_3A into AMP and AMP-2′,3′-cyclic phosphate, and Ap_4A into AMP and ADP-2′,3′-cyclic phosphate (Mukai *et al.*, 1989, 1991). Finally, a specific Ap_4A hydrolase converts Ap_4A into AMP and ATP in crude extracts from higher eukaryotic cells (Warner and Finamore, 1965b; Vallejo *et al.*, 1973; Cameselle *et al.*, 1982; Höhn *et al.*, 1982; Ogilvie and Antl, 1983; Jakubowski and Guranowski, 1983; Vallejo and Leon, 1989; Prescott *et al.*, 1989; Pinto *et al.*, 1991). However, evidence for such activity has yet to be found in micro-organisms.

A. SYMMETRICAL Ap_4A HYDROLASES

Enzymes splitting Ap_4A into two ADP molecules (EC 3.6.4.41) have been isolated from two eubacteria, namely *E. coli* and *Acidaminococcus*

fermentans (Guranowski *et al.*, 1983; Plateau *et al.*, 1985), from the thermophilic archaebacterium *Pyrodictum occultum* (Guranowski *et al.*, 1983) and from the slime mould *Physarum polycephalum* (Barnes and Culver, 1982). The same enzyme activity has also been detected in *Methanococcus vannielii* and in *Rhodopseudomonas spheroides* (Guranowski and Sillero, 1992). However, an Ap_4A-degrading activity could not be detected in crude extracts of *Methanobacterium wolfei*, *Methanolobus* sp., *Clostridium symbiosum* (Guranowski and Sillero, 1992) or *Clostridium acetobutylicum* (Balodimos *et al.*, 1988).

Properties of the enzyme from *E. coli* were studied in depth. All dinucleoside oligophosphates behave as substrates provided they contain at least three phosphate residues (Ap_4A, Ap_4G, Ap_4C, Gp_4G, Ap_3A, Ap_3G, Ap_3C, Gp_3G, Gp_3C, Ap_5A, Ap_6A and dAp_4dA are substrates; Ap_2A, NAD^+ and $NADP^+$ are not) (Guranowski *et al.*, 1983; Plateau *et al.*, 1985). The mononucleoside triphosphates, ATP, ADP, AMP, GTP, CTP, UTP, dATP, dGTP, dCTP and dTTP are not substrates, although the mononucleoside tetraphosphate Ap_4 is. One of the products of the reaction is always a nucleoside diphosphate (Guranowski *et al.*, 1983; Plateau *et al.*, 1985). Thus, Ap_3A is cleaved into a mixture of ADP and AMP, Gp_4G into two GDP molecules, and Ap_5A into a mixture of ADP and ATP.

Addition of cobaltous ions (50 μM) to the reaction buffer (50 mM Tris-HCl, pH 7.8, 50 μM Ap_4A, 0.5 μM EDTA, 37°C) strongly stimulates Ap_4A hydrolysis (2500-fold) (Guranowski *et al.*, 1983; Plateau *et al.*, 1985). With 50 μM manganous ions, stimulation reaches 900-fold. Calcium, ferrous and magnesium ions have no effect. The K_m value for Ap_4A is 25 μM with cobaltous ions (Guranowski *et al.*, 1983; Plateau *et al.*, 1985; McLennan *et al.*, 1989) and 12 μM with manganous ions (Plateau *et al.*, 1985). Hydrolysis of Ap_3A into a mixture of ADP and ATP is stimulated by these metal ions. However, the extent of stimulation by cobaltous ions is smaller than with Ap_4A hydrolysis, while stimulation brought by manganous ions in Ap_3A hydrolysis is nine-fold greater than that brought by cobaltous ions (Plateau *et al.*, 1985).

By using $[^{18}O]H_2O$ as a substrate in the hydrolysis reaction, it was shown that ^{18}O was always incorporated into ADP and not into AMP or ATP during degradation of Ap_3A or Ap_5A, respectively (Blackburn *et al.*, 1991). This finding demonstrates that the water molecule attacks the phosphate residue in the second position of the oligophosphate chain. The same type of experiment showed that the R_p and S_p isomers of $Ap_4A\alpha S$ were attacked by water at the unmodified $P_{\beta'}$ phosphate residue yielding ^{18}O-labelled ADP and an unlabelled R_p or S_p isomer of ADPαS (Guranowski and Sillero, 1992). The mechanism of action of the hydrolase from

TABLE 2. Phosphonate analogues of Ap_4A behaving as substrates and/or inhibitors of the Ap_4A hydrolase from *Escherichia coli*[a]

Compound	V_{rel} (%)	K_i (μM)
$AppCF_2ppA$	2	15
AppCHFppA	0.8	8
$AppCCl_2ppA$	0	16
AppCHClppA	0	10
$AppCH_2ppA$	4	7
AppCH=CHppA	0	21
$AppCH_2CH_2ppA$	0.3	30
$ApCF_2ppCF_2pA$	0.2	25
ApCHFppCHFpA	0	130
$ApCCl_2ppCCl_2pA$	0	66
ApCHClppCHClpA	0	125
$ApCH_2ppCH_2pA$	0	15
$ApCH_2CH_2ppCH_2CH_2pA$	0	250
$ApppCH_2pA$	0.3	3

[a] Data are from McLennan *et al.* (1989) and Guranowski *et al.* (1987). Initial rates of hydrolysis were assayed at 30°C in a 20 mM Bicine-KOH buffer (pH 8.4) containing 2 mM magnesium acetate and 100 μM cobalt chloride, with the exception of $ApppCH_2pA$ which was assayed at 30°C in a 50 mM Hepes-KOH (pH 8.2) buffer containing 100 μM cobalt chloride and 10 μM dithiothreitol. In these two buffer conditions, K_m values for Ap_4A were 28 and 25 μM, respectively. Rates (V_{rel} values) are quoted as percentages of the rate measured with Ap_4A. Values for K_i were measured using the same assay conditions, in the presence of 10 or 50 μM Ap_4A.

E. coli was also investigated with the help of Ap_4A analogues (Table 2). Unexpectedly, the β,β′-methylene analogue of Ap_4A ($AppCH_2ppA$) is cleaved by the enzyme into AMP and $AppCH_2p$ (McLennan *et al.*, 1989). Thus, the Ap_4A hydrolase from *E. coli* would adapt the configuration of its active centre to switch the position of the cleavage site from the ββ′ bond to the adjacent bond when the ββ′ bond is refractory to breakdown. Table 2 shows that, among the various αβ,α′β′-disubstituted analogues assayed, only $ApCF_2ppCF_2pA$ behaves as a substrate for the enzyme. The other analogues, although not substrates, were potent competitive inhibitors for Ap_4A hydrolysis.

The specificity of the enzyme from *P. polycephalum* towards putative substrates is similar to that of the enzyme from *E. coli*. Substrates Ap_3A, Ap_4A, Ap_5A, Ap_6A, Ap_4, Gp_3G, Gp_4G, Gp_5G, Gp_6G, Gp_4 are hydrolysed. Neither ATP, ADP, AMP, Ap_2A, ADP-ribose nor the cap dinucleotides m^7Gp_3Am and m^7Gp_3Cm are changed by the enzyme (Garrison *et al.*, 1982). In contrast to the hydrolase from *E. coli*, that from *P. polycephalum* does not require exogenous divalent metal ions for activity. The enzyme remains 50% active in the presence of a 10 mM concentration

of the chelators EDTA or EGTA (Garrison *et al.*, 1982). The cations Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and other divalent cations, inhibit the activity of the enzyme with 40–80% inhibition occurring at a concentration of 0.5 mM. The kinetics of Ap_4A hydrolysis related to Ap_4A concentration are biphasic with apparent K_m values of 2.6 μM and 37 μM (Barnes and Culver, 1982).

B. Ap_4A PHOSPHORYLASES

In *Sacch. cerevisiae*, two isoenzymes degrading Ap_4A through a phosphorolysis rather than through a hydrolysis have been discovered (Guranowski and Blanquet, 1985; Plateau *et al.*, 1989, 1990). The reaction catalysed is:

$$Ap_4A + P_i \leftrightarrow ADP + ATP$$

The systematic name of these enzymes is "ADP:ATP adenylyltransferase" (EC 2.7.7.53). If radioactively labelled inorganic phosphate is used as a substrate, the label is recovered in ADP (Guranowski and Blanquet, 1985; Plateau *et al.*, 1990). Divalent cations such as Mn^{2+}, Mg^{2+} or Ca^{2+} are required as cofactors. In addition to Ap_4A, nucleotides such as Ap_4C, Ap_4G, Ap_4U, Ap_5A, Gp_4G and Gp_4U behave as substrates for the two phosphorylases, a nucleoside diphosphate being always one of the products (Guranowski and Blanquet, 1985; Plateau *et al.*, 1990). For instance, Ap_5A and Gp_4G are converted into ADP and Ap_4 and GDP and GTP, respectively. However, Ap_3A, ATP and GTP are not metabolized.

The phosphorolytic reactions are likely to proceed through formation of a covalent enzyme–nucleoside monophosphate complex. This mechanism would explain why Ap_4A phosphorylases catalyse also an exchange reaction between inorganic phosphate and the β-phosphate of a nucleoside diphosphate (Npp + p* ↔ Npp* + p), as well as conversion of adenosine 5′-phosphosulphate (AMPS) into ADP according to the scheme: AMPS + P_i ↔ ADP + sulphate (Guranowski and Blanquet, 1986a,b; Plateau *et al.*, 1990). Therefore, Ap_4A phosphorylases are likely to be responsible for the ADP sulphurylase activity previously described in *Sacch. cerevisiae* (Robbins and Lipmann, 1958; Grunberg-Manago *et al.*, 1966; Adams and Nicholas, 1972; Burnell and Anderson, 1973; Nicholls, 1977). In contrast to phosphorolysis of dinucleotides, NDP-P_i exchange and AMPS phosphorolysis do not require the presence of divalent cations (Guranowski and Blanquet, 1986a; Plateau *et al.*, 1990).

Since phosphorolysis of Ap_4A maintains constant the number of energy-rich bonds, the reaction is reversible. Consequently, incubation of Ap_4A phosphorylase in the presence of both ATP and ADP leads to the appearance of Ap_4A (Guranowski and Blanquet, 1985; Brevet *et al.*,

1987). The equilibrium constant of the Ap_4A phosphorolytic reaction (K value, $[Ap_4A][P_i]/[ATP][ADP]$) is very sensitive to pH value (Brevet *et al.*, 1987). Synthesis of Ap_4A is favoured at low pH values (K value 0.0003 at pH 8.0; K value 0.028 at pH 5.5), in agreement with consumption of protons when ATP and ADP are converted into Ap_4A and phosphate.

In general, Ap_4A phosphorylases in *Sacch. cerevisiae* support synthesis of various Np_4N' nucleotides from NTP and N'DP:

$$NTP + N'DP \leftrightarrow Np_4N' + P_i$$

The NTP site on the enzyme is specific for purine ribonucleotides (N = A, G) whereas the other subsite accommodates N'DPs without marked specificity (N' = A, C, G, U or dA) (Brevet *et al.*, 1987). The Ap_4A phosphorylases can also use AMPS as a precursor of Ap_4N (AMPS + NTP $\rightarrow$ Ap_4N + sulphate; N = A or G) (Guranowski *et al.*, 1988a). Because the free energy of AMPS is very high, the latter reactions appear to be irreversible (Guranowski *et al.*, 1988a).

C. Ap_3A HYDROLASES

An enzyme that specifically hydrolyses Ap_3A (EC 3.6.1.29) was characterized in extracts of *E. coli* (Hurtado *et al.*, 1987). This enzyme has a strict requirement for magnesium ions. Other cations such as Mn^{2+}, Ca^{2+} or Co^{2+} are without effect. The products of the reaction are AMP and ADP. The K_m value for Ap_3A is about 12 μM (50 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 37°C). Neither the dinucleoside oligophosphates Ap_4A, Ap_5A and Ap_6A, nor NAD^+, ATP, ADP and AMP are substrates of the enzyme (Hurtado *et al.*, 1987).

A specific dinucleoside triphosphatase was also detected in *Sacch. cerevisiae* (Brevet *et al.*, 1991). Its activity is strongly stimulated by the presence of Mn^{2+}, Mg^{2+}, Co^{2+} or Ca^{2+}. The K_m value for Ap_3A is 5.4 μM (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 37°C). Dinucleoside oligophosphates are substrates of this enzyme, provided they contain more than two phosphate residues and that at least one of the two base residues in the substrate is a purine: Ap_3A, Ap_3G, Ap_3C, Gp_3G, Gp_3C, m^7Gp_3A, m^7Gp_3G, Ap_4N (N= A, C, G or U), Gp_4G and Ap_5A are substrates, AMP, ADP, ATP, Ap_2A and Cp_4U are not. A nucleoside monophosphate is always one of the products. This enzyme is likely to be responsible for the residual Ap_4A degradation activity that is still measurable in mutant yeast strains devoid of any Ap_4A phosphorylase activity (Brevet *et al.*, 1991). In contrast to the dinucleoside triphosphatase of rat liver (Costas *et al.*, 1986; Bernet *et al.*, 1991), the enzyme

in *Sacch. cerevisiae* was not associated with mitochondria (A. Brevet, P. Plateau and S. Blanquet, unpublished results).

D. NON-SPECIFIC CLEAVAGE OF DINUCLEOSIDE OLIGOPHOSPHATES

In addition to the enzymes already described which are specifically dedicated to catabolism of dinucleoside oligophosphates, several micro-organisms contain non-specific phosphodiesterases or nucleotidases capable of hydrolysing dinucleoside oligophosphates. These enzymes always liberate a nucleoside monophosphate as one of their products. In *P. polycephalum*, two such enzymes have been detected (Robinson and Barnes, 1986). On the basis of their substrate specificities, they were named nucleotide pyrophosphatase and phosphodiesterase I. Nucleotide pyrophosphatase hydrolyses Ap_nA (n = 3 to 6), Ap_4G, Gp_nG (n = 3 to 5), NAD^+, ATP or GTP. Phosphodiesterase I hydrolyses the same nucleotides except NAD^+. It also hydrolyses typical substrates of phosphodiesterases I such as *bis*-nitrophenyl phosphate (Robinson and Barnes, 1986).

The 5′-nucleotidase of *E. coli* is an enzyme which is secreted into the periplasm of the bacterium. In addition to AMP, ADP, ATP, ribose-5′-phosphate or *bis*-nitrophenyl phosphate, it hydrolyses Ap_nA (n = 2 to 4) and Gp_3G (Ruiz *et al.*, 1989). The *ushA* gene encoding a 5′-nucleotidase in *E. coli* has been cloned, sequenced and mapped at 11 min on the genome of *E. coli* (Burns and Beacham, 1986; Bachmann, 1990).

V. Catabolism of Dinucleoside Oligophosphates *in vivo*

After *in vitro* characterization of the enzymes capable of degrading dinucleoside oligophosphates, it was essential to confirm that these enzymes were actually involved in *in vivo* catabolism of these dinucleotides. This was carried out using genetic experiments in *E. coli* and *Sacch. cerevisiae*.

A. CLONING AND CHARACTERIZATION OF THE APA*H* GENE ENCODING Ap_4A HYDROLASE IN *ESCHERICHIA COLI*

The *apaH* gene encoding Ap_4A hydrolase in *E. coli* (Mechulam *et al.*, 1985) forms part of a complex operon including at least four other cistrons namely *surA*, *pdxA*, *ksgA* and *apaG* (Blanchin-Roland *et al.*, 1986; Roa *et al.*, 1989; Tormo *et al.*, 1990). This operon is located at one min on the chromosome map of *E. coli* (Bachmann, 1990). The *surA* cistron is an essential gene for survival of *E. coli* in the stationary phase of growth

(Tormo *et al.*, 1990), while *pdxA* encodes a 35.1 kDa protein involved in biosynthesis of pyridoxine (Roa *et al.*, 1989). The *ksgA* cistron encodes a 30.4 kDa methyltransferase that specifically methylates two adjacent adenosine residues at the 3'-end of the rRNA (van Buul and van Knippenberg, 1985). Finally, *apaG* codes for a 13.8 kDa protein whose function is not yet identified (Blanchin-Roland *et al.*, 1986). However, using radiolabelled 8-azido-Ap_4A, it was shown that *apaG* gene products can bind Ap_4A *in vitro* (Kitzler *et al.*, 1992). Expression of the *surA-pdxA-ksgA-apaG-apaH* operon is governed by at least three promoters; P_{up} directs transcription of a transcript probably encompassing the five genes (Tormo *et al.*, 1990; Kitzler *et al.*, 1992), while P_1 is located near the 3'-end of the *pdxA* gene and expresses an mRNA covering the *ksgA*, *apaG* and *apaH* genes (Blanchin-Roland *et al.*, 1986; Roa *et al.*, 1989). Finally, a third promoter, P_2, directs expression of the two distal cistrons, *apaG* and *apaH* (Blanchin-Roland *et al.*, 1986).

High intracellular concentrations of Ap_4A hydrolase have been obtained as a result of transformation of *E. coli* with a high copy-number plasmid harbouring the *apaH* gene. A 30-fold overproduction of the hydrolase in exponentially growing cells is accompanied by a 10-fold decrease in the concentration of Ap_4N (Mechulam *et al.*, 1985; Plateau *et al.*, 1987a). Strains of *E. coli* unable to express *apaH* were obtained by inserting a kanamycin-resistance cassette in the *apaH* chromosomal gene (Farr *et al.*, 1989; Lévêque *et al.*, 1990a). In crude extracts of such strains, Ap_4A-degrading activity is decreased to less than 1% of the value in the parental *apaH*$^+$ strain. Concomitantly, cellular Ap_4N concentration is increased 150-fold, reaching a value of 500 μM (Lévêque *et al.*, 1990a). Concentrations of the other Np_4N' species are also enhanced through inactivation of the *apaH* gene (Lévêque *et al.*, 1990a).

All of these results demonstrate that Ap_4A hydrolase is the main enzyme responsible for catabolism of dinucleoside tetraphosphates in *E. coli*. Disruption of the *apaH* gene does not affect Ap_3A concentration (Lévêque *et al.*, 1990a), in agreement with the occurrence of a specific Ap_3A hydrolase in *E. coli* (Hurtado *et al.*, 1987).

B. CLONING AND CHARACTERIZATION OF *APA1* AND *APA2* GENES ENCODING Ap_4A PHOSPHORYLASES IN *SACCHAROMYCES CEREVISIAE*

The *APA1* and *APA2* genes encoding two Ap_4A phosphorylases in *Sacch. cerevisiae* have been cloned and sequenced (Plateau *et al.*, 1989, 1990; Kaushal *et al.*, 1990). They encode proteins of M_r 36.5 and 36.8 kDa, respectively. The amino acid-residue sequences deduced from DNA sequences are 60% identical, suggesting a common origin for the two genes

(Plateau *et al.*, 1990). The *APA1* gene is located around kbp 38 on chromosome III (Garrison *et al.*, 1992; Oliver *et al.*, 1992), while *APA2* maps to chromosome IV, just upstream of the *QCR7* gene encoding subunit VII of ubiquinol:cytochrome *c* oxidase (De Haan *et al.*, 1984; Schoppink *et al.*, 1989). Interestingly, the *QCR7* gene of *Kluyveromyces lactis* is preceded by a gene sharing 51% identity with the *APA2* gene in *Sacch. cerevisiae* (J. H. de Winde, W. Mulder and L. Grivell, personal communication).

Strains of *Sacch. cerevisiae* that do not express either *APA1* or *APA2*, or both genes, have been constructed by interrupting the corresponding gene(s) with a selectable marker gene (Plateau *et al.*, 1989, 1990). No differences in growth rates of *APA1 APA2*, *apa1 APA2*, *APA1 apa2* and *apa1 apa2* strains were observed, showing that these genes are not essential for cell viability. In the *apa1 apa2* double mutant, the Ap_4N concentration is 50-fold higher than in the parental *APA1 APA2* strain (Plateau *et al.*, 1990). Therefore, it is likely that Ap_4A phosphorylases behave *in vivo* as Ap_4A-degrading enzymes rather than as synthesizing enzymes. In addition, catabolism of dinucleoside tetraphosphates seems to be mainly achieved by these enzymes in *Sacch. cerevisiae*. However, the biological role of Ap_4A phosphorylases might be more complex since, unexpectedly, overproduction of the *APA1* gene from a multicopy plasmid is not accompanied by a decrease in intracellular Ap_4A concentration (Avila *et al.*, 1991). A 10- or 90-fold increase in Ap_4A phosphorylase I activity causes a 2- or 15-fold increase in the concentration of the Ap_4A metabolite, respectively. Concentrations of Ap_4G and Gp_4G also increase in response to Ap_4A phosphorylase overproduction (Avila *et al.*, 1991). This accumulation of dinucleoside tetraphosphates may be explained if we assume that the concentrations of precursor nucleotides, inorganic phosphate or protons vary inside the yeast cell. In this context, it is conceivable that a fraction of cellular Ap_4A phosphorylase I molecules are sequestered in a micro-environment or in a cell compartment in which it works in the anabolic direction. Accordingly, an increase in Ap_4A phosphorylase concentration in such a cell locus might result in an overall increase in total dinucleoside tetraphosphate concentration, masking possible decreases in the dinucleotides in other parts of the cell.

VI. Possible Physiological Roles of Ap_4N

A. ABSENCE OF A CAUSAL RELATIONSHIP BETWEEN Ap_4N ACCUMULATION AND THE HEAT-SHOCK PROTEIN RESPONSE INDUCTION

Hyperthermia as well as oxidative stress give rise to an accumulation of Ap_4N and of heat-shock proteins. However, it now seems clear that there

is no causal relationship between the two responses. Thus, *E. coli rpoH* mutants that fail to express heat-shock proteins at high temperature accumulate Ap_4N during a heat shock in amounts comparable to those measured in the *rpoH*$^+$ strain (Lee *et al.*, 1983b). Moreover, Ap_4N accumulation can be considerably lowered in *E. coli* strains overproducing Ap_4A hydrolase. However, the pattern of induction of heat-shock proteins remains normal (Plateau *et al.*, 1987a). It was also shown that the time-course of Ap_4N accumulation under various stress conditions lagged behind induction of heat-shock proteins (vanBogelen *et al.*, 1987). Finally, concentrations of heat-shock proteins are not modified during exponential growth of *E. coli* Δ*apaH* mutants, although the Ap_4N concentration in such mutants is about 150-fold higher than in the corresponding wild-type strain (Farr *et al.*, 1989; Lévêque *et al.*, 1990a). All of these results serve to demonstrate that a high Ap_4N concentration is neither sufficient nor necessary to trigger expression of heat-shock proteins. However, a concerted action of both Ap_4N and heat-shock proteins in cell homeostasis may still be envisaged.

B. CHARACTERIZATION OF PHENOTYPES ASSOCIATED WITH AN ARTIFICIALLY OBTAINED HIGH INTRACELLULAR Ap_4N CONCENTRATION

A phenotype specifically related to occurrence of a constitutively high intracellular Ap_4N concentration was revealed by studying *E. coli apaH* null mutants. As already stated, the *apaH* gene belongs to the same operon as *ksgA*, a gene encoding a specific 16S rRNA methyltransferase. Mutants in *ksgA* are resistant to the aminoglycosidic antibiotic kasugamycin (Helser *et al.*, 1971). More precisely, *ksgA* mutants can grow in the presence of 600 μg kasugamycin ml^{-1}, whereas the growth of the wild-type strain is inhibited by 150 μg of the antibiotic ml^{-1} (Lévêque *et al.*, 1990a). Surprisingly, *apaH ksgA* double mutants do not grow in the presence of 600 μg kasugamycin ml^{-1} (Lévêque *et al.*, 1990a). Growth inhibition is accompanied by filamentation of bacteria and an increase in the Ap_4N concentration (up to 4 mM). This phenotype is related to the absence of Ap_4A hydrolase since sensitivity to kasugamycin is abolished by complementation of the double mutant with a phage or a plasmid carrying the intact *apaH* gene (Lévêque *et al.*, 1990a).

This phenotype is possibly explained by an effect of the high level of Ap_4A on the DnaK protein. Indeed, overproduction of the protein DnaK from a multicopy plasmid also abolishes sensitivity of *apaH ksgA* double mutants to kasugamycin (Lévêque *et al.*, 1990a). The protein DnaK is a major heat-shock protein of *E. coli* (Tilly *et al.*, 1983). It belongs to the Hsp70 family, a class of proteins highly conserved during evolution

(Bardwell and Craig, 1984; Pelham, 1986). The Hsp70 family are chaperone proteins thought to be involved in an ATP-dependent refolding of proteins (Georgopoulos, 1992). *Escherichia coli dnaK* null mutants have severe defects in cell division and grow only slowly and as filaments (Paek and Walker, 1987; Bukau and Walker, 1989). Because it has been reported that the ATPase activity of DnaK is completely inhibited *in vitro* by 170 μM Ap_4A (Bochner *et al.*, 1986), it is conceivable that the kasugamycin-induced increase in Ap_4A concentration (greater than 850 μM) in *apaH ksgA* mutants inhibits DnaK *in vivo* and, consequently, contributes to the filamentation of cells.

Several other results suggest a modulation of DnaK activity by Ap_4N. By using labelled 8-azido Ap_4A in photocrosslinking experiments, Johnstone and Farr (1991) showed that, in crude extracts, Ap_4A binds to several *E. coli* proteins including heat-shock and oxidative-stress proteins DnaK, GroEL, E89, C45 and C40. Moreover, the high concentration of Ap_4N in *apaH* mutants causes delayed λ phage morphogenesis in a λcI^{ts} lysogen (Farr and Kogoma, 1991; Kitzler *et al.*, 1992), a process that requires both DnaK and GroEL (Zylicz *et al.*, 1989).

In this context, it is tempting to establish a link between possible inhibition of DnaK by Ap_4A *in vivo* and a role for Ap_4A in the cellular response to stress. During the early phase of the response, the activity of DnaK would become blocked by simultaneous accumulation of Ap_4N. These nucleotides would be useful to delay expression of the activity of DnaK until the last phase of the stress response, i.e. when refolding of proteins that have been denatured by the stress becomes useful. Consistent with such ideas, a prolonged synthesis of DnaK was observed in *apaH* null mutants upon return of heat-shocked cells to 30°C (Farr *et al.*, 1989).

Other phenotypes associated with disruption of the *apaH* gene were described by Farr *et al.* (1989). These authors observed that *apaH* null mutants become non-motile, because they could no longer express genes required for motility and chemotaxis. These genes are regulated by the transcription factor σ^F, which is itself regulated by the cAMP-binding protein (CAP) (Bartlett *et al.*, 1988; Farr *et al.*, 1989). Expression of all genes regulated by the cAMP-CAP complex would be lowered in *apaH* mutants (Farr *et al.*, 1989). Thus, expression of both β-galactosidase and galactokinase would be decreased by 84%. These observations provide interesting prospects on possible cellular roles for Ap_4N. However, they need to be defined further.

C. OTHER SPECULATIVE PHYSIOLOGICAL ROLES OF Ap_4N

Several observations on higher eukaryotes indicate that Ap_4A might play a role in DNA repair. Firstly Ap_4A inhibits ADP-ribosylation of histone

H1 (Tanaka *et al.*, 1981; Suzuki *et al.*, 1987). This inhibition occurs because Ap_4A itself behaves as a substrate of poly(ADP-ribose) polymerase (Yoshihara and Tanaka, 1981). Secondly, ADP-ribosylated Ap_4A inhibits *in vitro* replication of SV40 (Baker *et al.*, 1987), while both poly(ADP-ribose) and Ap_4A accumulate in cultured mammalian cells submitted to stresses resulting in breaks in DNA strands (Baker and Ames, 1988a,b; Gilson *et al.*, 1988). Therefore, it was proposed that Ap_4A inhibits DNA replication following DNA damage and, consequently, favours DNA repair (Gilson *et al.*, 1988; Orfanoudakis *et al.*, 1990; Remy, 1992). In the context of such a working hypothesis, stimulation by Ap_4A of proteolytic processing of ADP-ribosylated poly(ADP-ribose) polymerase (Surowy and Berger, 1983, 1985) might be biologically significant by helping a return to the normal cellular state. Nevertheless, the latter observations have not yet been extended to eukaryotic micro-organisms.

Finally, Ap_4N were sometimes thought to play a role in control of cell proliferation (reviewed by Grummt, 1988). However, the observations which gave birth to this hypothesis never received support from further studies. In particular, the early observation of variations in Ap_4A concentration during the cell-cycle of mammalian cells (Weinmann-Dorsch *et al.*, 1984a) were not confirmed (Segal and Le Pecq, 1986; Baker and Jacobson, 1986; Orfanoudakis *et al.*, 1987; Moris *et al.*, 1987; Perret *et al.*, 1990) and binding of Ap_4A to replicative DNA polymerase (Grummt *et al.*, 1979; Rapaport *et al.*, 1981; Baril *et al.*, 1983; Vishwanatha *et al.*, 1986; Vishwanatha and Wei, 1992) appears to depend on the enzyme preparation used (McLennan and Zamecnik, 1992).

In *E. coli* and *P. polycephalum*, intracellular Ap_4N concentrations have been shown to remain constant during the cell cycle (Garrison *et al.*, 1986; Plateau *et al.*, 1987b). Moreover, growth rates of *E. coli* as well as of *Sacch. cerevisiae* are not sensitive to a genetically created 100- or 50-fold increase in Ap_4N concentrations, respectively (Farr *et al.*, 1989; Lévêque *et al.*, 1990a; Plateau *et al.*, 1990). Consequently, the idea that Ap_4N might have some influence on proliferation rate of a micro-organism is discounted.

VII. Conclusion

In spite of the numerous studies performed during the last decade, the biological role of dinucleoside oligophosphates remains obscure. The artificial elevation of these nucleotides as a consequence of the over-production in *E. coli* of Ap_4A hydrolase did not modify the response of the bacterium at the level of the so-called heat-shock proteins. The

phenotypes associated with Ap_4A could only be observed following disruption of the *apaH* gene and accumulation of huge amounts of the dinucleotides in a bacterium. Such an artificial situation was probably never previously encountered by *E. coli* and it is dangerous to derive any conclusion as to the normal function of Ap_4A from dysfunctions provoked by such abnormally high dinucleotide levels.

In this context, it must still be envisaged that dinucleoside oligophosphates are unavoidable by-products of the action of aminoacyl-tRNA synthetases and that variations in the concentrations of these nucleotides simply reflect the metabolism of the adapting cell. As pointed out by McLennan and Zamecnik (1992), it is even possible that some dinucleoside oligophosphates arise chemically within the cell, through activation of an NTP or NDP nucleotide by some cellular nucleophile, followed by a reaction of the activated nucleotide with another nucleotide. Following this route, dinucleoside oligophosphates would obviously be potential poisons for the cell. As an example, it is interesting that millimolar concentrations of methylene *bis*-phosphonate (PCP) inhibit growth of *D. discoideum* cells, probably because PCP triggers accumulation of $AppCH_2p$ and of undegradable $AppCH_2ppA$ within the cell (Klein *et al.*, 1988). Consequently, elimination of dinucleoside oligophosphates has required specific catabolic pathways. These pathways have evolved in the direction of enzymes able to economize as much as possible the energy of the phosphate–phosphate bonds invested in dinucleotides. With *Sacch. cerevisiae*, the occurrence of phosphorylases renders possible recovery of one ADP and one ATP molecule from one Ap_4A molecule.

Assuming that production of dinucleoside oligophosphates could not be avoided in a living cell, it may be speculated that these nucleotides appeared early during evolution and were later selected to play some physiological functions. The important and ubiquitous variations of dinucleoside oligophosphate concentrations in response to several stresses underline the potential for these nucleotides as regulatory molecules. In order to elucidate possible roles of dinucleoside oligophosphates, future studies will benefit from the recent synthesis of numerous analogues of these unusual nucleotides (Blackburn *et al.*, 1992). These compounds, mimicking Np_nN', will be potentially useful to identify the cellular targets of dinucleoside oligophosphates, in particular because some of these analogues are resistant to degradation by cellular enzymes. When available, new working hypotheses on the possible roles of dinucleoside oligophosphates will be easily probed with the help of the *E. coli* and *Sacch. cerevisiae* strains in which Ap_4N concentration can be varied at will.

REFERENCES

Adams, C. A. and Nicholas, D. J. D. (1972). *Biochemical Journal* **128**, 647.

Andersson, M. (1989). *International Journal of Biochemistry* **21**, 707.

Avila, D. M., Robinson, A. K., Kaushal, V. and Barnes, L. D. (1991). *Journal of Bacteriology* **173**, 7875.

Bachmann, B. J. (1990). *Microbiological Reviews* **54**, 130.

Baker, J. C. and Ames, B. N. (1988a). *Mutation Research* **208**, 87.

Baker, J. C. and Ames, B. N. (1988b). *Journal of Inherited Metabolic Disease* **11**, 221.

Baker, J. C. and Jacobson, M. K. (1986). *Proceedings of the National Academy of Sciences of the United States of America* **83**, 2350.

Baker, J. C., Smale, S. T., Tijan, R. and Ames, B. N. (1987). *Journal of Biological Chemistry* **262**, 14 855.

Balodimos, I. A., Kashket, E. R. and Rapaport, E. (1988). *Journal of Bacteriology* **170**, 2301.

Baltzinger, M., Ebel, J. P. and Remy, P. (1986). *Biochimie* **68**, 1231.

Bambara, R. A., Crute, J. J. and Wahl, A. F. (1985). *Cancer Investigation* **3**, 473.

Bardwell, J. C. A. and Craig, E. A. (1984). *Proceedings of the National Academy of Sciences of the United States of America* **81**, 848.

Baril, E., Bonin, P., Burstein, D., Mara, K. and Zamecnik, P. (1983). *Proceedings of the National Academy of Sciences of the United States of America* **80**, 4931.

Baril, E. F., Coughlin, S. A. and Zamecnik, P. C. (1985). *Cancer Investigation* **3**, 465.

Barnes, L. D. and Culver, C. A. (1982). *Biochemistry* **21**, 6123.

Bartkiewicz, M., Sierakowska, H. and Shugar, D. (1984). *European Journal of Biochemistry* **143**, 419.

Bartlett, D. H., Frantz, B. B. and Matsumura, P. (1988). *Journal of Bacteriology* **170**, 1575.

Bernet, D., Pinto, R. M., Sillero, A. and Cameselle, J. C. (1991). *FEBS Letters* **283**, 286.

Biryukov, A. I., Zhukov, Y. N., Lavrik, O. I. and Khomutov, R. M. (1990). *FEBS Letters* **273**, 208.

Biryukov, A. I., Ankilova, V. N. and Lavrik, O. I. (1991). *Nucleic Acids Research Symposium Series* **24**, 19.

Blackburn, G. M., Ashton, P. R., Guo, M. J., Rogers, M., Taylor, G. E., Guranowski, A. and Watts, D. (1991). *Heteroatom Chemistry* **2**, 163.

Blackburn, G. M., Guo, M. J. and McLennan, A. G. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 305–342. CRC Press, Boca Raton.

Blanchin-Roland, S., Blanquet, S., Schmitter, J. M. and Fayat, G. (1986). *Molecular and General Genetics* **205**, 515.

Blanquet, S., Plateau, P. and Brevet, A. (1983). *Molecular and Cellular Biochemistry* **52**, 3.

Bochner, B. R., Lee, P. C., Wilson, S. W., Cutler, C. W. and Ames, B. N. (1984). *Cell* **37**, 225.

Bochner, B. R., Zylicz, M. and Georgopoulos, C. (1986). *Journal of Bacteriology* **168**, 931.

Brevet, A., Coste, H., Fromant, M., Plateau, P. and Blanquet, S. (1987). *Biochemistry* **26**, 4763.

Brevet, A., Chen, J., Lévêque, F., Plateau, P. and Blanquet, S. (1989). *Proceedings of the National Academy of Sciences of the United States of America* **86**, 8275.

Brevet, A., Chen, J., Fromant, M., Blanquet, S. and Plateau, P. (1991). *Journal of Bacteriology* **173**, 5275.

Bukau, B. and Walker, G. C. (1989). *Journal of Bacteriology* **171**, 2337.

Burnell, J. N. and Anderson, J. W. (1973). *Biochemical Journal* **133**, 417.

Burns, D. M. and Beacham, I. R. (1986). *Nucleic Acids Research* **14**, 4325.

Cameselle, J. C., Costas, M. J., Gunther Sillero, M. A. and Sillero, A. (1982). *Biochemical Journal* **201**, 405.

Cameselle, J. C., Costas, M. J., Sillero, M. A. G. and Sillero, A. (1984). *Journal of Biological Chemistry* **259**, 2879.

Costas, M. J., Montero, J. M., Cameselle, J. C., Günther Sillero, M. A. and Sillero, A. (1984). *International Journal of Biochemistry* **16**, 757.

Costas, M. J., Cameselle, J. C. and Sillero, A. (1986). *Journal of Biological Chemistry* **261**, 2064.
Coste, H., Brevet, A., Plateau, P. and Blanquet, S. (1987). *Journal of Biological Chemistry* **262**, 12 096.
De Haan, M., van Loon, A. P. G. M., Kreike, J., Vaessen, R. T. M. J. and Grivell, L. A. (1984). *European Journal of Biochemistry* **138**, 169.
Denisenko, O. N. (1984). *FEBS Letters* **178**, 149.
Eriani, G., Delarue, M., Poch, O., Gangloff, J. and Moras, D. (1990). *Nature* **347**, 203.
Farr, S. B. and Kogoma, T. (1991). *Microbiological Reviews* **55**, 561.
Farr, S. B., Arnosti, D. N., Chamberlin, M. J. and Ames, B. N. (1989). *Proceedings of the National Academy of Sciences of the United States of America* **86**, 5010.
Federn, H. and Ristow, H. (1986). *Chromatographia* **22**, 287.
Finamore, F. J. and Warner, A. H. (1963). *Journal of Biological Chemistry* **238**, 344.
Garrison, P. N. and Barnes, L. D. (1984). *Biochemical Journal* **217**, 805.
Garrison, P. N. and Barnes, L. D. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 29–61. CRC Press, Boca Raton.
Garrison, P. N., Roberson, G. M., Culver, C. A. and Barnes, L. D. (1982). *Biochemistry* **21**, 6129.
Garrison, P. N., Mathis, S. A. and Barnes, L. D. (1986). *Molecular and Cellular Biology* **6**, 1179.
Garrison, P. N., Mathis, S. A. and Barnes, L. D. (1989). *Journal of Bacteriology* **171**, 1506.
Garrison, P. N., Koob, M. and Barnes, L. D. (1992). *Gene* **111**, 131.
Georgopoulos, C. (1992). *Trends in Biochemical Sciences* **17**, 295.
Gilson, G., Ebel, J. P. and Remy, P. (1988). *Experimental Cell Research* **177**, 143.
Goerlich, O. and Holler, E. (1984). *Biochemistry* **23**, 182.
Goerlich, O., Foeckler, R. and Holler, E. (1982). *European Journal of Biochemistry* **126**, 135.
Grummt, F. (1983). *Plant Molecular Biology* **2**, 41.
Grummt, F. (1988). *Modern Cell Biology* **6**, 29.
Grummt, F., Waltl, G., Jantzen, H. M., Hamprecht, K., Huebscher, U. and Kuenzle, C. C. (1979). *Proceedings of the National Academy of Sciences of the United States of America* **76**, 6081.
Grunberg-Manago, M., Del Campillo-Campbell, A., Dondon, L. and Michelson, A. M. (1966). *Biochimica et Biophysica Acta* **123**, 1.
Guédon, G., Ebel, J. P. and Remy, P. (1987). *Biochimie* **69**, 1175.
Günther Sillero, M. A. and Cameselle, J. C. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 205–228. CRC Press, Boca Raton.
Guranowski, A. and Blanquet, S. (1985). *Journal of Biological Chemistry* **260**, 3542.
Guranowski, A. and Blanquet, S. (1986a). *Journal of Biological Chemistry* **261**, 5943.
Guranowski, A. and Blanquet, S. (1986b). *Biochimie* **68**, 757.
Guranowski, A. and Sillero, A. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 81–133. CRC Press, Boca Raton.
Guranowski, A., Jakubowski, H. and Holler, E. (1983). *Journal of Biological Chemistry* **258**, 14 784.
Guranowski, A., Biryukov, A. I., Tarussova, N. B., Khomutov, R. M. and Jakubowski, H. (1987). *Biochemistry* **26**, 3425.
Guranowski, A., Just, G., Holler, E. and Jakubowski, H. (1988a). *Biochemistry* **27**, 2959.
Guranowski, A., Starzynska, E. and Wasternack, C. (1988b). *International Journal of Biochemistry* **20**, 449.
Guranowski, A., Sillero, M. A. G. and Sillero, A. (1990). *FEBS Letters* **271**, 215.
Harnett, S. P., Lowe, G. and Tansley, G. (1985). *Biochemistry* **24**, 2908.
Helser, T. L., Davies, J. E. and Dahlberg, J. E. (1971). *Nature (New Biology)* **233**, 12.
Hirel, P. H., Lévêque, F., Mellot, P., Dardel, F., Panvert, M., Mechulam, Y. and Fayat, G. (1988). *Biochimie* **70**, 773.
Hirshfield, I. N., Bloch, P. L., Van Bogelen, R. A. and Neidhardt, F. C. (1981). *Journal of Bacteriology* **146**, 345.

Holler, E. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 9–28. CRC Press, Boca Raton.
Höhn, M., Albert, W. and Grummt, F. (1982). *Journal of Biological Chemistry* **257**, 3003.
Holmes, R. P. and Stewart, P. R. (1979). *Biochimica et Biophysica Acta* **545**, 94.
Hurtado, C., Ruíz, A., Sillero, A. and Günther Sillero, M. A. (1987). *Journal of Bacteriology* **169**, 1718.
Hutkins, R. W. and Kashket, E. R. (1986). *Applied and Environmental Biology* **51**, 1121.
Jakubowski, H. (1983). *Acta Biochimica Polonica* **30**, 51.
Jakubowski, H. and Guranowski, A. (1983). *Journal of Biological Chemistry* **258**, 9982.
Johnstone, D. B. and Farr, S. B. (1991). *EMBO Journal* **10**, 3897.
Kaushal, V., Avila, D. M., Hardies, S. C. and Barnes, L. D. (1990). *Gene* **95**, 79.
Kitabatake, S., Dombou, M., Tomioka, I. and Nakajima, H. (1987). *Biochemical and Biophysical Research Communications* **146**, 173.
Kitzler, J. W., Farr, S. B. and Ames, B. N. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 135–149. CRC Press, Boca Raton.
Klein, G., Martin, J. B. and Satre, M. (1988). *Biochemistry* **27**, 1897.
Kramer, G. F. and Ames, B. N. (1987). *Journal of Bacteriology* **169**, 2259.
Kramer, G. F., Baker, J. C. and Ames, B. N. (1988). *Journal of Bacteriology* **170**, 2344.
Lazewska, D. and Guranowski, A. (1990). *Nucleic Acids Research* **18**, 6083.
Led, J. J., Switon, W. K. and Jensen, K. F. (1983). *European Journal of Biochemistry* **136**, 469.
Lee, P. C., Bochner, B. R. and Ames, B. N. (1983a). *Journal of Biological Chemistry* **258**, 6827.
Lee, P. C., Bochner, B. R. and Ames, B. N. (1983b). *Proceedings of the National Academy of Sciences of the United States of America* **80**, 7496.
Lévêque, F., Blanchin-Roland, S., Fayat, G., Plateau, P. and Blanquet, S. (1990a). *Journal of Molecular Biology* **212**, 319.
Lévêque, F., Plateau, P., Dessen, P. and Blanquet, S. (1990b). *Nucleic Acids Research* **18**, 305.
Lévêque, F., Gazeau, M., Fromant, M., Blanquet, S. and Plateau, P. (1991). *Journal of Bacteriology* **173**, 7903.
Lowe, G. (1991). *Nucleosides and Nucleotides* **10**, 181.
Lüthje, J. and Ogilvie, A. (1987). *European Journal of Biochemistry* **169**, 385.
Mayaux, J. F. and Blanquet, S. (1981). *Biochemistry* **20**, 4647.
McLennan, A. G. and Zamecnik, P. C. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 1–7. CRC Press, Boca Raton.
McLennan, A. G., Taylor, G. E., Prescott, M. and Blackburn, G. M. (1989). *Biochemistry* **28**, 3868.
Mechulam, Y., Fromant, M., Mellot, P., Plateau, P., Blanchin-Roland, S., Fayat, G. and Blanquet, S. (1985). *Journal of Bacteriology* **164**, 63.
Moris, G., Meyer, D., Orfanoudakis, G., Befort, N., Ebel, J. P. and Remy, P. (1987). *Biochimie* **69**, 1217.
Mukai, J. I., Ikuta, R., Shoji, T. and Hirashima, A. (1989). *Nucleic Acids Research Symposium Series* **21**, 37.
Mukai, J. I., Hirashima, A. and Mikuniya, T. (1991). *Agricultural and Biological Chemistry* **55**, 117.
Nakajima, H., Tomioka, I., Kitabatake, S., Dombou, M. and Tomita, K. (1989). *Agricultural and Biological Chemistry* **53**, 615.
Neidhardt, F. C. and VanBogelen, R. A. (1981). *Biochemical and Biophysical Research Communications* **100**, 894.
Nicholls, R. G. (1977). *Biochemical Journal* **165**, 149.
Ogilvie, A. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 229–273. CRC Press, Boca Raton.
Ogilvie, A. and Antl, W. (1983). *Journal of Biological Chemistry* **258**, 4105.
Ogilvie, A. and Jakob, P. (1983). *Analytical Biochemistry* **134**, 382.
Ogilvie, A., Lüthje, J., Pohl, U. and Busse, R. (1989). *Biochemical Journal* **259**, 97.

Oliver, S. G., van der Aart, Q. J. M., Agostoni-Carbone, M. L., Aigle, M., Alberghina, L., Alexandraki, D., Antoine, G., Anwar, R., Ballesta, J. P. G., Benit, P., Berben, G., Bergantino, E., Biteau, N., Bolle, P. A., Bolotin-Fukuhara, M., Brown, A., Brown, A. J. P., Buhler, J. M., Carcano, C., Carignani, G. *et al.* (1992). *Nature* **357**, 38.

Orfanoudakis, G., Baltzinger, M., Meyer, D., Befort, N., Ebel, J. P., Befort, J. J. and Remy, P. (1987). *Molecular and Cellular Biology* **7**, 2444.

Orfanoudakis, G., Gilson, G., Wolff, C. M., Ebel, J. P., Befort, N. and Remy, P. (1990). *Biochimie* **72**, 271.

Paek, K. H. and Walker, G. C. (1987). *Journal of Bacteriology* **169**, 283.

Pálfi, Z., Surányi, G. and Borbély, G. (1991). *Biochemical Journal* **276**, 487.

Pelham, H. R. B. (1986). *Cell* **46**, 959.

Perret, J., Hepburn, A., Cochaux, P., Van Sande, J. and Dumont, J. E. (1990). *Cellular Signalling* **2**, 57.

Pinto, R. M., Costas, M. J., Fernández, A., Canales, J., García-Agundez, J. A. and Cameselle, J. C. (1991). *FEBS Letters* **287**, 85.

Plateau, P. and Blanquet, S. (1982). *Biochemistry* **21**, 5273.

Plateau, P. and Blanquet, S. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 63–79. CRC Press, Boca Raton.

Plateau, P., Guéron, M. and Blanquet, S. (1981a). *Biochimie* **63**, 827.

Plateau, P., Mayaux, J. F. and Blanquet, S. (1981b). *Biochemistry* **20**, 4654.

Plateau, P., Fromant, M., Brevet, A., Gesquière, A. and Blanquet, S. (1985). *Biochemistry* **24**, 914.

Plateau, P., Fromant, M. and Blanquet, S. (1987a). *Journal of Bacteriology* **169**, 3817.

Plateau, P., Fromant, M., Kepes, F. and Blanquet, S. (1987b). *Journal of Bacteriology* **169**, 419.

Plateau, P., Brevet, A. and Blanquet, S. (1987c). *In* "Handbook of Nucleic Acids and Related Compounds" (A. M. Krstulovic, ed.), pp. 183–192. CRC Press, Boca Raton.

Plateau, P., Fromant, M., Schmitter, J. M., Buhler, J. M. and Blanquet, S. (1989). *Journal of Bacteriology* **171**, 6437.

Plateau, P., Fromant, M., Schmitter, J. M. and Blanquet, S. (1990). *Journal of Bacteriology* **172**, 6892.

Plesner, P., Stephenson, M. L., Zamecnik, P. C. and Bucher, N. L. R. (1979). *In* "Specific Eukaryotic Genes" (J. Engberg, V. Leick, H. Klenow and J. H. Thaysen, eds), pp. 383–394. Alfred Benzon Symposium 13, Munksgaard, Copenhagen.

Prescott, M., Milne, A. D. and McLennan, A. G. (1989). *Biochemical Journal* **259**, 831.

Prescott, M., Thorne, N. M. H., Milne, A. D. and McLennan, A. G. (1992). *International Journal of Biochemistry* **24**, 565.

Randerath, K., Janeway, C. M., Stephenson, M. L. and Zamecnik, P. C. (1966). *Biochemical and Biophysical Research Communications* **24**, 98.

Rapaport, E., and Zamecnik, P. C. (1976). *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3984.

Rapaport, E., Svihovec, S. K. and Zamecnik, P. C. (1975). *Proceedings of the National Academy of Sciences of the United States of America* **72**, 2653.

Rapaport, E. Zamecnik, P. C. and Baril, E. F. (1981). *Proceedings of the National Academy of Sciences of the United States of America* **78**, 838.

Rauhut, R., Gabius, H. J., Engelhardt, R. and Cramer, F. (1985). *Journal of Biological Chemistry* **260**, 182.

Remy, P. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 151–204. CRC Press, Boca Raton.

Roa, B. B., Connoly, D. M. and Winkler, M. E. (1989). *Journal of Bacteriology* **171**, 4767.

Robbins, P. W. and Lipmann, F. (1958). *Journal of Biological Chemistry* **233**, 681.

Robinson, A. K. and Barnes, L. D. (1986). *Archives of Biochemistry and Biophysics* **248**, 502.

Rodriguez-Pascual, F., Torres, M., Rotllán, P. and Miras-Portugal, M. T. (1992). *Archives of Biochemistry and Biophysics* **297**, 176.

Ruiz, A., Hurtado, C., Meireles Ribeiro, J., Sillero, A. and Günther Sillero, M. A. (1989). *Journal of Bacteriology* **171**, 6703.

Schoppink, P. J., Berden, J. A. and Grivell, L. A. (1989). *European Journal of Biochemistry* **181**, 475.
Segal, E. and Le Pecq, J. B. (1986). *Experimental Cell Research* **167**, 119.
Sillero, M. A. G., Villalba, R., Moreno, A., Quintanilla, M., Lobatón, C. D. and Sillero, A. (1977). *European Journal of Biochemistry* **76**, 331.
Sillero, M. A. G., Guranowski, A. and Sillero, A. (1991). *European Journal of Biochemistry* **202**, 507.
Silverman, R. H. and Atherly, A. G. (1979). *Microbiological Reviews* **43**, 27.
Surowy, C. S. and Berger, N. A. (1983). *Journal of Biological Chemistry* **258**, 579.
Surowy, C. S. and Berger, N. A. (1985). *Biochimica et Biophysica Acta* **832**, 33.
Suzuki, H., Tanaka, Y., Buonamassa, D. T., Farina, B. and Leone, E. (1987). *Molecular and Cellular Biochemistry* **74**, 17.
Tanaka, Y., Matsunami, N. and Yoshihara, K. (1981). *Biochemical and Biophysical Research Communications* **99**, 837.
Terracciano, J. S., Schreurs, W. J. A. and Kashket, E. R. (1987). *Applied and Environmental Microbiology* **53**, 782.
Tilly, K., McKittrick, N., Zylicz, M. and Georgopoulos, C. (1983). *Cell* **34**, 641.
Tormo, A., Almirón, M. and Kolter, R. (1990). *Journal of Bacteriology* **172**, 4339.
Traut, T. W. (1987). *Molecular and Cellular Biochemistry* **75**, 15.
Vallejo, C. G. and Leon, P. (1989). *International Journal of Biochemistry* **21**, 1223.
Vallejo, C. G., Infante, J. M., Renart, J. and Sillero, A. (1973). *Biochemical and Biophysical Research Communications* **51**, 113.
vanBogelen, R. A., Kelley, P. M. and Neidhardt, F. C. (1987). *Journal of Bacteriology* **169**, 26.
van Buul, C. P. J. J. and van Knippenberg, P. H. (1985). *Gene* **38**, 65.
Vishwanatha, J. K. and Wei, Z. (1992). *Biochemistry* **31**, 1631.
Vishwanatha, J. K., Coughlin, S. A., Wesolowski-Owen, M. and Baril, E. F. (1986). *Journal of Biological Chemistry* **261**, 6619.
Wang, D. and Shatkin, A. J. (1984). *Nucleic Acids Research* **12**, 2303.
Warner, A. H. (1992). *In* "Ap_4A and Other Dinucleoside Polyphosphates" (A. G. McLennan, ed.), pp. 275–303. CRC Press, Boca Raton.
Warner, A. H. and Finamore, F. J. (1965a). *Biochimica et Biophysica Acta* **108**, 525.
Warner, A. H. and Finamore, F. J. (1965b). *Biochemistry* **4**, 1568.
Warner, A. H. and Huang, F. L. (1974). *Canadian Journal of Biochemistry* **52**, 241.
Warner, A. H., Beers, P. C. and Huang, F. L. (1974). *Canadian Journal of Biochemistry* **52**, 231.
Wasternack, C., Hess, S., Löffler, A. and Guranowski, A. (1989). *International Journal of Biochemistry* **21**, 1089.
Weinmann-Dorsch, C., Hedl, A., Grummt, I., Albert, W., Ferdinand, F. J., Friis, R. R., Pierron, G., Moll, W. and Grummt, F. (1984a). *European Journal of Biochemistry* **138**, 179.
Weinmann-Dorsch, C., Pierron, G., Wick, R., Sauer, H. and Grummt, F. (1984b). *Experimental Cell Research* **155**, 171.
Yoshihara, K. and Tanaka, Y. (1981). *Journal of Biological Chemistry* **256**, 6756.
Zamecnik, P. C. (1969). *Cold Spring Harbor Symposia on Quantitative Biology* **34**, 1.
Zamecnik, P. (1983). *Analytical Biochemistry* **134**, 1.
Zamecnik, P. C. and Stephenson, M. L. (1968). *In* "Regulatory Mechanisms for Protein Synthesis in Mammalian Cells" (A. San Pietro, M. R. Lamborg and F. T. Kenney, eds), pp. 3–16. Academic Press, New York.
Zamecnik, P. C. and Stephenson, M. L. (1969). *In* "The Role of Nucleotides for the Function and Conformation of Enzymes" (H. M. Kalckar, H. Klenow, A. Munch-Petersen, M. Ottesen and J. H. Thaysen, eds), pp. 276–291. Alfred Benzon Symposium I, Munksgaard, Copenhagen.
Zamecnik, P. C., Stephenson, M. L., Janeway, C. M. and Randerath, K. (1966). *Biochemical and Biophysical Research Communications* **24**, 91.

Zamecnik, P. C., Janeway, C. M., Randerath, K. and Stephenson, M. L. (1967). *In* "Regulation of Nucleic Acid and Protein Biosynthesis" (V. V. Koningsberger and L. Bosch, eds), pp. 169–176. Elsevier, Amsterdam.
Zylicz, M., Ang, D., Liberek, K. and Georgopoulos, C. (1989). *EMBO Journal* **8**, 1601.

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Nematophagous Fungi: Physiological Aspects and Structure–Function Relationships

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I. Introduction

Nematophagous fungi are natural enemies of nematodes since they may attack living nematodes or their eggs and utilize them as a source of nutrients. For this purpose, usually special morphological adaptations of the fungal mycelium are necessary. Three different strategies have been

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encountered so far. Firstly, there are predatory (or nematode-trapping) fungi which produce special mycelial nematode-trapping structures that may be either adhesive or non-adhesive, and by which nematodes are efficiently captured: secondly, there exist endoparasitic fungi which use adhesive or non-adhesive spores that adhere to the surface of nematodes or are ingested by them, respectively. Finally, there are fungal parasites of cyst and root-knot nematodes which attack eggs or females of these nematodes by in-growth of vegetative hyphae during which an appressorium-like swelling may be involved. The fungi differ in their dependency on nematodes as hosts. Predatory fungi generally possess a comparatively good saprophytic ability, while endoparasitic fungi are often obligate parasites. Among the egg parasites, both saprophytic and parasitic species have been encountered. The saprophytic–parasitic ability of nematophagous fungi is important in relation to biocontrol of nematodes by fungi.

Because of the role these fungi may play in natural or applied biocontrol of nematodes, and also because of the interesting biology and biochemistry associated with the capturing, penetration and digestion processes, over the years these fungi have attracted much attention, in particular with regard to their ecology, physiology and structure–function relationships. The ecology of nematophagous fungi, especially that of predatory and endoparasitic fungi, and their activities in soil including recognition of prey and survival strategies, were reviewed recently (Nordbring-Hertz, 1988a,b; Gray, 1988; Dackman *et al.*, 1992). In a recent review, Barron (1992) evaluated the possible ecological basis for the nematode-trapping habit and emphasized the role of a proper carbon:nitrogen balance (see Section III). Ecological and biocontrol aspects, especially of the cyst and root-knot nematode parasites, have been covered in several reviews (e.g. Mankau, 1980; Kerry, 1984; Stirling, 1988). Mechanisms governing adhesion of nematodes to the infection structures of fungi and the infection events have also been reveiwed in recent years (Nordbring-Hertz and Chet, 1986; Jansson and Nordbring-Hertz, 1988; Tunlid *et al.*, 1992a,b). For general information on all aspects of the biology of these fungi, the reader is referred to Barron's book (1977).

The purpose of this review is to summarize physiological, biochemical and ultrastructural aspects of both predatory and endoparasitic nematophagous fungi and to discuss current views on the role of the fungi in natural ecosystems. Special emphasis will be given to the contribution that microscopic techniques have made to our understanding of the physiology of these fungi. This refers both to development of infection structures and their subcellular components, properties of the surface coat of these structures and to mechanisms of penetration of the nema-

tode cuticle and subsequent invasion, colonization and digestion of the nematode.

II. Pioneering Work

Although one of the best known nematophagous fungi, *Arthrobotrys oligospora*, was isolated during the mid-nineteenth century, its nematode-trapping ability was first described towards the end of the century (Zopf, 1888). However, only from the 1930s onwards was this phenomenon further studied. Charles Drechsler, the pioneer taxonomist in this field, over a period of almost 50 years isolated and described a large number of nematophagous fungi. His detailed descriptions and excellent drawings, now classical mycological literature, have been a source of information for all later investigators in this field (Drechsler, 1937, 1941, 1950). Since then, interest in different aspects of the biology of these fungi has grown. Duddington took up field and laboratory studies on them, especially from the point of view of biological control, and summarized the increasing knowledge in several excellent reviews (Duddington, 1955, 1962). David Pramer, (working in Rutgers University, NJ, USA) paved the way for later physiological and biochemical work. As a biochemist he asked the question: how are nematodes capable of inducing trap formation in nematode-trapping fungi? He partially purified a substance from nematodes which he called nemin that could induce trap formation in the absence of nematodes (Pramer and Stoll, 1959). Nemin was thought to be a small peptide or perhaps even an amino acid (Pramer and Kuyama, 1963). Pramer stressed the importance of more insight into the nutrition and physiology of these fungi in order better to understand their function in soil and their role as antagonists of nematodes (Pramer, 1964). He also raised many foresighted and critical questions concerning their detection and function in soil or in the rhizosphere (Eren and Pramer, 1966), suggested a role for a collagenase in penetration of nematodes (Schenck *et al.*, 1980) and touched upon the role of heavy metals for growth, morphogenesis and collagenase activity of the fungi (Rosenzweig and Pramer, 1980). These studies invariably were based on comprehensive knowledge of the nutritional and physiological characteristics of the fungi. Ecological studies of them were initiated by Cooke in the early 1960s (Cooke, 1962, 1963a,b, 1964), and these have since been a basis for all subsequent ecological considerations. They later led to a rather pessimistic view of the possibilities of the fungi to be used as regulators of the soil nematode population in biocontrol. The reason for this view was a lack of knowledge of the ecology of the fungi (Cooke, 1968), and this was

addressed again in his book on the biology of symbiotic fungi (Cooke, 1977). Barron (1977) elegantly summarized existing knowledge of the biology of nematode-destroying fungi and succeeded in extending our knowledge of the endoparasitic fungi by continuous detection and description of new species. His book (Barron, 1977) and his later reviews (Barron, 1981, 1982) are valuable information for all who want to learn more about these fungi.

III. Growth Strategies

A. MODE OF NUTRITION

In soil, where starvation conditions occasionally prevail, the ability to attack living nematodes and to exploit them as a source of nutrients may provide nematophagous fungi with a survival advantage. Fungi that possess this ability differ in their dependency on nematodes as hosts. The need for nematodes is most obvious for those nematophagous fungi which are obligate parasites and develop a vegetative mycelium only inside the host. Nematode-trapping or predatory fungi, on the other hand, also grow in a saprophytic mode. For both types of fungi, the living, and moving, nematode is most important. By its movements it is brought into contact with spores of endoparasitic fungi and with traps of the predatory fungi, leading to trapping and invasion of the nematode. In addition, living nematodes also induce trap formation in the latter fungi, thereby increasing the chances of encounter between traps and nematodes.

Both predatory and endoparasitic fungi share the property that they produce sophisticated morphological structures that enable them to attack living nematodes. Some predatory fungi form trapping structures only after induction by external stimuli (e.g. the presence of living nematodes); others develop traps spontaneously. In view of these properties, the latter species are considered to be more dependent on nematodes as a source of nutrients. Gray (1985) observed that spontaneous trap formers were more abundant in soils containing enhanced contents of organic matter compared with non-spontaneous trap formers. These observations led him to suggest that the ability to form traps spontaneously may offer these predators a competitive advantage over non-spontaneous trap formers in soils with a rich microbial flora and fauna.

Barron (1992) considers that the nematode-trapping habit confers an advantage on organisms living in nitrogen-limiting habitats. In such environments with a high carbon:nitrogen ratio, nematodes may serve as an important source of nitrogenous nutrients during growth of fungi on

carbohydrates. This view was originally based on the observation that lignolytic and wood-decaying fungi ultilize their nematode prey as a nitrogen source to compensate for the low levels of nitrogen in wood (Thorn and Barron, 1984; Barron and Thorn, 1987). Barron and his colleagues argue that the same may be true for predatory and endoparasitic nematophagous fungi; similar thoughts were developed by Cooke (1977). It appears, therefore, that saprophytic species which can all grow on cellulose and produce cellulases may utilize nematodes primarily as a nitrogen source while the more specialized endoparasites, lacking cellulase activities, are dependent on nematodes as a source of carbon and nitrogen. Between these two extremes, a continuum of intermediate responses probably exists (Barron, 1992).

B. GROWTH ON LABORATORY MEDIA

To understand the behaviour of various fungi as antagonists to nematodes, methods for their growth on laboratory media have been developed. Generally, nematode-trapping fungi can be easily grown on laboratory media; in contrast, endoparasites generally show poor growth under these conditions (Jaffee and Zehr, 1982; Lohmann and Sikora, 1989). With respect to predatory fungi, early studies on the nutrition of *Arthrobotrys conoides* failed to reveal unique nutritional requirements (Coscarelli and Pramer, 1962). Blackburn and Hayes (1966) and Hayes and Blackburn (1966) evaluated the effect of different sources of carbon and nitrogen on the saprophytic and predaceous phases of *A. oligospora* and *A. robusta*, respectively. These studies essentially confirmed the results already described. However, Hayes and Blackburn (1966) indicated that the predaceous activity is not necessarily correlated with hyphal length since only certain mycelial adaptations are responsible for nematode-destroying activity. Recently, it was shown that *A. oligospora* grows well in mineral media containing oleic acid or D-alanine as the sole source of carbon and energy (Dijksterhuis *et al.*, 1993a). Rapid growth and production of abundant mycelium are two important factors for spread and survival of predatory fungi in natural environments. Another is development of infection structures. As already described, it appears that heavy mycelial development is not necessarily correlated with a high nematode-destroying activity (Dackman *et al.*, 1987; Jansson, 1982a). Indeed, growth rates may vary considerably among different predatory fungal species; generally, non-spontaneous trap formers grow faster than spontaneous trap formers. Isolation of a natural auxotrophic strain of *A. dactyloides* indicates that some trap formers may need special growth factors (i.e. vitamins and/or amino acids) and that differences in this respect

may occur even within species (Zachariah, 1983a; Zachariah and Victor, 1983).

C. NEMATODES AS NUTRIENTS

The role of nematodes in nutrition of fungi is not at all clear. For instance, the growth response following addition of substantial numbers of killed (autoclaved) nematodes as the sole source of nutrients to either two nematode-trapping species or to a non-predaceous soil fungus revealed no significant differences compared with controls (Schenck and Pramer, 1976). All three fungi were able to invade and utilize nematode carcasses. However, dead nematodes appeared to be invaded solely by normal vegetative hyphae through their natural orifices, a process which proceeds much more slowly than trap-mediated invasion of living nematodes (Nordbring-Hertz and Stålhammar-Carlemalm, 1978). Furthermore, killed nematodes placed in the vicinity of isolated germinating adhesive knobs, ring cells and conidia of different nematophagous fungi caused directed growth of hyphae, resulting in formation of a sparse mycelium around the dead body (Zachariah, 1981, 1983b).

However, for exploitation of nematodes as a source of nutrients, a living nematode is probably more useful, particularly if, during penetration of the animal, leakage of the nematode contents into its environment can be prevented. Development of *A. oligospora* on individual living nematodes was studied by Veenhuis *et al.* (1989a). Single nematodes which had been captured by a trap were cut out and placed in a droplet of water immediately after capture. The main result was that the fungus was able to grow in the absence of external nutrients solely at the expense of the captured nematode. Under these conditions, a branched mycelium was formed which originated from cells in the trap. In a recent investigation, Jaffee *et al.* (1992) studied growth of four nematode-trapping fungi on single nematodes in extracts of different soils. Not only two fungi which are known to form traps spontaneously on agar, namely *Monacrosporium ellipsosporum* and *M. cionopagum*, but also *A. oligospora* and *A. dactyloides* lacking this ability, were able to grow apparently at the expense of the nematode and, in addition, to form a mycelium with plenty of traps. Traps were formed, but to a lesser extent, from parasitized nematodes in controls in 2 mM potassium chloride. The authors suggest that, in soil, there may be no need for induction of trap formation in the latter fungi, as in laboratory media. The main difference between this study and the one already referred to (Veenhuis *et al.*, 1989a) is the chemical composition of the liquid in which the interaction was studied. Soil extract probably contains nutrients and/or trap-inducing compounds which are especially

TABLE 1. Taxonomic classification of nematophagous fungi; representative genera and functional classes[a]

Division	Genus	Interaction	Infection structure
Chytridiomycetes	*Catenaria*	Endoparasitic, female parasite	Zoospores
Oomycetes	*Myzocytium*	Endoparasitic	Zoospores
	Nematophthora	Endoparasitic	Zoospores
Zygomycetes	*Stylopage*	Nematode-trapping	Adhesive hyphae
	Cystopage	Nematode-trapping	Adhesive hyphae
Deuteromycetes	*Arthrobotrys*	Nematode-trapping	(Non-)adhesive traps
	Monacrosporium	Nematode-trapping	Adhesive traps
	Dactylaria	Nematode-trapping	(Non-)adhesive traps
	Dactylella	Nematode-trapping, egg parasite	Adhesive traps, hyphal tips
	Nematoctonus	Nematode-trapping, endoparasitic	Adhesive traps, adhesive conidia
	Harposporium	Endoparasitic	Non-adhesive conidia
	Drechmeria	Endoparasitic	Adhesive conidia
	Hirsutella	Endoparasitic	Adhesive conidia
	Verticillium	Endoparasitic, egg parasite, female parasite	Adhesive conidia, hyphal tips
	Paecilomyces	Egg parasite	Hyphal tips
	Cylindrocarpon	Egg parasite, female parasite	Hyphal tips
	Fusarium	Egg parasite, female parasite	Hyphal tips
Basidiomycetes	*Hohenbuhelia* (teleomorph of *Nematoctonus*)	Nematode-trapping, endoparasitic	
	Pleurotus	Nematode-trapping, toxic	Adhesive traps, toxic droplets
Ascomycetes	*Atricordyceps* (teleomorph of *Harposporium*)	Endoparasitic	

[a] Reprinted from Dackman *et al.* (1992) by courtesy of Marcel Dekker Inc.

effective on a young mycelium (Dackman and Nordbring-Hertz, 1992). To evaluate the nature and the amount of fungal biomass formed during the infection process, endoparasites offer an especially attractive system as the number of conidia formed during the interaction may readily be counted. During the interaction between the fungus *Drechmeria coniospora* (Gams and Jansson, 1985) and the nematode *Panagrellus redivivus*, approximately 10 000 conidia were formed at the expense of a single nematode (Dijksterhuis *et al.*, 1991). Obviously, the number of spores produced is dependent on both the fungal and nematode species; Jaffee and Zehr

(1983) observed a yield of maximally 700 conidia for each nematode when *Hirsutella rhossiliensis* parasitized *Criconemella xenoplax*.

IV. Morphological Adaptations

A. INTRODUCTION

Parasites of nematodes are found among all major fungal taxonomic groups. The genera, their functional relationships with nematodes (mode of nutrition) and their morphological adaptations (mode of capturing) are summarized in Table 1 (from Dackman *et al.*, 1992). Most of the infection structures produced by predatory fungi possess an adhesive layer, which mediates a firm attachment to the nematode surface. The structures include hyphal nets (*A. oligospora*; Fig. 1(a)), branches (*Monacrosporium cionopagum*; Fig. 1(b)), knobs (*Dactylaria candida*; Fig. 1(c)) and unmodified hyphae (*Stylopage* sp. and *Cystopage* sp.). Others are mechanical as with non-constricting (*D. candida*) or constricting ring formers (*D. brochopaga* and *A. dactyloides*; Figs 1(d) and (e)). Conidia of endoparasitic fungi also show sophisticated morphological adaptations to enable their interaction with nematodes. Conidia of both *D. coniospora* (Fig. 1(f)) and *Verticillium balanoides* contain a distinct adhesive layer to mediate adhesion to the nematode cuticle, whereas zoospores of *Catenaria anguillulae* show an adhesive stage of development. Conidia in species of the genus *Harposporium*, on the other hand, lodge in the digestive tract of the animal as a result of their special shape. A striking mechanism of penetration has been observed with conidia of *Haptoglossa* spp. which inject a so-called infection particle directly under the cuticle of the host by a process resembling explosive discharge of ascospores (Barron, 1989). Several of these fungi are described in a film which illustrates the different strategies used by fungi and the results of these strategies (Nordbring-Hertz *et al.*, 1993). In spite of differences in morphology among different species with respect to their infection structures, several remarkable similarities exist in the processes of capture, penetration and digestion of the nematodes. This suggests that the fungi possess similar basic solutions that enable them to feed on nematodes and survive in the soil ecosystem.

B. INDUCTION OF TRAP FORMATION

As already indicated, for most predatory fungi, development of devices that enable an interaction with their prey is a prerequisite for feeding on nematodes. The compounds and/or factors responsible for induction of

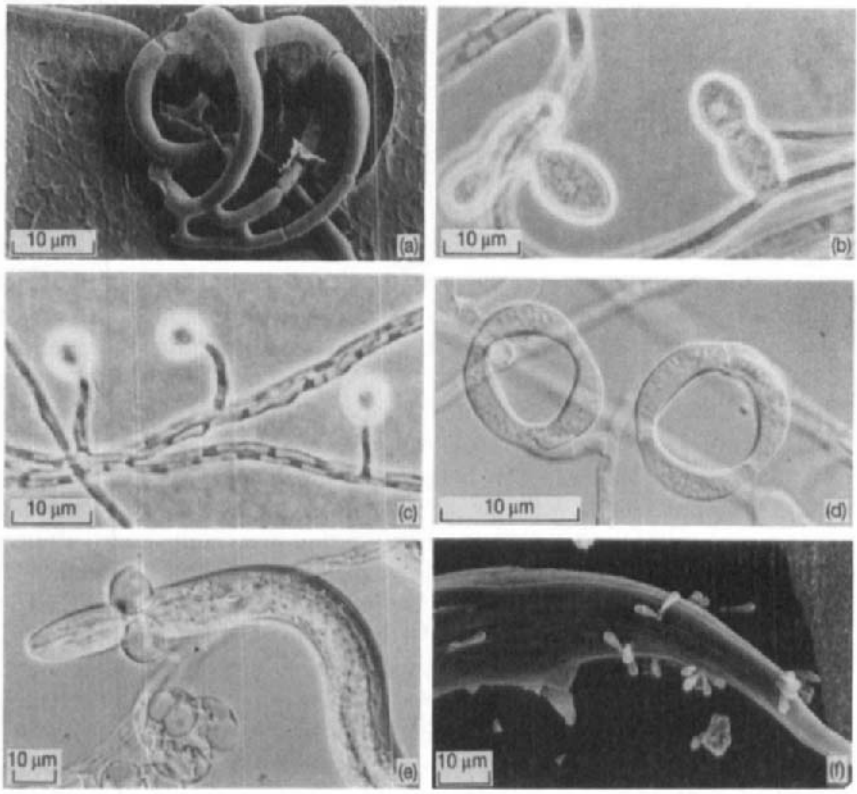

FIG. 1. Trapping devices of different nematophagous fungi. (a) Adhesive network of *Arthrobotrys oligospora* after low-temperature scanning electron microscopy. (b, c) Light micrographs showing adhesive branches of *Monacrosporium cionopagum* (b) and adhesive knobs of *Dactylaria candida* (c). (d, e) Constricting rings of *Dactylaria brochopaga* before (d) and after closure (e). The ring, shown at the lower part of the figure, is inflated though a nematode is not captured. (f) Survey of conidia of *Drechmeria coniospora* adhered to the tail of a male *Panagrellus redivivus* nematode. (b–e) are reproduced by courtesy of Institute of Scientific Films, Göttingen, Germany. Bars = 10 µm. All micrographs are taken of permanganate or aldehyde–permanganate fixed samples, unless otherwise stated. Abbreviations (Figs 1–7): A, adhesive layer; C, cuticle; Co, conidiophore; Cw, cell wall; Er, endoplasmic reticulum; L, lipid droplet; M, microbody; Mt, mitochondrion; N, nucleus; Ne, nematode; S, septum; V, vacuole.

trap formation have been a matter of interest for several decades. In the early 1960s, Pramer and his coworkers isolated "nemin" from culture broths of the nematode *Neoaplectana glaseri* (see Section II p. 113). Subsequently, Wootton and Pramer (1966) grew the fungi on yeast extract and

found that valine, leucine or isoleucine were the components in these cultures which induced trap formation in *A. coniodes*. Nordbring-Hertz (1973) and Nordbring-Hertz and Brinck (1974) isolated and partly characterized several small peptides from enzyme-hydrolysed casein which markedly increased trap formation in *A. oligospora* and which were also present in excudates from the nematode *Panagrellus redivivus* (Nordbring-Hertz, 1977). The peptides possessed a high proportion of non-polar and aromatic amino-acid residues and the potency of the peptides was much more pronounced than the effect of individual component amino acids. A prerequisite for significant trap formation in a Petri-dish assay was that the fungus was grown on a low-nutrient mineral salts medium (Nordbring-Hertz, 1973). This might indicate that nutrient limitation favours initiation of trap formation in this system.

A further improvement to induce trap formation by *A. oligospora* in liquid cultures used modified separatory funnels with vigorous aeration from the bottom, and was also based on a dilute medium (0.01% soya peptone) supplemented with the amino acids valine and phenylalanine (Friman *et al.*, 1985). Trap formation on the thin mycelium started after 2 days, and a high trap-to-hyphae ratio was routinely observed within 5–7 days of cultivation. Inclusion of phosphate (24 μM–12 mM; E. Friman, unpublished results; Tunlid *et al.*, 1991a) in the medium inhibited trap formation completely. Although the dilute medium employed does not allow production of much biomass, this method of cultivation has been a valuable tool in studies of various aspects of fungal–nematode interactions. These include studies on morphology and capture of nematodes after various treatments (Friman *et al.*, 1985; Friman, 1993; Tunlid *et al.*, 1991a), surface polymers (Tunlid *et al.* 1991a), lectins (Borrebaeck *et al.*, 1984; Rosén *et al.*, 1992) and proteases (Tunlid and Jansson, 1991; Persson and Friman, 1993).

That adverse environmental conditions may be an important factor in induction of trap formation is also indicated by development of traps on germinating conidia (so-called conidial traps) following inoculation of cow dung-containing medium (Dackman and Nordbring-Hertz, 1992). Furthermore, formation of traps in the presence of the chitin-synthetase inhibitor polyoxin D (Persson *et al.*, 1990) or after a shift of vegetative mycelium to a liquid medium containing D-alanine as the sole carbon source (Dijksterhuis *et al.*, 1993a) support this view. However, factors other than the presence of peptides or adverse environmental conditions are also involved in the induction process. This is convincingly illustrated by the observation that addition of living nematodes to cultures of *A. oligospora* is by far the most powerful trigger of adhesive-trap formation in this species (Nordbring-Hertz, 1977). This author suggests that volatile

compounds might also play a role in this process (Nordbring-Hertz and Odham, 1980). In the fungi *A. conoides* and *Dactylaria brochopaga*, induction of trapping devices is enhanced in the presence of living nematodes in combination with selected bacterial strains (Rucker and Zachariah, 1987). Rosenzweig and Pramer (1980) showed that, out of seven fungal species belonging to the genera *Arthrobotrys* and *Monacrosporium*, some were blocked in trap formation during growth in media containing different concentrations of heavy metals (e.g. cadmium, zinc and lead). However, with most fungi, the decreased capacity to form traps was correlated with inhibition of growth. It is, however, clear that a shift from a saprophytic to a predaceous growth mode is a subtle process which is dependent on a range of parameters and is, most probably, species- or even strain-dependent. For instance, *A. conoides* forms more traps when grown on a corn meal-agar surface than on media with a low nutrient status (Rosenzweig, 1984). This is in contrast to our laboratory strain of *A. oligospora* in which the opposite response has been observed (Nordbring-Hertz, 1973). Another example is described in the report by Jaffee *et al.* (1992) which clearly showed enhanced trap formation in soil extracts under their experimental conditions.

C. MORPHOLOGY OF TRAPPING DEVICES

As already stated, all devices that mediate fungal–nematode interactions are either specialized hyphal structures or differentiated cells (e.g. conidia). These "special-purpose" structures are equipped to establish a firm contact with the prey and, subsequently, to mediate penetration of the cuticle. In the following paragraphs the various morphological adaptations employed in nematode-capturing are described in more detail.

1. Adhesive Trapping Structures

One general mode of establishing fungal–nematode interaction, which is observed in hyphal traps and conidia of a relatively large group of nematophagous fungi, is the use of adhesive layers located outside the fungal cell wall. Generally, this layer is not preserved by conventional transmission electron microscopy except at the site of contact with the nematode. This is most probably the result of dissolution and/or disruption of this layer during preparation of the samples for microscopical investigation (Veenhuis *et al.*, 1985a). However, after negative staining procedures, applied on intact cells, adhesive layers on traps of *A. oligospora* are visible. Adhesive layers were also evident in scanning electron micrographs from captured nematodes as well as on empty traps (Nordbring-Hertz, 1972;

Wharton and Murray, 1990). Furthermore, remnants of adhesives were detected by scanning and transmission electron microscopy after generation of protoplasts from traps of *A. oligospora* (Nordbring-Hertz *et al.*, 1989a). This indicates that at least part of the layer is formed on the trap cell before adhesion to the nematode which is in contrast to the view that adhesive is produced as a result of contact with the prey. An exception to this is the behaviour of *Acaulopage pectospora* which possesses hyphal branches that quickly eject an adhesive after being rubbed by the prey (Saikawa and Morikawa, 1985). This resembles the mechanism observed with the water mould *Zoophagus insidians* when consuming rotifers (Whisler and Travland, 1974; Saikawa *et al.*, 1988). The adhesive is most probably present in large vesicles which occupy a major part of the cytoplasmic space of the branch (Saikawa and Morikawa, 1985). The structure and composition of the adhesive layer are quite complex and, in *A. oligospora*, the structure is present as an electron-dense fibrillar layer containing residues of neutral sugars and uronic acids as well as proteins (Tunlid *et al.*, 1991a). Interestingly, freeze-fracture techniques applied to trap-containing hyphae of *A. oligospora* revealed the presence of a rodlet pattern on their surface which was not present on normal hyphae (Veenhuis *et al.*, 1985a). Comparable structures have been observed on many aerial

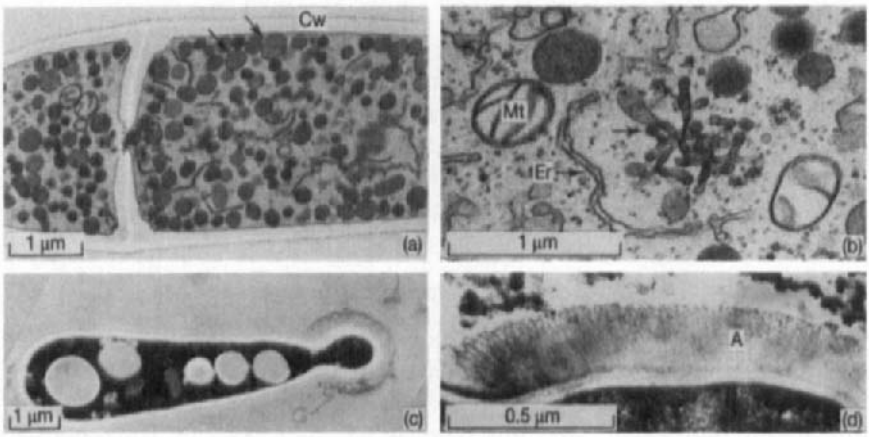

FIG. 2. Ultrastructure of trapping devices of certain nematophagous fungi. (a) Adhesive trap of *Arthrobotrys oligospora* containing numerous dense bodies (arrows). (b) Detail of a young trap cell in *Arthrobotrys oligospora* showing the specialized region of the endoplasmic reticulum involved in dense-body development (arrow). (c) Median section through a conidium of *Drechmeria coniospora* showing the adhesive knob with the adhesive layer. (d) High magnification of the adhesive knob of *Drechmeria coniospora* illustrating the fibrillar nature of the adhesive layer. Abbreviations are explained in Fig. 1.

hyphal structures (e.g. conidia). However, their significance in nematode-capturing is not yet understood. One explanation is that they may add to the rigidity of the adhesive layers. However, the role of the rodlet layer has to be further investigated in connection with other components such as surface polymers (Tunlid *et al.*, 1991a) and proteins (Rosén *et al.*, 1992). For further detailed information on adhesive layers we refer readers to a recent review (Tunlid *et al.*, 1992a).

2. *Dense Bodies*

A common feature, observed in all traps of predatory fungi, is the presence of numerous cytosolic organelles, the so-called dense bodies (Fig. 2a,b). Generally, the organelles are surrounded by a single membrane, with the possible exception of that in *Dactylella cionopaga* (Dowsett *et al.*, 1984), in which bounding membranes were not observed. The organelles may contain a dense core (e.g. *Dactylella lysipaga*; Wimble and Young, 1984); in *A. oligospora* they are probably peroxisomal in nature since they contain catalase and D-amino acid oxidase activity (Veenhuis *et al.*, 1984). Furthermore, many electron-dense inclusions were observed in the constricting rings of *A. dactyloides* and *D. brochopaga* (Heintz and Pramer, 1972; Dowsett *et al.*, 1977) where they are contained in the labyrinthine matrix at the luminal side of the organ. Traps are strongly differentiated fungal cells; they are formed on normal vegetative hyphae which invariably lack dense bodies. Biogenesis of these organelles has been studied in detail in *A. oligospora*. In this organism, dense bodies develop from specialized regions of the endoplasmic reticulum (Fig. 2b); multiplication by fission of existing dense bodies, as is common for normal fungal microbodies (Veenhuis *et al.*, 1984), was not observed. The same mechanism of dense-body development most probably also occurs in the fungus *Dactylella arcuata* (Scheuer and Webster, 1990; J. Dijksterhuis, unpublished observations). In *A. oligospora*, dense bodies are already present upon initiation of trap development, and can be detected in the first cell of the trap to be formed. It is interesting to note that this cell already displays all of the properties of a mature trap in that it is able effectively to capture and penetrate nematodes. Upon further development of the trap, the initial hyphal branch bends and grows in the shape of a loop with a diameter of approximately 20–25 μm before it meets and fuses with a short outgrowth of the vegetative hypha on which it develops (Nordbring-Hertz *et al.*, 1989b). This event (anastomosis) is accompanied by an intense traffic of cellular components between the fusing cells. Furthermore, the presence of numerous microfilaments is evident in these cells. By this mechanism of hyphal growth and anastomosis, complex three-dimensional hyphal nets

are formed (Veenhuis *et al.*, 1989c). Generally, adhesive knobs, hyphal branches and nets have a very similar subcellular morphology (Dowsett and Reid, 1977; Tzean and Estey, 1979; Wimble and Young, 1983; Veenhuis *et al.*, 1984, 1985b).

3. *Mechanical Traps*

In contrast to adhesive-containing capturing organs, inflating rings such as those formed on mycelia of *A. dactyloides* and *D. brochopaga* possess a highly-ordered ultrastructure. This is thought to reflect the ability of the cells suddenly to expand, resulting in an irreversible capture of a nematode. These rings consist of three cells, connected with the vegetative mycelium by a two-celled stalk. From the luminal to the outer side of the ring, four zones are discerned namely: (i) a thickened, mainly fibrillar cell wall; (ii) dense bodies which constitute the complex labyrinthine matrix; (iii) a vacuolated zone containing the other cell organelles; and (iv) an outer cell wall of normal appearance (Tzean and Estey, 1979). Dowsett and Reid (1983) observed multilaminate bodies at the inner side of the ring, and suggested that these function as a supply of membranous material to be delivered to the cell membrane during the sudden volume increase during capturing. The cytoplasm of uninflated cells displays a relatively high electron density (Heintz and Pramer, 1972). This is also observed in cells of *Haptoglossa mirabilis*, which possesses another trapping device using high osmotic pressure as a means to enter a host (Robb and Lee, 1986a,b; for further details see p. 125).

4. *Endoparasites*

In contrast to the traps of predatory fungi, conidia of endoparasitic fungi belonging to the genera *Drechmeria*, *Verticillium*, *Hirsutella* and *Harposporium* do not contain dense bodies. Generally these conidia contain, apart from the usual cell organelles (e.g. nucleus, mitochondria, microbodies and endoplasmic reticulum), storage materials such as lipid droplets and glycogen (Fig. 2c). Some of the endoparasitic species form conidia with adhesive layers present at one side of the cell (for example, *D. coniospora* and *V. balanoides*) or covering the whole spore (as in *H. rhossiliensis*). The structure of the adhesive layer may vary, and ranges from a highly-regular, fibrillar structure (seen in *D. coniospora*, Fig. 2d, and *V. balanoides*; Saikawa, 1982a,b; Dijksterhuis *et al.*, 1991; Sjollema *et al.*, 1993) to a large droplet of a fibrous nature (as in the basidiomycete *Nematoctonus pachysporus*; Saikawa and Arai, 1986), or it may be relatively thin and electron dense as reported for aplanospores of *Gonimochaete*

pyriforme, an Oomycete (Saikawa and Anazawa, 1985). Inside spores of the last of these fungi, numerous dense bodies are again evident. However, not all fungal nematode-capturing organs are immediately capturing-competent during their development. For instance, with conidiospores of *D. coniospora*, the adhesive knob, located at the distal part of the spore, is generally only formed after liberation of the cell from the conidiophore (Jansson *et al.*, 1984; Dijksterhuis *et al.*, 1991). Also, only a few conidia, located at the periphery of clusters of spores, form adhesive knobs. van den Boogert *et al.* (1992) showed that, in cultures of *D. coniospora* on an agar surface, only approximately 8% of the conidia possessed an adhesive knob. Further experiments showed that maturation of these spores is probably density-dependent since, upon suspension of clusters of spores in water, all conidia formed a knob within three days of incubation (van den Boogert *et al.*, 1992). In contrast, all spores of *V. balanoides* form an adhesive directly following liberation from the phialide (Sjollema *et al.*, 1993). Furthermore, spores of *G. pyriforme* form knobs after liberation from the sporangium (Saikawa and Anazawa, 1985). Conidia of *Harposporium* sp. mostly infect nematodes after lodging in the digestive tract (buccal cavity, oesophagus or lower gut). Spores of *Harposporium oxicoracum* have a highly-elongated shape, ending in a sharp hook with fibrous material present on the other side of the cell (Saikawa *et al.*, 1982). A unique morphology is observed with the fungus *Haptoglossa mirabilis* in which an invagination of the cell wall of the conidiospore forms a curved tube which is part of a mechanical penetration device. Inside the tube, a sharp projectile is present. Robb and Lee (1986b) suggest that this structure pierces the nematode cuticle when it evaginates rapidly after contact with the putative host. This injection process is thought to be driven by turgor present in the spore (Robb and Lee, 1986a; Barron, 1987).

V. Nematode–Fungal Interactions

A. METHODS

Current knowledge of the complex nematode–fungus interaction is mainly descriptive in nature and is primarily based on various light- and electron-microscopical methods as major tools. Light microscopy and video-enhanced contrast microscopy have been used to study interactions at the cellular level using living organisms, and have proved especially useful to investigate kinetics of the infection process. For instance, the significance of the characteristic wave pattern of the invading trophic hyphae of *D. coniospora* became clear from studies on living, infected nematodes, by

using video-enhanced contrast light microscopy (Dijksterhuis *et al.*, 1991). Using the same methods, mechanisms of outgrowth of conidiophores also became clear and provided useful indications of the strategy used by the fungus to exploit nematode contents (Dijksterhuis *et al.*, 1991). Transmission electron microscopy has been very useful for providing a further understanding of various mechanisms involved in nematode capturing and digestion. Application of these methods was significantly advanced by the development of the "flat embedding technique" which allowed an accurate estimate of the plane of sectioning. Growth of the fungus on dialysis membranes was a further improvement (Nordbring-Hertz *et al.*, 1984; Dijksterhuis *et al.*, 1990). Studies in which these methods were employed revealed several novel ultrastructural characteristics of nematode digestion, such as the ubiquitous presence of lipid droplets and microbodies in trophic hyphae during digestion of the nematode contents (Veenhuis *et al.*, 1989b). Also, detailed new information on the initial stages of nematode infection (e.g. formation of an appressorium on spores of *D. coniospora*; Dijksterhuis *et al.*, 1990) as well as on the significance of dense bodies during nematode infection by *A. oligospora* (Veenhuis *et al.*, 1989c) was obtained in this way. Scanning electron microscopy has been successfully applied to gather information about surface properties of the fungal cell wall; the presence of an additional adhesive layer on the appressorium of infecting conidia of *D. coniospora* was visualized in this way (Dijksterhuis *et al.*, 1990). Low-temperature scanning electron microscopy, allowing observation of samples in a frozen-hydrated state, is a further instrumental improvement and enables studies on highly fragile structures. An example of this is production of conidiospores on conidiophores of *D. coniospora* which adhere to each other only by a thin mucus layer present on their cell wall (Dijksterhuis *et al.*, 1991).

B. INITIAL STAGES

1. Recognition, Host Specificity and Adhesion

Contact between the nematode and the trapping structure by way of adhesives, or in the absence of any adhesive, is a prerequisite for initiation of the infection process (Tunlid *et al.*, 1992a). In order for this to occur, nematodes might be attracted to the mycelium and/or trapping devices of the fungus (Field and Webster, 1977). Hyphal traps show increased attractiveness to the prey compared with vegetative mycelium (Field and Webster, 1977; Jansson, 1982a). This capacity is shown to be species-dependent; spontaneous trap-forming fungi and endoparasites attract

nematodes more efficiently than other nematophagous fungi (Jansson and Nordbring-Hertz, 1979). In addition, adhesive spores appear to attract nematodes while non-adhesive ones do not (Jansson, 1982b). In other cases fungal growth or movement directed towards nematodes is observed. Hyphae of *Pleurotus ostreatus* grow towards nematodes which have previously been immobilized by means of a strong toxin, borne on specialized cells of the fungus (Thorn and Barron, 1984). Motile zoospores of *Catenaria anguillulae* are attracted to the natural orifices of the organism (Jansson and Thiman, 1992) or to nematode eggs after penetration of the lipid layer of the egg (Wyss *et al.*, 1991). Subsequently, an intimate contact between the nematode cuticle and the fungal cell wall is established (Figs 3a, b).

With adhesive layers, effective adhesion seems to depend on the presence of specific compounds on the outside of the cuticle (Rosenzweig and Ackroyd, 1983). Hence, the composition of the cuticle, which varies among different nematode species, may define the host-specificity of nematophagous fungi. For instance, nematode species of the order Dorylaimida such as *Xiphinema americanum* and *X. index* are not captured by the endoparasites *D. coniospora* and *V. balanoides* (Jansson *et al.*, 1985;

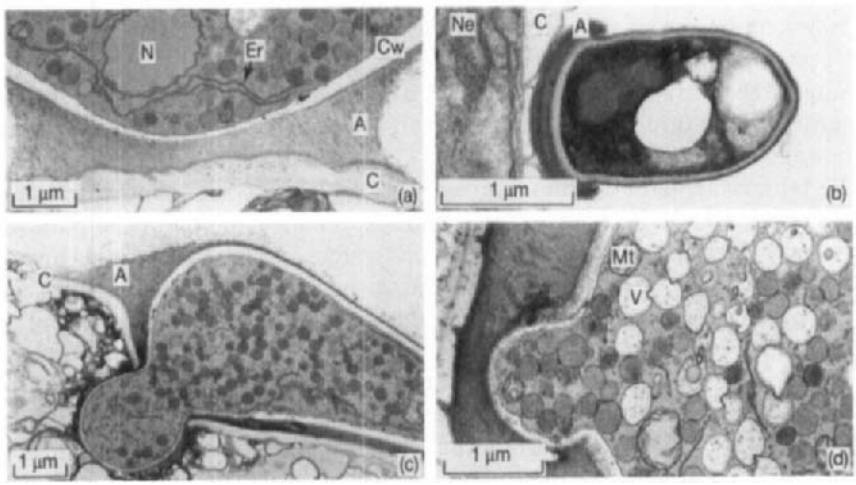

FIG. 3. Adhesion of fungi to the nematode and initial stages of infection. (a) Ultrathin section of the region of attachment between nematode and fungal trap (*Arthrobotrys oligospora*). Note the orientated fibrils present in the adhesive layer. (b) A conidium of *Verticillium balanoides* adhered to the nematode cuticle by means of an adhesive present on one side of the cell. (c, d) Germinated traps of *Arthrobotrys oligospora* piercing the cuticle of *Panagrellus redivivus* (c); in this trap numerous vacuoles (arrows) are present suggesting proteolytic degradation of individual dense bodies (d). Abbreviations are explained in Fig. 1.

Hashem, 1988) whereas zoosporic fungi such as *C. anguillulae* do so (Jaffee, 1986; Jaffee and Shaffer, 1987). Furthermore, the predatory fungus *Monacrosporium ellipsosporum* fails to capture Dorylaimid nematodes while other species belonging to the genera *Arthrobotrys*, *Monacrosporium* and *Dactylaria* capture and digest *Xiphinema index* successfully (Gaspard and Mankau, 1987; Rosenzweig *et al.*, 1985). In general, fungal-trapping devices adhere randomly to the nematode surface. However, adhesion of conidia of *D. coniospora* to *P. redivivus* is restricted to specific sites on the cuticle, namely those predominantly near the sensory organs located around the head and tail of the animals (Jansson and Nordbring-Hertz, 1983). Adhesion of this fungus may, furthermore, depend on the life-stage of the nematodes, as was observed with entomophagous nematodes (Poinar and Jansson, 1986; Timper and Kaya, 1989) and the free-living soil inhabitant *Acrobeloides buetschlii* (Dijksterhuis *et al.*, 1993b). That differences in surface properties of nematodes play a role was further demonstrated in a study of adhesion of spores of *V. balanoides* to ten nematode species (Dürschner-Pelz and Atkinson, 1988). Some differences in binding-site preferences were established, and fluorescent-lectin binding confirmed such differences in two of these nematodes.

During studies on nematode adhesion to traps of *A. oligospora*, a lectin–carbohydrate recognition mechanism was originally suggested by Nordbring-Hertz and Mattiasson (1979), the lectin being located on the surface of the fungus (Borrebaeck *et al.*, 1984). The mechanism of attachment of nematodes to three model fungi, namely *A. oligospora*, *D. coniospora* and *C. anguillulae*, was recently reviewed (Tunlid *et al.*, 1992a). In this review, the fungus–nematode interaction was viewed in a broader context because it is considered to be related to mechanisms involved in other host–parasite systems. In the nematophagous fungus system, attachment to the nematode surface is just one step in the infection process which may be preceded by chemotaxis of nematodes (Jansson and Nordbring-Hertz, 1988) and be accompanied by both morphological changes and secretion of adhesives and enzymes before penetration of the nematode cuticle can occur. Based on results from both ultrastructural and biochemical studies, a model for the molecular mechanisms involved in attack by several nematophagous fungi, in particular *A. oligospora*, was proposed. According to this model, adhesion starts with a physical contact between the living nematode and the trap. This contact leads to several events including a lectin–receptor interaction, a reorganization of surface polymers and secretion of specific enzymes. These studies have indicated that adhesion between fungal-infection structures and nematodes is a complex process involving both protein- and carbohydrate-containing polymers on the fungi (Tunlid *et al.*, 1991a,b). For further details, we refer readers

to several reviews (Nordbring-Hertz, 1984, 1988a,b; Jansson and Nordbring-Hertz, 1988; Tunlid *et al.*, 1992a,b; Zuckerman and Jansson, 1984).

It is clear from the above that the recognition process is not understood in detail. For instance, Rosenzweig *et al.* (1985) observed that certain simple carbohydrates, which inhibited adhesion and therefore may play a role in recognition, were not detected on the cuticle of nematodes which nevertheless were successfully captured by the fungus. Furthermore, sialic acid, which partially inhibits adhesion of conidia of *D. coniospora* to *P. redivivus* (Jansson and Nordbring-Hertz, 1984), was not detected on the cuticle of this nematode (Bacic *et al.*, 1990; Reuter *et al.*, 1991). These and other results suggest that more complex recognition phenomena may occur in this fungus (Jansson, 1993).

Recently, a new approach towards understanding the entire adhesion mechanism has been undertaken. In these studies not only lectins (Rosén *et al.*, 1992) but also other surface polymers (Tunlid *et al.*, 1991a) and enzymic activities have been taken into account (Tunlid and Jansson, 1991; Persson and Friman, 1993). In addition to (bio)chemically-defined recognition mechanisms, the motility of nematodes also influences the probability of encounter between parasite and host. This refers both to trap formation (Jansson and Nordbring-Hertz, 1980) and to capture of nematodes. For a quantitative estimation of the trapping ability of *A. oligospora*, the ratio between the number of traps to the number of nematodes should preferably be at least four (Friman, 1993). Furthermore, we observed that individuals of *P. redivivus*, which displayed retarded motility as a result of initial infection with *D. coniospora*, were captured in lower numbers after addition of the nematodes to trap-containing cultures of *A. oligospora* (Dijksterhuis *et al.*, 1993c). Infection structures without an adhesive may also show host-specificity; stylet-bearing nematodes are not infected by conidia of the genus *Harposporium* which are too large to enter the digestive tract of these animals. On the other hand, mechanical infection mechanisms may result in a relatively broad range of host organisms, as is the case with several species of the genus *Haptoglossa* which, besides nematodes, can also infect other small soil inhabitants such as rotifers and tardigrades (Barron, 1989).

2. *The Constricting-Ring Mechanism*

Completely different from all of the mechanisms described so far involves closure of constricting rings around nematodes which touch the luminal side of the trap. Barron (1979) suggested that the three cells constituting the ring are in a state of high osmotic pressure and that, after breakage of

a line of weakness present at the inner side of the ring, rapid closure occurs. The relatively thick inner cell-wall bulges inwards through the outer wall thereby strangling the nematode. After closure, the inner cell-wall appears much thinner, but dense bodies may be still present (as in *D. brochopaga*; Dowsett *et al.*, 1977) or may have completely disappeared (as in *A. dactyloides*; Heintz and Pramer, 1972); inflated cells characteristically contain large vacuoles. Most probably the volume of cells constituting the ring expands following rapid uptake of water. Insell and Zachariah (1978) punctured individual cells of so-called mega-traps of mutants of *D. brochopaga*, rings that are eight times larger than normal traps, and observed release of a droplet of liquid at the expense of cell volume. Atmospheric water may readily enter or leave these cells as judged by modifying the humidity of the environment. Furthermore, a correlation exists between the humidity of the environment and rate of ring closures. This is suggested from experiments by Rudek (1975) which arrested inflation of rings using air currents. Therefore, the source of water is most probably not endogenous in that it is originating from stalk cells or mycelium; this view is strengthened by the finding that rings may still successfully inflate after being removed from the stalk on which they arose (Insell and Zachariah, 1978; Barron, 1989). In addition, movements of intracellular components were not observed in the stalk cells during closure, suggesting that water is taken up from the environment (Barron, 1979). This suggestion is not unreasonable, since living nematodes are generally surrounded by a thin film of water that may provide sufficient liquid for ring closure. Besides inflation of traps caused by physical means, such as by touch, increased temperature or electrical stimulation (Higgins and Pramer, 1967), chemical induction of ring closure has also been observed in *D. brochopaga*. Cultures of this fungus, when exposed to solutions containing methanol, ethanol, propanol or butanol or to chlorobutanol vapour, showed inflation of all traps within 10–15 s. However, chloroform, ether and benzene had no effect. Mechanisms triggering this remarkable phenomenon are not yet known (Zachariah, 1989).

3. Cuticle Penetration

Germination of traps or conidiospores after contact with a nematode results in formation of a penetration tube which pierces the nematode cuticle (Fig. 3c). The predatory-trap former, *A. oligospora*, can form such an outgrowth from every trap cell that is in contact with the host in a process that includes the following stages. Firstly, there is local dissolution of the trap cell wall and simultaneous formation of the cell wall of the initial tube. This is followed by growth of the tube through the adhesive

layer and penetration of the cuticle by mechanical and enzymic means. Mechanically, the cuticle is obviously stretched prior to actual penetration; during this process the developing tube is held in a position perpendicular to the cuticle which is mediated by a tight connection between nematode and fungus involving the adhesive layer. Cytochemically, the presence of hydrolytic enzyme activity (such as acid phosphatase; Toth *et al.*, 1980) was detected at the site of penetration (Veenhuis *et al.*, 1985a). However, information on the requirement for specific enzymes, such as proteases or collagenases, involved in partial weakening of the cuticle is not available. It is of interest to note that the presence of proteases, including a chymotrypsin-like enzyme, has been demonstrated immunocytochemically in the region of penetration in studies on infection of insect larvae by the pathogen *Metarrhizium anisopliae* (St Leger *et al.*, 1989; Goettel *et al.*, 1989). Also, *A. oligospora* has been shown to produce different extracellular proteases including a serine protease (Tunlid and Jansson, 1991). The possible role of such proteases in nematode infection was examined by treatment of the fungus with various protease inhibitors. Serine-protease inhibitors did not affect nematode adhesion, but decreased subsequent immobilization of captured nematodes. However, trap-specific proteases have not been detected so far (Persson and Friman, 1993). Electron-microscope studies furthermore strongly suggested that the penetration site on the cuticle is effectively sealed. The objective of this is most probably to prevent leakage of nematode contents into the environment which may result from internal pressure of the infected, but living, nematode (Veenhuis *et al.*, 1985a; Dijksterhuis *et al.*, 1991).

Subsequently, once inside the nematode, the penetration tube swells to form a large infection bulb. This structure might be considered as an intermediate between the highly-differentiated trap cell and the trophic infection hyphae which all develop from this bulb. Inside the mature bulb cell, the usual cell organelles can be observed; the endoplasmic reticulum in particular is strongly developed. Dependent upon the nutrient status of the medium outside traps, dense bodies disappear inside the trap cell upon development of the infection bulb. In the absence of external nutrients, dense bodies are rapidly and actively degraded (Veenhuis *et al.*, 1989c; Fig. 3d). This process is characterized by sequestering of individual dense bodies from the cytosol by membranes which subsequently fuse with vacuoles. This mechanism resembles that of degradation of microbodies in yeast (Veenhuis *et al.*, 1983). Alternatively, in the presence of sufficient external nutrients, organelles are found scattered throughout the cytoplasm of the infection bulb as a result of dilution. The authors suggest that dense bodies play a crucial role and supply building stones to facilitate the initial stages of infection, in particular penetration and initial

formation of trophic hyphae. The initial patterns of nematode infection of other predatory fungi which use adhesive layers for capturing, are, albeit less thoroughly studied, largely similar to those described for *A. oligospora*. This is particularly true for the penetration process (*D. scaphoides*; Dowsett and Reid, 1979; *D. cionopaga*, Dowsett *et al.*, 1984; *D. lysipaga*, Wimble and Young, 1984), development of an infection bulb, and disappearance of dense bodies during the initial stages of infection. The latter two phenomena are observed in all electron-microscope studies reported so far on nematophagous fungi. In constricting rings of *A. dactyloides*, penetration is predominantly effected by the cell not neighbouring the stalk cell (in 95% of 40 infection events; Estey and Tzean, 1981). Penetration of the cuticle by two or more ring cells was never observed. In some fungi, exemplified by *D. scaphoides* and the endoparasites *D. coniospora* and *V. balanoides*, the first outgrowth on the infection structure is not dedicated to penetration of the nematode. With the endoparasites, an appressorium apparently is a prerequisite to facilitate effective infection (Dijksterhuis *et al.*, 1990; Sjollema *et al.*, 1993). The need for such an intermediate structure is most probably explained by the restricted area of contact between the host and the rounded knob of the spore which, especially with *D. coniospora*, is relatively small compared to the zone of contact in the predatory fungi. In the absence of an appressorium, the contact zone between the adhesive of the spore and the cuticle will be strongly diminished when the developing penetration tube reaches the cuticle. Consequently, mechanical forces exerted during penetration may become too strong to maintain proper contact between the spore and the cuticle. Therefore, formation of an appressorium, which enables a more intimate contact between the spore wall and the nematode cuticle over a larger area, may overcome this problem. Further adaptations to prevent loss of contact during initial stages of the interaction between host and infective spore include the presence of an additional adhesive at the site of contact between the appressorium and the cuticle, and the fact that penetration is achieved by a very thin penetration tube thereby decreasing the zone of adhesion only minimally during penetration (Dijksterhuis *et al.*, 1990).

Conidiospores of *D. coniospora* do not, or only rarely, germinate autonomously except after adhesion to the nematode indicating that an as yet unknown stimulus is required to induce germination. This is further exemplified by the finding that only approximately 0.01% of the cells germinate and form vegetative hyphae on solid or liquid artificial media (van den Boogert *et al.*, 1992). However, the course of interaction of *D. coniospora* with nematodes is species-dependent and, in several instances, successful infection is not achieved. This may be due to a variety of

reasons. Firstly, adhesion is not necessarily followed by formation of an appressorium. This has been observed for instance in several nematode species where conidia adhere to the cuticle without subsequent infection of the prey (Jansson *et al.*, 1985, 1987). Secondly, although germination of the spore is induced, penetration frequencies may be very low. This has been observed for instance in *Acrobeloides buetschlii* where failure to penetrate effectively is most probably due to properties of the nematode cuticle (Dijksterhuis *et al.*, 1993b). Surprisingly, when formation of an appressorium was not followed by penetration, a new adhesive knob was generally formed which, in turn, could germinate and, in a number of cases, penetrate the cuticle. In contrast to *D. coniospora*, *V. balanoides* produces conidiospores which do not show this response but germinate to form vegetative hyphae, irrespective of contact with the host. After successful penetration, endoparasites do not generally form an infection bulb like predatory fungi, but immediately invade the prey. Until now, only *V. balanoides* is known to form a small infection bulb; it is yet unknown if this structure is functionally analogous to the large infection bulb characteristic of trap-forming fungi.

C. COLONIZATION AND DIGESTION OF THE NEMATODE

Following entrance into the fungus, the nematode body cavity subsequently becomes invaded by trophic hyphae which develop at the expense of the body contents of the host (Figs 4a, b). As will be described later, studies on different organisms indicate that mechanisms involved in nematode colonization and digestion are similar among nematophagous fungi (Wimble and Young, 1984; Veenhuis *et al.*, 1989b; Dijksterhuis *et al.*, 1991). With *A. oligospora*, trophic hyphae develop from mature infection bulbs. These bulbs generally lack dense bodies and show all of the morphological characteristics of a normal vegetative cell. Also, developing trophic hyphae typically contain normal cell organelles, endoplasmic reticulum being particularly well developed. We have interpreted these results to indicate that the initial stages of infection are solely dedicated towards formation of invading hyphae. Subsequently, the growth rate of these hyphae is retarded (12–24 h after infection). At this stage numerous lipid droplets accumulate in all hyphal cells except the hyphal tip cell. This process is associated with development of many microbodies (Fig. 4b). These organelles have to be considered as peroxisomes since they contain, besides catalase, enzymes from the β-oxidation pathway. At this stage, development of fungal mycelium outside the nematode body is initiated. New hyphae develop from the original trap in which the nematode was captured, suggesting that an efficient intramycelial

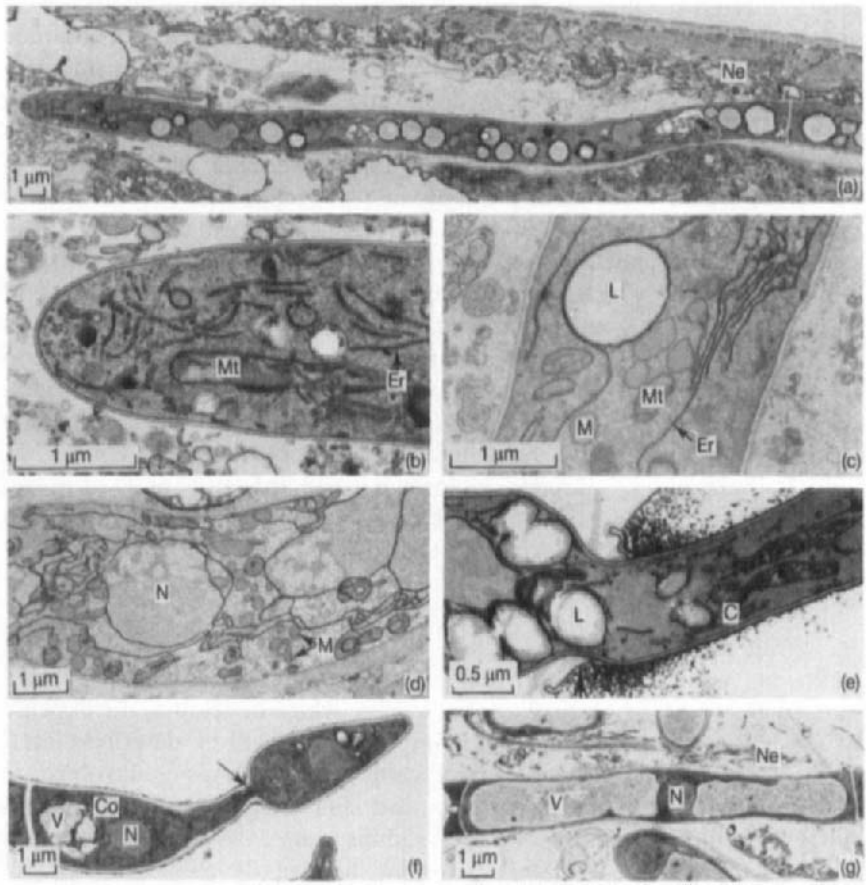

FIG. 4. Colonization and degradation of the nematode *Panagrellus redivivus* by different nematophagous fungi. (a) Apical hyphal cell of *Drechmeria coniospora* developing inside the nematode. The tip of this cell (b) typically contains vesicles and a proliferated endoplasmic reticulum. (c) Development of lipid droplets and accumulation of microbodies inside trophic hyphae of *Arthrobotrys oligospora*. (d) At later stages of infection by *Arthrobotrys oligospora*, numerous microbodies remain while the lipid droplets have disappeared. (e) Outgrowth of conidiophores of *Drechmeria coniospora* through the nematode cuticle. Note the presence of electron-dense material at the sites of outgrowth (arrow). (f) Formation of a conidium on an apical conidiogenous cell of *Drechmeria coniospora*. Note the initial septum formation prior to liberation of the spore (arrow). (g) Trophic hyphae of *Drechmeria coniospora* present at terminal stages of infection. Abbreviations are explained in Fig. 1.

translocation system of metabolic intermediates exists to account for this growth. In this process microbody-mediated metabolic pathways may play a distinct role. In an attempt to elucidate further the possible function of

these organelles, we studied growth of *A. oligospora* in mineral media known to induce microbody formation in other fungi. The results indicated that *A. oligospora* was indeed able to grow well on oleic acid as a sole source of carbon and energy; under these conditions β-oxidation enzymes were induced which were located in microbodies (Dijksterhuis *et al.*, 1993a). Proliferation of microbodies and accumulation of storage materials are characteristic phenomena during infection of nematodes by other endoparasites, such as *D. coniospora* and *V. balanoides* (Dijksterhuis *et al.*, 1991; Sjollema *et al.*, 1993) and may be a general and important aspect of host utilization in nematode–fungal interactions.

Branch formation on tropic hyphae of *A. oligospora* has never been observed; extensive examination by video-enhanced contrast microscopy revealed that hyphal fusions (anastomoses) between trophic hyphae were, however, common. The infection bulb and the anastomosed hyphae together may be regarded as a trophic system that converts nematode contents into storage materials (lipid droplets) which are subsequently metabolized to deliver nutrients to vegetative mycelium developing outside the nematode. Degradation of nematode contents may, at least in part, be achieved by activation of the animal's own autolytic system which may be induced by means of a boost of digestive activity during the initial stages of infection (Veenhuis *et al.*, 1985a). As infection proceeds, lipid droplets disappear again although numerous peroxisomes remain inside hyphae (Fig. 4d). In the final stages of infection, hyphae become vacuolated and are still enwrapped in remnants of the cuticle. The infection process is usually complete in 48–60 h (Veenhuis *et al.*, 1989b).

In contrast to *A. oligospora*, *D. coniospora* invades directly upon penetration by means of typically sinusoidal-shaped hyphae during the initial stages of infection. Invasion invariably proceeds by way of the pseudocoelom. Subsequently, hyphal branches are formed, but anastomosis was never observed. In this stage, accumulation of storage materials, proliferation of microbodies as well as development of conidiophores (Fig. 4e) occurred simultaneously. Initiation of their development generally occurred approximately 40–48 h after onset of infection. It should be emphasized that, also in this case, development of fungal biomass outside the nematode starts long before colonization of the nematode is completed. Therefore, conidia are formed at a relatively early stage of infection. A putative disadvantage of an early multiple penetration of the cuticle (from the inside of the nematodes) by numerous conidiophores is that it may result in a premature leakage of nutrients into the environment where bacteria and other soil inhabitants compete with the endoparasite. However, electron-microscope observations of these stages of the interaction strongly suggest that, similar to penetration from the outside, the site

of penetration from inside out is also effectively sealed especially at the base of the developing conidiophore (Fig. 4e). New conidia are subsequently formed on conidiophores (Fig. 4f); they develop in successive order from the same stalk, where they often form large clusters (Fig. 5a). After massive conidiospore production, often for at least 48 h following conidiophore emergence, cells of the trophic hyphae appear largely empty and typically contain two large vacuoles and a central spherical nucleus (Fig. 4g; Dijksterhuis *et al.*, 1991).

During the first 12–24 h after attachment of spores of *D. coniospora*, nematodes are able to move freely although they contain a considerable mass of trophic hyphae. Even nematodes on which the first conidiophores emerge often showed some movement. This is in contrast to observations on nematodes captured by conidia of a *Nematoctonus* species which revealed that the prey became immobilized prior to invasion; these findings suggest the possible involvement of a toxin in interaction of this fungus with nematodes (Giuma and Cooke, 1971; Kennedy and Tampion, 1978). In the predatory fungi *A. oligospora* and *D. candida*, on the other hand, immobilization of nematodes occurs soon after penetration of the nematode cuticle. Friman (1993) found no evidence for the presence of a toxin in this interaction. It therefore appears that use of compounds that immobilize or even kill nematodes prior to infection is species-dependent. The free mobility of nematodes, initially captured by *D. coniospora*, also implies that they remain possible candidates for subsequent infection, possibly by a different fungus. This aspect has been studied in more detail (Dijksterhuis *et al.*, 1993c) by subjecting nematodes infected with *D. coniospora* to subsequent infection with *A. oligospora*. The results showed that the outcome of the infection by the second predator was dependent on the stage of the initial infection by *D. coniospora*. During concurrent dual infection, trophic hyphae of both fungi developed inside the host, followed by production of exogenous hyphal mass (spores with *D. coniospora* and vegetative mycelium of *A. oligospora*). However, when *A. oligospora* was added during later stages of infection with *D. coniospora* generally after 12 h) it could still enter the nematode body but its trophic hyphae collapsed upon contacting hyphae of *D. coniospora*. These therefore function as antagonists, giving rise to the speculation that endoparasitic fungi may have a certain advantage over saprophytic organisms with respect to their interaction with nematodes in the natural environment (Dijksterhuis *et al.*, 1993c).

D. INFECTION STRATEGIES

A careful analysis of several published studies, together with our own kinetic data, revealed that development of trophic hyphae is invariably

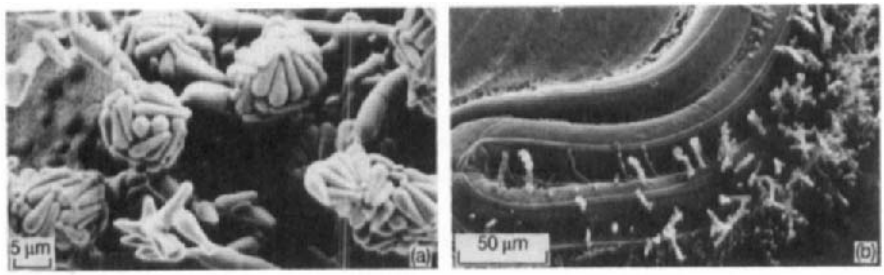

FIG. 5. Spore production by *Drechmeria coniospora* at the expense of *Panagrellus redivivus*. (a) Sequential production of conidia on conidiophorous pegs leads to formation of clusters of spores. (b) Infected nematode with many conidiophores.

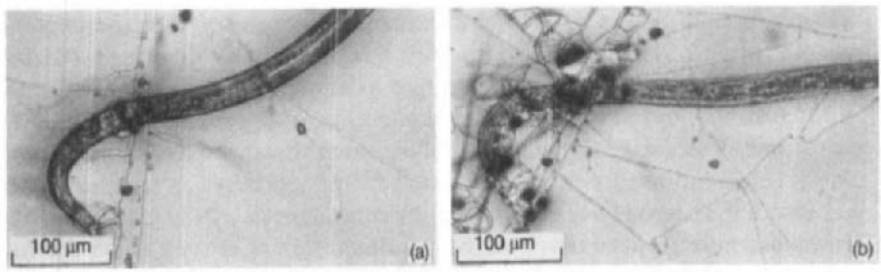

FIG. 6. Light micrographs illustrating the strategy of infection of *Panagrellus redivivus* by *Arthrobotrys oligospora*. Development of numerous traps, together with an increase in vegetative mycelium, is observed after nematode infection on an agar surface, illustrated on the same nematode. Pictures are taken at 0 (a) and 24 h (b) after infection.

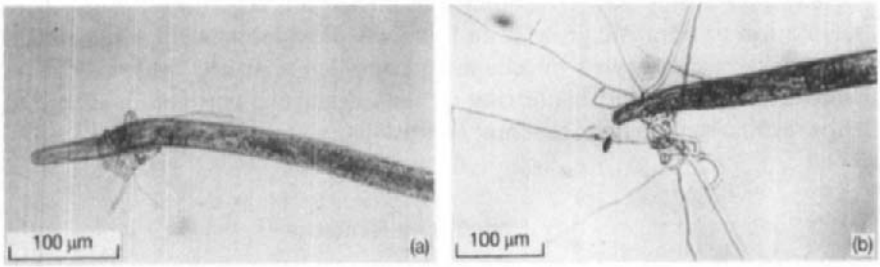

FIG. 7. Vegetative mycelium (b), formed at the expense of the host after placing a captured nematode in an environment lacking external nutrients (a) 24 h following initiation of infection.

repressed at a relatively early stage of infection in favour of development of fungal mass outside the nematode. Consequently, the main function of trophic hyphae is probably to digest nematode contents and convert its carbon-containing compounds into storage polymers and supply the vegetative mycelium and/or conidiophores with appropriate nutrients. The strategy of infection may be directed to production of either vegetative mycelium, as with *A. oligospora* (Veenhuis *et al.*, 1989a), or numerous conidiospores as with *D. coniospora* (Fig. 5b, Dijksterhuis *et al.*, 1991). In line with this view is the observation that maturation of spores of *D. coniospora* is confined to free spores after they are liberated from the conidiophorous peg. It can readily be envisaged that this mode of autonomous-knob formation decreases the time required for production of individual spores thus increasing the efficiency of spore formation at the expense of the prey (van den Boogert *et al.*, 1992).

It should be emphasized that conclusions from studies of interactions between nematophagous fungi and nematodes may vary with the experimental conditions employed. This view is convincingly demonstrated by results from initial experiments designed to study the interaction between *A. oligospora* and the nematode *Panagrellus redivivus*. These studies, performed on solid-agar media or dialysis membranes, revealed that upon digestion of captured nematodes, apart from vegetative mycelium there was also a massive formation of traps (Veenhuis *et al.*, 1989a, Figs 6a, b). However, later experiments, in which the interaction was studied with isolated captured nematodes which were removed from the agar surface after rupture of the trap cell from the mycelium on which it had developed, showed no further development of the original trap. Instead, vegetative mycelium was formed solely at the expense of the nematode (Veenhuis *et al.*, 1989a; Figs 7a, b). These results suggest that trap development, observed in the initial experiments, may be due to nutrients still available from the agar medium. Therefore, as already indicated, one strategy of *A. oligospora* may be to maintain the fungus in a vegetative form before adaptation to parasitic growth. In this view, development of traps should be considered a survival mechanism, complementary to others, such as vegetative spores, thus conferring on the organism a certain advantage to cope with adverse environmental conditions.

VI. Concluding Remarks

During the past decade, significant advances have been made in our understanding of the various functional and structural aspects of fungal–nematode interactions. Most of the published work has been of a descriptive

nature which is quite understandable because, in laboratory cultures, it is technically difficult to grow sufficient amounts of biomass of many of the fungi concerned to enable detailed biochemical studies. Thus, our knowledge of the early events in the interaction, including attachment of fungal spores to living nematodes and capture of living nematodes by specialized structures, is detailed at the (ultra)structural level but insufficient with respect to biochemical and molecular mechanisms. This is also true for subsequent events which involve penetration of a living nematode followed by proliferation of trophic hyphae inside the animal leading to its death. Attempts are now underway to exploit the tools of molecular biology for unravelling the biochemistry involved in various events that characterize the interaction between nematophagous fungi and their prey. These include isolation and molecular characterization of mutants and development of transformation systems that enable studies of the functional role of genes and gene products in various aspects of the interaction. These studies should also address the host specificity that many nematophagous fungi display, since these are of importance for their possible utility in biological control.

The ecology of these fungi is poorly understood. They occur in relatively small numbers in all soils. Although several can grow saprophytically, their competitive advantage probably resides in their ability to attack living nematodes. There is little doubt that nematodes can serve as a source of food for nematophagous fungi and that biomass (either mycelium and/or spores) is produced during the digestion of the contents of the animal. However, before these predators can be routinely and successfully applied in the biological control of nematodes, or natural control be attributed to them, detailed information on the ecology and ecophysiology of these remarkable fungi is required. In view of the potential of this type of biological control and the loss of agricultural productivity, these studies should be given a high priority.

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REFERENCES

Bacic, A., Kahane, I. and Zuckerman, B. M. (1990). *Experimental Parasitology* **71**, 483.
Barron, G. L. (1977). "The Nematode-Destroying Fungi". Canadian Biological Publications, Guelph, Ontario, Canada.

Barron, G. L. (1979). *Canadian Journal of Botany* **57**, 187.
Barron, G. L. (1981). *In* "Biology of Conidial Fungi", Vol. 2 (G. T. Cole and B. Kendrick, eds), pp. 167–200. Academic Press, New York.
Barron, G. L. (1982). *In* "Experimental Microbial Ecology" (R. G. Burns and J. H. Slater, eds), pp. 533–552. Blackwell Scientific Publications, Oxford.
Barron, G. L. (1987). *Mycologia* **79**, 877.
Barron, G. L. (1989). *Canadian Journal of Botany* **67**, 1645.
Barron, G. L. (1992). *In* "The Fungal Community. Its Organization and Role in the Ecosystem" (G. C. Carroll and D. T. Wicklow, eds), 2nd edn., pp. 311–326. Marcel Dekker, New York.
Barron, G. L. and Thorn, R. G. (1987). *Canadian Journal of Botany* **65**, 774.
Blackburn, F. and Hayes, W. A. (1966). *Annals of Applied Biology* **58**, 43.
Borrebaeck, C. A. K., Mattiasson, B. and Nordbring-Hertz, B. (1984). *Journal of Bacteriology* **159**, 53.
Cooke, R. C. (1962). *Annals of Applied Biology* **50**, 507.
Cooke, R. C. (1963a). *Annals of Applied Biology* **51**, 295.
Cooke, R. C. (1963b). *Annals of Applied Biology* **52**, 431.
Cooke, R. C. (1964). *Annals of Applied Biology* **54**, 375.
Cooke, R. C. (1968). *Phytopathology* **58**, 909.
Cooke, R. C. (1977). "The Biology of Symbiotic Fungi". John Wiley & Sons, London.
Coscarelli, W. and Pramer, D. (1962). *Journal of Bacteriology* **84**, 60.
Dackman, C. and Nordbring-Hertz, B. (1992). *Mycological Research* **96**, 194.
Dackman, C., Olsson, S., Jansson, H.-B., Lundgren, B. and Nordbring-Hertz, B. (1987). *Microbial Ecology* **13**, 89.
Dackman, C., Jansson, H.-B. and Nordbring-Hertz, B. (1992). *In* "Soil Biochemistry", Vol. 7 (G. Stotzky and J. M. Bollag, eds), pp. 95–130. Marcel Dekker, New York.
Dijksterhuis, J., Veenhuis, M. and Harder, W. (1990). *Mycological Research* **94**, 1.
Dijksterhuis, J., Wyss, U., Veenhuis, M. and Harder, W. (1991). *Mycological Research* **95**, 873.
Dijksterhuis, J., Harder, W. and Veenhuis, M. (1993a). *FEMS Microbiology Letters* **112**, 125.
Dijksterhuis, J., Veenhuis, M. and Harder, W. (1993b). *FEMS Microbiology Letters* **113**, 183.
Dijksterhuis, J., Sjollema, K. A., Veenhuis, M. and Harder, W. (1993c). *Mycological Research*. In press.
Dowsett, J. A. and Reid, J. (1977). *Canadian Journal of Botany* **55**, 2963.
Dowsett, J. A. and Reid, J. (1979). *Mycologia* **71**, 379.
Dowsett, J. A. and Reid, J. (1983). *Mycologia* **75**, 1094.
Dowsett, J. A., Reid, J. and van Caeseele, L. (1977). *Canadian Journal of Botany* **55**, 2945.
Dowsett, J. A., Reid, J. and Hopkin, A. A. (1984). *Canadian Journal of Botany* **62**, 674.
Drechsler, C. (1937). *Mycologia* **29**, 447.
Drechsler, C. (1941). *Biological Reviews of the Cambridge Philosophical Society* **16**, 265.
Drechsler, C. (1950). *Mycologia* **42**, 1.
Duddington, C. L. (1955). *Botanical Review* **21**, 377.
Duddington, C. L. (1962). *In* "Viewpoints in Biology" (J. D. Carthy and C. L. Duddington, eds), pp. 151–200. Butterworths, London.
Dürschner-Pelz, V. V. and Atkinson, H. J. (1988). *Journal of Invertebrate Pathology* **51**, 97.
Eren, J. and Pramer, D. (1966). *Soil Science* **101**, 39.
Estey, R. H. and Tzean, S. S. (1981). *Canadian Journal of Plant Science* **61**, 785.
Field, J. I. and Webster, J. (1977). *Transactions of the British Mycological Society* **68**, 467.
Friman, E. (1993). *Journal of General Microbiology* **139**. In press.
Friman, E., Olsson, S. and Nordbring-Hertz, B. (1985). *FEMS Microbiology Ecology* **31**, 17.
Gams, W. and Jansson, H.-B. (1985). *Mycotaxon* **22**, 33.
Gaspard, J. T. and Mankau, R. (1987). *Revue Nématologie* **10**, 241.
Giuma, A. Y. and Cooke, R. C. (1971). *Transactions of the British Mycological Society* **56**, 89.

Goettel, M. S., St. Leger, R. J., Rizzo, N. W., Staples, R. C. and Roberts, D. W. (1989). *Journal of General Microbiology* **135**, 2233.
Gray, N. F. (1985). *Soil Biology and Biochemistry* **17**, 499.
Gray, N. F. (1988). *In* "Diseases of Nematodes", Vol. 2 (G. O. Poinar and H.-B. Jansson, eds), pp. 3–38. CRC Press, Boca Raton, Florida.
Hayes, W. A. and Blackburn, F. (1966). *Annals of Applied Biology* **58**, 51.
Hashem, M. H. (1988). Dissertation: University of Kiel, Germany.
Heintz, C. E. and Pramer, D. (1972). *Journal of Bacteriology* **110**, 1163.
Higgins, M. L. and Pramer, D. (1967). *Science* **155**, 345.
Insell, J. P. and Zachariah, K. (1978). *Protoplasma* **95**, 175.
Jaffee, B. A. (1986). *Journal of Nematology* **18**, 87.
Jaffee, B. A. and Shaffer, R. L. (1987). *Nematologica* **33**, 220.
Jaffee, B. A. and Zehr, E. I. (1982). *Phytopathology* **72**, 1378.
Jaffee, B. A. and Zehr, E. I. (1983). *Plant Disease* **67**, 1265.
Jaffee, B. A., Muldoon, A. E. and Tedford, E. C. (1992). *Phytopathology* **82**, 615.
Jansson, H.-B. (1982a). *Microbial Ecology* **8**, 233.
Jansson, H.-B. (1982b). *Transactions of the British Mycological Society* **79**, 25.
Jansson, H.-B. (1993). *Journal of General Microbiology* **139**, 1899.
Jansson, H.-B. and Nordbring-Hertz, B. (1979). *Journal of General Microbiology* **112**, 89.
Jansson, H.-B. and Nordbring-Hertz, B. (1980). *Nematologica* **26**, 383.
Jansson, H.-B. and Nordbring-Hertz, B. (1983). *Journal of General Microbiology* **129**, 1121.
Jansson, H.-B. and Nordbring-Hertz, B. (1984). *Journal of General Microbiology* **130**, 39.
Jansson, H.-B. and Nordbring-Hertz, B. (1988). *In* "Diseases of Nematodes", Vol. 2 (G. O. Poinar and H.-B. Jansson, eds), pp. 59–72. CRC Press, Boca Raton, Florida.
Jansson, H.-B., von Hofsten, A. and von Mecklenburg, C. (1984). *Antonie van Leeuwenhoek* **50**, 321.
Jansson, H.-B., Jeyaprakash, A. and Zuckerman, B. M. (1985). *Applied and Environmental Microbiology* **49**, 552.
Jansson, H.-B., Dackman, C. and Zuckerman, B. M. (1987). *Nematologica* **33**, 480.
Jansson, H.-B. and Thiman, L. (1992). *Mycologia* **84**, 109.
Kennedy, N. and Tampion, J. (1978). *Transactions of the British Mycological Society* **70**, 140.
Kerry, B. R. (1984). *Helminthological Abstracts, Series B: Plant Nematology* **53**, 1.
Lohmann, U. and Sikora, R. A. (1989). *Nematologica* **35**, 97.
Mankau, R. (1980). *Annual Review of Phytopathology* **18**, 415.
Nordbring-Hertz, B. (1972). *Physiologia Plantarum* **26**, 279.
Nordbring-Hertz, B. (1973). *Physiologia Plantarum* **29**, 223.
Nordbring-Hertz, B. (1977). *Nematologica* **23**, 443.
Nordbring-Hertz, B. (1984). *In* "The Ecology and Physiology of the Fungal Mycelium" (D. H. Jennings and A. D. M. Rayner, eds), pp. 419–432. Cambridge University Press, Cambridge.
Nordbring-Hertz, B. (1988a). *In* "Advances in Microbial Ecology", Vol. 10 (K. C. Marshall, ed.), pp. 81–114. Plenum, New York.
Nordbring-Hertz, B. (1988b). *Microbiological Sciences* **5**, 108.
Nordbring-Hertz, B. and Brinck, C. (1974). *Physiologia Plantarum* **31**, 59.
Nordbring-Hertz, B. and Chet, I. (1986). *In*: "Microbial Lectins and Agglutinins: Properties and Biological Activity" (D. Mirelman, ed.), pp. 393–408. John Wiley & Sons, New York.
Nordbring-Hertz, B. and Mattiasson, B. (1979). *Nature* **281**, 477.
Nordbring-Hertz, B. and Odham, G. (1980). *Microbial Ecology* **6**, 241.
Nordbring-Hertz B. and Stålhammar-Carlemalm, M. (1978). *Canadian Journal of Botany* **56**, 1297.
Nordbring-Hertz, B., Veenhuis, M. and Harder, W. (1984). *Applied and Environmental Microbiology* **47**, 195.
Nordbring-Hertz, B., Fehne-Persson, K., de Vries, O. M. H. and Veenhuis, M. (1989a). *Mycological Research* **92**, 50.
Nordbring-Hertz, B., Friman, E. and Veenhuis, M. (1989b). *Antonie van Leeuwenhoek* **55**, 237.

Nordbring-Hertz, B., Jansson, H.-B., Friman, E., Persson, Y., Dackman, C., Hard, T. and Poloczek, E. (1993). Film V 2603, Institut für den Wissenschaft lichen Film, Göttingen, Germany.
Persson, Y. and Friman, E. (1993). *Experimental Mycology* **17**, 182.
Persson, Y., Nordbring-Hertz, B. and Chet, I. (1990). *Mycological Research* **94**, 196.
Poinar, G. O. and Jansson, H.-B. (1986). *Journal of Nematology* **18**, 225.
Pramer, D. (1964). *Science* **144**, 382.
Pramer, D. and Kuyama, S. (1963). *Bacteriological Reviews* **27**, 282.
Pramer, D. and Stoll, N. R. (1959). *Science* **129**, 966.
Reuter, G., Aumann, J., Wyss, U., Jansson, H.-B. and Schauer, R. (1991). *Experimental Parasitology* **73**, 389.
Robb, J. and Lee, B. (1986a). *Protoplasma* **135**, 102.
Robb, J. and Lee, B. (1986b). *Canadian Journal of Botany* **64**, 1935.
Rosén, S., Ek, B., Rask, L. and Tunlid, A. (1992). *Journal of General Microbiology* **138**, 2663.
Rosenzweig, W. D. (1984). *Canadian Journal of Microbiology* **30**, 265.
Rosenzweig, W. D. and Ackroyd, D. (1983). *Applied and Environmental Microbiology* **46**, 1093.
Rosenzweig, W. D. and Pramer, D. (1980). *Applied and Environmental Microbiology* **40**, 694.
Rosenzweig, W. D., Premachandran, D. and Pramer, D. (1985). *Canadian Journal of Microbiology* **31**, 693.
Rucker, C. J. and Zachariah, K. (1987). *Canadian Journal of Botany* **65**, 1160.
Rudek, W. T. (1975). *Mycopathologia* **55**, 193.
Saikawa, M. (1982a). *Canadian Journal of Botany* **60**, 2019.
Saikawa, M. (1982b). *Journal of Electron Microscopy* **31**, 276.
Saikawa, M. and Anazawa, T. (1985). *Canadian Journal of Botany* **63**, 2326.
Saikawa, M. and Arai, K. (1986). *Transactions of the Mycological Society of Japan* **27**, 33.
Saikawa, M. and Morikawa, C. (1985). *Canadian Journal of Botany* **63**, 1386.
Saikawa, M., Totsuka, J. and Morikawa, C. (1982). *Canadian Journal of Botany* **61**, 893.
Saikawa, M., Yamaguchi, K. and Morikawa, C. (1988). *Mycologia* **80**, 880.
Schenck, S. and Pramer, D. (1976). *Nematologica* **22**, 312.
Schenck, S., Chase, T. Jr., Rosenzweig, W. D. and Pramer, D. (1980). *Applied and Environmental Microbiology* **40**, 567.
Scheuer, C. H. and Webster, J. (1990). *Mycological Research* **94**, 718.
Sjollema, K. A., Dijksterhuis, J. and Veenhuis, M. (1993). *Mycological Research* **97**, 479.
Stirling, G. R. (1988). *In* "Diseases of Nematodes", Vol. 2 (G. O. Poinar and H.-B. Jansson, eds), pp. 93–139. CRC Press, Boca Raton, Florida.
St Leger, R., Butt, T. M., Staples, R. C. and Roberts, D. W. (1989). *Experimental Mycology* **13**, 253.
Thorn, R. G. and Barron, G. L. (1984). *Science* **224**, 76.
Timper, P. and Kaya, H. K. (1989). *Journal of Invertebrate Pathology* **54**, 314.
Toth, R., Toth, D. and Nordbring-Hertz, B. (1980). *Mycologia* **72**, 813.
Tunlid, A. and Jansson, S. (1991). *Applied and Environmental Microbiology* **57**, 2868.
Tunlid, A., Johansson, T. and Nordbring-Hertz, B. (1991a). *Journal of General Microbiology* **137**, 1231.
Tunlid, A., Nivens, D. E., Jansson, H.-B. and White, D. C. (1991b). *Experimental Mycology* **15**, 206.
Tunlid, A., Jansson, H.-B. and Nordbring-Hertz, B. (1992a). *Mycological Research* **96**, 401.
Tunlid, A., Rosén, S. and Nordbring-Hertz, B. (1992b). *Journal de Mycologie Medicale* **2**, 36.
Tzean, S. S. and Estey, R. H. (1979). *Canadian Journal of Plant Sciences* **59**, 785.
van den Boogert, P. H. J. F., Dijksterhuis, J., Velvis, H. and Veenhuis, M. (1992). *Antonie van Leeuwenhoek* **61**, 221.
Veenhuis, M., Douma, A. C., Harder, W. and Osumi, M. (1983). *Archives of Microbiology* **134**, 193.
Veenhuis, M., Nordbring-Hertz, B. and Harder, W. (1984). *FEMS Microbiology Letters* **24**, 31.

Veenhuis, M., Nordbring-Hertz, B. and Harder, W. (1985a). *Antonie van Leeuwenhoek* **51**, 385.
Veenhuis, M., Nordbring-Hertz, B. and Harder, W. (1985b). *Antonie van Leeuwenhoek* **51**, 399.
Veenhuis, M., Sjollema, K. A., Nordbring-Hertz, B. and Harder, W. (1989a). *Antonie van Leeuwenhoek* **55**, 361.
Veenhuis, M., Harder, W. and Nordbring-Hertz, B. (1989b). *Antonie van Leeuwenhoek* **56**, 241.
Veenhuis, M., Wijk, C. Van, Wyss, U., Nordbring-Hertz, B. and Harder, W. (1989c). *Antonie van Leeuwenhoek* **56**, 251.
Wharton, D. A. and Murray, D. S. (1990). *Journal of Microscopy* **158**, 81.
Whisler, H. C. and Travland, L. B. (1974). *Archives of Microbiology* **101**, 95.
Wimble, D. B. and Young, T. W. K. (1983). *Transactions of the British Mycological Society* **80**, 515.
Wimble, D. B. and Young, T. W. K. (1984). *Nova Hedwigia* **40**, 9.
Wootton, L. M. O. and Pramer, D. (1966). *Bacteriological Proceedings*, p. 75.
Wyss, U., Voss, B. and Jansson, H.-B. (1991). *Fundamental Applied Nematology* **15**, 133.
Zachariah, K. (1981). *Protoplasma* **106**, 173.
Zachariah, K. (1983a). *Antonie van Leeuwenhoek* **49**, 563.
Zachariah, K. (1983b). *Canadian Journal of Microbiology* **29**, p. 1295.
Zachariah, K. (1989). *Protoplasma* **148**, 87.
Zachariah, K. and Victor, J. R. (1983). *Canadian Journal of Botany* **61**, 3255.
Zopf, W. (1888). *Nova Acta Leopoldino-Carolinae* **52**, 314.
Zuckerman, B. M. and Jansson, H.-B. (1984). *Annual Reviews of Phytopathology* **22**, 95.

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Non-Invasive Concepts in Metabolic Studies

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I. Introduction

In the classical biological science disciplines, such as biochemistry, microbiology, molecular and cell biology, there exist analytical concepts for investigation of key structural elements of metabolism and its control. These methods involve, among others, preparation of genes, enzymes, substrates, cosubstrates and products as well as their *in vitro* kinetic analysis after recombination, representing basically an artificially simplified system. These types of procedure provide a powerful approach for investigation of biocatalytical and regulatory activities including functions and structures. Genetics plays a central role in identification of functional genes and affiliation of gene products. Modification of genes, mutation or recombination permit a direct proof of either the presence or absence of

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individual metabolic steps. As a result, construction of entire metabolic sequences can be described in detail. But this image is basically static, and the time behaviour can at its best be sketched only with poor resolution. Consequently, the dynamic behaviour of life, *in vivo*, cannot be investigated satisfactorily with these concepts. Only more recent developments of extended high precision on-line analytical tools (sensors, analytical systems and software) are capable of spanning this gap. First, results from fully functional and undisturbed populations describing their natural dynamics precisely open new aspects concerning the potential, and obviously underestimated, rates of *in vivo* biological reactions.

Nowadays, it is even possible to alter, intentionally, individual metabolic control elements in a living cell specifically by means of genetic engineering methods; this is called metabolic engineering (Bailey, 1991). It is an important tool for investigating cellular functions, and the practical application consequences are new products. As a result, new properties can be implanted into many different host organisms, selectivity or activity of biocatalysts can be altered, and the quantity of expressed biocatalysts and/or products can be varied. Presently, the most serious problems associated with this approach are deregulation of metabolism, poor stability of the genetic constructs and a need for re-evaluation of optimal microenvironmental conditions. Therefore, benefits from all of these achievements for industrial biotechnology are still below expectations. Metabolic networks are quite rigid (Stephanopoulos and Vallino, 1991) and provide, to the living cell, a series of control mechanisms which would probably not allow a straight-forward metabolic engineering alteration to work as expected. The entire network, which comprises both static and dynamic interrelations, and no matter whether in a recombinant or in a wild-type strain, is obviously not yet sufficiently well understood. This probably derives from the fact that classical methodologies over-emphasize investigation of isolated biological sub-systems, for example enzymes or relatively short enzymically driven metabolic sequences such as glycolysis or respiration—*in vitro*. Isolation of a sub-system from its natural environment, into which it was originally embedded, always results in loss of information concerning interactions. After reconstitution of several isolated subsystems, one will hardly get the same functional and regulatory information as compared to a direct investigation of the undisturbed and intact system (Fig. 1). This is a reason of paramount importance for promoting and exploiting non-invasive techniques—in addition to classical ones and not as a substitute—for basic biological research.

The concept of qualitative and quantitative perfection of the numerous state variables in an intact biosystem is not yet well accepted everywhere. Arguments of economy, feasibility or even necessity are confusing the

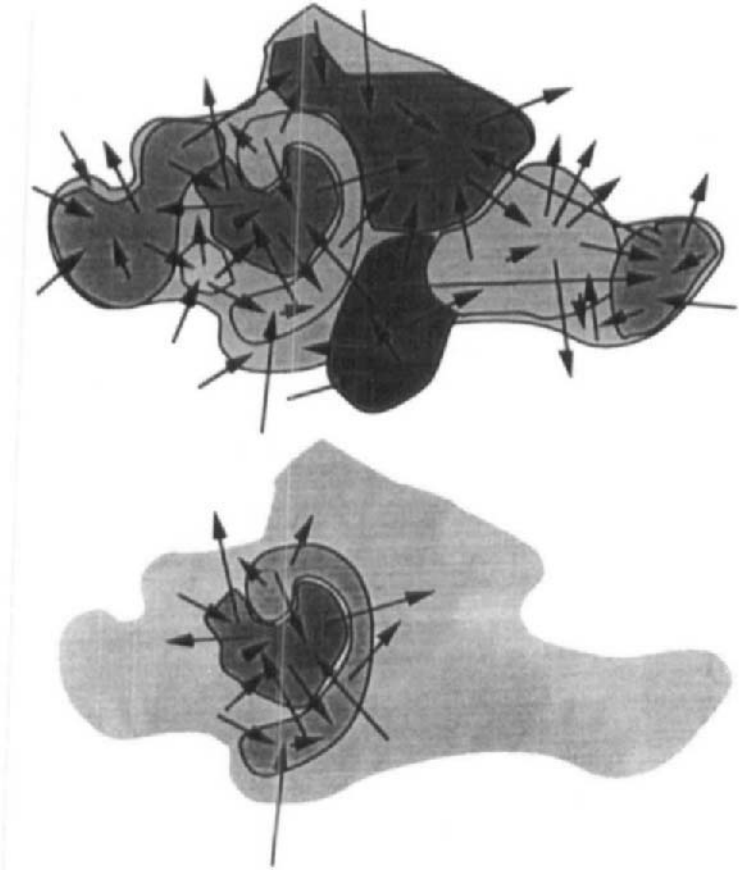


FIG. 1. In system theory, a *system* is a part of reality. It has its internal properties and interacts with the outside world (the properties of which are external to the system) via input-output properties. The upper sketch represents such a system, for example a whole cell or an entire population *in vivo*, which consists of several individual subsystems. If one or a few subsystems, such as some genes or enzymes or sequences thereof, are isolated from the original system, their characteristic properties may change; for example internal properties may become input/output or even external properties (lower sketch). Information about a system is generally altered or even lost if it is broken into subsystems and reconstituted partially from these. In fact, this is the most important general reason for the often recognized mismatches between *in vivo* and *in vitro* results in bioresearch. In metabolic research, this reflects frequently found statements, randomly taken from Thevelein (1992), such as ". . . function is unclear because of unusually high K_m *in vitro* . . ." or ". . . enormous discrepancy between the capacity to synthesize cAMP and the actual intracellular concentration of cAMP". As a consequence, in order to compensate these weak points, non-invasive methods must be promoted to study undisturbed biosystems *in vivo*.

situation and cementing the belief that *biology as such* is not an exact science, that it lacks the necessary reproducibility, and that it is of poor economic relevance in practical applications. However, the examples given in this review are convincing and raise rewarding expectations for the future. Perfection of synchronous growth has been reached only recently (Münch *et al.*, 1992) after substantial improvement of bioreactors and control of relevant culture parameters. For the first time, stable oscillations can be maintained independent of time. This perfection was obtained only after raising accuracy and precision of measurements and control loops to a significantly refined level compared with earlier efforts (e.g. unstable frequency and amplitude of oscillations as reported by Meyer and Beyeler (1984) were due to a methodological problem and not a biological effect).

Non-invasive approaches for studies of metabolic kinetics and regulation, however, permit identification and quantification of responses of intact populations of microbes or cells to imposed variations in micro-environmental conditions with respect to physical, chemical or biological variables. As in modern brain research, it is important to keep the object of the research intact and fully functional. The great advantages in studies in microbial physiology are the high number of individual cells observed in measuring a response, thereby making data obtained statistically analysable. There exists also the opportunity to investigate the intact entity of intra- and intercellular dynamic relationships. A significant disadvantage is still the lack of suitable sensors and analytical instruments suitable for this discipline. However, appropriate equipment has been developed and successfully applied in recent years, which helps to raise optimism (Scheper, 1991; Schügerl, 1991). High-performance bioreactor systems with a sophisticated degree of automation permit reliable and reproducible cultivation of cells and biotransformations (Sonnleitner and Fiechter, 1992). Novel sensors and high-performance analytical instruments have been adapted to interface monoseptic bioprocesses yielding a continuous, or at least highly frequent, image of the kinetics of important physical and crucial chemical state variables such as extracellular concentrations of substrates, metabolic intermediates, products and enzymes involving mass spectrometry, gas or liquid chromatography, flow injection analyses and electrophoreses. Even some intracellular components such as NAD(P)H and DNA have become accessible on-line (culture fluorescence; spontaneous or after specific staining). For a survey of these developments see Locher *et al.* (1993).

It took many years to improve the accuracy and precision of equipment to the present state of the art, the improvement being one or two orders of magnitude with respect to the errors. But, beneficially, these developments helped to overcome the established opinion that bio-

processes are slow, imprecise and irreproducible, which was always associated with perjorative terms such as "soft science". The dramatically improved reproducibility, together with the high frequency or even continuity, of observations provides the opportunity to revise current understanding of microbial and cellular relaxation times and interactions, that is population dynamics. There also exists now the opportunity to quantify timely developments rather than establishing just the sequences of events (which is the strength of molecular biological approaches), and to discriminate between cause and effect. In short, this is to recognize the dynamics *in vivo* of the complex but intact functionality of the living cell. Two examples of non-invasive metabolic studies will be discussed in this review, demonstrating the scientific efficiency, power and performance of the non-invasive experimental methods.

II. Studies with Microbes and Animal Cells

Sugars as carbon and energy sources, especially glucose, fructose, and their metabolic derivatives, are responsible for a variety of regulatory actions. They play on two different levels. Firstly, there is regulation of gene expression (induction, repression or derepression of transcription), and secondly modification of enzymes, involving activation, inhibition, disassociation of subunits, covalent modification or digestion. The underlying reactions are basically well known today but are not always well understood on a regulatory molecular basis. The substrates are the primary and also autonomous effectors for the intact cell, representing the driving force for activating or arresting mass transformations. For instance, the mechanism of signal transmission originating just from the presence or absence of a carbon and/or energy source in the medium or a cell, and the resulting metabolic regulatory effects, namely cell proliferation, switching between different physiological states, assimilation or mobilization of storage materials or secondary metabolite formation, are of tremendous importance. Besides mass transformation as such, rates of reactions in an intact cell are of paramount importance for the overall physiological result. Dynamics of metabolism are often underestimated and reach surprisingly high rates within the intact cell. The time scale in glycolytic oscillatory control may well be of the order of fractions of seconds (Aon *et al.*, 1992). This implies, further, that adequate analytical techniques must be employed in order to avoid artefacts. Recently, Benthin *et al.* (1992) pointed out that kinetics of glucose uptake can differ considerably with various cell types because cells may be anomerically specific for either α-or β-D-glucose.

Pulsed addition of glucose to a carbon-limited continuous culture of a wild-type baker's yeast results in a dramatic decrease of intracellular ATP concentration within only a few seconds. This effect was predicted by Nielsen *et al.* (1991) and was ascertained experimentally only very recently because of the necessity to withdraw, cool and pretreat samples within a second (or even less time; Theobald *et al.*, 1993) prior to analysis. On a longer time scale, that is within a few tens of seconds, the culture fluorescence, concentration of carbon dioxide in the exhaust gas, and extracellular ethanol concentration also increase (Beyeler *et al.*, 1981; Beyeler, 1982). Only later, within the first few minutes after glucose addition, does the intracellular cAMP concentration increase (Thevelein, 1991). Obviously, both uptake of glucose by cells and phosphorylation are not rate limiting and very rapid (ATP-sink; kinetic control) whereas transfer of information concerning the presence of glucose through cAMP and cAMP-dependent protein kinase is slower and possibly delayed. And it is not specifically glucose which can signal the presence of carbon and energy sources. Ethanol, acetic acid or pyruvic acid can also successfully and reproducibly be used to force entry of a yeast sub-population into a new round of the cell cycle (Münch *et al.*, 1992). The cAMP-dependent protein kinase is known to activate key enzymes of glycolysis and to mobilize storage carbohydrates, inactivate key enzymes of gluconeogenesis and synthesis of storage carbohydrates, and to play a significant role in initiation of a new cell cycle (Entian and Barnett, 1992; Thevelein, 1992). However, the mechanism of information transfer from these nutrients is still unclear or even unknown.

In the following section, we describe recent developments in biotechnological measurements and controls which can effectively investigate and identify these missing links. Not only operational parameters, such as temperature, pressure and flow rates but also chemical and biological variables can reliably be measured and, at least partly, controlled. *In situ* sensors and intelligent analytical subsystems (such as flow injection analysis or gas chromatography, see Filippini *et al.*, 1991, 1992) can deliver continuous or highly frequent information without significantly disturbing populations; they are non-invasive with respect to the population. Contamination problems can be overcome by sound mechanical constructions and careful maintenance of hardware. The same holds true for improved mixing and foam control, although critical validation on a regular basis is highly recommended. Even integrated process operation must be regarded as a reliable and reproducible state of the art today. This basic concept of exploiting high-technology cultivation equipment with near-perfect performance opens a new and additional access to biological research, which is important because genetical and biochemical methods alone (i.e.

work with mutants and isolated enzymes *in vitro*) cannot finally explain all dynamic phenomena observed in growing cells.

A. THE CONCEPT OF METABOLIC OVERFLOW

Many microbes and cells are known to form and excrete by-products of primary metabolism when growing under so-called balanced growth conditions, that is not when limited by carbon substrates. For instance, a wide variety of bacteria, such as *Escherichia coli, Bacillus* or lactic acid bacteria, excrete acetic or lactic acid when grown on readily available carbon sources. Also, many yeasts produce ethanol, glycerol, short-chain fatty acids and even propionic acid, whereas mammalian cells excrete predominantly lactic acid but also many amino acids. This by-product formation is obviously a very common phenomenon under growth conditions involving high specific substrate consumption rates. In principle, only a little of the reducing power generated during catabolism is needed for synthesis of new cells. Reducing equivalents can be scavenged by reacting with accumulated carbon intermediates (Ratledge, 1991). Under conditions of restricted respiration, a large formation of reduced carbon compounds is therefore needed to provide a small amount of energy. This is the reason why the respective pathways were termed "reductive" (Sonnleitner and Käppeli, 1986). In generalizing this phenotypic effect, we identify common properties which, in turn, might well be based on equivalent (or even common) and (highly) conserved molecular principles.

It is plausible to speculate that the same principal mechanism is responsible. An economic survival strategy for organisms is likely to be the objective of selection during evolution. Limiting environmental conditions are the rule, while abundant substrate availability is rather the exception in natural habitats. Rapidly depleting the environment of readily available carbon- and energy sources in order to out-compete potential rival organisms is one strategy. But saving most of the carbon and energy in a less accessible and preferably inhibitory form for later use when excreted, on the other hand, also seems a reasonable ploy. An alternative, but limited, solution is intracellular storage of reserve material. The first strategy requires only a few catabolic enzymes to be present in sufficient quantity and with potentially high activity all of the time. It allows cells to save a great amount of resources for enzymes on other catabolic and anabolic pathways during growth and/or survival under limiting environmental conditions. As a consequence, a considerable fraction of the carbon flux through initial catabolic steps must be excreted, most probably at the level of C_3 or C_2 molecules, as long as catabolic and anabolic sequences are rate limiting; this is what we refer to as a metabolic overflow reaction.

It is in the scope of the organism to reconsume excreted compounds later under conditions of limiting supply of primary substrate(s). It is very reasonable and economic for a cell to regulate the necessary or unnecessary pathways by controlling the amounts of enzyme activities. For instance, there can be mutual exclusion of opposite pathways in order to minimize so-called futile cycles. For instance, inactivation of gluconeogenetic enzymes starts 5–10 min after addition of glucose and is completed within 1 h (Entian and Barnett, 1992). However, mitochondrial cytochromes do not show a significant change during this period (Petrik *et al.*, 1983).

Yeasts provide an appropriate model system to study the overflow reaction because they possess various regulatory mechanisms. Characteristic representatives are *Saccharomyces* species which produce overflow products in response to glucose and oxygen, *Candida* species which are sensitive towards oxygen but not glucose, and *Trichosporon* species, which are strictly aerobic and do not produce overflow products in significant amounts (Fiechter and Seghezzi, 1992). Strict coupling of substrate consumption to respiration of the last type is not yet established but supposedly is linked with regulation of sugar uptake (Fuhrmann, 1992).

In *Saccharomyces cerevisiae*, the molecular bottleneck in flooded catabolism has not yet been elucidated but it is thought to be linked to respiratory pathways (respiratory bottleneck; Sonnleitner and Käppeli, 1986). This approach is a compromise between unstructured black-box modelling and attempts to include all accumulated molecular biological knowledge into a very large and complex model (see fig. 1 in Sonnleitner, 1991). Essentially, those detailed models cannot yet be formulated in a closed form (describing growth and product formation of an active population) because there are still too many unknowns (unfortunate missing links between numerous well-known sequences). For example the precise signal for glucose repression is still unknown (Entian and Barnett, 1992) even though these authors had come to the finding that "glucose repression . . . is directly associated with hexose phosphorylation by hexokinases PI and PII" (Rose *et al.*, 1991). Other benefits of such an approach are that the relatively simple model predicts the phenotypic behaviour of a population and it can be quantified even on-line on a powerful process computer. Furthermore, the otherwise weak points of this type of model can be scientifically exploited to identify key functional elements within a rigid metabolic control network just by simulating model variants (mechanistic alternatives) and experimental discrimination.

Biomass yields are significantly higher for respiration-linked growth than for anaerobic growth:

$$Y_{\text{X/glucose}}^{\text{respiratory}} \approx 0.5 \text{ g g}^{-1}$$
$$Y_{\text{X/ethanol}}^{\text{respiratory}} \approx 0.7 \text{ g g}^{-1}$$

whereas

$$Y_{\text{X/glucose}}^{\text{non-respiratory}} \approx 0.05\text{–}0.1 \text{ g g}^{-1}$$

which is similar to the value found under anaerobiosis. The only optimal solution when the objective is to maximize growth (or yield) for a given specific substrate flux (q_S) is a preference for respiration-linked pathways as long as the respiratory bottleneck is not saturated (in a chemostat, the dilution rate, D, is less than D_R, that is the dilution rate at which the regulatory switch occurs). It should be noted that the specific growth rate μ is correlated with the specific substrate consumption rate q_S via the yield $Y_{X/S}$:

$$\mu = q_S^{\text{respiratory}} \times Y_{\text{X/glucose}}^{\text{respiratory}} + q_S^{\text{non-respiratory}} \times Y_{\text{X/glucose}}^{\text{non-respiratory}}$$

If both the primary substrate (glucose) and product(s) of the overflow reaction (ethanol) are present in the culture, the primary substrate is consumed with clear priority. If the concentration of the primary substrate is high enough to permit an even greater substrate flux (supercritical; in a chemostat: $D > D_R$), the optimal solution is complete exploitation of the respiratory capacity together with utilization of the non-respiratory pathway thereby resulting in a metabolic overflow.

A clear advantage for the organism is excretion of an overflow product that inhibits substrate consumption and/or growth of rival organisms. However, it is normally only a question of concentration that discriminates rivals from the producer organism with respect to sensitivity or tolerance. Ethanologens also suffer from ethanol inhibition as do acid producers from acid inhibition. It could be shown for *Sacch. cerevisiae* that the overflow product, ethanol, decreases respiratory capacity (Käppeli *et al.*, 1988; Fig. 2). Generalized, this implies that a metabolic overflow reaction amplifies itself (negative feedback control). This fact explains the empirical experience accumulated, for instance in yeast factories, that simply a decrease in feed rate (to $0 < D < D_R$) is not an appropriate means to force an ethanol-producing culture back into the fully respiratory state after it had started formation of the overflow product. The method of choice was always to stop substrate feed entirely ($D = 0$) and restart it only after the overflow product has been completely consumed. In other words, a culture displaying metabolite overflow tends to adhere to this physiological state. But there are indications from older studies (Barford *et al.*, 1981) as well as from recent, reliably reproduced experiments (manuscript in preparation) with highly-improved equipment that cells

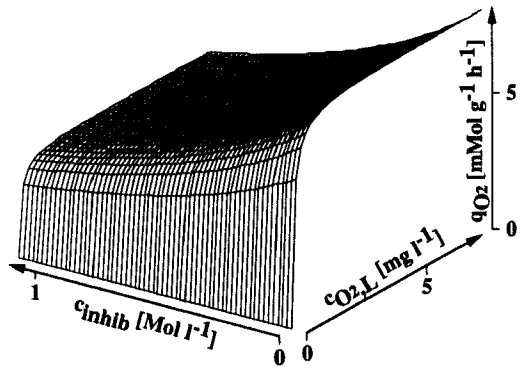
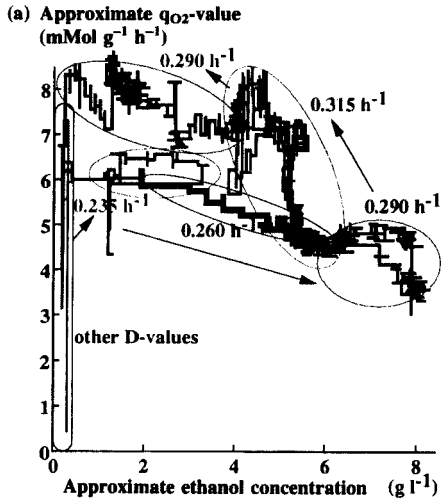
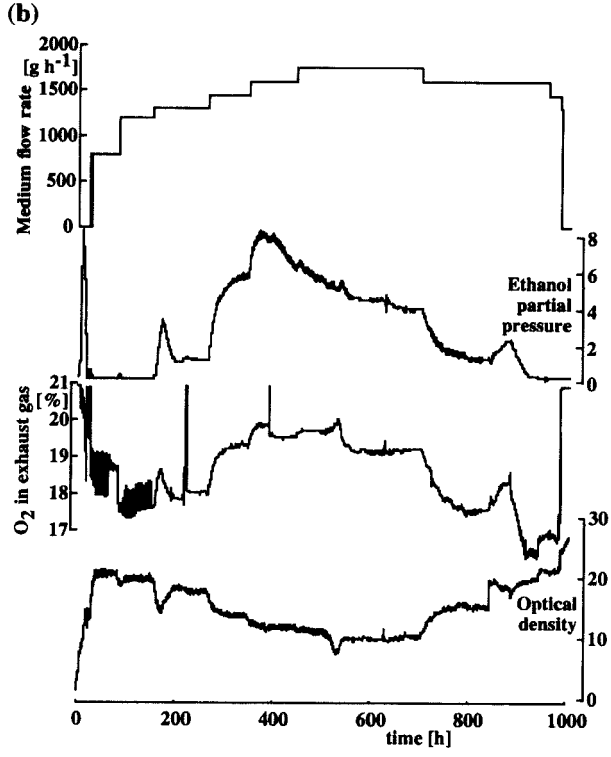


FIG. 2. Respiratory capacity (expressed as potential maximal q_{O2}; axis upwards) of *Saccharomyces cerevisiae* depends both on oxygen availability (limitation branch; axis towards the right: dissolved oxygen concentration $c_{O2,L}$. Monod-type limitation is assumed) and the presence of an overflow metabolite (inhibition branch; axis towards the left: concentration of the inhibiting substance c_{inhib}. Non-competitive inhibition type is assumed). The K_0 value (saturation parameter) has so far not been precisely identified but seems to be in the order of less than 0.1 mg l^{-1}. The K_I value (inhibition parameter for ethanol) has been determined by Käppeli *et al.* (1988) to be 1 mol l^{-1} from steady-state chemostat experiments at a D value of 0.35 h^{-1} with ethanol being endogenously produced from various substrate-input concentrations (10–100 g l^{-1}).

FIG. 3 (opposite). Development of specific oxygen uptake rate (q_{O2}) over a long-term experiment (more than 1000 h) as a function of ethanol concentration in the culture of *Saccharomyces cerevisiae* (a). The respective raw data are shown in (b). Dilution rate was increased from values permissive for spontaneous oscillation up to 0.315 h^{-1} and then decreased again (direction indicated roughly by arrows). In spite of the long duration and a well-tuned control loop for dilution rate, steady states have obviously not been reached under operating conditions where ethanol is present in the culture. Individual data presented are therefore surrounded by oval areas instead of being plotted as distinct points (dilution rates are indicated nearby). Interestingly, the culture seems to have adapted to increased respiration in the presence of ethanol. The first (i.e. during shift-up) operating levels at D values of 0.235, 0.260 and 0.290 h^{-1} were characterized by increasing ethanol concentrations and decreasing respiration values, whereas the typical steady-state areas during shift-down showed characteristically lower ethanol concentrations and higher respiration values; the respective D value of 0.26 h^{-1}, for instance, was definitely in the pure oxidative state, and is hidden in the leftmost area labelled with other D values. Dilution rate was under closed-loop control. Flow rate of fresh medium was controlled better than ± 15 g h^{-1} (which is less than ±1% at a D value of 0.315 h^{-1}) and culture volume better than ± 1.5% (i.e. ± 90 g for a 5.6 kg culture but around 100 kg total reactor weight). Absolute values given on the co-ordinates are only approximate because q_{O2} values were calculated using on-line data of optical density which do not correlate perfectly linearly over the entire operation range, while ethanol concentration was estimated from partial pressure measurements obtained with a membrane-inlet mass spectrometer where permeation properties of the membrane are known to vary with culture composition and with time. However, the orders of magnitude and ratios of values are significant. (b) Time-courses of dilution rate, ethanol partial pressure, oxygen in the exhaust gas and optical density. Aeration, pressure, pH value and stirrer as well as foam-separator speeds were kept constant at 1.0 volume air per volume liquid culture per minute, 1.10 bar (abs.), pH 5.0, 1500 and 2000 min^{-1}, respectively.


(a) Approximate q_{O2}-value
(mMol g^{-1} h^{-1})
0.290 h^{-1}
0.315 h^{-1}
0.290 h^{-1}
0.260 h^{-1}
other D-values
Approximate ethanol concentration (g l^{-1})


(b)
Medium flow rate [g h^{-1}]
Ethanol partial pressure
O_2 in exhaust gas [%]
Optical density
time [h]

may adapt to the adverse conditions created by excreted ethanol over very long periods. Obviously, sensitivity towards ethanol decreases with time, and respiration increases towards the strain-specific maximal respiratory capacity. However, the long time required (a typical experiment is shown in Fig. 3) seems to be beyond the timescale required for metabolic regulation at the transcriptional level. We have not consistently been able to establish stable steady-states within a few 100 h periods although cultivation parameters were under tight closed-loop control (accuracy and precision are given in Sonnleitner, 1991) and dilution rate was controlled at better than ± 1% of the preset value. It might well be that two alternative metabolic strategies, namely maximizing growth yield and energy efficiency, perform comparably well under these conditions and the cell has no significant basis to decide which of the strategies to choose. If the advantage of one strategy over the other is only marginal, then the driving force to approach the respective steady state is non-essential and the time required is prominent. The presence of an electron-transport chain results in more energy dissipation for each mole of carbon in biomass, compared with its absence. Electron-transport phosphorylation requires further biochemical reactions resulting in additional Gibbs energy dissipation of some 50–150 kJ for each mole of carbon (Heijnen and van Dijken, 1992).

A quantitative theoretical analysis of the bottleneck model extended with ethanol inhibition leads to the conclusion that multiple (at least two) steady states exist in a narrow window of specific growth rate ($\mu = D < D_R$) depending on the trajectory used to establish this distinct specific growth rate; however, the existence of steady state when D is greater than D_R is not excluded. Some experimental evidence is given by Axelsson *et al.* (1992) while another example is shown in Fig. 4. It is worthwhile noting that the predicted time required to reach a steady state is eventually around 100 generations. Although the bottleneck model is very simple and does not consider any regulation at the transcriptional or evolutionary levels, it just contains stationary rate equations including a negative feedback loop caused by ethanol inhibition. A rough estimate of dominating time constants using a linearized (i.e. further simplified) model revealed that typical time constants when D is less than D_R may be expected to be of the order of the mean residence time but, when D is greater than D_R, they should be at least ten times larger (Axelsson *et al.*, 1992). Jørgensen *et al.* (1992) confirmed this finding with a different experimental approach. They used an ethanol-auxostat, which is a continuously operated culture in which the dilution rate is no longer the independent variable. Ethanol concentration is the control variable and is held constant by tuning the dilution rate. Using this apparatus, they increased the set

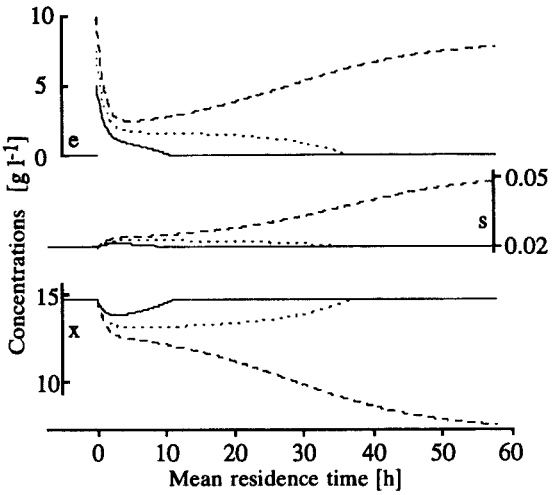


FIG. 4. Simulation of the effect of various ethanol pulses (5, 7.5, 10 g l^{-1}) on a culture of *Saccharomyces cerevisiae* growing in steady state when D is less than D_R. The simple bottleneck model was modified only by non-competitive ethanol inhibition (see Fig. 2). It is important to note that two different steady states can be reached in these experiments, depending on the extent of ethanol inhibition. At low concentrations, the original, fully respiratory steady state is reached, at high concentrations of ethanol, however, a new steady state with mixed metabolism is established (Axelsson *et al.*, 1992). Most experimentalists would misinterpret the intermediate horizontal trajectories of the state variables as steady states because the duration is approximatley 20 mean residence times. However, the entire experiment (200 h each) is unlikely to be undertaken due to the time required. (a), Ethanol concentration; (b), glucose concentration; (c), biomass concentration; ——, 5 g l^{-1} pulse; . . . , 7.5 g l^{-1} pulse; - - - , 10 g l^{-1} pulse.

point for ethanol concentration stepwise. First, the dilution rate increased above D_R, then it decreased slightly and, finally, increased again indicating the existence of three distinct steady states in a narrow range around the value for D_R.

B. REGULATION OF THE YEAST CELL CYCLE

Saccharomyces cerevisiae is an interesting model system for investigation of dynamics and control of the cell cycle. This yeast is a simple eukaryote with a complete cell cycle. Molecular biology has shown that the most important mechanisms of cell-cycle control are highly conserved among all eukaryotes (Nurse, 1992). But, most profitable for experimental studies, this yeast can be easily grown in spontaneously synchronized continuous culture.

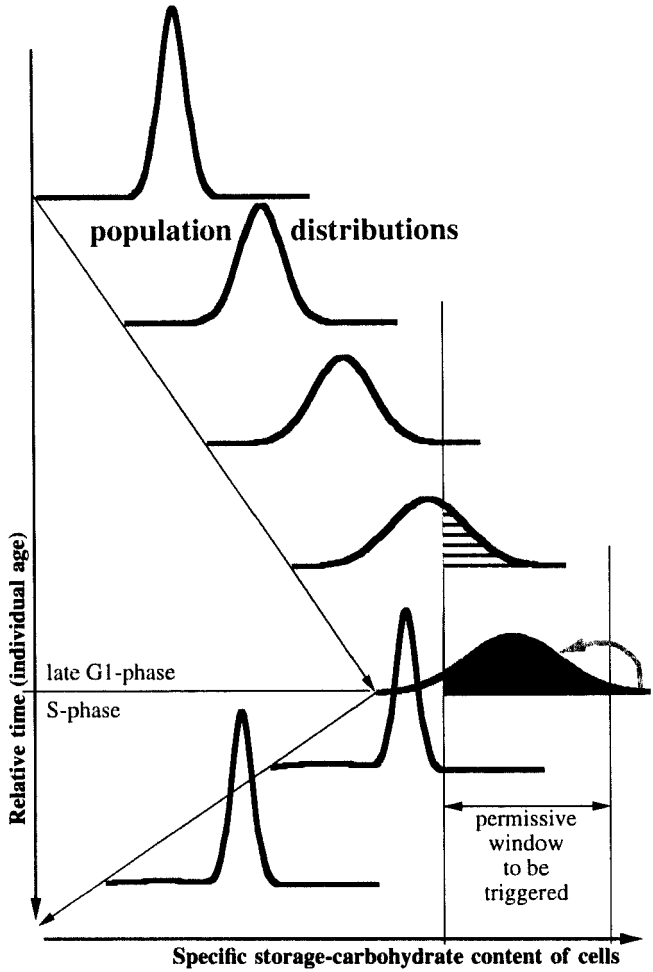


FIG. 5. Schematic development of population distributions during the cell cycle of *Saccharomyces cerevisiae*. The abscissa denotes the specific storage carbohydrate content of cells as a function of characteristic properties decisive for progression in cell cycle. The right border of the permissive window is thought to be the critical level of storage material content triggering entry into S-phase autonomously. Cells in the permissive window, however, are susceptible to external triggers such as extra carbon-containing compounds and/or energy supplies. As a synchronous population (or subpopulation) develops with time (down the ordinate), its distribution variance increases (indicated as broadening distribution curves). This would end up in an ideally asynchronous population distribution (horizontal line) if there were not some re-synchronization signal. This signal is thought to be generated by cells in the very leading head of the broadening distribution as they reach the upper limit of specific storage carbohydrate content. They enter the S-phase sponta-

The current understanding of spontaneously synchronizing chemostat cultures of *Sacch. cerevisiae* involves several key elements. Before describing these elements, it should be recalled that spontaneous synchronization occurs in a permissive range of dilution rates (D) which lie above the range of dominating maintenance metabolism but in the range of pure oxidative growth. Approximate values, here for strain H1022 at pH 5 and 30°C, are $0.1\ h^{-1} < D_{permissive} < 0.22\ h^{-1} << D_R \approx 0.3\ h^{-1}$. Turning to the elements, this strain's metabolism is significantly determined by a metabolic overflow reaction, in that ethanol is excreted whenever the respiratory bottleneck is overloaded (either by a supercritical glucose flux or by oxygen limitation) while ethanol can be reconsumed as an extra carbon and energy source whenever available and when the respiratory pathways are not saturated. This strain is known to build up carbon storage compounds even under carbon-limited growth conditions and to remobilize them during the cell cycle. Specifically, the storage carbohydrates, namely trehalose and glycogen, are utilized by cells during their S-phase. Küenzi and Fiechter (1969, 1972) have shown that a high content of trehalose is necessary for a cell to procede from G1- to S-phase. Trehalose is accumulated during early G1-phase; this process is non-permissive for initiation of transition to S-phase even by external triggers, but the late G1-phase is permissive (Münch *et al.*, 1992). A reasonable explanation for this mechanism is that it provides sufficient carbon compounds and energy for completion of DNA replication even under, possibly, adverse environmental growth conditions; the S-phase is the most subtle and decisive phase of the cell cycle. The dominating aspect of carbon metabolism is catabolized through the non respiration-linked pathway and, thus, results in formation and excretion of ethanol. In turn, most cells within the population, which are not in their S-phase at this time, are able to consume this extra substrate. They do this rapidly when D is very much less than D_R and this is the reason why only little ethanol (i.e. 100 to 150 mg l^{-1}) accumulates in the medium.

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neously and excrete ethanol and other metabolites to a smaller extent. The ethanol produced, in turn, signals and triggers the fraction of cells lying in the permissive window, which immediately respond by entering the S-phase (indicated by the bent arrow). The result is a sudden decrease in the variance of the distribution of this subpopulation, that is a resynchronization. This effect is rapid and efficient enough to explain the rapid occurrence of synchronous oscillations when a continuous culture is shifted to permissive conditions (i.e. basically $0.1\ h^{-1} \leqslant D \leqslant 0.22\ h^{-1}$ at pH 5.0 and 30.0°C under conditions of glucose limitation). Acceleration of cells belonging to another subpopulation, as assumed and modelled by Strässle *et al.* (1989), seems to be a minor side effect but acts in the same direction by narrowing the distribution of that population.

This acceleration function of ethanol is sufficient to explain both the occurrence of oscillations even from an ideally asynchronous population start point and the character of once established oscillations (Strässle *et al.*, 1988). However, this model predicts the evolution of oscillations to be much slower than observed experimentally (Strässle *et al.*, 1989). More recent studies (Münch *et al.*, 1992) have indicated that several classes of cells located just before the start-event into the S-phase are sensitive to triggers. Although the storage contents of these cells has not yet reached a maximum value which spontaneously initiates transition into the S-phase, the cells obviously sense an enriched environment (i.e. more carbon and energy source in the medium than under steady state conditions) and respond immediately. The cells exploit this fortunate (that is less adverse than normal) condition and enter the S-phase prematurely. As indicated in Fig. 5, this mechanism narrows the naturally broadening population distribution repetitively (every cycle) and is one cause for the pertinent character of culture synchrony. Münch *et al.* (1992) have shown that ethanol is obviously the natural, but not a specific, trigger. Glucose, acetic acid or pyruvic acid could be used as well to force the respective subpopulation into the S-phase. Faint pulses of these compounds applied at stated time intervals, are a suitable means to shorten the cell-cycle stably and reproducibly (within limits; Fig. 6). The independence of this effect on the nature of the added carbon and energy source indicates that there exist cellular sensing systems for many of these compounds. This could explain the very strong and pertinent self-synchronizing character of oscillating yeast cultures on a molecular basis: the intracellular sensor for the carbon or energy source might activate a kinase, which is identical with, or acts on, $p34^{cdc2}$. This protein kinase (MW = 34 000) is encoded by gene *cdc2* and is known to be very highly conserved in evolutionary terms (Nurse, 1992) and, in an activated form together with other factors such as G1 cyclins, to be responsible for controlling further downstream factors regulating the start event and S-phase. A positive feedback control loop among some of these factors could easily lead to very rapid activation of $p34^{cdc2}$ in response to slightly changing environmental conditions by self-amplification. Such a cascade would be practically equivalent to a digital (off/on-switching) control system (Johnston and Lowndes, 1992). This specific system might prove useful as an example for other regulatory switches.

Thevelein (1992) describes a general glucose-sensing complex in detail. He speculates that a cAMP-dependent protein kinase is likely to be an integrator for general nutrient availability, but does not provide any evidence for cellular sensors other than for glucose. Release of phosphate limitation by a phosphate pulse to a chemostat culture results in an increase

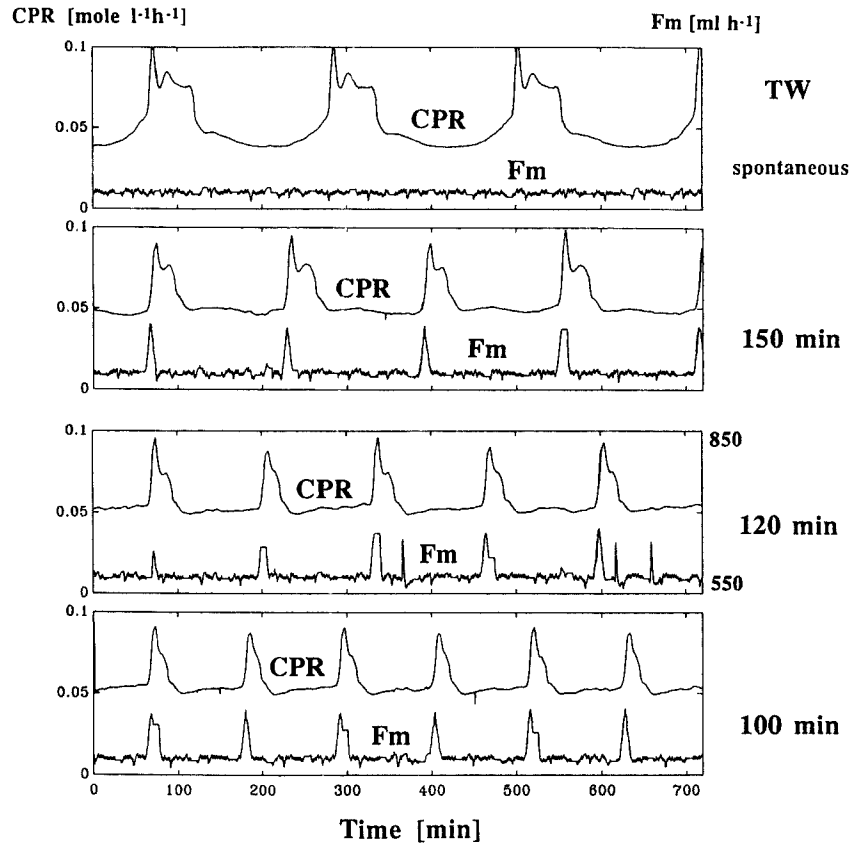

FIG. 6. Synchronized cultures of *Saccharomyces cerevisiae* can stably be forced to adapt the cell cycle period to an extracellular trigger. The upper plot shows the spontaneous behaviour of this wild type yeast at $D = 0.13\ h^{-1}$ and pH 4.0; the medium flow rate (F_m) is controlled at 550 ± 4 ml h^{-1} (which is better than $\pm$ 1%). If the medium flow rate is increased for a short period (for 10 min in the example shown) by approximately 10% (maximum), the culture adapts to the period of trigger events after two or three oscillations only (transients not shown) and remains stably oscillating under the new conditions (forcing periods as indicated on the right margin: 150 min; 120 min; 100 min). It is worth mentioning that the extra amount of glucose added during the phases of increased flow rate is as low as 60 mg l^{-1} (i.e. the integral over the roughly triangular peak) whereas the constant substrate feed contains 30 g l^{-1}. The flow rate increase can be substituted by an equivalent pulsed addition of solutions of either ethanol, pyruvic or acetic acid and the culture responds equally (data not shown). From Münch *et al.* (1992). CPR, carbon dioxide production rate.

in glucose consumption rate within less than three minutes. This could be experimentally verified only recently by using a sensitive glucose-flow injection analysis operated at a frequency of one measurement each minute (Rothen, 1992). Although the specific trehalase activity decreased by 70–80% during the first hour of an oscillation in a synchronous yeast culture, when determined in a crude protein extract, after treatment with cAMP-dependent protein kinase, it increased by approximately 30% (Weikert, 1992). These findings confirm the idea of a nutrient availability sensing mechanism based on and acting by regulation via post-translational modification rather than via *de novo* enzyme synthesis.

The method of choice in these investigations is to study large proliferating populations. Experimentally, this can be achieved by analysing a statistically relevant large number of individual cells in an undisturbed culture *in vivo*. High-performance bioreactor equipment (Sonnleitner, 1991) and on-line process analyses can provide a suitable hardware basis. Theoretically, structured and segregated models are capable of reflecting key elements in the cell-cycle dynamics and control. One objective of this highly automated non-invasive methodology is, so far, a causal-analytical search for triggering events and principal mechanisms of cell-cycle control with non-destructive methods.

Prerequisites for such an enterprise are highly efficient cultivation systems including sensitive measuring devices and control hardware and software. These must be suitably designed to reflect relevant relaxation times in the cultures under investigation. These include sampling systems and sample pretreatments compared with direct *in situ* and continuous measurements. Analytical methods used must allow quantification with appropriate resolution and reliability. Studying transient-system states rather than steady states only is essential in order to elucidate population dynamics. Forcing the culture is advantageous in addition to studying its spontaneous behaviour. Synchronously dividing yeast continuous cultures are therefore an ideal model system.

Desirable analytical tools include direct hardware sensors and instruments which permit both structured and segregated analyses of cell populations. Optical detection and quantification of specifically marked cellular properties are preferred; these include for example flow cytometry. Unfortunately, this is presently available as an off-line method only. However, cultivation as well as analytical techniques must be highly reproducible and reasonably able to cope with the relevant time constants of the culture.

The methodology referred to is a valuable additional, but not superseding, alternative to current molecular and developmental biological methods for cell-cycle research. One of its strengths is that it provides an opportunity

to elucidate the dynamics of cell cycle, that is its quantitative kinetic behaviour and not the simple sequence of events. It permits identification of macroscopic driving forces, such as cause and effect mechanisms, in the cell cycle, as well as metabolic control network.

C. RESEARCH STRATEGIES

All of the items already described have at least one aspect in common, namely the time constant for a cultivation is of the order of many hours (or days or even weeks). However, the relevant time constants for physiological control mechanisms are often in the range of seconds or minutes. As a consequence, a microbial or cellular culture must be observed with a frequency that is an order of magnitude greater than the frequency of the most rapid physiological phenomenon being studied. Further, the method of observation must neither disturb the culture nor influence its behaviour significantly. These considerations call for undelayed, continuous or highly frequent, non-invasive and automated supervision, i.e. measurement and control, in order to elucidate the dynamics of microbial and cellular growth, proliferation and product formation.

Trautmann *et al.* (manuscript in preparation) have successfully demonstrated that a well-established and widely accepted off-line method for estimating viable cell number concentration in cell culture (Chinese hamster ovary (CHO) cells counted after vital staining) is inaccurate. In a flow injection analysis, they determined the activity of lactate dehydrogenase in the supernatant as well as the total lactate dehydrogenase activity of the culture after forced lysis of cells in the continuously removed sample stream. Activity of the dehydrogenase in the supernatant was found to correlate perfectly with the proportion of non-viable cells in the culture. Viability predictions made from these data, unlike those from off-line data, coincided well with product-formation kinetics. This new automatic method is likely to raise general revision of methods used to measure cell viability and activity. Further, this is an excellent paradigm showing that even well-established off-line methods may be inappropriate tools for validation of measurements in general, of new methods and of plausible results. Research programmes should exploit the benefits of automation such as better resolution, higher reproducibility, constant accuracy, minimization of human error, efficient use of given time, and the most objective methods of validation or effective extraction of information from a flood of raw and reference data.

III. Experimental Background

A. CONTAINERS FOR CELL GROWTH

Containers for cell cultures have various tasks. First of all, they have to provide a sterile barrier which is necessary to maintain an uncontaminated culture over extended periods. The more closed such a system is, the easier is the realization of this goal. In other words, a fed-batch, continuous or even a continuous cell-recycle system is much more tedious to design and maintain than a container just for a simple batch operation. On the other hand, the more such a system is closed, the less information can be gained about its exact state and dynamic behaviour. But using sensors and sampling interfaces in classical analytical methods means that the system has to be opened more, increasing the probability of destroying the sterile barrier. However, there is no alternative to increasing knowledge about the processes, only the freedom of choice of more or less appropriate analytical equipment.

So-called culture parameters must be under sound supervision. It is not a good practice to decide at the beginning of a culture that conditions such as temperature, pH value and aeration should be kept constant and to rely blindly on the control loops from there on. Much better practice is to treat every measured signal *a priori* as variable. On-line during a cultivation, one may decide whether a signal actually behaves as variable (that is it changes with time) or as parameter which remains constant. Only this procedure ensures that any, even very short, deviation of any intentional parameter from the desired setpoint is trapped and can be documented. It is also a necessary requirement for tracing back and analysing any unexpected process behaviour. The example shown in Fig. 7 gives evidence that even small deviations from a setpoint may cause considerable variations in chemical and biological variables, and that return to the expected steady state may be quite delayed.

Besides these benefits, the advantage of applying this technique to all signals generated and collected is in general an effective decrease in data collection. Locher *et al.* (1991a) described a simple algorithm which works perfectly with window widths selected according to the expected noise of individual signals and the specified quality of the sensors and other instruments, whichever is the larger. Also, data provided in Fig. 7 have been treated with this algorithm and it is obvious that not any even rapid transient is lost.

Another decisive task with bioreactors is to provide sufficient capacity of mass and energy transfer. It is of tremendous importance to know safely what is measured in these bioprocesses, either kinetics of the cell popula-

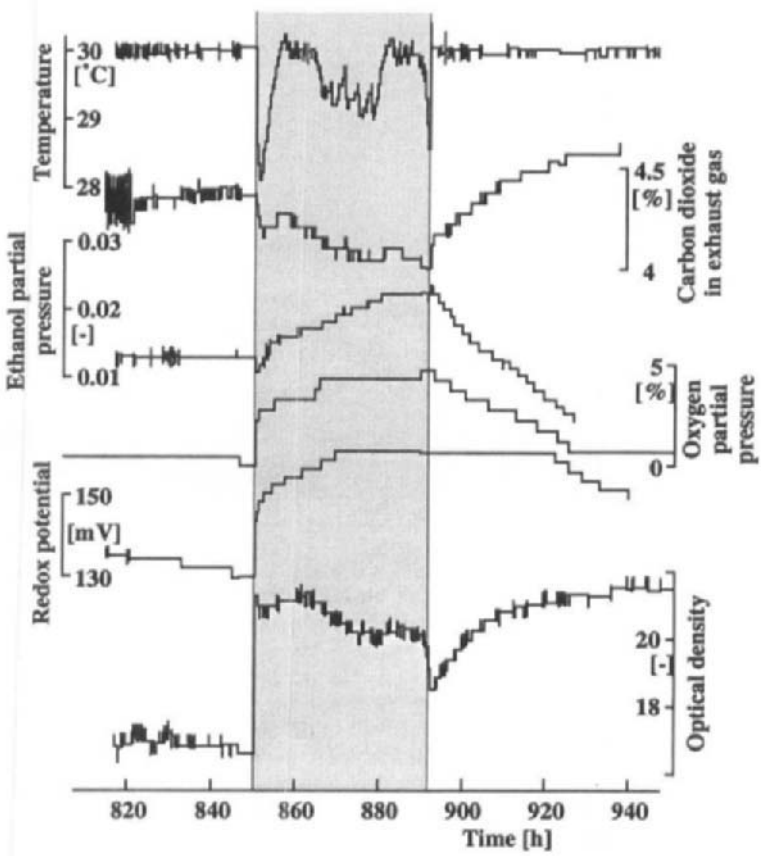

FIG. 7. Some properties of a portion of a continuous culture of *Saccharomyces cerevisiae* growing in a defined glucose-containing medium at $D = 0.32\ h^{-1}$, pH 5 and 30.0 ± 0.1°C. Until 850 h, the culture was obviously in a steady state. Due to maintenance works, the steam supply was then shut off for 2 days resulting in a temporary temperature decrease of less than 2°C (the controller was not able to operate equally without steam). It took more than 50 h after re-establishing the correct culture temperature to approach the original steady state again as indicated by the time-course of some sensor signals (carbon dioxide in the exhaust gas, ethanol partial pressure estimated from mass signal 31 determined by membrane inlet mass spectrometer, partial pressure of oxygen, redox potential and optical density determined with an Aquasant probe). The data illustrate the importance of precise control of culture parameters.

tion or a superposition of kinetics and physical transport phenomena. The target in biological research is clear. Therefore, reactors must have a high performance and approximate to an ideal reactor as closely as possible. Figure 8 conveys an idea of the consequences of this aspect. Responses

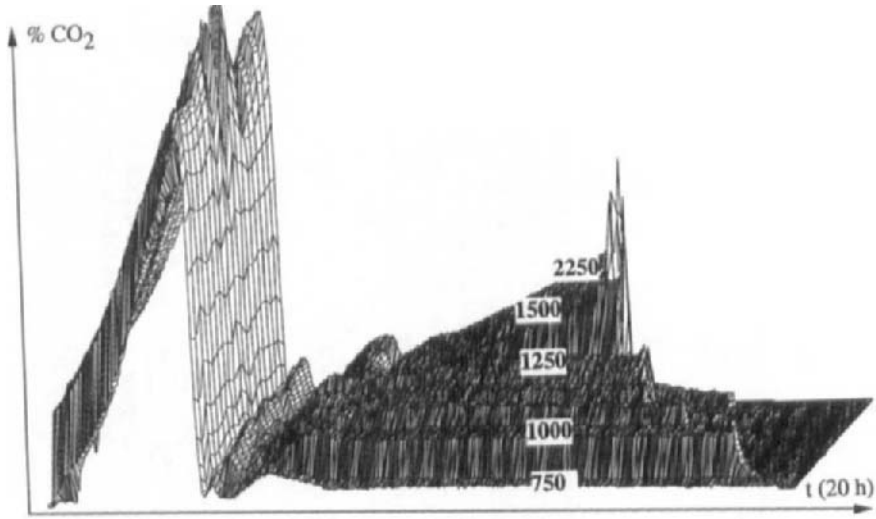

FIG. 8. Comparison of sensitive biological responses of *Saccharomyces cerevisiae* to physical mass transfer and inherent biological rate limitations. The yeast was cultivated in repetitive batch culture using a defined glucose-containing medium at different stirrer speeds (rev. min^{-1}) (as indicated by values on the figure) in a compact loop reactor. For simplicity, only the percentage of carbon dioxide in the exhaust gas is shown as a function of time for the individual experiments (third dimension of mesh plot). All other cultivation parameters were well controlled: T = 30.0 ± 0.05°C; pH value = 5.0 ± 0.05; aeration: 1.00 ± 0.01 vv^{-1} m^{-1}; pressure: 1.05 ± 0.01 bar. The culture was retained as an inoculum after harvest (0.20 ± 0.02 kg) and fresh medium (5.00 ± 0.02 kg) added using a batch stored at 15°C. Any decrease in slope of the time-course for evolution of carbon dioxide before the final peak indicates oxygen transfer limitation in culture(s). The minimal stirrer speed in this particular reactor system which permits investigation of growth kinetics rather than a badly resolvable superposition with the mass transfer capacity of the reactor is 1500 min^{-1}. From Sonnleitner and Locher (1992).

of a biological system to even faint variations in mass transfer are unequivocally sensitive and significant as long as this transfer is limiting.

B. OFF-LINE AND ON-LINE ANALYSES

Off-line analysis means that the procedure used involves partially or entirely manual operations, while on-line means that the complete measurement or procedure is fully automated. A significant advantage of automation is the elimination of human errors such as temporal variations in accuracy or precision during work. Currently, there are many automated analytical instruments commercially available for laboratory use, but suitable inter-

faces to *monoseptic* bio-processes are not. Development of these missing links must be energetically promoted in order to exploit the existing analytical know-how and hardware. In addition, procedures have to be established which guarantee the recalibration of instruments and sensors directly from the sterile interface. The interface itself may drift significantly, as with a microfiltration or pervaporation membrane, but to include this sterile barrier in the calibration does not seem to be realistic at present. Therefore, alternative analytical methods should be made available in order to validate results.

In situ means that a sensor or sampling device is mounted in the bioreactor; at-line is an alternative technical term. The opposite procedure involves withdrawal of samples (volume aliquots) which may be either discarded after analysis or, if the sterile barrier remains intact, recycled to the culture system. No matter to which of these classes an analytical method belongs, it may deliver either a continuous or a time-discrete signal.

C. CONTINUOUS AS DISTINCT FROM DISCRETE SIGNAL GENERATION

Sensors based on either electrical, optical or electromagnetic principles deliver normally a continuous signal; this is very convenient and also useful. The dynamics of the system being analysed can be resolved according to the time constants of the respective electronic amplification or indication equipment, so that data are most probably filtered and delayed, for instance of the order of milliseconds to several seconds. In the general case, however, the data will nowadays be digitized, that is discretized with respect to value. This information loss can normally be neglected because 12 or 16 bit converters are the state of art today. Further, data are stored in a computer at certain time intervals (e.g. every 15 s or 5 min) resulting in discretization with respect to time and concomitantly in a possible loss of information. Here, the already mentioned data reduction algorithm comes into beneficial play; raw data should be scanned with high frequency, say $\leqslant$ 15 s, but stored only with the necessary frequency, i.e. variably or not equidistant with respect to time.

Analytical instruments based on either physicochemical separation methods or biochemical reactions require a finite time to create results. These instruments are usually operated in repetitive, non-overlapping batch mode and deliver results with a true dead time; generally, data are produced with lower density, with a frequency, for instance, of the order of once per minute by a flow injection analysis or twice hourly by high-performance liquid chromatography.

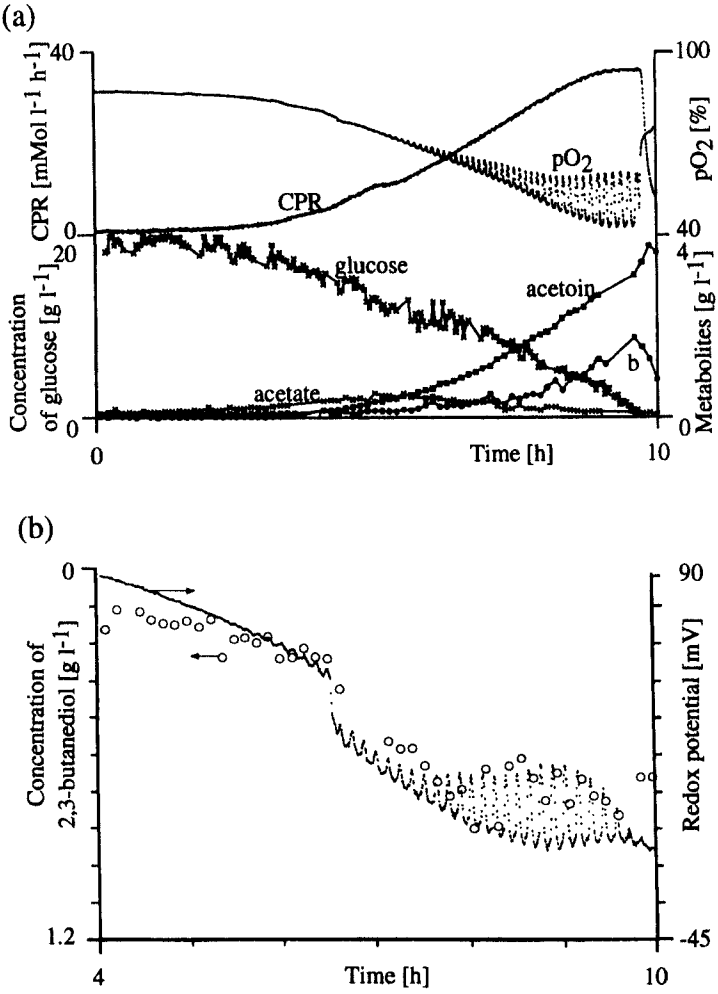

FIG. 9. Batch cultures of *Bacillus subtilis* growing in a defined minimal medium show a characteristic oscillatory behaviour, at least with respect to some continuously measured variables such as (pO_2) partial pressure of oxygen (**a**, according to Filippini, 1992) and redox potential (**b** according to Filippini *et al.*, 1992; note the inverse axis for butanediol). The typical period of an oscillation is approximately 6 min. However, the time necessary for on-line gas chromatographic analyses (concentrations of three metabolites shown: acetic acid, acetoin and 2,3-butanediol (abbreviated as 'b')) is 7–8 min. It is, therefore, impossible to resolve the oscillatory behaviour with respect to concentrations of excreted redox-compounds such as acetoin and butanediol. The scatter of the gas chromatographic data, which is significantly higher in the oscillation area, indicates a plausible correlation between redox potential or the partial pressure of oxygen and concentrations of the butanediol (O). The continuously measured state variables are plotted with dots at 15 s time intervals. CPR, carbon dioxide production rate.

It is merely a question of the dynamics of the biosystem under investigation as to whether time-discrete analyses will suffice to describe the behaviour with reasonable resolution or not. Typical examples are selected in Figs 9 and 10. This pitfall should be seriously considered in all areas of scientific research and at least during the early stages of industrial process development. A sensitivity analysis is highly recommended for each individual situation. Limiting substrate concentrations will most probably be incorrectly estimated if no *in situ* sensing device is available and/or procedures for sampling and sample pretreatment are inappropriate. The same must be expected for assay of metabolites with a short half-life, e.g. those excreted by one subpopulation and re-consumed by another one. Biomass concentration is certainly less prone to comparably erroneous determinations.

IV. Establishing Non-Invasive Strategies

A. MEDIUM DESIGN

Media for newly acquired microbes or cells are usually rich and nutritionally complex. Complex is the opposite of defined, which means that each of the medium components is chemically characterized (e.g. by a chemical formula and amounts of impurities) and accessible to analytical quantification. Causal analytical studies of metabolism, kinetics and regulation require the use of defined media. Conclusions arrived at are otherwise obscured due to ignorance of compounds that might act as effectors even in trace concentrations. In industrial bioprocesses, complex media are widely employed for economic reasons. However, extra costs resulting from removing unknown and unwanted compounds during downstream processing constitute a sound argument for substituting complex materials more and more by defined ones which have a proven essential function. Therefore, it seems worthwhile for both academia and industry to promote the design of defined media for microbial and cell cultures.

According to our experience, a very efficient method for substituting undesirable medium components, that is eliminating complex ones and introducing only the necessary defined ones, is based on evaluation of the responses of a population to transient disturbances. The basics of this method are quite old (Mateles and Battat, 1974) but its exploitation has remained scarce. When a population is cultivated under growth and/or production limitation by an undesirable medium component, pulsed or continuous addition of individual substances or defined mixtures thereof result in (a) a relaxation of limitation if this actually limiting, i.e. essential

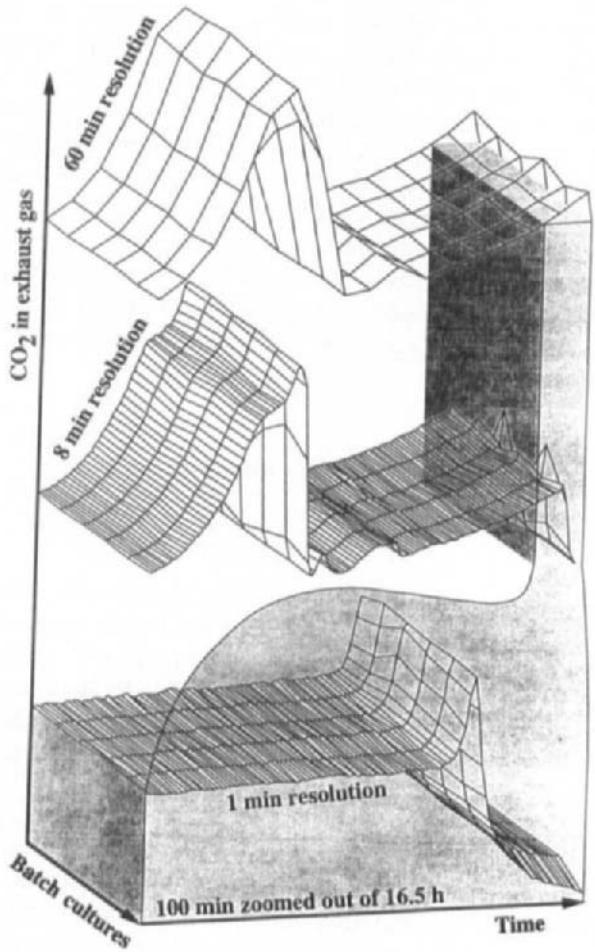

FIG. 10. Time courses of carbon dioxide appearance in the exhaust gas for six different batch cultures of *Saccharomyces cerevisiae*. The data from cultures using a defined glucose-containing medium are replotted with different time resolution: 60 min intervals on the top graph, 8 min in the middle and 1 min on bottom. The bottom plot, however, shows only the last 100 min (shaded area) of the above cultivations. It is important to realize that several fine structures of growth behaviour can only be resolved if the measurement frequency is high enough. Since the measurement of carbon dioxide evolution delivers a continuous signal, the graphs shown are only simulations. But they illustrate well how many details, such as non-exponential growth characteristics prior to complete glucose depletion in a chemically defined medium (the shoulder prior to maximum evolution of carbon dioxide; could be associated with partial synchronization of the culture) or re-

or promoting; (b) a measurable decrease in performance (e.g. washout of cells or product) if inhibitory or toxic; or (c) an indifferent response (e.g. just noisy but virtually constant behaviour of key variables) if non-essential or anyway present in excess.

Figure 11 summarizes these various situations. It is important to realize that all individual elements composing the objective function for optimization must be analysed. Normally, this means that concentrations of biomass, product(s), and limiting and pulsed substance(s) must be determined analytically in experiments and the corresponding reaction rates must be considered in the evaluation, i.e. μ (and/or q_P) and q_S must increase initially to justify the conclusion of a positive response. If only one increases and the other decreases, it is most likely that an alternative compound has been added for which cells have a higher affinity although the substance is not essential. Such situations have been met for instance by Laforce (1987: Fig. 12).

Even in the case of Chinese hamster ovary cells for instance, Messi (1991) succeeded in developing a serum- and protein-free medium by applying the concept, though manually and not using a chemostat. The main achievements were the implementation of a well-known nutrient limitation by constructing a fully-defined medium which is, however, complicated because it contains more than 40 individual compounds but is no longer complex. Serum and even protein additions could be avoided. It is important to realize that essential medium components can also exert an inhibitory effect. The most prominent example is probably the ammonium ion and, in equilibrium, free ammonia, both of which are created in deamination reactions. Ammonia was found to be a more potent inhibitor than the metabolic overflow product lactic acid, but there are good opportunities to remove ammonia on-line by in-situ application of pervaporation membranes. Even though such an optimized synthetic medium permits balanced growth of the culture, the minimal generation

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consumption of pyruvic and acetic acids excreted during growth on glucose (small peaks after local minimum of carbon dioxide evolution), must be overlooked using a sampling rate of 1 h^{-1}. This is most obviously the reason for naming the so-called diauxic lag phase as a lag phase although the next higher resolution gives proof for a dynamic behaviour. Even an 8 min resolution does not identify the final peaks reliably, they are due to depletion of acetic acid accumulated during growth in the presence of ethanol. The duration of the final peak, approximately 5 min, is only 0.5% of the duration of the entire cultivation but the event itself is significant and reproducible. In conclusion, appropriate analytical frequency is not determined by the duration of a culture (i.e. the overall specific growth rate of an organism) but rather specific for each particular proliferating, regulated biosystem. According to Locher *et al.*, 1992b.

(a)

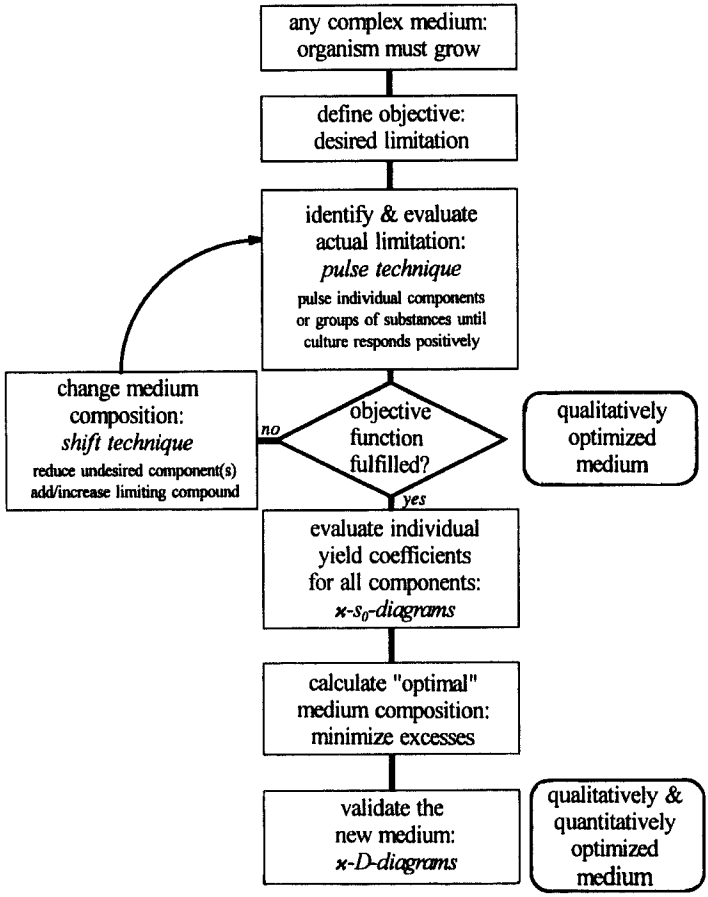
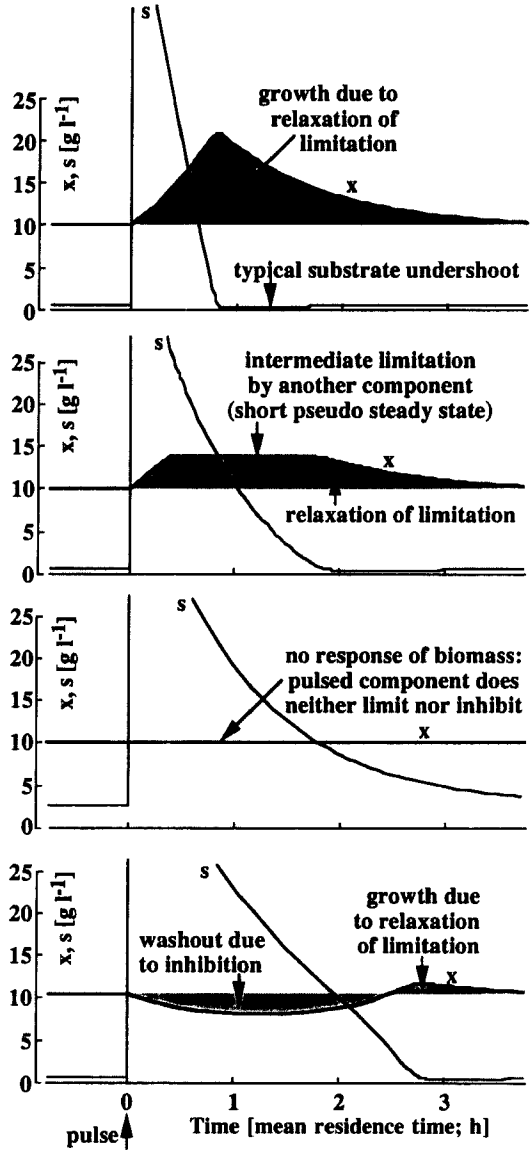


FIG. 11. Principles for the pulse and shift technique applied to medium development.

(a) Illustrates the concept of jointly exploiting different continuous-culture techniques, namely the pulse technique for positive identification of limiting medium components, the medium-shift technique for altering the quantitative medium composition and evaluating yield coefficients, and the classical technique of creating x-D-diagrams to investigate physiological properties. The starting point is a medium that just permits growth of the organism. The intermediate result is a qualitatively optimized medium, i.e. all unnecessary components are eliminated, a desired component limits growth, but the rest of essential components may be present in enormous excess. The final result is a quantitatively optimized medium with defined excesses of individual non-limiting substances.

(b)



(b) Depicts responses of cultures to a pulse of a potential medium component. The upper plot presents the situation which is easiest to identify in practice. The only limiting substrate is pulsed at zero time and immediately relaxes growth limitation resulting in accumulation of biomass. Substrate is both

times found in serum-containing media cannot yet be achieved. Decisive is the parity between growth factors spontaneously produced by cells during proliferation and intra- and intercellularly distributed (Gandor, 1993).

Locher *et al.* (1993; Fig. 13) have shown that such a procedure, including control of substance selection, addition and response evaluation, may be automated. This is reasonable to a limited extent only because preparation and modification of a series of pure sterile solutions is quite demanding. The crucial benefit is, however, that all 24 hours of a day can be exploited for experimentation. There are currently two further obstacles in employing it routinely. The first is that selection of compounds to be added, as well as their sequence or combination in mixtures, is still a matter of personal experience and decision rather than projectable by rules. Expert systems derived from successful data sets from a wide variety of organisms will certainly help in the future. Only a finite number of classes of compounds can reasonably be tested with this method, for instance mineral salts, trace elements, water-soluble vitamins or amino acids. These are all relatively stable in solution, do not adsorb to surfaces, are readily available and

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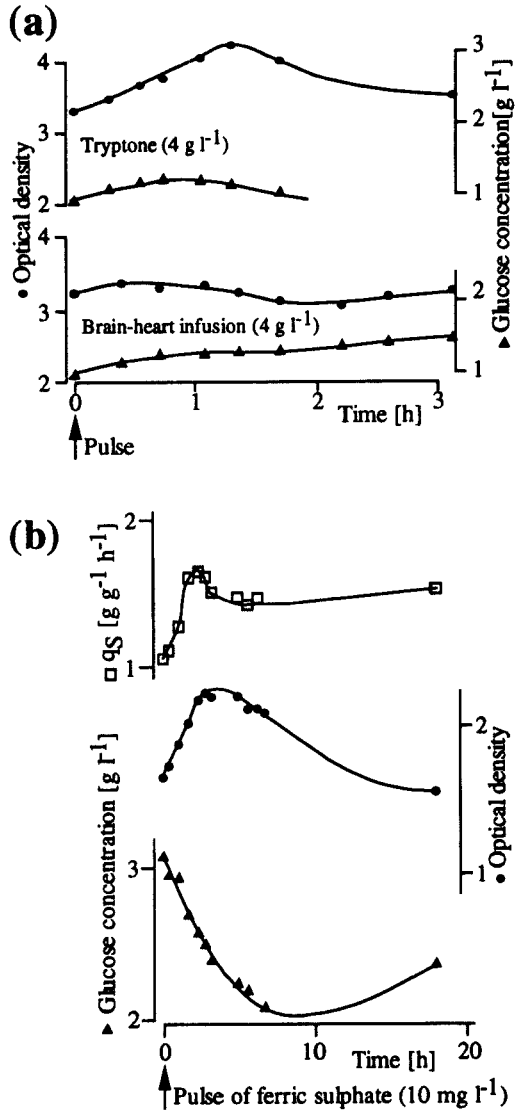
washed out and consumed by cells. There are too many cells (compared to the steady-state concentration) present when the substrate concentration becomes limiting again and approaches the steady-state value. Therefore, the volumetric substrate consumption rate is greater than in steady-state, while substrate concentration must fall below steady-state values which, in turn, lowers the specific growth rate (μ) of cells below the steady-state value (D). Cells then wash out and the system stabilizes at the uniquely stable steady-state. This undershoot of substrate concentration is typical but most likely never realized in experimental work, especially if the K_s value is low, i.e. around or below the limit of detection.

FIG. 12 (opposite). Examples of the pulse technique applied to medium development. From Laforce (1987).

(a) Shows two examples of negative pulses. Tryptone or brain–heart infusion were pulsed into a steady-state chemostat culture of *Bacillus acidocaldarius* grown in a glucose medium at a dilution rate of 0.4 h^{-1}, 65°C and pH 3.5. Both pulses (each at 4 g l^{-1}) resulted in an increase in biomass concentration (estimated by optical density; ●) *and* of glucose concentration (▲), i.e. the specific glucose-consumption rate decreased significantly after the pulse. This is sound proof for concluding that the chemically complex nutrients pulsed in did not contain the factor restricting complete glucose consumption. They were used by the organism as an alternative carbon source rather than as source of growth factors. The conclusion must be drawn that no component present in these complex nutrients was limiting growth of the culture and that the component pure compounds (e.g. amino acids) can be excluded from further experimentation. This device, namely to add complex mixtures of compounds, instead of pure compounds sequentially, can save a lot of time during experimentation. However, it does not necessarily result in such easy-to-interpret responses.



(b) Shows the results of pulsing a solution of ferric sulphate (10 mg l^{-1}) into the same culture, but using less yeast extract in the fresh medium. This addition relaxed an iron limitation positively as indicated by an increase of both biomass (estimated as optical density; ●) and specific glucose consumption rate (q_S).

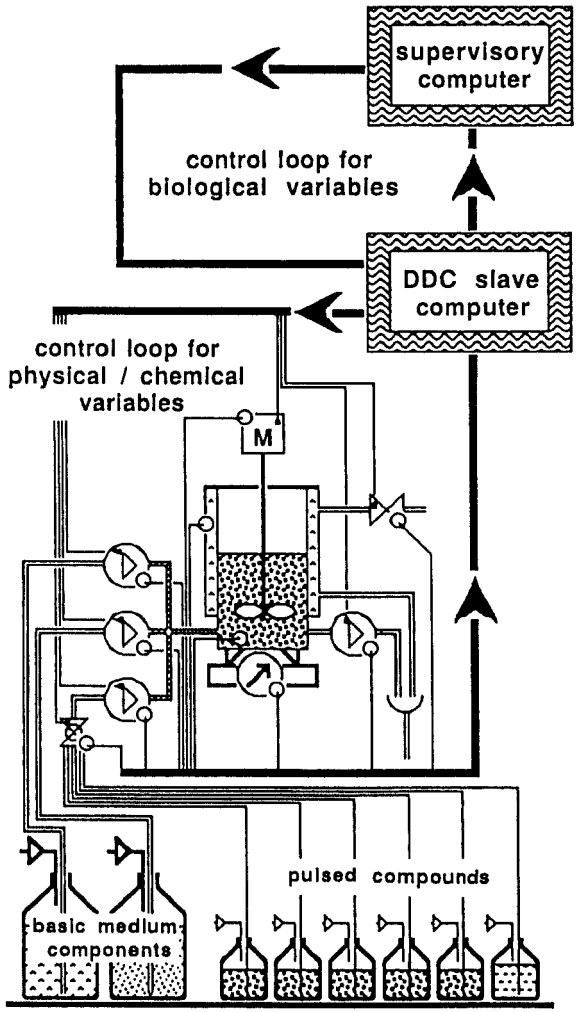


FIG. 13. Flow diagram of the apparatus required for automatic pulsing or adjusting medium composition on-line. Any particular component of the medium, such as carbon sources, vitamins or trace elements, can be stored as a pure solution or partial mixture and pumped into the reactor according to the pulsing or control strategy (determined either by the operator or by the supervisory computer). This allows identification of limiting and/or inhibiting compounds automatically or of compounds which influence cell metabolism, e.g. by switching carbon sources or establishing various other limitations. However, it must be emphasized that compounds can only be added; removal is normally not possible. Further, as this technique is based on flow of compounds into the reactor, it can only be exploited in fed-batch or continuous cultures. From Locher *et al.* (1992b).

inexpensive. Secondly, only a few compounds can presently be analysed on-line with sufficient reliability and this is a prerequisite for on-line evaluation and automated decision making.

The effectiveness of this method in testing individual compounds for either a promoting or an inhibitory regulatory effect has been demonstrated by Locher *et al.* (1991b). They applied repetitive pulses to batch cultures of micro-organisms in various growth phases and showed slightly variable responses of populations. Münch *et al.* (1992) exploited a variant of the repetitive-pulse technique to trigger premature entry of a yeast subpopulation into the S-phase of the cell cycle. Successful application and exploitation of these techniques require a sound experimental and theoretical concept, highly reliable and reproducible equipment (hardware) and appropriate software.

B. CLASSICAL COMPARED WITH INTEGRATED CULTIVATION AND PRODUCTION SYSTEMS

The two extreme modes of operation, namely batch and continuous culture, have both significant advantages as well as their disadvantages, a recent evaluation of which is provided by Heijnen *et al.* (1992). A practicable and reasonable combination results in a repetitive batch operation. Once the initial, classical batch culture is completed, only part of the culture is harvested and the rest, say 5%, is diluted with fresh medium thereby starting a new batch immediately. Naturally, the repetition can also be applied to fed-batch processes.

The mode of repetition saves dead times, permits distinction between individual runs and makes use of 24-hours per day. However, it is crucial, firstly, to determine the harvest time reliably, either in advance or on-line, and secondly to avoid any adverse conditions for the inoculum during harvesting and fill when it may easily happen that sensors, no longer submerged in the culture, generate irrelevant data and misfeed connected controllers.

Non-invasive measurements of a series of relevant state variables combined with pattern recognition have been shown to serve as an elegant and most reliable basis for automatic decision making (Locher, 1991). This method provides a sound basis for estimating and proofing the reproducibility of transient biotechnological processes.

Cell recycling is a proper means to prolong the productive period of a culture, increase the volumetric productivity of a culture, control specific growth rate fairly independent of dilution rate, which can be most useful for metabolite production. The specific growth rate (μ) is related to the dilution rate (D) by the recycle ratio R, provided the separated

supernatant flux is absolutely cell free, with R being (cell-free supernatant flux) / (total substrate feed flux) in the interval [0 1] ⟨0 ∝ classical chemostat operation; 1 ∝ total cell retention⟩: $\mu = (1 - R)\ D$. Cell recycling can provide alternative methods of selective product recovery, remove potentially toxic products thus eventually forcing relaxation of product inhibition, exploit membrane-mediated gas transfer and, most probably, improve handling of foaming systems as well.

Essential for the success of this mode of operation are closed-loop control of individual fluxes into and out of the reaction system, consideration of changing environmental and physical conditions, and tight control of mean residence time of cells in individual compartments of the system (e.g. well-mixed reactor, pump, filter or settler, void volume of tubing or piping, mechanical stress, oxygen depletion or product enrichment). If these aspects are appropriately considered and handled, a tremendous increase in productivity without sacrificing product quality can be achieved and maintained over extended periods (see for instance Nipkow *et al.*, 1986a, b; Leist, 1988; Gruber, 1991; Rohner *et al.*, 1992). The specific product-formation rate need not be necessarily coupled with the specific growth rate (μ). It might well depend also on the history of μ; this dynamic disadvantage, as it is called by Heijnen *et al.* (1992), can be elegantly overcome using a well tuned integrated process.

V. Conclusions and Outlook

This review demonstrates paradigmatically the application of new methods to approach biological objectives. The methods have predominantly been developed in bioprocess engineering and process control, but have proved beneficial also for scientific research. Prerequisites are, however, excellent cultivation equipment and impressive techniques for analysing biologically important components.

Adaptation for fully automatic bioprocessing is realistic, although some decisive aspects must be carefully considered. These include the robust sterile barrier between process and environment (e.g. cell inactivation after sampling, cell-free sampling, in-situ sensors), the possibly very small relaxation times of cultures, as low as one second, minimal volume aliquots or fluxes available for analyses (to minimize disturbances of a culture), reliability and long operating life of all hardware components, accuracy and precision of measurement and control elements.

Non-invasive cultivation methods can effectively contribute to a study of the regulation of central metabolic pathways. In general, substrate turnover rates determined *in vivo* have always been greater than those in

in vitro systems. The principle of the metabolic overflow reaction as a most rapid biological response to changing environmental conditions is therefore a powerful extension, not a substitution, of the regulation models derived from molecular biological disciplines for understanding fine tuning and dynamics of metabolic control in proliferating populations. A precise temporal resolution of individual reactions will undoubtedly allow identification of relative contributions of the different control mechanisms (kinetic control such as overflow, post-translational modification of biocatalysts, trancriptional control) to the observed *in vivo* phenotypic behaviour, and unify the presently still diverse views.

The new powerful quantitative methods enable us to describe certain states or effects in much more detail and with higher accuracy than hitherto. This provides a good opportunity to avoid unfortunately established imprecise and ambiguous terminology (such as "ferment . . .", "Crabtree effect", "derepressed", "late stationary" and the like: see for instance Ratledge, 1991) in the future and, instead, use quantitative state descriptions thus paving the way for a prosperous development from just fermentation to bioprocess sciences.

REFERENCES

Aon, M. A., Cortassa, S., Westerhoff, H. V. and van Dam, K. (1992). *Journal of General Microbiology* **138**, 2219.

Axelsson, J. P., Münch, T. and Sonnleitner, B. (1992). "ICCAFT 5 / IFAC-BIO 2", Computer Appl. in Ferm. Techn. and IFAC Symp. on Modelling and Control of Biotechnology Processes. p. 187. Pergamon Press, Keystone, CO.

Bailey, J. E. (1991). *Science* **252**, 1668.

Barford, J. P., Jeffery, P. M. and Hall, R. J. (1981). "Advances in Biotechnology I, Proceedings of 6th International Fermentation Symposium, London, Canada, 1980 (M. Moo-Young, C. W. Robinson and C. Vezina, eds), pp. 255–260. Pergamon Press, Oxford.

Benthin, S., Nielsen, J. and Villadsen, J. (1992). *Biotechnology and Bioengineering* **40**, 137.

Beyeler, W., Einsele, A. and Fiechter, A. (1981). *European Journal of Applied Microbiology and Biotechnology* **13**, 10.

Beyeler, W. (1982). *Chemie Ingenieur Technik* **54**, 498.

Entian, K. D. and Barnett, J. A. (1992). *Trends in Biochemical Sciences* **17**, 506.

Fiechter, A. and Seghezzi, W. (1992). *Journal of Biotechnology* **27**, 27.

Filippini, C. (1992). PhD thesis 9862, ETH Zürich.

Filippini, C., Moser, J. U., Sonnleitner, B. and Fiechter, A. (1991). *Analytica Chimica Acta* **255**, 91.

Filippini, C., Sonnleitner, B. and Fiechter, A. (1992). *Analytica Chimica Acta* **265**, 63.

Fuhrmann, G. F. (1992). *Journal of Biotechnology* **27**(1), special issue.

Gandor, C. R. (1993). PhD thesis 10087, ETH Zürich.

Gruber, T. (1991). PhD thesis, University of Stuttgart.

Heijnen, J. J. and van Dijken, J. P. (1992). *Biotechnology and Bioengineering* **39**, 833.

Heijnen, J. J., Terwisscha van Scheltinga, A. H. and Straathof, A. J. (1992). *Journal of Biotechnology* **22**, 3.

Johnston, L. H. and Lowndes, N. F. (1992). *Nucleic Acids Research* **20**, 2403.

Jørgensen, S. B., Møller, H. E. and Andersen, M. Y. (1992). "Harnessing Biotechnology

for the 21st Century: Proceedings of 9th International Biotechnology Symposium" (M. R. Ladisch and A. Bose, eds), pp. 364–369. ACS, Crystal City, VA.
Käppeli, O., Lorencez Gonzales, I., Kühne, A. and Sonnleitner, B. (1988). "Proceedings of 8th International Biotechnology Symposium, I" (G. Durand, L. Bobichon and J. Florent, eds), pp. 467–478 Société Française de Microbiologie, Paris.
Küenzi, M. T. and Fiechter, A. (1969). *Archiv für Mikrobiologie* **64**, 396.
Küenzi, M. T. and Fiechter, A. (1972). *Archiv für Mikrobiologie* **84**, 254.
Laforce, R. (1987). PhD thesis 8336, ETH Zürich.
Leist, C. (1988). PhD thesis 8537, ETH Zürich.
Locher, G. (1991). PhD thesis 9594, ETH Zürich.
Locher, G., Sonnleitner, B. and Fiechter, A. (1991a). *Journal of Biotechnology* **19**, 127.
Locher, G., Sonnleitner, B. and Fiechter, A. (1991b). *Journal of Biotechnology* **19**, 173.
Locher, G., Hahnemann, U., Sonnleitner, B. and Fiechter, A. (1993). *Journal of Biotechnology* **29**, 75.
Locher, G., Sonnleitner, B. and Fiechter, A. (1992a). *Journal of Biotechnology* **25**, 23.
Locher, G., Sonnleitner, B. and Fiechter, A. (1992b). *Journal of Biotechnology* **25**, 55.
Mateles, R. I. and Battat, E. (1974). *Applied Microbiology* **28**, 901.
Messi, F. (1991). PhD thesis 9559, ETH Zürich.
Meyer, C. and Beyeler, W. (1984). *Biotechnology and Bioengineering* **26**, 916.
Münch, T., Sonnleitner, B. and Fiechter, A. (1992). *Journal of Biotechnology* **24**, 299.
Nielsen, J., Nikolajsen, K. and Villadsen, J. (1991). *Biotechnology and Bioengineering* **38**, 1.
Nipkow, A., Sonnleitner, B. and Fiechter, A. (1986a). *Journal of Biotechnology* **4**, 35.
Nipkow, A., Sonnleitner, B. and Fiechter, A. (1986b). *Journal of Biotechnology* **4**, 49.
Nurse, P. (1992). *Biochemical Society Transactions* **20**, 239.
Petrik, M., Käppeli, O. and Fiechter, A. (1983). *Journal of General Microbiology* **129**, 43.
Ratledge, C. (1991). *Bioprocess Engineering* **6**, 195.
Rohner, M., Sonnleitner, B. and Fiechter, A. (1992). *Journal of Biotechnology* **22**, 129.
Rose, M., Albig, W. and Entian, K. D. (1991). *European Journal of Biochemistry* **199**, 511.
Rothen, S. (1992). Diplomarbeit, ETH Zürich.
Scheper, T. (1991). "Bioanalytik". Vieweg, Braunschweig.
Schügerl, K. (1991). "Analytische Methoden in der Biotechnologie". Vieweg, Braunschweig.
Sonnleitner, B. (1991). *Antonie van Leeuwenhoek* **60**, 133.
Sonnleitner, B. and Fiechter, A. (1992). *Advances in Biochemical Engineering/Biotechnology* **46**, 143.
Sonnleitner, B. and Käppeli, O. (1986). *Biotechnology and Bioengineering* **28**, 927.
Sonnleitner, B. and Locher, G. (1992). "Proceedings of the IFAC/IFIP/IMACS International Symposium on Artificial Intelligence in Real-Time Control", Delft, The Netherland, pp. 359.
Stephanopoulos, G. and Vallino, J. J. (1991). *Science* **252**, 1675.
Strässle, C., Sonnleitner, B. and Fiechter, A. (1988). *Journal of Biotechnology* **7**, 299.
Strässle, C., Sonnleitner, B. and Fiechter, A. (1989). *Journal of Biotechnology* **9**, 191.
Theobald, U., Mailinger, W., Reuss, M. and Rizzi, M. (1993). *Analytical Biochemistry* **214**(1), 31.
Thevelein, J. M. (1991). *Molecular Microbiology* **5**, 1301.
Thevelein, J. M. (1992). *Antonie van Leeuwenhoek* **62**, 109.
Weikert, C. (1992). Diplomarbeit, ETH Zürich.

The Two-Competing Site (TCS) Model for Cell Shape Regulation in Bacteria: the Envelope as an Integration Point for the Regulatory Circuits of Essential Physiological Events

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. . . *sapias, vina liques, et spatio brevi spem longam reseces*
(Horatius, Od., I, 11)

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I. Introduction

In this chapter we do not intend to review the literature on all aspects of bacterial cell shape, which has already been excellently reviewed in recent papers (Koch, 1988, 1992; Harold, 1990; Holtje and Glauner, 1990; Cooper, 1991; Nanninga, 1991; Romeis *et al.*, 1991; Thwaites and Mendelson, 1991; Wientjes and Nanninga, 1991) to which we refer the reader. Instead, we mainly describe a number of ideas which we have developed concerning mechanisms of key importance in cell physiology, such as regulation of cell shape and cell division, and interaction between DNA replication and cell division as well as evolution of such mechanisms. Most of these ideas are included in a model which we have named "two competing sites for shape regulation in bacteria" and which we refer to as the TCS model (Satta *et al.*, 1975a, 1979a; Lleó *et al.*, 1990). In this review we describe all aspects of this model in detail and review experimental data and relevant literature.

The TCS model describes how cell shape, cell division, the connection between surface expansion and cell division and interactions between DNA replication and septum formation are regulated in bacteria. The studies which have allowed us to construct this model have also allowed an understanding of a number of simple mechanisms on which the regulatory systems analysed are based and which, we believe, may also have a more general meaning and may be applied to aspects of cell biology other than those we have studied.

Basically, the TCS model suggests that the already-mentioned cellular functions are regulated by an integrated system of different mechanisms which started very simply allowing rough-and-ready regulation of cell shape, cell dimensions and cell division, upon which, at successive stages of evolution, others have superimposed themselves and integrated with each other leading to an efficient and complex regulatory system. According to the TCS model, the first very simple regulatory mechanism was in the cellular envelope and consisted in two competing reactions on whose

balance depended septum formation and expansion of the rest of the envelope. The purpose of the other regulatory mechanisms which appeared at later stages of evolution was to refine and complete this gross primordial regulatory mechanism in the envelope and link it in with other essential events for cell physiology such as DNA replication and macromolecular synthesis. In this sense, bacterial growth is regulated by specific mechanisms and the envelope is the integration point of the regulatory mechanisms that link together major cell-physiology functions.

Even when only very specific aspects of cell biology are studied, the ultimate goal of such studies is to understand how life started, how it is organized, and how and why it ends. Therefore, when similar studies are summarized and commented on, it is customary to locate them in a broad perspective and to try to describe possible conclusions or suggestions of general significance that can be drawn from them. This is our intention here. But instead of presenting the work performed and then describing the conclusions of general value that can be drawn, we intend first to present a few simple suggestions concerning general aspects of cell biology and cellular evolution that experimental work concerning the TCS model allows and, in our opinion, justifies. Therefore, we start by describing which type of essential regulatory mechanisms may have developed in primordial living entities and how they may have evolved. Special reference will be made to the crucial role of the envelope in the life and development of living organisms.

We then consider the possibility that the concept of superimposition of more complex and efficient regulatory mechanisms on the simple primordial one during evolution may apply to the still debated issue of whether bacterial growth is exponential or linear. After this we present the TCS model, starting with a discussion of the concept of competing reactions as a general system for simple gross regulation of physiological events which is an essential aspect of it. Finally, we compare the TCS model with all relevant available experimental data.

II. Development of Primordial Living Entities and Their Evolution: The Central Role of Envelopes

In its most elementary essence, life may be seen as a process of rapid transformation of matter occurring within a well-defined space. This process, once started, has continued ever since, continuously evolving its properties and, if interrupted, does not start again. The conditions that allowed it to start are complex and it is unlikely that chance will reproduce

such a combination. Also, if it started again, it would probably be overwhelmed by living organisms that, having evolved earlier, are better adapted to the present environment.

In all of the forms we see at present, living matter occupies defined spaces which are precisely delimited by a structure developed *ad hoc*, namely the envelope. At present, there is no life without envelopes. It is however possible that, initially, life did not involve the presence of such structures. The primordial form of life might have been a kind of gel possibly occupying a space with support from a denser material. Under these conditions strong selective pressures may have favoured development of envelopes. Without them living matter might still expand in the denser material but, under these conditions, expansion cannot be indefinite since the space in the denser support cannot be infinite. On the other hand, the absence of envelopes would make duplication of this type of living material difficult, as well as its transfer to places where, for instance, it could find novel sources of substances necessary for its growth. It should be noted that such a primordial living entity has no shape, but acquires the shape produced by the denser matter in which it is contained.

Acquisition of the first envelope proved extremely advantageous for primordial life particularly by defining its space more precisely, affording better possibilities of keeping together all the components necessary for the process, providing a simpler way of duplicating itself and allowing a better chance of moving from one place to another. Primordial envelopes probably did not confer a shape on living matter since they were probably not stably structured. Separation of matter wrapped in a stably structured envelope into two different entities would involve active participation of the envelope and would be much more complex than separation of entities wrapped in non-rigid envelopes into two different parts. The latter, in fact, could be split much more easily by physical forces generated outside the living entity. This, in contrast, cannot occur in a stably structured envelope that has to be able to split on its own and therefore through processes that are more complex. Only after development of stably structured envelopes did living matter acquire a shape. Although probably not essential for their existence, acquisition of a shape-conferring envelope was of the greatest importance for living forms, by providing greater efficiency in their continued expansion through proliferation, and for their evolution. The existence of a structured envelope makes proliferation a process which depends on its splitting. A process of proliferation governed by splitting of the envelope allows cell duplication to occur under conditions most favourable for the state of the envelope which, in turn, involves a better co-ordination of its metabolism with that of other functions essential for cell physiology. A stably structured envelope also gives cells a much better

chance of moving (or being moved) to different places where different and more advantageous conditions for growth could be found. This is also of great importance for cellular evolution since conditions in different environments into which cells could transfer may differ very considerably and exert selective pressures which favour development of different forms of life.

A structured envelope thus appears to play a major role in cell physiology. It is essential for a cell to exist as such and for it to proliferate in a way proportional to continued expansion of the living mass (the cellular cytoplasm). The need for living matter to be contained, through its continuous expansion, in an envelope, has most likely been the driving force behind essential cell regulatory mechanisms. The expanding cell mass has to be contained in the envelope. In order to achieve this, the envelope also has to expand continuously and duplicate in order to allow proliferation that prevents formation of giant cells where metabolic exchanges with the environment would be at a disadvantage. In addition, duplication of the envelope needs to allow sister cells each to receive at least one DNA molecule and therefore has to occur only after DNA has duplicated. In order to be able to regulate and co-ordinate all of these events with its expansion, the envelope must have developed an ability to regulate its own extension and duplication as well as to co-ordinate them with other essential events of cell physiology such as macromolecular synthesis and DNA duplication. The mechanisms whereby envelopes regulate their growth and duplication and co-ordinate these functions with others which are also essential for cell physiology is the subject of the TCS model and are described and discussed in detail later on.

III. Development and Evolution of Mechanisms Regulating Cell Growth

As occurred with the cell envelope, we believe that, in the beginning, cell growth must also have been regulated in the simplest possible way and that it subsequently developed more complex regulatory mechanisms needed to keep the cell mass inside an envelope and carry gene-containing material. The overall mechanism by which bacteria grow has yet to be clarified, and at present two major contrasting opinions exist as to how individual bacteria grow during the cell cycle. One view assumes that growth rate is continuously increasing (exponential growth models) (Cooper, 1988b), the other that the rate of cell growth is constant until a given cell age or cell size is reached at which time the growth rate doubles (linear or bilinear models) (Kubitschek, 1968, 1981). These models have been proposed on the basis of studies which measured cell growth, taking

into account parameters such as cell length, cell surface, cell volume, macromolecular synthesis (DNA, RNA, protein, peptidoglycan) and cell density.

Recently, a general model of the bacterial division cycle has been conceived which takes into account the biosynthetic pattern of three main categories of molecules (cytoplasmic molecules, DNA and cell-surface molecules) (Cooper, 1991). The central idea of this model is that relationships between different components in a cell are regulated by passive, local mechanisms, thus excluding a super-regulatory mechanism co-ordinating biosynthesis. Synthesis of cytoplasmic components is the driving force regulating synthesis of other cell components. The cytoplasm increases in response to an interaction of nutrients, precursors, energy sources and environmental factors. Synthesis of DNA responds to synthesis of cytoplasmic components; these are synthesized at a particular rate during the division cycle without regard to continued synthesis of cell cytoplasm or when DNA synthesis occurs during the division cycle. The cell surface grows or stretches to accommodate increased cytoplasm or cell mass (Koch, 1988). The resulting growth kinetics of individual cells is nearly exponential because these are the sum of kinetics of cytoplasm growth which is exponential, of DNA synthesis which is linear, and of the cell surface which is exponential only during the cell-elongation phase. This model is mainly supported by experiments carried out in cells synchronized by the membrane-elution technique which reveals an exponential increase in contents of RNA and protein during the cell cycle of *Escherichia coli* (Cooper, 1988a). All of the experimental data reported in the literature showing a linear increase in cell components have been rejected by advocates of this model on the basis of criticisms of the technique used which could have caused perturbations in cell metabolism.

The alternative linear (or bilinear) growth models predict that cells grow at a constant rate until a certain mass or volume or length is reached at which time growth rate doubles. If this event occurs during the division cycle, growth is bilinear; if it occurs at the time of cell division, growth is linear. An important implication of the linear (or bilinear) growth model is that there is a global control system which regulates mass synthesis. Comparison and implications of exponential and linear growth models were discussed by Kubitschek (1990). The methods and criteria used in these studies, including the membrane-elution technique, have also been critically reviewed. In a linear model (Kubitschek, 1990), it has been postulated that increase in cell mass during steady-state growth is limited and proportional to the net rate of transport of materials into the cell, which, in turn, is proportional to the number of functional transport sites on the cell surface. Two predictions of this model are supported by

experimental findings. The first is that a number of cell proteins associated with the surface double abruptly and that soluble pools of RNA and protein precursors increase to mid-cycle maxima, then decrease again to their initial values by the end of the division cycle. In addition, direct measurements of cell size in steady-state culture, and after a shift-up, have been put forward to support linear growth.

In spite of numerous theoretical and experimental studies, the way in which the individual cell grows and divides is still a matter of debate. The two modes of growth are hard to distinguish between in experiments. For instance, the slope of the exponential or linear growth curves is predicted to differ by only 6%. Thus, the method used must possess extraordinary sensitivity and reproducibility. Direct methods, such as those based on measurement of cell dimensions, are also too insensitive to allow precise differentiation between the two modes of growth or between the exponential and actual pattern of growth which is extremely close to being, but is not quite, exponential (Cooper, 1988b).

The great difficulty encountered in establishing experimentally which of the two models is the correct one justifies our attempt to solve the problem by speculating on what appears to be the most likely situation, considering possible analogies with regulation of other essential events in cell physiology whose regulatory mechanisms we believe have been understood (i.e. those concerning the TCS model). On the basis of the concept of a simple, primordial though basically gross and inefficient mechanism upon which others have superimposed themselves in successive stages of evolution, we believe that, in all probability, the two theories are both correct and each explains one part of the mechanism of bacterial growth.

It seems to us likely that living organisms originally were regulated by physical laws which probably governed uninterrupted exponential growth of all compounds at the same time. This way of growing is certainly simple, but is probably not the most efficient. It does not require interplay among various cell components which is important for a more efficient use of energy generated by the cell. This, in all probability, must have exerted a strong selective pressure towards development of biochemical controls over bacterial growth and proliferation which superimposed themselves upon the primordial mechanism based on physical laws. Such controls serve the purpose of determining an ordered interplay among various bacterial events during the cell cycle. In order to achieve this, cells have had to develop mechanisms that stop specific functions for determined periods of time thus performing the various functions they are capable of at specific times in the cell cycle. In a sense, evolution from primordial to evolved organisms has mainly occurred through development of mechanisms that stop potential continuous exponential growth of all cellular

components, according to precise schedules. This suggestion is supported by the fact that all of the most evolved cellular organisms have the ability to stop synthesis of certain polymers with critical functions in the cell cycle, such as DNA, when others continue to be synthesized, or they can stop their growth as a response to specific environmental conditions. Known examples of this are, for instance, spore-forming bacteria which stop growing and develop into spores when environmental conditions are unfavourable for continued growth, or vertebrate cells growing as monolayers on solid surfaces which stop proliferating and stop DNA synthesis when they come into close contact with each other. Other examples are vertebrate cells which generally do not proliferate when performing a specific function and stop performing this function when they resume proliferating. We should consider, for instance, nerve cells and lymphocytes which have to revert from differentiation in order to proliferate and differentiate again into cells which do not proliferate when they return to generate and conduct nerve stimuli or start forming antibodies. It is likely that the pressure for developing these controls was exerted by the fact that, in order to perform all (or more than one) essential functions efficiently at the same time, cells would need to generate an amount of energy that they are not capable of producing. In addition, if cells were to perform all functions at the same time, the functions that require more energy would be at a disadvantage as compared to those which require less.

IV. Foundations of the TCS Model

The TCS model is basically founded on the idea that some primordial general mechanisms that may constitute the basis for most (or all) of the events essential for cell physiology should exist. All events that are essential for cells to exist as such ought to be present in cells from their very first development. Such controls may more easily appear all at the same time in a cell if they are based on a fundamental principle that is common to all of them. A simple way to regulate events that are critical, and may often need to be adjusted, is to have contrasting reactions that would promote events which are mutually exclusive. Under similar conditions, the timing and the extent of the events to be regulated should be determined by the balance between the contrasting reactions. This appears to be the simplest way to set up a regulatory mechanism which can be fairly loose, but is so simple as to be likely to develop in a single evolutionary step. Such contrasting reactions allow a good level of regulation even in the absence of genes specifically devoted to their modulation. Reactions that contrast with each other may be virtually self-regulating since, the

more one reaction is performed at the expense of the other, the greater the chance that environmental conditions may be modified in such a way as to favour the start of the contrasting one. It could be, for instance, that the amount of precursors needed for two different reactions is continuously lowered while one of them is being performed, thus favouring start of the opposite reaction. It is important to note that events based on such contrasting reactions are more prone to be influenced by the environment or by other cellular events. Many factors could easily shift the balance of the competition, by hampering or favouring one or other of the two competing reactions. We, of course, do not imply that all of the regulatory mechanisms that exist in a cell are based on the principle of two contrasting reactions. We suggest that, on this principle, are based only the primordial ones, that is those that had to be present right from the beginning in order to guarantee minimal necessary regulation for the most critical cell-cycle events of primordial cells. Development of similar contrasting reactions does not appear unlikely since enzymes are known which are capable of catalysing opposite reactions depending on the conditions under which they are allowed to work (Chipman and Sharon, 1969). Therefore, contrasting reactions do not even necessarily imply the existence of different sets of enzymes. In cellular metabolism too, for any pathway that builds a compound there exists one that destroys it.

As already stated, regulatory mechanisms based on reactions that contrast with each other are practically self-regulated, but they are by no means strict and therefore cell-cycle events that occur under their domain are unlikely to be identical each time they are determined. In addition, since such regulatory mechanisms based on contrasting reactions can be more easily influenced by intracellular and extracellular factors, they provide cells with a certain degree of adaptability to environmental conditions and the possibility of re-adjusting events regulated by these mechanisms in response to the particular physiological status, also remodulating them in the course of subsequent generations, if necessary. In this way, events over-expressed or under-expressed in one generation could be corrected in successive cell cycles. On the other hand, this regulation mechanism allows an easier interaction of events which they regulate with other events of the cell cycle which could easily interfere with the balance of the contrasting reactions, either stimulating or hampering one or the other of them. They also allow an easier integration of these regulatory mechanisms with others developed at later stages of evolution which refine them, making them stricter and more precise. It is likely that variability in the phenotype of genetically-identical cells, the virtual impossibility of finding completely identical organisms in nature, and phenotypic variations with which living organisms are capable of responding to very different

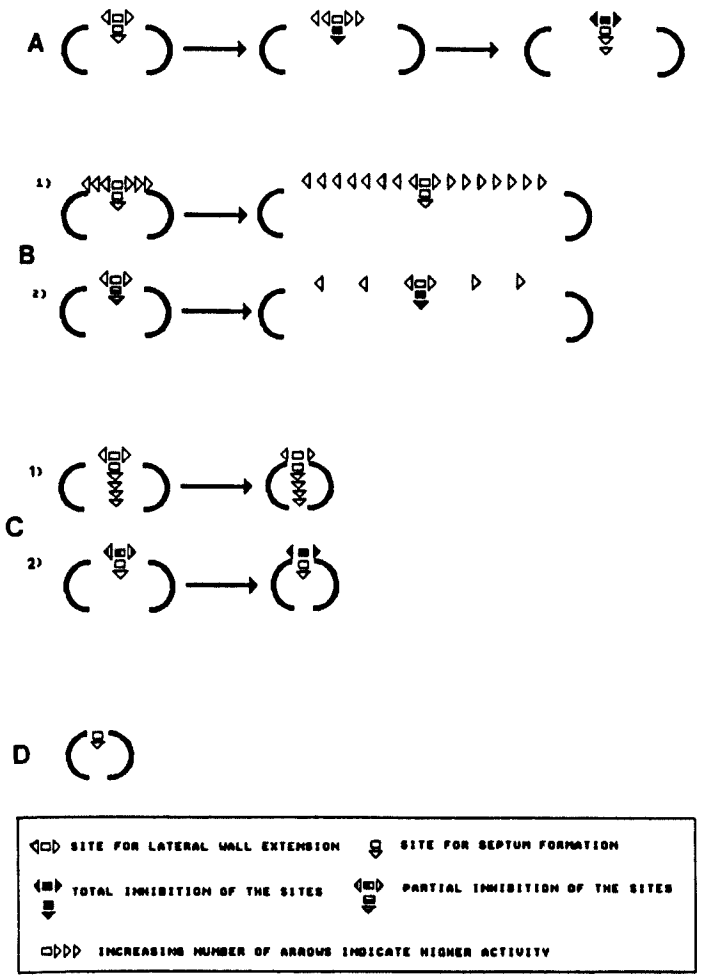

FIG. 1. Schematic depiction of the TCS model for shape regulation in bacteria. In this model, some bacteria possess two sites for peptidoglycan assembly, which compete with each other. In these bacteria (A), normal rod shape depends on regulated alternation of the activities of the sites. (B) Relative hyperactivity of the site for lateral-wall elongation leads to formation of long rods or filaments (and inhibition of cell division); such relative over-activity can be due either to increased activity of the site (B1) or to decreased activity or inhibition of the site for septum formation (B2). (C) Relative over-activity of the site for septum formation leads to formation of coccobacilli or cocci; such relative over-activity can be due either to a greater efficiency of the site for septum formation (C1) or to a lower activity or inhibition of the site for lateral-wall extension (C2). (D) Other bacteria possess only one site for peptidoglycan assembly and can only grow as cocci. From Lleó *et al.* (1990).

environmental conditions, also depend on the fact that major events in cellular physiology are regulated by mechanisms of the type described here.

V. The TCS Model

The TCS model suggests that bacteria can carry only one or two mechanisms (sites) for performing the terminal reactions of peptidoglycan synthesis and assembly. When two mechanisms exist, one is devoted to septum formation and the other to lateral wall elongation. They compete with each other so that substantially no extension of the lateral wall occurs during septum formation and vice versa. Bacteria which possess only one mechanism for peptidoglycan assembly form only septa, since septum formation is essential for cell propagation. They expand their surface only as an aspect of septum formation and are spheroidal in shape (cocci). In contrast, in bacteria which possess two mechanisms for peptidoglycan assembly, cell shape depends on the balance between the two competing sites. These cells could be rods of various lengths depending on the relative strength of the two reactions, or they could also be cocci when septum formation constantly overcomes lateral wall extension (Fig. 1).

Since septum formation is an essential event for cell division and proliferation, the model also infers that the balance between the two sites governs cell division and cell proliferation (obviously only in bacteria that carry the two sites). Due to this link between cell division and cell shape, the model proposes that additional controls exist which regulate cell shape, more strictly, also link cell proliferation to it and vice versa (LED control: *L*ateral wall *E*longation control over *D*ivision). This control occurs, in particular, in bacteria such as *E. coli* and *Klebsiella pneumoniae*, which maintain a fairly constant rod shape under different growth conditions (Fig. 2).

The model also suggests that synthesis of major macromolecules, such as DNA, RNA, and proteins, is linked to envelope metabolism in that expansion of the cell surface is necessary for newly-formed macromolecules to find space to fit into. This implies that a control exists which blocks such macromolecular synthesis when expansion of the envelope is completely prevented. Moreover, septum formation is linked to DNA replication, since products of DNA metabolism can interfere with the balance between the two competing sites and cause the septum-formation site to be unable to overcome the lateral wall-elongation site until one round of DNA replication has been accomplished (*D*ivision *I*nhibition by *D*NA *I*nterference or DIDI control).

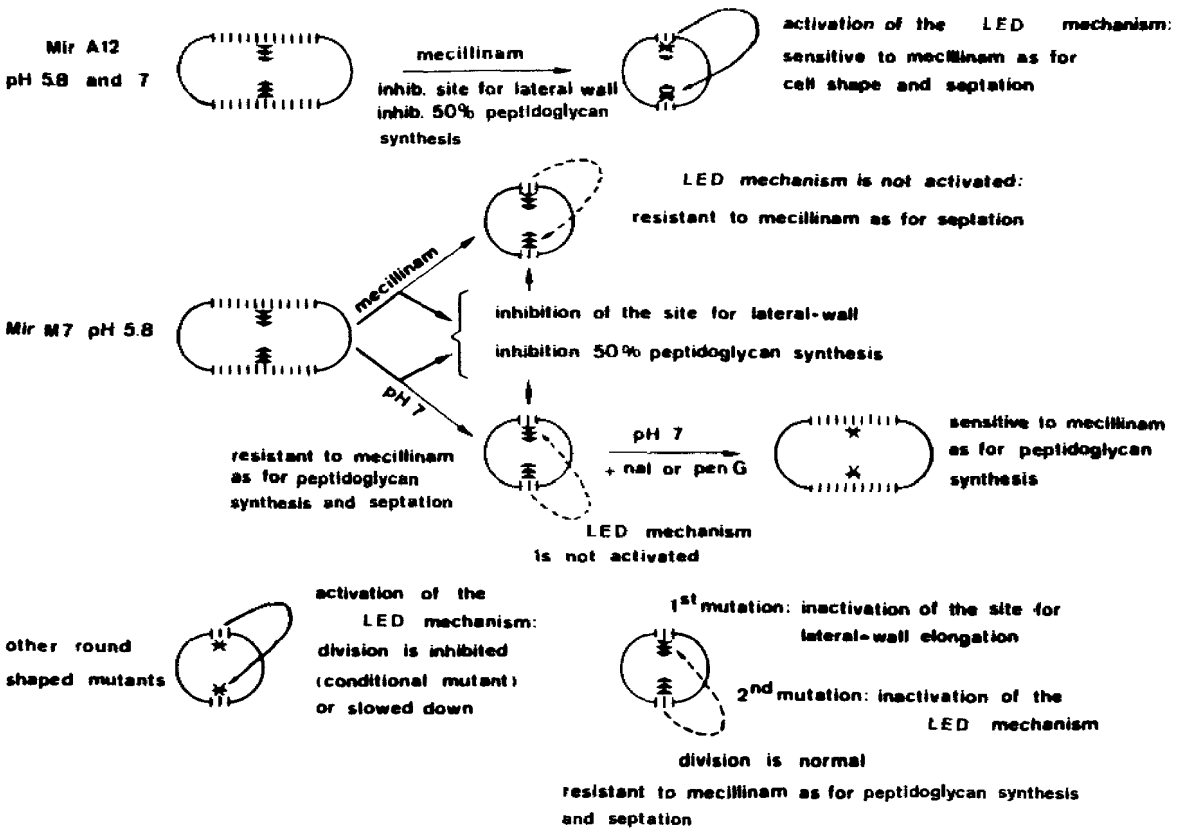
Mir A12
pH 5.8 and 7
mecillinam
inhib. site for lateral wall
inhib. 50% peptidoglycan
synthesis
activation of the LED mechanism:
sensitive to mecillinam as for
cell shape and septation
LED mechanism is not activated:
resistant to mecillinam as for septation
Mir M7 pH 5.8
mecillinam
pH 7
inhibition of the site for lateral-wall
inhibition 50% peptidoglycan synthesis
resistant to mecillinam
as for peptidoglycan
synthesis and septation
pH 7
+ nal or pen G
sensitive to mecillinam
as for peptidoglycan
synthesis
LED mechanism
is not activated
other round
shaped mutants
activation of the
LED mechanism:
division is inhibited
(conditional mutant)
or slowed down
1st mutation: inactivation of the site for
lateral-wall elongation
2nd mutation: inactivation of the
LED mechanism
division is normal
resistant to mecillinam as for peptidoglycan synthesis
and septation

FIG. 2

Finally, the model views determination of rod shape (in normally rod-shaped bacteria) and its maintenance during the cell cycle as dependent on the interplay that occurs between the lateral wall-elongation site, the septum-formation site and the control that blocks septum formation when lateral wall extension is inhibited (LED control) and describes such interplay as follows. Coccoid cells first elongate until they reach the size of a rod at birth, then they start a septum which is prevented from being completed by the LED control. This allows another round of lateral wall elongation to be accomplished at the end of which the septum is completed and a new site for septum formation is activated in each of what will become the newly born cells.

At this stage the model does not precisely specify the biochemistry of the two competing reactions, but suggests that a major role may be played by penicillin-binding proteins (PBPs). Although it is generally held that PBPs play a major role in cell division and in determining cell shape of bacteria (Spratt, 1975), the TCS model does not consider each of these to be a specific component of one of the two sites, or to perform a constant and fixed function. In contrast, it considers PBPs as contributing to competition between the two reactions, suggests that they interact with each other in accomplishing their various functions and considers them capable of also performing different functions depending on the physiological status of the cells. According to this view, similar effects on cell physiology could be obtained through either inactivation of one PBP or through hyperproduction of another while damage to one PBP could be

FIG. 2 (opposite). The existence of a control of lateral wall extension over septation (the LED control) as derived from studying the effect of mecillinam on cell shape and division of normal rods and of morphology mutants. A normal rod as in this case the *Klebsiella pneumoniae* Mir A12 strain, in the presence of mecillinam undergoes rod-to-coccus transition as a consequence of specific inhibition of lateral wall synthesis and stops dividing for the presence of the LED control. As a consequence of this, lateral wall defective mutants (round shaped mutants) should have two mutations to be able to grow normally and divide as cocci: one mutation responsible for loss of lateral wall extension, and one responsible for inactivation of the LED control. Strains defective in lateral wall extension but carrying an active LED control would stop dividing (conditional mutants) or grow slowly after becoming cocci. Mutants that carry only the mutation inactivating the LED control are resistant to mecillinam and could grow as both cocci or rods because in the former the site for septation could continuously prevail over the one for lateral wall elongation, while sometimes external stimuli could allow the site for lateral wall elongation to overcome the septum prevalence. This is the case of the mutant of *Klebsiella pneumoniae* Mir M7 which grows as cocci at pH 7 and as rods at pH 5.8, but rods can be obtained at pH 7 in the presence of nalidixic acid (nal) or penicillin G (pen G), or cocci at pH 5.8 in the presence of mecillinam. From Satta *et al.* (1980b).

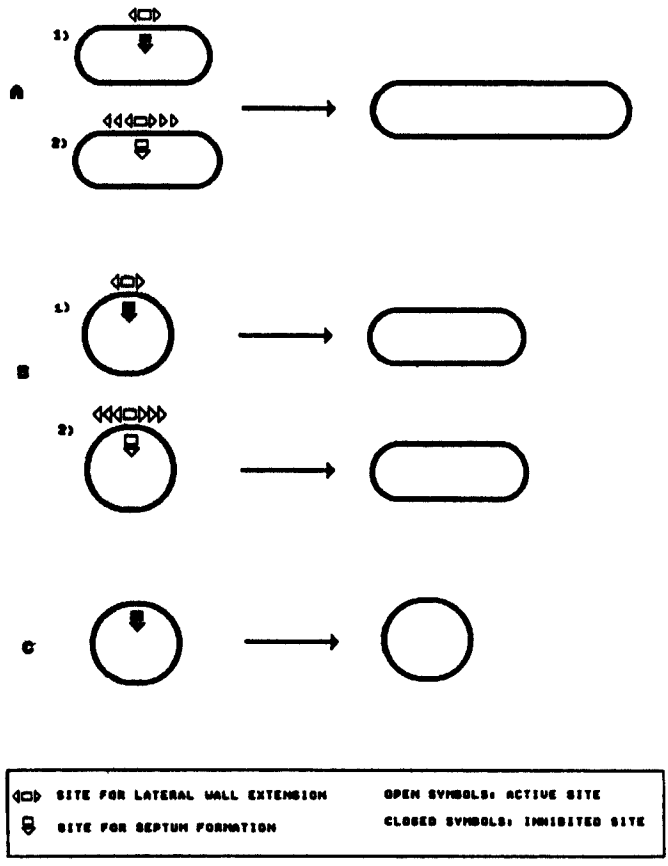

FIG. 3. Some predictions allowed by the TCS model for shape regulation in bacteria. These predictions refer only to the effects of interferences of various nature on cell shape and cell division of coccoid and rod-shaped bacteria. (a) When, in rod-shaped bacteria, the septum is inhibited or impaired by mutations (or increased gene dosage) or antibiotics (or other chemicals) (a1), or when the activity of the lateral wall-elongation site is enhanced by mutations (or increased gene dosage) or by antibiotics (or other chemicals) (a2), filaments are formed and cell division is inhibited. (b) When, in coccoid bacteria that carry two sites for peptidoglycan assembly, septum formation is inhibited by mutations (or increased gene dosage) or by antibiotics (or other chemicals) (b1), or when the lateral wall-elongation site is enhanced by mutations (or increased gene dosage) or by antibiotics (or other chemicals) (b2), cocci are converted into rods. (c) When, in coccoid bacteria that carry only one site for peptidoglycan assembly, septum formation is inhibited by mutations (or increased gene dosage) or by antibiotics (or other chemicals), cell-surface extension is blocked and the coccal shape is maintained. From Lleó *et al.* (1990).

compensated for by alterations to others. All this is an obvious consequence of the concept of competing reactions, of which, as already stated, PBPs are an essential part.

A. EVOLUTION OF BACTERIAL SHAPE IN THE TCS MODEL

The TCS model proposes that bacteria that have only one site for peptidoglycan assembly evolved before others and that, in the latter bacteria, development of the second site occurred gradually, first by doubling the original one, then through its gradual development towards the ability to form peptidoglycan oriented in such a way as to cause lateral wall elongation, and finally acquiring the progressive ability to compete with the septum-formation site. Once the second site was fully evolved, other controls were developed and links were established between competing sites for peptidoglycan assembly and other essential cell physiology events (see above).

Selective pressure for development of a second site may have arisen from the great advantage cells acquired with their ability to continue to expand the cell surface even when the septum-formation site was blocked (Fig. 3). While inhibition of the septum makes it impossible for the cell surface to expand in cells that carry only one site, thus causing inhibition of macromolecular synthesis, in bacteria that carry two sites, cell-surface expansion can proceed due to activity of the second site. Development of the rod shape may provide a further advantage. For instance, in cells where this site has evolved, septum inhibition leads to formation of cells of various shapes (generally of a spheroidal type) which progressively increase in size. Under these conditions, macromolecular synthesis is not inhibited due to continued expansion of the cell surface. However, the ratio of cell surface to cell mass decreases continuously, thus creating disadvantageous conditions for cell metabolism. In addition, owing to the increase in cell diameter when the septum block is removed, a septum larger than usual has to be formed. This septum has to be made in a cell whose shape is distorted, and this renders its formation highly critical as well as making resumption of proliferation more difficult. In bacteria which have developed the ability to form a cylindrical wall, septum inhibition causes continued elongation of the lateral wall and formation of filaments. In these bacteria, the cell wall continues to expand at rates which are not much decreased compared with untreated cells. In addition, owing to the fact that, under these conditions, cells grow with an approximately cylindrical shape (rather than with a spherical one), continued growth in the absence of cell division does not cause any significant progressive alteration of the ratio of cell surface to cell mass. Filamentous cells which

form under these conditions also maintain the same diameters as the original cells so that, when septum formation resumes, they do not encounter any abnormal condition capable of hampering its completion.

These advantages might be of great importance, since it is quite likely that bacteria in the environment will encounter many conditions that cause direct or indirect temporary inhibition of the septum. We need only mention, for instance, the large number of physical and chemical agents capable of damaging DNA, and the fact that, in all bacteria so far studied, a constant feature of such damage is inhibition of septum formation. Most bacteria can repair damaged DNA, but, to do so, genes that are normally repressed have to be derepressed and novel proteins have to be synthesized. For this to occur it is necessary that protein synthesis be allowed to continue after DNA has been damaged and the septum inhibited.

B. MAIN FEATURES OF THE TCS MODEL

The TCS model has features deriving from its being based on novel concepts which were not expounded in previous proposals. The first of these is the idea of considering shape determination (in most bacteria) and cell division as dependent on competition between two different biochemical reactions (sites). This concept allows the TCS model to make predictions that are not possible with other models and to explain experimental data which cannot be explained by other models.

The model takes evolutionary development into account, indicating selective pressures that may reasonably have favoured evolution. It also describes the shape and division regulation of bacteria as developed in successive steps by identifying some of the regulatory mechanisms that have superimposed themselves, and also indicating those which developed first and those which developed later.

The fundamental mechanism on which regulation of shape and septum formation is based can easily be viewed as being liable to further modulation, not only by additional controls, but also by various intracellular and extracellular factors which may, in turn, act in opposite ways, either by stimulating one of the two competing sites or by blocking the other. Therefore, many events occurring in the cell or outside it may influence cell division and cell shape. This allows the model to link cell shape to septum formation, septum formation to DNA replication and overall macromolecular synthesis to cell-surface expansion, thereby embracing all of the fundamental events of cell physiology which appear interlinked in bacteria.

Being based on grossly-autoregulated competing reactions endows the model with other unique properties such as the fact that complete

inhibition of septum formation (in most bacteria) still allows cells to continue to extend their surface and mass through lateral wall elongation. Continued expansion of the cell surface under conditions that block septum formation allows cellular metabolism to continue in spite of a lack of proliferation (see above) and this, on the one hand, helps cells to remain alive and, on the other, gives them the possibility of expressing mechanisms capable of eliminating or repairing damage that inhibited septum formation (see above). The above-mentioned phenomenon also makes it unnecessary to define a precise balance that has to be reached between the septum and lateral wall for the cells to be able to grow normally. This balance, on the contrary, can be shifted indifferently in favour of one or other of the two competing sites in response to various physiological interferences which may vary in one and the same cell under different growth conditions and in different bacterial strains. This property gives single bacterial cells the possibility of modulating their shape in response to variations in growth conditions by continuously re-adjusting their shape whenever the general physiological conditions of the cell make this necessary. This explains the individual variability in cell shape and length, of rod-shaped bacteria, in cells of the same clone and the different shape of cells of bacterial species which are all determined by the same regulatory mechanism. It also explains the fact that growth conditions can grossly alter cell shape particularly in certain species such as *Proteus mirabilis*. Finally, a peculiar feature of the model is that it is able to explain by the same mechanism shape determination in wild-type bacteria that appear as coccobacilli, as very short rods or rods of all possible lengths.

C. RELATIONSHIP BETWEEN THE TCS MODEL AND PREVIOUS PROPOSALS

The TCS model is based on, and fully integrates, the previous suggestion made by others (Schwarz *et al.*, 1969) that rod-shaped bacteria have two different mechanisms for peptidoglycan assembly, one responsible for lateral wall elongation and the other for septum formation. It expands on this suggestion and states it more precisely, indicating that the two mechanisms compete with each other so that cell shape and cell division depend on the balance between them.

The new concept of competition between the two sites creates a major difference between the TCS model and the one previously suggested. In addition, it also considers cocci, which it suggests as being of two types, one carrying only one site and one carrying two competing sites. This suggestion which concerns cocci differs from that of others (Higgins and Shockman, 1971) who proposed that cocci carry only one site for peptidoglycan synthesis which is responsible for septum formation. The difference

between these two suggestions is by no means minor, since in the previous model all cocci are viewed as being equal and none of them should be able to expand its surface after septum inhibition, being unable to undergo transition to a rod shape under any circumstances. The TCS model, in contrast, predicts the existence of two different types of wild-type cocci, one of which can expand the cell surface, even when synthesis of lateral wall is inhibited, and can assume the rod shape upon septum inhibition (see below).

The concept of two different mechanisms for peptidoglycan assembly was also strongly supported by observations which are very different from those already described. By analysing the functions of PBPs of *E. coli*, it was suggested that some of them were needed for lateral-wall elongation and some for septum formation (Spratt, 1975). However, in this case too, the concept of mechanisms that compete with each other is entirely lacking, and different PBPs are each thought to perform a given specific and non-variable function. The TCS model takes up the proposal that different PBPs are involved in the two mechanisms, but it considers them as capable of modulating each other's activity and as being prone to interference by various physiological factors capable of stimulating or hampering their activity. As a result, none of them performs a constant fixed function, but each performs functions that may also depend on the physiological status of cells, being capable in particular of taking over one of the functions of the other.

D. MAJOR EXPERIMENTAL DATA ON WHICH FUNDAMENTAL ASPECTS OF THE TCS MODEL ARE BASED

The existence, in bacterial rods, of a biochemical mechanism responsible for lateral wall formation and one responsible for septum formation was first supported by data showing that low concentrations of penicillin inhibited formation of septa in *E. coli*, but allowed the lateral wall to be extended, thus leading to formation of filaments (Schwarz *et al.*, 1969). Further support for the concept of two different mechanisms came from work on PBPs from *E. coli* showing that mutants which lacked one of these proteins (PBP3) were unable to form septa, while those lacking another (PBP2) formed spheres (Spratt, 1975; Spratt and Pardee, 1975), thus indicating that each of the two events, namely septum and lateral-wall formation, could be blocked without directly disturbing the other. Additional support finally came from the demonstration that, among the different β-lactam antibiotics, some specifically (or preferentially) inhibit lateral-wall elongation, while others inhibit septum formation in *E. coli* and other bacterial species (Spratt and Pardee, 1975; Lorian and Atkinson,

1977; Spratt *et al.*, 1977; Jones *et al.*, 1981). The concept of competition between the mechanisms responsible for lateral-wall elongation and septum formation is strongly supported by the properties of a morphology mutant of *K. pneumoniae* (strain Mir M7) which, depending on environmental conditions, can grow indifferently either as cocci or as rods of various lengths or can form filaments (Satta and Fontana, 1974a,b; Fontana *et al.*, 1979; Satta *et al.*, 1979a, 1980b, 1981, 1985; Canepari *et al.*, 1984; Lleó *et al.*, 1990). The existence of a control that refines and completes regulation of cell shape and septum formation by preventing formation of new septa until the lateral wall has elongated (the LED control) is supported by the observations that morphology-conditional mutants of *E. coli*, under non-permissive conditions, stop dividing immediately after converting into cocci and by the finding that mecillinam, an antibiotic which makes *E. coli* assume a coccal shape, also causes inhibition of cell division, but only after cells have assumed a coccal shape (Spratt *et al.*, 1977; Satta *et al.*, 1985). The idea that PBPs perform functions which may vary depending on the physiological status of cells was supported by data demonstrating that, in *Streptococcus faecalis* ATCC 9790 (a strain subsequently classified as *Streptococcus faecium* and more recently as *Enterococcus hirae*) growth conditions heavily influence both the PBP pattern and sensitivity of bacteria to β-lactam antibiotics (Fontana *et al.*, 1980, 1983a). Further support is also provided by the finding that, in the same micro-organism, PBPs that function as the primary target of β-lactam antibiotics may be different when bacteria are grown under different conditions (Fontana *et al.*, 1980), and by the fact that inability to grow caused by loss or alterations of PBPs 1, 2, or 3 may be suppressed by hyperproduction of PBP 5 (Fontana *et al.*, 1983b; Canepari *et al.*, 1987).

VI. Testing Various Proposals of the TCS Model Against Known Experimental Observations

A. EVOLUTION OF BACTERIAL MORPHOLOGY

As mentioned above, the TCS model states that, among bacteria that are coccal shaped, some carry only one site for peptidoglycan assembly while others carry two sites. To carry two sites is a necessary condition for a bacterium to be able to form rods, but it is not enough in itself since, in order to do so, the second site must acquire the ability to form a peptidoglycan which is oriented perpendicular to that of the septum. This is likely to involve various evolutionary steps (Fig. 4). Therefore, cocci that carry two sites for peptidoglycan assembly ought to include bacteria

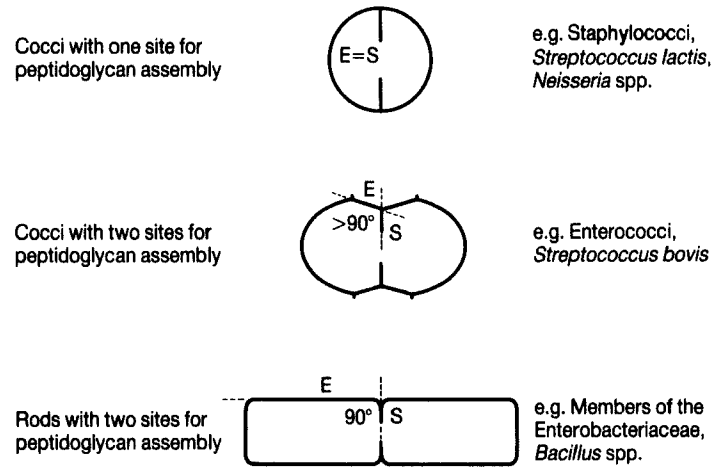

FIG. 4. Evolution of sites for surface extension from cocci to rods. E indicates surface extension; S septum.

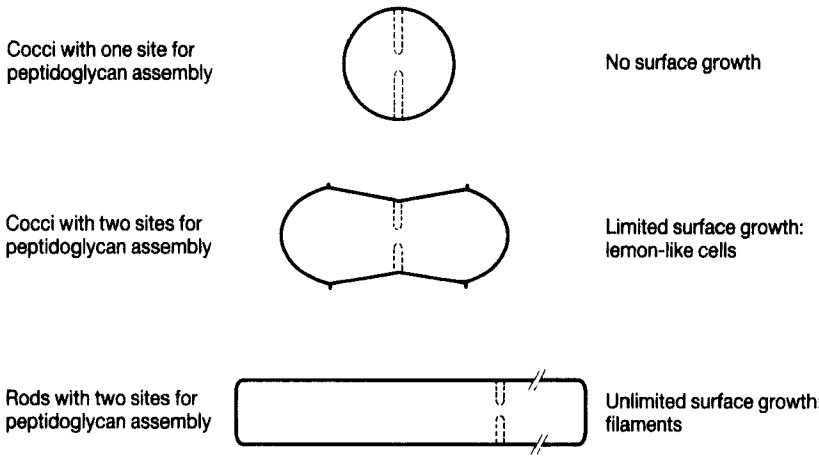

FIG. 5. Effect of inhibition of the site for septum formation on surface extension of cocci and rods. Dashed lines indicate inhibition of septum formation.

that are at different stages of this evolutionary process. On this basis, it is possible to predict that inhibition of the septum in a variety of different wild cocci would cause different effects on cell shape (Fig. 5). In those cocci that carry only one site for peptidoglycan assembly, inhibition of the septum would completely block cell-wall extension, and no shape changes

should be observed. In cocci where two sites for peptidoglycan assembly are present, inhibition of septum formation should allow peptidoglycan to go on being formed by the second site. The amount of peptidoglycan synthesized after septum formation has been blocked, and the morphological changes observed, would depend on the degree of evolution of the second site towards an ability to form cylindrical walls in the absence of septum synthesis. Therefore, of these cocci, some would form a peptidoglycan whose orientation is such as to allow only a relatively limited extension of the cell surface with formation of cells with altered shape resembling that of a lemon. In such lemon-shaped cells it could be that the second site makes a peptidoglycan that is not yet perpendicularly oriented with respect to that of the septum, but makes it at an angle higher than 90°, which causes activity of the second site to be stopped when the two sides of the wall converge. In other cocci, the second site may be more evolved allowing peptidoglycan to be formed in a direction perpendicular to that of the septum, thus causing formation of a cylindrical wall with a consequent transition from coccal to rod shape upon inhibition of septum formation. In similar bacteria, the length the rods are capable of attaining should be greater, the more the second site has developed its ability to function independently of the septum-formation site. All these predictions have been tested (Lleó *et al.*, 1990). It was found that certain cocci, such as staphylococci, *Neisseria* species and some streptococci such as *S. lactis and S. disgalactiae*, do not expand cell surface and do not modify their shape upon inhibition of the septum. Other streptococci such as *S. bovis, S. agalactiae, Enterococcus faecium* and *Streptococcus sanguis* elongated, forming rod-shaped cells after 90 min and long rods after 240 min incubation in the presence of septum inhibitors.

B. LATERAL WALL ELONGATION CONTROL OVER SEPTUM FORMATION (LED CONTROL)

As already stated, the TCS model suggests that the advantages conferred by ability to form a lateral wall have favoured the imposition, on the fundamental mechanism of shape regulation based on the two grossly-autoregulated competing reactions, of an additional control, which shifts the balance of the two reactions in favour of lateral-wall elongation by preventing septum formation in cells that have not extended their lateral wall. This is known as LED control (Fig. 2).

The existence of a control linking cell division to lateral-wall elongation was first indicated by studies on the morphology mutant *K. pneumoniae* Mir M7 (Satta and Fontana, 1974a,b; Satta *et al.*, 1979, 1980a,b, 1981, 1985a; Fontana *et al.*, 1979; Canepari *et al.*, 1984; Lleó *et al.*, 1990). This

strain forms cocci at pH 7 and above, and rods at pH 5.8 and below. It was the first mutant to be described which was capable of dividing normally also in non-permissive conditions as cocci. In addition, on treating rods (i.e. cells grown at pH 5.8) of the mutant Mir M7 and cells of the parent with mecillinam, it was observed that the antibiotic caused a transition from rod to coccal shape using the same concentration and at the same rate (Satta *et al.*, 1980b, 1981). However, while the parental strain, immediately after assuming coccal shape, stopped increasing in cell number, the mutant did not but continued to grow and divided faster than it had before undergoing the shape change (Fig. 6). That inhibition of cell division in the parent was, most probably, a consequence of the rod-to-coccus shape transition was indicated by the observation that, in the same bacteria treated with different concentrations of mecillinam, and in strains of different species treated with the same concentration of the antibiotic, inhibition of cell division occurred only after transition to a coccal shape (Satta *et al.*, 1981). Also when mecillinam was removed from cultures of wild-type strains of *K. pneumoniae* or *E. coli* which had become cocci in the presence of the antibiotic, the cells reshaped gradually and re-assumed a rod configuration after two generation times (Satta *et al.*, 1993). Throughout this period, cell number did not increase, but cells started dividing again only after having once again assumed a clear rod shape. In contrast to this, when mecillinam was removed from cultures of the morphology mutant Mir M7 grown at pH 5.8, the cells did not stop dividing but reshaped into rods while continuing to proliferate. In a previous paper (Satta *et al.*, 1980b), we showed that addition of mecillinam to exponentially growing cultures of wild-type *E. coli* or *K. pneumoniae* did not cause inhibition of cell division for at least 60–90 min (depending on the growth medium). If the antibiotic was removed from the culture 30–40 min after its addition (when cells still appeared as rods and divided), transition to the coccal shape still occurred exactly 60–90 min after addition of the antibiotic. Under these conditions and immediately after transition to a coccal shape, cell division stopped. This occurred in spite of the fact that, at the time of shape change and cell-division inhibition, the antibiotic had been removed from the culture for at least 30 min. In these experiments, too, the cells started dividing again only 60–80 min after the shape change and therefore only 90–120 min after removal of the antibiotic, indicating that the crucial factor for resuming division was not the time elapsing after antibiotic removal but the time after transition to a coccal shape. If mecillinam was removed 10 min earlier than already indicated, a transition to coccal shape was not observed and cell division was not inhibited. Thus, mecillinam interfered with cell division only under conditions in which it was able to determine transition to the coccal shape; recovery of cell

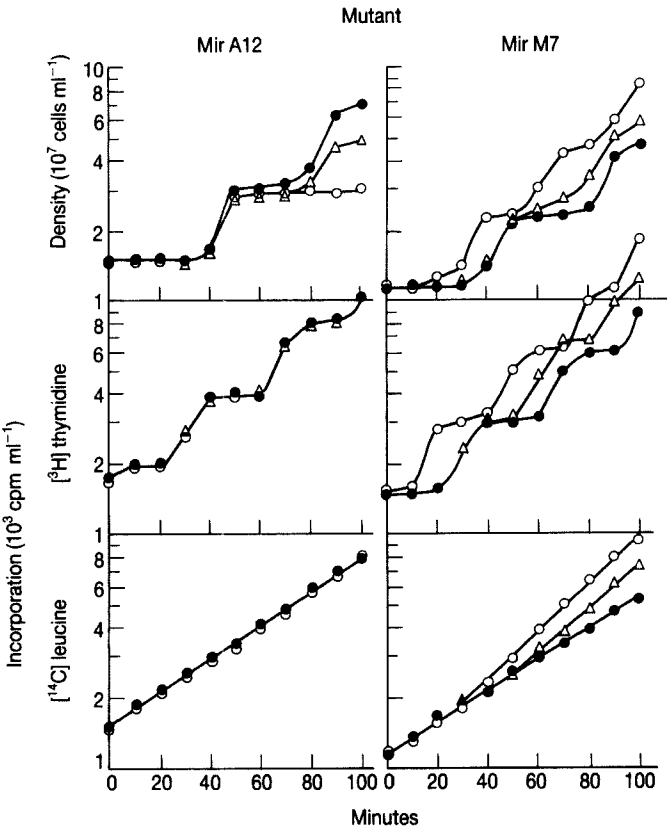

FIG. 6. Effect of the addition of 0.7 μg ml^{-1} mecillinam on cell division and DNA and protein synthesis during different times of the cell cycle in *Klebsiella pneumoniae* Mir A12 (parent) and Mir M7 (mutant) both growing synchronously in a minimal-salt medium supplemented with glucose at pH 5.8. Cells collected from a sucrose gradient were diluted in pre-warmed medium supplemented with [^{3}H]thymidine (2 μCi ml^{-1}) and [^{14}C]leucine (0.1 μCi ml^{-1}) and immediately subdivided into three parts. One was the untreated control (●). Mecillinam (0.7 μg ml^{-1}) was added to the others at time zero (○) and at 30 min (△). Every 10 min, samples were withdrawn, to evaluate cell number with a Coulter counter, and isotope incorporation. From Satta *et al.* (1981).

division, after removal of mecillinam, took a time which was exactly the same as that required for the cells that had become cocci to reshape into rods. It is important to note that this time is much longer than that necessary for the same cells to recover from cell-division inhibition caused by other β-lactam antibiotics which do not cause transition to a coccal shape (Craig and Gudmundsson, 1991).

These observations were interpreted as indicating that bacterial rods, such as *E. coli* and *K. pneumoniae*, carry a control which blocks formation of new septa when lateral-wall elongation has been inhibited (LED control) and that, as a result of this control, cells which have become coccoid cannot divide until the lateral wall elongates again and they re-acquire rod shape (Satta *et al.*, 1980b; Fig. 2). We also concluded that mecillinam is a specific inhibitor of the site for lateral-wall elongation with no direct effect on septum formation (Satta *et al.*, 1980b, 1985). In fact, cells in which mecillinam inhibited lateral-wall elongation for one generation time, thereafter, when they are ready to divide, have the length of a newborn cell. These short cells, after splitting into two, end up with a length which is very close to the width, thus assuming a coccal configuration. The fact that cells fatten slightly during division also contributes to the final coccal shape (Trueba and Woldringh, 1980).

The fact that cells divide even if mecillinam has fully inhibited lateral-wall elongation does not contradict the hypothesis that a control blocks septum formation when lateral-wall elongation is inhibited (LED control) since, in cells that have the length of newborn cells, lateral-wall elongation has occurred and the septum can be formed at least once (see below).

On the basis of such findings, it was proposed that mecillinam inhibits bacterial proliferation by a mechanism which is highly peculiar for this β-lactam antibiotic family and is indirect and can only be effective in those cells where the control is present that blocks cell division when lateral-wall elongation has been prevented (Satta *et al.*, 1980b, 1981). Since PBP 2 is the only one of these proteins that mecillinam binds (even at concentrations 1000-fold greater than the minimum inhibitory concentration), it was concluded that PBP2 has an essential role in lateral-wall elongation and is a major component of the site for lateral-wall elongation (Satta *et al.*, 1980b). The conclusion that mecillinam specifically inhibits lateral-wall elongation and that PBP2 plays an important role in it contrasts with previous proposals which suggested that PBP1 was responsible for lateral-wall elongation and described PBP2 as necessary for acquisition of rod shape (Spratt, 1975; Spratt and Pardee, 1975). According to this latter view, the effect of mecillinam on cell shape is described as being due to its interference with the morphogenetic effect of PBP2. Note that these proposals do not explain the inhibitory effect of mecillinam on cell division. Others have proposed that PBP2 causes a triggering of lateral-wall elongation, which is then continued by PBP1 (Matsuhashi, 1990). However, although this proposal is closer to that of the TCS model, it too is unable to explain the inhibitory effect mecillinam has on cell division.

The suggestions already made enable us to predict that mutants which are incapable of forming a lateral wall should not be able to divide unless

they carry a second mutation that abolishes the function of the LED control (Fig. 2). They also predict the existence of a class of mutants in which the two sites for lateral-wall and septum formation are unaltered, but have lost the control that inhibits septum formation when lateral-wall elongation has been inhibited. These mutants may appear as rods, but growth conditions should influence cell shape much more than parental strains, also making them grow as cocci. Such strains should be sensitive to mecillinam as regards cell shape but not cell division (Fig. 2).

A typical mutant of this type is the morphology mutant Mir M7 (Satta *et al.*, 1980b) where, unfortunately, in spite of several attempts (Satta *et al.*, 1978) it was not possible to map the mutation. More recently two mutations (*lov 1, lov B*) conferring such behaviour were identified and mapped in *E. coli* (Bouloc *et al.*, 1988, 1989; Vinella *et al.*, 1992). Strains that carry these mutations appear as rods which are shorter than the parent cells (Bouloc *et al.*, 1988) and, in the presence of mecillinam, become cocci, which, however, as opposed to the parent, continue to grow in this shape.

Several authors have described coccoid mutants of *E. coli* (Adler *et al.*, 1967; Normark, 1969; Henning *et al.*, 1972; Matsuzawa *et al.*, 1973; Matsuhashi *et al.*, 1974; Westling-Haggstrom and Normark, 1975; Kumar, 1976; Spratt, 1977; Iwaga *et al.*, 1978; Spratt *et al.*, 1980; Wachi *et al.*, 1987; Jaffè *et al.*, 1990). A few of them were short ovoid cells (Westling-Haggstrom and Normark, 1975), while most were real morphology mutants. Some constitutive mutants which appeared as cocci grow very slowly in comparison to the parent and in the case of one of them (SP 6), a second mutation conferring mecillinam resistance and necessary for them to divide as cocci was postulated (Spratt *et al.*, 1980). Accurate analysis of data concerning growth of conditional morphology mutants and timing of the shape change under non-permissive conditions, as well as repetition in our laboratory of the experiments with some of them (SP45 and SP52) (Lleó *et al.*, 1990), has allowed us to conclude that, after transition to the non-permissive temperature, transition to the coccal shape takes 60–80 min. During this time, cells divide twice and full transition to the coccal shape is only observed after these divisions have occurred. After this, in contrast, cells no longer proliferate. This clearly indicates that, in these mutants, the process whereby transition to the coccal shape is determined is of the same type as the one we have described as occurring in bacterial rods treated with mecillinam. It also indicates that these mutants do not divide under coccal shape.

If the inability of morphology mutants to form septa under the coccal shape were due to the block imposed by the control exercised by lateral-wall elongation over septum formation, acquisition of the mutation that

causes loss of such control should make these mutants capable of growing and dividing at the non-permissive temperature even after they have become cocci. A double mutant of this type has been constructed by transferring the *lov* mutation which confers resistance to mecillinam in a strain containing a *ts* PBP2 allele (Ogura *et al.*, 1989) which causes cells to become coccal and to stop dividing at 42°C. The resulting strains grew as spheres even at 42°C. Although the concept that a mutation eliminating control of lateral-wall elongation over septum formation (LED control) was necessary for rod-shaped bacteria to be able to grow as cocci had been proposed by us in 1981, and the mutant Mir M7 of *K. pneumoniae* had already been described as the first morphology mutant that carried this mutation in addition to the one causing them to become cocci at pH 7, the authors who described the properties of the above-mentioned double mutants (Ogura *et al.*, 1989) do not interpret their findings from the standpoint of the TCS model, but suggest that PBP2 plays a dual role in cell growth, namely a non-essential activity required for cell-wall elongation, and an essential activity in cell division which can be bypassed in *lov* mutants (Ogura *et al.*, 1989). The *lov* mutations have recently been mapped and found to lie within genes of aminoacyl-tRNA synthetase (*argS, alaS*). Inactivation of these genes causes a decrease in the charge ratio of a tRNA species which is known to activate the stringent response and to increase the content of ppGpp which is an RNA polymerase effector causing shut-off of transcription of rRNA and tRNA operons. *Lov* mutants contain an increased amount of ppGpp. A model is proposed whereby the existence of elements which transmit information on the state of PBP2 to the septum machinery is postulated. The nucleotide ppGpp may act as a regulator of the gene involved in this transmission, and its overproduction may cause lack of the control that links cell division to normal activity of PBP2. This control is postulated as also involving ribosome activity, since a number of mutations in the rpsL gene, which alter the S12 ribosomal protein and cause acquisition of streptomycin resistance, suppress the mecillinam resistance phenotype associated with *lov* mutations.

Regarding possible links between PBP2 and macromolecule synthesis, we have shown that addition of mecillinam to rods of the morphology mutant of *K. pneumoniae* Mir M7 made synchronous cells initiate new rounds of DNA replication significantly earlier than controls not treated with the antibiotic (Satta *et al.*, 1981; Fig. 6). Since this effect was observed in rods of the mutant, but not in those of the wild-type strains which also become cocci in the presence of the antibiotic, we believe that the connection between PBP2 and DNA replication is not a direct one, but is indirect through alterations which the antibiotic causes in the cell wall. It seems possible that, in this connection, alterations in cell-wall components

with which DNA has been reported to be associated (Gudas *et al.*, 1976; Schlaeppi and Karamata, 1982; Schlaeppi *et al.*, 1985) may be involved. In our view, the effect that an altered PBP2 was found to have on synthesis of proteins and RNA is also indirect and, as already mentioned, can be determined by a block in cell-surface expansion occurring when bacteria which cannot form a lateral wall become cocci and, as a result of the LED control, also stop forming septa. The TCS model suggests that, in these cells, macromolecule synthesis has to be inhibited due to lack of space to fit them in the cell.

The existence of a control that inhibits septum formation when lateral-wall formation is prevented and therefore forces these cells to maintain a rod configuration is well supported by the data already referred to. This supports our proposal that regulation of cell shape and septum formation in bacteria has evolved in steps starting from a mechanism capable of exerting a loose regulation (the grossly-autoregulated competing reactions) which was subsequently refined through development of additional controls, developed at a later stage in evolution, which have been superimposed upon it. Of course, if this proposal were correct, we would expect that, in nature, bacteria exist which possess the two competing sites, but have not yet evolved the control mechanism that blocks septum formation when lateral-wall formation is inhibited. In these bacterial species, cell shape may be more variable and may be much more influenced by growth conditions than that in other species, such as *E. coli* and *K. pneumoniae* where this control is present. Examples of these bacteria may be *Arthrobacter* spp. which grow as rods in the exponential phase of growth but appear as cocci in the stationary phase of growth (Krulwich and Plate, 1971). A wild-type strain of *E. coli* and one of *Proteus mirabilis* which were rod-shaped and, in the presence of mecillinam, became coccoid and grew in this form, have also been described (Lorian and Atkinson, 1977; Barbour *et al.*, 1981). These could be either strains carrying a spontaneous mutation in the control that links formation of septa to lateral-wall elongation or wild-type strains where the control already referred to has not yet evolved (possibly the *P. mirabilis* strain).

C. PEPTIDOGLYCAN CONTENT AND SYNTHESIS IN RODS AND COCCI OF MORPHOLOGY MUTANTS

In conditional morphology mutants of rod-shaped bacteria, under non-permissive conditions where they grow as cocci, only one site for peptidoglycan synthesis should be active. This implies that, as compared with rods, cocci of such mutants should have a decreased amount of peptidoglycan, and that transition from coccal to rod shape should be associated with an

increase in peptidoglycan synthesis and vice versa. In addition, such cocci, in which the site for lateral-wall elongation is assumed to be inactive, should synthesize peptidoglycan only during that part of the cell cycle which coincides with the time that precedes cell separation, whereas rods should make this polymer throughout the entire cell cycle.

All of these predictions have been tested using the morphology mutant of *Klebsiella pneumoniae* M7, which exhibits rod morphology at pH 5.8 and coccal shape at pH 7. We have found that cocci contain 61% less peptidoglycan in each cell (dry weight) (Satta *et al.*, 1979a). In addition, transition from rod to coccal configuration was accompanied by an approximate 50% reduction in the rate of peptidoglycan synthesis while, during the reverse shape transition, this rate of synthesis approximately doubled (Satta *et al.*, 1979a). Synchronized rods of this mutant synthesized peptidoglycan throughout the cell cycle; specifically, they showed linear synthesis for the first 30 min and exponential synthesis for the following 20 min, coinciding approximately with the increase in cell number (Fig. 7). In contrast, in cocci the rate of synthesis of this polymer increased exponentially immediately before and during septum formation, and decreased exponentially to virtually zero after the end of division (Satta *et al.*, 1979a).

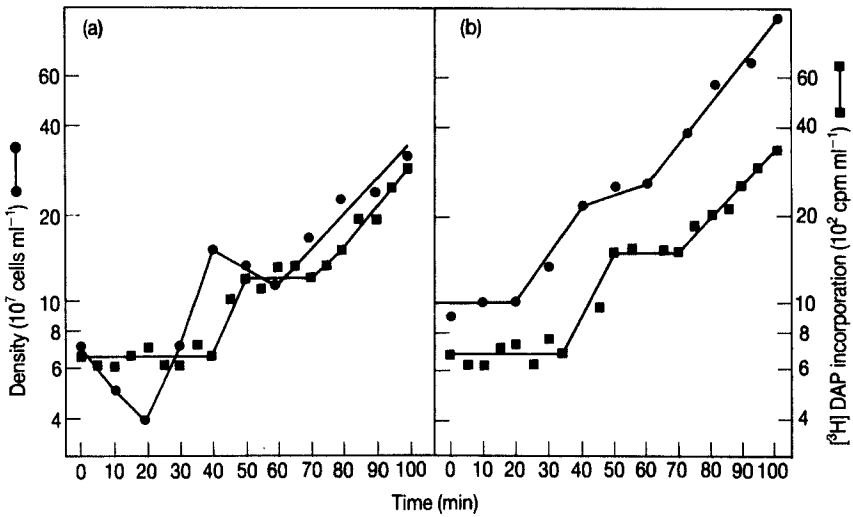

FIG. 7. Incorporation of [^{3}H]diaminopimelic acid into cells of synchronized *Klebsiella pneumoniae* Mir M7 grown at pH 7 (a) or pH 5.8 (b). Exponentially-growing cells were synchronized with the sucrose-gradient method. Every 10 min, duplicate samples (1 ml) were pulse-labelled with [^{3}H]diaminopimelic acid. From Satta *et al.* (1979a).

As already stated, when mecillinam is added to the cultures of the mutant Mir M7 grown as rods at pH 5.8, cells undergo transition to coccal shape and continue to proliferate as cocci. Under these conditions, soon after addition of mecillinam, a drop in peptidoglycan synthesis is observed which is followed by an exponential increase which starts slightly before cells start dividing and stops slightly before the end of the first synchronous division (Satta *et al.*, 1985; Canepari *et al.*, 1993). After this, cells become cocci and an immediate exponential decrease in the rate of peptidoglycan synthesis is observed, and this is followed by another exponential increase slightly preceding the beginning of the second synchronous division. This behaviour is identical with that of cocci grown at pH 7.

D. EFFECTS OF DIFFERENT β-LACTAM ANTIBIOTICS ON CELL DIVISION, CELL SHAPE AND PEPTIDOGLYCAN SYNTHESIS OF WILD-TYPE STRAINS

It is generally accepted that β-lactam antibiotics inhibit bacterial growth by interfering with the terminal stages of peptidoglycan synthesis (Ghuysen, 1977; Spratt, 1979; Waxman and Strominger, 1983). As this antibiotic group includes molecules with many different structures, in the light of the TCS model it is possible that some β-lactam antibiotics may act (at least preferentially) on one of the two sites, i.e. only the one for septum formation or only the one for lateral-wall elongation. This inference from the model finds support in a very early study (Schwarz *et al.*, 1969) which proposed that bacteria have functionally different systems for peptidoglycan synthesis, one of which is active during septum formation and the other during lateral-wall elongation. These systems were revealed by their differential susceptibility to penicillin. Later on, biochemical and genetic evidence for such a different susceptibility to β-lactams of the systems involved in cell elongation and cell division was provided (Spratt, 1975; Spratt and Pardee, 1975). It was shown that, in *E. coli*, among various β-lactam antibiotics tested some preferentially bound PBP2 and forced the cells to become cocci and others bound PBP3 and caused formation of long unseptated cells (filaments), while yet others bound PBP1 and inhibited cell division and elongation without causing shape alterations. As already described, it was suggested that PBP1 is necessary for cell-wall elongation, PBP3 for septum formation and PBP2 for bacteria to grow as rods (Spratt, 1975; Spratt and Pardee, 1975). Although these findings were not explicitly discussed in terms of the two reactions, one responsible for lateral-wall and the other for septum formation, previously proposed by others, they clearly indicate that different β-lactam antibiotics may interfere preferentially with one or the other of the reactions.

The possibility that different β-lactam antibiotics may act on the

different sites for peptidoglycan assembly was first raised by studying the susceptibility of the morphology mutant *K. pneumoniae* Mir M7 to these antibiotics, under conditions causing it to grow as cocci or as rods (Satta *et al.*, 1979a). It was found that the minimal inhibitory concentration (MIC) of antibiotics, such as cephalexin, that inhibit cell division and induce filament formation were much lower for cocci than for rods of the mutant. In contrast, cocci were fully resistant to mecillinam which inhibited growth of rods at very low concentration whereas antibiotics such as penicillin had fairly similar MICs for cocci and rods.

The prediction that β-lactam antibiotics may preferentially inhibit one of the two sites for peptidoglycan synthesis is also supported by the numerous observations that, in Gram-negative rods, some of these antibiotics, such as piperacillin, azlocillin, mezlocillin and aztreonam, at concentrations up to 50 to 100 times greater than the MIC, inhibit septum formation without interfering with cell elongation (Iida *et al.*, 1978; Curtis *et al.*, 1979). In contrast, antibiotics such as mecillinam and others which, at the MIC, bind PBP2 only, such as thienamycin, clavulanic acid, 6-aminopenicillanic acid, penicillanic acid, and penicillanic acid sulphone (Spratt *et al.*, 1977; Curtis *et al.*, 1979; Ishiguro *et al.*, 1979) completely block lateral-wall elongation. However, these antibiotics still allow cells to form septa and divide, only once, in cells carrying the LED control and indefinitely in strains which do not have it or have lost it.

Again, as regards the effects of β-lactam antibiotics on bacterial cells, another implication of the model is that antibiotics which interfere with the site for septum formation should be active against both rods and cocci, since both are able to make septa. On the other hand, those which inhibit lateral-wall elongation should be active only against rod-shaped microorganisms, in which they cause transition to coccal shape. However, these latter drugs should inhibit growth only of those rod-shaped bacteria bearing the control that links septum formation to lateral-wall elongation (which among pathogenic rod-shaped bacteria are by far the vast majority). In addition, antibiotics which block only one of the sites should cause only partial inhibition of peptidoglycan synthesis, while those that block both sites should cause considerable inhibition of peptidoglycan synthesis. All of these implications have been experimentally proved by the finding that mecillinam has no effect on coccoid wild-type bacteria or on morphology mutants of Gram-negative rods when they are grown as cocci, while the same mutants are fully sensitive to mecillinam, as regards the effect on cell shape, when grown as rods (Satta *et al.*, 1980b; Spratt *et al.*, 1980). As already mentioned, Gram-negative rods without control of lateral-wall elongation over septum formation undergo shape alteration, but are not inhibited as far as cell growth is concerned. In these rods,

peptidoglycan synthesis is inhibited by mecillinam by approximately 50% until they become cocci (Satta *et al.*, 1985; Canepari *et al.*, 1993). After the shape change has occurred, no further decrease in the rate of synthesis is observed. All of these findings together with others already described, prompted us to suggest, as already stated, that mecillinam causes formation of cocci through its ability specifically to inhibit activity of the site for lateral-wall elongation. We also suggested that PBP2 was essential for bacteria to acquire a rod shape as a result of its crucial role in determining formation and lengthening of the lateral wall.

Unlike β-lactam antibiotics, which are specific inhibitors of synthesis of the lateral wall in rods but not in cocci, antibiotics which inhibit septum formation, such as piperacillin, aztreonam and cephalexin, have been shown to inhibit growth of wild-type bacteria and mutants completely, irrespective of whether they grow as rods or as cocci (Satta *et al.*, 1985; Canepari *et al.*, 1993). As far as the effect of these antibiotics on peptidoglycan synthesis is concerned, it has been shown that piperacillin, at concentrations as high as 50 times the MIC, has no apparent effect on incorporation of labelled diaminopimelic acid into TCA-precipitable material or into material insoluble in SDS (Iida *et al.*, 1978). With this antibiotic, as with aztreonam which also inhibits septum formation without interfering with lateral-wall elongation, we were unable to observe any clear inhibition of peptidoglycan synthesis (measured as already indicated) in exponentially-growing cells (unpublished observation). These findings are rather unexpected since they indicate that these antibiotics, at concentrations that saturate PBP3 and also probably at least partially saturate other PBPs, inhibit septum formation without grossly impairing overall peptidoglycan synthesis (Iida *et al.*, 1978). Of course, these observations are inexplicable on the basis of current models of the mode of action of β-lactam antibiotics and shape regulation, even if the concept of there being two different reactions, one for the septum and the other for the lateral wall, is now accepted. However, in contrast, the findings can be fully integrated and explained with the aid of the TCS model. In fact, this model predicts that, if ability to form the septum is impaired, the balance between the competing reaction is shifted in favour of the lateral-wall elongation site, so that all peptidoglycan synthetic activity of cells is directed towards formation of the lateral wall. Therefore, antibiotics that prevent septum formation without any significant overall inhibition of peptidoglycan synthesis can do so because they have the peculiar property of shifting the balance of the two competing reactions in favour of the one for lateral-wall elongation without grossly interfering with the biosynthetic capability of either of the two competing sites.

E. MORPHOLOGY AND CELL-DIVISION MUTANTS

1. Different Classes of Mutants with Impaired Site for Septum or Lateral-wall Formation

An original prediction of the TCS model is that two different types of morphology and cell-division mutants of rod-shaped bacteria may exist. As regards morphology mutants, the model predicts that coccal shape may depend on two different types of damage. It may be that cells have lost the ability to form a lateral wall or that, though still maintaining this ability, their site for lateral-wall extension is constantly overridden by the septum formation site. This condition may depend either on excessive strength of the site for septum formation or weakness of the site for lateral-wall elongation. In cell-division mutants, inability to form septa may be due either to loss of functionality of the site for septum formation or to the fact that this site is constantly overridden by the lateral-wall elongation site. Such a condition may again depend either on decreased activity of the site for septum formation or on hyperactivity of the site for lateral-wall extension. Morphology mutants which have lost the function of the site responsible for lateral-wall formation should not be able to regain rod shape unless the activity of this site is restored. In contrast, in mutants where the same shape damage depends on a constant imbalance between the activities of the two competing reactions, additional mutations or antibiotics which slow down or block activity of the site for septum formation should enable cells to regain rod shape. In addition, such mutants should also be converted into rods by antibiotics, mutations or by an increase in the amount of specific gene products which stimulate or reinforce activity of the site for lateral-wall elongation. Similarly, division mutants which have lost activity of the site for septum formation cannot have their division capability restored unless they regain the lost activity. In contrast, division mutants in which the site for lateral-wall elongation overrides the septum site can start dividing again either in the presence of antibiotics which interfere with the site for lateral-wall elongation or in the presence of compounds which stimulate the site for septum formation. Similar effects should be obtained also by mutations which weaken the site for lateral-wall elongation or by mutations (or by an increase in the amount of specific gene products) which reinforce the site for septum formation.

According to the TCS model, mutations which impair activity of the site for septum formation should also influence the morphology of some, but not all, wild-type cocci. In fact, in cocci with two sites for peptidoglycan assembly, impairment of the site for septum formation may allow the second site to start operating, possibly causing formation of a lateral wall

with consequent transition from coccal to rod shape. In cocci that carry only one site for peptidoglycan assembly, the same mutation should impair cell division without causing any gross shape alteration.

The effect of inhibiting septum formation in cocci of the pH-dependent conditional morphology mutant of *K. pneumoniae* Mir M7 was studied (Lleó *et al.*, 1990). It was found that antibiotics, such as nalidixic acid, mitomycin, aztreonam and piperacillin, at a minimal concentration which completely inhibits cell division, cause cocci first to become rods and then to tend to form filaments. In the same study, cocci of the mutant were also treated with sub-inhibitory concentrations of nalidixic acid. Under these conditions, DNA synthesis and cell number increase were inhibited by no more than 30% within the first 150 min and only slightly more during the following 30 min. In this period of time, the strain underwent approximately three cell-division cycles. Under these conditions, the cells became rod shaped within 60 min and divided in this shape. Since the mutant cannot divide at high temperatures, probably due to a defect in DNA synthesis of the *dna*A type, cocci of this strain were incubated at pH 7 and 41°C (Satta and Fontana, 1974a). It was observed that cells underwent coccus-to-rod transition within 80 min. Altogether, these results indicate that the coccal morphology exhibited by the mutant at pH 7 is not due to inactivation of the site of lateral-wall elongation, but to hyperactivity of the site for septum formation or to a decrease in the activity of the lateral wall site.

A similar approach (Lleó *et al.*, 1990) was also used to study properties of some genetically well-characterized morphology mutants of *E. coli*. The strains used were: *E. coli* SP 6 (*pbpA*) which does not synthesize PBP2 and has a round shape at 32°C and 42°C (Spratt *et al.*, 1980); *E. coli* SP45 (*pbpA*-ts) which does not synthesize PBP2 at 42°C and has a round shape at this temperature (Spratt *et al.*, 1980); *E. coli* SP52 (*rodA*, ts) which forms a normal PBP2, but does not synthesize the *rodA* protein at 42°C and has a round shape at this temperature (Spratt *et al.*, 1980); and *E. coli* *env*B which has a round shape at 32°C and 42°C (Westling-Haggstrom and Normark, 1975). It was found that cocci of strains SP52 and *envB* underwent transition to rod shape in the presence of antibiotics which inhibit septum formation either through interference with DNA synthesis, such as nalidixic acid and mitomycin, or by directly interacting with PBP3, such as aztreonam. In contrast, cocci of mutants SP6 and SP45, under the same conditions, maintained their coccal shape (Table 1.).

These results clearly demonstrate that two types of morphology mutants do indeed exist, as predicted by the TCS model, one of which has irreversibly lost its ability to regain rod shape while the other can acquire the normal rod configuration upon inhibition or impairment of septum

TABLE 1. Effect of septum inhibition by antibiotics on cell shape of various morphology mutants of *Escherichia coli*

Strain[a]	Relevant properties	Antibiotic (μg ml^{-1}) and growth conditions[b]	Shape after drug treatment for[c]		
			60 min	120 min	240 min
SP6	*pbpA*[d] PBP2$^-$, cocci at 32 and 42°C	Nal(20) at 32 or 42°C	Cocci	Cocci	Cocci
		MitC(5) at 32 or 42°C	Cocci	Cocci	Cocci
		Aztr(5) at 32 or 42°C	Cocci	Cocci	Cocci
SP45	*pbpA* PBP2$^-$(Ts), cocci at 42°C	Nal(20) at 42°C	Cocci	Cocci	Cocci
		MitC(5) at 42°C	Cocci	Cocci	Cocci
		Aztr(5) at 42°C	Cocci	Cocci	Cocci
SP52	*rodA* RodA$^-$(Ts), cocci at 42°C	Nal(20) at 42°C	Rods	Rods	Rods
		MitC(5) at 42°C	Rods	Rods	Rods
		Aztr(5) at 42°C	Rods	Rods	Rods
envB	*envB* EnvB$^-$, cocci at 32 and 42°C	Nal(20) at 32 or 42°C	Rods	Rods	Rods
		MitC(5) at 32 or 42°C	Rods	Rods	Rods
		Aztr(5) at 32 or 42°C	Rods	Rods	Rods

[a] The strains were incubated for 120 min at 42°C before addition of antibiotics to allow full expression of altered morphology. Behaviour of SP6 and envB, both constitutive mutants, was the same at both temperatures.

[b] The concentration used was the minimal concentration inhibiting cell number increase. Nal indicates nalidixic acid, MitC mitomycin C, and Aztr, aztreonam.

[c] All cells were cocci at zero time.

[d] The gene encoding PBP2.

formation. In addition, these findings also support the suggestion that PBP2 plays a major role in lateral-wall elongation since its presence in morphology mutants appears to be necessary for them to be able to form a lateral wall. In this respect, it is also important to note that, if incubation of cocci that carry PBP2 (SP52 and envB) in the presence of aztreonam is long lasting, these cells form filaments, in agreement with the suggestion that PBP2 is responsible for formation of rods (Canepari, P., Lleó, M. M., Fontana, R., Satta, G., unpublished observations).

Inhibition of the septum formation should cause resumption of peptidoglycan synthesis in cocci that elongate and become rods, but not in those that do not. If the peptidoglycan which is made during transition to rod shape were associated with lateral-wall elongation and with PBP2 activity, its synthesis should be inhibited by mecillinam. These predictions were confirmed on studying *in vitro* synthesis of peptidoglycan in toluene-permeabilized cells (Satta *et al.*, 1983). We found that the amount of peptidoglycan synthesized by toluene-permeabilized cells of Mir M7 grown as cocci at pH 7 was nearly 50% less than that synthesized by cells of this mutant grown as rods at pH 5.8. The parent strain Mir A12, synthesized the same amount of peptidoglycan as rod-shaped cells of strain Mir M7.

When cocci of strain Mir M7 grown at pH 7 were converted to rods by treatment with nalidixic acid, *in vitro* peptidoglycan synthesis was restored to the level of that in rods of this strain at pH 5.8. When rods of strain Mir M7 grown at pH 5.8 were converted to cocci by mecillinam, the amount of peptidoglycan synthesized was equal to that of cocci grown at pH 7. Moreover, when mecillinam was added to permeabilized rods of the mutant, an approximately 50% decrease in peptidoglycan synthesis was observed.

2. *Effect of Mecillinam on Division Mutants*

Mecillinam, which a large body of evidence clearly indicates to be a specific inhibitor of the site for lateral-wall elongation, was used to test predictions of the TCS model concerning different classes of cell-division mutants. In particular, the effect of this antibiotic on several *ts* cell-division mutants of *E. coli*, such as BUG6 (*fts, ts*; Reeve *et al.*, 1970), AX655 (*fts*I-2158, *ts*; Walker *et al.*, 1975) and AX621 (*fts*A-1882, *ts*; Walker *et al.*, 1975) that form filaments at the non-permissive temperature was evaluated (Canepari, *et al.*, 1984; Fig. 8). It was found that the antibiotic restored the ability to divide at 42°C to strains BUG6 and AX655 but not to strain AX621. In strain AX655, ability to divide was restored only if the antibiotic was added immediately after shifting to the non-permissive temperature, whereas with strain BUG6 the rise in cell number was observed also when mecillinam was added after 90 min of incubation at 42°C. These findings clearly show that at least two different classes of cell-division mutants exist and that inability of bacteria to form septa and divide is not necessarily linked to inactivation of the site for septum formation, but may also be due to the relative hyperactivity of the site for lateral-wall elongation. Under these conditions, inhibition of this site by an antibiotic restores the ability to form septa and divide.

3. *Properties of Double Mutants Which Carry One Mutation Altering Cell Shape and One Impairing Septum Formation*

a. Double mutants which recover normal shape and ability to divide. The observations already described also allow us to predict that in mutants that do not divide due to relative hyperactivity of the site for lateral-wall elongation, mutations which impair this site should restore the ability to form septa and divide. Similarly, in morphology mutants which appear as cocci, due to relative hyperactivity of the site for septum formation, mutations which impair this site should restore normal rod shape.

Several years after the effect of antibiotics on morphology and cell-

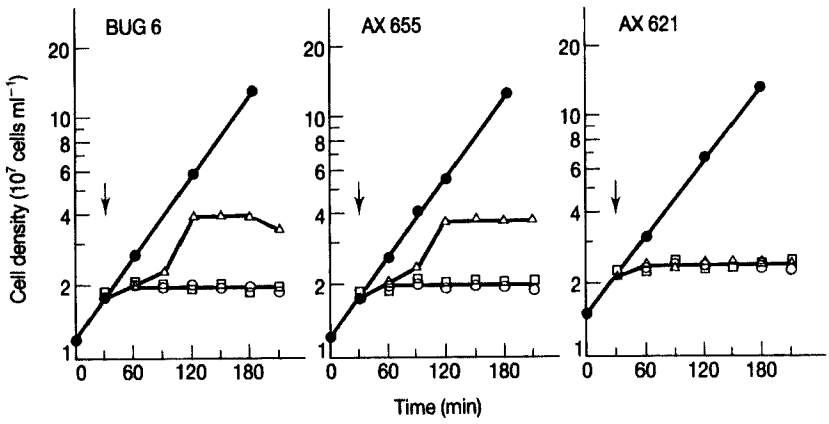

FIG. 8. Effect of mecillinam (5 μg ml^{-1}) on cell division of *Escherichia coli* strains BUG6, AX655 and AX621 grown at 42°C. Cultures of the three strains growing exponentially at 30°C (●) were subdivided at the time indicated by the arrows into four parts. One was incubated at 30°C; the others were incubated at 42°C without antibiotic (○), with mecillinam (△), or with mecillinam and an excess of penicillinase (□). At regular intervals, samples were withdrawn from all of the cultures to evaluate the cell number with a Coulter counter. From Canepari *et al.* (1984).

division mutants already described had been published, a double mutant (strain I4) of *E. coli* carrying one mutation in the *rod*A gene (*sui, ts*) which impairs lateral wall elongation and causes formation of cocci at 42°C, and one in the *ftsI* gene (*ftsI23*) which impairs septum formation and causes formation of filaments at 42°C, was isolated (Begg *et al.*, 1986). This double mutant was found to divide normally and to form rod-shaped cells even at 42°C, a temperature which in the strains carrying only one of the two mutations was not permissive either for cell shape or for cell division. A further characterization of the *rod*A (*sui*) mutation showed that it caused production of a lower content of wild-type *rod*A protein accompanied by an increase in the contents of both PBP2 and PBP5, which are also coded for by genes in the *rodA* operon. This latter finding strongly supports the suggestion from the TCS model that PBPs do not each perform a constant and specific function but interact with one another, also modulating one another's activity. The round shape of cells of the *rod*A (*sui*) mutant was explained by overproduction of PBP5 which caused excessive removal of terminal D-alanine residues from peptidoglycan precursors, converting them to a form which cannot be utilized by the biosynthetic system involved in cell elongation, but which is the preferred substrate for the cell-division system (Markiewicz *et al.*, 1982; Begg *et al.*,

1990). It was postulated that the *rodA* (*sui*) mutation restored cell division because it permitted activity of the damaged PBP3 (*ftsI23* mutation) by indirectly providing an excess of substrate for this protein. This hypothesis, however, does not explain recovery of correct morphology. On the contrary, behaviour of this double mutant is explained by a prediction of the TCS model, namely that morphology mutants which appear as cocci may carry a mutation which causes hyperactivity of the site for septum formation (e.g. the *sui* mutation) and that mutations which impair this site (e.g. *ftsI23* mutation) can restore normal morphology by re-establishing the correct balance between the two competing sites. The behaviour of the double mutant *E. coli* I4 also confirms the existence of mutants in which cell division is restored by impairing activity of the site for lateral-wall elongation by mutations specifically affecting it.

As regards these aspects of the TCS model, it appears to be of great importance that mecillinam, which causes indirect inhibition of septum formation in wild-type Gram-negative rods, restores cell division in mutants which are unable to divide (Canepari *et al.*, 1984). This apparently contradictory effect of the antibiotic would be inexplicable without the TCS model, which clearly predicts and fully explains it. Also of great importance is the behaviour of the double mutant *E. coli* I4. This strain too has allowed findings which could not have been predicted on the basis of any hypothesis regarding cell shape or cell-division regulation other than the TCS model which, in contrast, fully and clearly predicted the possibility of isolating these mutants long before they were found (Canepari *et al.*, 1984). Both the effect of mecillinam on cell-division mutants and the properties of the double mutant already described very strongly support the concept that reactions responsible for lateral wall and septum formation indeed compete with each other. As already stated this concept is peculiar and exclusive to the TCS model.

It was unfortunate that the workers who performed experiments on the double mutants of *E. coli* did not refer to the TCS model, but interpreted their results in the light of the observation that, among the various PBPs, some are specifically (and exclusively, according to the concept they quote) involved in cell elongation and others in septum formation. This was unfortunate since the existence of PBPs, each with a specific function in lateral-wall elongation or in septum formation, is not in itself enough to explain the fact that mutations impairing septum formation are corrected by mutations damaging lateral-wall elongation, unless one accepts that the reactions responsible for the two events compete with each other. As already stated, the concept of competition is peculiar and exclusive to the TCS model both with regard to overall activity at the two sites and to activity of PBPs. Instead of considering competition between septum and

lateral-wall formation, they (Begg *et al.*, 1986) consider these as alternating processes as indeed they unquestionably are. However, their alternation is merely a consequence of a balanced competition between the two sites, but is a concept which does not in itself imply competition and the behaviour of the double mutant *E. coli* I4 is not explicable without the competition concept.

b. Double mutants which do not recover normal shape and ability to divide. Other double mutants carrying a *ts* mutation impairing cell shape (*pbpA, rodA*) and a *ts* mutation damaging septum formation (*ftsQ, ftsA, ftsZ,* and *ftsI*) were also constructed (Begg and Donachie, 1975). All of these double mutants, as opposed to *E. coli* I4, when incubated at the non-permissive temperature for both shape and division, did not recover correct shape or ability to septate, but only grew in cell mass and formed lemon-shaped cells with or without a partial constriction, depending on the *fts* mutation. These results were interpreted as disproving the existence of two reactions, one responsible for lateral-wall elongation and the other for septum formation. However, in spite of this clear-cut conclusion, the properties described of these double mutants do absolutely nothing to disprove the TCS hypothesis regarding two reactions being involved in peptidoglycan synthesis. They can be fully explained in light of the concept of competition between the two mechanisms which, as we have described, is peculiar to the TCS model. In fact, first of all, it has to be considered that, in double mutants, shifting to the non-permissive temperature causes an immediate inhibition of septum formation, but an appreciably delayed inhibition (around 100 min) of the site for lateral-wall elongation. It is very likely that, during these 100 min, the cell wall continues to expand due to activity of the as yet not fully inhibited site for lateral-wall elongation. The lemon shape may be caused by the as yet uninhibited activity of the site for lateral wall formation in cells where the site for septum formation is completely inactive. In addition to this, it has to be considered that, for the TCS model, coccal shape or inability to form septa does not imply that the respective sites are necessarily unable to perform their function under all conditions. But in a mutant unable to form a lateral wall, the site for this reaction may be activated by a mutation or by an antibiotic which inhibits septum formation; and, vice versa in mutants unable to form a septum, the relevant site may be activated by antibiotics or mutations that interfere with lateral-wall elongation (see above). It may therefore be that, in all of the above-mentioned double mutants each carrying a mutation in the *pbpA* or *rodA* genes, combined with one mutation in one of the *fts* genes, a mutation causing formation of cocci does not irreversibly inactivate the site for lateral-wall elongation, but predisposes it to being constantly over-

ridden by the site for septum formation. Consequently, the presence in the same strain of a mutation that blocks septum formation could cause re-activation of the lateral-wall site, thus allowing the cell surface to expand at the non-permissive temperature because of resumption of activity at this site. That this was indeed correct was proved by the fact that mutants carrying only the *rodA* or *pbpA* mutation form regular rods at the non-permissive temperature when septum formation is inhibited by nalidixic acid or by aztreonam (Lleó *et al.*, 1990). On the other hand, we found that strains that carry the *ftsZ* mutation, which was used to construct the double mutant KJB 20 (Begg *et al.*, 1986), causing inability to divide at 42°C, divide at the non-permissive temperature in the presence of mecillinam.

Finally, in order to confirm that the ability of double mutant KJB 20 to continue to expand its cell surface under conditions which are apparently not permissive for either septum or lateral-wall formation was due to the fact that, in this strain, inhibition of septum formation allowed the lateral-wall site to operate at the non-permissive temperature, we incubated the double mutant at the permissive temperature until it formed cocci and shifted the cocci to 42°C. Under these conditions, cells did not expand their surface or change their shape as long as mecillinam was kept in the culture to block the site for lateral-wall elongation. When, however, the antibiotic was removed cells started expanding their surfaces and changing their shape. This clearly indicates that, in the double mutant KJB 20, the site for lateral-wall elongation is indeed active at the non-permissive temperature, when the site for septum formation is blocked.

4. *Effects of Alterations in* mre *Genes on Cell Shape and Cell Division*

Other authors have shown that mutations causing round-cell formation also mapped in a region (*mre*) containing multiple genes (*mreB, mreC, mreD*), none of which apparently codifies for peptidoglycan-synthesizing enzymes. Two mutants were isolated, namely *mreBI29* which is spherical and overproduces PBP3, and △-*mre-678*, which is spherical and overproduces PBP1B and 3 (Wachi *et al.*, 1987; Doi *et al.*, 1988). Transformation of these mutants with plasmids carrying the wild-type *mreB* gene makes them acquire rod shape, whereas introduction of additional copies of *mre* genes causes inhibition of cell division and formation of filaments (Wachi and Matsuhashi, 1989). These shape-determining genes are thought to have a negative influence on cell division and a positive function on elongation of cells to give the correct rod shape.

All of the properties of the strains already described are also predicted by the TCS model and only by that model. They are typical expressions

of a situation in which septum formation and cell division can be inhibited without directly interfering with activity of the relevant site, by increasing the relative strength of the site for lateral-wall formation (in this instance probably through increased synthesis of a gene product). Such an effect, which is by no means logical or predictable without the aid of the concept of competition between the two mechanisms responsible for peptidoglycan synthesis of bacterial rods, emerges as self-evident in light of the competition model. The authors have perhaps underestimated the significance of work on the TCS model including one paper (Satta *et al.*, 1980b) whose title (Control of cell septation by lateral wall extension in a pH-conditional morphology mutant of *Klebsiella pneumoniae*) expresses the very concept that lateral-wall extension controls septum formation, as in the title of their own work published several years later (Wachi and Matsuhashi, 1989).

F. EFFECTS OF SIMULTANEOUS INHIBITION OF LATERAL-WALL ELONGATION AND SEPTUM FORMATION ON MACROMOLECULE SYNTHESIS

As has already been described, the TCS model states that each of the two sites for peptidoglycan synthesis can be inhibited without interfering with the other, so that, on the one hand, the lateral wall can still continue to be formed when septum formation is inhibited and, on the other, the site for septum formation can continue to function when the lateral-wall site is inhibited. An important consequence of this is that bacteria with two sites for peptidoglycan assembly, as opposed to those with only one, continue to expand their surface even after one of the two sites is blocked. According to the TCS model, this is of crucial importance since the model suggests that a control mechanism exists in bacteria that blocks macromolecule synthesis when cell-surface expansion is prevented. This control mechanism depends on the fact that cells which do not expand their surface do not create additional intracellular space to fit in newly synthesized macromolecules. Obviously, as a result and according to the TCS model, complete simultaneous inhibition of the two sites for peptidoglycan assembly should cause complete block of cell-surface expansion with consequent activation of the control mechanism which, under these conditions, gives rise to inhibition of macromolecule synthesis.

The inferences from the model allow several predictions to be made which can be easily tested. The first is that, in wild coccoid bacteria, antibiotics such as β-lactams or mitomycin and nalidixic acid, which have no direct effect on macromolecule synthesis other than that on peptidoglycan and DNA, should also cause rapid inhibition of other macromolecules whose synthesis is not their direct target at the MIC. Under

similar conditions, in wild-type cocci which also possess the site for lateral-wall formation, the same antibiotics should not interfere significantly with synthesis of macromolecules which are not their target. Secondly, in bacterial rods, antibiotics such as the β-lactam aztreonam and the quinolones, which at the MIC cause inhibition of septum formation through two different mechanisms, should not inhibit synthesis of macromolecules which are not their respective targets. However, if these bacteria are treated, at the same time, with one of these antibiotics and with mecillinam, rapid inhibition of overall macromolecule synthesis should be observed. Thirdly, in morphology mutants which grow as cocci, quinolones and β-lactam antibiotics (which specifically inhibit septum formation) should have a different effect on those mutants which, on inhibition of septum, form rods and continue to expand their surface as well as on those which are unable to do so. In the former strains, these antibiotics should completely inhibit only the specific syntheses which are their targets, (DNA or peptidoglycan) while, in the latter strains, at the MIC they should cause virtually simultaneous inhibition of synthesis of all macromolecules. In cell-division conditional mutants of rod-shaped bacteria, which grow as filaments, addition of mecillinam (at the MIC) should completely inhibit synthesis of macromolecules. Cell-division conditional mutants of wild-type cocci should not synthesize macromolecules under non-permissive conditions if isolated from species with only one site for peptidoglycan assembly, but should continue to expand the cell surface and form macromolecules if isolated from species with two sites for peptidoglycan synthesis. Finally strains with two mutations, one causing irreversible inactivation of synthesis of the lateral-wall site and one inactivating synthesis of the septum formation site should be unable to form macromolecules in spite of the fact that mutants with only one of these sites are capable of synthesizing such compounds. As regards this specific prediction, it is important to note that this does not mean that a combination of one mutation that causes transition to coccal shape with one which causes formation of filaments results in an inability to expand the cell surface. Such mutations do not necessarily cause irreversible inactivation of specific sites for peptidoglycan synthesis, but may also cause an imbalance between the competing sites, both of which still remain potentially active. Accordingly, one site which is inactive due to the fact that it is constantly overridden by the other may become active if the site that overrides it is impaired or if its own activity is reinforced in some way (e.g. through an increase in the number of copies of a specific gene; see above).

In order to challenge the first prediction, we studied the effect of antibiotics which inhibit septum formation by interacting with peptidoglycan synthesis (methicillin for Gram-positives) or with DNA replication

(mitomycin C for Gram-positive strains, and nalidixic acid for Gram-negative strains) on macromolecule synthesis and cell-surface extension of wild-type cocci of *Staphylococcus aureus, Streptococcus lactis, Strep. disgalactiae, Strep. bovis, Strep. agalactiae, Enterococcus faecium, Strep. sanguis, Neisseria gonorrhoeae* and *N. catarrhalis* (Canepari *et al.*, 1983; M. M. Lleó, P. Canepari, R. Fontana and G. Satta, unpublished observation). It was found that in *Strep. bovis, Strep. agalactiae, E. faecium* and *Strep. sanguis* (four species in which inhibition of septum formation causes formation of rods, indicating the presence of a site for lateral-wall elongation) when treated with mitomycin or methicillin, protein synthesis and surface extension continued for at least 3 h, whereas in *Staph. aureus, Strep. lactis, Strep. disgalactiae, N. gonorrhoeae* and *N. catarrhalis* (five species in which inhibition of septum formation does not cause any shape change, thus indicating the absence of a second site for peptidoglycan assembly), when treated with nalidixic acid, protein synthesis and surface extension were immediately blocked.

As regards the second prediction, we treated *E. coli* with a specific inhibitor of lateral-wall elongation (mecillinam) for 120 min and then added aztreonam (or nalidixic acid) at the minimal concentration which inhibits cell division. Under these conditions, an immediate block of cell-surface expansion and inhibition of protein and DNA synthesis was observed as was when both sites were inhibited by β-lactam antibiotics (Canepari *et al.*, 1983; M. M. Lleó, P. Canepari, R. Fontana and G. Satta, unpublished observation).

The interesting aspect of these findings is that it is well established that β-lactam antibiotics have no direct effect on protein and DNA synthesis, while quinolones have no direct effect on protein synthesis. In the experiments in which they completely inhibited expansion of the cell surface through simultaneous inhibition of the two sites for peptidoglycan assembly, the antibiotics caused complete inhibition of synthesis of these macromolecules. Such antibiotic activity can only be indirect and is likely to be merely a consequence of their effect on cell-surface expansion.

As regards the third prediction, we studied (M. M. Lleó, P. Canepari, R. Fontana and G. Satta, unpublished observation) cell-surface expansion and macromolecule synthesis in mutants which were constitutively or conditionally unable to form a lateral wall, such as *E. coli* SP6 (*pbp*A$^-$, PBP2, constitutive; Spratt *et al.*, 1980), *E. coli* SP45 (*pbp*A, *ts*, PBP2$^-$; Spratt *et al.*, 1980), *E. coli* SP52 (*rod*A, *ts*, *rod*A$^-$; 441 C), *E. coli* envB (constitutive; Westling-Haggstrom and Normark, 1975) and *K. pneumoniae* Mir M7 (pH-dependent; Satta and Fontana, 1974a,b). In strains SP6 and SP45, in which synthesis of the lateral-wall site is irreversibly inactive, treatment with specific inhibitors of septum formation (either

with aztreonam or nalidixic acid) inhibited surface extension, while macromolecule synthesis was immediately blocked. In contrast, in strains SP52, *env* and Mir M7, in which upon inhibition of septum formation, the site for lateral wall synthesis starts functioning again, these antibiotics allowed protein synthesis to continue together with surface extension. In other experiments, we evaluated the effect on protein and DNA synthesis of *ts* cell-division mutants of *E. coli* incubated for 60 min at 32°C (the permissive temperature), in the presence of mecillinam which blocks lateral-wall formation, and then shifted cultures to 42°C to inhibit septum formation. An immediate and complete arrest of DNA and protein synthesis was observed immediately after shifting to 42°C. Finally *ts* mutants for cell division obtained from *Staph. aureus, Strep. lactis, Strep. disgalactiae, N. gonorrhoeae* and *N. catarrhalis* were found not to alter their coccal shape at the non-permissive temperature and to be unable to synthesize macromolecules under these conditions. In contrast, mutants isolated from *Strep. bovis, Strep. agalactiae, Strep. faecium* and *Strep. sanguis* assumed rod shape at the non-permissive temperature, due to activation of the second site for peptidoglycan assembly, and continued both to expand their surface and to synthesize macromolecules.

Regarding predictions for macromolecule synthesis and surface extension in double mutants carrying a mutation in the site for septum formation and one in the site for lateral-wall elongation, it should be recalled that findings already referred to (Begg and Donachie, 1985) showing that double mutants continued to expand their surface at the non-permissive temperature do not clash with the prediction from the TCS model, since one of the two mutations in these strains was not an irreversible one. As a result, one of the two sites became active in the double mutant and allowed the cell surface to be expanded at the non-permissive temperature. However, as already described, when we analysed the cell shape of cells of the *pbpA* (*ts*) *ftsZ* (*ts*) double mutant KJB 20, first by growing them at 32°C in the presence of mecillinam (until it changed to coccal shape) in order fully to inhibit synthesis of the lateral wall site, and then shifting them to the non-permissive temperature, we found that they maintained their coccal shape (Lleó *et al.*, 1990). Under these conditions, no increase in DNA and protein synthesis was observed in the double mutant KJB 20.

It is also important to recall and stress that, while many cell-division mutants have been described in rod-shaped bacteria, none has been described in coccoid strains. This fact is explained by the TCS model, but cannot be explained by any of the other models and hypotheses concerning cell shape and cell-division regulation in bacteria. Cell-division mutants are defined as those which, under non-permissive conditions, do not form complete septa and do not increase in cell number, but continue forming

macromolecules at approximately normal rates. Virtually all attempts to isolate cell-division mutants from wild-type cocci were made using bacteria such as staphylococci or micrococci which are micro-organisms with only one site for peptidoglycan assembly, where, according to the TCS model, inability to form septa abolishes the only mechanism whereby cells can expand their surface. The TCS model predicts that, as a consequence of this, these mutants also become unable to form macromolecules under conditions that prevent septum formation and, therefore, that cell division mutants which match up to the above-mentioned requirements cannot be isolated from these species. We were the first (Canepari *et al.*, 1983, 1987, 1989; Lleó *et al.*, 1990) to attempt to isolate cell-division mutants from wild-type cocci such as *Strep. bovis, Strep. agalactiae, Strep. faecium* and *Strep. sanguis* which carry two sites for peptidoglycan assembly. We were fully successful in finding several mutants which, under conditions which inhibited septum formation and increase in cell number, continued to undergo macromolecule synthesis at a normal rate. These mutants continued to expand their surface, under these conditions, through formation of a lateral wall that obliged them to undergo coccus-to-rod shape transition (Lleó *et al.*, 1990).

G. EFFECTS OF PBP ALTERATIONS ON CELL SHAPE AND DIVISION AND THEIR NUMBER IN COCCI AND RODS

1. Mutual Compensation Among Damaged PBPs

Among cellular components involved in the process leading to septum formation and lateral wall elongation, a key role is played by PBPs (Spratt, 1975; Spratt and Pardee, 1975; Matsuhashi *et al.*, 1990). Since the balance between the two competing reactions requires modulation of the activity of these proteins, the model predicts that similar damage to cell shape or cell division may be caused by inactivation of one PBP or by overproduction of another. Conversely, abnormal cell physiology caused by damage to one of these proteins can be corrected by decreased activity (or complete inactivation) of another PBP. A large amount of experimental evidence corroborates these predictions. Mutants which carry altered PBPs or which overexpress one of these proteins have been constructed. It was found that, in *E. coli*, overproduction of either PBP5 or PBP6 causes the same damage as inactivation of PBP2, that is to say formation of spherical cells (Markiewicz *et al.*, 1982; Begg *et al.*, 1990). It has also been shown that inability of cells to grow without PBP1 is corrected in cells that form an increased amount of PBPs 1A and 2 (Matsuhashi *et al.*, 1990).

The proposal that PBPs can mutually modulate their functions indirectly derives from the demonstration that, under particular conditions, certain PBPs can take over functions of others. It was shown that in *Strep. faecium* ATCC 9790, growing at an optimal temperature, saturation of PBP3 (or 2 and 3) by a β-lactam antibiotic caused inhibition of growth (Fontana *et al.*, 1980, 1983a). In contrast, if cells were grown at suboptimal temperatures or at the optimal temperature in a diluted medium, saturation of all PBPs (including PBPs 2 and 3), but not PBP5, allowed cells to grow normally (Fontana *et al.*, 1983b). In mutants lacking PBP5, saturation of PBP3 was always sufficient to cause growth inhibition at all temperatures and under all growth conditions (Fontana *et al.*, 1985). In contrast, *Strep. faecium* ATCC 9790 mutants which overproduced PBP5 were capable of growing normally also at the optimal temperature in the presence of antibiotic concentrations that saturated all PBPs except for the overproduced PBP5 (Fontana *et al.*, 1983b). Mutants with damaged PBP1 or lacking PBPs 2 or 3 were unable to grow at the optimal temperature, but grew well at lower temperatures (Canepari *et al.*, 1987). These same mutants also grew well at the optimal temperature if an additional mutation made them overproduce PBP5. It was also shown that staphylococci which carry a novel PBP, designated as PBP2a, are capable of growing normally in the presence of a β-lactam antibiotic concentration that completely saturates all of the other PBPs, thus indicating that this PBP alone can take over the function of all of the others (Brown and Reynolds, 1980; Rossi *et al.*, 1985).

2. PBP Number in Cocci and Rods

According to the TCS model, the level of complexity in regulation of cell shape has increased as one goes from cocci to rods. Since PBPs are thought to play a major role in shape determination and regulation, it appears likely that the number of PBPs should be higher in rods that maintain their shape under all conditions, low in cocci which do not elongate upon inhibition of septum formation and in between in content in cocci that elongate under such conditions. In accordance with this prediction, it was first shown that cocci such as *Neisseria* spp. have only three PBPs, whereas rods such as *E. coli* have at least seven PBPs (Spratt, 1979). A correlation between the PBP number in cells and the level of evolution towards the ability to grow as rods with a well-defined and regulated shape has also been shown in studies on several species of cocci and rods. We studied PBPs from several staphylococcal species and found that the number of these proteins ranged from one (*Staph. hyicus*) to four (*Staph. aureus*) (Canepari *et al.*, 1985). In addition, we have analysed PBPs of different

species of streptococci, some with two sites for peptidoglycan synthesis and others with only one site. Those unable to elongate (*Strep. lactis* and *Strep. disgalactiae*) carried a maximum of four PBPs, whereas those which elongated after septum inhibition (*Strep. agalactiae, Strep. bovis, Strep. faecium, Strep. sanguis*) carried a minimum of six PBPs (Lleó *et al.*, 1990).

H. CHEMICAL COMPOSITION OF PEPTIDOGLYCAN

The idea that the cell wall in rods is made as a result of two different reactions implies the possibility that differences may be observed in the chemical composition of the septum and side-wall peptidoglycan. Attempts have been made to demonstrate structural or biochemical differences in peptidoglycan synthesized by these different systems. It was shown that spherical cells derived from *E. coli* with mutations in PBP2, mecillinam-treated wild-type strains (which become cocci) and mutants overproducing PBP5 (which assume a coccal shape) show a rise in total cross-linkage and a drop in the average length of the glycan strands (Schwarz and Glauner, 1988). On the contrary, other authors reported no difference in peptidoglycan composition in minicells compared with rods or of morphology mutants grown as spheres or filaments (Driehuis and Wouters, 1987).

The most recent studies are based on reverse-phase HPLC separation of muropeptides. In this methodology, peptidoglycan is completely digested with muramidase and the resulting products consisting of disaccharide units linked together by peptide side chains connected by covalent bonds are separated (Glauner, 1988). This technique has revealed a high degree of structural complexity in the peptidoglycan from *E. coli* involving 80 different components, which are the result of the combination of seven different peptide side chains (dipeptide, tripeptide, tripeptide-Lys-Arg, tetrapeptide, tetrapeptide-Gly4, pentapeptide, pentapeptide-Gly5) with two types of peptide cross-linking (D-Ala-m-A2pm, m-A2pm-m-A2pm) giving rise to dimeric, trimeric and tetrameric compounds. The peptidoglycan of cells taken at different stages of the cell cycle was examined, but no differences were observed in septating or elongating cells (de Jonge *et al.*, 1989). Also, no differences in peptidoglycan composition were found in isogenic morphological mutants (de Jonge, 1990) of differently cell-shaped bacteria. These results have been interpreted as supporting the hypothesis that the chemical composition of peptidoglycan does not determine the shape of bacteria.

Analysis by HPLC of muropeptides has also been used to study the mode of insertion of new peptidoglycan during the cell cycle. Synchronized cells were pulse-labelled with radioactive diaminopimelic acid, after which peptidoglycan was purified and digested with muramidase. Measurement

of radioactivity recovered in the different fractions provides an indication of the extent of cross-linking in peptidoglycan synthesized in different phases of the cell cycle. Using this technique, an increase in cross-linking was reported in septating as compared with elongating cells (Woldringh *et al.*, 1977). However, in a subsequent paper (de Jonge *et al.*, 1989), the same authors corrected this value by the acceptor-donor radioactivity ratio and concluded that the apparent increase in cross-linking was due to the fact that, in separating cells, new strands function as both donor and acceptor whereas, in lateral-wall elongation, they act only as donor. The conclusion was that there was no increase in cross-linking in septating cells, but a different type of insertion of new strands.

A new insight into the study of peptidoglycan in the septum and side wall came from other studies which focussed (Romeis *et al.*, 1991) on the length of glycan strands associated with the septum and cell wall. These authors completely digested peptidoglycan with an amidase which removed the peptide side chain leaving the glycan strands intact. This study was applied to peptidoglycan from filaments in which synchronous septation was induced, peptidoglycan from mecillinam-treated cells and peptidoglycan from an *envA* mutant altered in the process of septum splitting. An increase was found in short strands associated with filament decatenation, with round cell formation induced by mecillinam and with blocking of septum splitting in the *env*A mutant. A model has been proposed in which PBP3 synthesizes such short strands, which causes septum constriction. This preseptum may be stabilized by the activity of PBP2, which eventually inserts new strands by the same mechanism used to make side-wall peptidoglycan. As the preseptum has a very short life, the amount of septum specific peptidoglycan is very limited and cannot be detected in normal cells harvested in different phases of the cell cycle.

According to the TCS model, the lack of any difference in peptidoglycan composition in the septum and side wall is acceptable, since competition between the two reactions cannot relate to synthesis of a compound with a different gross composition, but, for instance, to one with a different orientation of the glycan chains. A change in orientation of the direction of peptidoglycan extension may occur as a result of intervention of components not involved in peptidoglycan synthesis (these might be mechanoproteins) or changes in the composition of a small fraction of precursors which have to be inserted in a very narrow area. The amount of polymer with a different chemical composition may be so small that it cannot be detected by chemical analysis of septum and side-wall peptidoglycan. Detection of this peptidoglycan with a different composition may be difficult, amongst other things due to the fact that, in exponentially growing cells, peptidoglycan with a changed direction of growth might be inserted

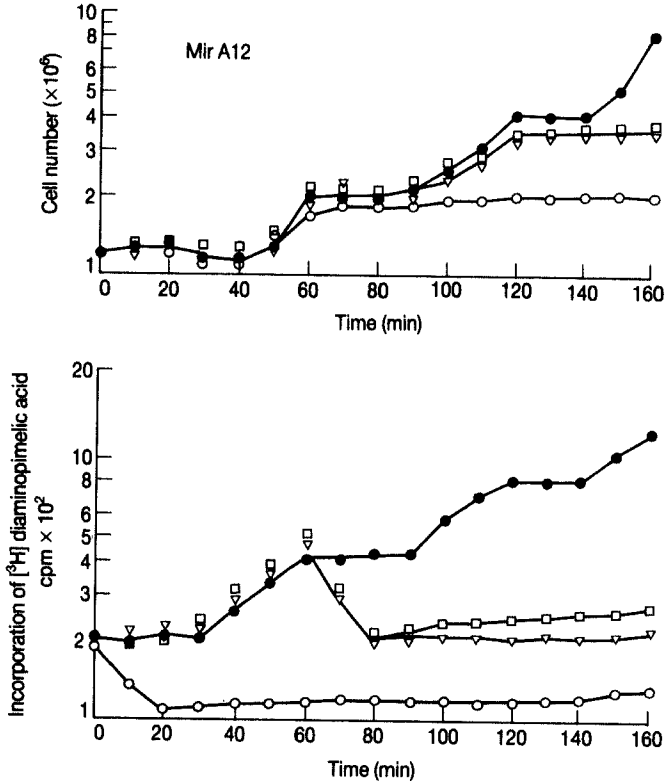

FIG. 9. Effect of mecillinam (1 μg ml^{-1}) added at various times to synchronously dividing cultures of *Klebsiella pneumoniae* Mir A12 on peptidoglycan synthesis and cell number. Symbols: untreated control (●); mecillinam added at zero time (○) after 10 min (▽) and after 20 min (□). From Canepari *et al.* (1993).

in the cylindrical part of the wall long before septum formation starts. As a consequence, cells without any evident constriction may have already formed this peptidoglycan, which may subsequently trigger septum formation.

I. TEMPORAL RELATIONSHIPS BETWEEN LATERAL WALL AND SEPTUM FORMATION DURING THE CELL CYCLE

1. Mutual Exclusion between Lateral-Wall Elongation and Septum Formation

Since reactions that lead, on the one hand, to septum formation and, on the other, to lateral-wall elongation, are thought to compete with each

other, the TCS model implies that bacteria do not elongate while they are forming septa and vice versa. Such mutual exclusion allows a much simpler regulation of septa and lateral-wall formation than that required for bacteria to expand their surface in the septum and in the lateral wall at the same time. In this latter situation, peptidoglycan precursors ought to be correctly inserted either in the septum or in the lateral wall throughout the entire cell cycle, while enzymes involved in synthesis and processing of the two structures should be regulated in order to function correctly in the peptidoglycan for which they are specific.

The suggestion that lateral-wall elongation and septum formation mutually exclude each other can be tested by adding mecillinam, which is known to inhibit synthesis of the lateral-wall elongation site, but not the septum formation site, in synchronously dividing *E. coli*. This antibiotic should inhibit peptidoglycan synthesis if added at the beginning of the cell cycle until septum formation starts, but not after that; that is during lateral-wall elongation, but not during cell division. For reasons which are still unclear, in Gram-negative wild-type rods mecillinam inhibits peptidoglycan synthesis only when added to cultures at the very beginning of the cell cycle (Fig. 9) so that the above-mentioned prediction cannot be tested using this approach. We solved this problem (Satta *et al.*, 1985; Canepari *et al.*, 1993) by using the *E. coli* GC 2702 (carrying the *lov* mutation) and the M7 strains of *K. pneumoniae* which have both lost the LED control linking septum formation to lateral-wall elongation. The cell cycle of these *E. coli* and *K. pneumoniae* strains, when grown in a glucose-minimal salts medium, lasts 40–50 min during which cells elongate without increasing in number for 25–30 min and duplicate in the following 15–20 min. It was found that, in these strains, the inhibitory effect of mecillinam on peptidoglycan synthesis is not restricted to a very short period of the cell cycle as in the parents, but is exerted at any time during lateral wall elongation (Fig. 10).

As predicted by the TCS model, mecillinam in these strains, too was completely devoid of any effect when added to cultures of cells that had started to form septa or were about to do so. In other experiments, synchronized cells of the mutants of both *K. pneumoniae* and *E. coli* were taken at the very beginning of the cell cycle, or slightly before the start of the increase in cell number, and treated for 20 minutes with mecillinam (Canepari *et al.*, 1993). The cell length of cells so treated was compared with that of untreated controls. Cells exposed to antibiotic at the very beginning of the cell cycle were 30% shorter than controls after the 20 min treatment, while those exposed to antibiotic during septum formation had exactly the same length as controls. This further confirms that mecillinam inhibits lateral-wall elongation, and that no elongation occurs during septum formation either in the mutants or in wild-type rod-shaped strains.

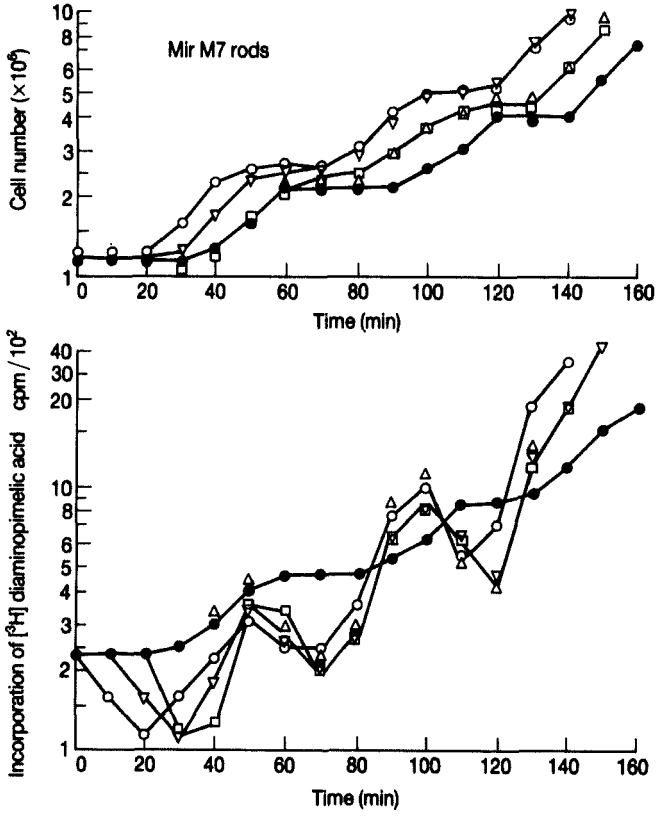

FIG. 10. Effect of mecillinam (1 μg ml^{-1}) added at various times to a synchronously dividing culture of rod-shaped *Klebsiella pneumoniae* Mir M7 on peptidoglycan synthesis and cell number. Symbols: untreated control (●); mecillinam added at zero time (○), at 10 min (▽), 20 min (□) and 30 min (△). From Canepari *et al.* (1993).

In order to exclude the possibility that apparent mutual exclusion of the two sites may be a property linked to the mutation present in the two strains, experiments of a similar type were also performed with wild-type strains of *K. pneumoniae* and *E. coli* (Satta *et al.*, 1985; Canepari *et al.*, 1993). In these experiments, mecillinam was added to synchronously dividing cells at the very beginning of the cell cycle, the only period during which cells are sensitive to the antibiotic. At 10 min intervals, samples were taken and cell length measured and compared with that of control cells. During the first 30 min, differences in length between the two cel-

lular populations increased with time. Thereafter, however, it stopped increasing and remained constant for the following 20 min, corresponding to the time during which cells septated and divided, thus indicating that no lateral-wall elongation occurs during septum formation in wild-type strains.

On the other hand, if cells were to elongate during septum formation and cell number doubling, immediately after cell division they ought to be significantly longer than half the length they had been immediately before cell division started. If, on the contrary, cells did not elongate during septum formation, newborn cells should have a length that is half the length they had 25–30 min after the beginning of their cell cycle. This experiment performed with wild-type strains of both *E. coli* and *K. pneumoniae* showed that, in both strains, immediately after division the average cell length is exactly half the length of cells measured before starting to increase in number, but are longer than half the length they had before that time.

2. *Incorporation of Radioactive Peptidoglycan Precursors into Lateral Wall and Septum During the Cell Cycle*

It was long debated whether elongation of the lateral wall in rod-shaped bacteria occurred through localized or diffuse insertion of peptidoglycan precursors. This problem has been tackled by measuring the rate of peptidoglycan synthesis along the cell surface using combined electron microscope and autoradiographic analysis of cells pulse-labelled with radioactive diaminopimelic acid (Ryter *et al.*, 1973). This analysis has been applied to cells synchronized using sucrose-gradient centrifugation (Wientjes and Nanninga, 1989) and to cell-division mutants (Woldringh *et al.*, 1987, 1988). It was found that, in synchronized wild-type populations, radioactivity was uniformly distributed along the lateral wall in elongating cells whereas, in dividing cells, a greater amount of radioactivity was found at the centre of the cell at the point where the septum was being formed although radioactivity was still also incorporated along the lateral wall. Similarly, in filaments of *ts* mutants, radioactivity was uniformly distributed along the lateral wall while, in dividing cells of the same mutants, up to 90% of label was incorporated at the centre of the cell at the point where the septum was growing. The residual incorporation into the lateral wall was interpreted by the authors as an indication of simultaneous synthesis of the lateral wall and septa (polar caps), thus contradicting alternation and competition between the two reactions responsible for peptidoglycan synthesis. This conclusion can easily be questioned by stressing that the fact that the TCS model claims that the lateral wall is

substantially not elongated during septum formation and vice versa does not imply in any way that a relatively small amount of radioactive peptidoglycan precursors cannot be incorporated in the lateral wall during septum formation (and vice versa). First of all, it appears quite possible that reactions which are common to both lateral-wall and septum formation occur throughout the cell cycle (this is in no way excluded by the model), and obviously this leads to simultaneous insertion of radioactive precursors in both the septum and side wall. In addition, as has been shown in Gram-positive bacteria (Pooley, 1976a,b), peptidoglycan in Gram-negative rods undergoes turnover (Goodell, 1985; Goodell and Schwarz, 1985) and there is no reason for thinking that turnover of the lateral wall has to be blocked during septum formation. Moreover, it is now accepted that peptidoglycan is not arranged in a single layer, and this may imply that, during septum formation, peptidoglycan in the lateral wall may be thickened in order to allow it to elongate faster immediately after the septum is formed. Some authors have also claimed that bacterial cells become wider during septum formation (Trueba and Woldring, 1980). Extension of the transverse diameter should require incorporation of radioactive precursors in the lateral wall although the cells are not elongating. Finally, it should be recalled that two different steps have been identified in septum formation, namely initiation and extension. We provide later in this review data to support the suggestion that septa are initiated some time before they begin to be formed. Radioactivity that was found in the lateral wall of septating cells could also be due to initiated septa.

In conclusion, since the TCS model does not take into account all possible events capable of justifying incorporation of radioactive precursors into the non-elongating lateral wall, but only assumes that, elongation being substantially blocked during septum formation, at this time in the cell cycle most of the newly made peptidoglycan has to be inserted at the centre of the cell wall, while the opposite might occur during elongation. Therefore, the findings already described confirm the competition between the two sites for peptidoglycan synthesis and provide strong support for an important component of the TCS model.

J. SHAPE MAINTENANCE DURING THE CELL CYCLE

Hypotheses have also been put forward regarding the way that rod shape is determined and maintained during the cell cycle, and the temporal relationship between septum formation and lateral-wall elongation. These hypotheses were based fundamentally on the study of effects of mecillinam on division of rod-shaped bacteria (Satta *et al.*, 1980b) and on the

reshaping of round cells formed in the presence of mecillinam by wild-type rods (Satta *et al.*, 1983, 1985). As far as the former approach is concerned, it was observed that wild-type synchronous rods divide once after addition of mecillinam. After this division, cells become spherical and no longer divide. It was also observed that, after antibiotic addition, the rate of peptidoglycan synthesis decreases with a reduction of as much as 50% and then remains constant even when the cells are forming the septum and doubling in number. This 50% decrease in the rate of peptidoglycan synthesis during transition from rod to coccal shape can be explained by inhibition of one of the two mechanisms for peptidoglycan synthesis and matches up entirely with predictions of the TCS model.

As regards the study of reshaping of mecillinam-induced spherical cells, it was shown that, if mecillinam is added to cultures of cells that grow synchronously at the very beginning of the cell cycle and removed after 30 min, just before control cells start to divide, treated cells divide normally at the same time as the control cells. After this division, which occurs in the absence of mecillinam, they become cocci and thereafter start reshaping and elongating. At 20–30 min after this division, the bacteria appear to have regained the rod shape and are similar to newborn rods. These short rods, which appear at approximately the time that control cells undergo the second synchronous division, skip the division, continue to elongate until they reach the length of control cells at division, and synchronously divide for the second time approximately at the same time as the third division of control cells. Such newborn cells have a shape which is indistinguishable from that of control cells.

A study of peptidoglycan synthesis carried out in the same experiment shows that removal of mecillinam just before cells start forming septa is not followed by an immediate resumption of peptidoglycan synthesis, as occurs when the antibiotic is removed some time before septum formation starts (Canepari *et al.*, 1993). However, after 15–20 min, at a time corresponding to the end of the increase in cell number and the reshaping of treated cells, peptidoglycan synthesis resumes at an exponential rate for a time encompassing the period required for control cells to elongate and divide for the second time. After this, peptidoglycan continues to be synthesized at a constant rate as in control cells. If antibiotic, after its removal, is added again to the reshaping cells, peptidoglycan is inhibited by 50% for the first 40 min following the first division, corresponding to the time during which untreated control cells elongate and undergo the second synchronous division. Cells in cultures from which mecillinam was removed reshape into apparently newborn rods. Thereafter, while cells continue elongating, the antibiotic has no effect until immediately after the second division. This latter behaviour is identical with that of normal cells

which divide synchronously when mecillinam is added 10 min after cell division, while the previous behaviour is identical with that of mutants that lack the LED control linking septum formation to lateral-wall elongation. It therefore appears that formation of lateral wall in cocci that become rods is different from that occurring after the skipped division at a time corresponding to resumption of lateral-wall elongation after division in untreated control cells.

These findings suggest that, after removal of mecillinam, spherical cells activate a novel site for lateral-wall elongation and become short rods. At a time corresponding to division of control cells, in reshaping cells a septum is started but not completed, possibly because the lateral wall starts elongating. After this second elongation process is accomplished, the initiated septum can be completed. Two new septa are then initiated and soon blocked by the start of lateral-wall elongation. Separation of daughter cells can only occur after two new septa have been started and after this elongation has begun. In this way, newborn cells always carry a septum which is initiated but blocked by the start of cell elongation. This explains why newborn cells treated with mecillinam are capable of dividing once, although elongation of the lateral wall has been blocked. After this division has taken place, no other division occurs since the LED control blocks completion of new septa if lateral-wall elongation does not occur. The suggestion that the septum is first initiated, then stopped, and completed only after the lateral wall has elongated allows several predictions. If this hypothesis is correct, addition of antibiotics such as piperacillin which specifically block septum completion during lateral-wall elongation should not normally cause any delay in cell division, but should cause a delay if added at the end of the process of lateral-wall elongation. Another prediction is that, if piperacillin is added to reshaping mecillinam-induced spheres, as already described, and then removed before the end of elongation, no delay in cell division should occur. On the contrary, piperacillin should cause a delay in division if added between the end of elongation and the start of increase in cell number (Fig. 11). Addition of mecillinam to cells that have already started synthesizing a new septum and are elongating should allow them to divide in spite of the fact that completion of elongation is prevented. These predictions have been fulfilled, since piperacillin has no effect on cell division if added to synchronously dividing cultures of wild-type cells during elongation or on cocci that reshape into rods after removal of mecillinam, while it delays division in both situations when added after lateral-wall elongation. In addition, mecillinam, when added to cells which reshape 20 min after its removal, inhibits peptidoglycan synthesis by approximately 50% (as already indicated), but does not prevent cells from dividing once as with cells not previously treated with the antibiotic.

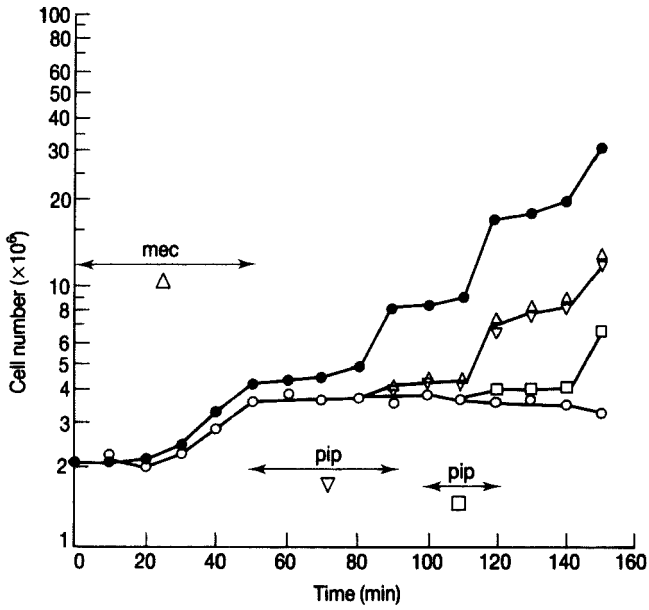

FIG. 11. Effect of addition of piperacillin (pip) for two distinct periods of time (▽, □) to a synchronous culture of *Klebsiella pneumoniae* Mir A12 pretreated for 50 min (△) with mecillinam (mec). Untreated control (●), mecillinam added at zero time (○). From Satta *et al.* (1993).

It has been suggested (Nanninga, 1991) that septum formation involves two different steps with different sensitivities to β-lactam antibiotics, namely initiation of the septum, which is insensitive to these antibiotics, and septum extension which is sensitive. The protein PBP1 has been reported to be involved both in lateral-wall elongation and in septum extension (de la Rosa *et al.*, 1985; del Portillo *et al.*, 1985; del Portillo and de Pedro, 1990; Wientjes and Nanninga, 1991). If this is so, antibiotics which specifically inactivate PBP1, when added to synchronous cultures of *E. coli* at the beginning of the cell cycle and removed after 20 min, should interfere only with lateral-wall elongation. They should also cause a delay in cell division which is not longer than 20 min, since the first septum is already initiated in newborn cells, and septum extension has not yet started, so that the only event that the antibiotic can block is lateral-wall elongation. On the contrary, if antibiotic is added and removed in the 20 min period which includes the time when the cells finish elongating and then form septa, the delay in division should be longer than 20 min; in particular, if new septa are initiated at the end of septum completion and

their initiation precedes lateral-wall elongation. This prediction is also fulfilled, since cephaloridine causes a 20 min delay in cell division when added for the first 20 min of the cell cycle, but causes a 40 min delay when added for the same time period but corresponding to the first division of synchronous cells (Satta *et al.*, 1985). In addition to these predictions, one might also predict that, in reshaping cells after removal of mecillinam at the time of the skipped division, a septum should be inserted which may be observed either by electron microscopy or by autoradiography.

K. INTERACTION BETWEEN DNA AND THE BACTERIAL ENVELOPE

As already indicated, according to the TCS model the essential role played by the competition between two sites for peptidoglycan assembly in regulation of septum formation allows a simple basis whereby cell division can be co-ordinated with DNA replication. In order to achieve this co-ordination, all that is needed is that the balance between the competing sites be shifted in favour of lateral-wall extension throughout DNA replication and that the pressure in favour of this site should stop after termination of replication. The first indication that such a mechanism may exist comes from the observation that septum formation is invariably inhibited when DNA repair is induced. It was suggested (Satta, 1984; Satta *et al.*, 1980a) that the turnover to which DNA is most likely subjected during its replication induces, albeit at a lower level, functions of the same type or of a type similar to those responsible for inhibition of cell division during DNA repair. It was also suggested that the rate of turnover, and therefore the amount of the compound that indirectly or directly shifts the balance in favour of lateral-wall composition, is proportional to the number of replicating forks active at any time in cells. When a round of DNA replication has finished, replicating forks cease their activity causing a deceleration of turnover and of compounds that interfere with the balance between the two competing sites and prevent septum formation. Before new forks start up again, the decreased cellular content of turnover products causes the balance between the two competing sites to be shifted in favour of septum formation. Once completion of the septum has been initiated, restarting of new forks cannot block it. However, products associated with turnover which interfere with the two sites accumulate during septum formation and ensure that, after formation of each septum, the site for lateral-wall elongation will prevail and cells will consequently elongate. In bacteria which have only one site for peptidoglycan assembly, there is no competition, and the products generated in connection with turnover may more easily inhibit septum formation. In these cells, competition may arise between products needed for septum formation and

DNA turnover products. At the end of each round of DNA replication, the amount of turnover-associated products drops, while the amount of products needed for septum formation continues to increase as it overcomes the inhibiting activity of DNA turnover and starts septum formation and cell division (DIDI control). This implies that the material necessary for septum formation (and, in these bacteria, also for cell-wall extension) is continuously produced in an amount which has to be proportional to the amount of other macromolecules which are also continuously synthesized and have to find intracellular space into which to fit. Therefore, the greater the number of replicating forks cells have, the larger will be the cell mass at division.

This model accepts the previous proposal (Donachie *et al.*, 1976) that the bacterial envelope and DNA have regulatory circuits which are fundamentally independent of one another. Although capable of independently regulating their duplication, these two bacterial components interact loosely in two stages. The first interaction serves mainly to avoid excess accumulation of nucleoids and occurs at DNA initiation. This control does not need to be strict since excess nucleoids, on the one hand, tend to be lost due to the disadvantages they cause to cells and, on the other hand, are compatible with bacterial survival. According to the TCS model, bacteria which carry excess nucleoids should generate a greater amount of the turnover products that inhibit septum formation. This should cause a delay in cell division until enough material inducing septum formation has accumulated and consequently, cells need to be larger at division. The larger cell mass may provide the energy and the metabolites necessary for duplicating all of the nucleoids to be produced in the cell. However, the greater dimensions are disadvantageous for the cells, particularly for those of coccal shape. The loose interaction between DNA and the envelope at initiation stems from the fact that DNA initiates its replication, fundamentally regardless of the status of the envelope, but can be accelerated by events occurring in the envelope such as termination of lateral-wall elongation or initiation of septa. Alternatively its start, although not blocked, may be delayed by events in the cell wall.

The other interaction is tighter but not strict. It consists in the fact that septum formation is antagonized by a number of DNA-turnover products whose relative amounts decrease after each round of DNA replication and serves the function of assuring the proper DNA content in daughter cells. In this situation, the link consists in the fact that, although the envelope expands independently of DNA replication, it does not divide before DNA is replicated. This does not mean that the envelope is bound to duplicate after each round of DNA replication, since envelope duplication can only occur after the independent regulatory mechanism involved in envelope

growth starts septum formation. But once this event has been triggered, it can be expressed only if one round of DNA replication has been completed.

Possible loose interactions between the envelope and DNA replication at DNA initiation and termination are supported by several findings and allow a number of predictions to be made that have already been confirmed and others that can be tested. As regards the first link involving possible interactions between septum and DNA replication at DNA initiation, it has previously been suggested that new replication forks induce initiation of new septa or that newly formed septa trigger initiation of new replicating forks (Helmsetter and Pierucci, 1968). These suggestions, however, were disproved by the observation that mutants unable to form septa produce normal nucleoids and that bacteria can divide in the absence of DNA replication (*dnaA* mutants; Bremer and Churchward, 1991). This indicates that the septum can be formed totally independently of DNA replication and vice versa, as suggested by others (Cooper, 1991; Bernander and Nordstrom, 1990). However, it was found that in rods of a morphology mutant of *K. pneumoniae* that lacks the LED control linking septum formation to lateral-wall elongation, addition of mecillinam to synchronous cultures of newborn cells causes an immediate 50% decrease in peptidoglycan synthesis followed by initiation of DNA replication that starts at least 10 min earlier than in control cells. This clearly indicates that inhibition of lateral-wall elongation, or possibly septum formation that results from blocking of lateral-wall elongation, can allow a new replicating fork to start earlier. However, in *lov* mutants of *E. coli* (Bouloc *et al.*, 1988, 1989) which behave like the *K. pneumoniae* mutant Mir M7, while the same treatment with mecillinam has the same effect on cell shape it does not influence DNA initiation and generation time. This indicates that inhibition of lateral-wall elongation does not necessarily induce initiation of DNA replication or may be insufficient for DNA replication to start. Most likely, however, once DNA is ready to initiate replication, this event cannot occur if cells are still elongating and therefore have not yet initiated completion of a new septum. This may be because initiation of DNA replication may require such a large amount of energy that it cannot occur if cells, at the same time, are performing functions that are additional to those essential for growth and division. Indeed, extension of the lateral wall may afford advantages for cells although it is in no way necessary for their proliferation since many bacteria grow as cocci and therefore do not elongate. It is possible that, in the rods of the Mir M7 mutant of *K. pneumoniae*, the time required for lateral-wall elongation is longer than that needed for starting new rounds of DNA replication while in the *lov* mutant of *E. coli*, it is the same or shorter. We should note that this

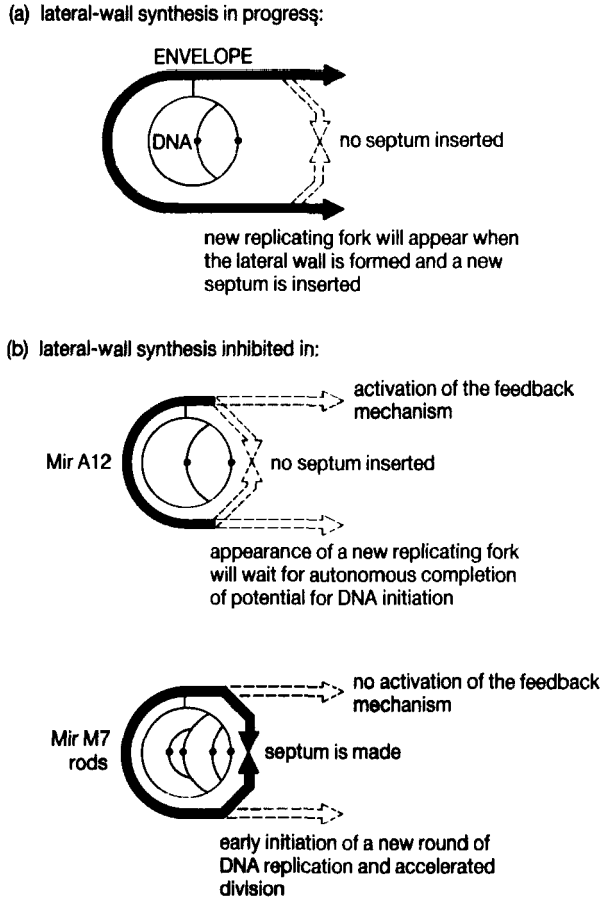

FIG. 12. Model showing the different responses of strains Mir M7 and Mir A12 of *Klebsiella pneumoniae* to shape alterations caused by mecillinam. In normal rods, no new septa can be initiated while lateral-wall synthesis is in progress, and initiations of new rounds of DNA replication will wait until complete formation of the lateral wall and insertion of a new septum (a). When lateral-wall formation is inhibited, for instance by mecillinam in strain Mir M7, the lateral-wall control preventing new septa is released. A new septum is quickly inserted, and consequently (see text) both early initiation of a round of DNA synthesis and accelerated division occur. In strain Mir A12, inhibition of lateral-wall formation causes activation of a negative control which prevents insertion of new septa, although the one already in can be completed in due time. As a result, neither faster initiation of new septa nor accelerated division occurs (b). In the figures, half sections of bacteria are represented. The dark envelope indicates the one being synthesized, and the dashed envelope indicates the one whose synthesis is inhibited. From Satta *et al.* (1981).

possibility is likely since *lov* mutants are shorter than normal *E. coli* rods and also significantly shorter than rods of the Mir M7 mutant. In rods which carry an active LED control, it is probably this control that prevents initiation of new septa when production of the lateral wall is inhibited and this prevents cells that do not elongate from starting their replication earlier (Fig. 12).

The suggestion that actively proliferating cells cannot perform additional functions that are essential to proliferation may be of general value and be applicable to all cells. It is, in fact, well known that differentiated cells do not proliferate in this state but, in order to do so, have to de-differentiate. Differentiated cells are those which, after proliferating, acquire the ability to perform specific functions. We need only think, for instance, of plasma cells and nerve cells which never proliferate while performing their specific functions. On the other hand, it is known that highly differentiated cells such as nerve cells and lymphocytes can also be induced to de-differentiate by relatively simple interaction of appropriate compounds with surface receptors and, immediately after de-differentiating, start dividing. This indicates that ability to proliferate in differentiated cells cannot be destroyed, but only temporarily repressed. It is likely that it has been easier for cells to evolve a control mechanism that inhibits initiation of DNA replication when they are performing functions not connected with proliferation, than to develop either mechanisms for extra energy production or mechanisms for co-ordinating the performance of their specific functions and their proliferation.

As regards the second link, the model predicts that cell mass at division will be proportional to the number of replicating forks present in chromosomal DNA. As a consequence, bacteria grown in a nutritionally poor medium, where they have fewer replicating forks, are smaller than those grown in a nutritionally rich medium when there are more replicating forks operating at the same time. It is known that a nutritional shift-up from a poor to a rich medium causes what is essentially an immediate increase in the rate of RNA and protein synthesis, but does not change the rate of increase of DNA synthesis for at least 60 min (Cooper, 1969). In contrast, after an opposite shift, the rate of RNA and protein synthesis soon decreases while DNA synthesis continues at a rate similar to that in the rich medium. In the former situation, the model predicts that cells divide earlier when they are smaller since more precursor compounds for septum formation are made while the number of forks remains for a while the same. In the other situation, cells should undergo a delay in cell division. Other predictions invoke data from *dnaA* mutants which are strains which, under non-permissive conditions, complete one round of DNA replication but do not start any new rounds (Hirota *et al.* 1968). For these mutants

the model predicts that, after the end of DNA replication, they will continue to divide making non-nucleated cells. In these cells, the number of divisions that occur will be greater the richer the medium in which the cells are grown. Several reports confirm that both of these predictions are correct (Spratt and Rowbury, 1970; Pardee *et al.*, 1973). In strains that carry the *cos* mutation (Kellenberger-Gujer *et al.*, 1978) an abnormally high number of replication forks are formed at the non-permissive temperature. In these strains, inhibition of cell division proportional to DNA turnover should be higher than normal and cell mass should be proportionally greater. It has been shown that cells are significantly longer at the non-permissive than at the permissive temperature. An increased number of replicating forks can also be detected in cells containing multiple copies of the chromosome-replication origin. These cells have been found to be longer the greater the number of replicating forks they carry (Donachie and Robinson, 1987). If inhibition of division, as proposed by the TCS model, was caused by products of DNA turnover similar to those associated with activation of DNA repair, this type of control should not exist in mutants that do not express DNA-repair function. It was found that *rec*A and *lex*A mutants which do not express this function continue to divide after DNA is damaged and its replication has been inhibited. When DNA is disturbed, they proliferate, generating high proportions of anucleated cells (Inouye, 1971; Witkin, 1976; Satta and Pardee, 1978; Satta *et al.*, 1979b).

It may very well be then that, under most conditions, DNA replication plays an important role in regulating the balance between lateral-wall elongation and septum formation. The turnover product may be capable ordinarily of blocking septum formation by shifting the balance of competition in favour of lateral-wall elongation. From a more general point of view, septa may be initiated independently of DNA replication. After initiation they should not be completed because turnover products produced by replicating forks shift the balance of competition in favour of lateral-wall elongation. When the replicating forks are not produced, in the period of time that precedes their restarting, inhibition of septum formation is removed and an initiated septum can be completed, so leading to cell division. According to this hypothesis, DNA initiates replication, continues and completes it, substantially regardless of events that occur in the envelope, except for the possibility of facilitating initiation of DNA replication in connection with initiation of new septa (see above).

The envelope also initiates its duplication and expands substantially and independently, except that DNA has the ability ordinarily to delay envelope splitting until the end of its duplication. In bacteria with two sites for peptidoglycan synthesis capable of competing with each other, one

leading to splitting of the envelope and the other to its expansion without splitting, this goal is achieved by shifting the balance between the two competing sites in favour of envelope expansion. Strong support for the suggestion that products formed during DNA repair, and probably turnover, are capable of interfering with the balance between the two competing sites comes from the observation that treatment with β-lactam antibiotics of morphology mutants of *E. coli* in which septum formation overrides lateral-wall elongation, and a *rec*A mutation is present, causes transition to rod-shaped cells, while treatment of the same cells by compounds such as nalidixic acid is devoid of any effect on cell shape.

A form of DNA control over septum formation different from the DIDI control has recently been proposed (Woldringh *et al.*, 1990). In this model, it has been suggested that nucleoids exert a negative control over toporegulation of cell division because its presence may affect local peptidoglycan synthesis. Abrupt arrest of DNA-synthesizing replication forks at termination and subsequent separation of daughter nucleoids are considered by these authors to be internal signals to which membrane proteins respond by synthesizing a division site. This model does not conflict with the DIDI control we proposed previously since more evolved bacteria may carry both controls. Of these, DIDI control is certainly simpler and is likely to have evolved first; the other may have appeared at a later stage in evolution to reinforce and refine the primordial one.

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REFERENCES

Adler, H. I., Fisher, W. A., Cohe, A. and Hardigree, A. A. (1967). *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8849.

Barbour, A. G., Mayer, L. W. and Spratt, B. G. (1981). *The Journal of Infectious Diseases* **143**, 114.

Begg, K. J. and Donachie, W. D. (1985). *Journal of Bacteriology* **163**, 615.
Begg, K. J., Spratt, B. G. and Donachie, W. D. (1986). *Journal of Bacteriology* **167**, 1004.
Begg, K. J., Takasuga, A., Edwards, D. H., Dewar, S. J., Spratt, B. G., Adachi, H., Obta, T., Matsuzawa, H. and Donachie, W. D. (1990). *Journal of Bacteriology* **172**, 6697.
Bernander, R. and Nordstrom, K. (1990). *Cell* **60**, 365.
Bouloc, P., Jaffè, A. and D'Ari, R. (1988). *Review of Infectious Diseases* **10**, 90S.
Bouloc, P., Jaffè, A. and D'Ari, R. (1989). *EMBO Journal* **8**, 317.
Bremer, H. and Churchward, G. (1991). *Microbiological Reviews* **55**, 459.
Brown, F. S. and Reynolds, P. E. (1980). *FEBS Letters* **122**, 275.
Canepari, P., Botta, G. and Satta, G. (1984). *Journal of Bacteriology* **157**, 130.
Canepari, P., Di Stefano, F., Lleó, M. M. and Satta, G. (1993). *Microbiologica* **16**, 165.
Canepari, P., Lleó, M. M., Fontana, R., Satta, G., Daneo-Moore, L. and Shockman, G. D. (1983). *In* "The Target of Penicillin" (R. Hachenbek, J.-V. Holtje and H. Labischinski, eds), pp. 141–146. Walter de Gruyter, Berlin.
Canepari, P., Varaldo, P. E., Fontana, R. and Salta, G. (1985). *Journal of Bacteriology* **163**, 796.
Canepari, P., Lleó, M. M., Satta, G. and Fontana, R. (1987). *Journal of Bacteriology* **169**, 2432.
Canepari, P., Lleó, M. M., Satta, G., Fontana, R., Shockman, G. D. and Daneo-Moore, L. (1989). *Journal of Bacteriology* **163**, 796.
Chipman, D. M. and Sharon, N. (1969). *Science* **165**, 454.
Cooper, S. (1969). *Journal of Molecular Biology* **43**, 1.
Cooper, S. (1988a). *Journal of Bacteriology* **170**, 436.
Cooper, S. (1988b). *Journal of Bacteriology* **170**, 5001.
Cooper, S. (1991). *Microbiological Reviews* **55**, 649.
Craig, W. A. and Gudmundsson, S. (1991). *In* "Antibiotic in Laboratory Medicine" (V. Lorian, ed.), pp. 403–431. Williams and Wilkins, Baltimore.
Curtis, N. A. C., Orr, D., Ross, G. W. and Boulton, M. G. (1979). *Antimicrobial Agents and Chemotherapy* **16**, 533.
de Jonge, B. L. M., Wlenrjes, F. B., Jurida, T., Driehuis, F., Wouters, J. T. M. and Nanninga, N. (1989). *Journal of Bacteriology* **156**, 136.
de Jonge, B. M. M. (1990). *Journal of Bacteriology* **172**, 4682.
del Portillo, F. G. and de Pedro, M. A. (1990). *Journal of Bacteriology* **172**, 5863.
del Portillo, F. G., Pisabarro, A. G., de la Ross, E. J. and de Pedro, M. A. (1985). *Journal of Bacteriology* **169**, 2410.
de la Rosa, E., de Pedro, M. A. and Vasquez, D. (1985). *Proceedings of the National Academy of Science of the United States of America* **82**, 5632.
Doi, M., Wachi, M., Ishino, F., Tomioka, S., Ito, M., Sakagami, Y., Suzuki, A. and Matsuhashi, M. (1988). *Journal of Bacteriology* **170**, 4619.
Donachie, W. D., Begg, K. J. and Vicente, M. (1976). *Nature* **264**, 328.
Donachie, W. D. and Robinson, A. C. (1987). *In "Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology" (J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger, eds), pp. 1578–1593. American Society for Microbiology, Washington DC.
Driehuis, F. and Wouters, J. T. M. (1987). *Journal of Bacteriology* **169**, 97.
Fontana, R., Canepari, P. and Satta, G. (1979). *Journal of Bacteriology* **139**, 1028.
Fontana, R., Canepari, P., Satta, G. and Coyette, J. (1980). *Nature* **278**, 70.
Fontana, R., Canepari, P., Satta, G. and Coyette, J. (1983a). *Journal of Bacteriology* **154**, 916.
Fontana, R., Cerini, R., Longoni, P., Grossato, A. and Canepari, P. (1983b). *Journal of Bacteriology* **155**, 1343.
Fontana, R., Grossato, A., Rossi, L., Cheng, Y. R. and Satta, G. (1985). *Antimicrobial Agents and Chemotherapy* **28**, 678.
Glauner, B. (1988). *Analytical Biochemistry* **177**, 451.
Ghuysen, J.-M. (1977). *Journal of General Microbiology* **101**, 13.
Goodell, E. W. (1985). *Journal of Bacteriology* **163**, 305.

Goodell, E. W. and Schwarz, U. (1985). *Journal of Bacteriology* **162**, 391.
Gudas, L. J., James, R. and Pardee, A. B. (1976). *Journal of Biological Chemistry* **251**, 3470.
Harold, F. M. (1990). *Microbiological Reviews* **54**, 381.
HelmsFelter, C. E. and Pierucci, O. (1968). *Journal of Bacteriology* **95**, 1627.
Henning, V., Rehn, K., Braun, V., Höhn, B. and Schwarz, U. (1972). *European Journal of Biochemistry* **26**, 570.
Higgins, M. and Shockman, G. D. (1971). *Critical Review of Microbiology* **1**, 29.
Hirota, Y., Ryter, A. and Jacob, F. (1968). *Cold Spring Harbor Symposium on Quantitative Biology* **33**, 677.
Holtje, J. V. and Glauner, B. (1990). *Research in Microbiology* **141**, 75.
Iida, K., Hirata, S., Nakamuta, S. and Koike, M. (1978). *Antimicrobial Agents and Chemotherapy* **14**, 257.
Inouye, M. (1971). *Journal of Bacteriology* **106**, 539.
Ishiguro, E. E., Iwaga, M. and Turley, W. (1979). *Antimicrobial Agents and Chemotherapy* **16**, 849.
Iwaga, M., Jones, C. W., Khorana, J. and Strominger, J. L. (1978). *Journal of Bacteriology* **133**, 196.
Jaffè, A., Boye, E. and D'Ari, R. (1990). *Journal of Bacteriology* **172**, 3500.
Jones, M. V., Dawson, H., Wells, V. E. and Perkins, H. R. (1981). *Journal of General Microbiology* **124**, 281.
Kellenberger-Gujer, G., Podhajska, A. J. and Caro, L. (1978). *Molecular and General Genetics* **162**, 9.
Koch, A. L. (1988). *Microbiological Reviews* **52**, 337.
Koch, A. L. (1990). *Research in Microbiology* **141**, 119.
Krulwich, T. A. and Plate, J. L. (1971). *Journal of Bacteriology* **105**, 408.
Kubitschek, H. E. (1968). *Biophysic Journal* **8**, 792.
Kubitschek, H. E. (1981). *Journal of Bacteriology* **148**, 730.
Kubitschek, H. E. (1990). *Journal of General Microbiology* **136**, 599.
Kumar, S. (1976). *Journal of Bacteriology* **125**, 545.
Lleó, M. M., Canepari, P. and Satta, G. (1990). *Journal of Bacteriology* **172**, 3758.
Lorian, V. and Atkinson, B. (1977). *Antimicrobial Agents and Chemotherapy* **11**, 541.
Markiewicz, Z., Brome-Smith, K., Schwarz, U. and Spratt, B. G. (1982). *Nature* **297**, 702.
Matsuhashi, M., Kamiryo, T., Blumberg, P. M., Linnett, P., Willoughby, E. and Strominger, J. L. (1974). *Journal of Bacteriology* **117**, 578.
Matsuhashi, M., Wachi, M. and Ishino, F. (1990). *Research in Microbiology* **141**, 89.
Matsuzawa, H., Hayakawa, K., Sato, T. and Imahori, K. (1973). *Journal of Bacteriology* **115**, 436.
Nanninga, N. (1991). *Molecular Microbiology* **5**, 791.
Normark, S. (1969). *Journal of Bacteriology* **98**, 1274.
Ogura, T., Bouloc, P., Niki, H., D'Ari, R., Hirg, S. and Jaffè, A. (1989). *Journal of Bacteriology* **171**, 3025.
Pardee, A. B., Wu, P. C. and Zusman, D. R. (1973). *In* "Bacterial Membrane and Walls" (L. Leive, ed.), pp. 357–389. Marcel Dekker, New York.
Pooley, H. M. (1976a). *Journal of Bacteriology* **125**, 1127.
Pooley, H. M. (1976b). *Journal of Bacteriology* **125**, 1139.
Reeve, J. N., Groves, D. J. and Clark, D. J. (1970). *Journal of Bacteriology* **104**, 1052.
Romeis, T., Kohlrausch, V., Burgdorf, K. and Holtje, J. V. (1991). *Research in Microbiology* **142**, 325.
Rossi, L., Tonin, E. A., Cheng, Y. R. and Fontana, R. (1985). *Antimicrobial Agents and Chemotherapy* **27**, 828.
Ryter, A., Hirota, Y. and Schwarz, U. (1973). *Journal of Molecular Biology* **78**, 185.
Satta, G. (1984). *L'Igiene Moderna* **81**, 974.
Satta, G. and Fontana, R. (1974a). *Journal of General Microbiology* **80**, 51.
Satta, G. and Fontana, R. (1974b). *Journal of General Microbiology* **80**, 65.
Satta, G. and Pardee, A. B. (1978). *Journal of Bacteriology* **133**, 1492.
Satta, G., Botta, G., Canepari, P. and Fontana, R. (1981). *Journal of Bacteriology* **148**, 10.

Satta, G., Canepari, P. and Boaretti, M. (1993). *Microbiologica* **16**, 135.
Satta, G., Canepari, P., Botta, G. and Fontana, R. (1980a). *EMBO Workshop on* "Duplication of Bacteria". Book of Abstracts. Session IIIB.
Satta, G., Canepari, P., Botta, G. and Fontana, R. (1980b). *Journal of Bacteriology* **142**, 43.
Satta, G., Canepari, P. and Fontana, R. (1983). *In* "The Target of Penicillin" (R. Hachenbek, J.-V. Holtje and H. Labischinski, eds), pp. 135–140. Walter de Gruyter, Berlin.
Satta, G., Canepari, P., Maurici, R. and Marcialis, M. A. (1985). *Annales of the Pasteur Institute/Microbiology* **135A**, 85.
Satta, G., Fontana, R., Canepari, P. and Botta, G. (1979a). *Journal of Bacteriology* **137**, 727.
Satta, G., Gudas, L. and Pardee, A. B. (1979b). *Molecular and General Genetics* **168**, 69.
Satta, G., Pruzzo, C., Debbia E. and Fontana, R. (1978). *Journal of Virology* **28**, 772.
Schlaeppi, J. M. and Karamata, D. (1982). *Journal of Bacteriology* **152**, 1231.
Schlaeppi, J. M., Schaefer, O. and Karamata, D. (1985). *Journal of Bacteriology* **164**, 130.
Schwarz, U. and Glauner, B. (1988). *In* "Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function" (P. Actor, L. Daneo-Moore, M. H. Higgins, M. R. Salton, G. D. Shockman, eds), pp. 33–40. American Society for Microbiology, Washington DC.
Schwarz, U., Asmus, A. and Frank, H. (1969). *Journal of Molecular Biology* **41**, 419.
Spratt, B. G. (1975). *Proceedings of the National Academy of Sciences of the United States of America* **72**, 2999.
Spratt, B. G. (1977). *Antimicrobial Agents and Chemotherapy* **3** (Suppl. B), 1319.
Spratt, B. G. (1979). *Science Progress (Oxford)* **65**, 101.
Spratt, B. G., Boyd, A. and Stoker, N. (1980). *Journal of Bacteriology* **143**, 569.
Spratt, B. G., Jobanputra, V. and Zimmerman, W. (1977). *Antimicrobial Agents and Chemotherapy* **12**, 406.
Spratt, B. G. and Pardee, A. B. (1975). *Nature* **254**, 516.
Spratt, B. G. and Rowbury, R. J. (1970). *Journal of General Microbiology* **64**, 127.
Thwaites, J. J. and Mendelson, N. H. (1991). *Advances in Microbial Physiology* **32**, 174.
Trueba, F. J. and Woldring, C. L. (1980). *Journal of Bacteriology* **142**, 869.
Vinella, D., D'Ari, R. and Bouloc, P. (1992). *The EMBO Journal* **11**, 1493.
Wachi, M. and Matsuhashi, M. (1989). *Journal of Bacteriology* **171**, 3123.
Wachi, M., Doi, M., Tamaki, S., Park, W., Nakajima-Iijima, S. and Matsuhashi, M. (1987). *Journal of Bacteriology* **169**, 4939.
Walker, J. R., Kovarik, A., Allen, J. S. and Gustafson, R. A. (1975). *Journal of Bacteriology* **123**, 693.
Waxman, D. J. and Strominger, J. L. (1983). *Annual Review of Biochemistry* **52**, 825.
Westling-Haggstrom, B. and Normark, S. (1975). *Journal of Bacteriology* **123**, 75.
Wientjes, F. B. and Nanninga, N. (1989). *Journal of Bacteriology* **171**, 3412.
Wientjes, F. B. and Nanninga, N. (1991). *Research in Microbiology* **142**, 333.
Witkin, E. M. (1976). *Bacteriological Review* **40**, 869.
Woldringh, C. L., de Jonge, M. A., Vandenberg, W. and Koppes, L. (1977). *Journal of Bacteriology* **131**, 270.
Woldringh, C. L., Huls, P., Nanninga, N., Pas, E., Tascner, P. E. M. and Wientjes, F. B. (1988). *In* "Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function" (P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton and G. D. Shockman, eds), pp. 66–78. American Society for Microbiology, Washington DC.
Woldringh, C. L., Huls, P., Pas, E., Brakenhoff, G. J. and Nanninga, N. (1987). *Journal of General Microbiology* **133**, 575.
Woldringh, C. L., Mulder, E., Huls, P. G. and Vischer, N. (1990). *Research in Microbiology* **142**, 113.

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Respiratory Chains and Bioenergetics of Acetic Acid Bacteria

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I. Introduction

Acetic acid bacteria are obligate aerobes and well known as vinegar producers. They produce acetic acid from ethanol by two sequential catalytic reactions of membrane-bound alcohol dehydrogenase (ADH) and

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aldehyde dehydrogenase (ALDH). Besides alcohols and aldehydes, acetic acid bacteria are able to oxidize various sugars and sugar alcohols such as D-glucose, glycerol and D-sorbitol (Kondo and Ameyama, 1958). Such oxidation reactions are called "oxidative fermentations", since they involve incomplete oxidation of alcohols or sugars accompanied by accumulation of the corresponding oxidation products in huge amounts in the growth medium. Bacteria capable of effecting oxidative fermentations are called "oxidative bacteria", of which the most prominent ones are acetic acid bacteria.

The oxidation reactions are carried out by membrane-bound dehydrogenases, such as ADH or ALDH, linked to the respiratory chain located in the cytoplasmic membrane of the bacterium. The membrane-bound dehydrogenases can be divided into quinoproteins and flavoproteins that have pyrroloquinoline quinone (PQQ) and covalently-bound flavin adenine dinucleotide (FAD) as prosthetic groups, respectively. These quinoprotein and flavoprotein dehydrogenases have been shown to function by linking to the respiratory chain which transfers electrons to the final electron acceptor, oxygen, and generating energy for growth. In addition, the enzymes are involved in production of the oxidation products, carboxylic acids and sugar acids, in the culture media. Thus, acetic acid bacteria are also important for the fermentation industries to produce biomaterials such as vinegar and L-sorbose.

Acetic acid bacteria are classified into two genera, *Gluconobacter* and *Acetobacter* of the family Acetobacteraceae. *Gluconobacter* species catalyse highly active oxidation reactions on ethanol or D-glucose, including also oxidative reactions on sugars such as D-gluconic acid, D-sorbitol, and glycerol. By contrast, *Acetobacter* species have a highly active ethanol-oxidizing system but not enzymes for sugar oxidation. Thus, although species in the two genera exhibit differences in their oxidizing systems, both function by linking tightly their oxidizing systems with their aerobic respiratory chains, which have many similarities. The respiratory chain in *Gluconobacter* spp. consists of a large amount of cytochrome *c*, ubiquinone and a cytochrome *o* terminal ubiquinol oxidase; it also has a cyanide-insensitive alternative oxidase. The respiratory chain in *Acetobacter* spp. has ubiquinone, cytochrome *b*, cytochrome *c*, and a terminal ubiquinol oxidase which is either cytochrome a_1 or cytochrome *o*. In this review, characteristics of these alcohol- and sugar-oxidizing respiratory chains of acetic acid bacteria are described, and discussed.

II. Alcohol- and Sugar-Oxidizing Systems of Acetic Acid Bacteria

Acetic acid bacteria, especially *Gluconobacter* species, possess various dehydrogenases which participate in oxidation of alcohols and sugars.

TABLE 1. Comparison of activities of NAD(P)$^+$-dependent and -independent enzymes in acetic acid bacteria

Location	Cofactor	Direction	Specific activity[a]	Optimum pH value	References
Ethanol ⇄ Acetaldehyde (*Acetobacter* spp.)					
membrane	PQQ[b]	→	1.4	4.0	Adachi *et al.* (1978a)
cytoplasm	NAD^+	→	0.005	8.0	Adachi *et al.* (1978a)
	NADH	←	0.35	6.0	
Acetaldehyde ⇄ Acetate (*Acetobacter* spp.)					
membrane	PQQ	→	3.5	5.0	Ameyama *et al.* (1981a,c)
cytoplasm	$NADP^+$	→	0.54	9.5	Adachi *et al.* (1980a)
	NADPH	←	n.d.[c]	n.d.	
D-Glucose ⇄ D-Gluconate (*Gluconobacter* spp.)					
membrane	PQQ	→	1.5	6.0	Ameyama *et al.* (1981a,e)
cytoplasm	$NADP^+$	→	0.08	8.5–9.0	Adachi *et al.* (1980b)
	NADPH	←	0.003	7.0	
D-Gluconate ⇄ 2-Keto-D-gluconate (*Gluconobacter* spp.)					
membrane	FAD	→	2.7	6.0	Shinagawa *et al.* (1984)
cytoplasm	$NADP^+$	→	0.08	10.5	Ameyama and Adachi (1982)
	NADPH	←	0.2	6.0	
D-Fructose ⇄ 5-Keto-D-fructose (*Gluconobacter* spp.)					
membrane	PQQ	→	0.8	6.0	Ameyama *et al.* (1981d)
cytoplasm	$NADP^+$	→	0.006	8.5–10.0	Ameyama *et al.* (1981b)
	NADPH	←	0.8	6.5	

[a] Activities of PQQ enzymes in membrane, or of NAD(P)$^+$-enzymes in cytosol [μmole of substrate oxidized min^{-1} (mg protein)$^{-1}$].
[b] PQQ indicates pyrroloquinoline quinone.
[c] n.d. indicates that the value was not determined.

TABLE 2. Summary of membrane-bound dehydrogenases purified from acetic acid bacteria

Dehydrogenase	Solubilization conditions	Subunit molecular weight (k)	Prosthetic group	Optimum pH value	Origin	References
Alcohol	Triton X-100 (1%)	I(80) II(50) III(14)	PQQ and heme *c* Heme *c*	4.5	*Acetobacter aceti*	Adachi *et al.* (1978a) Muraoka *et al.* (1982b)
Alcohol	Triton X-100 (1%)	I(80) II(48) III(14)	PQQ and heme *c* Heme *c*	5.0–6.5	*Gluconobacter suboxydans*	Adachi *et al.* (1978b) Ameyama *et al.* (1981a)
Alcohol	Triton X-100 (1%)	I(72) II(44)	PQQ and heme *c* Heme *c*	5.0–6.0	*Acetobacter polyoxogenes*	Tayama *et al.* (1989)
Alcohol	Triton X-100 (0.5%)	I(79) II(53) III(12)	PQQ and heme *c* Heme *c*	5.0–7.0	*Acetobacter methanolicus*	Matsushita *et al.* (1992c)
Aldehyde	Triton X-100 (1.5%)	I(86) II(55)	PQQ Heme *c*	4.0	*Gluconobacter suboxydans*	Adachi *et al.* (1980c) Ameyama *et al.* (1981a)
Aldehyde	Triton X-100 (5%) with cetylpyridinium chloride (0.2%)	I(78) II(45) III(14)	PQQ Heme *c*	5.0	*Acetobacter aceti*	Ameyama *et al.* (1981c) Muraoka *et al.* (1981)
Aldehyde	Triton X-100 (3%) with *N*-lauroyl sarcosinate (2%)	I(75K) II(19K)	PQQ	7.0	*Acetobacter polyoxogenes*	Fukaya *et al.* (1989)
Aldehyde	Triton X-100 (1%)	I(78) II(66)	PQQ	5.1	*Acetobacter rancens*	Hommel and Kleber (1990)
D-Glucose	Triton X-100 (1%) with KC1(0.3 M)	I(87)	PQQ	6.0	*Gluconobacter suboxydans*	Ameyama *et al.* (1981a,e) Matsushita *et al.* (1989b)

D-Gluconate	Triton X-100 (2%)	I(64) II(45) III(21)	FAD (covalent) Heme *c*	6.0	*Gluconobacter dioxyacetonicus*	Shinagawa *et al.* (1984)
2-Keto-D-gluconate	Cholate (2%) with KCl(0.2 M)	I(61) II(47) III(25)	FAD (covalent) Heme *c*	4.0	*Gluconobacter melanogenus*	Shinagawa *et al.* (1981)
D-Sorbitol	Triton X-100 (1%) with KCl(0.1 M)	I(63) II(51) III(17)	FAD (covalent) Heme *c*	4.5	*Gluconobacter suboxydans* subsp. α	Shinagawa *et al.* (1982)
L-Sorbose	Triton X-100 (0.3%)	I(58)	Possibly flavin (covalent)	7	*Gluconobacter melanogenus*	Sugisawa *et al.* (1991b)
D-Mannitol	Triton X-100 (1%) with KCl(0.1 M)	I(63) II(52) III(20.5)	Heme *c*	5.0?	*Gluconobacter melanogenus*	Cho *et al.* (1990)
Glycerol	Anhitol (0.5%)	I(80)	PQQ	4.0	*Gluconobacter industrius*	Ameyama *et al.* (1985)
D-Fructose	Triton X-100 (1%)	I(67) II(50) III(20)	PQQ Heme *c*	4.0	*Gluconobacter industrius*	Ameyama *et al.* (1981d)

Earlier studies mainly dealt with soluble $NAD(P)^+$-dependent dehydrogenases (Asai, 1968). However, in addition to the $NAD(P)^+$-dependent dehydrogenases located in the cytoplasm, other dehydrogenases independent of $NAD(P)^+$ had been shown to be located in the membrane. Table 1 shows the comparison of specific activities and optimum pH values of both $NAD(P)^+$-dependent and -independent enzymes that catalyse similar reactions in *Acetobacter* or *Gluconobacter* species. As well as NADH oxidase activity, alcohol- or sugar-oxidizing activity comparable to that in intact cells can be measured using the membrane fraction of *Gluconobacter suboxydans* (Daniel, 1970; Ameyama *et al.*, 1987). Subsequently, it has been shown that almost all of the primary dehydrogenases involved in alcohol- and sugar-oxidizing systems are located in the membranes of organisms. These membrane-bound dehydrogenases have been solubilized by detergent treatment, and purified and characterized as shown in Table 2. The purified primary dehydrogenases so far are either quinoproteins, which contain PQQ, or flavoproteins which bind FAD covalently. Dehydrogenases which oxidize alcohol, aldehyde, D-glucose, D-fructose and glycerol are quinoproteins, while D-gluconate, 2-keto-D-gluconate, D-sorbitol, and L-sorbose are oxidized by flavoproteins.

A. LOCALIZATION OF ALCOHOL- AND SUGAR-OXIDIZING SYSTEMS

Acetic acid bacteria are Gram-negative and, thus, the membrane fraction consists of outer and inner cytoplasmic membranes. In general, all respiratory components of Gram-negative bacteria are localized in the cytoplasmic membrane including most of the primary dehydrogenases, such as ADH, ALDH, glucose dehydrogenase (GDH), lactate and NADH dehydrogenases as well as cytochrome components (Matsushita *et al.*, 1985). In *G. suboxydans*, intracytoplasmic membranes have been observed to appear when there is an increase in glycerol oxidation (Claus *et al.*, 1975), in which the membranes may be formed by invagination of the cytoplasmic membrane. Furthermore, the topology of the primary dehydrogenases involved in alcohol- and sugar-oxidizing systems of *G. suboxydans* has been investigated by using spheroplasts which can be prepared successfully with sodium chloride instead of sucrose as an osmolite (Matsushita *et al.*, 1981a). Since ferricyanide is an impermeable electron acceptor for dehydrogenases, one can detect ferricyanide reductase activity among the enzymes if the active site is located on the outer surface of the spheroplasts. Alcohol dehydrogenase, GDH and glycerol dehydrogenase (GLDH) exhibited a higher ferricyanide reductase activity in intact spheroplasts than in lysed ones (the activity in the spheroplast is 113% for ADH, 142% for GDH, or 143% for GLDH, of that in the lysed one), while the

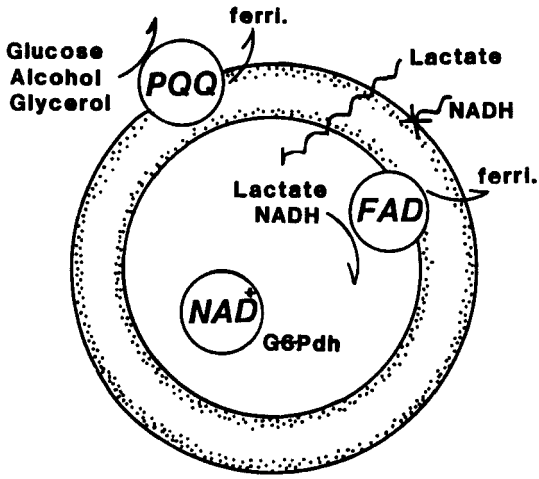

FIG. 1. Localization of primary dehydrogenases involved in alcohol- and sugar-oxidizing systems in spheroplasts of *Gluconobacter suboxydans*. Spheroplasts were prepared as described (Matsushita *et al.*, 1981a) and passed through a French press to produce the lysed spheroplasts. Ferricyanide (ferri.) reductase activities for glucose, alcohol and glycerol dehydrogenases can be detected in intact spheroplasts, while ferricyanide reductase activities for lactate and NADH dehydrogenases and also glucose-6-phosphate dehydrogenase (G6Pdh) activity were detected only in lysed spheroplasts. Lactate dehydrogenase activity is increased by the addition of the uncoupler, CCCP.

activities of cytoplasmic enzymes, such as glucose-6-phosphate dehydrogenase and 2-ketogluconate reductase, and of NADH dehydrogenase of the NADH oxidase system, were detected only after lysis (8% or 9% for glucose-6-phosphate dehydrogenase or NADH dehydrogenase, respectively). Lactate dehydrogenase is somewhat peculiar, showing an intermediate activity (26%) which is diminished to 3% by addition of the proton uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), which may inhibit active transport of lactate into the cytoplasm. Thus, as shown in Fig. 1, the active sites of at least ADH, GDH and GLDH are thought to be located on the outer side of the cytoplasmic membranes while those for NADH and lactate dehydrogenases are on the inner face of the membrane. With respect to the topology of GDH, more substantial evidence has been obtained using membrane vesicles of *Escherichia coli* (Matsushita *et al.*, 1986). When right-side out and inside out membrane vesicles of *E. coli* are treated with trypsin, digestion of GDH to a smaller fragment occurs only in the right-side out but not inside out vesicles, which could be seen by immunoblotting with antibody raised against the enzyme. Furthermore, as

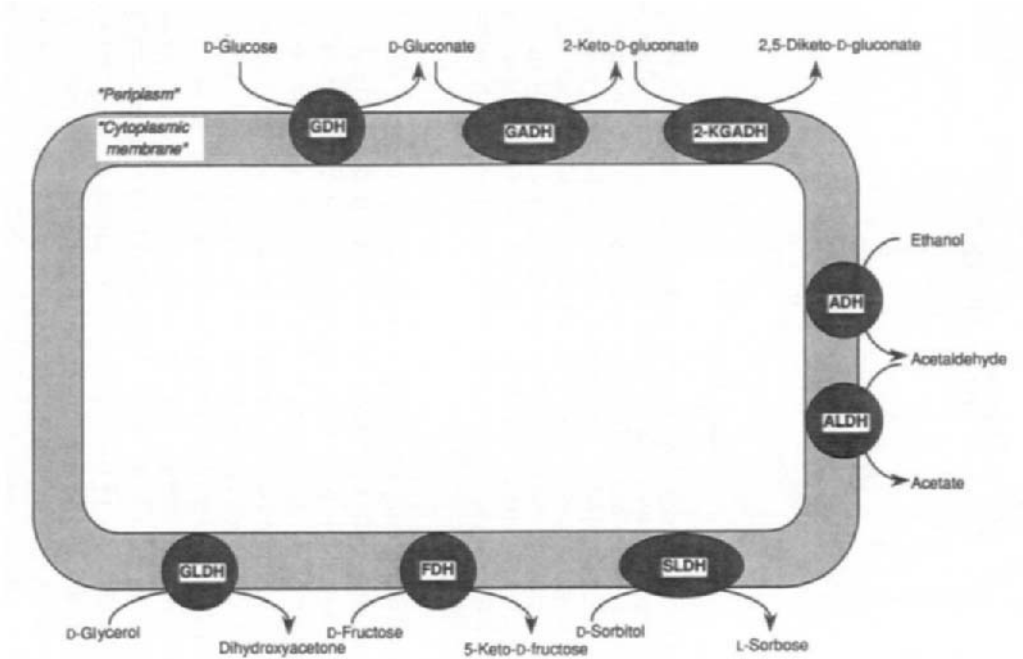

FIG. 2. Diagram showing primary dehydrogenases in alcohol- and sugar-oxidizing systems of acetic acid bacteria. Circles show quinoproteins which include D-glucose dehydrogenase (GDH), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and glycerol dehydrogenase (GLDH). Ellipses show flavoproteins including D-gluconate dehydrogenase (GADH), 2-keto-D-gluconate dehydrogenase (2-KGADH) and D-sorbitol dehydrogenase (SLDH).

will be described later, a periplasmic location for ADH or GDH in the cytoplasmic membrane has been predicted based on data obtained from the DNA sequences.

Thus, almost all primary dehydrogenases involved in the alcohol- and sugar-oxidizing systems of acetic acid bacteria seem to be localized in cytoplasmic membranes with the substrate site facing into the periplasm (Fig. 2). Some dehydrogenases may consist of membrane components with periplasmic subunits firmly bound to them. This notion is supported by the presence of a signal sequence with its *N*-terminus of amino acid residues predicted from the nucleotide sequence of either ADH or ALDH gene.

B. PRIMARY DEHYDROGENASES IN ETHANOL-OXIDIZING SYSTEMS

Alcohol and aldehyde dehydrogenases dependent on $NAD(P)^+$ have been isolated and characterized from the cytoplasm of both *Acetobacter* and *Gluconobacter* species (Table 1), whereas $NAD(P)^+$-independent ADH and ALDH are present in the cytoplasmic membrane and contain PQQ as a prosthetic group (Fig. 3). Enzymes dependent on $NAD(P)^+$ exhibit pH optima different from the pH value in the cytoplasm in oxidation reactions, and specific activities are much lower than those of quinoprotein enzymes (Table 1). Thus $NAD(P)^+$-dependent enzymes seem not to be involved in vinegar production, where the quinoproteins ADH and ALDH play a main role. Although distribution of ADH activities among strains is almost the same between *Acetobacter* and *Gluconobacter* species, high quinoprotein ALDH activities can be detected much more often in *Acetobacter* species than in *Gluconobacter* species (Table 3). Moreover, the ADH of *Acetobacter aceti* is more active and stable under acidic conditions than that of *G. suboxydans* (Adachi *et al.*, 1978a). Together with the high resistance of *Acetobacter* species to acetic acid, this may explain why productivity of acetic acid is higher with *Acetobacter* species than *Gluconobacter* species.

Quinoprotein ADHs have been solubilized and purified in the presence of detergent from membranes of *G. suboxydans* (Adachi *et al.*, 1978b) and *A. aceti* (Adachi *et al.*, 1978a; Muraoka *et al.*, 1982b). The purified enzymes are composed of three subunits, namely I of 78–80 kDa, II (cytochrome c_{553}) of 48–53 kDa, and III of 14–17 kDa. Tayama *et al.* (1989) purified ADH from *Acetobacter polyoxogenes* which consisted of subunits I and II but not subunit III. The enzyme contains as its prosthetic groups one mole of PQQ which binds non-covalently, together with three moles of heme *c*, per mole of enzyme complex (Ameyama *et al.*, 1981a; Matsushita *et al.*, 1992d; K. Matsushita, T. Yakushi, E. Shinagawa,

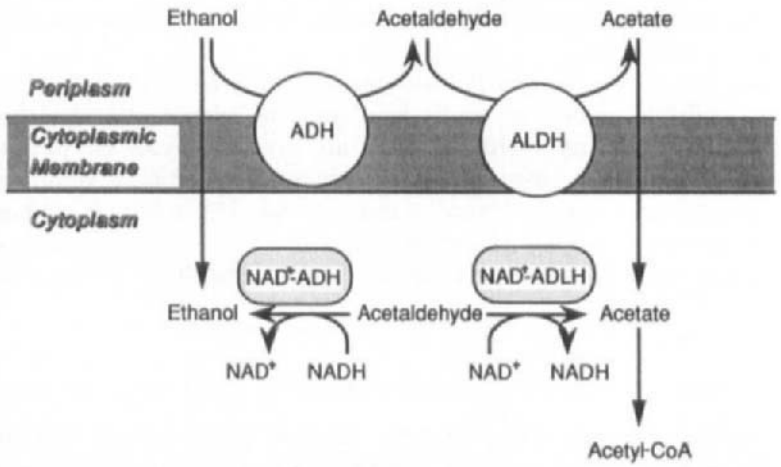

FIG. 3. Alcohol-oxidizing systems in acetic acid bacteria. Quinoprotein alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are located on the outer surface of the cytoplasmic membrane, while NAD^+-dependent alcohol dehydrogenase (NAD^+-ADH) and aldehyde dehydrogenase (NAD^+-ALDH) are in the cytoplasm).

TABLE 3. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities in particulate fractions of *Acetobacter* and *Gluconobacter* species

Activities in particulate fraction		*Acetobacter* species[a]	*Gluconobacter* species[b,c]
Enzyme (Specific activity[d])		No. of strains (%)	No. of strains (%)
ADH	(> 2)	2 (9)	7 (10)
	(1–2)	4 (18)	5 (12)
	(< 1)	16 (73)	31 (72)
ALDH	(> 2)	3 (14)	2 (5)
	(1–2)	15 (68)	14 (33)
	(< 1)	4 (18)	27 (63)

[a] Adachi *et al.* (1978a).
[b] Adachi *et al.* (1978b).
[c] Adachi *et al.* (1980c).
[d] Activities of ADH and ALDH in particulate fractions [μmole of substrate oxidized min^{-1}(mg protein)$^{-1}$]

H. Toyama and O. Adachi, unpublished observation). Heme *c* moieties are bound to both subunits I and II (Matsushita *et al.*, 1992d), which is consistent with the notion from its predicted amino acid-residue sequence obtained from the cloned gene, and PQQ is probably bound to subunit I. Alcohol dehydrogenase shows a lower optimum pH value, more restricted substrate specificity, and 10-times higher enzyme activity than the soluble quinoprotein alcohol dehydrogenases from other oxidative bacteria, which contain either only PQQ or PQQ and heme *c* (Matsushita and Adachi, 1993). Dissociation of subunit II from the ADH complex results in a decrease in activity, while re-association restores the activity (K. Matsushita, T. Yakushi, E. Shinagawa, H. Toyama and O. Adachi, unpublished observations). Therefore, complex formation is indispensable for the higher enzyme activity. The enzyme is able to donate electrons to long-chain ubiquinones, Q_6 or Q_9, as well as several artificial dyes such as ferricyanide, phenazine methosulfate (PMS), dichlorophenolindophenol (DCIP) and short-chain ubiquinone homologue, Q_1 or Q_2 (Matsushita *et al.*, 1992d). Alcohols with a chain length of C_2–C_6, formaldehyde and acetaldehyde can be oxidized by the enzyme, but methanol, and secondary and tertiary alcohols cannot (Matsushita *et al.*, 1992d).

Genes from *A. aceti* encoding subunits I and II of quinoprotein ADH have been cloned and sequenced (Inoue *et al.*, 1989, 1992), as have genes from *A. polyoxogenes* (Tamaki *et al.*, 1991), and *G. suboxydans* (Takeda and Shimizu, 1991). The predicted polypeptide structures of both subunits I and II have a signal sequence consisting of 23–35 amino acid residues at their *N*-termini, which is consistent with the notion that ADH is located on the periplasmic side of the cytoplasmic membrane. Of the total amino acid-residue sequence of subunit I, a region of about 600 residues from the *N*-terminus exhibits a homology with sequences of the quinoprotein methanol dehydrogenases (MDHs) from *Paracoccus denitrificans, Methylobacterium organophilum* and *Methylobacterium extorquens*, while the following residues on the *C*-terminal side contain the sequence for a heme *c*-binding site (Cys-X-X-Cys-His) (Anthony, 1992). Thus, subunit I of ADH is considered to be a conjugated product, with a quinoprotein region and a cytochrome *c* region. Subunit II is predicted to have three heme *c*-binding sequences (Inoue *et al.*, 1989; Tamaki *et al.*, 1991; Takeda and Shimizu, 1991). Alcohol dehydrogenase contains three moles of heme *c* in each enzyme complex; therefore, one of the three heme *c*-binding sites in subunit II could be unoccupied. Since the predicted amino acid-residue sequences of both subunits I and II lack plausible membrane-binding regions, subunit III may play an important role in binding to the cytoplasmic membrane. A plausible PQQ-binding region is proposed by comparison of the sequences of quinoprotein ADH, MDH and GDH (Inoue *et al.*, 1989; Anthony, 1992).

Quinoprotein ALDHs are present in large amounts in the membrane (Table 1). The enzyme plays a key role in vinegar production, because aldehyde liberation is not observed during alcohol oxidation. They have been purified from *G. suboxydans* (Adachi *et al.*, 1980c), *A. aceti* (Ameyama *et al.*, 1981c; Muraoka *et al.*, 1981), *A. polyoxogenes* (Fukaya *et al.*, 1989), and *Acetobacter rancens* (Hommel and Kleber, 1990). Aldehyde dehydrogenase binds to the membrane more tightly than ADH, and it is unstable after solubilization so that addition of benzaldehyde is needed to stabilize it for purification. Hommel *et al.* (1985) reported that phospholipids, especially phosphatidylglycerol, stabilize the enzyme and stimulate activity. The reported subunit structure of quinoprotein ALDH is somewhat confusing (see Table 2). The enzyme can oxidize a wide range of aliphatic aldehydes except for formaldehyde. The gene for ALDH from *A. polyoxogenes* has been cloned and sequenced (Fukaya *et al.*, 1989; Tamaki *et al.*, 1989). There is a single open-reading frame encoding 773 amino acid residues, of which 44 residues from the *N*-terminus may be removed during processing. There seems to be no similarity to sequences in quinoprotein ADH, even when compared with the plausible PQQ-binding region already referred to (Tamaki *et al.*, 1989).

Unlike other acetic acid bacteria, the methylotrophic acetic acid bacterium *Acetobacter methanolicus* produced either quinoprotein MDH or ADH when cultured with methanol or glycerol as a carbon source (Loffhagen and Babel, 1984). The MDH, composed of two 60 kDa- and two 10 kDa-subunits (Elliott and Anthony, 1988; Chan and Anthony, 1991b), is a soluble enzyme and is totally different from ADH which is membrane-bound, composed of three subunits, and does not react with methanol (Matsushita *et al.*, 1992c).

C. PRIMARY DEHYDROGENASES IN GLUCOSE-OXIDIZING SYSTEMS

Figure 4 shows the primary dehydrogenases taking part in glucose oxidation in acetic acid bacteria. Besides the $NAD(P)^+$-dependent enzymes in the cytoplasm, such as D-glucose dehydrogenase (Adachi *et al.*, 1980a) and 2-keto-D-gluconate reductase (2KGR) (Ameyama and Adachi, 1982), the $NAD(P)^+$-independent membrane-bound GDH, gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2KGADH) were also purified and characterized (Table 2). Gluconic acid production by *Gluconobacter oxydans* has been shown to be mainly due to the activity of membrane-bound GDH as the activity is 30-fold higher than that of the $NADP^+$-dependent GDH (Pronk *et al.*, 1989; see Table 1). D-Glucose and 2-keto-D-gluconate are thought to be transported into the cytoplasm and converted to D-gluconate by cytoplasmic enzymes, because the reactions

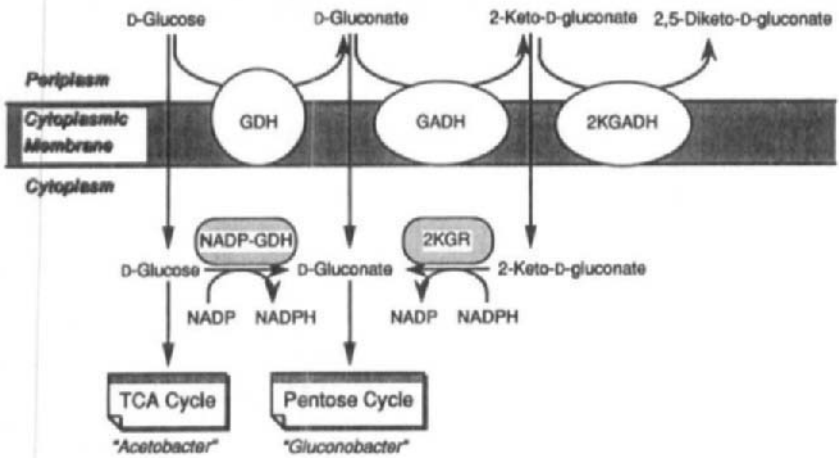

FIG. 4. Glucose-oxidizing systems in acetic acid bacteria. Quinoprotein D-glucose dehydrogenase (GDH), and flavoproteins D-gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2KGADH) are located on the outer surface of the cytoplasmic membrane. NADP-dependent D-glucose dehydrogenase (NADP-GDH) and NADP-dependent 2-keto-D-gluconate reductase (2KGR) are working in the cytoplasm.

of both $NADP^+$-dependent GDH and 2KGR favour production of D-gluconate at neutral pH values (see Table 1). Sugars in the cytoplasm are thought to be metabolized through the Embden–Meyerhof–Parnas pathway and TCA cycle in *Acetobacter* species, but through the pentose-phosphate cycle in *Gluconobacter* species which lack a TCA cycle (Asai, 1968).

The $NAD(P)^+$-independent GDH (quinoprotein GDH) catalyses direct oxidation of D-glucose to D-gluconate at the outer surface of the cytoplasmic membrane. The enzyme is found in a wide variety of Gram-negative bacteria including facultative anaerobes such as enteric bacteria and *Zymomonas* spp., and strictly aerobic bacteria such as pseudomonads, as well as acetic acid bacteria. This enzyme is involved in the metabolic pathway known as the alternative pathway to the phosphotransferase system of bacteria to effect D-glucose assimilation (Midgley and Dawes, 1973). Immunological studies have demonstrated that these GDHs are highly homologous to each other (Matsushita *et al.*, 1986). Glucose dehydrogenase purified from *G. suboxydans* is a single polypeptide of 87 kDa, at least in the presence of detergent (Ameyama *et al.*, 1981e). The enzyme binds PQQ non-covalently and so tightly that PQQ cannot be removed by EDTA dialysis (Ameyama *et al.*, 1981a,e). In contrast, PQQ in GDHs of other bacteria can be readily removed and the inactivated

apoenzyme can be successfully reconstituted with PQQ in the presence of calcium or magnesium ions (Imanaga *et al.*, 1979; Duine *et al.*, 1983; Ameyama *et al.*, 1985). The enzyme is able to donate electrons to a long-chain ubiquinone (Q_6 or Q_9) as well as to several artificial dyes and short-chain ubiquinone homologues. Recently, Cleton-Jansen *et al.* (1991) cloned and sequenced the GDH gene of *G. oxydans* and reported that a single-base substitution resulting in replacement of His-787 by Asn is responsible for a change in substrate specificity. Such a mutation seems to occur often naturally, since the membrane-bound D-xylose-oxidizing enzyme from *G. oxydans* (Buchert, 1991) seems to be the same enzyme as GDH from *G. suboxydans*, although it is more active with D-xylose (Ameyama *et al.*, 1981e). The amino acid-residue sequence deduced from the nucleotide sequence shows that GDH of *G. suboxydans* is a 87 kDa protein with 808 amino acid residues and homologous to those from both *Acinetobacter calcoaceticus* and *E. coli* (Cleton-Jansen *et al.*, 1988, 1990; Anthony, 1992). Glucose dehydrogenase contains five hydrophobic domains in the *N*-terminal region, comprising 145 amino acid residues which might serve as membrane-spanning segments and also as a ubiquinone-reacting site, while the residual *C*-terminal region of GDH is thought to be located on the periplasmic side, following results from a fusion experiment with a reporter gene (Yamada *et al.*, 1993).

Gluconate dehydrogenase, which catalyses production of 2-keto-D-gluconate from D-gluconate, has been purified from *Gluconobacter dioxyacetonicus* (Shinagawa *et al.*, 1984) and characterized (Table 2). It shows almost the same properties as those of purified enzymes from other oxidative bacteria, including subunit structure, heme content and optimum pH value (Matsushita *et al.*, 1982c). 2-Keto-D-gluconate dehydrogenase, which catalyses synthesis of 2,5-diketo-D-gluconate from 2-keto-D-gluconate, has been purified and characterized from *Gluconobacter melanogenus* (Shinagawa *et al.*, 1981; Table 2). Gluconate dehydrogenase and 2KGADH have almost the same substructure consisting of three subunits; namely flavoprotein, cytochrome *c*, and a third subunit whose function is unknown. It has been shown that FAD is bound through the methyl group at the C^8 site with the N^3 site of a histidine residue in the flavoprotein subunit (McIntire *et al.*, 1985). Both enzymes are highly specific for D-gluconate and 2-keto-D-gluconate, respectively. Some *Gluconobacter* strains, e.g. *G. suboxydans*, have been reported to produce 5-keto-D-gluconate as well as 2-keto-D-gluconate (Shinagawa *et al.*, 1983). The $NADP^+$-dependent 5-keto-D-gluconate reductase has been purified from *G. suboxydans* (Adachi *et al.*, 1979), but the optimum pH value for oxidation of D-gluconate is around 10. Another enzyme oxidizing D-gluconate to 5-keto-D-gluconate may be present in the cytoplasmic membrane of these strains.

D. PRIMARY DEHYDROGENASES IN SORBITOL-OXIDIZING SYSTEMS

Figure 5 shows the dehydrogenases involved in the metabolism of D-sorbitol, and production of L-sorbose and 2-keto-L-gulonic acid (Sugisawa *et al.*, 1991a). The enzymes D-sorbitol dehydrogenase (SLDH) and L-sorbose dehydrogenase (SDH) are located in the cytoplasmic membrane. In the cytoplasm, there are an $NADP^+$-dependent D-sorbitol dehydrogenase (L-sorbose reductase) and an $NAD(P)^+$-dependent L-sorbosone reductase (Hoshino *et al.*, 1991). D-Sorbitol seems to be utilized through the pentose-phosphate cycle via D-fructose (Asai, 1968).

D-Sorbitol dehydrogenase has been purified from *G. suboxydans* (Shinagawa and Ameyama, 1982). The enzyme contains three subunits; namely a flavoprotein (63 kDa), cytochrome *c* (51 kDa) and a third one of 17 kDa. The cytochrome *c* is not reduced by addition of D-sorbitol unless coenzyme Q_1 is present. D-Mannitol is also oxidized at a rate of 5% compared with D-sorbitol. The enzyme can use ferricyanide, PMS, DCIP and Wurster's blue (WS), but not $NAD(P)^+$ or molecular oxygen as an electron acceptor. Cho *et al.* (1990) purified a membrane-bound dehydrogenase from *G. melanogenus*, which reacts with polyols having a D-lyxo configuration such as D-mannitol and D-sorbitol. The enzyme seems to contain no flavin, while D-mannitol is oxidized faster than D-sorbitol. The second subunit (52 kDa) shows an absorption spectrum characteristic of oxidized cytochrome *c*, and is rapidly reduced by D-mannitol in the enzyme complex.

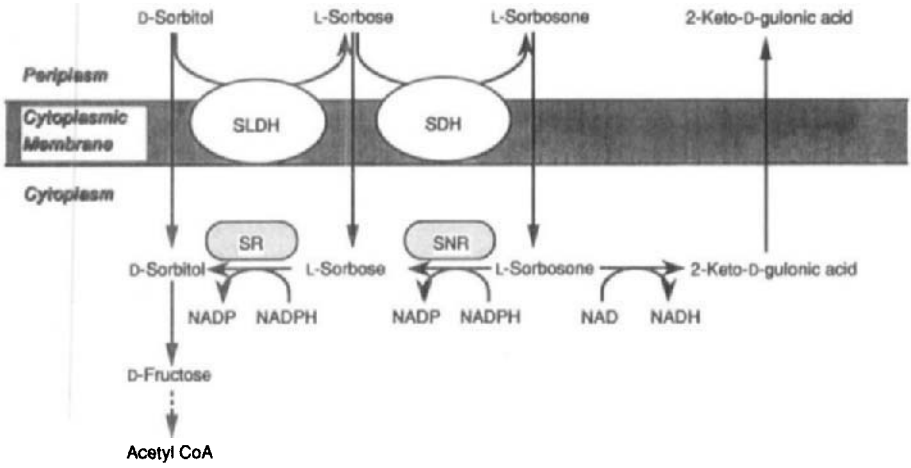

FIG. 5. Sorbitol-oxidizing systems in acetic acid bacteria. The flavoproteins D-sorbitol dehydrogenase (SLDH) and L-sorbose dehydrogenase (SDH) are located on the outer surface of the cytoplasmic membrane. The $NADP^+$-dependent L-sorbose reductase (SR) and L-sorbosone reductase (SNR) function in the cytoplasm.

L-Sorbose dehydrogenase has been purified and characterized by Sugisawa *et al.* (1991b). The enzyme has a single polypeptide (58 kDa) and only reacts with L-sorbose. Sugisawa *et al.* (1991b) also reported that an enzyme catalysing oxidation of L-idonic acid to 2-keto-L-gulonic acid is present in membranes of *G. melanogenus*. The enzyme can be solubilized from membranes separately from SDH, and should be different from D-gluconate dehydrogenase which is incapable of oxidizing L-idonic acid (Shinagawa *et al.*, 1984).

E. PRIMARY DEHYDROGENASES IN OTHER SUGAR-OXIDIZING SYSTEMS

Gluconobacter species can oxidize glycerol to dihydroxyacetone, which is a useful character in identifying *Gluconobacter* species. De Ley and Dochy (1960) reported that an enzyme catalysing oxidation of glycerol yielding dihydroxyacetone, and independent of ATP or NAD^+, is present in the particulate fraction from *Gluconobacter liquefaciens*. This enzyme is an $NAD(P)^+$-independent glycerol dehydrogenase (GLDH), which has been purified and characterized from *Gluconobacter industrius* by Ameyama *et al.* (1985). The enzyme is highly hydrophobic and, in the absence of 0.1% dimethyldodecylamine oxide and 10% glycerol, its inactivation proceeds rapidly with formation of a huge aggregate. A methanol extract of the purified enzyme activated the apo-GDH from *E. coli*, showing that it contains PQQ as a prosthetic group. When the membrane fraction was treated with 2 mM EDTA, GLDH activity was lost completely, but was restored to the original level by addition of PQQ in the presence of magnesium ions, indicating that the affinity of this enzyme for PQQ is relatively low. Glycerol dehydrogenase catalyses oxidation of other polyhydroxyl alcohols such as *meso*-erythritol and D-arabitol at similar rates. D-Sorbitol, D-mannitol, polyethyleneglycol, adonitol and dulcitol are also oxidized by the enzyme but at relatively low rates. D-Glucose, D-fructose, ethanol and acetaldehyde are not oxidized. The oxidation products of glycerol, D-sorbitol and D-mannitol were identified as dihydroxyacetone, L-sorbose and D-fructose, respectively. Neither $NAD(P)^+$ nor molecular oxygen is an electron acceptor, PMS-DCIP being the most effective.

D-Fructose dehydrogenase, yielding 5-keto-D-fructose, has been purified and characterized from membranes of *G. industrius* (Ameyama *et al.*, 1981d). The enzyme is specific for D-fructose but has little affinity for substrate analogues such as D-glucose, D-fructose-6 phosphate, D-fructose-1,6-bisphosphate and 5-keto-D-fructose. This character is advantageous in using the enzyme as a quantitative reagent for D-fructose microdetermination. The enzyme consists of three subunits, these being subunits I

(67 kDa), II (50 kDa), and III (20 kDa). Subunit I contains PQQ as a prosthetic group, while subunit II is cytochrome *c*. Ferricyanide, PMS and DCIP are good electron acceptors for D-fructose dehydrogenase, but $NAD(P)^+$ and molecular oxygen do not function. 5-Keto-D-fructose reductase is found in the cytoplasm (Table 1); 5-keto-D-fructose seems to be utilized after conversion by this enzyme to D-fructose.

III. Terminal Oxidases of Acetic Acid Bacteria

The bacterial respiratory chain has been separated into two classes; one has cytochrome *c* oxidase at the terminal end and the other ubiquinol oxidase (Anraku, 1988). The former class is found in the so-called "oxidase positive" bacteria such as species of *Paracoccus, Pseudomonas* and *Azotobacter*, where cytochromes aa_3, caa_3 or *co* function as the terminal oxidase. The latter class occurs in oxidase negative bacteria including *E. coli* and acetic acid bacteria, the respiratory chains of which contain cytochromes *o*, a_1, aa_3 or *d* (Table 4). Recent evidence has shown that there are more divergences in some bacterial respiratory chains where both categories of terminal oxidases occur in a single strain (Lauraeus *et al.*, 1991; Ludwig, 1992).

Acetic acid bacteria have been shown only recently to have the same type of terminal oxidase, namely ubiquinol oxidase, but they are very divergent with respect to the cytochrome species (Table 5). Bächi and Ettlinger (1974) divided acetic acid bacteria into three groups based on the α absorption peak of their cytochromes. The first group (*peroxydans* group) of *Acetobacter* strains exhibits peaks corresponding to both cytochrome a_1 and cytochrome *d*, while the second group of *Acetobacter* (*oxydans* and *mesoxydans* groups) exhibits only the peak of cytochrome a_1. The third group of bacteria contains *Gluconobacter* strains which do not show any peaks corresponding to cytochrome a_1 or cytochrome *d*. In his classic work, Warburg had shown the occurrence of cytochrome a_1 in *Acetobacter pasteurianus* (see Poole, 1983; Matsushita *et al.*, 1990), while Williams and Poole (1987) reported that the same strain has cytochrome *d* but not cytochrome a_1. Daniel (1970) has clearly shown that *G. suboxydans* contains cytochrome *o* as a terminal oxidase. Several other earlier observations suggested the presence of the same kinds of cytochrome oxidases in acetic acid bacteria. Thus, the terminal oxidase of acetic acid bacteria seems to be divided into three types; cytochrome *o* observed mainly in *Gluconobacter* spp., cytochrome a_1 seen in many species of *Acetobacter* and cytochrome *d* in some other species of *Acetobacter* (Table 5). Terminal oxidases of both *Gluconobacter* and *Acetobacter*

TABLE 4. Examples of the two classes of bacterial terminal oxidases

Oxidase species	Hemes	Bacterial strains from which they have been purified	References
I. Cytochrome c *oxidase*			
Cytochrome aa_3	two hemes *a*	*Paracoccus denitrificans*	Ludwig and Schatz (1980)
		Thiobacillus novellus	Yamanaka and Fujii (1980)
			Poole (1988) as a review
Cytochrome caa_3	heme *c* and two hemes *a*	Thermophilic bacterium PS3	Sone and Yanagita (1982)
		Thermus thermophilus	Fee *et al.* (1980); Hon-nami and Oshima (1980)
			Poole (1988) as a review
Cytochrome *co*	hemes *b* and *c*	*Pseudomonas aeruginosa*	Matsushita *et al.* (1982b)
		Azotobacter vinelandii	Jurtshuk *et al.* (1981)
			Poole (1988) as a review
II. Ubiquinol oxidase			
Cytochrome *o*	hemes *b* and *o*	*Escherichia coli*	Kita *et al.* (1984a); Matsushita *et al.* (1984)
		Gluconobacter suboxydans	Matsushita *et al.* (1987b)
			Poole (1988) as a review
Cytochrome *d*	hemes *b* and *d*	*Escherichia coli*	Miller and Gennis (1983); Kita *et al.* (1984b)
		Photobacterium phosphoreum	Konishi *et al.* (1986)
Cytochrome a_1	hemes *b* and *a*	*Acetobacter aceti*	Matsushita *et al.* (1990)
		Paracoccus denitrificans	Ludwig (1992)
Cytochrome aa_3	two hemes *a*	*Sulfolobus acidocaldarius*	Anemüller and Schäfer (1990)
		Bacillus subtilis	Lauraeus *et al.* (1991)

TABLE 5. Terminal oxidases which have been detected in acetic acid bacteria

Bacterial species	Oxidase	Prosthetic group	References
I. *Gluconobacter* species	Cytochrome *o*	heme *b*	Daniel (1970)
		heme *o*	Bächi and Ettlinger (1974)
		Cu^{2+} [a]	Ameyama *et al.* (1987)
			Matsushita *et al.* (1987b)
II. *Acetobacter* species	Cytochrome a_1	heme *b*	Poole (1983) as a review
		heme *a*	Bächi and Ettlinger (1974)
		Cu^{2+}	Matsushita *et al.* (1990)
	Cytochrome *o*	heme *b*	Matsushita *et al.* (1992a)
		heme *o*	Matsushita *et al.* (1992b)
		Cu^{2+}	
III. Other *Acetobacter* species	Cytochrome *d*	heme b^a	Bächi and Ettlinger (1974)
Acetobacter pasteurianus		heme d^a	Williams and Poole (1987)
(*Acetobacter peroxydans*)	Cytochrome *o*	heme b^a	Williams and Poole (1988)
		heme o^a	
		Cu^{2+a}	
Acetobacter methanolicus	Cytochrome *co*	heme *b*	Chan and Anthony (1991a)
		heme *c*	Matsushita *et al.* (1992c)
	Cytochrome *o*	heme *b*	Matsushita *et al.* (1992c)
		heme *o*	
		Cu^{2+} [a]	

[a] Speculative but not determined.

spp. have been isolated and characterized (Matsushita *et al.*, 1987b, 1990, 1992a).

A. CYTOCHROMES *O* OF *GLUCONOBACTER* AND *ACETOBACTER* SPECIES

Cytochrome *o* of acetic acid bacteria was detected in *G. suboxydans* (originally called *Acetobacter suboxydans*) by Smith (1954) and King and Cheldelin (1957). The occurrence of cytochrome *o* was substantiated by Daniel (1970) using a membrane fraction from *G. suboxydans*. The cytochrome *o* has been solubilized and isolated from membranes (Ameyama *et al.*, 1987) and further purified to homogeneity (Matsushita *et al.*, 1987b). Cytochromes *o* have also been detected in other acetic acid bacteria, namely *A. aceti* and *Acetobacter methanolicus*, where this terminal oxidase occurs in cells grown under specific growth conditions. This is somewhat different from *G. suboxydans* where cytochrome *o* is detected in cells grown under any growth conditions. *A. aceti* is able to produce cytochrome *o* as the terminal oxidase only when grown in static cultures (Matsushita *et al.*, 1992b). Unlike the common acetic acid bacteria, *A. methanolicus* can grow on methanol and induce cytochrome *co*

as a terminal oxidase functioning for methanol oxidation (Chan and Anthony, 1991a). When grown under non-methylotrophic conditions, however, *A. methanolicus* produces cytochrome *o* extensively as the terminal oxidase for ubiquinone in a respiratory chain which is similar to that of *Gluconobacter* strains (Matsushita *et al.*, 1992c).

Cytochromes *o* of acetic acid bacteria have been purified, after solubilization from membranes, by a single-step column chromatography on DEAE-Toyopearl (Matsushita *et al.*, 1987b, 1992a,c). All purified cytochromes *o* are highly active ubiquinol oxidases having closely similar structural and functional properties to each other, as summarized in Tables 4 and 5. These purified oxidases consist of four non-identical subunits and two *b*-type cytochromes, one of which at least can bind with carbon monoxide, thereby showing it to be a cytochrome *o*. The enzymes from *A. aceti* and *A. methanolicus* have been shown to contain the newly identified heme *o* (Puustinen and Wikström, 1991) in addition to heme *b* in a 1:1 ratio, and are termed cytochromes *bo* (Matsushita *et al.*, 1992a,c). With the enzyme from *G. suboxydans*, however, both cytochromes seem to bind with carbon monoxide, and thus cytochrome *o* may contain two hemes *o* in the molecule or the cytochrome *b* component may bind with carbon monoxide artificially. The ubiquinol oxidation activity of the oxidase is inhibited by cyanide or quinone analogues. The purified cytochrome *o* can be reconstituted with phospholipids into proteoliposomes in which it generates a proton electrochemical gradient (inside negative and alkaline) of about −140 mV during ubiquinol oxidation. Thus, cytochrome *o* is regarded as a ubiquinol oxidase functioning as an energy generator.

The cytochrome *o* of acetic acid bacteria has a closely similar structure to cytochrome *o* of *E. coli*, which also consists of four non-identical subunits and contains heme *b*, heme *a* and a copper atom as the prosthetic group (Kita *et al.*, 1984a; Matsushita *et al.*, 1984; Chepuri *et al.*, 1990; Puustinen and Wikström, 1991). It has been shown that cytochrome *o* from *A. aceti* consists of one each of heme *b* and *o* and one copper atom (Matsushita *et al.*, 1992a). Furthermore, subunit I of cytochrome *o* from *E. coli* has been deduced from DNA sequence and site-directed mutagenesis to have a heme and a heme-copper binuclear centre (Minagawa *et al.*, 1992; Lemieux *et al.*, 1992), which seems to be the same as in cytochrome *c* oxidase. Cytochrome *o* may have a redox-linked proton pump involved in energy generation similar to that of cytochrome *c* oxidase (Puustinen *et al.*, 1989, 1991; Verkhovskaya *et al.*, 1992). This proton-pump mechanism has recently been proposed to counter the original notion that cytochrome *o* may generate an electrochemical proton gradient by catalysing scalar release of protons from ubiquinol on the outer surface

of the membrane, vectorial transfer of electrons from the outer to the inner surface, and scalar utilization of protons on the inner surface to reduce oxygen (Matsushita *et al.*, 1984).

Cytochrome *o* might function as at least one of the terminal oxidases in almost all acetic acid bacteria, since cytochrome *o* has also been detected even in *A. pasteurianus* which has cytochrome *d* as the terminal oxidase (Williams and Poole, 1988) besides *A. aceti* and *Gluconobacter* species.

B. CYTOCHROME a_1 OF *ACETOBACTER* SPECIES

Cytochrome a_1 has a long history since the classic work by Warburg showed that *Acetobacter pasteurianum* (syn. *A. pasteurianus*) exhibits a weak band at 589 nm which is intensified by cyanide and shifts to 592 nm in the presence of carbon monoxide (Keilin, 1966; Poole, 1983, 1988). The cytochrome peak around 590 nm in extracts of *A. pasteurianus* was also observed by Smith (1954) and King and Cheldelin (1957). The earlier findings have been confirmed by Chance who showed that cytochrome a_1 works as a terminal oxidase in *A. pasteurianus* (Chance, 1953a,b; Castor and Chance, 1955). *Acetobacter* species, especially the *oxydans* and *mesoxydans* groups, may contain cytochrome a_1, although there is some confusion as discussed later.

1. Cytochrome a_1 *of* Acetobacter aceti

A cytochrome a_1-like ubiquinol oxidase was shown to be formed by *A. aceti* in shaking cultures (Matsushita *et al.*, 1992b) and, subsequently, the cytochrome was purified and characterized (Matsushita *et al.*, 1990, 1992a). The ubiquinol oxidase has been shown to be cytochrome a_1, judged by several criteria. These are: (i) the oxidase contains heme *a* in addition to heme *b*; (ii) the enzyme exhibits a weak and broad α band, which is largely intensified and changed to a sharp band at 587 nm by addition of cyanide; cyanide-induced intensification of the α band, which may be due to formation of a compound between ferrous heme and cyanide, is characteristic of cytochrome a_1 as well as the weak α band (Poole, 1983); (iii) the cytochrome *a* component of the enzyme reacts with carbon monoxide. Thus, the terminal oxidase of *A. aceti* turns out to be the first enzyme purified as cytochrome a_1; this is also a reconfirmation of the original evidence that the classic cytochrome a_1, originally found by Warburg, occurs in the respiratory chain of *Acetobacter* species.

Cytochrome a_1 of *A. aceti* is distinct from other *a*-type cytochrome terminal oxidases in terms of spectroscopic properties, heme composition, and its electron donor. Cytochrome aa_3-type cytochrome *c* oxidases, such

as those from *Paracoccus denitrificans* and thermophilic bacterium PS3, exhibit a sharp α peak around 605 nm in the reduced state, mainly due to heme *a* but not heme a_3 in the absence of carbon monoxide or cyanide, which is clearly a different feature from cytochrome a_1. Thus, cytochrome a_1 seems to lack the heme *a* moiety, reacting with reduced cytochrome *c* present in cytochrome aa_3, but instead to have the heme *b* moiety reacting with ubiquinol. Recently, a similar species of cytochrome has been purified from *Thermus thermophilus* and termed cytochrome ba_3. This cytochrome was shown to consist of a single polypeptide of 35 kDa, containing one heme *a* molecule, one heme *b* molecule and two copper ions (Zimmermann *et al.*, 1988). Moreover, a ubiquinol oxidase consisting of heme *b* and heme *a* molecules has been purified from *P. denitrificans* (Ludwig, 1992). Thus, such a cytochrome a_1 may be widely present in other "oxidase negative" bacteria exhibiting an a_1-like spectrum or having heme *a*.

Cytochrome a_1 of *A. aceti* is a cytochrome *ba* oxidase, catalysing ubiquinol oxidation, which is closely related to cytochrome *o* ubiquinol oxidase. Both terminal oxidases have similar four polypeptides and the only variation is that the carbon monoxide-binding component is heme *a* instead of heme *o*. Furthermore, the oxidase has been shown to be capable of generating an electrochemical proton gradient, a function that has also been found in other ubiquinol oxidases. Thus, cytochrome a_1 of *A. aceti* is an energy-generating ubiquinol oxidase like cytochrome *o*.

2. *Homology Between Cytochrome* a_1 *and Cytochrome* o

Cytochrome a_1 is produced in *A. aceti* grown in shaking culture, while cells grown in static culture produce an alternative oxidase, namely cytochrome *o* (Matsushita *et al.*, 1992b). Cytochrome a_1 and cytochrome *o* show little difference in kinetics for ubiquinol oxidation, although both enzymes exhibit different kinetics for oxygen (Table 6), suggesting that both oxidases have a twin structure with a similar electron-accepting site but a different oxygen-reduction site. Cytochrome a_1 and cytochrome *o* purified from *A. aceti* were shown to have the same heterotetrameric substructure, in which the subunits have identical molecular weights (Matsushita *et al.*, 1992a). Subunits I and II of both oxidases are indistinguishable immunochemically and have no differences in their peptide mapping. Furthermore, the amino-terminal sequence of subunit II from cytochrome a_1 is identical with that of cytochrome *o* in their first ten residues. Thus, cytochrome a_1 and cytochrome *o* of *A. aceti* are highly homologous in structure or even may share the same polypeptides. Furthermore, there is some evidence that both oxidases arise from a single structural gene (K. Matsushita,

TABLE 6. Terminal oxidases purified from acetic acid bacteria

Characters	Cytochrome *o* from			Cytochrome a_1 from *Acetobacter aceti*[d]
	Gluconobacter suboxydans[a]	*Acetobacter methanolicus*[b]	*Acetobacter aceti*[c]	
Subunits (kDa)				
I	55[e] (76)	55[e]	55[e]	55[e]
II	30[e] (35)	30[e]	35	35
III	21	21	22	21
IV	15	15	18	18
Q_2H_2 oxidation activity				
optimum pH value	6.5	6.5	6.5	6.5
K_m (μM)	22	–	16	19
specific activity[f]	165	123	120	144
Electron acceptor				
K_m for oxygen (μM)	33[g]	–	16.9	4.4
Inhibitor				
K_i for carbon monoxide (μM)	3.0 and 13	–	3.7	4.6
K_i for cyanide (μM)	2.8	2.2	38	74
K_i for HQNO (μM)	0.4	0.5	2.7	4.8

[a] Matsushita *et al.* (1987b).
[b] Matsushita *et al.* (1992d).
[c] Matsushita *et al.* (1992a).
[d] Matsushita *et al.* (1990).
[e] Apparent molecular weight (the value after correction by a Ferguson plot).
[f] μmol of quinol oxidized min^{-1}(mg protein)$^{-1}$.
[g] Daniel (1970).

M. Yamada, H. Toyama and O. Adachi, unpublished observations) and the gene for cytochrome a_1 cloned from *A. aceti* consists of four open-reading frames which are similar to the *cyo* operon of *E. coli* and has a high homology to the genes (K. Tayama, unpublished observations).

Thus, both cytochrome a_1 and cytochrome *o* of *A. aceti* are ubiquinol oxidases with closely similar structural properties except for the moiety in the copper–heme binuclear centre, where heme *a* in cytochrome a_1 is replaced by heme *o* in cytochrome *o*. Since heme *b* may be located similarly at the quinol-reacting site of both oxidases, it is reasonable that both enzymes exhibit a similar K_m value and turnover number for quinol. In contrast, the differences in cyanide sensitivity and oxygen affinity between cytochrome a_1 and cytochrome *o* could be explained by the oxygen-reacting site being occupied by different heme moieties, heme *a* and heme *o*, respectively. Since both oxidases contain two iron and one copper atom in each molecule, the oxygen-reacting site in cytochrome a_1 and cytochrome *o* of *A. aceti* may be constituted by heme *a* (or heme *o*) and copper, as expected in cytochrome *o* of *E. coli* or cytochrome aa_3 from *Rhodobacter sphaeroides* where heme *o* or heme *a* and copper may constitute a heme-copper binuclear centre (Minagawa *et al.*, 1992; Shapleigh *et al.*, 1992).

Furthermore, one may speculate that the two ubiquinol oxidases of *A. aceti* may accept either heme *a* or heme *o* at the oxygen-reacting binuclear centre and, thus, exchange of oxidases can be regulated by synthesis of heme *a* from heme *o* without altering the apoprotein moiety. Aside from the mechanism, such an exchange of terminal oxidase from one species to the other has been observed in many bacterial strains. The most prominent example is *E. coli* where cytochrome *o* is exchanged by the enzyme having the same ubiquinol oxidase activity but being a completely different protein, namely cytochrome *d* (Anraku and Gennis, 1987). Similar phenomena have been observed among cytochrome *c* oxidases of several bacterial species, where cytochrome caa_3 can be replaced by a similar cytochrome oxidase, namely cytochrome aa_3, cytochrome ba_3 or cytochrome *cao*. *Thermus thermophilus* produces cytochrome ba_3 whose structure is different from cytochrome caa_3 (Zimmerman *et al.*, 1988). In *Bacillus subtilis*, there is a cytochrome caa_3 exhibiting cytochrome *c* oxidase activity, and a cytochrome aa_3 ubiquinol oxidase (Lauraeus *et al.*, 1991), both of which consist of four non-identical subunits generated by highly homologous but different sets of genes (Van der Oost *et al.*, 1991; Santana *et al.*, 1992). The thermophilic bacterium PS3 contains cytochrome *cao* which can be replaced by cytochrome caa_3 having the same oxidase activity and the same substructure in which the molecular size of each of the four subunits is identical in both oxidases (Sone and Fujiwara,

1991). In *Bacillus cereus*, cytochrome caa_3 can also be exchanged by cytochrome aa_3 in which subunits I, but not subunits II, in both oxidases are indistinguishable immunochemically (Garcia-Horsman et al., 1991). Thus, the exchange mechanism of terminal oxidases in *T. thermophilus*, *B. subtilis* and *E. coli* might be different from that in *A. aceti*, since these bacterial strains produce two completely different sets of terminal oxidases with similar prosthetic groups. Instead, the situation in *A. aceti* may be similar to that in thermophilic bacterium PS3 or *B. cereus* where only a heme moiety is possibly replaced in two terminal oxidases.

C. CYTOCHROME *d* OF *ACETOBACTER* SPECIES

As already described, Williams and Poole (1987) could not demonstrate the presence of cytochrome a_1 as a terminal oxidase in *A. pasteurianus* NCIB 6428. Instead, they have shown that a_1-like cytochrome donates electrons to the cytochrome *d* terminal oxidase in this organism. Probably, this apparent controversy is due to differences in the strains used, because the original strain used by Warburg and subsequently by other researchers had been lost. Since Bächi and Ettlinger (1974) could not detect cytochrome *d* in *A. pasteurianus* NCTC 6428, the same type culture as NCIB 6428, there seems to be some confusion even in the type culture collections.

In any case, there might be cytochrome *d* as well as a_1-like cytochromes in *Acetobacter peroxydans* used by Bächi and Ettlinger (1974) or *A. pasteurianus* used by Williams and Poole (1987). The a_1-like cytochromes of these *Acetobacter* strains may be a component of cytochrome *d*, since a_1-like cytochrome copurified with cytochrome *d* has been shown to be ascribable to heme *b* being contained in the cytochrome *d* terminal oxidase complex of *E. coli* (Lorence *et al.*, 1986). Therefore, cytochrome a_1 may not be present, but instead cytochrome *d* may work as the terminal oxidase in these *Acetobacter* strains. In *E. coli*, cytochrome *d* is produced under limited-oxygen conditions with cytochrome *o* being synthesized under conditions of high oxygen tension (Anraku and Gennis, 1987). Since cytochrome *o* seems to be present in almost all *Acetobacter* species including ones having cytochrome *d* as already described, these *Acetobacter* strains may produce cytochrome *d* in addition, or instead, of cytochrome *o* under conditions of low aeration.

IV. Respiratory Chains of Acetic Acid Bacteria

Acetic acid bacteria are strict aerobes having highly active oxidase systems for metabolizing several alcohols and sugars. Although a considerable

amount of earlier work had been done on sugar metabolism of acetic acid bacteria, little was known of the respiratory chain of these bacteria and of the relation with sugar-oxidation reactions (Asai, 1968). As described in Section II, although some of this metabolism may link indirectly to the respiratory chain through NADH dehydrogenase, most oxidation reactions of acetic acid bacteria seem to link directly to the membrane-bound respiratory chain of the organisms. In the following sections of this review, respiratory chains of *G. suboxydans*, *A. aceti* and *A. methanolicus* are described mainly in relation to the alcohol- and sugar-oxidizing systems of these bacteria.

A. THE RESPIRATORY CHAIN OF *GLUCONOBACTER SUBOXYDANS*

1. A Branched Respiratory Chain with Cyanide-Sensitive and Cyanide-Insensitive Terminal Oxidases

Earlier studies on the respiratory chain of *G. suboxydans* suggested, on the basis of cytochrome difference spectra, that the organism contains cytochromes *c*, *b*, *o* and a_1 (Smith, 1954; King and Cheldelin, 1957; Iwasaki, 1960). More detailed analysis of the respiratory chain of *G. suboxydans* has been undertaken with membrane preparations by Daniel (1970) who showed that the organism has a highly active membrane-bound respiratory chain that contains only cytochromes *c* and *o* but no cytochrome *b*, *d* or *a*. He found moreover that the respiratory chain contains ubiquinone, two kinds of *c*-type cytochromes and two kinds of *o*-type cytochromes being involved in this order in an NADH oxidase system (Fig. 6). Many aspects of his observations were confirmed and further improved by subsequent studies including kinetics, solubilization and characterization of the membrane-bound cytochrome components, which confirmed more unambiguous evidence for the respiratory chain of *G. suboxydans* (Matsushita *et al.*, 1981b, 1987b; Ameyama *et al.*, 1987).

Gluconobacter suboxydans, especially when grown in a sugar-rich medium, possesses extremely high oxidase activities for D-glucose, D-gluconate, glycerol, D-sorbitol, ethanol, acetaldehyde and lactate, which are all detected in membranes together with NADH oxidase activity (Table 7). The membrane-bound respiratory chain has a high ubiquinol oxidase activity but lacks cytochrome *c* oxidase activity as the terminal oxidase. Furthermore, in membranes of *G. suboxydans*, there are at least six cytochromes *c*, almost all of which are components of primary dehydrogenases working in alcohol- and sugar-oxidizing systems, as shown in Table 8, and also as described in Section II. Major cytochromes *c* present in the

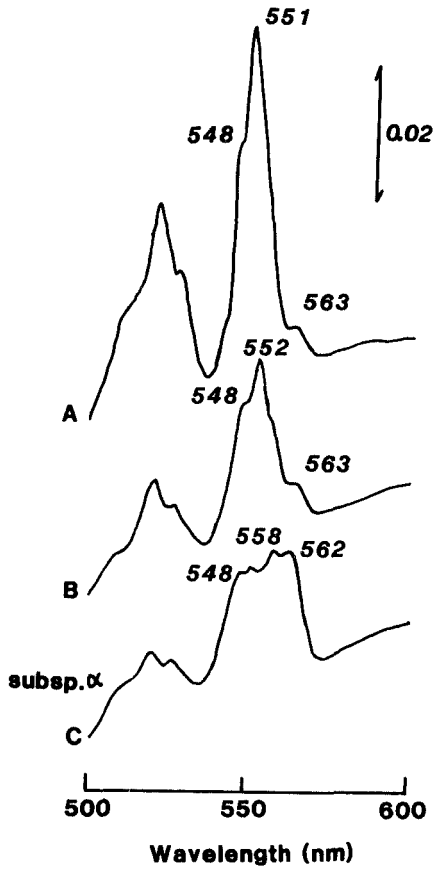

FIG. 6 Low-temperature reduced minus oxidized difference spectra of membranes of *Gluconobacter suboxydans*. The bacterium was grown in a sugar-rich medium around pH 6(A) or pH 4(B), while *Gluconobacter suboxydans* subsp. α(C) was also grown in a sugar-rich medium (Ameyama *et al.*, 1987). Membranes prepared from these cells were suspended in 50 mM potassium phosphate buffer (pH 6.5) at protein concentrations of 2.5, 2.5, and 7.2 mg ml^{-1}, respectively. Dithionite-reduced minus ferricyanide-oxidized difference spectra were taken in a cuvette of 2 mm light path in liquid nitrogen.

membranes of *G. suboxydans* are cytochrome c_{553} (ADH), cytochrome c_{553} (CO), cytochrome c_{551} (ALDH) and cytochrome c_{551} (CO), of which the first two are the most prominent (Matsushita *et al.*, 1989a). Since cytochrome c_{553} (CO) has been shown to be identical to the second subunit of ADH (Takeda and Shimizu, 1991; Matsushita and Adachi, 1993), cytochrome c_{553} (ADH), which includes two *c*-type cytochromes of a

TABLE 7. Respiratory chain-linked enzyme activities and cytochrome contents of membranes from *Gluconobacter suboxydans* and *Acetobacter aceti*

	Gluconobacter suboxydans[a]		*Acetobacter aceti*[b]	
		subsp. α	shaking culture	static culture
Oxidase (μmol of substrate oxidized $min^{-1}mg^{-1}$				
Glucose	1.14	0.77	0.28	0.23
Gluconate	0.30	–	–	–
Glycerol	1.56	0.50	n.d.	n.d.
Sorbitol	0.39	0.55	–	–
Ethanol	1.64	0.00	0.79 (1.30)[c]	0.45 (1.11)
Acetaldehyde	–	0.47	0.36 (1.60)	0.18 (1.57)
Lactate	0.49	–	0.76	0.48
NADH	2.20	1.08	0.66	0.29
Q_2H_2	2.91	4.47	5.00	4.80
Cytochrome (nmol mg^{-1})				
Cytochrome *c*	1.42	0.18	0.60	0.60
Cytochrome *o*	0.27	0.23	n.d.	0.25
Cytochrome *a*	n.d.	n.d.	0.24	n.d.
Heme *b*	0.38	0.25	0.55	0.51
Heme *c*	1.35	0.21	0.41	0.44
Heme *a*	n.d.	n.d.	0.12	n.d.

[a] *Gluconobacter suboxydans* IFO 12528 and *Gluconobacter suboxydans* subsp. α IFO 3254 were grown in a sugar-rich medium (Ameyama *et al.*, 1987; Matsushita *et al.*, 1991).

[b] *Acetobacter aceti* IFO 3284 grown on a glycerol medium (Matsushita *et al.*, 1992b).

[c] When grown on an ethanol-containing medium (K. Matsushita, unpublished data).

n.d. indicates that the component was not detected.

TABLE 8. Properties of cytochromes *c* found in the membranes of acetic acid bacteria

Cytochromes *c*	Absorption maxima (nm) Reduced	Oxidized	Molecular weight (k)	Function	Strains	References
Cytochrome c_{553} (ADH)[a]	553,522,417	410	80, 48	Subunits I and II of ADH	*Gluconobacter suboxydans*	Adachi *et al.* (1978b) Ameyama *et al.* (1987)
Cytochrome c_{553} (ADH)	553,522,417	409	78, 50	Subunits I and II of ADH	*Acetobacter aceti*	Adachi *et al.* (1978a) Matsushita *et al.*(1992d) Inoue *et al.* (1989, 1992)
Cytochrome c_{553} (ADH)	553,523,418	–	72, 44	Subunits I and II of ADH	*Acetobacter polyoxogenes*	Tayama *et al.* (1989) Tamaki *et al.* (1991)
Cytochrome c_{554} (ADH)	554,523,417	–	79, 53	Subunits I and II of ADH	*Acetobacter methanolicus*	Matsushita *et al.* (1992c)
Cytochrome c_{551} (ADH)	551,523,418	410	55	Subunit II of ALDH (+135 mV)	*Gluconobacter suboxydans*	Adachi *et al.* (1980c) Ameyama *et al.* (1987)
Cytochrome c_{551} (ALDH)	551,523,417	411	45	Subunit II of ALDH	*Acetobacter aceti*	Ameyama *et al.* (1981c)
Cytochrome c_{553} (CO)	553,522,418	409	48	Subunit II of ADH (+60 mV)	*Gluconobacter suboxydans*	Matsushita *et al.* (1981b) Takeda and Shimizu (1991)
Cytochrome c_{551} (CO)	551,523,417	412	72	unknown (−25 mV)	*Gluconobacter suboxydans*	Ameyama *et al.* (1987)
Cytochrome c_{551} (SLDH)	551,522,417	–	51	Subunit II of SLDH	*Gluconobacter suboxydans*	Shinagawa *et al.* (1982)
Cytochrome c_{552} (MLDH)	552,522,418	–	52	Subunit II of MLDH	*Gluconobacter melanogenus*	Cho *et al.* (1990)
Cytochrome c_{553} (GADH)	553,523,418	–	45	Subunit II of GADH	*Gluconobacter dioxyacetonicus*	Shinagawa *et al.* (1984)
Cytochrome c_{554} (2KGADH)	554,523,417	411	47	Subunit II of 2KGDH	*Gluconobacter melanogenus*	Shinagawa *et al.* (1981)
Cytochrome c_{553} (FDH)	553,523,417	409	51	Subunit II of FDH	*Gluconobacter industrius*	Ameyama *et al.* (1981d)

[a] Terms in parentheses indicate the enzyme with which the cytochrome is associated, or the inhibitor with which it reacts. Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CO, carbon monoxide; SLDH, sorbitol dehydrogenase; MLDH, mannitol dehydrogenase; GADH, gluconate dehydrogenase; 2KGADH, 2-ketogluconate dehydrogenase; FDH, fructose dehydrogenase.

quinohemoprotein (80 kDa) and a diheme cytochrome *c* (48 kDa), turns out to be the most prominent cytochrome *c* of the respiratory chain in *G. suboxydans*. On the basis of redox kinetics, the *c*-type cytochrome(s) can possibly work between ubiquinone and cytochrome *o* in the respiratory chains of the NADH oxidase system (Daniel, 1970) and also of the alcohol- and sugar-oxidizing systems (Ameyama *et al.*, 1987). However, the terminal oxidase purified from *G. suboxydans* is a cytochrome *o* capable of directly oxidizing ubiquinol but not reduced cytochrome *c* (Matsushita *et al.*, 1987b). This is something peculiar because, in most bacteria containing cytochrome *c*, the predominant terminal oxidases are enzymes able to oxidize cytochrome *c*. Furthermore, cytochrome c_{553} (ADH) can be extracted from membranes with a low concentration of detergent with almost no effect on the activity of the glucose oxidase system, one of the sugar-oxidizing systems (Ameyama *et al.*, 1987). Thus, unlike the notion obtained from kinetic data, *c*-type cytochrome(s) may not be located between ubiquinone and cytochrome *o*, or even be not involved in the glucose oxidase system with ubiquinone donating electrons directly to the terminal oxidase.

The peculiar properties of the respiratory chain in *G. suboxydans* already referred to can be rationalized by supposing that one part of cytochrome *c* works near the substrate side of the respiratory chain while the other part works on the terminal end. This notion can be further supported by the finding that depletion of cytochrome c_{553} (ADH) from membranes causes the cyanide-insensitive glucose oxidase system to become cyanide-sensitive (Ameyama *et al.*, 1987). Besides this glucose oxidase system, other alcohol- and sugar-oxidizing systems and also the NADH oxidase system are resistant to cyanide, a potent inhibitor of cytochrome *o*, in native membranes of *G. suboxydans* (Fig. 7). Furthermore, the aerobic steady state of *c*-type cytochrome is not increased by addition of cyanide, suggesting that at least a part of cytochrome *c* can react with oxygen without passing through cytochrome *o*. Thus, the respiratory chain of *G. suboxydans* branches at ubiquinone, with cyanide-sensitive and -insensitive terminal oxidases, of which the cyanide-sensitive one is cytochrome *o*. The alternative, cyanide-insensitive, terminal oxidase system may contain some cytochrome *c*, probably cytochrome c_{553} (ADH), as one component. Daniel (1970) has shown that there are two oxygen-reacting sites, with high and low affinity, in the respiratory chain of *G. suboxydans*, while he further suggested that the two terminal oxidases correspond to two cytochromes, o_{558} and o_{565}, with low and high affinity for carbon monoxide. However, since both cytochrome *o* components can be detected in purified cytochrome *o* (Matsushita *et al.*, 1987b), *G. suboxydans* seems to contain a single cytochrome *o* as a cyanide-sensitive

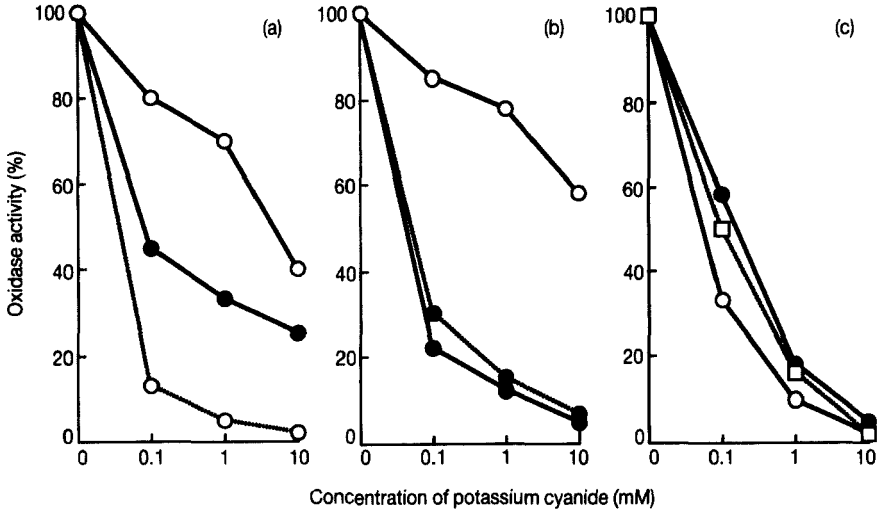

FIG. 7. Effect of cyanide on several oxidase activities in membranes of *Gluconobacter suboxydans*, *Gluconobacter suboxydans* subsp. α, and *Acetobacter aceti*. *Gluconobacter suboxydans* was grown in a sugar-rich medium around pH 6 or around pH 4, *Gluconobacter suboxydans* subsp. α was also grown in a sugar-rich medium (Ameyama *et al.*, 1987), while *Acetobacter aceti* was grown in glycerol-containing medium in shaking or static conditions (Matsushita *et al.*, 1992b). Membranes were prepared from these cells, and ethanol, glucose and Q_2H_2 oxidase activities measured in the presence of different concentrations of potassium cyanide. Furthermore, Triton-washed membranes were prepared from membranes of *Gluconobacter suboxydans* grown around pH 4 by treating them with 0.2% Triton X-100 followed by ultracentrifugation. (a) Shows ethanol oxidase activities of membranes from *Gluconobacter suboxydans* grown around pH 4 (○——○) or pH 6 (●——●), and Q_2H_2 oxidase activity of membranes from *Gluconobacter suboxydans* subsp. α (○···○). (b) Shows glucose oxidase activities of membranes from *Gluconobacter suboxydans* grown around pH 4 (○——○) and of Triton-washed membranes (●——●), and of *Gluconobacter suboxydans* subsp. α (●···●). (c) Shows ethanol (●——●) and Q_2H_2 (□···□) oxidase activities of membranes from *Acetobacter aceti* grown in shaking cultures, and ethanol oxidase activity of membranes from bacteria grown in static cultures (○——○).

terminal oxidase. Thus, the two terminal oxidases suggested by Daniel (1970) seem to correspond to the cyanide-sensitive cytochrome *o* and to cyanide-insensitive bypass oxidase (Fig. 8).

2. *Energetic Aspect of the Respiratory Chains*

The energetics of the respiratory chain in *G. suboxydans* can be examined by measurement of the H^+:O ratio in resting cells, the value for which has

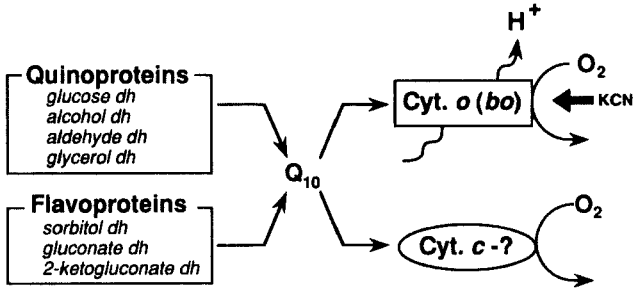

FIG. 8. The respiratory chain of *Gluconobacter suboxydans*. Quinoproteins, including D-glucose, alcohol, aldehyde, glycerol, and D-fructose dehydrogenases, and flavoproteins including D-gluconate, 2-keto-D-gluconate, sorbitol and sorbose dehydrogenases, donate electrons directly to ubiquinone (Q_{10}), after which ubiquinol transfers electrons to either a cyanide (KCN)-sensitive energy-generating cytochrome *o* (Cyt. *o*) or cyanide-insensitive bypass which consists of cytochrome *c* (Cyt. *c*) and an unknown enzyme.

been shown to vary from 1.1 to 2.3 depending on the nature of the respiratory substrates and on the extracellular pH value of the growth medium (Matsushita *et al.*, 1989a). Since a cyanide-sensitive terminal oxidase, namely cytochrome *o*, has been shown to generate an electrochemical proton gradient by oxidation of ubiquinol (Matsushita *et al.*, 1987b), the respiratory chain containing cytochrome *o* as a sole terminal oxidase is expected to yield an H^+:O ratio of two or four depending on the mechanism for proton translocation across the cytoplasmic membrane. Since cytochrome *o* catalyses oxidation of ubiquinol (QH_2) to ubiquinone (Q), there is a scalar proton release from QH_2 outside a membrane and a concomitant proton uptake inside a membrane to reduce oxygen ($\frac{1}{2}O_2$) to water (H_2O) (Matsushita *et al.*, 1984). Thus, the scalar reaction produces a H^+:O ratio of 2 (a H^+:e^- ratio of 1). Recently, cytochrome *o* from *E. coli* has been reported to have an ability to pump protons (Puustinen *et al.*, 1989, 1991; Verkhovskaya *et al.*, 1992). If cytochrome *o* of *G. suboxydans* effects such a vectorial proton translocation, the oxidase is expected to exhibit a H^+:O ratio of 4 (a H^+: e^- ratio of 2). Therefore, the observed H^+:O ratio is lower than the value expected with only cytochrome *o* working as the terminal oxidase. As already described, however, there is an alternative cyanide-insensitive bypass oxidase besides the cyanide-sensitive cytochrome *o* in the respiratory chain of *G. suboxydans*. Furthermore, a membrane potential generated during oxidation of glucose or ethanol in membrane vesicles of *G. suboxydans* has been shown to be dissipated completely with 1 mM potassium cyanide that is able to block cytochrome *o* completely but not the cyanide-insensitive oxidase

(Matsushita *et al.*, 1989a). Thus, it is suggested that the cyanide-insensitive terminal oxidase has no ability to generate an electrochemical proton gradient and that it works at a significant rate in the respiratory chain of *G. suboxydans*. If the cytochrome *o* branch is assumed to give the H^+:O ratio of 4, judging from the observed value, that is H^+:O ratio of 1.1 to 2.2, the cyanide-insensitive bypass may contribute half or more to the respiratory activity of *G. suboxydans*. As described in the next section, although the H^+:O ratio in cells grown at low extracellular pH values is lower than that in cells grown at higher pH values, the membrane potential generated in membrane vesicles of *G. suboxydans* is extremely sensitive to cyanide regardless of the external pH value during growth of cells. This can be also explained by a non-energy generating cyanide-insensitive bypass oxidase only being changed by extracellular pH values.

3. Variations in Cyanide-Insensitive Respiratory Chains

The cytochrome *c* content of membranes of *G. suboxydans* has been shown to change greatly depending on the extracellular pH value during growth as shown in Fig. 6 (Matsushita *et al.*, 1989a). Lowering the external pH value increases the cytochrome *c* content as well as the activity of ADH, while the cytochrome *o* content and other enzyme activities of the respiratory chain remain constant. The increase in cytochrome *c* content is mainly due to the increase in a cytochrome *c* component of about 50 kDa, which may correspond to the second subunit of ADH or to cytochrome c_{553} (CO). Concomitant with such changes, the cyanide sensitivity of the respiratory activity in membranes of *G. suboxydans* is decreased by lowering the extracellular pH value. As already described, since the respiratory chain of *G. suboxydans* branches with a cyanide-sensitive cytochrome *o* and a cyanide-insensitive bypass oxidase, while the cyanide-insensitive oxidase may contain cytochrome c_{553} (ADH) as one component, it is reasonable to conclude that the cyanide-insensitive respiratory activity changes in parallel to the level of cytochrome *c*.

The same kind of phenomena can be seen in subspecies of *Gluconobacter* strains, named *G. suboxydans* subsp. α, which are characterized by their inability to oxidize ethanol despite having the ability to oxidize sugars and sugar alcohols (Table 7). *Gluconobacter suboxydans* subsp. α strains have a characteristic common feature in the respiratory chain, which is totally different from those of other *Gluconobacter* strains (Matsushita *et al.*, 1991). Membranes from these strains have extremely low ADH and ethanol oxidase activities and a very low cytochrome *c* content (Fig. 6), and they exhibit cyanide- or azide-sensitive respiratory acitivity (Fig. 7). Furthermore, the first and second subunits of ADH are also lower and

deficient, respectively, in membranes of *G. suboxydans* subsp. α. Since cyanide insensitivity of the respiratory activity has been known to be diminished by removing cytochromes *c* or ADH from membranes of *G. suboxydans* as already described, it is reasonable to conclude that the respiratory chain of ADH-deficient *G. suboxydans* subsp. α is sensitive to cyanide or azide. Thus, the second subunit cytochrome *c* of the ADH complex is thought to be involved in the pathway of the cyanide-insensitive respiratory chain bypass of *Gluconobacter* species.

B. THE RESPIRATORY CHAIN OF *ACETOBACTER ACETI*

1. The Respiratory Chain of Acetobacter aceti *Grown in Shaking Culture*

The respiratory chain of *Acetobacter* species has not been well characterized except for the terminal oxidase as described in Section III and for a recent study of *A. aceti* (Matsushita *et al.*, 1992b). When grown in shaking cultures as well as static culture, *A. aceti* has a membrane-bound respiratory chain exhibiting relatively high oxidase activities for glucose, ethanol, acetaldehyde and lactate. Ethanol and acetaldehyde oxidase activities involved in acetic acid production are largely increased in cells grown on ethanol (Table 7). Neither glycerol, sorbitol nor gluconate oxidase activities, specific for *Gluconobacter* species, are detectable. Furthermore, the membranes show a high ubiquinol oxidase activity but lack cytochrome *c* oxidase activity. Membranes of *A. aceti* grown in shaking culture have *b*- and *c*-type cytochromes, and also cytochrome a_1 which can be seen in the spectrum as a broad peak at 580–600 nm shifted to a sharp peak at 587 nm in the presence of cyanide (Fig. 9). The respiratory chain looks peculiar, similar to that in *G. suboxydans*, since the organism has cytochrome a_1, capable of oxidizing only ubiquinol, as a sole terminal oxidase in spite of having cytochrome *c*. However, the membrane residues solubilized with a relatively low concentration of detergent retain almost all glucose oxidase activity as well as ubiquinol oxidase activity, irrespective of their having been depleted of almost all cytochromes *c* and ADH, which led to a total loss of ethanol oxidase activity (Fig. 9). Therefore, it is conceivable that cytochromes *c* of *A. aceti* are not electron donors to the terminal oxidase but are constituents of ADH and ALDH (Table 8). Thus, the respiratory chain of *A. aceti* seems to be constituted of primary dehydrogenases, ubiquinone (Q_9), and terminal oxidase (Fig. 10), which is similar to the cyanide-sensitive respiratory chain of *G. suboxydans* described in the previous section.

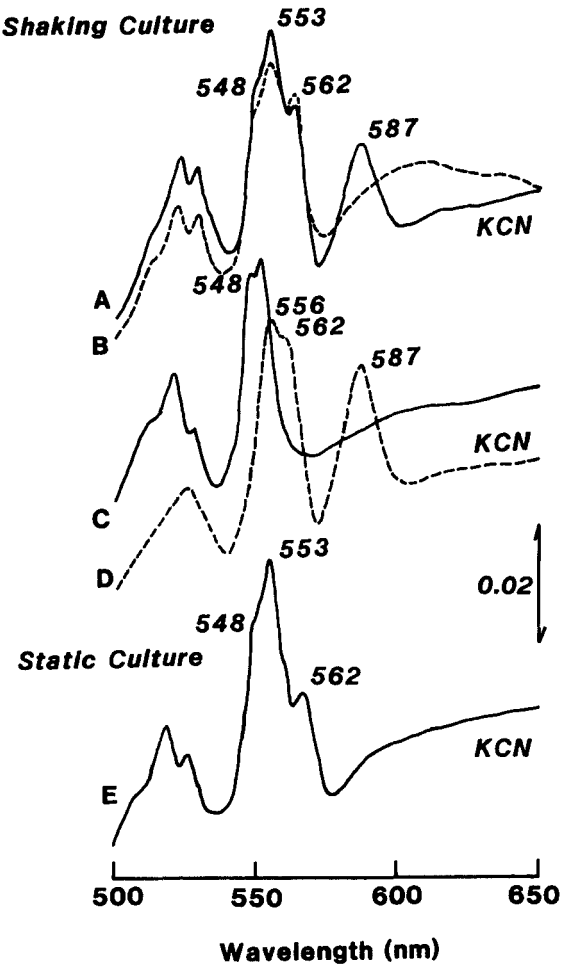

FIG. 9. Low-temperature reduced minus oxidized difference spectra of membranes from *Acetobacter aceti* grown in shaking and static cultures. Membranes prepared from cells grown in shaking cultures were suspended in 50 mM potassium phosphate buffer, pH 6.5 (A and B; 10.5 mg protein ml^{-1}), or alternatively were solubilized with 0.3% Triton X-100 and separated by ultracentrifugation to give a supernatant (C; 5.0 mg protein ml^{-1}) and residual membranes (D; 9.6 mg protein ml^{-1}). Membranes were also prepared from cells grown in static culture (E; 7.4 mg protein ml^{-1}). Dithionite-reduced minus ferricyanide-oxidized difference spectra were measured with (A, D, and E) or without (B and C) 1 mM potassium cyanide (KCN) in a cuvette of 2 mm light path in liquid nitrogen.

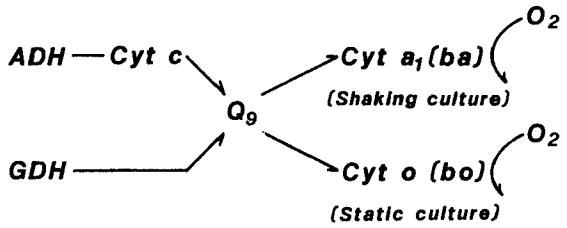

FIG. 10. The respiratory chain of *Acetobacter aceti*. Alcohol dehydrogenase (ADH), containing cytochrome *c* (Cyt *c*), and glucose dehydrogenase (GDH) are shown to donate electrons directly to ubiquinone (Q_9), and then to cytochrome a_1 (Cyt a_1) or cytochrome *o* (Cyt *o*).

However, unlike the respiratory chain of *G. suboxydans*, that in *A. aceti* seems not to have a cyanide-insensitive bypass oxidase because there is no difference in the cyanide-sensitivity between the terminal ubiquinol oxidase and ethanol-oxidizing system (Fig. 7). Furthermore, the value of H^+:O ratio in resting cells is close to 4.0 (K. Matsushita, unpublished observation), which is an ideal value when cytochrome a_1 has an ability to pump protons as previously described. In *A. aceti* grown in shaking cultures, it is suggested that cytochrome a_1 is a sole terminal oxidase functioning in the respiratory chain.

2. *Exchange between Cytochrome* a_1 *and Cytochrome* o *in* Acetobacter aceti *grown in Shaking and Static Cultures*

It is well known that several *Acetobacter* species have the ability to float on the surface of a static culture, when they form a pellicle in order to maintain floating. Such a property is observed characteristically in *Acetobacter xylinum*, in which cellulose biosynthesis involved in pellicle formation has been extensively studied (Ross *et al.*, 1991). Likewise, *A. aceti* is able to grow well in static cultures as well as in shaking cultures, in contrast to strains of *Gluconobacter* species, which are incapable of growing in static culture. Since the degree of aeration varies considerably between cultures, the respiratory chains of cells grown on static culture are expected to be different from those of the cells grown on shaking culture. Regardless of the culture conditions, however, *A. aceti* has almost the same respiratory chain except for the terminal oxidase, which changes from cytochrome a_1 to cytochrome *o* (Fig. 9; Matsushita *et al.*, 1992b). As described in Section III, the terminal oxidase present in cells grown in the shaking cultures is a cytochrome a_1, that is a cytochrome *ba* functioning as ubiquinol oxidase, while the terminal oxidase of cells from static cultures is a cytochrome *bo* ubiquinol oxidase. Thus, the respiratory chain of

A. aceti can be depicted as shown in Fig. 10, in which the terminal oxidase can be changed from cytochrome a_1 to cytochrome *o* by altering culture conditions from shaking to static.

The exchange mechanism of the terminal oxidase is rather complicated. In static culture, after inoculation, a large number of cells are unable to float and to grow rapidly. But some of them are able to float and to form a pellicle, and thus the contents of cytochrome a_1 is gradually decreased and cytochrome *o* appears. Conversely, when a static culture is shaken, no change in cytochrome components is detected after a single batch culture but is detected on repeated shaking, during which the content of cytochrome *o* is decreased and that of cytochrome a_1 increased. Thus, the terminal oxidase may not be changed simply from cytochrome a_1 to cytochrome *o* by a change in culture conditions from shaking to static and vice versa. There have been shown to be two different types of cells, one of which forms a smooth-surfaced colony while the other forms a rough-surfaced colony (Matsushita *et al.*, 1992b). Cells forming rough-surfaced colonies can grow forming a pellicle on the surface of static culture, while cells forming smooth-surfaced colonies cannot grow in the static culture but predominate in shaking cultures. Furthermore, each colony, once isolated, seems not to be interconvertible at least after several repeated cultures. Thus, the smooth-surfaced cells produce cytochrome a_1 in the shaking cultures while the rough-surfaced cells produce cytochrome *o* even in shaking cultures as well as in the static cultures (Fig. 11).

Thus, *A. aceti* can produce two types of ubiquinol oxidase, namely cytochrome a_1 and cytochrome *o*, exchange between which is suggested to be accompanied by formation of cellulosic materials enabling cells with cytochrome *o* to float in static culture, although the mechanism of the cell change remains unresolved to date. Since cytochrome a_1 has a higher affinity for oxygen than cytochrome *o* (Matsushita *et al.*, 1992a), it is suspected that such cellulosic materials or the resulting floating state may affect an oxygen environment which induces exchange of the terminal oxidase.

3. *Instability of the Ethanol-oxidizing Respiratory Chain of* Acetobacter aceti

As already described, spontaneous mutation is observed at high frequencies in acetic acid bacteria, which leads to deficiencies in various physiological properties such as acetic acid resistance, ethanol-oxidizing ability and cellulose formation. These mutations may be related to the genetic instability of *Acetobacter* species (Carr, 1958; Shimwell and Carr, 1964).

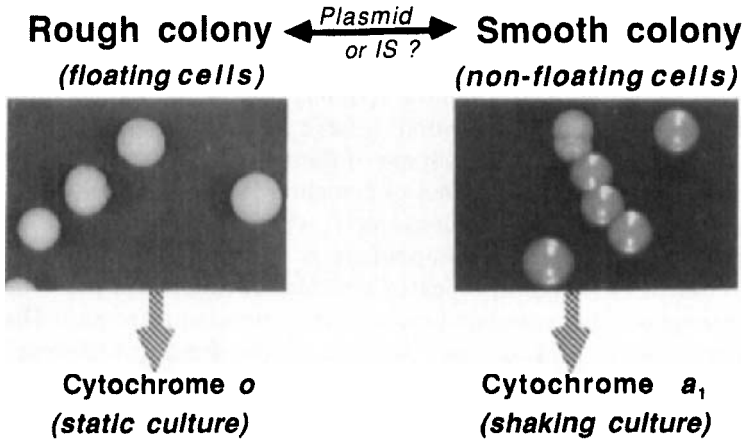

FIG. 11. Two different types of colonies of *Acetobacter aceti* from cells grown in static or shaking cultures. Cells forming rough-surfaced colonies can grow floating on the surface of the culture and thus predominate in static cultures. Cells forming smooth-surfaced colonies are incapable of floating and thus grow only in shaking cultures. The two types of cell produce cytochrome *o* and cytochrome a_1, respectively, as the terminal oxidase.

Acetobacter aceti has been shown to lose easily the ability to produce acetic acid in shaking culture (using an ethanol-containing medium) by a transient stop of aeration when the acetic acid concentration in the culture is more than 6% (Muraoka *et al.*, 1982a), which seems to be accompanied by damage to the cell structure due to oxygen deficiency. Likewise, prolonged shaking of cultures of *A. aceti* leads to a significant proportion of cells in the culture having a decreased ability to oxidize ethanol and a lowered resistance to acetic acid (Ohmori *et al.*, 1982). Although at a relatively low frequency, spontaneous mutants incapable of oxidizing ethanol can be obtained in cultures subjected to prolonged aeration. Such a mutation is probably caused by ethanol or its product, acetic acid, in the culture medium. Taken together with both observations (Muraoka *et al.*, 1982a; Ohmori *et al.*, 1982), such a frequency of mutation of *A. aceti* seems to be caused by a combination of exposure to concentrations of high acetic acid and low oxygen tensions. Spontaneous mutation leading to organisms defective in acetic acid resistance and ethanol-oxidizing ability is reminiscent of a phenomenon caused by curing of extrachromosomal DNA, which has not been substantiated (Ohmori *et al.*, 1982).

The same kind of mutation has been observed in *A. pasteurianus*, in which spontaneous mutants deficient in ethanol oxidation can be obtained during prolonged shaking of cultures containing ethanol (Takemura *et al.*,

1991). The deficient mutants appear at high frequency when viable cells decrease from 10^9 per ml to less than 10^3 per ml, accompanied by an accumulation of high concentrations of acetic acid. All the mutants also show a complete loss of ADH activity and a decreased resistance to acetic acid, concomitant with a deficiency in ethanol oxidation. The genetic origin of the spontaneous mutation can be demonstrated in mutants of *A. pasteurianus* where a 1.5 Kb DNA fragment is inserted in the gene for the cytochrome *c* subunit of ADH (Takemura *et al.*, 1991). Although the insertion element, IS 1380, is not necessarily detected in the ADH gene of all mutants defective in ethanol oxidation, transposition of IS 1380 to other related genes of ADH can be suspected to cause a deficiency in ethanol oxidation. Thus, since IS 1380 is detected in various *Acetobacter* species but not in *Gluconobacter* species, transposition of the insertion sequence has been suggested to be involved in genetic instability of *Acetobacter* species including instability of the ethanol oxidase respiratory system. In general, such a genetic instability in *Acetobacter* species would be induced by an insertion sequence element because the IS element is also known to cause a spontaneous cellulose deficient mutation in *A. xylinum* (Coucheron, 1991).

C. THE RESPIRATORY CHAIN OF *ACETOBACTER METHANOLICUS*

Acetobacter methanolicus is an acidophilic methylotrophic bacterium which has been described as a new species of acetic acid bacteria (Uhlig *et al.*, 1986). The organism is able to grow on methanol as well as on glycerol or glucose as carbon and energy sources. Thus, *A. methanolicus* is a unique acetic acid bacterium which has a methanol oxidase respiratory chain, as seen in methylotrophs, in addition to its ethanol oxidase respiratory chain (Loffhagen and Babel, 1984). Cells grown on methanol exhibit a high methanol oxidase activity and contain large amounts of methanol dehydrogenase and soluble cytochromes *c*, while cells grown on glycerol have higher ethanol, but little methanol, oxidase activities and large amounts of membrane-bound ADH. The respiratory chain of *A. methanolicus* contains only *c*- and *b*-type cytochromes but not *a*- or *d*-type cytochromes (Elliott and Anthony, 1988) and two different terminal oxidases, cytochrome *c* and ubiquinol oxidases. Cytochrome *c* oxidase predominates in cells grown on methanol while ubiquinol oxidase predominates in cells grown on glycerol. Both terminal oxidases have been solubilized from membranes and separated from each other, and shown to be a cytochrome *co* and a cytochrome *o*, respectively (Chan and Anthony, 1991a; Matsushita *et al.*, 1992c).

The methanol oxidase respiratory chain is somewhat different from

other respiratory chains, such as ethanol, glucose or NADH oxidase systems which are all retained in membranes and are stable to cell disruption procedures. Methanol dehydrogenase, and cytochromes c_L and c_H, which together constitute the methanol oxidase system, are all located in the periplasm. Thus, any interaction of these components with the membrane-bound respiratory chain is easily disrupted even in the intact cells by treatment with a high concentration of salt or EDTA. Only when cells are grown on methanol, furthermore, is cytochrome *co* produced together with methanol dehydrogenase and soluble cytochromes *c*. By analogy with other methylotrophs (Anthony, 1988), it thus seems reasonable to suggest that the methanol oxidase respiratory chain of *A. methanolicus* may consist of methanol dehydrogenase, cytochromes c_L and c_H, and a cytochrome *co* terminal oxidase.

In contrast, the ethanol oxidase system of *A. methanolicus* consists only of membrane-bound components. Alcohol dehydrogenase purified from membranes of glycerol-grown cells is closely similar to ADH purified from *G. suboxydans* or *A. aceti* and can be reconstituted together with Q_{10} and cytochrome *o* into proteoliposomes exhibiting ethanol oxidase activity (Matsushita *et al.*, 1992c), as will be described.

Thus, it seems that *A. methanolicus* has two independent respiratory chains for methanol and ethanol. The methanol oxidase respiratory chain appears to operate by linking methanol dehydrogenase to cytochrome *c* oxidase, cytochrome *co*, through soluble cytochrome(s) *c*, while the ethanol oxidase respiratory chain consists of ADH, ubiquinone and cytochrome *o* ubiquinol oxidase. Although there is no substantial evidence as in the case with the ethanol oxidase system, the glucose and NADH oxidase respiratory chains of *A. methanolicus* may also be constituted from a primary dehydrogenase portion and a terminal ubiquinol oxidase portion consisting of Q_{10} and cytochrome *o*.

V. Reconstitution of Alcohol- and Sugar-oxidizing Respiratory Chains

A. THE CYANIDE-SENSITIVE RESPIRATORY CHAINS

As described in the previous section, respiratory chains in acetic acid bacteria are relatively simple and the cyanide-sensitive respiratory chain is suggested to consist of a primary dehydrogenase, ubiquinone and terminal ubiquinol oxidase (Figs 8 and 10). The primary dehydrogenases of the alcohol- and sugar-oxidizing respiratory chains are bound to membranes and function by linking to a membrane-bound respiratory chain where the terminal ubiquinol oxidase, either cytochrome a_1 or cytochrome *o*, and

ubiquinone are all embedded in membranous phospholipids. If one intends to construct the respiratory chain *in vitro*, therefore, all of the components have to be reconstituted with phospholipids into liposomes. This can be performed by the detergent-dialysis method using octylglucoside as the detergent (Viitanen *et al.*, 1986), which is depicted in Fig. 12. Thus, of the respiratory chains of acetic acid bacteria, the glucose and alcohol oxidase respiratory chains have been reconstituted successfully into proteoliposomes (Matsushita *et al.*, 1989b, 1992c,d).

1. Reconstitution of the Glucose Oxidase Respiratory Chain of Gluconobacter suboxydans

Membrane-bound quinoprotein GDHs from several oxidative bacteria, including *E. coli, Pseudomonas fluorescens* and *Acinetobacter calcoaceticus*, have been shown to be able to react with short side-chain ubiquinone homologues, Q_1 and Q_2, and even with longer-chain homologues in appropriate detergent solution (Matsushita *et al.*, 1982a; 1987a; 1989c). Likewise, GDH purified from the membranes of *G. suboxydans* is able to reduce the ubiquinone homologue with a long-side chain, Q_6, at a relatively low but a significant rate (Matsushita *et al.*, 1989b). Since such a hydrophobic electron acceptor is very difficult to use in aqueous solution, the enzyme reaction with ubiquinone should be effected ideally in a phospholipid environment. This can be performed by reconstitution of proteoliposomes, as already described, where a terminal oxidase capable of oxidizing the resultant ubiquinol must be reconstituted at the same time in order to know whether or not a proper electron transfer occurs from the enzyme to ubiquinone. Thus, GDH purified from membranes of *G. suboxydans* can be reconstituted into proteoliposomes together with Q_{10} and the cytochrome *o* terminal oxidase purified from *G. suboxydans* (Matsushita *et al.*, 1989b). The proteoliposomes thus reconstituted are able to catalyse oxidation of glucose at a reasonable rate, the electron-transfer reactions generating an electrochemical proton gradient at the site of the terminal oxidase. The apparent turnover of glucose oxidase with respect to GDH in proteoliposomes is comparable to that of the native membrane (Table 9). Since cytochrome *o* has been shown to catalyse oxidation of ubiquinol, such a reconstitution of glucose oxidase activity indicates that GDH is able to reduce Q_{10} to produce $Q_{10}H_2$, which is in turn oxidized rapidly by cytochrome *o* in proteoliposomes. Furthermore, the glucose oxidase system in proteoliposomes is cyanide-sensitive unlike native membranes, which is similar to properties found in membranes depleted of the cytochrome *c* of ADH. Thus, reconstitution experiments clearly demonstrate that the GDH of *G. suboxydans* donates electrons directly to

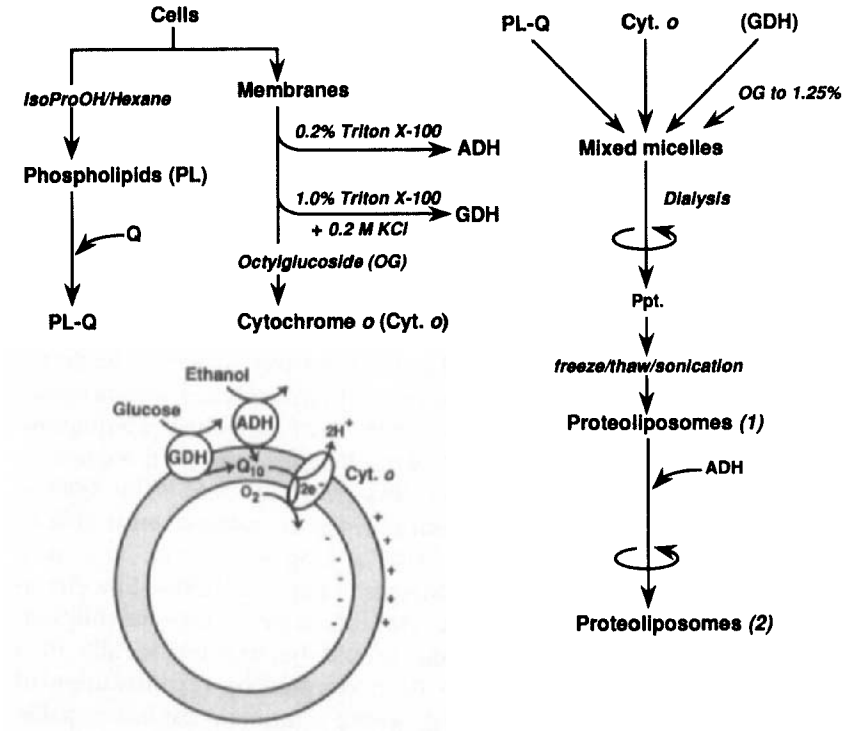

FIG. 12. Reconstitution of proteoliposomes using the octylglucoside-dialysis method. Phospholipids are extracted with an isopropyl alcohol (IsoProOH)–hexane mixture from cells and purified (Viitanen *et al.*, 1986). The appropriate ubiquinone (Q) is mixed in ether with the phospholipids, and the mixture is evaporated, suspended with water, and then sonicated. Alcohol dehydrogenase (ADH) and D-glucose dehydrogenase (GDH) are then solubilized from cell membranes with Triton X-100 sequentially and purified. From the residual membranes, cytochrome *o* (Cyt. *o*) is solubilized with octylglucoside (OG) and purified. The purified terminal oxidase and primary dehydrogenase (in the case of GDH) are mixed with phospholipids containing ubiquinone (PL-Q), and incubated on ice in the presence of OG. After the mixture has been dialysed against an appropriate buffer, proteoliposomes are collected by ultracentrifugation, suspended in a minimal volume of the same buffer, and frozen rapidly in liquid nitrogen. Just prior to use, the proteoliposomes are thawed at room temperature and then sonicated for 10–20 s with a bath-type sonicator. Alcohol dehydrogenase (ADH) is added to the solution of proteoliposome (1) and incubated at room temperature, and the preparation centrifuged to obtain proteoliposomes (2) containing ADH.

ubiquinone in the respiratory chain, and that the cyanide-sensitive glucose oxidase respiratory chain of *G. suboxydans* consists of GDH, Q_{10} and cytochrome *o* oxidase (Fig. 13). This notion is also consistent with the idea,

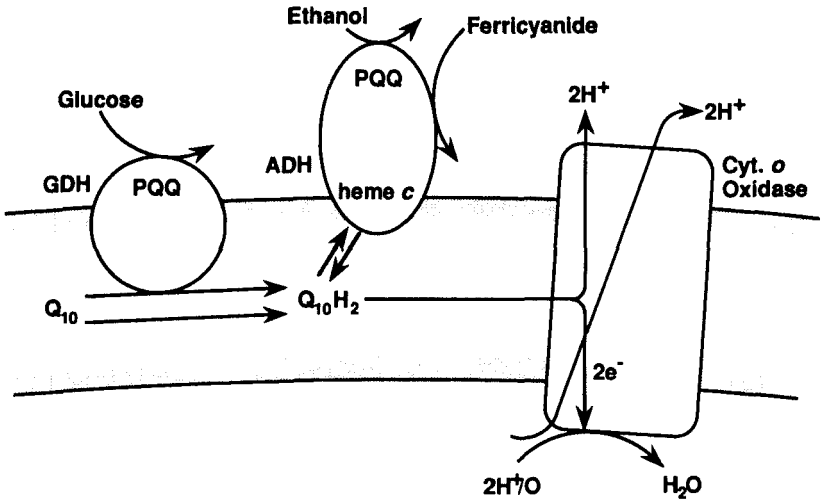

FIG. 13. Electron-transfer reactions in proteoliposomes reconstituted with glucose dehydrogenase (GDH), alcohol dehydrogenase (ADH) and cytochrome *o* from *Gluconobacter suboxydans*. Ferricyanide is able to accept electrons from glucose dehydrogenase via ubiquinone (Q_{10}) and the cytochrome *c* (heme *c*) component of alcohol dehydrogenase. Ubiquinol ($Q_{10}H_2$) can be oxidized directly by cytochrome *o* oxidase to generate an electrochemical proton gradient.

described in the previous section, that the cyanide-sensitive branch of the glucose oxidase respiratory chain of *G. suboxydans* does not contain any cytochrome *c* in spite of the respiratory chain containing a high content of cytochrome *c*.

2. *Reconstitution of Ethanol Oxidase Respiratory Chains of Acetic Acid Bacteria*

As with GDH, ADHs purified from *G. suboxydans* and *A. aceti* have been shown to react directly with ubiquinone (Matsushita *et al.*, 1992d). They are able to reduce the respective native ubiquinone, Q_9 or Q_{10}, solubilized with octylglucoside at a relatively low but significant rate as well as Q_1 in an aqueous solution. Furthermore, the quinone reductase activity of these ADHs is inhibited by ubiquinone analogues such as 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and 3-undecyl-2-hydroxy-1,4-naphthoquinone. These findings suggest that the ADH of acetic acid bacteria is able to react with ubiquinone *in vivo*. The reactivity of ADH with ubiquinone in the native-membrane environment has been further confirmed by reconstituting ADHs into proteoliposomes which had been reconstituted from ubiquinone, Q_9 or Q_{10}, and a terminal oxidase,

TABLE 9. Properties of glucose- and ethanol-oxidizing respiratory chains of acetic acid bacteria reconstituted into artificial proteoliposomes[a] or into membrane vesicles

Reconstituted sugar-oxidizing systems	Dehydrogenase activity[b]	Q_2H_2 oxidase activity[b]	Oxidase activity[b]	Turnover number[c]	Cyanide sensitivity
Glucose oxidase system of *Gluconobacter suboxydans* (Matsushita *et al.*, 1989b)					
Native membranes	2.86	2.69	1.08	676	Insensitive
Proteoliposomes	40.0	25.5	9.00	435	Sensitive
Ethanol oxidase system of *Gluconobacter suboxydans* (Matsushita *et al.*, 1992d)					
Native membranes	3.21	3.42	1.22	210	Insensitive
Proteoliposomes	23.0	40.0	3.81	92	Sensitive
Ethanol oxidase system of *Acetobacter aceti* (Matsushita *et al.*, 1992d)					
Native membranes	2.40	3.30	0.76	289	Sensitive
Proteoliposomes	24.7	57.3	4.06	150	Sensitive
Ethanol oxidase system of *Gluconobacter suboxydans* subsp. α (Matsushita *et al.*, 1991)					
Native membranes	0.01	4.47	0.00	0	Sensitive[d]
Membrane vesicles reconstituted with ADH from *Gluconobacter suboxydans*	2.32	3.99	1.61	383	Insensitive
Membrane vesicles reconstituted with ADH from *Acetobacter aceti*	2.05	3.99	1.59	707	Sensitive

[a] Proteoliposomes were prepared by reconstituting glucose dehydrogenase or alcohol dehydrogenase (ADH) and a terminal oxidase (cytochrome a_1 in *Acetobacter aceti* or cytochrome *o* in *Gluconobacter suboxydans*) purified from the respective acetic acid bacteria into phospholipids containing ubiquinone (Q_9 in *Acetobacter aceti* or Q_{10} in *Gluconobacter suboxydans*) by the octylglucoside-dialysis method. Purified ADHs were also bound to membrane vesicles derived from *Gluconobacter suboxydans* subsp. α by the freeze–thaw and sonication method.

[b] Number of μmoles of substrate oxidized min^{-1} (mg of protein)$^{-1}$.

[c] Number of $e^- s^{-1}$ (moles of dehydrogenase)$^{-1}$.

[d] Other oxidase activities such as sorbitol, glucose or glycerol oxidases.

cytochrome a_1 or cytochrome *o* (Matsushita *et al.*, 1992d). By contrast with the situation with GDH, ADH can be incorporated successfully into preformed proteoliposomes, which may be explained by there being a difference in hydrophobicity between the two dehydrogenases. Proteoliposomes thus prepared exhibited an ethanol oxidase activity comparable to the activity in intact membranes (Table 9). Since cytochrome a_1 or cytochrome *o* has been shown to catalyse oxidation of ubiquinol, reconstitution of ethanol oxidase activity indicates that ADH is able to reduce native ubiquinone dissolved in a phospholipid bilayer to produce ubiquinol, Q_9H_2 or $Q_{10}H_2$, which is in turn oxidized rapidly by the respective ubiquinol oxidase in the proteoliposomes. Since the ethanol oxidase system in proteoliposomes has almost the same ability as that in the native membrane vesicles to generate an electrochemical proton gradient as well as to transfer electrons from ethanol to oxygen, it is reasonable to conclude that the ethanol oxidase respiratory chain of *A. aceti* or *G. suboxydans* consists of ADH, ubiquinone and a terminal ubiquinol oxidase. Likewise, ADH purified from *A. methanolicus* has been shown to be reconstituted together wtih Q_{10} and cytochrome *o* purified from the same organism into proteoliposomes capable of reproducing an ethanol oxidase respiratory chain of *A. methanolicus* (Matsushita *et al.*, 1992c).

As described in the previous section, the ethanol oxidase respiratory chain in native membranes of *G. suboxydans* is insensitive to cyanide. The second subunit of ADH may play an especially important role in the cyanide-insensitive bypass activity of the respiratory chain. Since no cyanide-insensitive respiration is observed in proteoliposomes reconstituted with the components from *G. suboxydans* or from *A. aceti*, another respiratory component may be required in addition to ADH to transfer electrons to oxygen in the cyanide-insensitive bypass respiratory chain of *G. suboxydans*. Therefore, ethanol oxidase activity reconstituted from ADH, Q_{10} and cytochrome *o* possibly reflects only the cyanide-sensitive branch of the ethanol oxidase respiratory chain of *G. suboxydans*.

B. THE CYANIDE-INSENSITIVE RESPIRATORY CHAINS

1. Electron Transfer from GDH to ADH through Ubiquinone in the Respiratory Chain

Quinoproteins GDH and ADH of *G. suboxydans* function as primary dehydrogenases for the glucose and ethanol oxidase respiratory chains, where both enzymes donate electrons to ubiquinone embedded in

membranes. As already described, when membranes from *G. suboxydans* are treated with a relatively low concentration of detergent, ADH can be solubilized while GDH is retained in membrane residues. Although both GDH and ADH can reduce ferricyanide in native membranes, GDH loses the ability to react with the artificial dye after solubilization while ADH does not. Therefore, there is expected to be some mediator(s) for electron transfer between GDH and ferricyanide in the membranes. Because the solubilized supernatant containing ADH, and also ADH by itself, can restore ferricyanide reductase activity to GDH in residual membranes (Shinagawa *et al.*, 1990), ADH is possibly such a mediator. This is also supported by the findings that ADH is able to confer ferricyanide reductase activity of GDH on membranes of *G. suboxydans* subsp. α strain in which both ferricyanide reductase activities of GDH and ADH are deficient (Matsushita *et al.*, 1991). However, ADH alone is not sufficient as a mediator because there is no ferricyanide reductase activity of GDH when GDH and ADH are simply mixed or reconstituted together into proteoliposomes. Ferricyanide reductase activity can be detected in proteoliposomes reconstituted from GDH, ADH, and further ubiquinone Q_{10}. Since reconstitution of ferricyanide reductase activity of GDH cannot be accomplished from only GDH and Q_{10}, all three components are indispensable for electron transfer. Thus, electron transfer from GDH to ferricyanide seems to be mediated by ubiquinone and ADH in membranes of *G. suboxydans*. Since GDH and ADH are able to react directly with ubiquinone in a phospholipid bilayer, it is possible that GDH donates electrons to ubiquinone first and that ubiquinol then reacts directly with ADH which subsequently reduces ferricyanide by way of its heme component (Fig. 13). Although the electron-transfer reaction to ferricyanide is not physiological, these observations provide an important clue for understanding electron-transfer reactions in which GDH and ADH may transfer electrons mutually through ubiquinone in the respiratory chain.

2. *Reconstitution of the Cyanide-insensitive Respiratory Chain of* Gluconobacter suboxydans

As already described, the cyanide-insensitive respiratory chain bypass of *G. suboxydans* is thought to contain ADH or the second subunit cytochrome c_{553} (CO), but it is lacking in *G. suboxydans* subsp. α strains in which ADH, especially the second subunit, is deficient. Thus, cyanide-insensitive respiratory bypass activity has been reconstituted in membranes of *G. suboxydans* subsp. α by an *in situ* reconstitution in which purified ADH is associated with membranes using a freeze–thaw and sonication method (Matsushita *et al.*, 1991) and also by an *in vivo* reconstitution using

a recombinant DNA technique (Takeda and Shimizu, 1991, 1992; Takeda *et al.*, 1992).

Ethanol oxidase activity can be reconstituted in inactive membranes of *G. suboxydans* subsp. α by binding purified ADH and also its second subunit (cytochrome *c*) to membranes. Furthermore, by the same method, cyanide- or azide-insensitive respiratory activity can be restored in the respiratory chain of *G. suboxydans* subsp. α, which is originally cyanide-sensitive, by binding ADH purified from *G. suboxydans* (Table 9). However, ADH purified from *A. aceti* is unable to restore the activity despite its being able to reconstitute an intense ethanol oxidase activity to membranes of *G. suboxydans* subsp. α (Table 9). This is interesting because the respiratory chain of *A. aceti* is sensitive to cyanide, while the second subunits (cytochrome *c*) of ADHs from *G. suboxydans* and *A. aceti* are immunologically distinct from each other (K. Matsushita, unpublished observation) despite a high sequence homology of 60% (Takeda and Shimizu, 1991). Therefore, the functions of the cytochromes *c* can be expected to be different. Thus, the second subunit of ADH from *G. suboxydans*, cytochrome c_{553} (CO), can be shown to be indispensable in the cyanide-insensitive respiratory chain bypass of *G. suboxydans*, where electrons from each primary dehydrogenase pass to cytochrome *c* through ubiquinone and then to oxygen through an unknown enzyme (Figs 8 and 13). This notion can be supported by the following findings as already described: (i) ADH is able to accept electrons from ubiquinol as shown by the interaction between GDH and ADH, and (ii) ethanol oxidase activity is cyanide-sensitive in proteoliposomes where it consists of ADH, ubiquinone and cytochrome *o*.

The gene encoding cytochrome c_{553} (CO) was cloned and the plasmid harbouring the gene transformed into *G. suboxydans* subsp. α strain (Takeda and Shimizu, 1992; Takeda *et al.*, 1992). The resulting transformants produce cytochrome c_{553} (CO) in membranes, which exhibit restored ADH and ethanol oxidase activities. Furthermore, cyanide- or azide-insensitive respiratory activity can be restored in the respiratory chain of transformants. This genetic approach strongly supports the evidence obtained by *in situ* reconstitution experiments already described. Interestingly, the genetic procedure confers additional evidence that other sugar-oxidizing activities, such as glucose, sorbitol and glycerol oxidases, are significantly strengthened in *G. suboxydans* subsp. α by transforming with the cloned gene, which could not be detected using *in situ* reconstitution. This is probably due to the genetic procedure that seems to enable more natural integration of cytochrome *c* into membranes. The increase in the activity of the alcohol- and sugar-oxidizing systems is clearly associated with an increase in cyanide-insensitive bypass oxidase activity,

which may imply that such a cyanide-insensitive bypass respiratory chain is not an artifact of *in vitro* experiments but could play a role in the respiratory chain of *G. suboxydans*.

VI. Functional Aspects of Alcohol- and Sugar-Oxidizing Respiratory Chains

A. PERIPLASMIC OXIDASE SYSTEMS

The periplasm and the periplasmic surface of the cytoplasmic membrane have only recently been recognized as an important location for metabolism, especially for electron transport, in Gram-negative bacteria. More than 20 types of electron-transport proteins have been assigned to this region (Ferguson, 1988). These periplasmic electron-transport proteins are coupled to the rest of the respiratory chain which is embedded in the membrane, constituting periplasmic oxidase systems. While, $NAD(P)^+$-dependent dehydrogenases located in the cytoplasm are coupled through NADH and NADH dehydrogenase to the membrane-bound respiratory chain, some membrane-bound flavoprotein dehydrogenases such as succinate and lactate dehydrogenases also interact with them. These flavoprotein dehydrogenases, including NADH dehydrogenase, are located at the inner surface of the cytoplasmic membrane, and thus constitute cytoplasmic oxidase systems, which contrast with periplasmic oxidase systems.

Of these periplasmic oxidase systems, the methanol- and methylamine-oxidizing systems of methylotrophs (Anthony, 1986, 1993a; Davidson, 1993) and also the alcohol-oxidizing systems of pseudomonads (Matsushita and Adachi, 1993) contain soluble quinoprotein primary dehydrogenases which are considered to be freely soluble in the periplasmic space or loosely bound to the periplasmic side of the cytoplasmic membrane. These primary dehydrogenases are coupled to a membrane-bound terminal oxidase through cytochrome *c* or a copper-containing protein, a typical periplasmic electron-transport protein. Thus, the terminal oxidase must be a cytochrome *c* oxidase which is either cytochrome *co* or cytochrome aa_3 capable of oxidizing cytochrome *c* (Anthony, 1988, 1993b).

In contrast, primary dehydrogenases involved in alcohol- and sugar-oxidizing systems of acetic acid bacteria are all bound tightly to the cytoplasmic membrane and react with substrates in the periplasm. Quinoprotein GDH and ADH donate electrons to ubiquinone embedded in membrane phospholipids, and the resulting ubiquinol is subsequently oxidized by a terminal oxidase in the membrane. Flavoprotein dehydrogenases, such as GADH or 2KGDH, also seem to donate electrons directly

to ubiquinone, although this has not been definitively established. Thus, the terminal oxidase involved in these systems must be a ubiquinol oxidase, activity of which has been found in cytochrome *o*, cytochrome *d* and cytochrome a_1.

Thus, respiratory chains in alcohol- and sugar-oxidizing systems of acetic acid bacteria are simple. Only the terminal oxidase may be involved in generation of an electrochemical proton gradient, which could be comparable to other periplasmic oxidase systems (Fig. 14). Such a truncated respiratory chain may be favourable for rapid oxidation of a large amount of substrate, a view which is also supported by the periplasmic location of primary dehydrogenases since there is no requirement for an energy-consuming transport of substrates into cells.

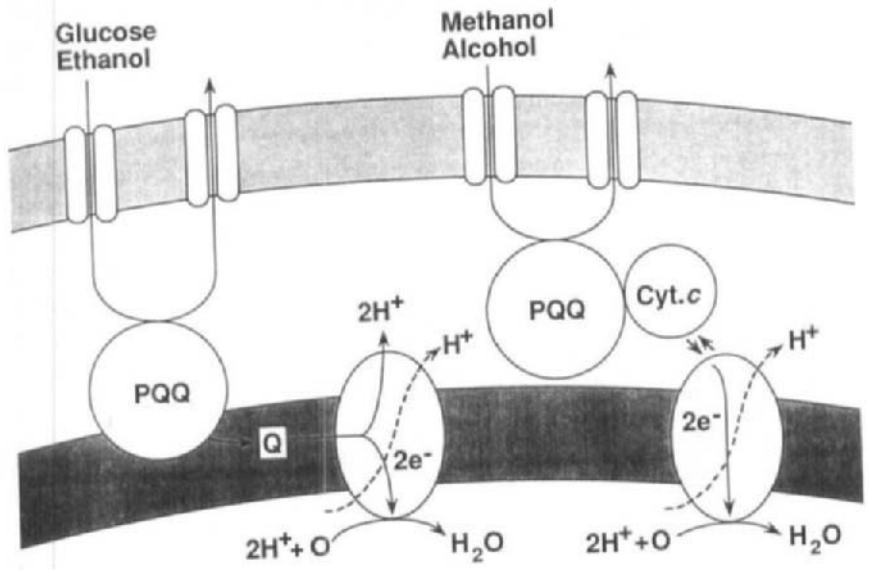

FIG. 14. Reactions carried out by periplasmic oxidase systems including alcohol- and sugar-oxidizing systems of acetic acid bacteria, methanol-oxidizing system of methylotrophs or alcohol-oxidizing systems of pseudomonads. Glucose or ethanol can be oxidized by the respective quinoprotein dehydrogenase (PQQ) which is tightly bound to the surface of the cytoplasmic membranes of acetic acid bacteria. The dehydrogenase then donates electrons to ubiquinone (Q) which in turn transfers them to a terminal ubiquinol oxidase. Methanol or alcohols (several kinds of alcohol such as ethanol, butanol, isopropanol) can also be oxidized by the respective quinoprotein dehydrogenase (PQQ) which is freely soluble in the periplasmic space of methylotrophs and pseudomonads. This dehydrogenase donates electrons to a terminal cytochrome *c* oxidase through a soluble cytochrome *c* (Cyt. *c*). The terminal oxidases can generate an electrochemical proton gradient either by charge separation or a proton pump or both.

B. RELATION BETWEEN OXIDATION REACTIONS AND ENERGETICS

Acetic acid bacteria are able to accumulate large amounts of oxidative products outside cells. The oxidation reactions are performed by alcohol- and sugar-oxidizing systems coupled to the respiratory chain, in which electron transfer generates an electrochemical proton gradient across the cytoplasmic membranes that is used for ATP generation or bioenergetic events for cell growth. As a general principle, however, the proton gradient thus generated may suppress electron transfer by feedback control, which may turn out to disturb oxidation reactions. Thus, a rapid oxidation by the respiratory chain disturbs electron transfer by itself, which may be unfavourable for organisms that need to produce large amounts of oxidation products by the respiratory chain. There seem to be two ways to overcome such an apparently contradictory situation. One is that at least a part of the respiratory chain might have a route involving no energy generation; the other is that electron transfer and energy generation in the respiratory chain are uncoupled in some way.

The former scenario can be seen in *Gluconobacter* species which have a non-energy generating cyanide-insensitive bypass system in their respiratory chain, so that a rapid alcohol or sugar oxidation can be carried out with generation of a little energy which may not interfere significantly with electron transfer. In fact, in *G. suboxydans*, the bypass oxidase system seems to be increased under conditions of low extracellular pH values which can be generated as a result of a large accumulation of oxidation products. Furthermore, in *G. suboxydans* subsp. α strain, introduction of a bypass oxidase system into cells by genetic manipulation increases production of oxidation products.

The latter instance, involving uncoupling of electron transfer and energy generation, could be expected in *Acetobacter* species which do not have a non-energy generating bypass unlike *Gluconobacter* species. The organisms grow in an environment containing a relatively high concentration of acetic acid which is known, in general, to be a powerful uncoupler and thus a well-known bactericidal agent. Therefore, although there is no evidence, the alcohol- and sugar-oxidizing systems of *Acetobacter* species may be affected by this acetic acid which may uncouple energy generation and electron transfer in the respiratory chain and thus increase the rate of alcohol and sugar oxidation.

Acetic acid bacteria inhabit the surface of flowers (the pistils), fruits and their fermented products such as vinegar, sake, wine or beer (Asai, 1968), and thus seem to be able to adapt to such a specific environment where high concentrations of sugars or alcohols exist under highly aerobic conditions. In such specific environments, there seem to be relatively few

competitors, since only yeast or lactic acid bacteria may share such a habitat, but they favour an anaerobic environment, so that acetic acid bacteria may not compete with these organisms. Therefore, acetic acid bacteria may not need to grow rapidly. Thus, the respiratory chains of these bacteria may have evolved to be highly aerobic and highly active for alcohols and sugars, thus allowing for rapid oxidation of a high concentration of substrates but not permitting a high rate of energy generation.

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REFERENCES

Adachi, O., Miyagawa, E., Shinagawa, E., Matsushita, K. and Ameyama, M. (1978a). *Agricultural and Biological Chemistry* **42**, 2331.

Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K. and Ameyama, M. (1978b). *Agricultural and Biological Chemistry* **42**, 2045.

Adachi, O., Shinagawa, E., Matsushita, K. and Ameyama, M. (1979). *Agricultural and Biological Chemistry* **43**, 75.

Adachi, O., Matsushita, K., Shinagawa, E. and Ameyama, M. (1980a). *Agricultural and Biological Chemistry* **44**, 155.

Adachi, O., Matsushita, K., Shinagawa, E. and Ameyama, M. (1980b). *Agricultural and Biological Chemistry* **44**, 301.

Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K. and Ameyama, M. (1980c). *Agricultural and Biological Chemistry* **44**, 503.

Ameyama, M., Matsushita, K., Ohno, Y., Shinagawa, E. and Adachi, O. (1981a). *FEBS Letters* **130**, 179.

Ameyama, M., Matsushita, K., Shinagawa, E. and Adachi, O. (1981b). *Agricultural and Biological Chemistry* **45**, 863.

Ameyama, M., Osada, K., Shinagawa, E., Matsushita, K. and Adachi, O. (1981c). *Agricultural and Biological Chemistry* **45**, 1889.

Ameyama, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1981d). *Journal of Bacteriology* **145**, 814.

Ameyama, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1981e). *Agricultural and Biological Chemistry* **45**, 851.

Ameyama, M. and Adachi, O. (1982). *Methods in Enzymology* **89**, 203.

Ameyama, M., Nonobe, M., Hayashi, M., Shinagawa, E., Matsushita, K. and Adachi, O. (1985). *Agricultural and Biological Chemistry* **49**, 1227.

Ameyama, M., Matsushita, K., Shinagawa, E. and Adachi, O. (1987). *Agricultural and Biological Chemistry* **51**, 2943.
Anemüller, S. and Schäfer, G. (1990). *European Journal of Biochemistry* **191**, 297.
Anraku, Y. (1988). *Annual Review of Biochemistry* **57**, 101.
Anraku, Y. and Gennis, R. B. (1987). *Trends in Biochemical Sciences* **12**, 262.
Anthony, C. (1986). *In* "Advances in Microbial Physiology" (A. H. Rose and D. W. Tempest, eds), pp. 113–210. Academic Press, London.
Anthony, C. (1988). *In* "Bacterial Energy Transduction" (C. Anthony, ed.), pp. 293–316. Academic Press, London.
Anthony, C. (1992). *International Journal of Biochemistry* **24**, 29.
Anthony, C. (1993a) *In* "Principles and Applications of Quinoproteins" (V. L. Davidson, ed.), pp. 17–45. Marcel Dekker, New York.
Anthony, C. (1993b) *In* "Principles and Applications of Quinoproteins" (V. L. Davidson, ed.), pp. 223–244. Marcel Dekker, New York.
Asai, T. (1968). *In* "Acetic Acid Bacteria", pp. 124–203. Tokyo University Press, Tokyo.
Bächi, B. and Ettlinger, L. (1974). *International Journal of Systematic Bacteriology* **24**, 215.
Buchert, J. (1991). *Journal of Biotechnology* **18**, 103.
Carr, J. G. (1958). **Nature 26**, 265.
Castor, L. N. and Chance, B. (1955). *Journal of Biological Chemistry* **217**, 453.
Chan, H. T. C. and Anthony, C. (1991a). *Journal of General Microbiology* **137**, 693.
Chan, H. T. C. and Anthony, C. (1991b). *Biochemical Journal* **280**, 139.
Chance, B. (1953a). *Journal of Biological Chemistry* **202**, 383.
Chance, B. (1953b). *Journal of Biological Chemistry* **202**, 397.
Chepuri, V., Lemieux, J., Au, D. C. -T. and Gennis, R. B. (1990). *Journal of Biological Chemistry* **265**, 11 185.
Cho, N. C., Kim, K. and Jhon, D. -Y. (1990). *Korean Biochemical Journal* **23**, 172.
Claus, G. W., Batzing, B. L., Baker, C. A. and Goebel, E. M. (1975). *Journal of Bacteriology* **123**, 1169.
Cleton-Jansen, A. -M., Goosen, N., Odle, G. and van de Putte, P. (1988). *Nucleic Acids Research* **16**, 6228.
Cleton-Jansen, A. -M., Goosen, N., Fayet, O. and van de Putte, P. (1990). *Journal of Bacteriology* **172**, 6308.
Cleton-Jansen, A. -M., Dekker, S., van de Putte, P. and Goosen, N. (1991). *Molecular and General Genetics* **229**, 206.
Coucheron, D. H. (1991). *Journal of Bacteriology* **173**, 5723.
Daniel, R. M. (1970). *Biochimica et Biophysica Acta* **216**, 328.
Davidson, V. L. (1993) *In* "Principles and Applications of Quinoproteins" (V. L. Davidson, ed.), pp. 73–95. Marcel Dekker, New York.
De Ley, J. and Dochy, R. (1960). *Biochimica et Biophysica Acta* **40**, 277.
Duine, J. A., Frank, J. and Jongejan, J. A. (1983). *Analytical Biochemistry* **133**, 239.
Elliott, E. J. and Anthony, C. (1988). *Journal of General Microbiology* **134**, 369.
Fee, J. A., Choc, M. G., Findring, K. L., Lorence, R. and Yoshida, T. (1980). *Proceedings of the National Academy of Sciences of the United States of America* **77**, 147.
Ferguson, S. J. (1988). *In* "Bacterial Energy Transduction" (C. Anthony, ed.), pp. 151–182. Academic Press, London.
Fukaya, M., Tayama, K., Okumura, H., Kawamura, Y. and Beppu, T. (1989). *Applied Microbiology and Biotechnology* **32**, 176.
Fukaya, M., Tayama, K., Tamaki, T., Tagami, H., Okumura, H., Kawamura, Y. and Beppu, T. (1989). *Applied and Environmental Microbiology* **55**, 171.
Garcia-Horsman, J. A., Barguera, B. and Escamilla, J. E. (1991). *European Journal of Biochemistry* **199**, 761.
Hommel, R. and Kleber, H. -P. (1990). *Journal of General Microbiology* **136**, 1705.
Hommel, R., Borneleit, P. and Kleber, H. -P. (1985). *Biomedica Biochemica Acta* **9**, 1315.
Hon-nami, K. and Oshima, T. (1980). *Biochemical and Biophysical Research Communications* **92**, 1023.

Hoshino, T., Sugisawa, T. and Fujisawa, A. (1991). *Agricultural and Biological Chemistry* **55**, 665.
Imanaga, Y., Hirano-Sawatake, Y., Arita-Hashimoto, Y., Itou-Shibouta and Y., K. -S., R. (1979). *Proceedings of Japan Academy* **55**, 264.
Inoue, T., Sunagawa, M., Mori, A., Imai, C., Fukuda, M., Takagi, M. and Yano, K. (1989). *Journal of Fermentation and Bioengineering* **70**, 58.
Inoue, T., Sunagawa, M., Mori, A., Imai, C., Fukuda, M., Takagi, M. and Yano, K. (1992). *Journal of Fermentation and Engineering* **73**, 419.
Iwasaki, Y. (1960). *Plant Cell Physiology* **1**, 207.
Jurtshuk, P., Mueller, Jr, T. J. and Wong, T. Y. (1981). *Biochimica et Biophysica Acta* **637**, 374.
Keilin, D. (1966). "The History of Cell Respiration and Cytochrome". Cambridge University Press, Cambridge.
King, T. E. and Cheldelin, V. H. (1957). *Journal of Biological Chemistry* **224**, 579.
Kita, K., Konishi, K. and Anraku, Y. (1984a). *Journal of Biological Chemistry* **259**, 3368.
Kita, K., Konishi, K. and Anraku, Y. (1984b). *Journal of Biological Chemistry* **259**, 3375.
Kondo, K. and Ameyama, M. (1958). *Bulletin of the Agricultural Chemical Society of Japan* **22**, 369.
Konishi, K., Ouchi, M., Kita, K. and Horikoshi, I. (1986). *Journal of Biochemistry* **99**, 1227.
Lauraeus, M., Haltia, T., Saracte, M. and Wikström, M. (1991). *European Journal of Biochemistry* **197**, 699.
Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J. and Gennis, R. B. (1992). *Journal of Biological Chemistry* **267**, 2105.
Loffhagen, N. and Babel, W. (1984). *Zeitzschrift für Allgemeine Microbiologie* **24**, 143.
Lorence, R. M., Koland, J. G. and Gennis, R. B. (1986). *Biochemistry* **25**, 2314.
Ludwig, B. (1992). *Biochimica et Biophysica Acta* **1101**, 195.
Ludwing, B. and Schatz, G. (1980). *Proceedings of the National Academy of Sciences of the United States of America* **77**, 196.
Matsushita, K. and Adachi, O. (1993). *In* "Principles and Applications of Quinoproteins" (V. L. Davidson, eds), pp. 47–63. Marcel Dekker, New York.
Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1981a). *Agricultural and Biological Chemistry* **45**, 1515.
Matsushita, K., Tayama, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1981b). *FEMS Microbiology Letters* **10**, 267.
Matsushita, K., Ohno, Y., Shinagawa, E., Adachi, O. and Ameyama, M. (1982a). *Agricultural and Biological Chemistry* **46**, 1007.
Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1982b). *FEBS Letters* **139**, 255.
Matsushita, K., Shinagawa, E. and Ameyama, M. (1982c). *Methods in Enzymology* **89**, 187.
Matsushita, K., Patel, L. and Kaback, H. R. (1984). *Biochemistry* **23**, 4703.
Matsushita, K., Nonobe, M., Shinagawa, E., Adachi, O. and Ameyama, M. (1985). *Agricultural and Biological Chemistry* **49**, 3519.
Matsushita, K., Shinagawa, E., Inoue, T., Adachi, O. and Ameyama, M. (1986). *FEMS Microbiology Letters* **37**, 141.
Matsushita, K., Nonobe, M., Shinagawa, E., Adachi, O. and Ameyama, M. (1987a). *Journal of Bacteriology* **169**, 205.
Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1987b). *Biochimica et Biophysica Acta* **894**, 304.
Matsushita, K., Nagatani, Y., Shinagawa, E., Adachi, O. and Ameyama, M. (1989a). *Agricultural and Biological Chemistry* **53**, 2895.
Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1989b). *Journal of Biochemistry* **105**, 633.
Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1989c). *Biochemistry* **28**, 6276.
Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1990). *Proceedings of the National Academy of Sciences of the United States of America* **87**, 9863.

Matsushita, K., Nagatani, Y., Shinagawa, E., Adachi, O. and Ameyama, M. (1991). *Journal of Bacteriology* **173**, 3440.
Matsushita, K., Ebisuya, H. and Adachi, O. (1992a). *Journal of Biological Chemistry* **267**, 24 748.
Matsushita, K., Ebisuya, H., Ameyama, M. and Adachi, O. (1992b). *Journal of Bacteriology* **174**, 122.
Matsushita, K., Takahashi, K., Takahashi, M., Ameyama, M. and Adachi, O. (1992c). *Journal of Biochemistry* **111**, 739.
Matsushita, K., Takaki, Y., Shinagawa, E., Ameyama, M. and Adachi, O. (1992d). *Bioscience Biotechnology and Biochemistry* **56**, 304.
McIntire, W., Singer, T. P., Ameyama, M., Adachi, O., Matsushita, K. and Shinagawa, E. (1985). *Biochemical Journal* **231**, 651.
Midgley, M. and Dawes, E. A. (1973). *Biochemical Journal* **132**, 141.
Miller, M. J. and Gennis, R. B. (1983). *Journal of Biological Chemistry* **258**, 9159.
Minagawa, J., Mogi, T., Gennis, R. B. and Anraku, Y. (1992). *Journal of Biological Chemistry* **267**, 2096.
Muraoka, H., Watabe, Y., Ogasawara, N. and Takahashi, H. (1981). *Journal of Fermentation Technology* **59**, 247.
Muraoka, H., Watabe, Y. and Ogasawara, N. (1982a). *Journal of Fermentation and Technology* **60**, 171.
Muraoka, H., Watabe, Y., Ogasawara, N. and Takahashi, H. (1982b). *Journal of Fermentation Technology* **60**, 41.
Ohmori, S., Uozumi, T. and Beppu, T. (1982). *Agricultural and Biological Chemistry* **46**, 381.
Poole, R. K. (1983). *Biochimica et Biophysica Acta* **726**, 205.
Poole, R. K. (1988). *In* "Bacterial Energy Transduction" (C. Anthony, ed.), pp. 274–278. Academic Press, London.
Pronk, J. T., Levering, P. R., Olijve, W. and Van Dijken, J. P. (1989). *Enzyme and Microbial Technology* **11**, 160.
Puustinen, A., Finel, M., Virkki, M. and Wikström, M. (1989). *FEBS Letters* **249**, 163.
Puustinen, A. and Wikström, M. (1991). *Proceedings of the National Academy of Sciences of the United States of America* **88**, 6122.
Puustinen, A., Finel, M., Haltia, T., Gennis, R. B. and Wikström, M. (1991). *Biochemistry* **30**, 3936.
Ross, P., Mayer, R. and Benziman, M. (1991). *Microbiological Review* **55**, 35.
Santana, M., Kunst, F., Hullo, M. F., Rapoport, G., Danchin, A. and Glaser, P. (1992). *Journal of Biological Chemistry* **267**, 10 225.
Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Gennis, R. B. and Ferguson-Miller, S. (1992). *Proceedings of the National Academy of Sciences of the United States of America* **89**, 4786.
Shimwell, J. L. and Carr, J. G. (1964). *Nature* **201**, 1051.
Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1981). *Agricultural and Biological Chemistry* **45**, 1079.
Shinagawa, E. and Ameyama, M. (1982). *Methods in Enzymology* **89**, 194.
Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1982). *Agricultural and Biological Chemistry* **46**, 135.
Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1983). *Journal of Fermentation Technology* **61**, 359.
Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1984). *Agricultural and Biological Chemistry* **48**, 1517.
Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, M. (1990). *Journal of Biochemistry* **107**, 863.
Smith, L. (1954). *Archives of Biochemistry and Biophysics* **50**, 299.
Sone, N. and Fujiwara, Y. (1991). *FEBS Letters* **288**, 154.
Sone, N. and Yanagita, Y. (1982). *Biochimica et Biophysica Acta* **682**, 216.
Sugisawa, T., Hoshino, T. and Fujiwara, A. (1991a). *Agricultural and Biological Chemistry* **55**, 2043.

Sugisawa, T., Hoshino, T., Nomura, S. and Fujiwara, A. (1991b). *Agricultural and Biological Chemistry* **55**, 363.
Takeda, Y. and Shimizu, T. (1991). *Journal of Fermentation and Bioengineering* **72**, 1.
Takeda, Y. and Shimizu, T. (1992). *Journal of Fermentation and Bioengineering* **73**, 89.
Takeda, Y., Shimizu, T., Matsushita, K., Adachi, O. and Ameyama, M. (1992). *Journal of Fermentation and Bioengineering* **74**, 209.
Takemura, H., Horinouchi, S. and Beppu, T. (1991). *Journal of Bacteriology* **173**, 7070.
Tamaki, T., Fukaya, M., Takemura, H., Tayama, K., Okumura, H., Kawamura, Y., Nishiyama, M., Horinouchi, S. and Beppu, T. (1991). *Biochimica et Biophysica Acta* **1088**, 292.
Tamaki, Y., Horinouhi, S., Fukaya, M., Okumura, H., Kawamura, Y. and Beppu, T. (1989). *Journal of Biochemistry* **108**, 541.
Tayama, K., Fukaya, M., Okumura, H., Kawamura, Y. and Beppu, T. (1989). *Applied Microbiology and Biotechnology* **32**, 181.
Uhlig, H., Karbaum, K. and Steudel, A. (1986). *International Journal of Systematic Bacteriology* **36**, 317–322.
Van der Oost, J., Von Wachenfeld, C., Hederstedt, L. and Saraste, M. (1991). *Molecular Microbiology* **5**, 2063.
Verkhovskaya, M., Verkhovsky, M. and Wikström, M. (1992). *Journal of Biological Chemistry* **267**, 14 559.
Viitanen, P., Newman, M. J., Foster, D. I., Wilson, T. H. and Kaback, H. R. (1986). *Methods in Enzymology* **125**, 429.
Williams, H. D. and Poole, R. K. (1987). *Journal of General Microbiology* **133**, 2461.
Williams, H. D. and Poole, R. K. (1988). *Current Microbiology* **16**, 277.
Yamada, M., Sumi, K., Matsushita, K., Adachi, O. and Yamada, Y. (1993). *Journal of Biological Chemistry* **268**, 12 812.
Yamanaka, T. and Fujii, K. (1980). *Biochimica et Biophysica Acta* **591**, 53.
Zimmerman, B. H., Nitsche, C. I., Fee, J. A., Ruswak, F. and Münk, E. (1988). *Proceedings of the National Academy of Sciences of the United States of America* **85**, 5779.

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Subject Index

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Abbreviations: ACDQ, (6-amino-7-chloro-5,8-dioxoquinoline); ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; GDH, glucose dehydrogenase; PBP, penicillin-binding protein.

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