Biotechnology of Antibiotics and Other Bioactive Microbial Metabolites

Giancarlo Lancini
and
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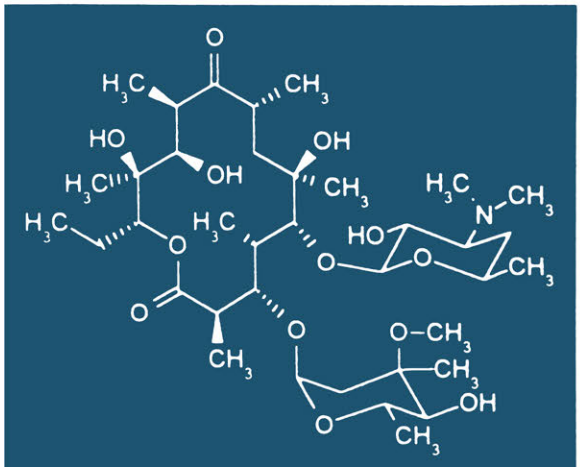

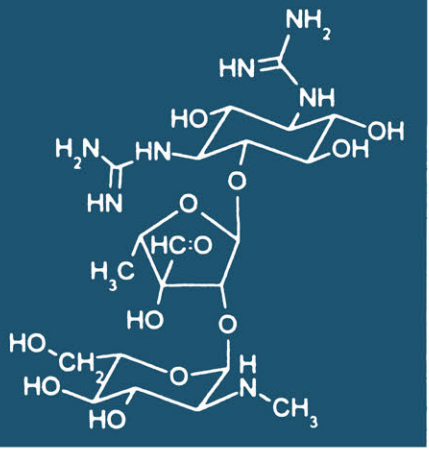

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To Carlotta, Valentina, and Lisa

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Preface

Antibiotics are the most prescribed drugs in human medicine. Almost every one of us will receive an antibiotic at some time in our lives for an infectious disease. As antimicrobial agents, antibiotics are also widely used in agriculture, animal husbandry, and the food industry. Several of the most efficacious antitumor agents are also antibiotics. Other microbial secondary metabolites are becoming more and more important for their pharmacological properties or as pesticide agents.

Studies of secondary metabolite biochemistry and genetics are greatly contributing to our understanding of microbial evolution and differentiation. The search for novel secondary metabolites, the development of the producing strains, and the improvement of industrial production involve several disciplines, such as basic and applied microbiology, microbial biochemistry and genetics, and molecular biology.

The aim of this book is to give up-to-date, concise information on these aspects, which we now refer to as biotechnology. The book has been conceived as a teaching aid for advanced undergraduate and graduate students, but I believe it may also provide useful background on this subject to junior staff members of research and industrial laboratories.

The major problem we encountered in writing the book was selecting, from the enormous literature, the most relevant material so as to make the book both informative and readable. Rather than providing long lists of products and tables, more suitable for review articles and treatises, we have chosen to use examples that could help the reader understand the progress made in the different disciplines. The references,

listed at the end of each chapter, should help the reader who needs additional information.

My co-author, Rolando Lorenzetti, and I wish to express our thanks to our colleagues who reviewed the different chapters, and in particular to Dr. William Higgins, who provided useful criticisms and comments, and to Ms. Karen Hutchinson Parlett, who patiently revised the English style.

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Contents

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Antibiotics and Bioactive Microbial Metabolites

1.1. Antibiotics

Everyone knows what an antibiotic is. However, the definition of an antibiotic has for several years been the subject of hot disputes among the experts in the field. The definition that we prefer, and that is accepted with minor variations by several authors, is that antibiotics are low-molecular-weight microbial metabolites that at low concentrations inhibit the growth of other microorganisms. The reason for this preference is that this class of natural products is clearly identified by its origin and by its biological activity. Nevertheless, some of the expressions used in the definition need further clarification.

The term *low-molecular-weight metabolites* refers to molecules of at most a few thousand daltons. Enzymes such as lysozyme, and other complex protein molecules that also have antimicrobial activity, are not considered to be antibiotics. Strictly speaking, only the natural products of microorganisms should be termed antibiotics; in practice we also include the products obtained by chemical modification of the natural substances in this category, under the name *semisynthetic antibiotics.*

Inhibition of growth of other microorganisms refers substantially to the inhibition of the cells to reproduce, and, consequently, to the growth of a microbial population, rather than of the individual cell. The inhibition can be permanent, and in this case the action is termed "cidal" (e.g., bactericidal, fungicidal), or lasting only while the antibiotic

is present, and is then termed "static" (e.g., bacteriostatic). The limiting phrase "at low concentrations" is included in the definition because, obviously, even normal cell components can cause damage at excessive concentrations. For instance, amino acids such as glycine or leucine may have an inhibitory effect on some bacteria when present at high concentration in the culture medium. For the same reason, the products of anaerobic fermentation, such as ethanol or butanol, cannot be considered antibiotics. The antimicrobial activity of typical antibiotics is very high and may be observed, at least on some bacterial species, at the micromolar (sometimes even nanomolar) level.

1.1.1. Antimicrobial Activity

The commonly used parameter by which antimicrobial activity is measured is the minimal inhibitory concentration (MIC). This is determined by adding decreasing concentrations of the antibiotic to a series of test tubes (today microwells are commonly used) containing a nutrient medium inoculated with the test organism. The MIC is defined as the lowest concentration of the antibiotic at which, after a suitable incubation period, no visible growth is observed. The MIC can also be determined in a solid medium. In this case decreasing concentrations of the antibiotic are incorporated in an agarized medium distributed in plates, on the surface of which a droplet of the test organism culture is added. The MIC is the lower antibiotic concentration at which there is no formation of a visible colony of the test organism. In both cases the MIC value is expressed as micrograms per milliliter.

It is evident that the MIC is a value that refers to one antibiotic and one microbial species, or more exactly to one microbial strain, since different strains of the same species can be inhibited by different antibiotic concentrations. The group of microbial species against which an antibiotic is active (i.e., those for which low MICs are observed) is called the spectrum of activity of the antibiotic. The spectrum of activity is quite different for different antibiotics. Some antibacterial antibiotics are active only against either gram-positive or gram-negative species and are said to have a narrow spectrum of activity. Others have a broad spectrum of activity, being inhibitory on a variety of bacterial or fungal species. The term *antitumor antibiotics* is justified by the fact that, for many years, these products were isolated on the basis of their antibac-

terial activity, and only subsequently tested for their cytostatic or cytocidal activity.

The most interesting aspect of the antibiotic activity is the variety of their mechanisms of actions. The mechanism of action of an antibiotic is the biochemical event by which the growth of a sensitive microorganism is inhibited. This is the result of the interference of the antibiotic with a molecule, called the target molecule, essential for the cell metabolism. Target molecules are normally macromolecules, such as DNA, RNA, and enzymes, but are occasionally small metabolites, such as substrates of enzymatic reactions or membrane components. Strictly speaking, understanding the mechanism of action of an antibiotic implies the identification not only of the target molecule, but also of the site and of the type of interaction. This has been determined for a large number of antibiotics. However, it is easier to identify the metabolic pathway that is blocked than the specific molecule involved, and from the practical point of view, this is often sufficient. For this reason one normally speaks of antibiotics that inhibit the synthesis of the cell wall, DNA replication or transcription, protein synthesis, or cell membrane functions.

The specificity of the mechanism of action is the main reason for the selectivity of action of the antibiotics. When, for instance, the target molecule of the bacterial cell has no equivalent in mammalian cells, or the composition of its mammalian counterpart is substantially different, the antibiotic will, in principle, be selectively active against bacteria and nontoxic for higher organisms.

1.1.2. Chemical Nature of Antibiotics

The chemical structure of several thousand antibiotics has been determined and published in the specialized literature. Because of the modern physicochemical techniques, hardly any new compound is reported without an accompanying paper on its structural elucidation. The first and most important conclusion that we can draw from this very large amount of available data is that antibiotics are, from the chemical point of view, a very heterogeneous group of substances.

The chemical system of antibiotic classification reported in Fig. 1.1 illustrates the variety of structures found. It must be added that different chemical groups, such as oxygenated functions, nitrogen func-

PRIMARY CODE NUMBER	FAMILY
1	**Carbohydrate Antibiotics**
1.1	Pure saccharides
1.2	Aminoglycoside antibiotics
1.3	Other (N- and C-) glycosides
1.4	Various sugar derivatives
2	**Macrocyclic Lactone (Lactam) Antibiotics**
2.1	Macrolide antibiotics
2.2	Polyene antibiotics
2.3	Other macrocycliclactone antibiotics
2.4	Macrolactam antibiotics
3	**Quinone and Similar Antibiotics**
3.1	Linearly condensed polycyclic compounds
3.2	Naphthoquinone derivatives
3.3	Benzoquinone derivatives
3.4	Various quinone-like compounds
4	**Amino Acid, Peptide Antibiotics**
4.1	Amino acid derivatives
4.2	Homopeptides
4.3	Heteromer peptides
4.4	Peptolides
4.5	High molecular weight peptides
5	**Nitrogen-containing Heterocyclic Antibiotics**
5.1	Non-condensed (single) heterocycles
5.2	Condensed (fused) heterocycles
5.3	Alkaloids with antibiotic (antitumor) activity
6	**Oxygen-containing Heterocyclic Antibiotics**
6.1	Furan derivatives
6.2	Pyran derivatives
6.3	Benzopyran derivatives
6.4	Small lactones
6.5	Polyether antibiotics
7	**Alicyclic Antibiotics**
7.1	Cycloalkane derivatives
7.2	Small terpenes
7.3	Oligoterpene antibiotics
8	**Aromatic Antibiotics**
8.1	Benzene compounds
8.2	Condensed aromatic compounds
8.3	Non-benzenoid aromatic compounds
8.4	Various derivatives of aromatic compounds
9	**Aliphatic Antibiotics**
9.1	Alkane derivatives
9.2	Aliphatic carboxylic acid derivatives
9.3	Aliphatic compounds with S or P content
0	**Miscellaneous antibiotics** (with unknown skeleton)

Figure 1.1. Chemical classification of antibiotics according to Bérdy (1974).

tions, halogen atoms, alkyl or acyl groups, are often present as substituents on the basic structures, increasing the variety of the molecules.

From this heterogeneity it is clearly a serious mistake to consider antibiotics as a class of chemical compounds, such as is done for proteins or steroids. There are no chemical features common to all antibiotics, nor to the more limited group of those used in medicine.

An informal classification, often practically used, is based on the concept of "family" of antibiotics. Although not exactly defined as a family (or class), this is a group of antibiotics having a common general structure and showing biological activities based on the same mechanism of action. We stress here the mechanism of action because the antimicrobial activity can be significantly different among members of the same family. Examples of well-known families of antibiotics are:

1. The β-lactam antibiotics, chemically characterized by the presence of a four-membered ring closed by an amide bond (Fig. 1.2), and that act by inhibiting the peptidoglycan synthesis of the bacterial cell wall. The β-lactams are divided in subfamilies, such as penicillins, cephalosporins, carbapenem, and monobactams, according to specific chemical features.
2. The aminoglycosides, constituted by an aminocyclitol (an alicyclic six-membered ring with hydroxyl and amino substituents) and by a few sugars or amino sugars (Fig. 1.3); these act by inhibiting ribosomal functions.
3. The tetracyclines, whose structure consists of four linearly condensed rings (Fig. 1.4), and which also inhibit protein synthesis at the ribosomal level.
4. The anthracyclines, also constituted by four condensed rings (Fig. 1.4), but acting at the DNA level. These interfere with the enzyme topoisomerase and are used as antitumor, rather than anti-infective, agents.
5. The antibacterial macrolides, characterized by a large lactone ring (Fig. 1.5); inhibitors of protein synthesis by binding to the large subunit of bacterial ribosomes.
6. The antifungal macrolides, or polyenes, constituted by a very large lactone ring (Fig. 1.6), and characterized by the presence on the cycle of a series of conjugated double bonds; they act by interfering with the sterol of the eukaryotic cell membrane.

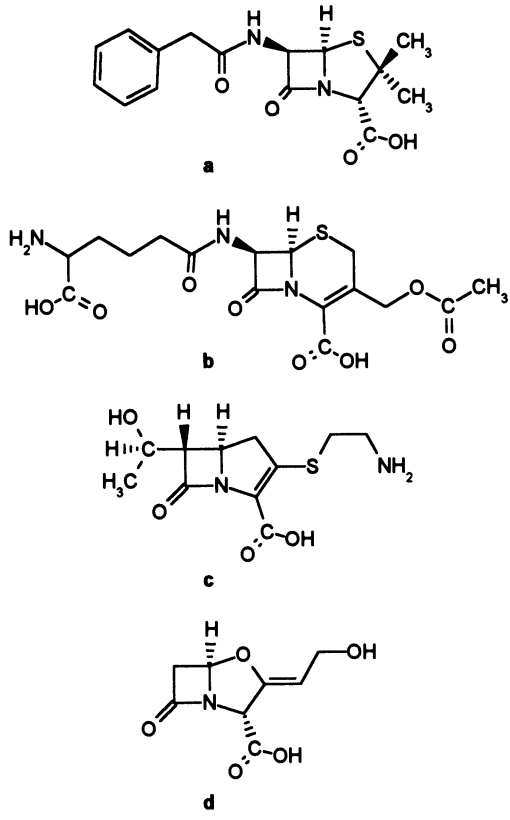


Figure 1.2. Examples of β-lactam antibiotics: (a) penicillin G; (b) cephalosporin C; (c) thienamycin; (d) clavulanic acid.

7. The ansamycins, in which an aromatic ring is spanned by an aliphatic chain closed by an amide bond (Fig. 1.7). An ansamycin subfamily, the rifamycins, are inhibitors of the enzyme RNA polymerase.

Some members of all of these families have clinical application. The chemical modification of natural products has generated a large number of semisynthetic derivatives, so that today a large variety of agents are available for the treatment of infectious diseases. It is noteworthy that the naturally occurring cephalosporins, monobactams, and rifamycins have no clinical usefulness and are important only as starting materials for the design and the preparation of clinically effective semisynthetic agents.

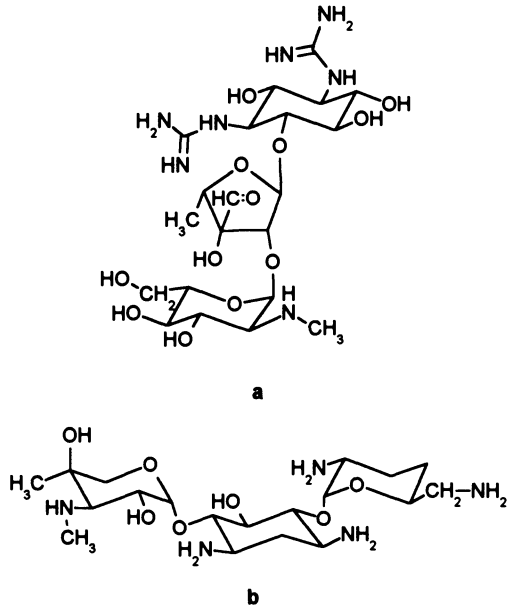


Figure 1.3. (a) Streptomycin, a streptidine-containing aminoglycoside; (b) gentamicin Cla, a deoxystreptamine-containing aminoglycoside.

Some families of antibiotics are not used in human medicine but are employed in agriculture, in veterinary medicine, or in animal husbandry. Largely used for the protection of plants from fungi are the nucleoside polyoxins, inhibitors of chitin biosynthesis in the fungal cell

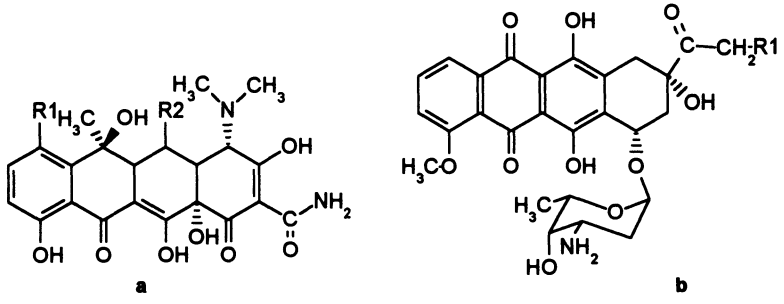


Figure 1.4. Tetracyclic antibiotics: (a) tetracycline (R1 = R2 = H), chlortetracycline (R1 = Cl, R2 = H), oxytetracycline (R1 = H, R2 = OH); (b) daunorubicin (R1 = H), doxorubicin (R1 = OH).

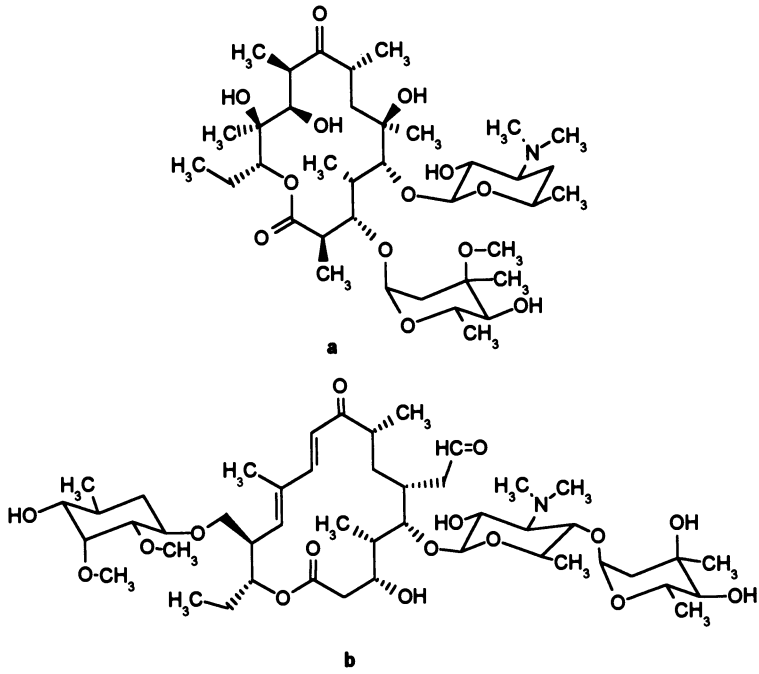


Figure 1.5. (a) Erythromycin, a 14-member macrolide; (b) tylosin, a 16-member macrolide.

wall. Important feed additives and veterinary agents are the ionophoric polyether antibiotics.

There are of course several useful antibiotics having different chemical structures that do not belong to any large family. These will be mentioned in the following chapter, in relation to the microorganism by means of which they are produced.

1.1.3. Antibiotic-Producing Microorganisms

Although the ability to produce antibiotics is widespread in nature, it is not uniformly or randomly distributed among the various taxonomic groups of microorganisms. Only a few genera are versatile producers: according to the literature data collected in our laboratory, there are about 6000 reported antibiotics produced by actinomycetes. Seventy-five percent of these have been isolated from strains of the

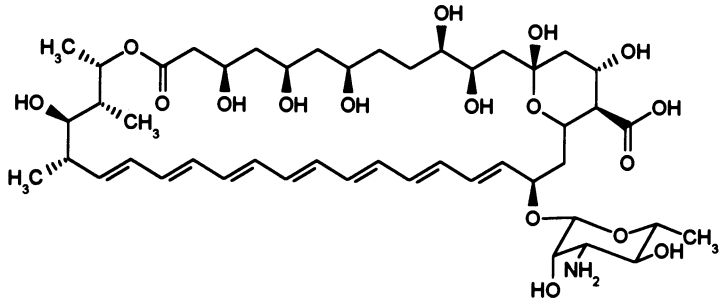


Figure 1.6. Amphotericin B, an antifungal polyene.

genus *Streptomyces.* About 1200 antibiotics are reported from eubacteria, one-half of which are produced by the genera *Bacillus* or *Pseudomonas.* From lower fungi, approximately 1500 antibiotics or other bioactive metabolites have been isolated, one-third of which come from either *Penicillium* or *Aspergillus* strains.

In evaluating these data, one has to take into account that some genera have been more thoroughly scrutinized than others. In fact, although less than 100 antibiotics have been reported from myxobacteria, this class of organism can be considered a very productive one, since a variety of novel structures have been found in the limited number of strains tested. However, the frequency at which antibiotic activity is observed in soil isolates in general agrees with the overall data. It has been our experience that about one-third of the actinomycetes isolated from soil demonstrate antimicrobial activity after growth in one stan-

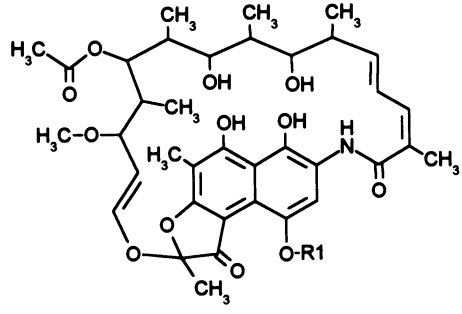


Figure 1.7. Ansamycins: rifamycin B (R1 = CH_2-COOH) and rifamycin SV (R1 = H).

dard medium. The frequency is about one in ten when molds are isolated and tested.

One could ask the question: what do the prolific genera have in common that distinguishes them from the other microorganisms? We do not have a simple and generally accepted answer to this question. Many versatile producers are microorganisms showing some form of differentiation, such as spore formation, and it is well known that most often antibiotic production is associated with a stage of morphological differentiation (the antibiotic production can itself be considered a form of biochemical differentiation). However, pseudomonads, for instance, are among the good producers and do not show any tendency to differentiate in their life cycle.

We may observe that the vast majority of the strains of all of the important producing genera have soil as their habitat and are the most efficient organisms in recycling decaying organic materials. Interesting antibiotics have been, for instance, obtained from gram-negative bacteria isolated from soil samples, whereas gram-negative enterobacteria do not produce true antibiotics but bacteriocins, toxinlike proteins selectively active against closely related strains. Within genera, such as *Pseudomonas,* that include species living in diverse habitats, the majority of antibiotic-producing species dwell in the soil, whereas the species that live in association with plants mostly produce toxins, not necessarily showing antimicrobial activity. A similar observation can be made for some fungal genera, such as *Penicillium.*

Whether these observations should be interpreted as evidence for an ecological role of the antibiotics is a moot question hotly debated in the scientific community.

1.2. Bioactive Secondary Metabolites

1.2.1. Secondary Metabolites

The term *secondary metabolites* was introduced by Bu'Lock in the early 1960s (the term had been previously used by plant physiologists to indicate plant alkaloids) to indicate microbial metabolites found as the products of differentiation in restricted taxonomic groups, and not essential for cell metabolism. These substances are thus clearly distin-

guished from primary metabolites—products, such as amino acids or nucleotides, participating in the basic cellular metabolism.

This expression has been questioned by several authors. The alternative term *specific metabolites,* as opposed to *general metabolites,* appears indeed more appropriate in that it underlines the fact that these products represent the individual diversity in the biochemistry of the microbial world. However, the term *secondary metabolites* is so widespread that it cannot practically be replaced.

Secondary metabolites are low-molecular-weight compounds with the following characteristics:

1. They are synthesized by only some microbial strains, and are the expression of the biochemical differentiation of the producing organism.
2. They have no obvious function in the growth of cultures or colonies. Strains able to make these molecules may lose, because of mutations, the capacity to synthesize them, without any apparent effect on their general metabolism.
3. They are often produced in connection with differentiation processes.
4. They are often made as families of similar products.

Not all of these characteristics are shared by all secondary metabolites. It is important to stress that "no obvious function in the growth" is different from "no function at all." Lack of essential metabolic function does not imply lack of selective function in evolutionary terms; for example, with respect to other microorganisms. The essential aspect of secondary metabolism is that it is a manifestation of the organism's individuality, i.e., of cellular differentiation, often expressed only under conditions of limited growth.

According to this definition, antibiotics are secondary metabolites. Studies aimed at uncovering a role as regulatory agents in the producer metabolism have been attempted for several antibiotics. In one case only, that of pamamycin, has a function been demonstrated—as an inducer of aerial mycelium formation in actinomycetes.

It is important to emphasize that many secondary metabolites, besides antibiotics, are produced by microorganisms. Examples are some pigments, numerous protease inhibitors, and toxins. Their frequency is difficult for us to estimate, since, as discussed in Chapter 3, the research

programs have been for many years targeted at the discovery of antimicrobial activities. However, in more recent times, the search for microbial metabolites endowed with different biological activities has revealed a high number of new products. Such inhibitors of physiological functions of higher organisms are termed bioactive microbial metabolites, and are discovered with a lower frequency, in comparison with antimicrobial substances.

1.2.2. Antimicrobial and Other Biological Activities

If we analyze the general biological activity of secondary metabolites in relation to their antimicrobial activity, the following different situations are observed:

1. Several typical antibiotics demonstrate, in addition to their antimicrobial activity, an appreciable activity on other biological systems. This is the case, for instance, of the cholesterol-lowering activity of rifamycins; the induction of gastric motility by erythromycin; and the ability of ristocetin to trigger platelet aggregation. These activities have no relation to the mechanism of action of the antibiotic and may be enhanced in semisynthetic derivatives, without a corresponding increase, or even with the disappearance, of the antimicrobial activity. We must therefore consider these types of activities as adventitious: in fact, the chances are that when thousands of molecules are tested on a high number of biological systems a few of them will show a positive or negative interaction. This is not surprising, in view of the experience of the pharmacological screening of synthetic compounds successfully performed for many years by the research laboratories of the pharmaceutical industry.

2. The same biological action can be effective both on the microbial cell and on cells or systems of higher organisms. This is the case for compounds such as mevinolin or compactin that, by inhibiting the isoprenoid biosynthesis, are active as fungistatic and are cholesterol-lowering agents in man. Analogous cases may be those of the immunosuppressant agents cyclosporin A and FK 506, which inhibit two different enzymes having a peptidyl-prolyl isomerase activity. The binding of these metabolites to corresponding fungal enzymes results in the formation of complexes toxic for the microorganism.

3. Many bioactive metabolites do not show any microbiological activity. Among these are cytostatic compounds, insecticides and antiparasites, and herbicides, but the vast majority result from the search for substances potentially active as pharmacological agents. These are predominantly inhibitors of physiologically (or pathologically) relevant enzymes, antagonists of hormones or other regulatory molecules that act by competing with the natural ligand for cellular receptors. There are also immunomodulators, antagonists of cell growth factors, and free radical scavengers.

It is not known whether some of these substances have an activity related to the producer cell metabolism, or a function in cell-to-cell or species-to-species communication. The variety of their biological activities and chemical structures certainly preclude any general interpretation of their relation to the producer organisms.

1.2.3. Bioactive Secondary Metabolites of Practical Interest

The demonstration of an inhibitory activity in an *in vitro* system, or of an effect on cell cultures, is just the first of the several characteristics that a substance must possess to become a useful biological agent. This activity must be high and selective. Further, potential drugs must be well absorbed and distributed in the human body, reach their site of action in sufficient concentrations, and not be toxic or present serious side effects. The requirements may be less stringent for products of agricultural or veterinary use, but still a good level of *in vivo* activity is necessary. Finally, it has to compare favorably with competitor substances from the economical point of view.

It is therefore not surprising that only a small fraction of the bioactive microbial compounds are developed to become industrial products. We give here a short description of substances that, in spite of these difficulties, have reached or appear close to reaching the commercial stage.

1.2.3.1. Immunomodulators

Bestatin, a dipeptide (Fig. 1.8), is one of the first products obtained from a screening directed at inhibitors of cell surface enzymes. It is an enhancer of the immune system and has been tried in clinical studies

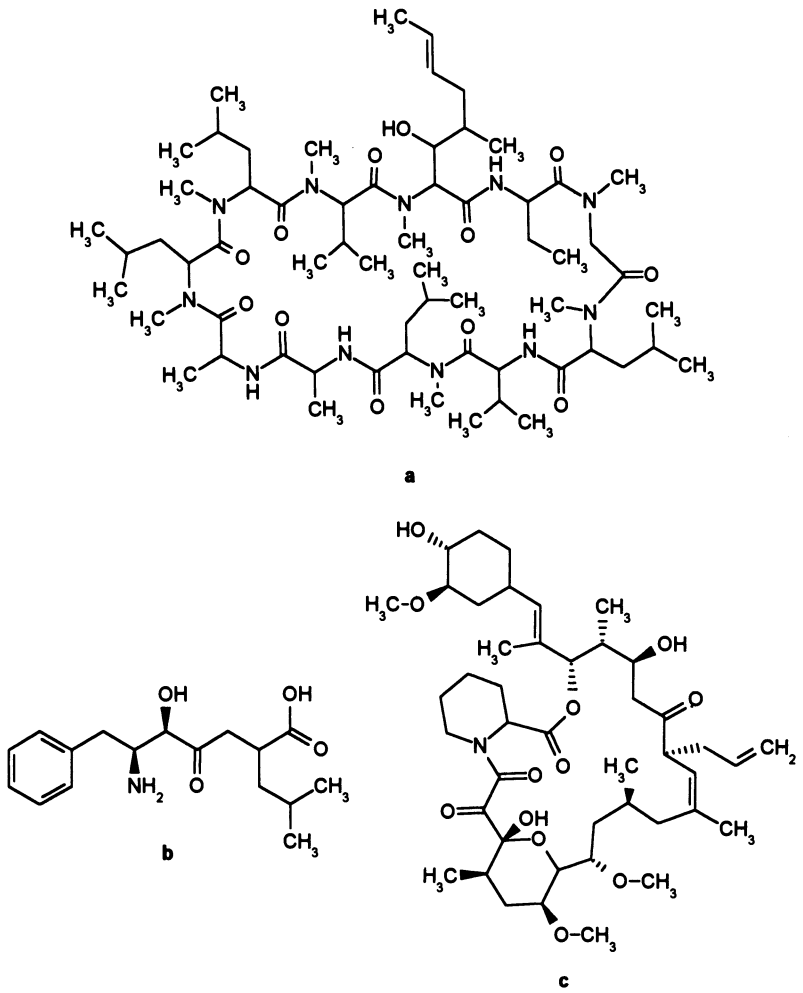


Figure 1.8. Immunomodulators of microbial origin: (a) cyclosporin A; (b) bestatin; (c) FK 506.

as an adjuvant in tumor therapy and in the treatment of *Candida* infections.

Cyclosporin A is a cyclic peptide composed of 11 amino acid residues (Fig. 1.8). Originally isolated for its antifungal activity, it was then developed for its immunosuppressive activity and has an application

in organ transplantation. It acts by inhibiting T-cell activation, by binding to cyclophilin, an enzyme with peptidyl-prolyl isomerase activity.

FK 506 is a 23-membered macrolactone, including an *N*-heterocyclic ring (Fig. 1.8). It also inhibits T-cell activation by binding to a peptidyl-prolyl isomerase that is, however, different from cyclophilin. Its immunosuppressant activity is therefore somewhat different from that of cyclosporin A. At present it is being evaluated in clinical trials.

1.2.3.2. Products with Pharmacological Activity

Acarbose, a pseudotetrasaccharide composed of three sugars and an aminocyclitol, is a selective inhibitor of the gastric α-amylase and sucrase. It has been proposed for the therapy of metabolic disorders as an antihyperglycemic drug, since it delays, or hinders the intestinal absorption of carbohydrates.

Lovastatin (previously named monacolin K and mevinolin) is a polyketide-derived molecule, comprising a reduced naphthalenic ring and an aliphatic chain ending with a lactone (Fig. 1.9). It is an inhibitor of methyl-hydroxy-glutarate reductase, one of the first enzymes in sterol

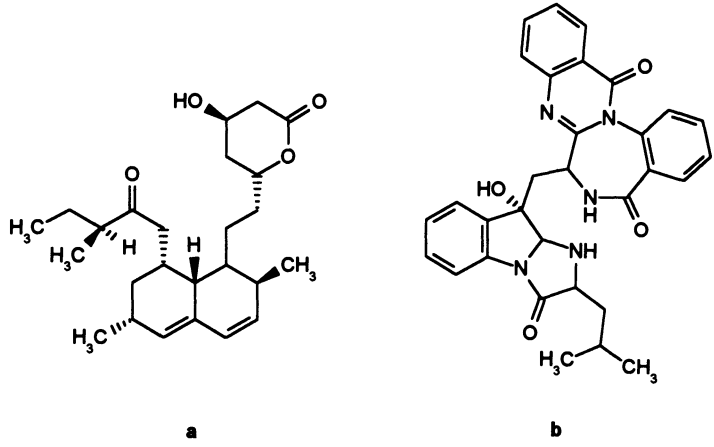
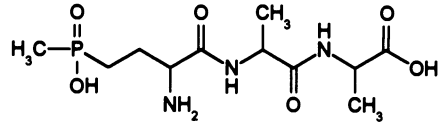


Figure 1.9. Pharmacologically active microbial metabolites: (a) lovastatin (previously termed mevinolin or monacolin K); (b) asperlicin.

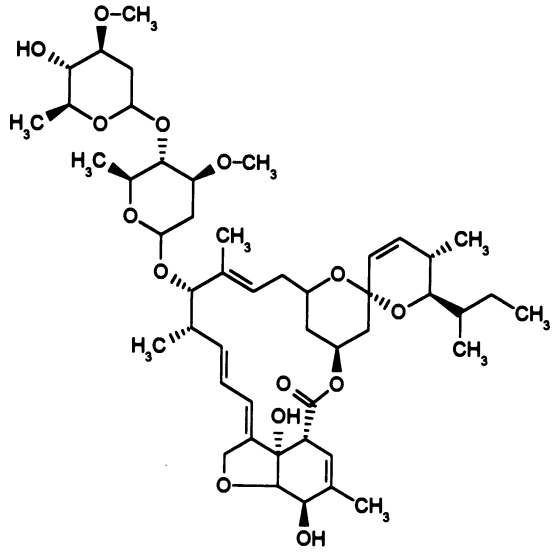
biosynthesis. It is used as a serum cholesterol-lowering agent. Two semisynthetic derivatives, simvastatin and pravastatin, have the same medical application.

Asperlicin is a complex amino acid-derived molecule, containing a benzodiazepine moiety (Fig. 1.9), which binds to the peripheral receptors of cholecystokinin, with high affinity. It is under evaluation, together with more potent derivatives, for the treatment of gastrointestinal disorders.

Lipstatin is a linear aliphatic chain, with an amino acyl substituent and a lactone moiety which is essential for its activity. Lipstatin is an



a



b

Figure 1.10. (a) bialaphos, an herbicide; (b) avermectin B_{1a}, an anti-parasite agent.

irreversible inhibitor of pancreatic lipase, and is under clinical evaluation for the treatment of obesity.

1.2.3.3. Products for Agriculture and Animal Husbandry

Avermectins are a family of sugar-substituted complex macrolides (Fig. 1.10), possessing strong anthelmintic, insecticidal, and acaricidal activity. They block neuromuscular transmission in the susceptible organisms by acting on the receptors of γ-aminobutyric acid. Practically used in veterinary medicine is ivermectin, a dihydro derivative of the natural product avermectin B_1.

Phosphinothricin (2-amino-4-methylphosphinyl-butyric acid) is a herbicidal compound, acting as a competitive inhibitor of chloroplast glutamine synthetase. A related metabolite is bialaphos, in which the phosphinothricin moiety is linked to two alanine residues. In contrast to the first compound, bialaphos can penetrate the bacterial cell, and demonstrates antimicrobial activity.

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2

Biology of Antibiotic-Producing Microorganisms

2.1. Genus Bacillus

The genus *Bacillus* comprises a heterogeneous group of unicellular rod-shaped bacteria, aerobes or facultatively anaerobes, whose prominent characteristic is the formation of endospores under unfavorable environmental conditions. Bacilli are gram-positive, generally motile by lateral or peritrichous flagella. Their main habitat is soil, where they live as saprophytes. However, one species, *B. anthracis,* is a human pathogen, and other species, notably *B. thuringiensis,* are insect pathogens.

2.1.1. Description and Taxonomy

The genus *Bacillus* belongs to the family Bacillaceae. It differs from the other rod-shaped, endospore-forming genus *Clostridium* because it is aerobic or facultative. Over 200 species are described in the literature; the type species is *B. subtilis.*

Although classified within one genus, the bacilli are a heterogeneous group of organisms. The G + C content of DNA ranges from 33.2 to 66.4%. The murein type is normally *meso*-DAP directly linked, but in a few species lysine has been detected. With one exception the membrane fatty acids are branched, either of the iso or anteiso type. Unsaturated fatty acids are also found in some species.

Endospores, which can be easily seen inside the mother cell because they are refractile under phase-contrast microscopy, are composed of

an electron-transparent core, an inner and outer membrane delimiting a primordial cell wall and a thick cortex, and protein coats. The cortex contains a peptidoglycan of unusual structure. Typical of the spores is the high content of calcium dipicolinate constituting up to 15% of the spore dry weight.

2.1.2. Physiology and Metabolism

There is a striking diversity in the range of nutritional requirements and growth conditions of the members of the genus *Bacillus*. Most of the strains attain maximum growth in media containing a simple sugar, amino acids, one or more B group vitamins, inorganic nitrogen, and minerals. Several species, including the well-known *B. subtilis*, do not require amino acids or vitamins. Others, however, only grow well in media containing unidentified factors, present in yeast or in soil extracts.

Under anaerobic conditions, several species ferment sugars producing 2,3-butanediol, glycerol, and CO_2. The final products of a few other species include H_2 and no glycerol.

Most species are mesophilic, while a few others grow preferentially at 10°C or are definitely thermophilic, requiring temperatures from 40 to 65°C for maximum growth.

The transformation of vegetative cells into endospores is a very complex process, lasting up to 8 h. It is triggered by environmental conditions such as a lack of nutrients or high cell density. The physiological signals, notably the lowering of the guanine nucleotide pool, are sensed by a protein kinase which sets off the regulatory cascade. The first stage of sporulation is the condensation of the nucleoid into a compact filament along the cell axis. This is followed by the formation of a membrane septum asymmetrically dividing the cell into an incipient forespore and a mother cell, both containing part of the cytoplasm and one chromosome. In subsequent stages the forespore is totally engulfed by an outgrowth of the membrane, the physical structure of which is modified with consequent modifications of its enzymatic and transport functions. The process continues with the differentiation of the membrane into an inner and an outer membrane, between which the primordial cell wall and the cortex are synthesized. Finally, the external layers or coats are formed, the core assumes its final homogeneous structure, and all metabolic activity of the spore is switched off.

The endospores are typically resistant to dryness, heat, and disinfectants, and remain vital in the dormant state for years. Germination and resumption of vegetative growth require an appropriate nutritional environment and may be triggered by one of several factors such as temperature or low pH.

2.1.3. Genetics

The knowledge of the chromosomal structure of bacilli is mainly derived from studies on *B. subtilis.* DNA transformation of this organism occurs easily, and has been used as a powerful tool in genetic analysis. Another widely employed technique is transduction with phage PBS1, allowing the transfer of longer DNA fragments. The overall result is a detailed linkage map of the *B. subtilis* chromosome, which is circular, and presents both similarities and differences with the other well-known bacterial chromosome, that of *Escherichia coli.*

The DNA homology between *B. subtilis* and other *Bacillus* species, as determined by DNA–DNA hybridization, is surprisingly low, and *B. subtilis* is not easily transformed by the DNA of other species. However, the limited genetic analysis of other bacilli revealed several linkage groups identical to those of *B. subtilis.* The overall picture suggests that there is conservation of gene order in closely related species, whereas in more distant species the conservation is partially lost because of translocation of large DNA fragments.

2.1.3.1. Endospore Development Genetics

In *B. subtilis,* more than 50 mutations, mapping in different loci of the chromosome, have been identified that prevent sporulation without affecting vegetative metabolism. The relative genes (*spo* genes) are classified according to the spore development stage at which their product intervenes from *spo0,* related to the initial events taking place in the vegetative cell, to *spoVI,* determining maturation.

The regulation of the metabolic events in sporulation is exceedingly complex, and many of the identified genes have a regulatory, rather than structural, function. The *spo* genes expressed after the first hour are generally transcribed by RNA polymerase associated with several specific σ factors, four of which have been identified. Two of these, σ^E

and σ^F, are expressed early and it has been suggested that σ^F might have a role in forespore gene expression, whereas σ^E should be active in the mother cell. Similarly, in later stages σ^G and σ^K are active in the forespore and in the mother cell, respectively.

The function of several other genes is to regulate σ factor expression both at the transcription and translation levels. A third class of genes encode for either positive or negative transcriptional regulators, contributing to the fine tuning of the process. Finally, the late genes appear to have structural functions determining, for instance, the synthesis of coat proteins and the maturation of the spore core.

2.1.3.2. Gene Cloning

Since *B. subtilis* plasmids were not available, initial cloning of the DNA of this organism was performed with *E. coli* phages or plasmid vectors. The Charon phage, a system derived from λ phage, was frequently employed, and using this method large libraries were prepared. Other chromosomal libraries have been prepared in *E. coli* plasmid pMB9 and in shuttle plasmids, hybrid between *E. coli* and *S. aureus* plasmids replicating both in *B. subtilis* and in *E. coli.* These have been widely used to study the signals regulating gene expression.

To clone genes that code for proteins of industrial relevance, present research efforts are centered on constructs that are also stable when they include large DNA fragments. For example, *B. subtilis* cryptic pTA1060 plasmid is more stable than *S. aureus* plasmids.

2.1.4. Industrial Applications

The primary industrial use of bacilli is in the production of enzymes. Characteristic of this genus is the ability to produce a variety of extracellular proteins mainly having hydrolytic activity. One of the major products, hundreds of tons of which are used each year in the detergent industry, is the alkaline protease subtilisin. Similarly large amounts of amylases are currently produced for the food and brewing industries. Of potential interest are the cellulases and the xylanases for the conversion of waste material into substrates for the industrial fermentation processes.

Another important large-scale application of bacilli is as biological insecticides: some species, notably *B. thuringiensis,* produce toxins accumulated as crystals in the cell, that are selectively lethal for insects.

The protein export capacity of *B. subtilis* makes it an attractive organism for the production of heterologous proteins for medical and diagnostic uses.

2.1.4. Antibiotic Production

With few exceptions, antibiotics produced by bacilli belong to a single class, the peptides. In addition to their smaller size, peptide antibiotics differ from proteins in the presence of unusual components, notably D-amino acids, aliphatic acids, and cyclic amino acids. Also striking is the difference in biosynthesis. With a few exceptions, bacillus peptides are not synthesized on ribosomes but by a multienzyme complex, a system dubbed thiotemplate mechanism.

It is noteworthy that, in contrast to streptomycetes, bacilli do not produce depsipeptides, molecules in which amide bonds alternate with ester bonds. Other differences are the frequency with which the polypeptide chain in antibiotics from bacilli are initiated by an acyl chain and the absence of methylated amino acids.

In contrast to their uniform biosynthetic origin, these antibiotics present a variety of antimicrobial activities and mechanisms of action. Most are inhibitors of gram-positive bacteria; some, such as the polymyxins, inhibit gram-negative bacilli. Others, such as the iturins (bacillomycin), are antifungal agents. Their mechanisms of action vary from the inhibition of polynucleotide functions (edeines), through impairing peptidoglycan synthesis (bacitracin), to the interference with the cytoplasmic membrane functions (gramicidins).

At present, only a few products have a clinical use, although they are historically important, because they were among the first antibiotics discovered (Fig. 2.1).

Bacitracin, isolated in 1945 from *B. licheniformis* (formerly classified as *B. brevis*), has a clinical application presently limited to topical infections. In the past it was used systemically against severe infections from staphylococci.

Gramicidin was initially isolated, together with tyrocidine, in 1939 from *B. brevis,* and the mixture was named tyrothricin. Today it is included in a number of antibacterial topical preparations.

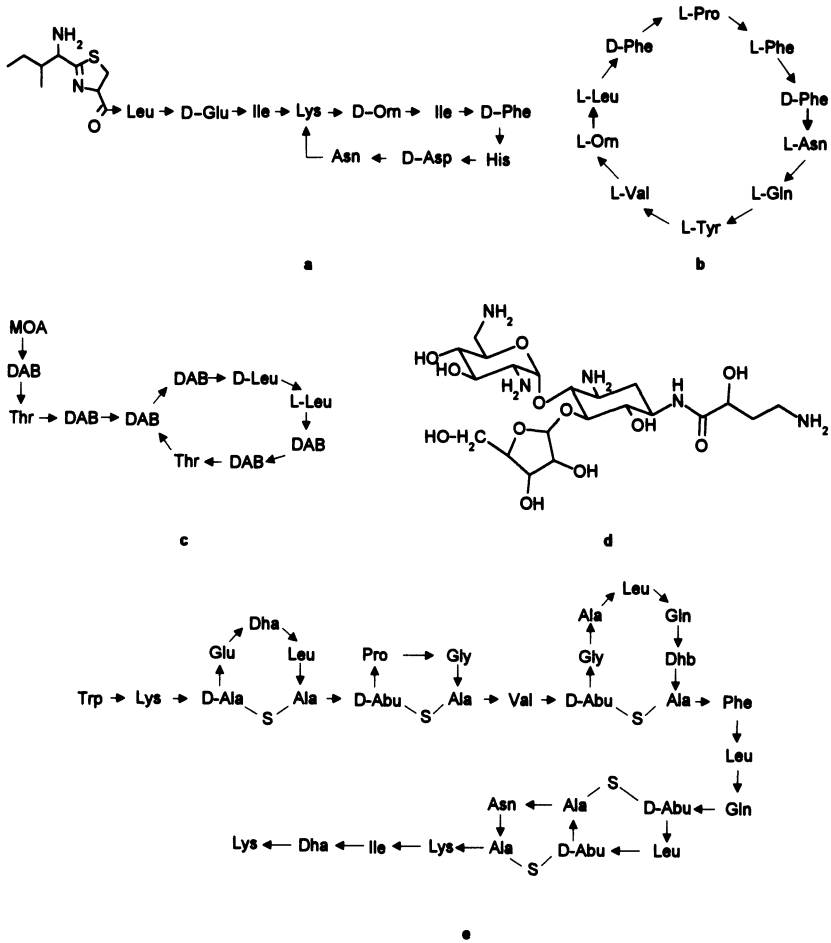


Figure 2.1. Antibiotics of interest produced by bacilli: (a) Bacitracin; (b) tyrocidine A; (c) polymyxin B (MOA, 6-methyloctanoic acid; DAB, diaminobutyric acid); (d) butirosin; (e) subtilin (Abu, aminobutyric acid, Dha, dehydroalanine, Dhb, dehydrobutyrine).

Polymyxin B, isolated from *B. polymyxa* in 1947, and colistin (polymyxin E), isolated from a variety of the same species in 1959, were originally used, as sulfate and as methane sulfonate, respectively, to treat severe *Pseudomonas* infections. At present, both are kept as reserve drugs because of the availability of less toxic agents.

Of the few nonpeptide antibiotics so far isolated from bacilli, butirosin, produced by *B. circulans,* belongs to the aminoglycoside family, and glucomycotrienin, an ansamycin, is produced by *B. megaterium.* Although butirosin is not used clinically, it is important because its structure led to methods to chemically modify other aminoglycosides to prevent inactivation by resistant bacterial strains. A β-lactone, SQ 26517, structurally similar to the β-lactams, was isolated from several *Bacillus* strains.

Among nonmedical applications, bacitracin is used as a feed additive, and subtilin, one of several antibiotics produced by *B. subtilis,* has a role in the dairy industry for food preservation.

2.2. Genus Pseudomonas

Bacteria of the genus *Pseudomonas* are gram-negative rods, 1 μm in diameter and 1.5–5 μm in length, normally motile by several polar flagella. They are aerobes, although some species may use nitrates instead of oxygen as the electron acceptor. No resting stages are known. Many species accumulate polyhydroxybutyrate as reserve material.

Pseudomonas strains can be isolated from very diverse habitats. Many dwell in soil and display great ability to degrade organic matter, notably aromatic compounds. Several species are associated with the rhizosphere or the leaves of plants. Two species, *P. mallei* and *P. pseudomallei,* are specific human and animal pathogens. *P. aeruginosa,* although normally living in the soil as a saprophyte, is a common etiological agent of nosocomial infections.

2.2.1. Taxonomy and Description

Pseudomonas cells can be distinguished from those of related genera, such as *Xanthomonas,* because they are multiflagellated, accumulate polyhydroxybutyrate, are often pigmented, and do not require growth factors.

The genus has been divided into five subgroups on the basis of rRNA homology. Group I is the largest one, including species common in soil such as *P. aeruginosa, P. putida,* and *P. fluorescens.* Characteristic of many of these is the production of fluorescent pigments, which can

chelate iron and allow growth on low-iron media. Species belonging to group II may be animal or plant pathogens, whereas group III is largely represented by species using CO_2 and H_2 as source of energy. A few species only, with rather diverse characteristics, belong to groups IV and V.

Although the presence of multiple polar flagella is included in the genus definition, at least one species is nonflagellated and others may also bear lateral flagella. Similarly to other gram-negative bacteria, the cell is surrounded by an outer membrane whose composition and functions have been extensively studied in *P. aeruginosa.* The membrane acts as an unusually effective permeability barrier. Channels allowing the permeation of small hydrophilic molecules are formed across the membrane by a specific protein, porin F.

2.2.2. Physiology and Metabolism

Almost all *Pseudomonas* strains have simple nutritional requirements. A medium consisting of low-molecular-weight carbon compounds, either nitrates or ammonium ions as nitrogen sources, and mineral salts, is adequate for good growth. Methane, however, and other one-carbon compounds are not utilized. At the same time their versatility in utilizing a large variety of carbon sources is remarkable. *P. putida,* for example, can grow on any of 78 compounds as the sole carbon and nitrogen source. This versatility relates to an unusual capacity to produce enzymes (oxygenases, dehalogenases, and amidases) by which many aliphatic or aromatic compounds are converted into suitable substrates for catabolic enzymes. The wide range of catabolic activities is also characteristic of the genus. Catechols, for instance, are degraded either by a chromosomally expressed 1,2-dioxygenase or by a plasmid-encoded 2,3-dioxygenase. Specific pathways are known for the degradation of polycyclic aromatics, amino acids, and terpenoids. The final products of catabolism are generally either acetate or intermediates of the Krebs cycle.

Glucose metabolism is noteworthy in some species. In contrast to the usual glycolytic pathway, glucose is oxidized to 2-keto-3-deoxygluconate which is subsequently split into pyruvate and triosephosphate, according to the so-called Entner–Doudoroff pathway.

2.2.3. Genetics

2.2.3.1. Genomic Structure

Classical conjugation studies, mainly on strains carrying the chromosome-mobilizing plasmid FP2, and transduction studies with phage F116, allowed the construction of a rather detailed linkage map of the circular chromosome of *P. aeruginosa.* The map revealed striking differences in its organization when compared with that of *E. coli.* Specifically, the genes of amino acid or purine biosynthesis are not clustered as they commonly are in the Enterobacteriaceae, although most of them are found in the same region of the chromosome. The genes of catabolic pathways are also dispersed.

A more limited map of the *P. putida* chromosome is also available with fewer genetic markers. Some differences in the order of the genes are evident: the catabolic genes are dominantly grouped in one region and the biosynthetic ones in another region of the chromosome. The gene disposition of both species suggests the presence of a "supra operon" mechanism for the regulation of transcription.

2.2.3.2. Plasmids

Many properties of *Pseudomonas* strains are related to the large number of plasmids they host. These plasmids have been classified into 13 incompatibility groups. Members of the same group often present similar phenotypic characteristics, have similar size, and, where examined, DNA homology has been found.

Widespread among the plasmids are the genes coding for antibiotic resistance, although normally the resistance observed in clinical isolates is not plasmid determined. Most important for the *Pseudomonas* metabolism are the plasmids coding for degradative metabolic pathways. Noteworthy are the TOL plasmids, which degrade aromatics such as xylene, toluene, and their derivatives; the plasmids, such as NAH7, metabolizing naphthalene to salicylate and pyruvate-acetaldehyde, and the CAM plasmid converting camphor to isobutyrate.

As mentioned previously, some plasmids have chromosome-mobilizing ability and are thus useful for the construction of linkage maps.

2.2.4. *Secondary Metabolite Production*

Pseudomonads produce several secondary metabolites that cannot properly be considered antibiotics. We have already mentioned the pigments, among which are the fluorescent pyoverdines and the siderophore pyochelin. Several phytotoxins are also produced, as are the numerous syringomycins and syringostatins from *P. syringae.*

A large proportion of the antibiotic substances are produced by strains belonging to one of two species: *P. aeruginosa* and *P. fluorescens.* Most of these antibiotics are nitrogen-containing heterocyclic molecules (derived biosynthetically from the catabolism of amino acids), such as the phenazine derivatives iodinin and pyocyanine.

A few interesting antibiotics isolated from *Pseudomonas* strains had previously been found among the metabolites of other organisms. Included among these are the peptidoglycan inhibitors cycloserine and fosfomycin, and the antiprotozoal azomycin. Only two antibiotics first produced by this genus have reached clinical use (Fig. 2.2).

Pyrrolnitrin, originally produced by *P. pyrrolnitrica* and *P. fluorescens,* is one of the few natural nitroderivatives. It shows a wide range of antifungal activities and is used for topical treatment of superficial infections. At present it is produced commercially by chemical synthesis.

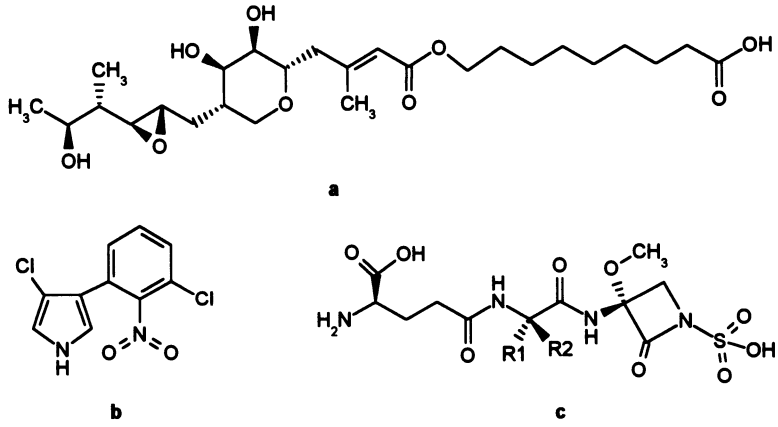


Figure 2.2. Antibiotics of interest produced by *Pseudomonas* strains: (a) mupirocin (pseudomonic acid); (b) pirrolnitrin; (c) sulfazecin (R1 = H, R2 = CH_3), isosulfazecin (R1 = CH_3, R2 = H).

Mupirocin, or pseudomonic acid A, is also a product of *P. fluorescens.* It is active on a broad spectrum of bacteria, including most of the gram-positives and several gram-negatives. It lacks systemic efficacy because it is rapidly metabolized in the body into an inactive derivative, monic acid. It is, however, used topically for the treatment of skin infections and for eradication of staphylococci from healthy carriers.

The monobactams sulfazecin and isosulfazecin (Fig. 2.2) were first isolated from cultures of *P. acidophila* and *P. mesoacidophila,* respectively. Although their activity is not sufficient for practical application, their structure has led to the synthesis of the therapeutically useful analogue azthreonam.

2.3. Streptomyces and Streptoverticillium

The genus *Streptomyces* belongs to the order Actinomycetales, the filamentous bacteria. Streptomycetes are gram-positive, aerobic, and grow in mats of branched filaments called hyphae which constitute both a substrate and an aerial mycelium. Characteristic of the genus is the segmentation of the aerial hyphae into long chains of nonmotile spores.

The genus *Streptoverticillium* is mentioned here since it is almost indistinguishable in most aspects of its biology from the genus *Streptomyces.* The main difference is morphological: the aerial hyphae bear the spore chains in verticils or umbels, that is, several chains branch from a single point of a hypha.

Streptomycetes are widely distributed in nature; their primary niche is the soil, where they play an important role in the degradation of organic polymers such as chitin, starch, and lignin. They may, however, also be found in freshwater or marine environments. One species, *S. scabies,* is a plant pathogen. In the soil their physical state is mostly as spores, rather than vegetative hyphae; from 10^4 to 10^7 colony-forming units can be isolated per gram of soil. The percentage of streptomycetes in the total microbial population is higher in dry soils than in damp ones. This has been attributed to the sensitivity of streptomycetes to CO_2 concentration, inhibitory when higher than 10%.

2.3.1. Taxonomy and Description

The genera of the order Actinomycetales are divided in groups according to the composition of the cell wall. Within a group the morphology of the aerial mycelium, the spore arrangement, and chemical characters are used to define the genus. *Streptomyces* belongs to the group containing L-diaminopimelic acid in the cell wall. As mentioned previously, the distinctive characteristic of the genus is the formation of chains of spores on the aerial mycelium. The spores are maintained in chains by a sheath which confers to them various types of surfaces: smooth, spiny, hairy, etc. The chains may be straight, flexuous, or may form open or closed spirals. The shape of the chains and the appearance of the spores are elements used for species determination. Other characters considered are the color of the surface and reverse of colonies, growth above or below 45°C, utilization of carbon compounds, and solubilization of specific substrates. The production of secondary metabolites, although of major practical importance, is not considered a determinant for speculation since it is well known that the same antibiotic can be produced by different microorganisms.

Although these criteria are widely accepted, species assignment within *Streptomyces* is a very complex problem. In patents and in the scientific literature, over 3000 strains have been named as species. Obviously this depends on different definitions of species and undoubtedly many of these are synonyms. Some order has been introduced by the International *Streptomyces* Project, a large cooperative study for the redescription of the species according to uniform criteria.

Distinctive features of *Streptoverticillium* are, besides the presence of verticils, the cottony appearance of the colonies and the "barbed wire" appearance of the mycelium when seen under moderate magnification.

Streptomyces hyphae are 0.5 to 2 μm thick. Nucleoids are distributed along the entire length of the hyphae; the very high G + C content, 69–78%, is characteristic of the genus. The overall cell wall composition is similar to that of other gram-positive bacteria; the peptidoglycan chains are joined by glycine bridges. Teichoic acids are also present but mycolic acids are absent. Menaquinones have chains of nine isoprene units, with three or four of the double bonds saturated. Linear saturated and branched fatty acids are present in the membrane lipids, the iso-16 and anteiso-15 and -17 usually predominating.

There are no remarkable differences in the fine structure of spores and vegetative hyphae. Spores are somewhat more resistant to heat and to desiccation but do maintain metabolic activities, albeit at a low level. Probably because of this, spores stored in dry conditions survive for years, whereas moisture induces a rapid loss of viability.

2.3.2. Growth Cycle

On solid substrates, streptomycetes grow in colonies whose life cycle begins either from a fragment of hypha or typically from a spore. Germination of the spores requires the presence of divalent ions, and may be hastened by a mild heat shock. It can be divided into three stages: darkening, swelling, and germ tube formation. The germ tube gives rise to a network of branched septated hyphae constituting the substrate mycelium. Changes in the environment (or genetic programming) trigger the formation of aerial hyphae that grow at the expense of the substrate mycelium, which lyses releasing the nutrients necessary for aerial mycelium development. In several cases the involvement of a low-molecular-weight autoregulator, such as the well-known A-factor, has been demonstrated in this process. The next step is the segmentation of the hyphae into spores; this appears to be genetically programmed at a given stage of development rather than induced by external factors. Sporulation begins with the simultaneous formation of septa in the aerial hyphae at regular intervals of about 1 μm. Both the wall and the septa thicken, and the spores assume their typical ellipsoid shape. Days later the sheath dissolves and spore dissemination completes the cycle.

The stages of development of streptomycetes growing in submerged cultures are not clearly defined. However, it is well known that many secondary metabolites are produced only at the end of vegetative growth, at a stage corresponding to the formation of aerial mycelium. Moreover, in some uncommon cases the formation of spores has been observed also in submerged cultures, indicating some similarity with the life cycle on solid substrates.

2.3.3. Physiology and Metabolism

Streptomycetes are obligate aerobes, chemoorganotrophs that need only an organic carbon source (such as glucose, starch, glycerol), an

inorganic nitrogen source, and a few mineral salts for growth. However, faster growth can be obtained in complex media containing, for instance, yeast extract, malt extract, or other organic nitrogen sources. Trace elements contained in tap water are generally sufficient, but addition of iron, manganese, zinc, and other ions can be beneficial.

The majority of species are neutrophilic and mesophilic: optimal conditions for growth are pH 6.8–7.5 and temperatures from 22 to 37°C, usually 28°C. However, one can isolate from acidic soils streptomycetes growing best at pH 4.5 and, from compost or manure, thermophilic or thermotolerant strains growing best at temperatures between 45 and 55°C. Thermophilic streptomycetes should not be confused with members of the genus *Thermoactinomycees,* microorganisms that are morphologically actinomycetes, but, in many biological aspects, are very similar to bacilli.

Few studies have been reported on the carbon compounds and nitrogen assimilation and metabolism in streptomycetes, and it is dangerous to extend to all the genus observations made on a single species. Constitutive active transport systems have been detected for common sugars and inducible ones for other sugars and glycerol. In some cases, cAMP-mediated glucose metabolite repression of inducible enzymes has been demonstrated. Regarding nitrogen assimilation, both glutamine synthetase and glutamate synthase have been detected in some species, and amino acid transport was found to be under both positive and negative feedback control.

Streptomycetes produce several extracellular enzymes, especially hydrolases such as amylases and proteases. There is evidence that their production is repressed when easily utilized nutrients are available. Cellulases and lignocellulases are chiefly found among thermophilic strains as are xylanases, although the latter are also produced by *S. lividans.*

2.3.4. Genetics

As mentioned previously, one characteristic of the genus is the high G + C content of the DNA. The genome size is estimated at about 7500 kb, approximately two times that of *E. coli.* The rate of replication, as determined in *S. granaticolor* and in *S. hygroscopicus,* is 600 bases per replication fork. This value is similar to that for *E. coli,* in agreement with the doubling time, which is around 1½ h at 28°C.

Streptomycetes, as other actinomycetes, are remarkable for a characteristic form of genetic instability. Many different traits, such as aerial mycelium and spore formation, antibiotic production and resistance, enzymes of arginine biosynthesis, are subject to an irreversible loss with a frequency of 0.1–1% of the progeny of plated colonies. This loss of function is the result of the deletion of large DNA regions, often associated with amplification of neighboring DNA segments. The mechanism underlying this and other forms of instability is at present being intensively studied but a complete picture is not available.

2.3.4.1. Chromosome Maps

Two techniques have been used to develop genetic linkage maps in several *Streptomyces* species: conjugation and more recently protoplast fusion.

Intraspecies conjugation has been observed in a number of strains but has been studied in detail only in *S. coelicolor, S. lividans,* and to a lesser extent in *S. rimosus.* In *S. coelicolor,* the major proportion of conjugational exchanges are caused by a plasmid, SCP1, either in its autonomous state or integrated in the chromosome. In strains cured of SCP1, a low level of fertility is still present and is attributed to a second plasmid, SCP2. In *S. lividans,* two analogous conjugative plasmids were originally identified, and named SLP2 and SLP3.

Protoplast fusion is a very powerful technique for the generation of recombinant strains with high frequency. Protoplasts are prepared by digestion of cell walls with lysozyme in the presence of an osmotic stabilizer. Fusion between the protoplasts is achieved by a brief treatment with polyethylene glycol in the presence of calcium ions. Regeneration is obtained by plating the fused protoplasts, preferably as a soft agar suspension, on a suitable solid medium.

By these techniques a detailed circular linkage map has been constructed for *S. coelicolor* and less detailed ones for several other strains (their number is steadily increasing) including *S. lividans, S. rimosus,* and *S. glaucescens.* The maps show many similarities between the different species; their main feature is the tendency of functionally related genes to be clustered together, frequently in two diametrically opposed regions of the map. This suggests a chromosomal duplication in the *Streptomyces* genome evolution.

Several loci have been identified that are related to differentiation. The products of one group, the *bld* genes (from "bald" mutants), are involved in aerial mycelium formation. One of these, *bldA*, determines the synthesis of a specific tRNA recognizing the rare codon TTA which codes for leucine. A second group, the *whi* genes (from "white" mutants), are needed for the conversion of aerial mycelium into spores. Among these, *whiG* codes for a sigma factor required for the translation of genes involved in the first steps of this process. Thus, it appears that there are two different mechanisms regulating differentiation: one at the translational level, that is, the expression of genes containing the TTA codon, and one at the transcriptional level, concerning the genes whose promoters are recognized by a specific sigma factor, as is the case for *B. subtilis.*

2.3.4.2. Transformation

Streptomycetes mycelium or spores are not transformed by foreign DNA. It was thus a major step for the advancement of genetic studies and manipulation of this genus when it was discovered that protoplasts, in the presence of polyethylene glycol, could take up DNA in a reproducible way. Plasmid monomers are taken up effectively by protoplasts and, after regeneration, transformants are obtained with high frequency. The most frequently used host for such transformations is *S. lividans* 66. As vectors, derivatives of plasmids SCP2*, SLP1.2, pIJ101 or of the temperate phage ΦC31 have been used by several laboratories. This technique has been widely used for gene cloning and transfer. Examples are given in the chapter on the genetics of antibiotics.

2.3.5. Industrial Production of Enzymes

Several enzymes produced by streptomycetes have been considered as potential industrial products. At present, D-xylose isomerase, popularly known as glucose isomerase, is largely exploited by the food industry for the conversion of glucose into fructose. Several streptomycetes, as well as other actinomycetes, are excellent sources of this enzyme.

Another industrially produced enzyme, or more precisely a complex of several enzymes, is pronase, routinely obtained as a byproduct

of *S. griseus* fermentations. Pronase is a mixture of several proteases and peptidases, ten of which have been purified and characterized. Similar to pronase is fradiase, obtained from *S. fradiae.*

Cholesterol oxidases, produced by *Streptomyces* or by *Nocardia* strains, have a clinical laboratory application for the quantitative determination of cholesterol in the blood serum.

Several other enzymes are under evaluation: one example we can mention here are the xylanases from *S. lividans,* which have been proposed as bleaching agents in paper manufacturing.

2.3.6. Secondary Metabolites

We have already mentioned the astounding ability of streptomycetes to produce thousands of secondary metabolites, most of which are endowed with a high and selective antimicrobial activity. No less impressive are the variety of the chemical structures produced, which are the result of a variety of biosynthetic pathways and of subtle variations within each pathway. Secondary metabolites are conveniently grouped in families, to some extent reflecting the biosynthetic pathway from which they derive. We may note here that (with a single exception) the only important metabolic pathway not used by streptomycetes is the condensation of isoprenoid units to make terpenoids and sterols. This is in contrast to lower fungi, which produce numerous metabolites of these classes.

2.3.6.1. Aminocyclitols

Antibiotics of this class are typically produced by actinomycetes, especially streptomycetes and micromonosporae. They are pseudosaccharides, containing an aminocyclitol (a six-carbon ring carrying hydroxy and amino or guanidino groups) and a few sugars, mainly aminosugars from which is derived their more common name, aminoglycosides.

Streptomycin (Fig. 1.3), the first antibiotic active against gram-negative bacteria and *Mycobacterium tuberculosis,* was isolated by Waksman's group from culture filtrates of *S. griseus.* It is still an important chemotherapeutic agent. A few related products, such as di-

hydrostreptomycin or mannosidostreptomycin, also produced by streptomycetes, do not have any practical application.

Neomycins. This is a large family of antibacterial antibiotics, chemically characterized by the aminocyclitol 2-deoxystreptamine substituted by sugars in positions 4 and 5 (Fig. 2.3). It includes neomycins B and C, produced by *S. fradiae,* the paromomycins (*S. rimosus*), the lividomycins (*S. lividus*), and ribostamycin (*S. ribosidificus*). Only the latter is used systemically for treating infections; because of toxicity problems the others are used topically.

Kanamycins and tobramycin. In this group 2-deoxystreptamine is substituted by sugars in positions 4 and 6 (Fig. 2.3). Kanamycin A, isolated from cultures of *S. kanamyceticus,* is used as an anti-gram-

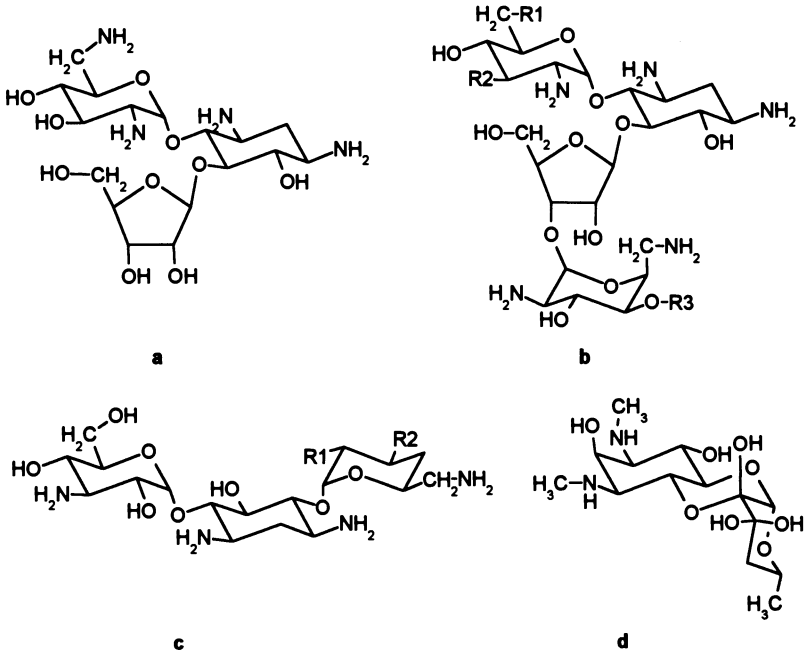


Figure 2.3. Therapeutically active aminoglycoside antibiotics from streptomycetes: (a) ribostamycin; (b) neomycin B (R1 = NH_2, R2 = NH_2, R3 = H), paromomycin I (R1 = R2 = OH, R3 = H), lividomycin A (R1 = OH, R2 = H, R3 = α-mannose); (c) kanamycin A (R1 = OH, R2 = OH), kanamycin B (R1 = NH_2, R2 = OH), tobramycin (R1 = NH_2, R2 = H); (d) spectinomycin.

negative and antimycobacterial agent. Tobramycin, a member of the nebramycin complex produced by *S. tenebrarius,* is important for its activity on *P. aeruginosa* and other difficult pathogens.

Destomycins. Hygromycin B, from *S. hygroscopicus,* and destomycin A, from *S. rimofaciens,* are also derivatives of 2-deoxystreptamine (Fig. 2.4). Because of their anthelmintic activity they have a veterinary use in poultry and swine.

Kasugamycin and validamycins. Produced by *S. kasugaensis* and *S. hygroscopicus,* respectively, these differ from the other antibiotics of the family in the structure of their cyclitols (Fig. 2.4). Both are used in agriculture, to prevent rice diseases caused by fungal infections.

2.3.6.2. Antibiotics Deriving from Polyketide Chains

A large number of metabolites from streptomycetes derive from polyketomethylene chains (polyketides), biosynthesized by condensation of acetate and malonate units. Two families have important therapeutic applications: the antitumor anthracyclines and the antibacterial tetracyclines (Fig. 1.4).

Chemically the antitumor anthracyclines are hydroxyanthraquinones carrying one or more sugar substituents. They were first isolated for their antibacterial activity but later the antileukemic properties of daunorubicin (formerly daunomycin), a product of *S. peucetius,* were observed and found therapeutic applications. A hydroxy derivative of daunorubicin, doxorubicin (formerly adriamycin), produced by a variant strain of *S. peucetius,* was found active against leukemia and several solid tumors. It is at present the agent with the largest spectrum of antitumor activity. Also clinically useful is the more recently discovered aclarubicin, produced by *S. galilaeus.*

The tetracyclines are antibiotics with a broad spectrum of antibacterial activity. Chemically they differ from the anthracyclines mainly by the absence of sugar substituents and the presence of a dimethylamino group on a ring. Originally two tetracyclines were isolated: chlortetracycline from *S. aureofaciens* and oxytetracycline from *S. rimosus.* Later tetracycline was obtained from cultures of a mutant strain of *S. aureofaciens.* Subsequently, several streptomycetes strains producing this antibiotic were reported, mainly in the patent literature. A fourth product, demethylchlortetracycline, is also produced by a mutant

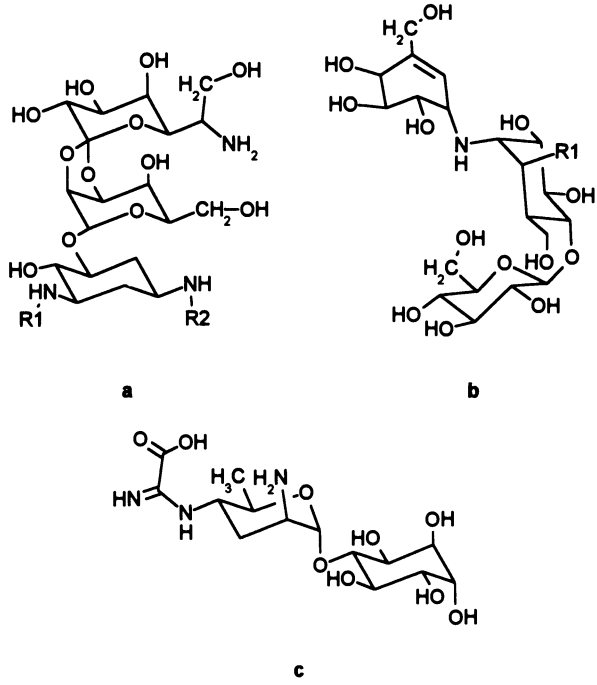


Figure 2.4. Aminoglycoside antibiotics of veterinary and agricultural use: (a) hygromycin B (R1 = H, R2 = CH_3), destomycin A (R1 = CH_3, R2 = H); (b) validamycin A (R1 = β-glucose); (c) kasugamycin.

of *S. aureofaciens*. At present, chlortetracycline is used only in animal husbandry. In human medicine, tetracycline is most commonly used, together with some semisynthetic derivatives, such as doxycycline and minocycline.

2.3.6.3. Metabolites Deriving from Substituted, Reduced, Polyketide Chains

Typical of streptomycetes biosynthetic ability is the formation of variants of the classical polyketide chain, either by the use of methylmalonate, rather than malonate as building block, or by a partial or total reduction of the majority of the carbonyl groups potentially present on the chain. Six main classes of secondary metabolites originate from

this pathway: the antibacterial macrolides, the ansamycins, the polyenes, the polyethers, the elfamycins, and the antiparasitic macrolides.

Antibacterial macrolides are branched macrocyclic lactones, with one or two sugars attached to the ring, active on gram-positive organisms. The ring size varies from 12 to 16 atoms (Figs. 2.5 and 1.5). About 100 compounds are known of which the following are industrially important. These are produced to be used as such, or as starting material for semisynthetic derivatives: erythromycin (*S. erythraeus*), oleandomycin (*S. antibioticus*), leucomycin (*S. kitasatoensis*), josamycin (*S. narbonensis*), spiramycin (*S. ambofaciens*), midecamycin (*S. mycarofaciens*), maridomycin (*S. hygroscopicus*), and tylosin (*S. fradiae*). A variant of the erythromycin producer, originally classified as a *Streptomyces,* has been reclassified into the genus *Saccharopolyspora;* similarly the leucomycin producer is now considered to be a *Streptoverticillium.* All of the above-mentioned macrolides are used in human therapy, with the exception of tylosin, which is used in veterinary medicine and as a growth promoter.

Ansamycins differ from the macrolides in being macrolactams, that is, macrocycles closed by an amide instead of an ester bond. In addition, the cyclic structure comprises an aromatic moiety. They are divided into four groups, differing in their biological activity, according to their ring size and the nature of the aromatic moiety. The only ansamycin industrially produced is rifamycin B (Fig. 1.7), belonging to the group with a naphthalene ring, which is the starting material for the synthesis of the therapeutic agent rifampicin. Rifamycin B is pro-

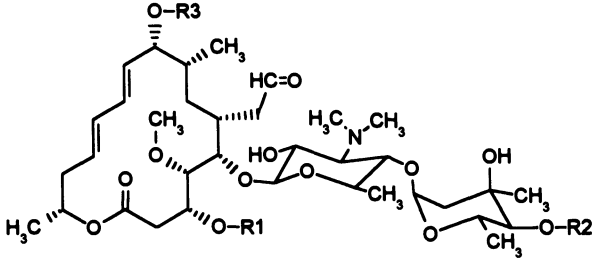


Figure 2.5. Antibacterial 16-membered macrolides: leucomycin A_5 (R1 = H, R2 = butyryl, R3 = H); Josamycin (R1 = acetyl, R2 = isovaleryl, R3 = H); midecamycin (R1 = R2 = propionyl, R3 = H); spiramycin (R1 = R2 = H, R3 = forosaminyl).

duced by a strain originally classified as *S. mediterranei* but later revised to *Nocardia mediterranei.* More recently a different classification, as *Amycolatopsis mediterranei,* has been proposed. Several other streptomycetes producing rifamycin congeners have been reported.

Polyenes are characterized by a large lactone ring (26–38 atoms) and a series of conjugated double bonds. Opposite the double bonds on the ring is a series of hydroxyl groups. Polyenes are subdivided according to the number of double bonds and are typically antifungal agents. Amphotericin B (Fig. 1.6), produced by *S. nodosus,* is used systemically to treat life-threatening mycosis. Other products such as nystatin, from *S. nursei,* and candicidin, from *S. griseus,* are used in topical infections.

Polyether antibiotics are linear aliphatic molecules, highly substituted with methyl or ethyl groups. Their main characteristic is the presence of a series of tetrahydropyran and tetrahydrofuran rings along the chain (Fig. 2.6). About 100 polyethers have been isolated from streptomycetes; many from other actinomycetes. The polyether antibiotics are active against several aerobic and anaerobic bacteria but their commercial usefulness relates to their anticoccidiostatic activity, for which they are used in veterinary medicine. In addition, they are used in animal husbandry because of their ability to increase feed conversion in ruminants. Industrial production is centered on monensin from *S. cinnamonensis,* lasalocid A from *S. lasaliensis,* salinomycin from *S. albus,* and narasin (methylsalinomycin) produced by *S. aureofaciens.*

Elfamycins also have a linear aliphatic structure, but differ from polyethers in having a smaller number of ether bonds, the presence of conjugated double bonds, and at least one nitrogen atom in the molecule. The name derives from their mechanism of action, inhibition of *el*ongation *fac*tor Tu. Useful in veterinary practice and as feed additives are aurodox, produced by *S. goldiniensis,* and mocimycin, produced by *S. ramosissimus* (Fig. 2.7). The latter had been previously isolated from cultures of *S. collinus* under the name kirromycin.

Antiparasite macrolides. The basic structure of antiparasite macrolides is a 16-atom cyclic lactone, which differs from that of the classical antibacterial macrolides in having oxygen heterocyclic rings fused with the macrocycle. They possess anthelmintic, insecticidal, and acaricidal activity, but no antibacterial activity. Two families are known: milbemycins, isolated from strains of *S. hygroscopicus,* and avermectins,

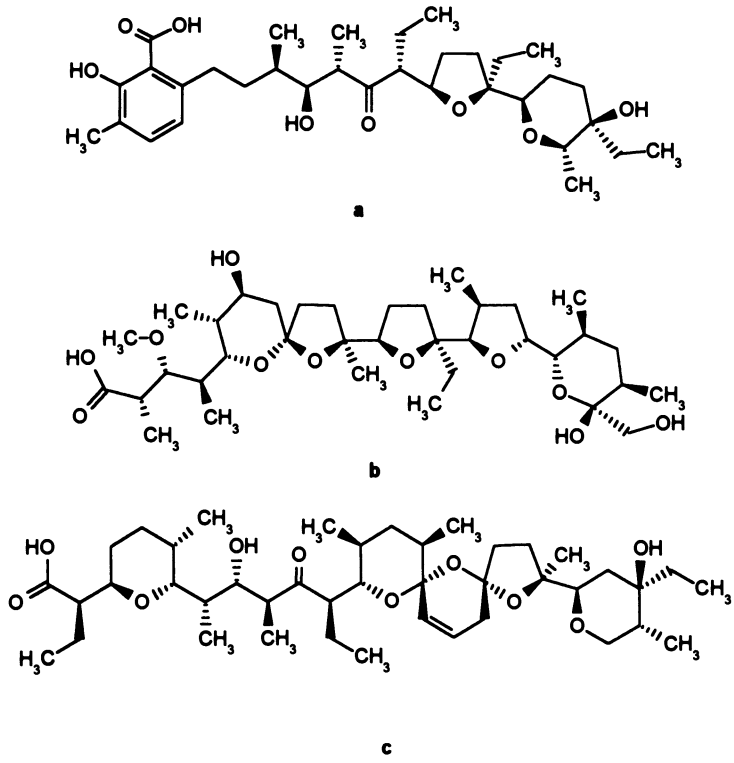


Figure 2.6. Polyether antibiotics: (a) lasalocid A; (b) monensin A; (c) salinomycin.

from *S. avermitilis*. One component of this last family, avermectin B_1 (Fig. 1.10), is used as the starting material for the production of ivermectin, a semisynthetic derivative widely used in veterinary practice.

2.3.6.4. Peptides

Peptide antibiotics are normally generated in streptomycetes by the so-called thiotemplate multienzyme mechanism. The great variety of metabolites produced are related to the variety of building blocks and to extensive postassembly modifications of the original chain. To the variety of structures corresponds a variety of biological activities, some of which are of practical importance.

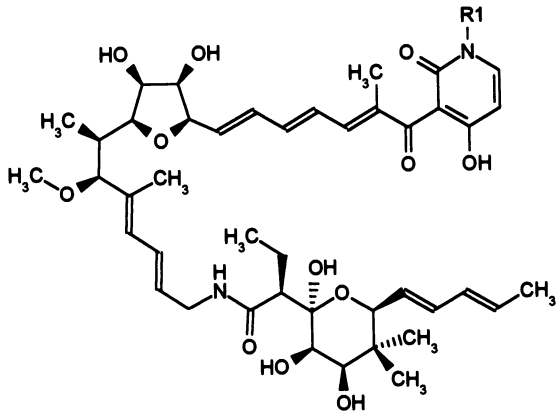


Figure 2.7. Elfamycins: aurodox (R1 = CH_3); mocimycin (R1 = H).

Antitumor peptides. Actinomycins, produced by *S. antibioticus,* have essentially a historical importance, since they were the first antibiotics isolated from a *Streptomyces* strain. Their structure contains a phenoxazinone chromophore and two cyclic peptides. Actinomycin D (Fig. 2.8) has been used for some time in the therapy of solid tumors. Bleomycins, isolated from *S. verticillus,* are complex glycosylated linear peptides containing heterocyclic moieties (Fig. 2.8). They are useful in the treatment of lymphomas and tumors of the skin.

Glycopeptides (dalbaheptides). The vancomycin–ristocetin family of antibiotics is characterized by a multiring core comprising seven amino acids, whose aromatic residues form a triphenylether and a diphenyl moiety. Sugars are attached at various positions on the rings, justifying the name of glycopeptides. Their alternative name, dalbaheptides, reflects their mechanism of action (D-alanyl-D-alanine binding) and the heptapeptide structure. A few dalbaheptides are produced by streptomycetes, many more by other actinomycetes. Among the first, avoparcin, isolated from *S. candidus* cultures, is used as a growth-promoting feed additive. Vancomycin, a clinically important drug, is produced by a strain originally described as *S. orientalis,* but now classified as *Amycolatopsis orientalis* (Section 2.4.2, Fig. 2.15). Another therapeutically useful product, teicoplanin, is produced by an *Actinoplanes* strain (Section 2.4.3, Fig. 2.16).

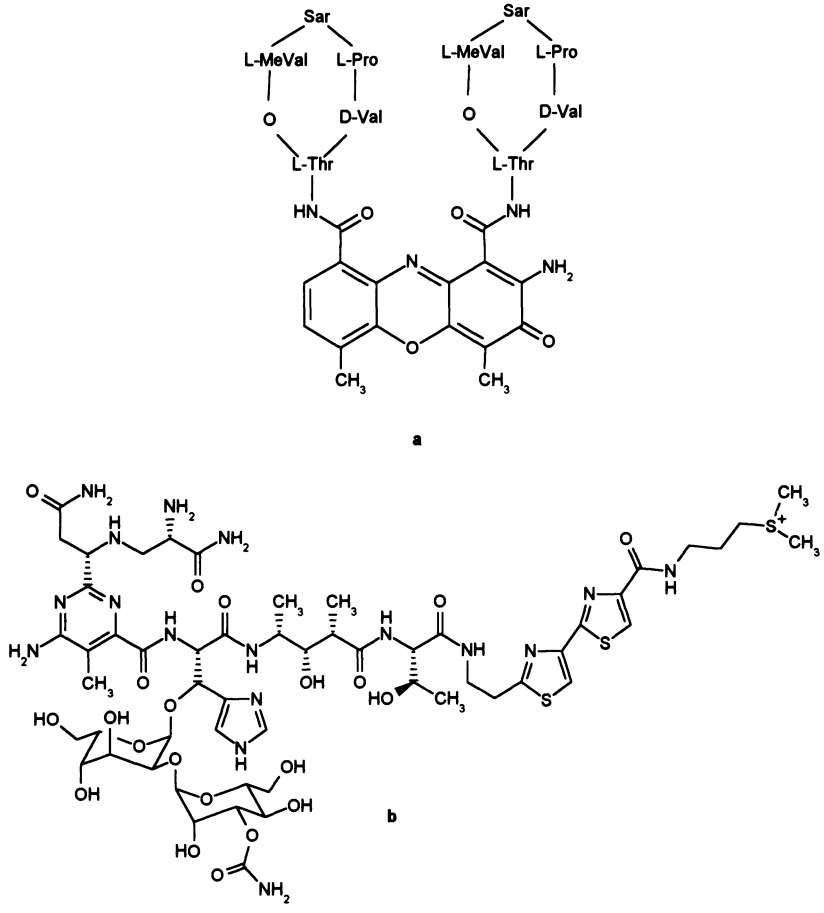


Figure 2.8. Antitumor peptides: (a) actinomycin D; (b) bleomycin A_2.

β-Lactams. For many years it was believed that β-lactams were exclusively produced by lower fungi. It was therefore surprising when it was discovered that *S. lipmanii* produced penicillin N and 7-methoxycephalosporin C. Several other methoxycephalosporins, differing in the substituent at position 3, were subsequently isolated from various strains, e.g., *S. clavuligerus,* and named cephamycins (Fig. 2.9). These are the starting material for the synthesis of therapeutically important antibiotics.

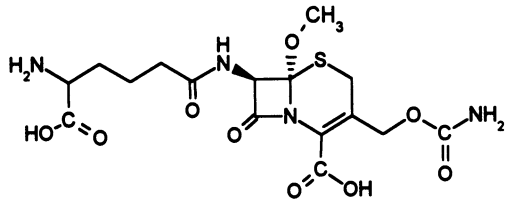


Figure 2.9. Cephamycin C.

The carbapenems also have a β-lactam structure, but are not derived from a tripeptide, like the classical penicillins and cephalosporins. In the carbapenem the β-lactam ring is fused with a five-member ring not containing sulfur atoms. The most important example, isolated from *S. cattleya,* is thienamycin (Fig. 1.2). This serves as starting material for the preparation of imipenem, an antibiotic with an exceptional broad spectrum of antibacterial activity.

In the clavam family the β-lactam ring is fused with an oxygen-containing ring. Clavulanic acid (Fig. 1.2), first isolated from *S. clavuligerus,* is almost devoid of antibiotic activity but is used in combination with other β-lactams because of its ability to inhibit bacterial β-lactamases.

Peptidase inhibitors and immunomodifiers. Several inhibitors of proteinases have been isolated from streptomycetes, mainly because of the pioneering work of Umezawa's laboratory. The inhibitors of serine proteases, such as leupeptin and β-MAPI, are tri- or tetrapeptides with the terminal carboxyl group reduced to an aldehyde. Their production is widespread among streptomycetes. Some inhibitors, acting on the proteases located on the cell surface, are reported to be enhancers of the immune response. The most studied is bestatin (Fig. 1.8), a dipeptide isolated from *S. olivoreticuli,* which has been clinically evaluated in leukemia and melanoma treatment.

Peptides used in agriculture and animal husbandry. Largely used as feed additives for swine and poultry are virginiamycin S_1 and virginiamycin M_1 produced by *S. virginiae.* The first is a cyclic hexapeptide, the second a peptidolactone, in which there is a combination of amide and ester linkages. Another family of antibiotics used as feed additives is that of thiostrepton and related substances (Fig. 2.10). These peptides are characterized by multiple thiazoles and other heterocyclic rings.

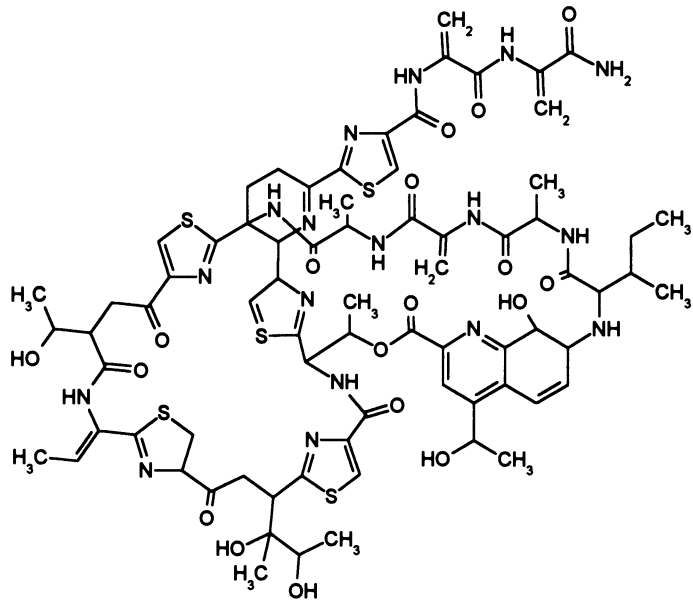


Figure 2.10. Thiostrepton.

Thiostrepton resistance is often used as a marker in genetic and molecular biology experiments. Bialaphos, first isolated from *S. viridochromogenes,* is a tripeptide analogue containing phosphinotrycin and two alanine molecules. It has both antibacterial and herbicidal activity, resulting from the inhibition of glutamine synthetase by the phosphinotrycin moiety.

Streptomycetes also produce a number of depsipeptides, molecules in which amide bonds alternate with ester bonds. A typical example is valinomycin, an ionophore with a cyclic structure selectively complexing potassium ions (Fig. 2.11).

2.3.6.5. Nucleosides

Over 200 nucleoside antibiotics are produced by *Streptomyces* or *Streptoverticillium* species. They have a variety of activities and modes of action but only a few have a practical application, mainly in agriculture.

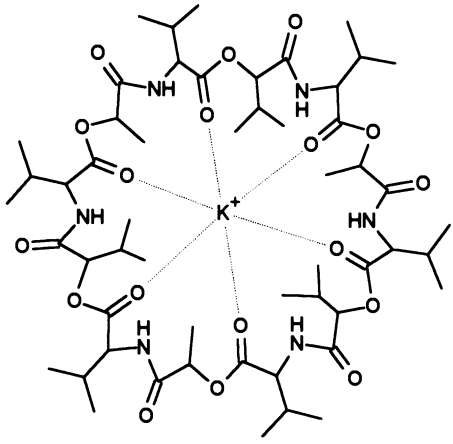


Figure 2.11. Interaction of valinomycin with potassium ions.

Vidarabine (adenosine arabinoside) is an antiviral agent, originally obtained by chemical synthesis, but also produced by *S. antibioticus.* It is clinically useful in the treatment of herpes infections (Fig. 2.12).

Blasticidin S is a pyrimidine nucleoside derivative, produced by *S. griseochromogenes.* Its main use is in the prevention of rice infection by *Piricularia oryzae.* The polyoxins are a complex of peptidyl derivatives of a modified uridine, produced by *S. cacaoi.* They possess a broad antifungal activity against plant pathogens (Fig. 2.12).

2.3.6.6. Other Bioactive Metabolites

Some antibiotics of therapeutic relevance do not belong to any of the biosynthetic classes mentioned above.

Chloramphenicol (Fig. 2.13), an aromatic amino-alcohol, is one of the few natural compounds with a nitro group. It was originally isolated from *S. venezuelae* cultures but is presently produced by chemical synthesis. It possesses a broad spectrum of antibacterial action, especially against gram-negative strains.

Lincomycin (Fig. 2.13) is a product of *S. lincolnesis* fermentations. The molecule is composed of a proline derivative linked to a modified sugar. Its biological activity is similar to that of erythromycin.

Novobiocin (Fig. 2.13) is a complex molecule, first isolated from *S. niveus,* comprising a coumarin, a sugar, and a benzoic acid derivative.

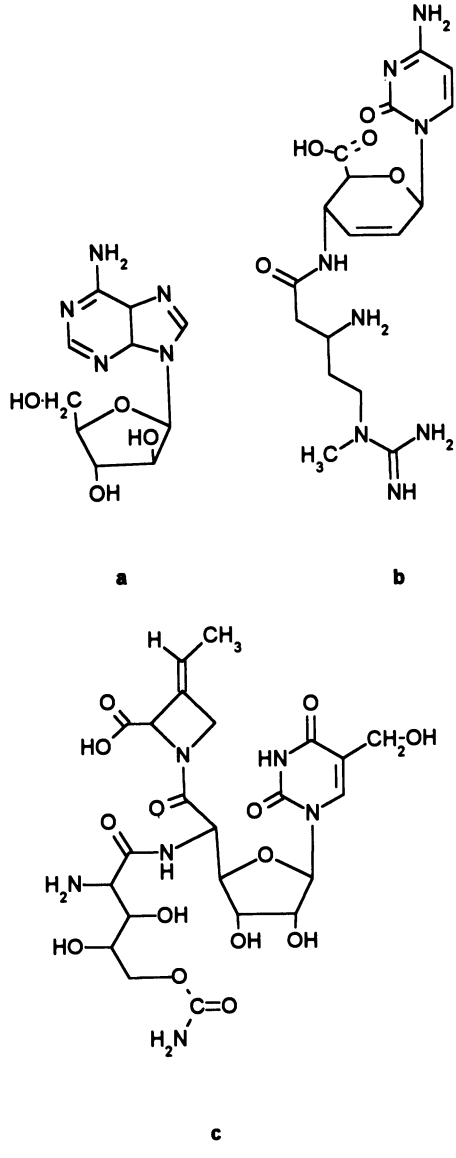


Figure 2.12. Nucleoside antibiotics: (a) vidarabine (Ara-A); (b) blasticidin S; (c) polyoxin A.

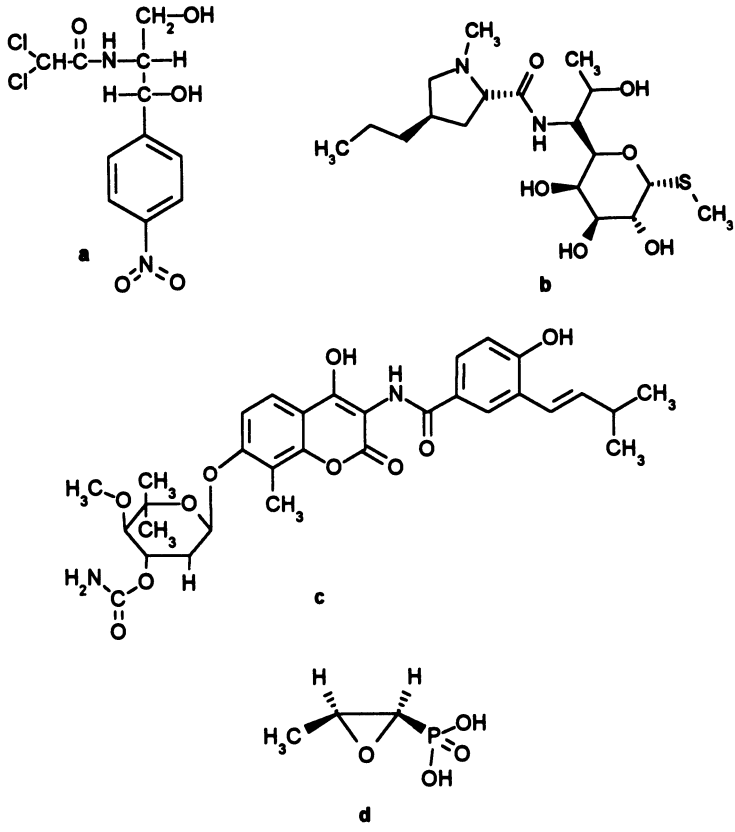


Figure 2.13. Miscellaneous therapeutically active antibiotics from *Streptomyces:* (a) chloramphenicol; (b) lincomycin; (c) novobiocin; (d) fosfomycin.

It has some value as an antistaphylococcal agent in human medicine, but is now mainly used in animal health care.

Fosfomycin (previously phosphonomycin, Fig. 2.13) is an inhibitor of peptidoglycan synthesis, produced by several streptomycetes, such as *S. fradiae.* Fosfomycin is moderately active on a wide range of gram-positive and gram-negative bacteria.

FK 506 is a product of *S. tsukubaensis* with a complex structure comprising a 23-member lactone (Fig. 1.8). It is undergoing advanced clinical trials for its very interesting activity as an immunosuppressive

agent. Several analogues, produced by other *Streptomyces* strains, are also being intensively studied.

2.4. Genera of Actinomycetales Other Than Streptomyces

Actinomycetes are a large group of diverse bacteria, having in common the characteristic (in some cases the simple tendency) to grow as filaments. They can be separated into two broad groups: the fermentative ones, generally living associated with man or animals, and the aerobic soil inhabitants.

We will deal here only with the genera of the last group in which antibiotic-producing species are frequent. These organisms are widespread in nature belying their classification as "rare actinos," which was related to being less easily detected than streptomycetes. The use of selective isolation techniques has demonstrated that some genera are not rare at all and that a number of strains can be found in any soil sample. No clear-cut habitat preference can be established for the different genera; however, some, such as *Micromonospora* or *Actinoplanes,* are more abundant in decaying plant material or muddy soils on freshwater shores. Thermophilic species are often found in natural warm habitats, such as compost or hay mounds.

2.4.1. Taxonomy

Information on the physiology, biochemistry, and genetics of actinomycetes is scant, generally limited to taxonomically relevant traits and properties related to applied microbiology. However, in most instances a similarity with *Streptomyces* biology can be assumed.

The taxonomy of Actinomycetales is very complex and rapidly evolving as new concepts and criteria are adopted by the specialists of this discipline. Traditionally the separation in genera has been based on morphological characteristics such as the presence or absence of aerial mycelium, the presence and the arrangement of spores and of spore-containing bodies (sporangia), the spore motility, and the formation of specialized structures. More recently, chemical properties have been accepted as valuable taxonomic characters. These include the cell membrane and wall composition and are especially useful in

organizing the Actinomycetales into groups, each comprising several related genera, roughly corresponding to the taxon "family," a term not universally used in actinomycetes taxonomy. We give here a brief description of the groups which include genera relevant for applied microbiology, essentially following the classification adopted by the 1989 edition of *Bergey's Manual of Systematic Bacteriology.*

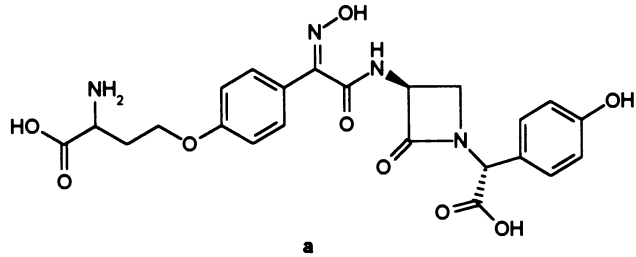
2.4.2. Nocardioform Actinomycetes

This is a very heterogeneous group that includes the actinomycetes which form a mycelium breaking up into coccoid or rod-shaped fragments. All of the organisms are gram-positive aerobes, but may differ in their chemical characteristics, except for the menaquinone structure which invariably contains eight or nine isoprene units. They are very common in soil, but some species are animal or plant pathogens.

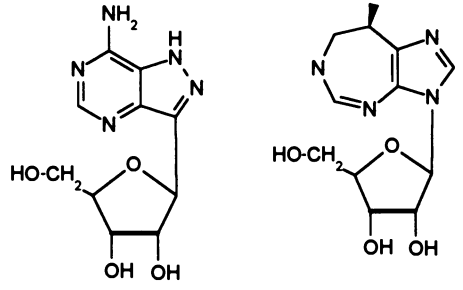
Nocardia. Morphological traits include the presence of both vegetative and aerial hyphae, sometimes rudimentary, fragmenting into nonmotile elements. Chains of nonmotile spores may be formed. Cell wall components include *meso*-diaminopimelic acid, arabinose, and galactose. Mycolic acids are present, with chains of 46–60 carbon atoms. Membrane fatty acids are linear or bear a methyl group at position 10.

Nocardiae are mesophilic and grow on simple media containing ammonia, nitrates, or amino acids as N sources and glucose or acetate as C sources. Growth is slow, the doubling time being about 5 h, at least for the species tested. Typical of several strains is the capability of utilizing, as C sources, long-chain alkanes and even gaseous hydrocarbons.

A variety of antibiotics are produced by nocardiae; however, the strains producing the most important ones, rifamycins, vancomycin, and ristocetin, have been recently attributed to a different genus (see below). Vice versa, the β-lactams cephamycin A, B, and C (Fig. 2.9) are produced by a strain originally considered a *Streptomyces* and later classified as *Nocardia lactamdurans.* A strain of *N. lactamdurans* also produces the elfamycin antibiotic efrotomycin (Fig. 2.14). Products of some interest for their antitumor activity are the ansamytocins, ansamycins produced by *Nocardia* species. The nocardicins (Fig. 2.14), monobactam antibiotics isolated from *N. uniformis,* are also interesting. Formycin and coformycin (Fig. 2.14) are two nucleoside antibiotics



a



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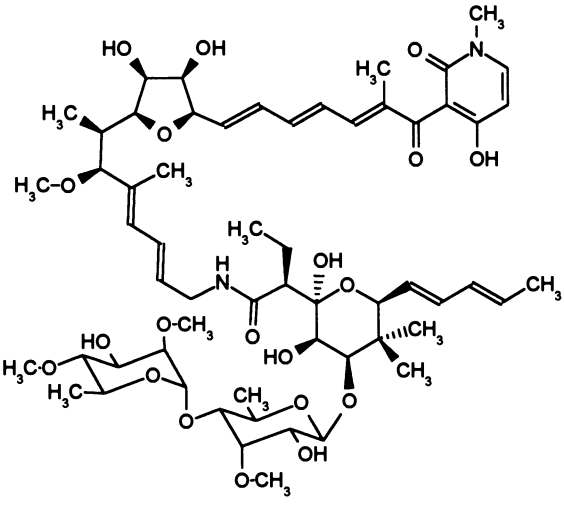

d

Figure 2.14. Antibiotics from *Nocardia:* (a) nocardicin; (b) formycin A; (c) coformycin; (d) efrotomycin.

produced by *N. interforma.* The latter has no antimicrobial activity, but is an inhibitor of the enzyme adenosine deaminase. Because of this property, coformycin potentiates the activity of vidarabine and other nucleoside antibiotics, by preventing their metabolic degradation. A similar activity is shown by 2-deoxycoformycin, a metabolite isolated from *S. antibioticus.*

Amycolatopsis. This genus has been recently proposed to accommodate species, formerly classified as *Nocardia,* which do not contain mycolic acids. The other taxonomic traits are similar to those of nocardiae, except for the presence of branched-chain fatty acids in the *Amycolatopsis* cell membrane. Several species have been described which produce antibiotics belonging to different biosynthetic classes. Glycopeptides (dalbaheptides) are rather frequent and among them the therapeutically important vancomycin (Fig. 2.15) is produced by *A. orientalis* and ristocetin by *A. orientalis* subsp. *lurida. A. orientalis* strains also produce an elfamycin antibiotic and the muraceins, muramyl peptide derivatives found to be inhibitors of angiotensin-converting enzyme.

A. mediterranei is the producer of the important ansamycins rifamycins. The original strain produces a complex of several compo-

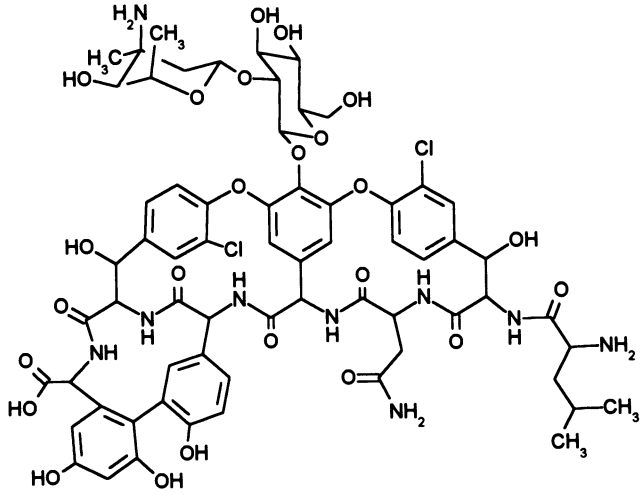

Figure 2.15. Vancomycin.

nents, among which rifamycin B (Fig. 1.7) was selected and used for the preparation of semisynthetic derivatives. Rifamycin SV was first prepared chemically from rifamycin B, but later was also isolated from a *Nocardia* species. *A. mediterranei* is one of the few actinomycetes whose genetics has been studied in some detail. Recombinants can be obtained with high frequency by conjugation of marked strains. By this technique a circular linkage map was constructed which, when compared with the much more complete *S. coelicolor* map, shows a good correspondence in the sequence of homologous markers. In addition, a method for the formation and regeneration of protoplasts has been described.

Saccharopolyspora. Organisms of this genus, which includes only two validated species, differ from streptomycetes in two respects: the tendency of their substrate mycelium to fragment, and the composition of the cell wall, containing *meso*-diaminopimelic acid, galactose, and arabinose. The species of interest is *S. erythrea* (formerly *Streptomyces erythreus*), the producer of the well-known antibiotic erythromycin (Fig. 1.5).

2.4.3. Actinoplanetes

The group actinoplanetes comprises actinomycetes producing motile spores enclosed in vesicles called sporangia. However, the genus *Micromonospora,* which bears nonmotile single spores, is also included in this group, because of similar chemical traits and the homology of nucleic acids. Actinoplanetes are gram-positive organisms, growing with branched septated hyphae; aerial mycelium is rarely developed and never abundant. The peptidoglycan of the cell wall contains *meso*-diaminopimelic or 3-hydroxydiaminopimelic acid and glycine rather than L-alanine. In the cell membrane iso- and anteiso-fatty acids predominate. Although the production of motile spores suggests an adaptation to aquatic habitats, actinoplanetes are found in any type of soil. They are aerobic and mesophilic, growing well at temperatures between 20 and 30°C and at a pH around 7.

Actinoplanes. Colonies of *Actinoplanes* are small, reflecting the slow growth of these organisms. The vast majority have a characteristic orange color, although brown, red, and blue strains have been described. The color is the result of carotenoid pigments. The sporangia are sub-

spherical, often borne on stalks called sporangiophores. Spore flagella are polar. *Actinoplanes* strains can grow on minimal media, in which sporulation is favored, but they are usually cultured in the rich media generally recommended for actinomycetes. Typically they utilize xylose or chitin as carbon sources; the enzyme xylose isomerase from *A. missourinensis* is used industrially for the conversion of glucose to fructose.

Genetics has been studied in *A. brasiliensis,* where methods for the selection of auxotrophic mutants and the formation and regeneration of protoplasts have been described.

More than 120 antibiotics have been isolated from *Actinoplanes* strains. Amino acid derivatives, such as peptides and depsipeptides, are prevalent, but polyene, aromatic, nucleoside, and chloro-heterocyclic compounds are also produced. Of clinical relevance is the lipoglycopeptide teicoplanin (Fig. 2.16), used in the therapy of gram-positive infection, isolated as a complex of five components from *A. teichomyceticus* cultures. Of potential therapeutic interest is ramoplanin, a macrocyclic peptide with sugar and fatty acid substituents, isolated from

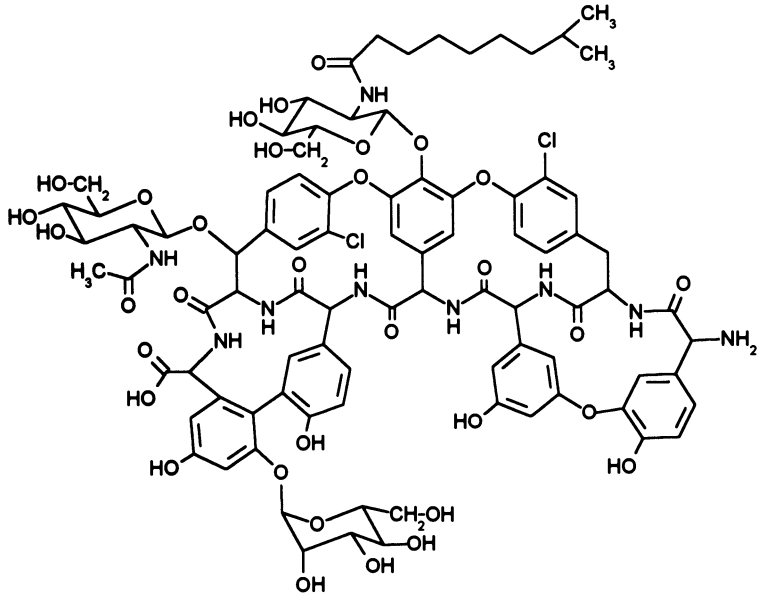

Figure 2.16. Teichomycin T-A2-2, the major component of teicoplanin.

Actinoplanes ATCC 33076. Other products of interest are the pleuracins, mixtures of macrocyclic lactones and depsipeptides, closely related to the virginiamycin group, isolated from *A. auranticolor* and *A. azureus;* lipiarmycin, an unusual macrolide inhibitor of RNA polymerase produced by *A. deccanenensis;* purpuromycin, a polyketide with a broad spectrum of antibacterial and antifungal activity produced by *A. ianthinogenes.*

A tetra-pseudosaccharide, produced by *Actinoplanes* SE 50, is a potent inhibitor of glucoamylases and has been developed for medical use in the treatment of metabolic diseases under the name acarbose.

Dactylosporangium. The name of this genus derives from the characteristic finger-shaped sporangia, arising from the substrate mycelium, each containing a row of three or four motile spores. Aerial mycelium is not formed. The chemical and biological properties are similar to those of *Actinoplanes;* however, not all of the strains grow on basal medium, and glycerol is not utilized as carbon source.

Although only 30 antibiotics from *Dactylosporangium* strains have been described, the variety of their structures indicate that this genus possesses a great biosynthetic capability. Noteworthy is the frequency of aminocyclitols, including sisomicin (Fig. 2.17) and *N*-formylsisomicin produced by *D. thailandense,* and dactimicin by *D. matsuzakiense.* An antimycobacterial peptide, capreomycin, previously isolated from *S. capreolus,* is produced by *D. variesporum,* and a polyether antibiotic, nigericin, by *D. salmoneum. D. aurantiacum* produces the family of tiacumicins, macrolides related to lipiarmycin. The polyketide-derived antibiotics dactylocycline A (4-hydroxy-8-methoxychlortetracycline) and DK-7814-A (hydroxypurpuromycin) have been isolated from cultures of *Dactylosporangium* SCC 1695 and *D. purpureum,* respectively.

Micromonospora. Colonies of *Micromonospora* strains are macroscopically similar to those of *Actinoplanes,* showing the same orange

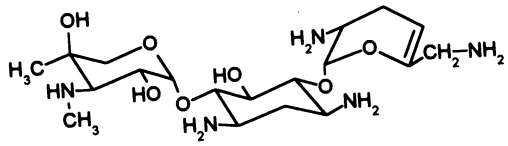

Figure 2.17. Sisomicin.

color, but can be easily distinguished microscopically by the absence of sporangia. Aerial mycelium is absent, spores are borne on sporophores on the vegetative mycelium and are often also observed in liquid cultures. Chemical characteristics are similar to those of *Actinoplanes.* Cultures require neutral pHs; common sugars and starch are suitable sources of carbon with ammonia or amino acids (preferably the acid or basic ones) serving as nitrogen sources. The preferred habitats are lake muds and river sediments, where they are sometimes the only actinomycetes present. Genetic studies have been reported on *M. echinospora* and *M. olivasterspora.*

Over 300 antibiotics have been described from *Micromonospora* strains. They belong to almost all of the classes found in streptomycetes. The most important ones are: the aminocyclitols, such as the gentamicins (Fig. 1.3), produced by *M. purpurea* and *M. echinospora;* sisomicin (Fig. 2.17) from *M. inyonensis;* fortimicin from *M. olivoasterospora.* Also of practical interest are the macrolides rosamicin, from *M. rosaria* and the mycinamicins isolated from *M. griseorubida.* A family of macrolides closely related to lipiarmycin, the clostomicins, are produced by *M. echinospora.* Ansamycins of the rifamycin family, the halomycins, have been isolated from *M. halophytica.* The polysaccharide everninomycin is a typical product of *M. carbonacea.*

2.4.4. Maduromycetes

Maduromycetes are a group of rather diverse actinomycetes, forming a substrate mycelium bearing aerial hyphae that differentiate into short chains of spores or into sporangia containing either motile or nonmotile spores. A characteristic trait is the presence of the sugar madurose in whole-cell hydrolysates; however, in some species this is present only in trace amounts. The cell wall contains *meso*-diaminopimelic acid and no characteristic sugar. Antibiotics have been isolated from several strains belonging to genera of this group, such as *Streptosporangium* or *Planobispora.* However, *Actinomadura* is the only genus producing a large variety of secondary metabolites, and thus will be described here.

Actinomadura. Colony appearance of strains of this genus is similar to that of streptomycetes. However, they can be distinguished microscopically: in contrast to streptomycetes, the spore chains are usually

short and the spore diameter noticeably exceeds that of the hyphae. Actinomadurae are rather slow-growing organisms: formation of spore-bearing aerial mycelium requires 10–14 days under optimal conditions. The natural habitat of actinomadurae is soil; the frequency is higher in the cultivated than in the uncultivated ones. Two species are pathogenic for man, causing actinomycetomas by infecting open wounds. Little is known about the physiology or the genetics of actinomadurae. Conditions for growth are similar to those of other actinomycetes. Sugars and inorganic salts are normally sufficient as nutrients; starch and proteins are hydrolyzed and can sustain growth. Unlike actinoplanetes, chitin and xylan are not utilized.

About 250 antibiotics have been isolated from *Actinomadura* strains. Most frequently found are the ionophoric polyethers, as the madurimicins produced by *A. yumaensis* and cationomycin produced by *A. azurea*. Also frequent are the antitumor anthracyclines, prominent among which are the carminomycins (Fig. 2.18), produced by *A. roseoviolacea* and other strains. Peptides of interest are: the dalbaheptides parvodicin (*A. parvosata*) and A-40926 (*Actinomadura* ATCC 39727) and the angiotensin-converting enzyme inhibitor I-5 B (*A. spiculosospora*). Characteristically classical antibacterial macrolides are not produced; however, structurally similar products, the macrolactams, have been isolated from *A. fulva* and other strains.

2.5. *The Myxobacteria*

The myxobacteria are a large group (order) of gliding gram-negative bacilli that, under starvation conditions, may aggregate to form complex

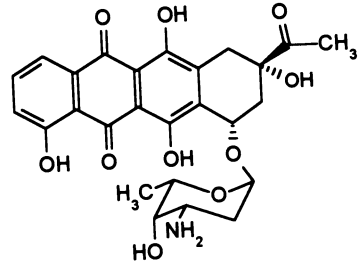

Figure 2.18. Carminomycin.

structures called fruiting bodies. The shape and the dimension of these structures are species specific; hundred of thousands of cells cooperate in their formation. Within the fruiting bodies the vegetative cells may assume a dormant state, transforming into myxospores. Myxobacteria are ubiquitous in the soil, in decaying plant material, and in the dung of herbivorous animals.

2.5.1. Taxonomy and Description

The myxobacteria are phylogenetically a homogeneous group, as indicated by the G + C content of DNA (which is between 67 and 71%) and the homology of 16 s rRNA sequences. Colonies are characteristically spreading; the edges of the swarm usually show flamelike protrusions and often a lacelike border zone. Another typical trait is their bright yellow to red color, produced by carotenoid pigments.

Within the myxobacteria two subgroups are recognized, the Cystobacterinae including, among others, the genera *Myxococcus* and *Corallococcus;* and the Soranginae comprising the genera *Sorangium* and *Nannocystis.* Organisms of the first group are slender rods with tapering ends; those of the second group are stout cylinders with blunt ends. Other morphological differences between the two groups are the shape of the swarm colonies and of the myxospores. Myxospores of *Myxococcus* are round refractile bodies, resistant to desiccation; those of the soranginae do not substantially differ in shape from the vegetative cells.

The size of the fruiting bodies varies from 60 to 600 μm; their shape also differs from species to species—from relatively simple spheres on a stalk to complex treelike structures.

2.5.2. Physiology and Metabolism

Myxobacteria are strictly aerobic, mesophilic, and grow at neutral pHs. The two above-mentioned subgroups differ noticeably in their nutritional requirements. Strains of *Myxococcus* and related genera live on proteinaceous material, including whole bacterial cells, which are hydrolyzed by a number of proteolytic and other bacteriolytic enzymes. The growth is cooperative since the concentration of hydrolytic enzymes needed to degrade the macromolecular material is dependent on cell density. Sugars are not metabolized but some strains can hy-

drolyze polysaccharides. In the laboratory they can be grown on peptone or casein media. Organisms related to *Sorangium* are able to degrade cellulose and require carbohydrates for growth. Ammonium ions are sufficient as nitrogen sources but nitrates may be preferred. It should be noted that myxobacteria stop growing when the concentration of ammonium ions reaches a high value because of amino acid catabolism.

The mechanism of gliding, that is, the ability to move on surfaces without the aid of flagella, is poorly understood. It is connected with the production of a slime, which leaves visible tracks on the agar surface, and it has been shown to be governed by two distinct multigene systems. A remarkable feature of myxobacteria, unique among prokaryotes, is the social behavior, which involves thousands of cells and is dependent on three interrelated properties: the secretion of specific molecules, the gliding motility, and cell-to-cell contact interaction. Social behavior is displayed in feeding, in swarm movement, and in development, that is, the formation of fruiting bodies and sporulation.

Spore formation within the fruiting bodies is a slow process, requiring up to 48 h. Sporulation can be rapidly induced by addition of glycerol to the medium of growing cultures. However, glycerol-induced spores are somewhat different from fruiting body spores, in that they have thinner coat layers with a different chemical composition.

2.5.3. Antibiotic Production

A systematic screening of myxobacteria for antibiotic production was initiated only in the late 1970s, and soon revealed the remarkable biosynthetic ability of these organisms. Activities were detected with a very high frequency and over 100 products have so far been identified. These include aromatic and heterocyclic derivatives, several lactam and lactone macrocycles, and peptides. Nucleosides or modified sugars have not been found. Most are novel compounds but althiomycin and pyrrolnitrin (both from *Myxococcus fulvus*) were previously described from streptomycetes and pseudomonads, respectively. Interesting antibacterial activities are shown by the sorangicins (Fig. 2.19), macrolactones produced by *Sorangium cellulosum,* and by the corallopyronins, isolated from *Myxococcus coralloides.* Ambruticin (Fig. 2.19), also produced by *S. cellulosum,* is an antifungal agent potentially active against human mycoses, and the macrocyclic lactones soraphens, iso-

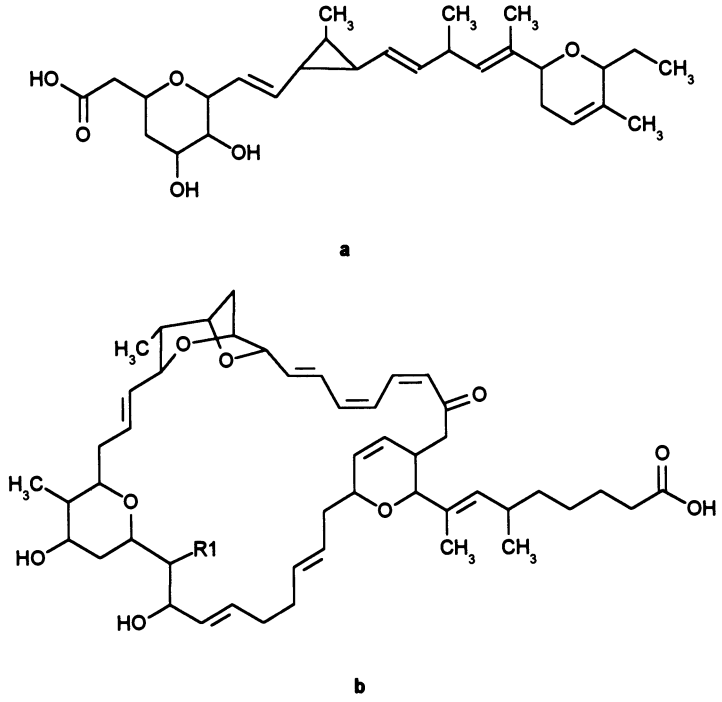

Figure 2.19. Examples of antibiotics from myxobacteria: (a) ambruticin; (b) sorangicin A (R1 = OH), sorangicin B (R1 = H).

lated from strains of the same species, have been considered as antifungal agents for plant protection.

2.6. *Genus Aspergillus*

Aspergilli are filamentous fungi, belonging to the Deuteromyces or Fungi Imperfecti, so denoted because they do not reproduce sexually. Characteristic of the genus is the conidial apparatus, a spore-bearing structure consisting of an elongated hypha terminating in a round vesicle from which chains of spores depart radially. The name derives from the shape of this apparatus, resembling that of an Aspergus, a device used in Catholic churches for sprinkling holy water.

Aspergilli are among the most diffused organisms in nature. The prevalent habitat is soil, especially in warm climates. Many species live on animal or plant residues, spoiling for instance stored grains. Some strains are opportunistic pathogens for animals and man, and can be found especially in the lungs.

2.6.1. Taxonomy and Description

The genus *Aspergillus* belongs to the family Moniliaceae, filamentous fungi with hyphae divided by incomplete septa, allowing cytoplasmic continuity within the mycelium. The genus was classified among the Fungi Imperfecti as lacking a sexual stage of reproduction. However, a major taxonomic problem arose when some species were found to produce sexual spores, contained in special bodies, the cleistothecia. Nomenclature experts proposed that the "perfect" form be classified in separate groups and genera. However, other taxonomists argued that it was simpler to maintain the same name for both the sexual (teleomorph) and imperfect (anamorph) forms. This last proposal has been largely adopted by industrial microbiologists and by many mycologists.

The general morphological and chemical features are similar to those of the other filamentous fungi: hyphae are on average 5 μm thick; the cell wall is composed of polysaccharides; fibrils of chitin constitute the rigid element. The cells are normally haploid but in some cases diploid cells develop as well. The conidial apparatus is composed of a stalk, the conidiophore, which is a vertical hypha enlarging at the top into a globose vesicle, the conidial head. The vesicle bears either on all its surface or on definite areas a single or double layer of elongated fertile cells, the phialides, from which chains of conidiospores depart. In sexually reproducing species, asci, each containing eight spores, are irregularly packed in a high number into baglike bodies called cleistothecia.

2.6.2. Physiology and Metabolism

Aspergilli grow, either as large colonies on solid media or as pellets in liquid media, on very simple nutrients. Chemically defined media may contain a sugar as the carbon source and nitrates or asparagine as

nitrogen sources. Suitable complex media may be based on peptone, potatoes, oak flakes, yeast extract, or extracts of hay, plum, etc. Aspergilli are strictly aerobes, and have a high oxygen requirement. For most species the optimal temperature is around 25°C but a few grow well at 37°C and over. A typical feature of ecological significance is the broad spectrum of pH tolerance, ranging from acidic to alkaline. The tolerance to osmotic pressure is also noteworthy: some strains are selectively isolated on media containing 7–8% NaCl.

Some species, such as *A. niger* and *A. terreus,* excrete organic acids when growing; this property may confer on them an ecological advantage since most of the soil bacteria do not grow in a low-pH environment. In addition to antibiotics, some species produce mycotoxins, of which the most studied (and dangerous) is the aflatoxin from *A. flavus.* Another important metabolic aspect is the production of large amounts of proteolytic and amylolytic enzymes, by which the growing hyphae degrade the complex organic constituents of plant or animal residues into simple nutrients.

2.6.3. Genetics

Aspergillus nidulans is one of the best characterized organisms and is a very good model system for the study of gene expression and gene regulation in eukaryotes. Asexually it reproduces by uninucleate conidia which are suitable for mutagenic treatment and the selection of mutants with easily recognizable markers, such as auxotrophy, spore color, and resistance to drugs.

In sexual reproduction a single event produces cleistothecia containing thousands of asci, each holding eight spores. *A. nidulans* is homothallic, that is, it is not divided into mating types: for genetic studies, parental strains with contrasting spore colors are used, to distinguish hybrid cleistothecia from those originated by self-fertilization.

A third important reproduction mechanism is the parasexual cycle. This consists of the following steps: (1) formation of a heterokaryon in which two haploid nuclei share the same cytoplasm; (2) fusion of the two nuclei into a heterozygous diploid; (3) mitosis. Mitotic crossing over can occur between homologous chromosomes during mitosis or, alternatively, nondisjunctive unstable nuclei with an abnormal number of chromosomes may be formed, which then revert to the haploid state.

The parasexual cycle has been demonstrated to occur in several *Aspergillus* species, facilitating the genetic study of these organisms.

The combination of mutagenesis on conidia, analysis of the sexual progeny (that reveals the linkage of genes within the chromosome), and analysis of the parasexual progeny (used mainly to assign genes to chromosomes) has allowed the construction of a detailed genetic map of *A. nidulans.*

Most of the subsequent studies have dealt with the regulation of gene expression. In contrast to *E. coli,* the genes coding for sequential metabolic function are seldom clustered. In general, each metabolic pathway for the utilization of nitrogen or carbon sources is under the control of a regulatory gene that responds to a specific inducer. For example, the *prnA* gene mediates proline induction of the enzymes of proline catabolism, and the *nirA* gene mediates the nitrate or nitrate induction of the activities involved in nitrate assimilation. In addition, there are wide-domain regulatory genes which control the expression of several pathways. For instance, the gene *areA* mediates the repression by glutamine of a number of enzymes involved in nitrogen metabolism and the negatively acting gene *creA* mediates the carbon catabolite repression of enzymes related to the utilization of several carbon compounds.

2.6.4. Industrial Applications

Aspergilli are among the most important organisms of industrial microbiology. Their products can be divided into two main classes: organic acids and enzymes. In addition, they are widely used for the preparation of Oriental food and beverages. Large amounts of citric, gluconic, and tartaric acids are produced by different strains of *A. niger;* and of itaconic acid by *A. terreus.* The enzymes α-amylase and glucoamylase, used in the starch industry, are produced by *A. oryzae.* Glucoamylases are also obtained from *A. awamory* and *A. niger.* Strains of this latter organism, and of *A. wentii,* are also used for the production of pectinases.

The possibility of using aspergilli as hosts for the commercial production of heterologous proteins has been widely studied. Two main systems are being developed: the *A. nidulans* system based on the *alcA* (alcohol dehydrogenase I) promoter and its regulator gene *alcR;* and

the *A. niger* var. *awamory* system, based on the promoter of the glucoamylase gene *gluA*.

2.6.5. Secondary Metabolites

Aspergilli produce secondary metabolites in a relative high frequency, although very few of them are of practical interest. Several are dangerous mycotoxins, such as the aflatoxins and tremorgen from *A. flavus;* cytochalasin E from *A. clavatus;* and ochratoxin A from *A. ochraceus.*

A large number of metabolites are polyketide derivatives. It should be noted that all of them are purely acetate–malonate derived; aspergilli do not produce the partially reduced, branched polyketide chains that are the origin of the useful antibacterial macrolides or the ionophoric polyethers. The only commercially important polyketide is mevinolin (lovastatin, Fig. 1.9), an inhibitor of cholesterol synthesis, isolated from cultures of an *A. terreus* strain.

Several terpenoids have been reported in the literature; among them is helvolic acid, an antibiotic structurally related to fusidic acid.

Amino acid-derived metabolites are also rather common; penicillins are produced by *A. nidulans,* which is not, however, a strain used for industrial production. *A. alliaceus* produces the asperlicins (Fig. 1.9), nonpeptide amino acid derivatives, under development as cholecystokinin antagonists. The small peptides aspergillomarasmine A and B, which present a moderate activity as angiotensin-converting enzyme inhibitors, were isolated from *A. oryzae* as well as from other fungal strains. Two species, *A. nidulans* and *A. rugulosus,* produce the lipopeptide antifungal agents echinocandins. Through biological and chemical modifications of one of these, echinocandin B (Fig. 2.20), the antifungal agent cilofungin, presently under development, has been synthesized.

2.7. Genus Penicillium

Organisms of the genus *Penicillium* are Fungi Imperfecti that grow in mycelial masses comprising septated hyphae and producing a characteristic spore-bearing apparatus, the conidiophore, resembling in

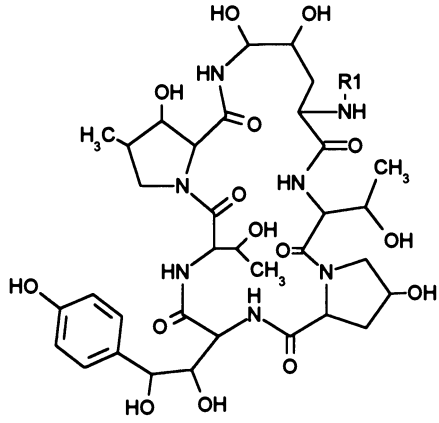

Figure 2.20. Echinocandin (R1 = linoleoyl).

shape a small artist's brush (*penicillus,* Latin for a small brush). The conidiophores consist of a stalk, arising from a submerged or aerial hypha, often branched and carrying on the tip a number of specialized cells, the phialides, from which chains of spores (conidia) originate.

Most *Penicillium* species are saprophytes, growing primarily in soil and on decaying vegetation. However, some species are associated with living tissue; for example, *P. expansus* grows on apples and *P. italicum* and *P. digitatum* on citrus fruits.

2.7.1. Taxonomy and Description

Penicillium hyphae are narrow, hyaline, divided by septa that contain pores allowing cytoplasmic continuity; the hyphal segments are polynucleated. When grown on agar, they form large colonies of different texture according to the species and the stage of development. Mature colonies usually appear gray-green to blue-green because of the presence of a large number of colored spores. The shape of the conidiophores (presence or absence of branches, number of branch levels and their symmetry) is the basis of the division of the genus into groups. Within groups species are defined according to various characters, such as: color, size, and texture of the colonies (including reverse color); form and surface of phialides and conidiospores.

As with the case for aspergilli, although by definition *Penicillium* organisms reproduce asexually, species producing sexual spores, in asci

contained in larger bodies, the ascomata or cleistothecia, have been identified. These were classified into two genera, the *Eupenicillium* and the *Talaromyces* according to the texture of the ascomata, which is hard in those of the first and soft in those of the latter.

2.7.2. Physiology and Metabolism

Members of the genus *Penicillium* are aerobic, mesophilic organisms. Normally they grow well at around 25°C; only a limited number of species can grow at either 5 or 37°C. Most species can grow over a wide range of pH and at low water activity, as for instance in media containing 25% glycerol.

Simple nutrients are generally required: sugars or polyalcohols are suitable sources of carbon and nitrates of nitrogen. However, several species can degrade cellulose or pectin; others produce lipases, such as the well-studied phospholipase B, allowing growth on fats. Amino acids are also utilized as nitrogen sources, and growth is usually more rapid in media containing peptone.

Growth in liquid media has been widely studied with the penicillin producer *P. chrysogenum,* using either batch or continuous culture. Dependent on the medium composition and cultural conditions are different morphologies, from a disperse mycelial form conferring a high viscosity to the medium to the formation of pellets resulting in lower viscosity and higher yields. Production of conidia can occur in liquid cultures; in *P. chrysogenum* this process is induced by high concentrations of calcium ions in the cultural medium.

2.7.3. Genetics

Most of the genetic studies have been performed with *P. chrysogenum,* in relation to penicillin production. These were, however, to some extent limited by the lack in this organism of a sexual cycle of reproduction. The overall base composition of total DNA is 51% G + C; the total genome relative mass has been estimated to be 11.5×10^9 D.

Mutagenic treatment of the spores can be performed with the usual agents. Mutants with a variety of genetic markers, such as spore color or resistance to drugs, are easily obtained. Auxotrophic mutants, par-

ticularly useful for DNA recombinant studies, can also be selected with somewhat more complex screening techniques.

Genetic mapping studies have been performed by exploiting the parasexual cycle, already mentioned in the section on aspergilli, in which recombination events occur during mitosis rather than meiosis. Subsequent studies have shown that heterokaryons and diploids can be easily obtained by protoplast fusion of strains carrying different genetic markers. Although recombination events are not as frequent as in sexual reproduction, the linkage of genetic markers in segregants from diploids allowed the construction of a partial genetic map, revealing the presence of six chromosomes.

Because of the lack of endogenous plasmids, the general strategy for the construction of cloning vectors for *Penicillium* is based on the use of *E. coli* as the host. The DNA recombinant process is performed in this organism. When the desired gene has been obtained, the recombinant plasmid is purified and used to transform the fungal strain.

2.7.4. Industrial Applications

The main industrial application of *Penicillium* strains, apart from antibiotic production, is in cheese manufacturing. *P. roqueforti* and *P. camemberti* are used for the ripening of blue cheese and camembert cheese, respectively. However, a few enzymes from *Penicillium* are industrially produced. These include β-glucanases from *P. emersonii,* pectinases from *P. funiculosum* and *P. simplicissimum,* and glucose oxidase from *P. amagasakiense.*

2.7.5. Secondary Metabolites

It is well known that the most important antibiotics, penicillins (Fig. 1.2), are produced by *P. chrysogenum, P. notatum,* and a variety of other fungal species. Biosynthetically they can be considered as an exception, since no other secondary metabolite derived from a tripeptide or longer peptide chain has been isolated from *Penicillium* strains.

Common among the metabolites derived from amino acids are the alkaloids resulting from modification of one or two molecules of tryptophan or other aromatic amino acid. Also common are the diketopiperazines, resulting from a double head-to-tail condensation of

two amino acid molecules. The largest biosynthetic group comprises the acetate-derived polyketides; derivatives from triketide to decaketide are known, presenting a variety of structures resulting from different folding patterns of the chains and from subsequent modifications. Of therapeutic relevance is the heptaketide griseofulvin (Fig. 2.21), isolated from cultures of *P. janczewskii* and *P. griseofulvum,* one of the very few antifungal antibiotics that can be used systemically.

Less frequent are the terpenoid metabolites. A few products have been identified that derive from modifications of fatty acids.

2.8. Genera Producing a Few Interesting Metabolites

2.8.1. Bacteria

In the late 1970s large-scale screening of gram-negative bacteria living in soil, or in aquatic environments, revealed that several strains were producers of antibiotics. All of these were β-lactams, because the screens were specifically designed to detect, with a high sensitivity, β-lactam molecules. The first interesting antibiotic isolated was SQ 26445, from a *Gluconobacter* species. It is a sulfonyl monocyclic β-lactam (monobactam), identical to sulfazecin (Fig. 2.2) independently isolated at the same time from a *Pseudomonas* strain. Later several other structurally related monobactams were found in strains of *Agrobacterium* and *Chromobacterium* and more recently from the gliding bacteria *Cytophaga johnsoniae* and *Flexibacter* species SC 11479 and SC 12681. Nonsulfonated monobactams of the nocardicin type, the formacidins, were also isolated from *Flexibacter alginoliquefaciens.* A classical β-lactam, deacetyl cephalosporin C, is produced by strains of *Flavobacterium* and *Xanthomonas.*

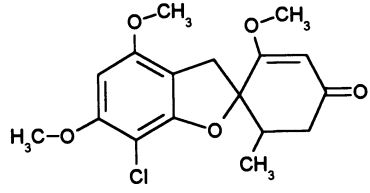

Figure 2.21. Griseofulvin.

2.8.2. Fungi

Cephalosporium and Acremonium. The producer of the most important cephalosporin antibiotic (Fig. 1.2) was initially termed *Cephalosporium acremonium,* but later the new names *Acremonium chrysogenum* or *A. strictum* were proposed for this strain. The genus *Acremonium* or *Cephalosporium* is a rather heterogeneous group of Fungi Imperfecti and the subject of considerable taxonomic dispute. It is characterized by the simple structure of the conidiophores, consisting of a single or a sparsely branched stalk bearing at the tip a small group, or "head," of conidia. The hyphae can also differentiate into another type of spore, the arthrospore. Strains are commonly isolated from soil but several species are associated with living organisms, such as plants and animals, and some strains are pathogenic for humans.

Nutritional requirements of *C. acremonium* are similar to those of *Penicillium:* a number of sugars, methyl oleate, or glycerol are utilized as carbon sources; inorganic nitrogen, amino acids or complex polypeptide as nitrogen sources. Genetic aspects have been studied mainly in relation to cephalosporin production. The presence of the parasexual cycle has been demonstrated but the formation of heterokaryons or diploids is a rare event. This limitation has been circumvented by the use of the protoplast fusion technique.

In addition to cephalosporins, only a few secondary metabolites, mainly polyketides or terpenoids, have been isolated from *Cephalosporium* or *Acremonium.* A hexaketide, cerulenin (Fig. 2.22), produced by *C. caerulens* is of interest since it is a specific inhibitor of fatty acid biosynthesis. The antistaphylococcal agent fusidic acid (Fig. 2.22) was first isolated from a strain identified as *Fusidium coccineum,* later renamed *Acremonium fusidioides.* It is also produced by several *Cephalosporium* species.

Trichoderma polysporum (later reclassified as *Tolypocladium inflatum*) is the producer of cyclosporin A (Fig. 1.8), an antifungal agent whose clinical value is, however, related to its immunosuppressive activity, exploited in organ transplantation.

Monascus ruber is the producer of monacolin K (lovastatin, Fig. 1.9), the inhibitor of cholesterol synthesis, identical to mevinolin independently isolated from *Aspergillus.* Monacolin K derivatives are also produced by other *M. ruber* strains.

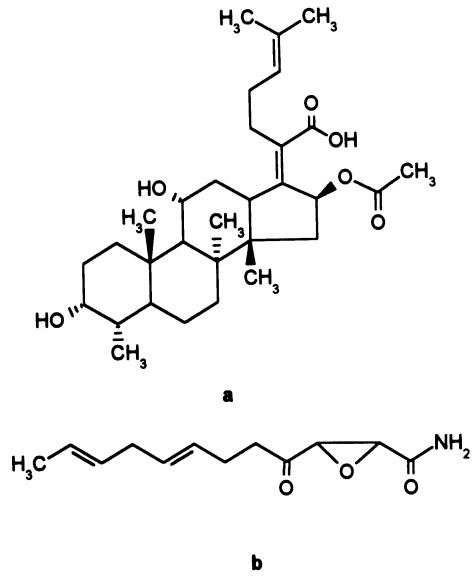

Figure 2.22. Antibiotics from *Cephalosporium:* (a) fusidic acid; (b) cerulenin.

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3

The Search for New Bioactive Microbial Metabolites

3.1. Basic Screening Methodologies

After the discovery of penicillin, systematic programs to search for new antibiotics were initiated. These resulted, in a short time, in the discovery of thousands of active molecules and, most importantly, of a number of clinically useful agents. The basic concepts of these programs, usually termed screening programs, were established by Waksman at Rutgers State University in the early 1940s. They are still valid today and are applied in many academic and industrial research laboratories all over the world.

3.1.1. Isolation and Fermentation of Potentially Producing Strains

In a screening program, microbial strains that are potential producers of bioactive metabolites are selected and isolated as pure colonies. In Chapter 2 we saw that by far the greatest number of antibiotic-producing organisms belong to species recycling organic material. For this reason soil samples, or decaying plant material from different environments, are collected and subjected to various procedures for the isolation of the desired class of microorganisms. The simplest procedure consists of shaking or stirring the sample in water and distributing, after dilution, portions of the supernatant on agarized media. Some of the colonies that develop are selected, cultured on solid medium, and used to inoculate flasks of complex nutrient liquid medium that are incubated

for several days. The broths are then tested for their antimicrobial or other desired biological activities.

3.1.2. Extraction and Purification of the Active Substances

Once any biological activity is detected, it is necessary to extract and partially purify the active substance in order to establish its novelty and to assess its potential in a series of preliminary biological tests. This process, known as dereplication, is done to avoid the unnecessary chemical effort required as a next step, should this activity be identical to known substances. Since the amount of active material produced by a wild strain is normally very small, in comparison with the total solids contained in a fermentation broth, the work necessary to obtain a pure sample of the active substance is difficult and time-consuming. In addition, the microorganism often produces several substances with either similar or totally different activities. Thus, the aim of the initial extraction and purification procedure is normally limited to obtaining a crude product in which the content of the active substance may be as low as 5 to 10%. This may be sufficient for testing, provided that only one active substance (or sometimes a complex of chemically and biologically closely related substances) is present.

Preliminary routine tests are initially carried out to identify a suitable extraction procedure. Primarily it is necessary to determine whether the activity is in the filtered broth or associated with the mycelium. If activity is in the filtered broth, tests of extraction with different solvents and of adsorption on different chromatographic materials are performed. The efficiency of the treatment is indicated by the extent of residual activity in the broth. If activity is associated with the mycelium, the substance is normally solubilized by suspending the mycelium in water-miscible solvents and the extracts are treated analogously to the filtered broth. On the basis of these preliminary results, an extraction and partial purification procedure is established.

3.1.3. Determination of the Novelty of the Antibiotic

A series of tests are performed on the crude material to identify its properties which are then compared with those of known products.

The principal properties useful for identification can be classified as follows:

1. *Biological properties*

- Antimicrobial spectrum of activity, spectrum of cross-resistance, effect on the activity of special conditions such as the presence of serum, pH of the medium, size of inoculum, ionic concentration.
- Effects on animals: the easiest to measure are the efficacy in curing experimental infections (determination of ED_{50}) and the toxicity (determination of the approximate LD_{50}) in the mouse. More important than the absolute values obtained is the ratio of the efficacious versus the toxic doses, which is normally independent of the purity of the sample, provided that it contains only one active substance.
- When the product is an inhibitor of an enzyme or of a specific metabolic activity, the task of showing novelty is much easier, since the comparison is restricted to the limited number of known compounds displaying the same action. This needs some caution, however, since it is always possible that the compound had been previously isolated on the basis of a different activity.

2. *Physicochemical properties*

Most of the physicochemical parameters used to identify a substance (melting point, polarized light rotation, infrared and NMR spectrum) cannot be determined on a heavily impure sample. In contrast, ultraviolet and visible light absorption can give useful indications even at an early stage of purification. Often the active substance is the heaviest molecule present in the sample; in this case the determination of the exact molecular weight by mass spectrometry may allow the immediate identification of the compound. Most important are the parameters related to the chromatographic behavior. Paper or thin-layer chromatography with different solvent systems can give information on the acidic or basic nature and lipophilicity of the substance. Moreover the migration values can be compared with published data, although now this is probably of historical significance since the widespread use of HPLC. The detection of the antibiotic activity is often accomplished by bioautography: the chromatographic plate is overlaid with an agar

medium containing the test microorganism and, after incubation, the position of the antibiotic is revealed by the presence of an inhibition zone in the agar film. Modern HPLC techniques are used for both diagnosis and separation. The different substances separated by the capillary column are generally scanned by ultraviolet absorption and with modern instrumentations can be compared on the basis of their spectra. The pure fractions can also be collected by semipreparative columns and tested for their biological activity.

Fundamental for the novelty determination is the availability of the published characteristics of all of the antibiotics previously discovered. Many laboratories have developed their own computerized systems of data collection. However, a very large data base is now commercially available, the Bioactive Natural Products Database (BNPD) developed by Berdy, with the collaboration of the NCI staff in Bethesda and Frederick, Maryland.

When a compound appears to be novel, and demonstrates an interesting biological activity, the purification process proceeds until a pure product is obtained and its physicochemical characteristics are accurately determined. With the advent of modern instrumentation, the elucidation of the chemical structure is normally carried out at the same time by techniques such as NMR and mass spectroscopy. However, it should be noted that for a patent application, the knowledge of the chemical structure is not absolutely necessary; analytical data establishing unequivocally the novelty of the product are sufficient.

3.2. Improving Screening Efficiency: Selection of Producing Organisms

The pioneering work of Waksman in the 1940s had shown that streptomycetes were the greatest source of antibiotics. As a consequence, through the 1950s the screening program in most laboratories were directed toward the isolation of these microorganisms. However, in the 1960s it became evident that, although new antibiotics were still consistently detected, the number of repetitions, that is, the isolation of products previously reported in the literature, was very high, resulting in a reduced efficiency. New types of microorganisms were then sought, exploring different geographical and ecological environments such as

marine sediments, soils from areas of extreme climate conditions, or soils of unusual composition. Alternatively, methods for selecting new organisms from ordinary soil samples were devised. This second approach has proved to be more fruitful, although both are still used today. We give here a brief account of the principal concepts and techniques applied.

3.2.1. Bacteria

Methods for the selective isolation of *Bacillus* strains from soil samples are simple and straightforward. They are essentially based on the high degree of resistance of the spores to heat or disinfectants. In fact, heating the soil sample at 70°C or suspending it for 1 h in 50% ethanol will kill most other organisms, and provide a population substantially enriched in bacilli. The number of gram-negative bacteria can be reduced by the addition of polymyxin to the agar medium used for growing the colonies. Species having specific requirements can also be isolated by the use of selective media; alkalophilic strains, for instance, are selected on a medium whose pH is between 8.5 and 11.5, and thermophilic strains by growing the colonies at 50°C or more.

Specific isolation of gram-negative bacteria can be obtained by plating aqueous extracts of fresh samples of soil or of plant material on one of the well-known media selective for the growth of these organisms such as MacConkey's or Brilliant Green. Actidione or other antifungal agents are added to retard the growth of molds.

3.2.2. Myxobacteria

Isolation of myxobacteria is based mainly on the visual recognition of their characteristic fruiting bodies or of swarm colonies. Isolation can be directly achieved from natural sources, e.g., soil, rotting wood, or animal dung. The samples are placed in petri dishes on layers of filter paper moistened with water, incubated at 30°C for 2 or 3 weeks, and examined under a microscope with a moderate magnification. Fragments of the fruiting bodies are transferred to a suitable agar medium with the aid of a fine-tipped glass rod.

Isolation of myxobacteria from soil can also be performed with baiting techniques. Autoclaved pellets of rabbit dung are placed on a

layer of moistened soil and incubated as described above. Myxobacterial cells migrate to the pellets where they develop fruiting bodies.

Another alternative is to place a small amount of soil or decaying plant material on a water–agar plate on which a living bacterial suspension is streaked. This constitutes a rather selective medium for the growth of myxobacteria which, in a few days, swarm over the plate and can be picked.

3.2.3. Actinomycetes

3.2.3.1. General Methods

Actinomycetes are very abundant in soil. By plating the supernatant of a soil suspension on simple agar media, the growth of typical colonies, mostly of *Streptomyces,* is easily observed. The system can be made more selective by air-drying the soil for a few days in order to reduce the population of nonsporulating bacteria, especially the gram-negative ones. Overgrowth of fungi can be hindered by addition of cycloheximide or nystatin to the agar medium. Since the spores of bacilli are resistant to desiccation, the growth of these organisms can be limited by the use of an isolation medium lacking peptone or the amino acids necessary for spore germination.

A methodology for eliminating nonactinomycete bacteria consists of placing a membrane filter on the agar medium; the surface of the filter is inoculated with the soil suspension. During the incubation the filamentous hyphae penetrate through the filter pores to the underlying agar, whereas the unicellular bacteria remain on the filter surface. Removal of the filter and incubation of the agar plate yields almost exclusively actinomycete colonies.

3.2.3.2. Selective Isolation of Actinomycetales Genera

As described above, the isolation of *Streptomyces* strains does not present any problem; therefore, in the search for novelty, strains with particular characteristics can be easily looked for, e.g., thermophilic strains or strains from acidic soils. In contrast, the enrichment of the population of other genera of Actinomycetales requires specific pro-

cedures that have been devised and improved over the years by different laboratories.

One general method consists of examining microscopically the colonies grown on the isolation medium. Strains showing unusual morphologies can be detected and picked with the aid of suitable devices. The method is not very efficient for a mass screening, but, in our experience, has led to the discovery of a few new genera.

A not very selective but useful method to enrich the population of various actinomycetes consists of air-drying the soils and then heating them at 100–120°C for 1 h. The number of bacterial and *Streptomyces* colonies is drastically reduced relative to those of other actinomycetes.

The following methods are considered useful for the specific enrichment of some important genera.

Streptoverticillium. Despite the similarity of this genus with *Streptomyces,* selective growth can be obtained by the use of lysozyme or oxytetracycline.

Nocardia. Isolation media containing demethylchlortetracycline or methacycline allow the preferential growth of these organisms.

Actinoplanes. A baiting technique is widely used for the isolation of *Actinoplanes* strains. Soil is placed in a petri dish covered with water on which pollen grains, reputed to be the best bait, float. After several days the characteristic sporangia can be seen developing on the grains' surface. A chemotactic method has also been described: the spores are attracted by a 2 M solution of KCl in a capillary tube and plated. It has to be noted that the motility of the spores can be exploited in other simple ways. For instance, a suspension of soil is centrifuged and left at rest for 1 h. Nonmotile spores are found in the pellet or at the air–water interface, whereas the motile spores of *Actinoplanes* or a few other genera are dispersed in the supernatant.

Dactylosporangium. Preferential growth of strains of this genus has been observed on colloidal chitin agar medium.

Micromonospora. Colloidal chitin agar medium is also used for the isolation of these organisms. Gentamicin and novobiocin added to the medium are effective selective agents.

Actinomadura. Selection of strains of this genus is obtained by air-drying and heating the soil according to the general procedure, and using an isolation medium containing antibiotics such as streptomycin or rifampicin.

3.2.4. Fungi

For the selective isolation of soil fungi, acidic agar media can be used. Chloramphenicol, a tetracycline, or other antibiotics are added to eliminate most of the bacterial cells. Preferential growth of fungi, with respect to actinomycetes, is also obtained by incubating the plates at temperatures around 20°C.

In the mycological literature, several media are reported suitable for the growth of different fungal genera; however, with some exceptions these are not very selective. There is some evidence that different classes of antibiotics are produced by fungi dwelling in different climatic areas, for example, tropical regions versus temperate ones. Moreover, it has been suggested that the production of pharmacologically active metabolites should be more frequent in fungi having a symbiotic relationship with higher organisms. Thus, the direct collection of specimens living on plants or insects is considered rewarding, and is performed, for instance, by Xenova laboratories.

To devise other procedures apt to enrich the population of the desired organisms is a task that, at present, appears entrusted to the experience and ingenuity of the research laboratories involved in screening programs.

3.3. Improving Screening Efficiency: Innovative Activity Tests

In the first decades of the screening for antibiotic producers, the fermentation broths were generally simply tested for their inhibitory activity against representatives of different classes of pathogens. Mainly antibacterial or antifungal agents were looked for. However, as the number of antibiotics described rapidly increased, the need for devising selective assays able to reduce the frequency of repetitions was perceived in several laboratories. At the same time, the possibility of testing the fermentation broths with more sophisticated systems was considered—systems that would reveal, in addition to the antimicrobial activity, some property relevant to the therapeutic application, such as the selectivity of action. Moreover, in some laboratories screening programs were initiated aimed at detecting nonantimicrobial activities, such as antitumor, herbicidal, or various pharmacological activities.

As a result, a very high number of novel test systems were proposed and reported in the literature. We present below some of the many that have been and are being applied to screening programs, following the specific objectives, experience, and the economic considerations of the different laboratories.

3.3.1. Assays for Antibacterial Agents

1. *Tests aimed at selecting novel compounds.* In some laboratories, the emphasis of the screening was placed on the discovery of new classes of compounds, irrespective of their potential therapeutic activity. The basic concept, applied mainly in Zahner's laboratory in Tübingen, involves selecting compounds "screened against" by the conventional procedures. In practice, fermentation broths showing activity against *S. aureus* or *B. subtilis* are discarded, since these two organisms have been largely employed as the primary test by most laboratories. An extreme example is the chemical screening by which the fermentation broths are examined for the presence of apparently inactive metabolites presenting specific chemical functions, such as nitro groups or chlorine atoms, or structural moieties, unusual in the primary metabolites, detectable by color reactions. Obviously, such compounds could not have been detected with the conventional screening based on antimicrobial activity. Another approach is to select products inactive on *S. aureus* or *B. subtilis,* but active against bacteria that are not normally employed, such as a *Clostridium.* By this method the kirromycin family of antibiotic was first discovered. A third approach consists in performing assays revealing the inhibition of some bacterial function, such as motility or formation of capsules, rather than inhibition of growth.

2. *Tests aimed at discovering congeners of known antibiotic families.* The concept here is almost the opposite of that mentioned above. It was considered that a novel member of a family that has an established clinical value should also have a high probability of being practically useful. Assays were thus devised aimed at a rapid detection of antibiotics belonging to well-known families. A simple way of performing this selection is to compare the activity of the fermentation broths on two isogenic strains, one of which is a well-characterized mutant selectively resistant to the antibiotics of the considered family. Alternatively, an organism insensitive to most antibiotics but very sensitive to those of

the class of interest can be used in the primary assay. For example, *Acholeplasma laidlawii* has been utilized for the detection of polyether antibiotics.

The major success of this screening approach has been in the discovery of new β-lactam antibiotics. Two techniques, among the several proposed, were especially successful. The first, by which nocardicins were discovered in Fujisawa laboratories, uses as the test organism a microbial strain hypersensitive to β-lactams but not to other antibiotics. A very weak activity can be detected and selected by comparison with the activity on a normal strain. Even more sensitive and specific is a test system, used at Squibb laboratories, based on the induction of β-lactamases. In some strains the production of these inactivating enzymes is specifically induced by very low concentrations of compounds having a β-lactam structure. The presence of even minute amounts of a β-lactam in a fermentation broth will induce the production of the enzyme that can be revealed microbiologically or by a chromogenic reaction. Obviously, both systems reveal known and novel substances; however, the identification is then simple and by these techniques important products have been discovered.

Another interesting example is the screening for the glycopeptide family of dalbaheptides. These antibiotics act by specifically binding to the D-alanyl-D-alanine terminus of oligopeptides. Addition of such an oligopeptide to plates on which a test organism is growing reverses the activity of these antibiotics and their presence can thus be revealed. A more efficient system, used in our laboratories, is based on affinity chromatography. Small columns are prepared in which the oligopeptide is linked to a Sepharose support. When the fermentation broth is passed through the column, the antibiotic is selectively retained and, after elution, can be revealed microbiologically.

3. *Target-oriented screening.* To be practically useful an antibacterial agent must be selectively active on bacterial cells, that is, it must act on structures or enzymes typical of prokaryotes and absent or different from those of mammalian cells. To avoid the isolation of nonselective and potentially toxic products, a growing number of assays, able to indicate the mechanism by which an unknown activity operates, have been devised and introduced into screening programs.

A typical example are assays aimed at detecting substances interfering with the formation of the bacterial cell wall. In our laboratory

this was simply done by comparing the activity of the broth on an *S. aureus* strain and on the L form (a mutant lacking the cell wall) derived from it. Inhibitors of cell wall formation are detected since these are only active on the normal strain. In other laboratories different assays have been applied, such as the inhibition of a cell-free system of peptidoglycan synthesis.

In more general terms any enzyme essential for the bacterial metabolism can be utilized to develop a screening test. Several techniques can be adopted. For instance, when a simple method for determining the enzymatic activity is available, a cell-free system may be utilized to reveal the presence of inhibitory substances. Inhibitors of RNA polymerase have been discovered by such a system. Microbiological assays can also be used. Important inhibitors of β-lactamases have been discovered by determining the ability of fermentation broths to dramatically increase the activity of a penicillin on a β-lactamase-producing bacterial strain.

In another alternative the desired activity can be indicated by the formation of a complex between the microbiologically active substance and a receptor. The formation of the complex can be revealed by the reversion of activity method mentioned above with respect to glycopeptide antibiotics. The antimicrobial activity of the fermentation broth is tested on two plates seeded with the same microorganism; to one plate a suitable concentration of the receptor is also added. An apparent inferior activity in this plate indicates that the receptor binds the active substance that is thus not available for penetration into the bacterial cell.

An elegant alternative for detecting substances active on a bacterial enzyme, but not active on the corresponding mammalian enzyme, consists of constructing, by recombinant DNA techniques, a strain containing both its natural enzyme and the equivalent mammalian one. If a product inhibits the normal strain but is inactive on the recombinant one, it must be selectively acting on the bacterial enzyme. This method has been applied in our laboratory to select fermentation broths active on dihydrofolate reductase, using a *B. subtilis* in which the human enzyme had been cloned.

It should be noted here that, although the principal aim of a target-oriented screen system is the detection of substances selectively active

on microorganisms, in the case where no described antibiotic acts on the chosen target the system will specifically select novel compounds.

3.3.2. Assays for Antifungal Activities

Although a high number of antifungal agents have been isolated from soil microorganism cultures, very few have found application in human medicine; a few more are used in agriculture. The obvious reason resides in the similarity of the fungal and the mammalian cells, and as a consequence most antifungal agents are toxic. Selective screening procedures have been tried, based on the identification of specific targets.

The search for fungal cell wall synthesis inhibitors was less successful than that for the bacterial cell wall. It is generally based on cell-free systems of chitin synthesis, for detecting inhibitors of the enzyme chitin synthetase. The antibiotics revealed by such a method, the polyoxins and the nikkomycins, proved useful as agents against fungal plant diseases, but not against *Candida* infections in humans. Not surprisingly, they have insecticidal activity also, because they inhibit the formation of the chitin tegument of insects. A more promising target is the inhibition of the synthesis of glucans, major components of the fungal cell wall. In fact, a derivative of echinocandin, a glucan synthase inhibitor, is currently undergoing clinical evaluation.

The clinical value of the polyene antibiotic amphotericin depends on the fact that it binds with higher affinity to ergosterol, present in the fungal membranes, than to cholesterol, present in membranes of mammalian cells. This suggests a differential assay, based on a higher reversion of activity when ergosterol, rather than cholesterol, is added to plates on which a test fungal strain is growing.

An interesting new target is the elongation factor EF-3, an essential component in the fungal protein synthesis system, for which there is no counterpart in the mammalian system. An assay revealing a specific inhibition of the activity of this protein could be very valuable for the selection of novel, nontoxic antifungal drugs.

Other targets that have been recently proposed are molecules that are not essential for the growth of the fungi, but are relevant for their pathogenicity. In particular, inhibitors of an aspartic proteinase of *Candida albicans,* or inhibitors of the binding of the adhesins (mannopro-

teins of the same organisms) to host cell receptors, should prevent the tissue invasion and virulence of this pathogen.

3.3.3. Assays for Antiviral Activity

The classical assay for antiviral activity is based on inhibition of lytic plaque formation in cell monolayers infected with viral particles. A suspension of infected cells (originally chick embryo fibroblast and Newcastle disease virus were used) is spread on an agar plate. On this, filter paper disks soaked with the test sample are placed; the plates are incubated and stained with neutral red. Antiviral activity is revealed by zones of colored cells around the disk, contrasting with the colorless lysed cells. An unstained zone around the disk followed by a stained zone indicates cytotoxicity at higher concentrations than those displaying antiviral activity. Microbial methods have also been proposed, based on similar procedures. This time, however, bacterial cultures and either DNA or RNA phages are used as test organisms.

Later, as viral enzymes were identified, target-oriented assays were developed. Typical is the assay, devised in the National Institute of Health in Tokyo, for inhibitors of the retroviral enzyme reverse transcriptase. This consists of measuring the DNA synthesis (determined as incorporation of [^{3}H]thymidine) catalyzed by murine leukemia virus proteins, using a poly(dT)poly(A) copolymer as the template. By this assay the reverse transcriptase inhibitor revistin was identified. It should be noted that this assay, as most of the assays based on the inhibition of enzymatic activities, may give false positives because of the presence of protease activities in the culture filtrates. These proteases can be inactivated by heating the filtrates at 100°C for a few minutes before the test.

Recombinant DNA techniques, allowing the cloning of viral proteins in bacterial cells and their production in relatively large amounts, provide the necessary material for a number of target-oriented test systems. These may be based on inhibition of enzymatic activities, such as reverse transcriptase or HIV viral proteases, or on competition for the binding of a viral protein to its cell receptor, as is the case for the HIV gp120 protein and the cell receptor CD4 (also obtained as a recombinant protein).

3.3.4. Assays for Antitumor Activities

In the first years of antibiotic research, no specific assays for antitumor activity were performed. Fermentation products isolated for their antimicrobial activity and too toxic for anti-infective applications were tested for antitumor activity, normally against experimental leukemia in mice. It was then realized that several antitumor agents act by interacting with DNA, and thus microbial assays able to reveal this action were introduced as a prescreen. Most of the assays proposed are based on prophage induction, which is a well-known effect of products binding to DNA or interfering with DNA synthesis. Prophage induction can be revealed by the formation of lytic plaques in agar cultures of a sensitive strain, or by biochemical methods, based on the induction of an easily measurable bacterial enzyme placed under the control of a prophage promoter.

Since another class of antitumor agents (e.g., methotrexate) are antagonists of coenzymes or amino acids, screens to detect antimetabolites were devised. A simple method consists of determining the activity of the samples on a bacterial strain grown on two different agar media: one a complex nutrient medium, the other a minimal medium containing inorganic nitrogen and glucose as the sole carbon source. A marked difference in the inhibition zones indicates the presence of an antimetabolite whose action is reversed by some component of the complex medium.

The most used screening tests at present are those based on the cytotoxicity on tumor cell lines. Inhibition of cell growth and cell mortality can be revealed by a variety of methods. Staining with various dyes is commonly used. Sulforhodamine B is recommended by NCI researchers for simplicity of use and possibility of quantitative determinations. Alternative methods are based on the determination of the uptake by the cells of radioactive nucleosides, particularly [^{3}H]thymidine. The advent of miniaturized and automatized systems has made this type of test suitable for the rapid screening of a large number of samples.

3.3.5. Screening for Products with Pharmacological Activity

The search for microbial metabolites potentially useful against noninfectious diseases is essentially a target-oriented screening aimed

at detecting specific biological responses. These could include inhibitors of enzymes involved in metabolic disorders, the ligand–receptor interaction of various kinds, and mediators of cell–cell interaction. It was initiated in the mid-1960s by Umezawa and co-workers at the Institute of Microbial Chemistry in Tokyo by testing thousands of culture filtrates for their capacity to inhibit the activity of various cell surface proteases. Several low-molecular-weight inhibitors were isolated, with different specificities. One of them, bestatin, an inhibitor of aminopeptidase B, is especially interesting for its activity as an immunomodulator.

The observation that cyclosporin A, a product originally isolated for its antifungal activity, has a clinically relevant immunosuppressant activity spurred research for other agents acting on the immunological system. An efficient test was introduced in Fujisawa laboratories screening program. Murine T lymphocytes are mixed with mitomycin-inactivated T lymphocytes, from a different mouse strain. These last cells act as antigens, stimulating the proliferation of the first population, which is revealed by the incorporation of labeled thymidine into the cells. By testing fermentation broths for their ability to inhibit thymidine incorporation, several immunosuppressant agents were identified. One of these, FK 506, is being developed as an alternative to cyclosporin in organ transplantation.

The number of tests proposed to detect inhibitors of physiologically relevant enzymes is very high. Many are concerned with the enzymes of adrenaline synthesis and metabolism, such as tyrosine hydroxylase, dopamine β-hydroxylase, monoamine oxidase, and catechol-*o*-methyl transferase. Several new active compounds have been isolated but none of these appear especially promising. Elastase is an enzyme believed to play a role in inflammatory processes. A specific screening led to the isolation of two interesting inhibitors, elasinin and elastinal. In the Bayer group, screening for inhibitors of pork pancreatic amylases and pork saccharases resulted in the identification of the useful oligosaccharide acarbose.

The screening for inhibitors of cholesterol biosynthesis has been highly rewarding. It has been performed by two groups, the Sankyo and Merck laboratories with different techniques. In the first culture filtrates were tested in a system of liver homogenate in which cholesterol synthesis was measured as conversion of labeled acetate into insoluble material. In the second group the inhibition of the hydroxy-methyl-

glutaryl reductase, one of the first enzymes of sterol synthesis, was directly measured. The same class of effective compounds, including compactin and mevinolin, was found by both groups.

A recent trend is to screen for products interfering with enzymes involved in the control of cell division. Typical targets are tyrosine kinase and protein kinase C. In theory, these products could be effective antitumor drugs. However, it has to be noted that there are several protein kinase C enzymes and that the inhibition of one or more of these may result in a range of unexpected biological effects.

Several tests for detecting antagonists of hormones or other physiological mediators have been devised, generally based on competition assays for the hormone receptor. An interesting example is the detection, as a result of such a screening method, of asperlicin and its analogues, antagonists of cholecystokinin, a peptide hormone regulating gastric and pancreatic secretion and gut motility.

3.3.6. Screening for Antiparasite and Agricultural Products

Antiprotozoal activity was not initially included in the screening programs. Later assays were introduced using as a main criterion the reduced motility of the test protozoa. A similar situation is true for insecticidal activities. As an indicator of this action the larvae of the shrimp *Artemia salina* have sometimes been used. The same organism was also used for a preliminary indication of the toxicity of mycotoxins. More specific biochemical tests are based on the inhibition of chitin synthetase and of chitinase.

A successful screening for products active on nematodes was carried out in Merck laboratories, with an *in vivo* assay on mice infected with the worm *Nematospiroides dubius.* The antiparasite activity of the avermectin family of metabolites, first isolated at the Kitasato Institute, was discovered by this assay.

A simple microbial assay for herbicidal products has been adopted at the Kitasato Institute in Tokyo. It is based on the observation that bialaphos, a herbicidal product originally isolated as an antimicrobial agent, acts by inhibiting the enzyme glutamine synthetase. Similar activities are detected by selecting fermentation broth inhibiting *B. subtilis* growth in a minimal medium but inactive when glutamine is added to the medium. At the same institute, cellulose biosynthesis has been pro-

posed as a target for herbicidal agents. Culture filtrates are screened for their inhibitory activity on *Phytophthora parasitica,* a fungal species containing cellulose in its cell wall, and on *Candida albicans* whose cell wall is composed of other polysaccharides. Selective activity on the first is taken as an indication of cellulose synthesis inhibition.

3.4. Screening Efficiency: Quantitative and Organization Aspects

The influence of the quantitative aspect on the results of a screening program is obvious. All other factors being equal, the more fermentation broths studied, the greater is the probability of finding a useful substance. The same reasoning applies to the number of different assays on which the broths are tested; the higher the number, the greater the probability of finding a new activity. Since the resources are obviously limited, a continual effort is needed to simplify and to streamline the operations involved. This is often accomplished by applying miniaturization and robotization of the assays.

It is, however, most important to find a correct balance between the different operations that constitute a screening program. Different laboratories may have different attitudes, based on their objectives and experience, with respect to the proportion of work and time that should be devoted to each set of operations. Some examples are worthy of discussion:

The first dilemma is whether to screen more strains grown using one culture condition or whether to screen a lesser number of strains grown under several culture conditions. It is in fact known that the medium composition and the culture conditions greatly influence (and may totally repress) the expression of biosynthetic genes. On the other hand, for a given genus of microorganism screened (or for certain classes of antibiotics), it is generally possible to find conditions allowing the production of the majority of its secondary metabolites. There is no universally accepted answer to this question, as demonstrated, for instance, by the fact that in our laboratories a single growth medium is used whereas in other laboratories each strain is grown in four different fermentation media. A consideration that may be used as a rule of

thumb is that it may be convenient to use one condition when the strains of the genera screened are abundant in the soil samples and are easily isolated. In contrast, when strains difficult to isolate or rare strains are looked for, it may be convenient to grow them on a variety of media.

A similar problem is related to the number of cultures examined and the number of assays in which each culture is tested. It is naturally desirable to test the cultures in as many assays as possible; but this may result in a reduction of the number of strains screened. A consideration that has to be made, in evaluating the correct balance between these two sets of operations, is that the probability of finding an active culture may be quite different for the different assays. For instance, an assay aimed at detecting inhibitors of protein synthesis, or of bacterial cell wall synthesis, will reveal, because of the large number of enzymes involved, active substances at a higher frequency than an assay for inhibitors of the elongation factor EF3 or of the enzyme D-alanyl-D-alanine synthase. As a consequence, one should screen a higher number of cultures when a very specific assay is employed; however, specific assays are often more laborious and time-consuming than the general ones. A compromise often adopted is to consider two sets of assays in a two-stage process: simple primary assays, such as those detecting an antimicrobial or a cytostatic activity, on which all of the cultures are tested, and secondary more complex ones (such as the biochemical assays on enzymatic activity) on which only the cultures that were positive in the primary screening are examined. This may reduce by fivefold the number of complex tests that have to be performed. Nevertheless, there is a serious drawback: novel substances active only in the secondary tests will be missed.

The final aspect of evaluating the efficiency of screens is the number of "hits" per, say, 10,000 strains screened. The two extremes to be avoided are *no* hits and *too many* hits. In both cases the specificity of the assay, the nature of the culture conditions, and several other factors must be reexamined.

The next complex decision, which must take into consideration several factors, is the amount and type of work that should be done when an extract of an active culture has been obtained. Often an evaluation of the biological activity can be performed on a crude sample. When the results are not particularly promising, it has to be decided

whether to continue with the purification and related chemistry, which is normally rather laborious, or to discard the product. Possibly the outcome will depend on the objectives of the laboratory. In an industrial laboratory, where only products promising for practical application should be pursued, the work could be discontinued; whereas in an academic institution the chemical characterization of a novel natural product could be considered of interest. Nevertheless, many well-characterized products, for which no industrial application is foreseen, have been reported in the literature by industrial laboratories. In the long term this attitude may be rewarding. As the case of cyclosporin A demonstrates, retesting old samples by a new assay may reveal interesting activities.

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4

Biosynthesis of Secondary Metabolites

The study of biosynthesis of secondary metabolites consists of the identification of the sequence of reactions by which the cell converts one or more primary metabolites into the final molecule. It also concerns the identification of the factors by which this process is regulated. In contrast to the great variety of chemical structures, the biological reactions involved in the biosynthesis of most secondary microbial metabolites can be grouped in a limited number of biosynthetic pathways. In this chapter we give a short description of these pathways, and summarize the methods commonly used for their identification.

4.1. Methods of Study

The elucidation of a biosynthetic pathway is a stepwise process that ideally comprises: (1) the identification of the "building blocks," that is, the primary metabolites from which the molecule is made; (2) the isolation of intermediates of the pathway, whose structure can suggest a reasonable hypothesis on the sequence of the reactions; (3) the identification and isolation of the enzymes that catalyze the single reactions.

A complete understanding of the biosynthetic process also includes the identification of the regulatory factors and of the biosynthetic genes. These two aspects will be discussed in the following chapters.

Logically, the above-mentioned steps should be performed in sequence. However, this is not always the case. In practice, it may happen

that a valuable clue to a biosynthetic pathway is given by the identification of a particular enzymatic activity before any intermediate compound is isolated. Sometimes, intermediates are quickly identified, as a consequence of mutagenic treatments made to improve production yields. However, the elucidation of a complex biosynthetic pathway is normally the result of a combination of all of the different approaches listed above.

4.1.1. Tracer Techniques

For the identification of the building blocks, tracer techniques are generally used. The possible precursors of the antibiotic, labeled with an isotope such as ^{14}C, ^{3}H, or ^{13}C, are added to cultures of the producing organism, preferably at the end of the growth phase. At the end of the production period the antibiotic is extracted, purified, and the incorporation of the isotope determined. When a radioactive label is used, simple counting of radioactivity gives indications about the extent of incorporation of the precursor. The results must be interpreted very carefully because: (1) the precursor could fail to be taken up by the cell, generating a false negative; (2) the precursor could be degraded to simpler molecules recycled through the general metabolic pathways thus giving a false positive. To some extent this aspect can be checked by using double-labeled tracers, e.g., molecules labeled with both ^{3}H and ^{14}C. In general, precursors specifically labeled in one or more atoms should be used and it is fundamental to determine, through the chemical degradation of the final molecule, in which atoms the radioactivity has been incorporated.

The chemical degradation of fairly large molecules into defined fragments is often difficult and time-consuming. Thus, alternative techniques to radioactive labeling have been developed. The most important of these techniques involves the use of ^{13}C as the label. This carbon isotope has a natural abundance of about 1.1% but simple molecules highly enriched in ^{13}C are available for labeling studies. Precursors whose carbons are enriched up to 99% with ^{13}C can be synthesized (many are commercially available) and added to the cultures of the producing strain. By nuclear magnetic resonance (NMR) it is possible to determine the ^{13}C fraction of each carbon atom in a molecule. The available instruments show it in the form of a resonance spectrum in which each

carbon is represented by a peak. After completion of fermentation and isolation of the antibiotic the incorporation of the label into the carbons of the final molecule is revealed as an increase in the height of the corresponding peaks in the magnetic resonance spectrum. Moreover, it is possible to use precursors labeled on two adjacent carbon atoms. A high-resolution analysis of the multiplicity of the resonance spectrum peaks, and of their coupling constants, can reveal whether the two atoms were linked together throughout the metabolic process or whether they had been separated and have been incorporated through different paths.

When the data are insufficient for making a reasonable hypothesis on the nature of the precursor, or when the desired labeled precursor is not available, it is possible to obtain important clues by using a generally metabolized molecule, such as glucose, or glycerol, specifically enriched with ^{13}C in one carbon atom. The identification of the specific carbon atoms in which the label is incorporated and the knowledge of the cellular intermediate metabolism can give important clues on the biosynthetic pathway.

NMR study of deuterium-labeled precursors can give useful information. The basis of this technique is that deuterium, in contrast to hydrogen, does not give a resonance signal in proton NMR. For the study of the mechanism of biosynthetic reactions, deuterium is specifically substituted for one or more hydrogen atoms in a biosynthetic intermediate which is then converted into the final molecule by cultures of the producing strain. NMR examination of the product reveals whether the atom has been retained or exchanged in the course of the enzymatic reaction.

4.1.2. Use of Blocked Mutants

The identification of the intermediates in the sequence of biosynthetic reactions can be done by submitting the producing organism to mutagenic treatment and selecting mutant strains that have lost the producing ability. When this is the result of a single mutation that inactivates one of the enzymes involved in the biosynthetic pathway, the strain is called a blocked mutant. The product that is the substrate for the blocked enzymatic reaction cannot be further transformed and often accumulates in the medium. It can then be isolated and identified. To prove that it is indeed an intermediate of the biosynthetic pathway,

it is necessary to verify the capacity of the original strain to transform it into the final product. This can be accomplished by radiotracer techniques. The radioactively labeled intermediate is prepared by feeding the mutant strain cultures with an appropriate labeled precursor. The intermediate is isolated, purified, and fed in turn to cultures of the producing strain. A high percentage of radioactivity incorporation into the final molecule demonstrates that the product belongs to the biosynthetic sequence. Alternatively, the conversion can be demonstrated by analytical methods, determining whether a substantial increase in the antibiotic production occurs when the intermediate is added to a suspension of cells of the parent organism in the absence of nutrients.

Many nonproducing strains are generally observed after mutagenic treatment. To facilitate the identification of those having a single mutation in the biosynthetic pathway, and that accumulate an intermediate in the fermentation broth, the cosynthesis method is used. This consists in growing the mutants two at a time in one flask. Strains that do not produce when grown singly but do produce when grown together are mutants blocked in two different steps of the sequence of the biosynthetic reactions: the inability of one to synthesize an intermediate is compensated by the ability of the other to accumulate it. It is also possible to determine which of the two strains accumulates the precursor and which utilizes it, by adding portions of the filtered broth of the culture of one strain to cultures of the other strain and vice versa. The strain whose producing capacity is restored by the addition of the filtrate is the acceptor strain, the other that accumulates the intermediate is the donor strain.

4.1.3. Enzyme Identification

The use of tracer techniques and the identification of intermediates are generally sufficient to determine the sequence of reactions through which the antibiotic is produced. The study, however, cannot be considered complete until it is shown that the microorganism possesses the enzymes able to catalyze the single reactions. This aspect is investigated using the usual biochemistry techniques. The enzymatic activity is sought in cell-free systems and when possible the enzyme is purified and its properties determined. In the presence of families of antibiotics, it is of interest to determine the substrate specificity of the enzymes,

which can clarify the precursor–product relationship between two metabolites. Sometimes the presence or absence of the enzyme in the producing and nonproducing variants of the microorganism is determined to confirm its role.

It should be noted that, by the recombinant DNA techniques discussed in Chapter 6, the biosynthetic genes may be identified without any previous knowledge of the corresponding enzymes. Since the sequence of a DNA segment is easier to establish than the amino acid sequence of a protein, the nature of the enzymes is often determined on the basis of the biosynthetic genes' sequence, and on their homology with known genes of primary or secondary metabolism.

4.2. Biosynthetic Reactions and Pathways

For several years the opinion was widespread, among students of secondary metabolism, that the biosynthesis of antibiotics could involve patterns and reactions quite different from those of primary metabolism. This notion was based on the complexity of the structure of many metabolites, whose formation could not be easily interpreted on the basis of known microbial biochemistry. In addition, several secondary metabolites contain chemical groups, such as chlorinated moieties or nitro groups, absent in primary metabolites. However, as more biosynthetic pathways were elucidated, similarities in the two groups of reactions were observed. Today we see that there is a close relationship between primary and secondary metabolism. In fact, it is reasonable to hypothesize that the enzymes now deputed to the synthesis of special metabolites evolved from those of general metabolism. Confirmation of this hypothesis, which is so far based only on the formal similarity of a number of reactions, will probably be obtained in the future by comparison of the corresponding gene sequences. The limited data today available are in agreement with this idea.

Apparently even an esoteric reaction such as the chlorination of biological molecules can be associated with common reactions when the relevant enzymes are examined. It has been found, for instance, that the enzyme that catalyzes the chlorination step in chloramphenicol biosynthesis is a heme protein. This has bromoperoxidase and catalase

activity, sharing several properties with conventional bacterial catalases, such as the one from *Micrococcus luteus.*

Classification of antibiotics according to their biosynthesis is generally based on the primary metabolites from which they are derived. We thus have antibiotics derived from sugars, amino acids, acetate, nucleotides, and so on. Although useful, this classification is insufficient to order the biosynthetic pathways in a meaningful way, since several unrelated series of reactions can occur from the same metabolite. Moreover, many antibiotics are composed of intermediates derived from different primary metabolites.

The system can be refined if we consider, in addition to the starting molecules, the pattern of reactions, which can be ordered according to the classical biochemical pathways of primary metabolism.

We can consider three classes of biosynthetic reactions:

1. Class I reactions are those by which a primary metabolite is transformed into an intermediate of the biosynthesis. These reactions can be divided according to the general metabolic pathway to which they are related, such as amino acid synthesis and catabolism, nucleotide metabolism, sugar transformation, or coenzyme synthesis. The biosynthetic intermediates can: (a) be further modified, thus giving rise to antibiotics derived from a single primary metabolite; (b) be condensed with one or two other intermediates, thus giving rise to more complex molecules, in analogy with the synthesis of some coenzymes, such as folic acid, coenzyme A, or the prosthetic group of quinoproteins; (c) be condensed to several similar metabolites by class II reactions.

2. Class II reactions are polymerization reactions by which several similar units are linked together to form larger molecules. They are the key step in the synthesis of a vast number of antibiotics. Four major types of polymerization reactions are recognized: (a) condensation of acetate–malonate (sometimes propionate–methylmalonate) units, by a mechanism analogous to that of fatty acid synthesis, denoted polyketide synthesis; (b) condensation of amino acids (sometimes hydroxy acids) to form peptide or depsipeptide antibiotics; the mechanism involved, denoted thiotemplate mechanism, has some similarity with that of polyketide synthesis; (c) condensation of isoprenoid units, as in the synthesis of terpenoids and sterols; (d) condensation of sugar units to give oligosaccharides. In addition, a few polypeptide antibiotics are

synthesized by the normal transcription–translation system of protein biosynthesis.

3. Further modifications of the assembled molecule are performed by class III reactions. These normally fall into a few categories quite common in primary metabolism; for example, oxidations or reductions, methylations, glycosylations. The halogenation reaction, mentioned previously, may occur either as an early or as a late biosynthetic step. The production, commonly observed in many strains, of families of closely related substances is frequently the result of class III reactions. Thus, a single culture may produce several metabolites presenting a common general structure but differing for instance in the degree of methylation, in the level of oxidation, or in the length of acyl substituents. This is considered the effect of a low level of substrate specificity of the enzymes involved in secondary metabolism, in contrast to the specificity of those of primary metabolism. Provided that all of the different molecules eventually resulting from the reaction sequences were similar in their biological activity, it is conceivable that there was no selective pressure operating during evolution favoring one substrate–enzyme relationship. It has to be remembered that, generally speaking, the enzyme specificity is not related to the entire molecular structure but to defined regions that fit in the enzyme active site. It is therefore likely that relatively large molecules, presenting some minor difference in their structure, may be substrates of the same enzymatic reaction, without implying a lack of enzymatic specificity.

In the following sections, to illustrate the variety of patterns by which secondary metabolites are made, a few examples of typical reactions of each class are given.

4.3. Class I Reactions: Transformation of Primary Metabolites into Biosynthetic Intermediates

4.3.1. Reactions Related to Amino Acid Metabolism

A metabolic pathway, common to several amino acids, consists of a transamination reaction to give the corresponding keto acid followed by oxidative decarboxylation yielding a carboxylic acid one carbon atom shorter. Hydroxy- and keto-acid constituents of depsipeptide

antibiotics originate through this pathway. This is for instance the case with D-hydroxy-isovalerate of both the ionophore valinomycin and enniantin B, and with the α-keto-β-methylvaleric moiety of pyridomycin. The same series of reactions, starting from tryptophan, provides the indole ring of the antibiotics carbazomycin A and B.

Threonine can be metabolized to lactate through a reaction sequence involving aminoacetone and ketopropanol. It has been shown that the lactic moiety of valinomycin, for example, originates in this fashion.

A pathway of lysine catabolism, which eventually gives acetoacetate, involves piperidine-2-carboxylate as an intermediate. There is evidence that the 3-hydroxypicolinic component of the peptide antibiotic etamycin derives from lysine by dehydrogenation of this intermediate.

An interesting reaction encountered in several biosynthetic pathways is the β-hydroxylation of amino acids. This reaction is unknown in primary metabolism (however, the β-hydroxylation of aliphatic acids is very common) but is frequently the first reaction in the pathways of secondary metabolism. It can be surmised that its function is to divert the amino acid from the pool of general metabolites to secondary metabolism. A few examples are: (1) the biosynthesis of *p*-hydroxyphenylglycine, a component of all dalbaheptide antibiotics and of nocardicin; this moiety derives from tyrosine through a series of reactions initiated by β-hydroxylation (Fig. 4.1); (2) the biosynthesis of the streptolidine moiety of streptothricin F; this heterocycle derives from arginine, by β-hydroxylation followed by oxidation to β-keto-arginine and cyclization (Fig. 4.2); (3) β-hydroxylation of *p*-aminophenylalanine is one of the first steps in chloramphenicol biosynthesis (Fig. 4.3); (4) β-hydroxyl amino acids are found as components of several antibiotics, such as β-hydroxytyrosine in dalbaheptides and β-hydroxyleucine in telomycin and leucostatins.

It should be noted that the β-hydroxylation of amino acids can also be postulated as the initial step in the biosynthesis of other antibiotics. However, poor absorption of exogenous β-hydroxy amino acids by the cells often precludes experimental confirmation.

A pathway of tyrosine catabolism, which eventually produces melanins, consists of hydroxylation of the aromatic ring to give dihydroxyphenylalanine, and of further reactions by which the nitrogen is retained to form the pyrrole ring. The alkylproline moieties of lincomycin and

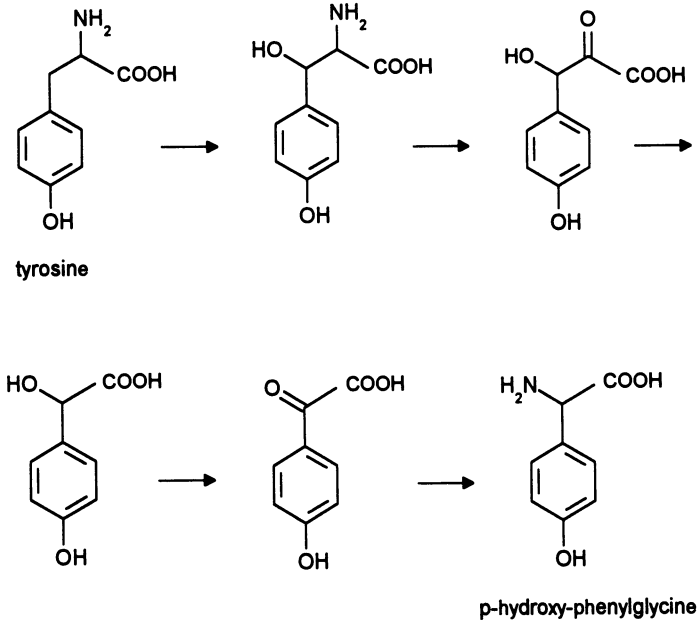

Figure 4.1. Tyrosine conversion into *p*-hydroxyphenylglycine. Presumptive pathway.

of the anthramycin family of antibiotics derive from tyrosine by a similar sequence of reactions (Fig. 4.4). The pyrrolidine (or pyrroline) ring originates from cyclization of the alanyl chain, whereas degradation of the aromatic ring provides the atoms of the aliphatic side chain.

4.3.2. Reactions Related to Nucleoside Metabolism

There is one interesting example of an antibiotic whose biosynthesis parallels the *de novo* synthesis of pyrimidine nucleotides. This is the deoxyriboside antibiotic PA 399 (5,6-dihydro-5-azathymidine). The biosynthetic sequence starts from the condensation of the carbamoyl group with glyoxylurea (formed by condensation of urea with glyoxylic acid) rather than to aspartic acid as is the case in uridine biosynthesis. All of the subsequent reactions such as ribosylation, decarboxylation, etc., are analogous to those of pyrimidine nucleotide biosynthesis.

Ribonucleotides are converted into deoxyribonucleotides by the reduction, catalyzed by the enzyme ribonucleotide reductase, of the

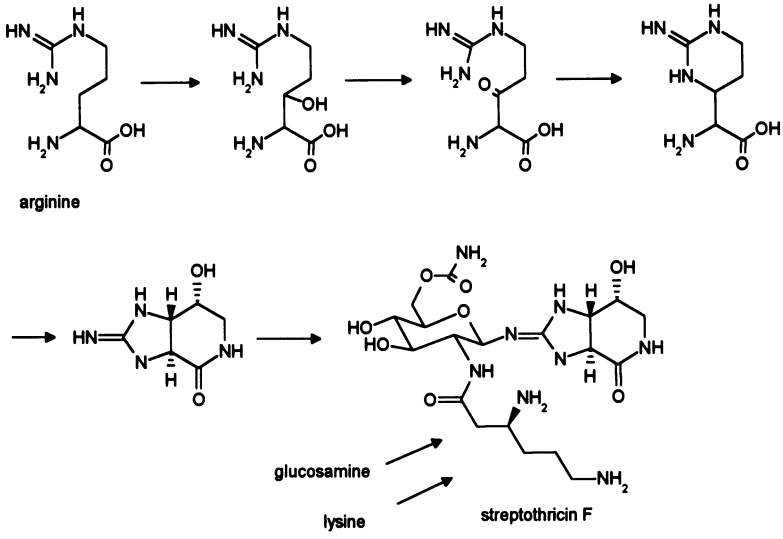

Figure 4.2. Biosynthetic pathway of streptothricin F.

hydroxy group in position 2′. Cordicepin (3′-deoxyadenosine) is synthesized by a modification of this process. In fact, it has been shown that it derives from adenosine and, moreover, that the reduction mechanism of the hydroxyl in 3′ is analogous to that operating in the formation of the 2′-deoxynucleotides.

Microorganisms can also synthesize nucleosides through the so-called salvage pathway, consisting of the ribosylation of exogenous purine or pyrimidine bases. Psicofuranine is made by an analogous reaction, that is, the glycosylation of adenine with the sugar psicose.

4.3.3. Reactions Related to Coenzyme Biosynthesis

Nicotinic acid, a precursor of nicotinamide adenine dinucleotide, is biosynthesized in mammals and in *Neurospora* from tryptophan through 3-hydroxyanthranilic acid, whereas in plants and in bacteria it originates from the condensation of aspartic acid and a three-carbon unit (glycerol or a closely related compound). Both patterns are found in the biosynthesis of antibiotic moieties. Two pyrimidine rings are present in the peptide antibiotic pyridomycin. Both derive from the

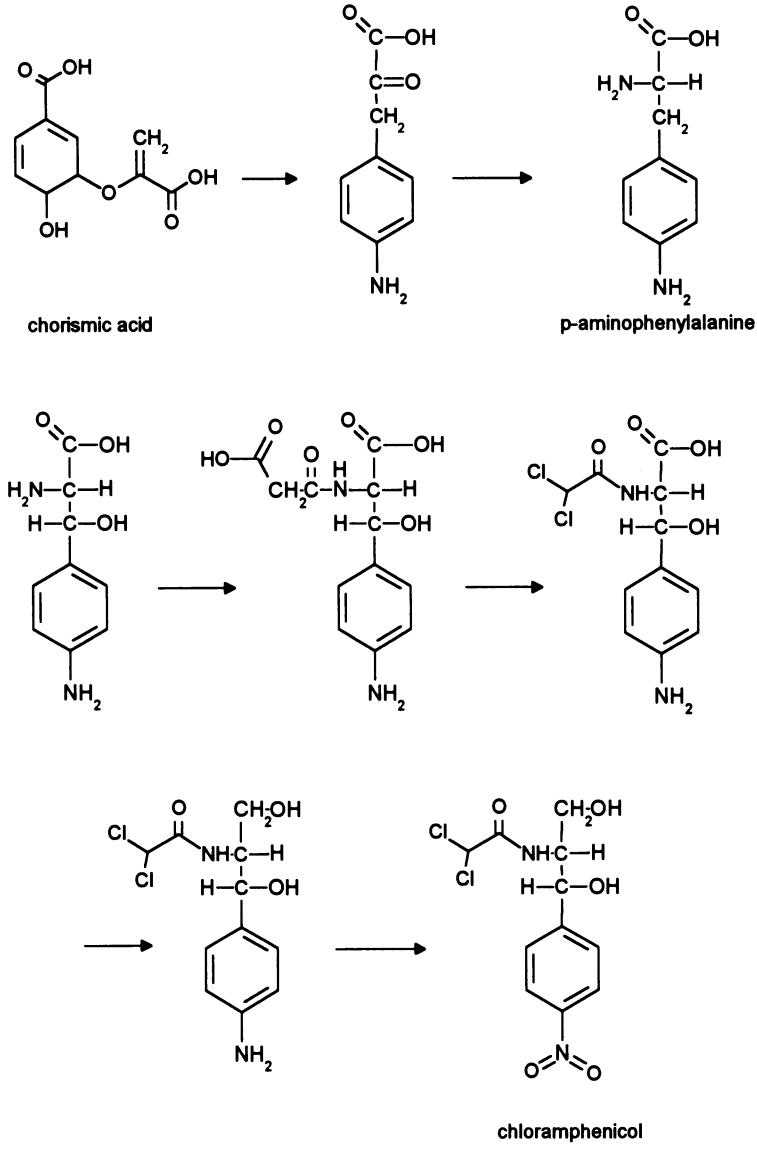

Figure 4.3. Biosynthesis of chloramphenicol.

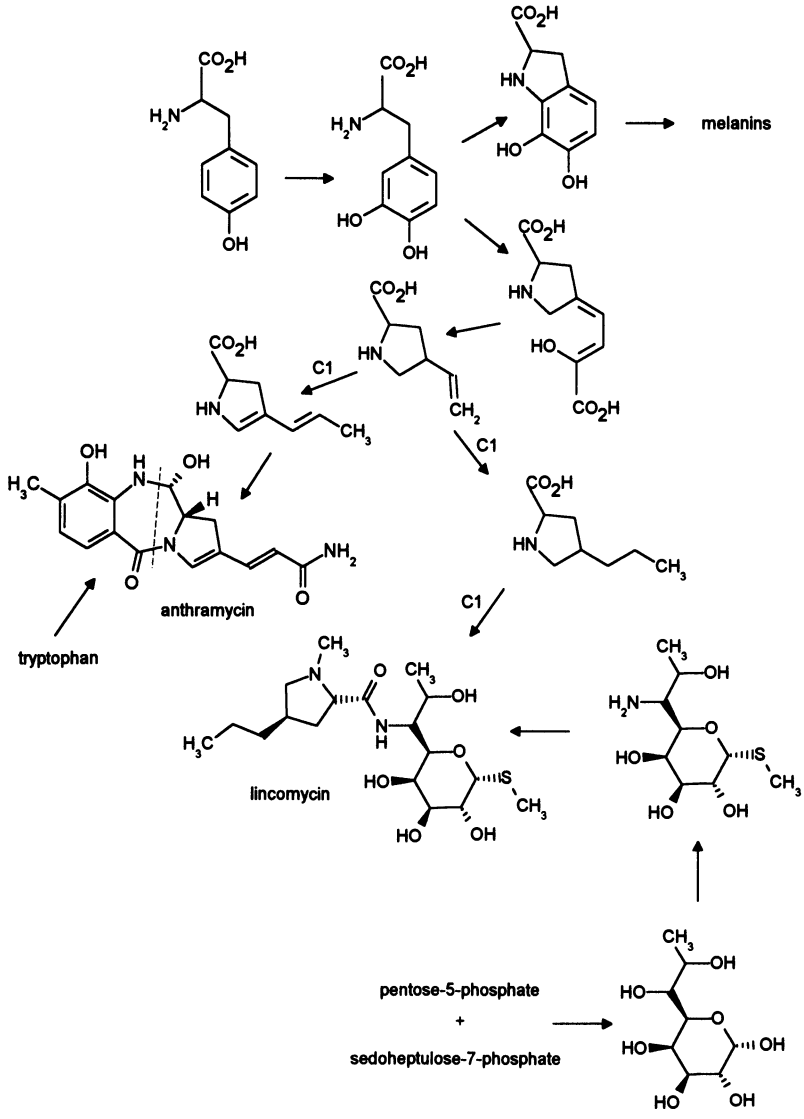

Figure 4.4. Biosynthesis of lincomycin and of the alkyl-dehydroproline moiety of anthramycin.

condensation of aspartate and a three-carbon unit, probably glyceraldehyde. The 3-hydroxy-4-methyl-anthranilic acid is a precursor of actinomycins and of anthramycin. This intermediate derives from tryptophan, through 3-hydroxykynurenine, which is also an intermediate in nicotinic acid biosynthesis in *Neurospora* (Fig. 4.5).

The pteridine moiety of both riboflavin and folic acid originates from guanosine triphosphate. The first reaction of the biosynthetic process is the removal, as formate, of carbon-8 of GTP. It is catalyzed by the enzymes GTP-cyclohydrolase I and II. The structure of the nucleoside antibiotics tubercidin, toyocamycin, and sangivamycin are characterized by a ribosyl-pyrrolopyrimidine ring. There is evidence that the pyrimidine moiety derives from ATP, and that the first reaction in the biosynthesis is the removal, as formate, of carbon-8 by the enzyme GTP-8-formylhydrolase. In addition, the carbon atoms of the pyrrole ring are provided by ribose, again paralleling folic acid biosynthesis in which ribose contributes to the formation of the pteridine's pyrazine ring (Fig. 4.6).

4.3.4. Sugar Transformations

Sugars are frequently present in antibiotic molecules. Oligosaccharide and aminocyclitol antibiotics are made entirely by sugar-derived units; many other antibiotics have glycosyl substituents as essential parts of their structure. Some of the sugars are very common ones, such as glucosamine or mannose. Others have unusual structures and are found only in secondary metabolites, although their biosynthesis present pathways analogous to the formation of the *O*-antigen.

Incorporation experiments have demonstrated that, as a general rule, the glycosidic moieties of antibiotics derive from glucose (or other common sugars such as mannose or glucosamine), without rearrangement of the carbon atoms. In primary metabolism, the interconversion of sugars normally occurs after the starting molecule has been activated as the nucleotide diphosphate ester at the anomeric carbon. In the small number of cases that have been investigated, the same activation has been observed in antibiotic glycoside biosynthesis, as the following examples demonstrate.

Mycarose is a deoxysugar present in tylosin and other macrolide antibiotics. The first two enzymes of its biosynthetic pathway have

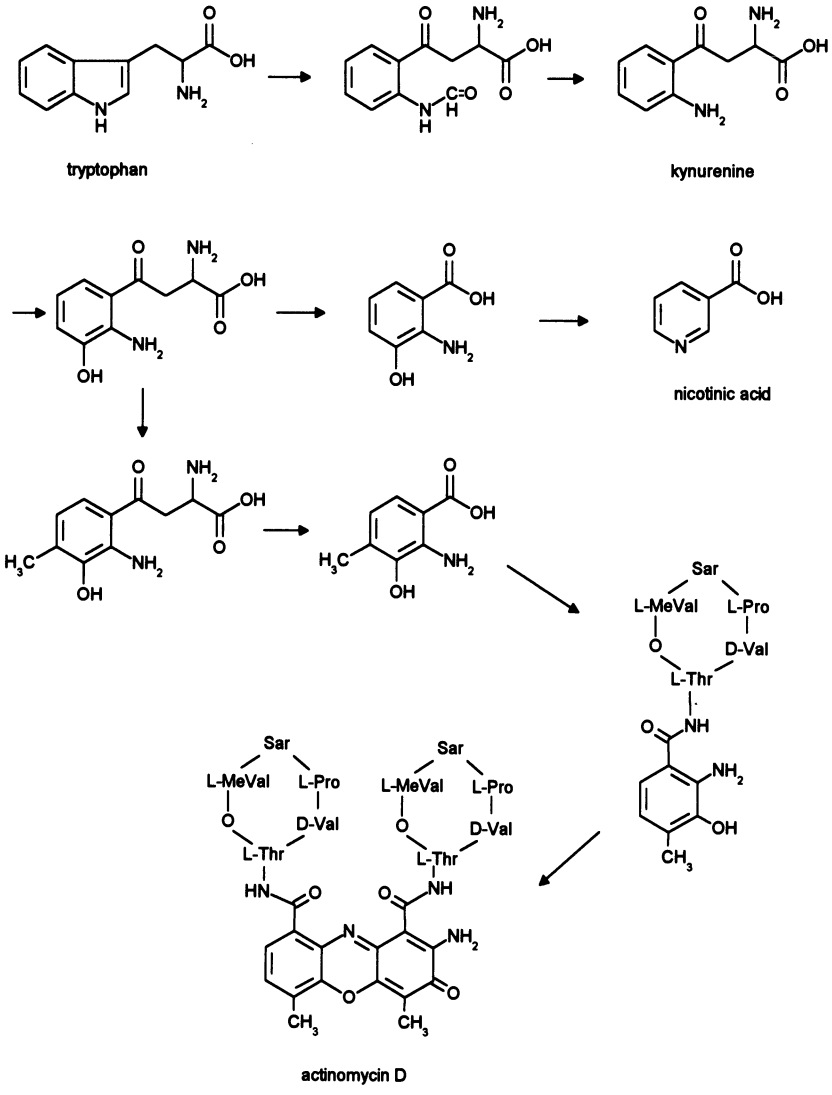

Figure 4.5. Comparison of the biosynthesis of the aromatic moiety of actinomycins and of nicotinic acid in *Neurospora.*

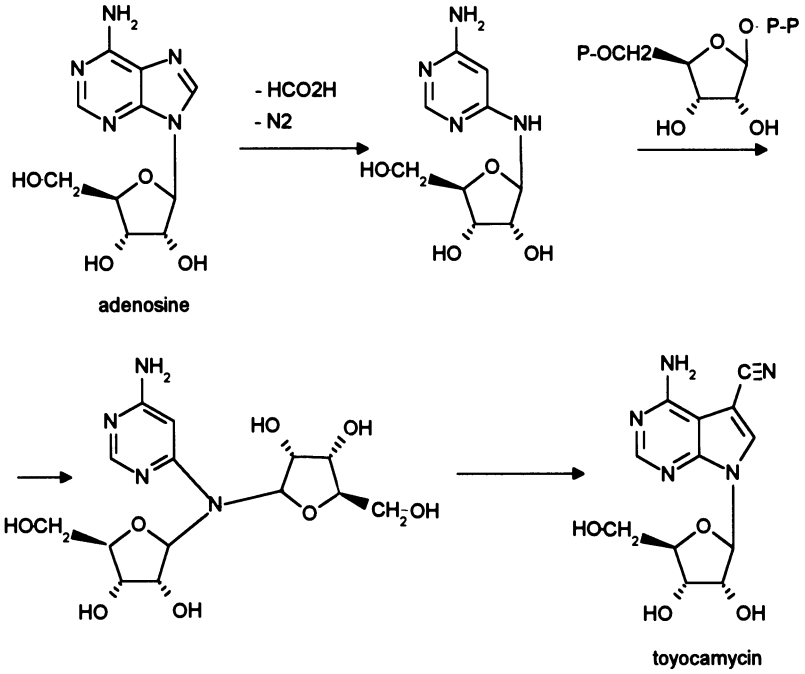

Figure 4.6. Presumptive biosynthetic pathway of toyocamycin. The phosphorylation level of adenosine and of the intermediates is unknown.

been identified. These are a dehydratase, converting dTDP-glucose to dTDP-4-keto-6-deoxyglucose, and an epimerase that converts the latter to dTDP-4-keto-L-rhamnose. The subsequent intermediates of the pathway have not been identified (Fig. 4.7).

More information is available on the biosynthesis of streptose, a component of streptomycin. The first two reactions are quite similar to those of mycarose biosynthesis, consisting of the dehydration of dTDP-glucose and the epimerization of the reaction product to dTDP-4-keto-L-rhamnose. This is then rearranged into dTDP-dihydrostreptose (Fig. 4.17). The oxidation of the latter to streptose occurs as a late step, after the assembly of the whole molecule. The other sugar component of streptomycin is methyl-L-glucosamine. It derives from D-glucosamine through a series of reactions, not fully understood, that formally involve the inversion of four chiral centers. It appears that D-glucosamine is

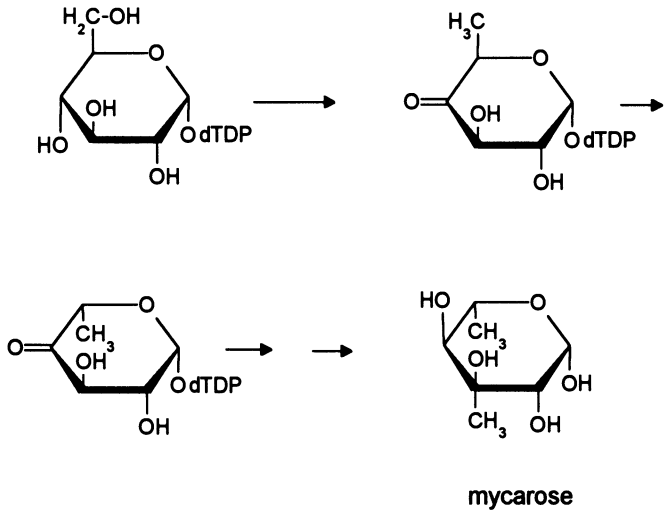

Figure 4.7. Biosynthesis of mycarose. The last intermediates of the pathway have not been identified.

activated as the UDP ester since UDP-methylglucosamine has been identified as an intermediate.

The aminocyclitols streptidine and 2-deoxystreptamine are essential components of several aminoglycoside antibiotics. Although both derive from glucose, and despite their structural similarity, they are biosynthesized through different pathways. A key intermediate in streptidine biosynthesis is D-*myo*-inositol, a common cellular metabolite. It is converted into the final molecule through two identical series of reactions (Fig. 4.8), each consisting of phosphorylation, oxidation of a hydroxy to a keto group, transamination of the latter, and conversion of the amino to the guanidino function (the group $-C(=NH)-NH_2$ being transferred from arginine).

In 2-deoxystreptamine biosynthesis, glucose, or its 6-phosphate, is converted to 2-deoxy-*scyllo*-inosose. The mechanism of this conversion is analogous to that of the dehydroquinate synthase reaction of aromatic amino acid biosynthesis, involving the oxidation and subsequent reduction of the hydroxy group in position 4. Transamination of the keto group, followed by oxidation and transamination of the hydroxy group

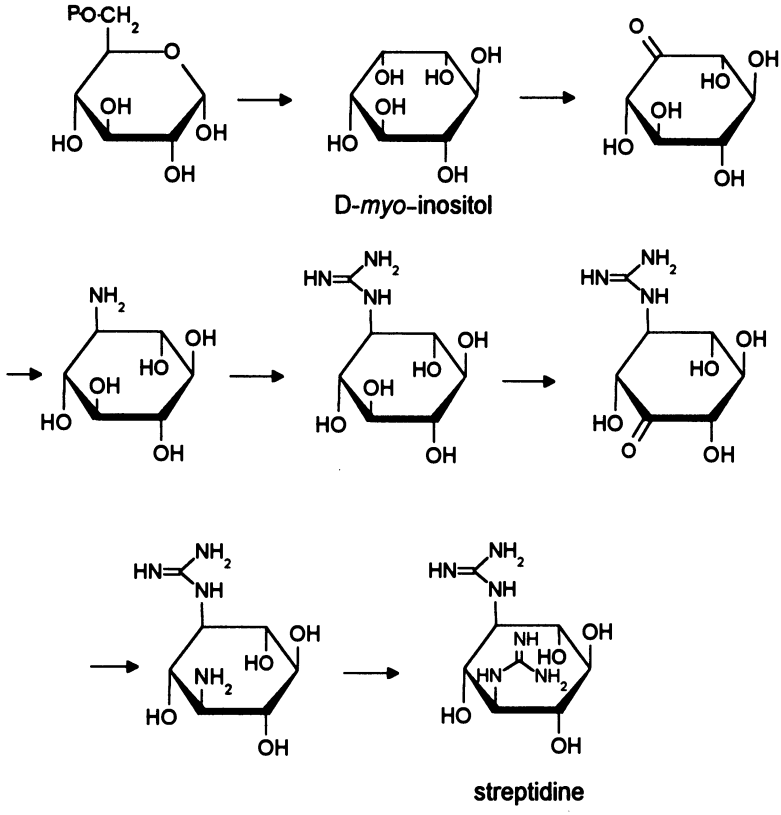

Figure 4.8. Biosynthetic pathway of streptidine, the aminocyclitol of streptomycin. For simplicity the phosphorylation and dephosphorylation steps have been omitted.

adjacent to the methylene carbon, produces the final molecule (Fig. 4.9).

4.4. *Class II Reactions: Polymerization of Small Metabolites*

4.4.1. *Polyketide Synthesis*

Fatty acid biosynthesis is a polymerization process, catalyzed by fatty acid synthase (FAS). In vertebrates, FAS is a large multifunctional

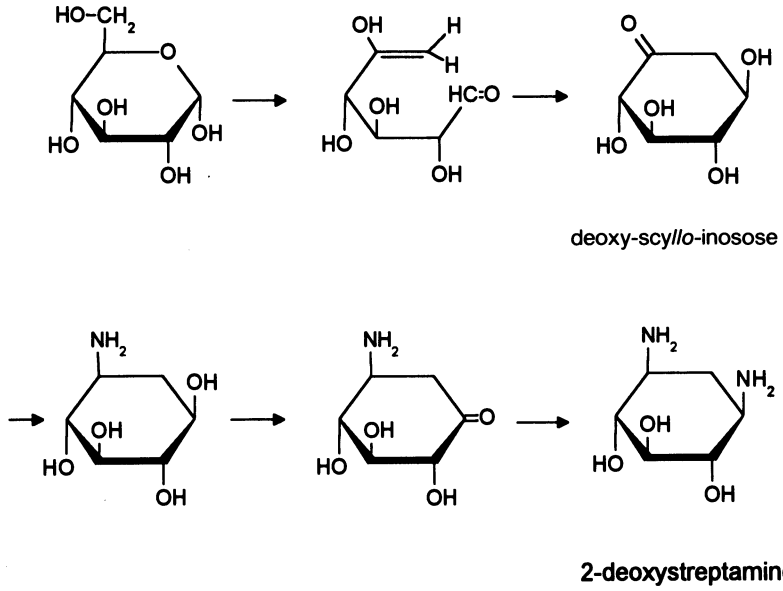

Figure 4.9. Biosynthesis of 2-deoxystreptamine, the aminocyclitol of the majority of aminoglycoside antibiotics.

polypeptide (type I FAS), whereas in bacteria and higher plants it is a multienzyme complex (type II FAS). The polymerization process has been studied in great detail, and its essential steps can be summarized as follows.

1. Acetate, the initiator molecule, and malonate, the extender unit, are linked to the synthase as thioesters [acetate to a subunit (or domain) denoted the condensing enzyme and malonate to the acyl carrier protein (ACP)].
2. The carboxyl carbon of acetate is condensed to the methylene carbon of malonate; at the same time the free carboxyl group of malonate is eliminated as CO_2. The resulting product is acetoacetate, bound as thioester to ACP.
3. The carbonyl group of acetoacetate is reduced to a hydroxy group. A subsequent dehydration step generates a double bond, which is then saturated by a second reduction step to give a butyrate moiety.

4. The growing chain is transferred to the condensing enzyme, another malonate unit is linked to the ACP, and the process is repeated until the proper length is reached. A thioesterase then releases the aliphatic acid.

If, during this process, all or most of the reducing steps of point 3 are omitted, the polymerization product is a polyketomethylene (or polyketide) chain, consisting of alternating carbonyl and methylene groups. This is the origin of the basic structure of a large number of antibiotics, produced by both actinomycetes and lower fungi (other secondary metabolites such as pigments also have a similar origin). Polyketide chains have a strong tendency to fold and are highly reactive. According to the nature of this folding, which may be enzymatically directed, and the length of the chain, a very large variety of structures can be formed. These mainly consist of aromatic rings because of steric and energetic reasons.

Examples of clinically useful antibiotics produced by this process are the tetracyclines, griseofulvin, and the antitumor anthracyclines, such as doxorubicin. It should be noted that, in some cases, the chain can be initiated by a different molecule, such as malonamide in tetracyclines or propionate in doxorubicin, rather than by acetate. The polyketide chains are too reactive and therefore too unstable to be isolated. However, their structure can be deduced from incorporation experiments and from the structure of early intermediates of the biosynthetic pathway (Fig. 4.10).

The general polymerization process has a limited number of variations in the biosynthesis of the bacterial fatty acids. These mainly consist of the omission of one reduction step resulting in the formation of unsaturated fatty acids, or in the use of other acids, instead of acetate, as the initiator molecule. In bacilli and in actinomycetes these initiators are frequently isobutyric or isovaleric acids, and the molecules that they give rise to are either iso or anteiso fatty acids.

In the biosynthesis of antibiotics, particularly those produced by actinomycetes, there is a much greater variation pattern. Frequently the initiator is propionate, but other aliphatic or aromatic acids are found in several antibiotics. Another source of variation is the extender molecule, which may be methylmalonate or sometimes ethylmalonate (rarely other malonate derivatives). When this is the case, the result is

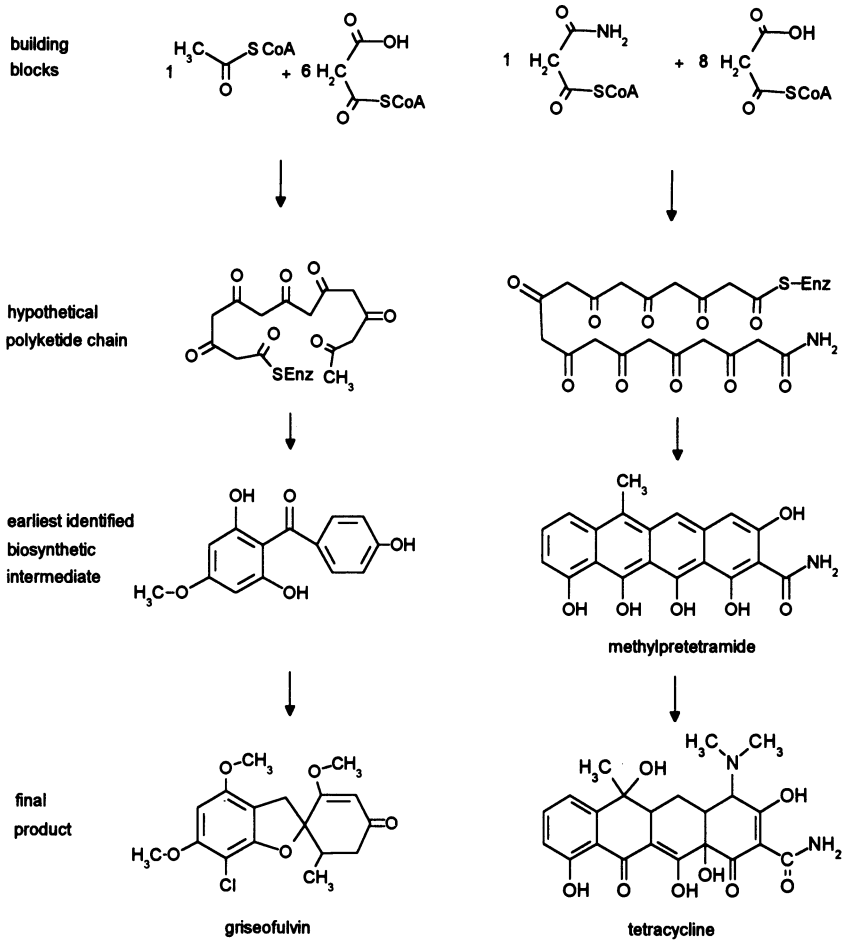

Figure 4.10. Basic scheme of the biosynthesis of typical polyketide derived antibiotics.

a chain carrying methyl or ethyl substituents, respectively—because the condensation of the growing chain always occurs at the carbon adjacent to the carboxyl group. It should be pointed out that acetate and propionate are easily converted in the cell into malonate and methylmalonate, respectively. Thus, incorporation of labeled acetate or propionate in a chain-extending position is generally taken as evidence of its origin from malonate or methylmalonate. A third important source

of variation is the omission of one of the reduction steps or of the dehydration step mentioned above in point 3. The chain then contains keto or hydroxy functions or double bonds. Because of this, and because of the presence of alkyl substituents, the chains do not contain a series of activated methylenes, and therefore cannot form aromatic rings by simple dehydration. The resulting structures are then either linear molecules or large cyclic molecules, closed by an amide (macrolactams), or by an ester bond (macrolactones).

In the synthesis of fatty acids or of aromatic polyketides, almost all elongation cycles are identical. In the biosynthesis of antibiotics derived from alkyl-substituted, partially reduced, polyketide chains, the chain assembly must be accurately programmed in order to insert the right extender unit with the right level of reduction in each position of the molecule. How this is accomplished has been demonstrated for the first time by studies on erythromycin biosynthetic genes. Erythromycin aglycone is made by propionate as the initiator, and by six molecules of methylmalonate as the extender units. Studies on chain formation show that the synthase enzymatic complex, as deduced from the gene arrangement and sequences, appears to be composed of three multifunctional proteins, resembling type I FAS. Two FAS-like functional domains are present in each protein. Thus, there are a total of six domains, each responsible for one of the six elongation cycles necessary for the completion of the chain. Each cycle includes the addition of one extending unit to the growing chain and the total or partial reduction of the β-carbonyl group. The growing chain is transferred from one domain to the next, so that the order in which the domains are arranged determines the order in which the different chemical functions produced in a cycle appear in the final molecule (Fig. 4.11).

The basic structures of several classes of important antibiotics produced by polymerization of this general type include:

1. Antibacterial macrolides. The basic structure of these products is a macrolactone comprising from 12 to 16 atoms carrying as substituents one or more sugars. The initiator molecule is either acetate or propionate, the extender units are mainly methylmalonate in 14-member macrolides and mainly malonate in the 16-member ones. In the latter group, one butyrate–ethylmalonate-derived unit is also present. The chain is highly

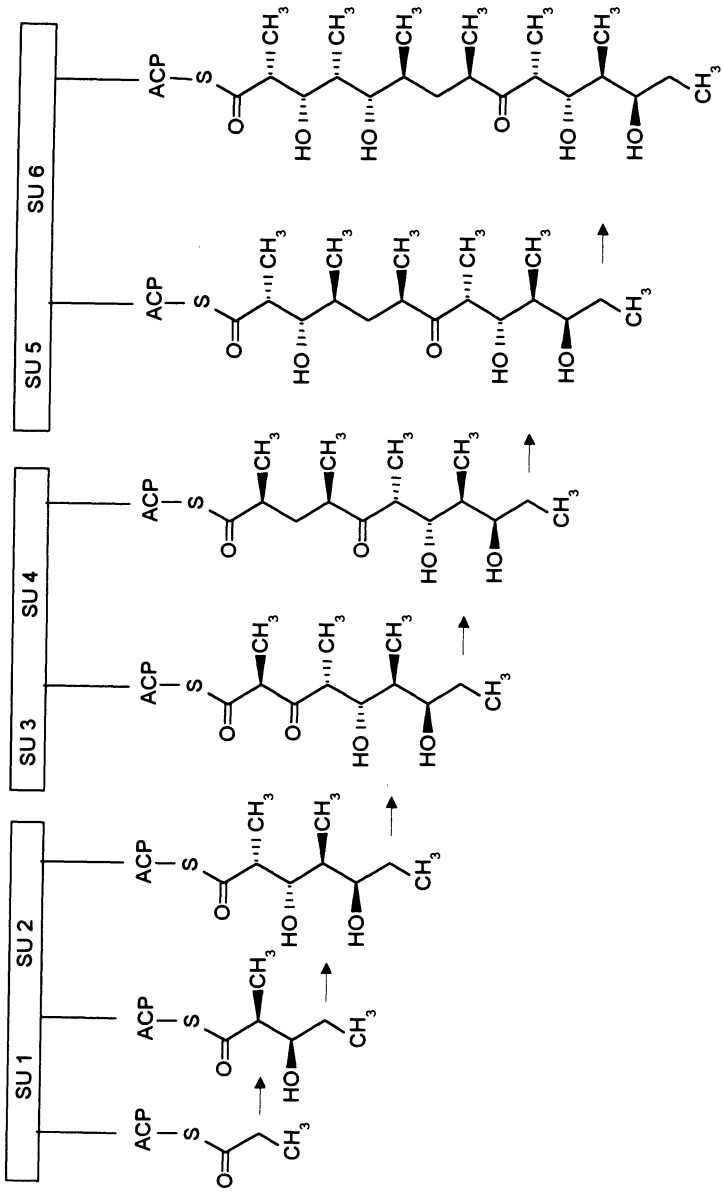

Figure 4.11. Example of a chain construction by type I polyketomethylene synthase. Biosynthesis of the chain that gives rise to 6-deoxyerythronolide B in *Saccharopolyspora erythrea*. SU, synthase unit.

reduced, carrying in most cases only one keto and a few hydroxy groups (Fig. 4.12).

2. The avermectins, an important family of secondary metabolites, discussed previously as antiparasites, are also 16-membered macrolides, but their structure includes reduced pyran and furan rings. Their biosynthesis is similar to that of other macrolides; the aglycone is derived from seven acetate (malonate) and five propionate (methylmalonate) units. The initiator molecule is either isobutyrate or 2-methylbutyrate, which are derived from valine and isoleucine, respectively.
3. Antifungal macrolides (polyenes). The biosynthesis of these antibiotics appears similar to that of antibacterial macrolides. An obvious difference is the length of the putative polyketide chain, giving rise to lactone rings of 26 to 38 atoms. Another difference is in the variety of the initiator molecule; in polyenes this is frequently *para*-aminobenzoic acid, isobutyric or 2-methylbutyric acid. The chain is mainly built from malonate units and a few methylmalonate units.
4. Ansamycins are macrolactams, containing either benzene or naphthalene aromatic rings. In all cases the biosynthetic chain is initiated by 3-amino-5-hydroxybenzoic acid, a molecule derived from a variation of the shikimate pathway, which is also found as a building block in the biosynthesis of other antibiotics, such as mitomycin. In rifamycins, a family of naphthalenic ansamycins, the extender units are two malonate and eight methylmalonate molecules. The naphthalene ring is formed by the initiator molecule with which a segment of the polyketide chains is condensed (Fig. 4.12).
5. Polyether antibiotics are linear molecules, made by malonate, methylmalonate, and ethylmalonate units. The name of the class results from the presence of pyran or furan rings, derived by the condensation of oxygen functions on the polyketide chain. A typical example is monensin, which is made by acetate as the initiator, four malonate, seven methylmalonate, and one ethylmalonate unit (Fig. 4.13).

4.4.2. The Thiotemplate Mechanism of Polypeptide Formation

There are three different mechanisms by which amino acids are condensed to give peptide secondary metabolites. (1) Amino acids are

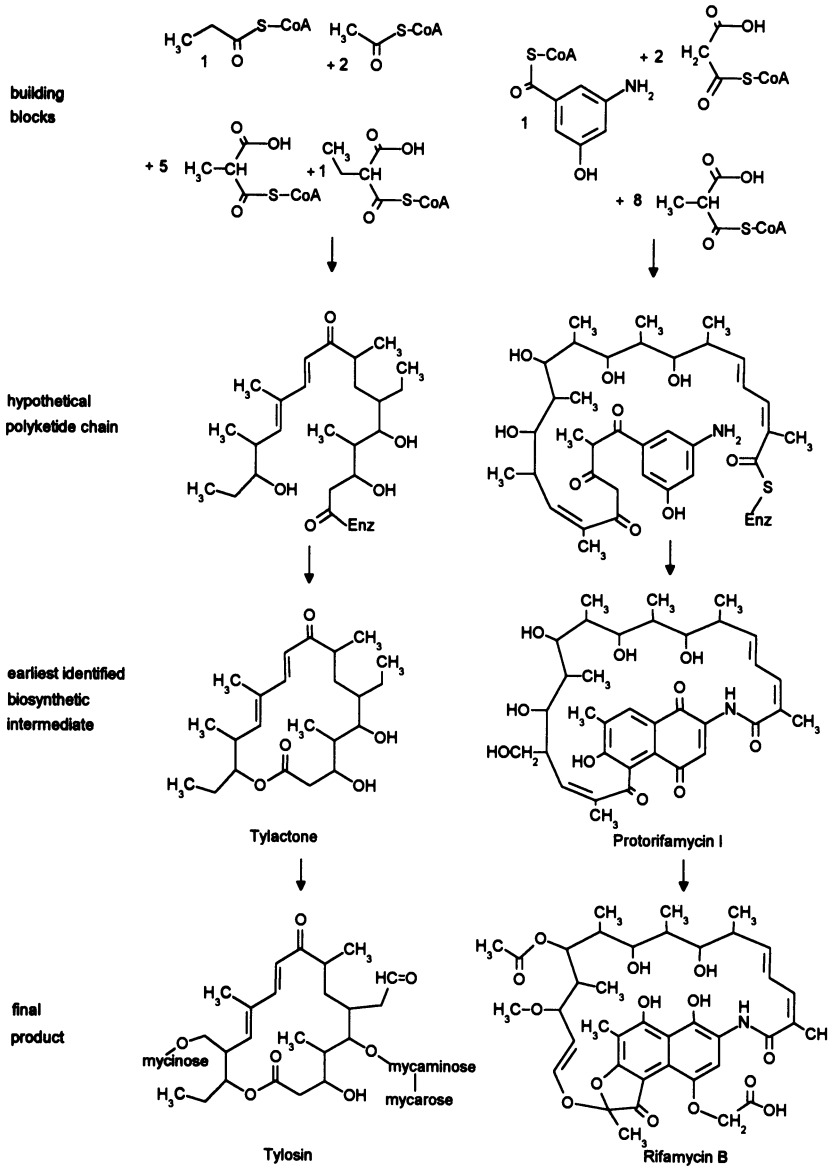

Figure 4.12. Basic scheme of the biosynthesis of typical antibiotics derived from substituted, reduced, polyketide chains.

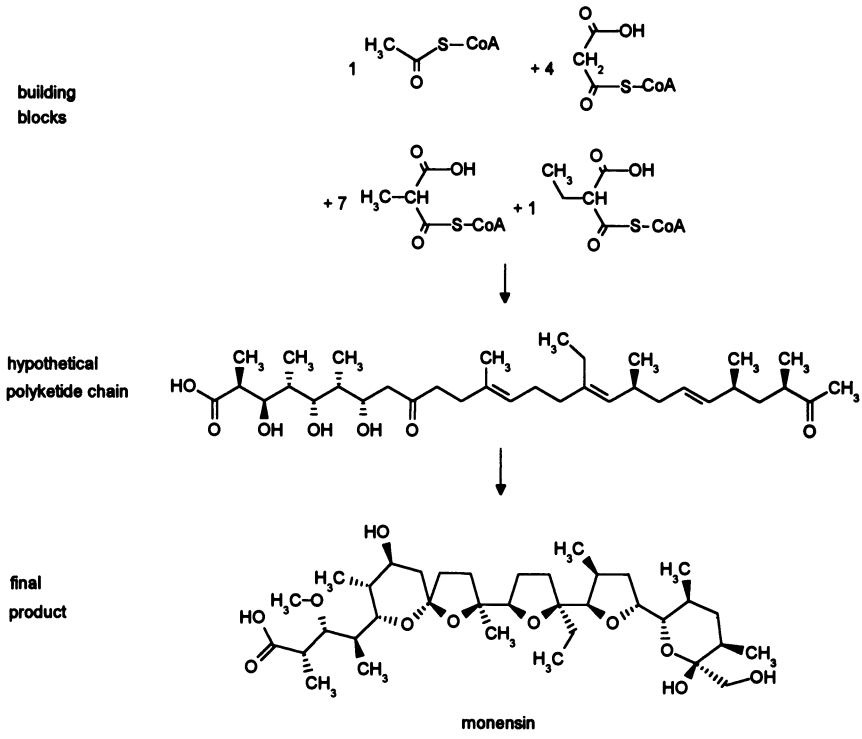

Figure 4.13. Basic scheme of a polyether (monensin) biosynthesis.

activated as phosphates and condensed by single specific enzymes. This mechanism, by which glutathione is made in primary metabolism, probably operates in the biosynthesis of some small peptides, such as bialaphos or the protease inhibitor leupeptin. (2) Large polypeptide chains are made by the common transcription–translation system of protein synthesis (see Section 4.4.5). (3) Amino acids are activated and condensed by a multienzyme complex (similar to the fatty acid synthases), according to the thiotemplate mechanism described next.

In general, the system includes: (1) activation of the amino acids by reaction with ATP and formation of the adenosyl monophosphate at the carboxyl group; (2) transfer of the amino acids to thiol groups on the enzymes, with formation of thioester bonds, in an order determined by the multienzyme complex structure; (3) formation of a peptide

bond between the carboxyl of the first amino acid and the amino group of the second one, the energy being provided by the breaking of the thioester bond; (4) similarly the thioester of the dipeptide breaks and the carboxyl group forms a peptide bond with the amine of the third amino acid; this step is repeated until the chain is completed.

The process is catalyzed by an enzymatic complex that may comprise up to four multienzymes. Each multienzyme performs the activation and the thioesterification of some of the amino acids and the peptide bond formation. Additional functions sometimes observed are the inversion of configuration from L to D of the amino acids, and the methylation of the amide nitrogen, involving *S*-adenosylmethionine as the methyl donor.

In the biosynthesis of depsipeptide antibiotics, in addition to amino acids, hydroxy acids are used as building blocks. The result is a chain in which ester bonds alternate with amide bonds.

As already stated, the mechanism presents similarities to that of polyketide synthesis. The analogy is enhanced by evidence indicating that the transfer of the substrates to the thiol groups is mediated by an enzyme-linked phosphopantetheine, a characteristic moiety of the FAS acyl carrier protein.

One of the best known multienzyme complexes is that which catalyzes gramicidin S synthesis (Fig. 4.14). Gramicidin S is a cyclic polypeptide, made by two identical pentapeptides joined head to tail. Its biosynthesis is performed by two soluble enzymes, GS1 and GS2, having a molecular weight of about 130,000 and 500,000, respectively. The lighter enzyme catalyzes the activation and racemization of phenylalanine, and the thioesterification of the D isomer, which is the starting molecule. The GS2 enzyme activates and carries the other four amino acids. By the process above described a pentapeptide is synthesized, two molecules of which are assembled head to tail, forming the final antibiotic.

Recent genetic studies have demonstrated that the synthesis of penicillins and cephalosporins is also initiated by a multienzyme using a thiotemplate mechanism. The enzyme, ACV synthase, has been purified from *Aspergillus nidulans, Cephalosporium acremonium,* and *Streptomyces clavuligerus.* It activates α-aminoadipic acid (at its δ-carboxyl group) cysteine and valine; binds the activated amino acids as thioesters; performs the inversion of configuration of L- to D-valine;

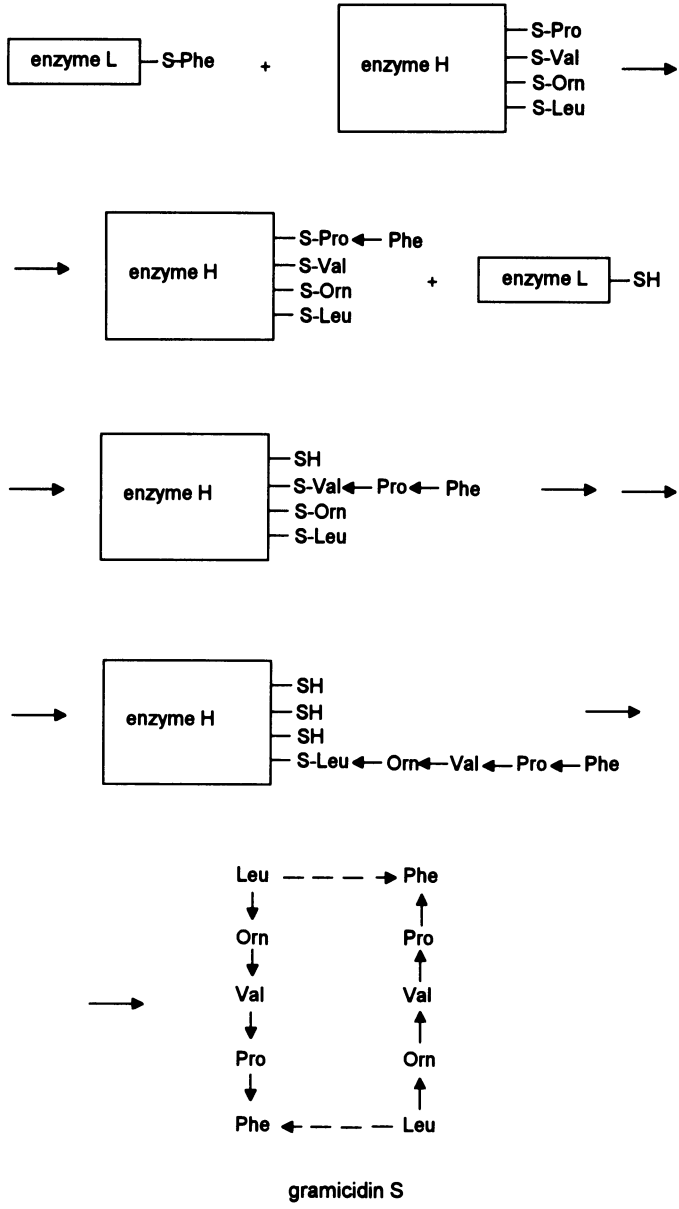

Figure 4.14. Biosynthesis of gramicidin S by the thiotemplate mechanism. Enzymes L and H represent the two polyfunctional enzymes GS1 and GS2 involved in the biosynthesis. The dashed arrows in the gramicidin S structure represent the bonds joining the two identical pentapeptides.

and polymerizes the amino acids to the tripeptide δ-aminoadipyl-cysteinyl-D-valine. In both penicillin and cephalosporin biosynthesis the tripeptide is then cyclized by the enzyme isopenicillin N synthase to form isopenicillin N, the last common intermediate of these β-lactam pathways (Fig. 4.15). The subsequent steps of the pathways are reported in Fig. 4.16.

4.4.3. Assembly of Oligosaccharides

The biosynthesis of aminoglycoside antibiotics (that are more exactly pseudosaccharides, containing an aminocyclitol) has been studied in some detail. Isolation of biosynthetic intermediates has demonstrated that the sugar units are in general linked stepwise, with the formation first of a disaccharide (often a pseudodisaccharide) to which one or more sugars are then added. The general process is quite similar to the synthesis of oligo- or polysaccharides present in bacterial and fungal

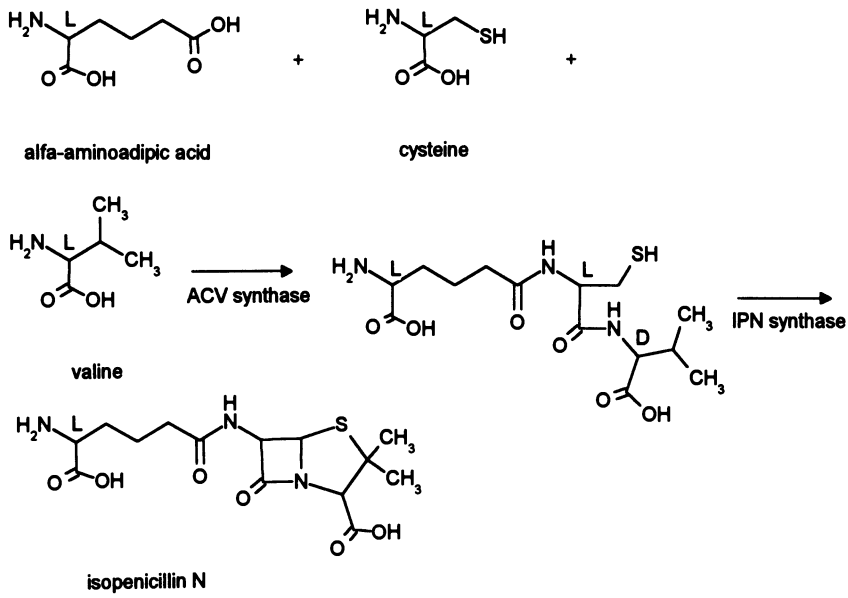

Figure 4.15. Biosynthesis of isopenicillin N, the common precursor of penicillins, cephalosporins, and cephamycins.

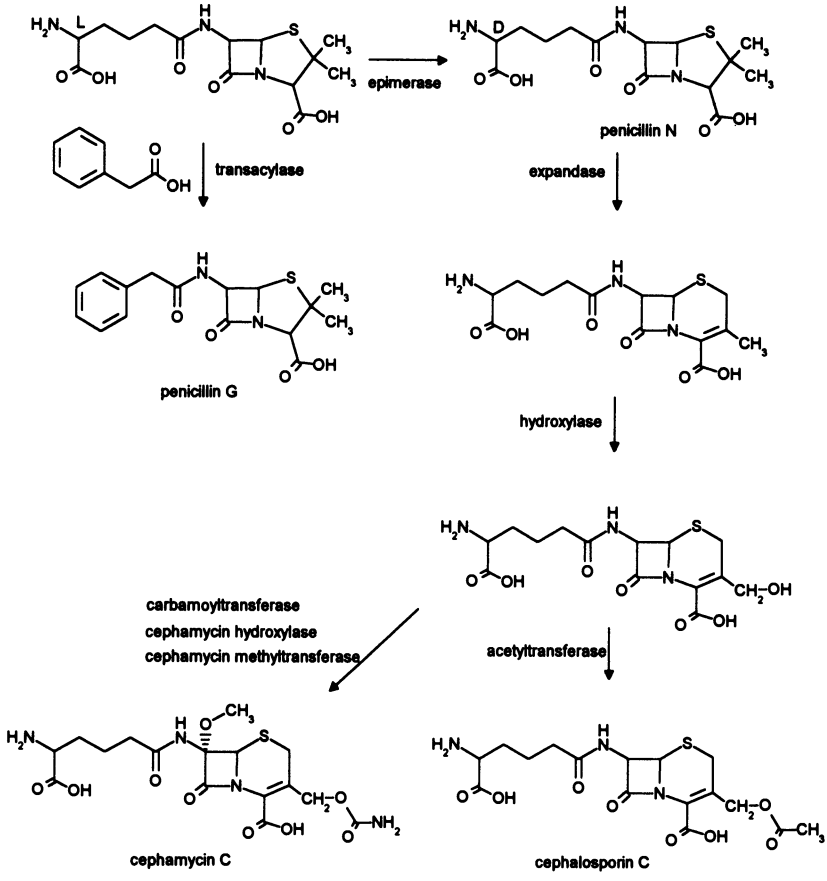

Figure 4.16. Final steps in β-lactam antibiotic biosynthesis.

cell walls. In analogy with the last process it is believed that the sugars are activated as nucleoside diphosphates at the anomeric carbon, prior to addition. However, this last point has only been demonstrated in a few cases.

The transformation of common sugars into the unusual ones constituting most of the aminoglycoside molecules, occurs frequently before the assembly of the units. Some examples have been reported in Section 4.2. In the formation of other antibiotics, major modifications occur at the disaccharide or trisaccharide level. The biosynthesis of

streptomycin and of gentamicins are representative of these different patterns.

The assembly of the streptomycin molecule begins with the glycosylation of streptamine phosphate by dihydrostreptose, activated as deoxythymidine diphosphate. *N*-Methyl-L-glucosamine, probably activated as uridine diphosphate, is then added to the 2′-hydroxyl of dihydrostreptose. Oxidation of the exocyclic hydroxy function of the latter and dephosphorylation complete the synthesis (Fig. 4.17).

In gentamicin biosynthesis, two normal sugars, D-glucosamine and D-xylose, are added stepwise to 2-deoxystreptamine, producing gentamicin A_2. Through a series of reactions the xylose of the latter is aminated and methylated, with formation of gentamicin X_2 the last common intermediate in the biosynthesis of several metabolites, including gentamicins C_{1a} and C_{2a}, and sagamicin (Fig. 4.18). These antibiotics are formed by the elimination of the hydroxyl functions of the D-glucosamine moiety, and differ in the presence and the position of methyl groups on this moiety.

4.4.4. Isoprenoid Synthesis

The basic structure of many fungal secondary metabolites is derived from the condensation of isoprene units, the common building block of primary metabolism. Derivatives most frequently found are the sesquiterpenes, made by the condensation of three units, the diterpenes, originating from four units, and the sterols, derivatives of squalene, an intermediate biosynthesized from the head-to-head condensation of two molecules of farnesyl pyrophosphate. The assembly of the isoprenoid antibiotic carbon skeleton follows the known pattern of the analogous primary metabolites. However, a great variety of structure is found because there are many ways in which the original chain can cyclize, and because of a number of rearrangements and other reactions frequently occurring.

The only therapeutically relevant antibiotic of this group is fusidic acid, whose biosynthesis is considered analogous to that of fungal membrane sterols.

4.4.5. Peptide Antibiotics of Ribosomal Origin

A handful of antibiotics are known which are directly made by the normal transcription and translation system of protein synthesis.

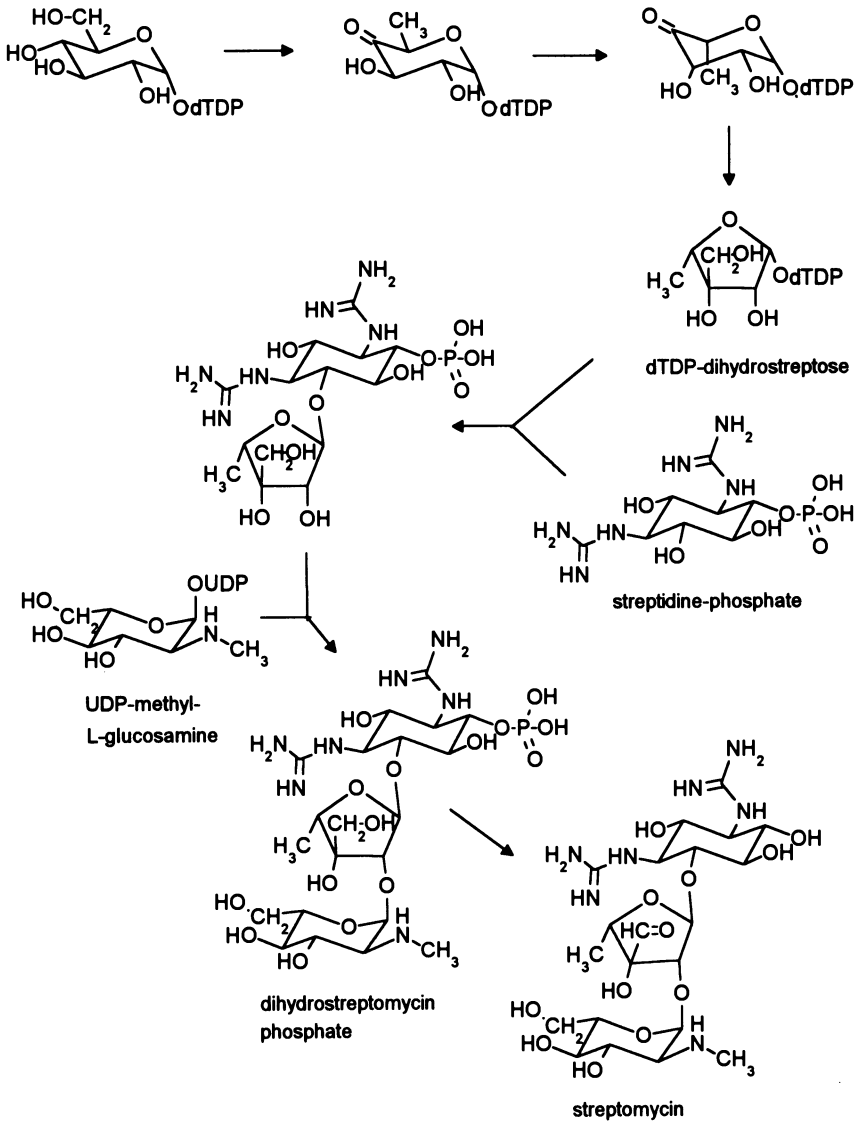

Figure 4.17. Conversion of dTDP-glucose into dTDP-dihydrostreptose, and biosynthesis of streptomycin.

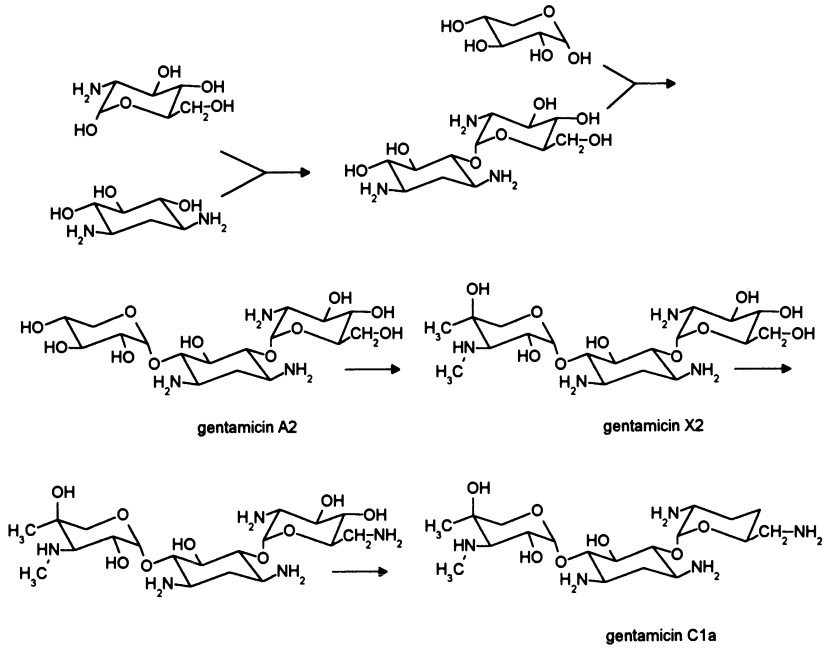

Figure 4.18. Biosynthetic pathway to gentamicin C_{1a}. Branches from the same pathway lead to the production of other gentamicins and of sagamicin.

Most of these are produced by bacteria, but a few have been isolated from actinomycetes. They are termed lantibiotics, because of the presence in the molecule of lanthionine residues, derived from the reaction of a cysteine thiol with a dehydroserine or dehydrothreonine residue. The ribosomally synthesized peptides are larger than the actual antibiotics, whose size can vary from the 19 residues of the ACE inhibitor ancovenin, to the 34 residues of the antibacterial nisin. In fact, the peptide precursor includes a leader sequence, which is then cleaved by posttranslational processing. Other posttranslation reactions, such as the above-mentioned lanthionine formation, modify some of the amino acid residues and give the molecule its final shape.

A few antibiotics, such as neocarzistatin, are composed of larger ribosomally synthesized peptides of over 100 residues, linked to a complex chromophore biosynthesized by different mechanisms. These are referred to as protein antibiotics.

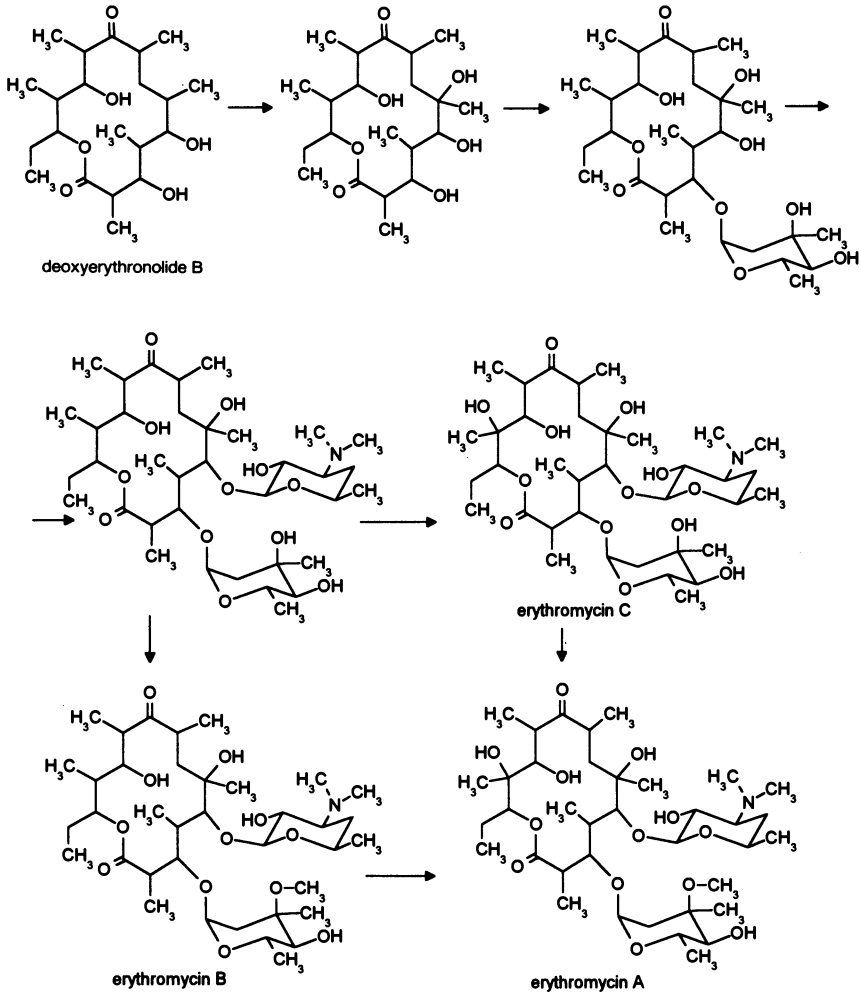

Figure 4.19. Final steps in erythromycin biosynthesis. Both pathways through erythromycin B or C are possible.

4.5. Class III Reactions: Modifications of the Basic Structure

In the vast majority of cases, the basic antibiotic structure, derived either by a single intermediate or by the assembly of several units, is further modified by enzymatic reactions to give the final molecules.

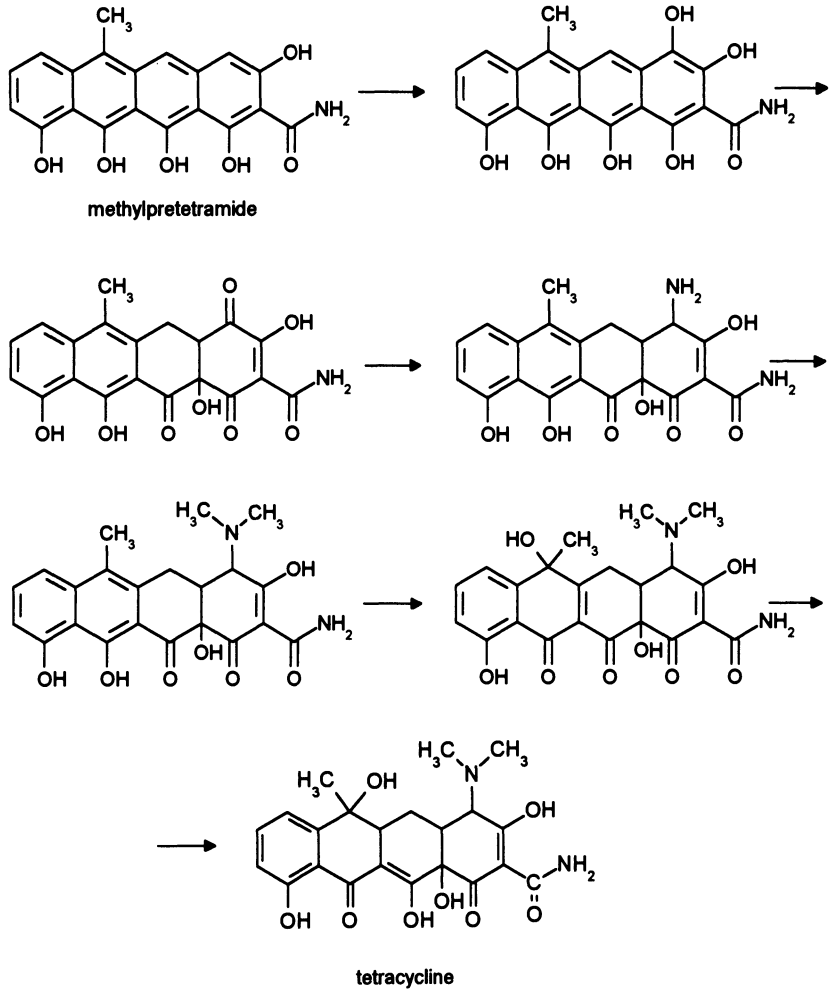

Figure 4.20. Biosynthetic reaction sequence from methylpretetramide to tetracycline.

The most frequent reactions are glycosylations, acylations, methylations, hydroxylations or reductions, and aminations. In most cases these modifications are essential for biological activity. When some of these reactions can be omitted without a significant loss of activity, or minor variations introduced, the result is the production of a number of similar substances denoted as an antibiotic complex.

For instance, during erythromycin biosynthesis we observe: hydroxylation of two carbons of the ring, the addition of two sugars, and the methylation of a sugar hydroxyl function (Fig. 4.19). In the biosynthesis of penicillins and cephalosporins, all reactions subsequent to the formation of isopenicillin N can be considered class III reactions. For penicillins these consist only in a transacylation by which other acyl moieties, notably phenylacetic acid or phenoxyacetic acid, are substituted for α-aminoadipic acid. For cephalosporins the pattern is more complex, including the inversion of configuration at the aminoacyl chain, the unusual ring expansion, and the hydroxylation and acetylation of the 3-methyl group (Fig. 4.16).

The number of possible examples is practically endless. Some essential reactions, such as dehydroxylation and introduction of amino functions, have been mentioned in reference to gentamicin synthesis. The conversion of methylpretetramide into tetracycline also represents an interesting series of oxidation, amination, and methylation reactions (Fig. 4.20). It is evident that a generalization is not possible and the variety of class III reactions is one of the main causes of the astounding variety of antibiotic chemical structure.

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5

Regulation of Antibiotic Biosynthesis

The principal mechanisms of regulation which apply to both primary and secondary metabolite biosynthesis are the repression of enzyme synthesis and the inhibition of enzyme activity. However, the fact that these operate in the control of secondary metabolite biosynthesis has only recently been well accepted. Some of these mechanisms are specific for antibiotic production but, remembering that secondary metabolism is a form of differentiation, it is not surprising that its regulation often falls under more general systems of control governing other forms of differentiation, such as the sporulation process or the formation of aerial mycelium.

5.1. Feedback Regulation

The assumption that secondary metabolites may regulate their own biosynthesis by feedback inhibition has been experimentally supported in several cases. Most of the evidence is based on the cessation of antibiotic production observed when the fermentation product is added to cultures of the producing organism. Other indirect evidence includes the increase in production yields achieved by continuously removing the produced antibiotic from the fermentation broth. This can be accomplished by adsorption onto a resin, or as in the case of cycloheximide production by *Streptomyces griseus,* by dialysis-extraction. This type of experiment cannot conclusively establish whether the underlying mechanism is repression of enzyme synthesis or inhibition of enzyme activity. However, at least in the case of aurodox production by *Strep-*

tomyces goldiniensis, the short time elapsing between addition of antibiotic and block of the synthesis suggests that inhibition, rather than repression, is the likely mechanism.

Only in a few cases has the biosynthetic enzyme involved been identified. In *Streptomyces venezuelae,* the producer of chloramphenicol, the addition to the fermentation medium of the final product, or of its *p*-methylthio analogue, inhibits the *de novo* formation of the antibiotic. This effect is caused by repression of arylamine synthetase, the enzyme converting chorismate to *p*-aminophenylpyruvate, which is the first specific metabolite in chloramphenicol biosynthesis.

In the puromycin producer *Streptomyces alboniger,* the activity of the last enzyme of the biosynthetic pathway, an *O*-methyltransferase, is inhibited by the presence of the end product. Similarly, in *Streptomyces fradiae* tylosin inhibits the *O*-methyltransferase involved in its own biosynthesis and in the mycophenolic acid producer, *Penicillium stoloniferum,* the last enzyme of the biosynthetic pathway, again an *O*-methyltransferase, is also inhibited by the end product.

Antibiotic production can be indirectly regulated by feedback inhibition of products of primary metabolism involved in their biosynthesis. For example, in *Penicillium chrysogenum,* lysine interferes with penicillin production by inhibiting the first enzyme involved in its own biosynthesis (homocitrate synthase) the product of which (L-α-aminoadipate) is also a precursor of the antibiotic. The decreased availability of the precursor molecule results in a corresponding decrease of the final product yields. Another example is, in *Streptomyces griseus* fermentation, the inhibition of candicidin biosynthesis by aromatic amino acids, tryptophan in particular. *p*-Aminobenzoic acid, a precursor of the aromatic moiety of candicidin, is synthesized through the shikimate pathway, subject to the feedback inhibition of the aromatic amino acids.

5.2. Regulation by Nutrient Concentration

When producing strains are cultured in nutritionally rich media, high levels of secondary metabolites are usually produced only after cellular growth is completed. On this basis the presence of a growth phase, called trophophase, distinct from a production phase, called idi-

ophase, has been considered a general characteristic of secondary metabolite fermentations. This is certainly an oversimplification, since some antibiotics, such as chloramphenicol, etamycin, and the rifamycins, are produced during the exponential growth phase also. Moreover, in defined media supporting only a slow growth rate, the two phases often overlap, since production is observed while growth is occurring.

It is clear that nutrient limitation can result in a slow growth rate and the expression of biosynthetic genes simultaneously. The link between the two phenomena is complex, and it is very difficult in most cases to distinguish between cause and effect. However, the direct interference of a particular nutrient level on antibiotic biosynthesis has been clearly demonstrated in several cases.

5.2.1. Carbon Source Repression

Glucose, usually an excellent carbon source, is known to interfere with the biosynthesis of many antibiotics, produced either by actinomycetes or by fungi and belonging to different biosynthetic families, such as aminoglycosides, polyketides, peptides, and β-lactams. In most cases the experimental support is limited to the difference of production observed when slowly metabolized carbon sources are substituted for glucose in the fermentation media. However, in some cases specific repression of the synthesis of enzymes of the antibiotic biosynthetic pathways has been demonstrated.

For example, in *Streptomyces antibioticus,* producer of actinomycin, phenoxazinone synthase is repressed by glucose. This enzyme is normally synthesized after cessation of cell growth and its presence increases following glucose depletion. Since the levels of the specific mRNA follow a parallel course, it appears that regulation is exerted at the level of transcription. All of the other enzymes of the actinomycin biosynthetic pathway so far studied also appear to be affected by glucose repression. Similarly, in *Nocardia lactamdurans* cephamycin biosynthesis is under glucose regulation, exerted through repression of at least two enzymes of the biosynthetic pathway, ACV synthase and expandase.

Other antibiotics produced by actinomycetes in which the effect of glucose on the biosynthesis has been studied are tetracycline, puromycin, and the aminoglycosides kanamycin, neomycin, and strepto-

mycin. In all of these cases the repression of one of the biosynthetic enzymes has been observed. Since in some cases this represents the only enzyme studied, it is quite possible that the repression also affects other, or all, enzymes of the pathway.

β-Lactam production in fungi is also regulated by rapidly utilized carbon sources. Penicillin production in *Penicillium chrysogenum* fermentations is depressed by glucose, fructose, and sucrose, as is depressed the production of cephalosporin in *Cephalosporium acremonium* by glucose or glycerol.

Glucose does not inhibit the activity of the penicillin-forming enzymes in *P. chrysogenum,* but represses the synthesis of the cyclase (isopenicillin N synthase) and to some extent that of the ACV synthase. In *C. acremonium* the cyclase is also moderately repressed by glucose or glycerol, whereas expandase is markedly depressed. This, and the fact that expandase is a labile enzyme, explains why intermediate concentrations of glucose result in a low cephalosporin production and in accumulation of isopenicillin N. Surprisingly, it has been found that in *Cephalosporium* the formation of ACV synthase is not depressed by carbon sources but that the activity of this enzyme is inhibited by glucose or by intermediates of the glycolytic pathway.

Since studies on the carbon source effect are normally performed on whole cells, it is not known whether glucose or one of its metabolites is the intracellular repressor. Carbon source regulation of antibiotic biosynthesis is often referred to as carbon catabolite repression, in analogy with the well-known repression of inducible enzymes in *E. coli* and other eubacteria. However, the mechanism by which the regulation is exerted appears to be different. The intracellular effector in *E. coli* is cyclic AMP. Both in actinomycetes and in fungi most of the accumulated evidence excludes participation of this molecule in the repressive mechanism.

5.2.2. Nitrogen Source Regulation

Depression of production by high concentrations of ammonium ions is a common feature of antibiotic fermentation. In fact, it has often been observed that antibiotic production starts only when most of the ammonium in the medium has been depleted. Evidence of this is also the increase of yields obtained when slowly metabolized nitrogen sources

are substituted for ammonia in some antibiotic fermentations. The classical example is streptomycin production by *Streptomyces griseus* that was enhanced threefold by substituting proline for ammonia as the sole nitrogen source in a chemically defined medium. Moreover, addition of magnesium phosphate, an ammonium ion trapping agent, to fermentations of macrolides or streptomycin also results in substantial increases in the yields of these antibiotics.

Kinetic studies on cephalosporin production by *Streptomyces clavuligerus* have suggested that the action involves repression of enzyme synthesis, rather than inhibition of enzyme activity, and this is consistent with observations made on other fermentations. However, there is no generally accepted hypothesis on the mechanism by which the repression is exerted. Contrasting results have been reported on the involvement of glutamine, whose synthesis is normally repressed by high concentrations of ammonium ions. In fungi, ammonia represses the enzymes involved in the utilization of a number of nitrogen sources. Specifically, in aspergilli it inhibits the expression of a gene, *areA,* coding for a protein which is a positive regulator of transcription. However, a link between this repression and inhibition of antibiotic biosynthesis has not been established.

In a few cases the biosynthetic enzymes affected by ammonium ions have been identified. In *C. acremonium* two enzymes of the cephalosporin biosynthetic pathway, ACV synthase and expandase, are repressed.

An interesting interpretation has been proposed for the negative effect of ammonia on macrolide production. The building blocks constituting the polyketide chain of these antibiotics, particularly propionate, derive from the degradation of branched amino acids. The first step of the degradation is the conversion of the amino acids to the keto acids, catalyzed by either valine dehydrogenase or valine transaminase. It has been demonstrated that ammonium ions both repress and inhibit valine dehydrogenase, suggesting that the lack of suitable precursors is the cause of antibiotic production depression.

5.2.3. Phosphate Control

Phosphate control of the formation of secondary metabolites is a commonly observed phenomenon among bacteria, fungi, and also

plants. The suppression of practically all of the secondary metabolites of a culture is most often observed at high concentrations of phosphate. However, the sensitivity to phosphate inhibition may differ from product to product, even when produced by the same cell, because of the differential expression of certain genes as a function of phosphate concentration. In this respect the effect of different phosphate concentrations on *S. clavuligerus* fermentation is noteworthy. This microorganism produces both clavulanic acid and a cephamycin. Whereas the biosynthesis of the first is inhibited by phosphate, the production of the second is practically unaffected, so that it is possible to dissociate cephamycin biosynthesis from that of clavulanic acid by adjusting the phosphate level in the culture medium. Phosphate control has been described for several groups of antibiotics, including aminoglycosides, tetracyclines, macrolides, polyenes, and polyether ionophores. Generally speaking, the biosynthesis of antibiotics directly assembled from amino acids tends to be less sensitive to phosphate regulation than that, for example, of polyketides and aminoglycosides.

As illustrated by the following examples, repression of enzyme synthesis appears to be the mechanism by which antibiotic production is normally depressed by phosphate.

5.2.3.1. Phosphatases

Phosphotransferases participate in the formation of aminoglycosides. Biologically inactive phosphorylated intermediates are synthesized in the biosynthetic pathway which are later enzymatically dephosphorilated to yield the final active product.

In *Streptomyces glaucescens* and *S. griseus,* the gene encoding streptomycin-6-phosphate phosphotransferase is physically linked to the other biosynthetic genes and is subject to phosphate repression. In *S. griseus,* an excess of phosphate leads to the accumulation of streptomycin-6-phosphate which is normally converted into the active molecule by the action of a specific phosphotransferase. In *S. fradiae,* phosphorylated intermediates of neomycin biosynthesis are converted into the biologically active end product by a phosphatase that is both inhibited and repressed by phosphate.

It is likely that similar phosphorylation–dephosphorylation reactions occur in the biosynthetic pathways of other aminoglycoside an-

tibiotics, and there are indications that these may also occur in the biosynthesis of antibiotics belonging to other families. The repression of the enzymes involved may be the unknown mechanism underlying the phosphate effect.

5.2.3.2. Synthetases

A number of other reactions, of which orthophosphate is neither a substrate nor a product, are catalyzed by enzymes repressible by phosphate. Some of these, related to antibiotics produced by actinomycetes, are anhydrotetracycline (ATC) oxygenase, *p*-aminobenzoic acid (PABA) synthase, and some tylosin-synthesizing enzymes.

In *S. aureofaciens* the ATC oxygenase catalyzes the penultimate reaction of the tetracycline biosynthesis. The endocellular specific activity of this enzyme is sensitive to the presence of phosphate in the culture medium. Since phosphate does not affect the activity of the purified enzyme, this suggests that a repression system rather than a specific inhibition is involved.

The formation of PABA synthase, involved in candicidin biosynthesis, is strongly repressed by phosphate. This inhibitory effect is mainly the result of a specific repression of the transcription of the corresponding gene, *pabS,* since total RNA synthesis is stimulated by the presence of phosphate in the production medium. Indeed, a phosphate-regulated promoter has been identified upstream to the *pabS* structural gene.

In *S. fradiae* during tylosin biosynthesis the conversion of protylonolide to the final product is also very sensitive to the presence of phosphate in the culture medium. At least three enzymes of tylosin biosynthesis have been shown to be repressed by phosphate, among which it is worth mentioning the macrocin *O*-methyltransferase, which is the enzyme, encoded by *tylF,* catalyzing the final reaction in tylosin biosynthesis.

It has recently been found that some enzymes of β-lactam biosynthesis in fungi are also repressed by phosphates. In *C. acremonium* the formation of all of the examined enzymes of the cephalosporin C pathway is repressed, namely ACV synthase, cyclase, and expandase. Phosphate also partially inhibits the activity of these enzymes.

5.2.3.3. *Proposed Mechanisms of Phosphate Control*

Whether the phosphate control of antibiogenesis is mediated by intracellular effectors has been the subject of extensive investigations.

Cyclic AMP. The presence of cAMP has been demonstrated in several *Streptomyces* and in some antibiotic-producing strains a parallel decrease of the level of cAMP and of the uptake of orthophosphate has been observed at the end of the trophophase. In particular, in *S. hygroscopicus* there is a parallel regulation of cAMP phosphodiesterase and phosphatases during turimycin fermentation. However, it has been demonstrated that cAMP is not involved in the phosphate control of streptomycin or candicidin production by *S. griseus*. Thus, it appears that in general cAMP is not an effector mediating the phosphate regulation of antibiotic biosynthesis, although in particular cases its involvement cannot be excluded.

Nucleoside triphosphates. The availability of inorganic phosphate normally increases the ATP levels in the cells, and it has been proposed that ATP is the intracellular effector of phosphate antibiogenesis control. Most of the evidence is based on the observation that after phosphate addition, first ATP levels increase, and then antibiotic production is depressed. Similarly, time course studies indicate that ATP levels normally decrease just before the onset of antibiotic synthesis. Detailed studies on tylosin and candicidin fermentations have indicated that the absolute intracellular level of ATP, rather than the energy charge (that is, the ratio of ATP versus ADP and AMP), affects the antibiotic production. However, a conclusive demonstration that the phosphate effect is mediated by the adenylates is so far lacking.

Nucleoside tetraphosphates. The role played by nucleoside tetra- and pentaphosphates (ppGpp and pppGpp) in the control of the stringent response in *E. coli* is now well understood and the presence of their adenine counterparts has been demonstrated during the transition from exponential growth to stationary phase in *Bacillus subtilis*.

The stringent response has been implicated as one of the triggers for antibiotic production and sporulation, also in *Streptomyces*. Depending on the species studied, both guanosine and adenine tetra- and pentaphosphate have been found in many *Streptomyces* strains. However, in this case also a clear correlation between antibiotic production, phosphate concentration, and nucleoside tetraphosphate level has not been established.

Mutants deficient in the stringent response, *relC,* have been isolated from different strains of *Streptomyces* which are also impaired in antibiotic production, thus suggesting that ppGpp may play a role in the onset of secondary metabolism. On the other hand, a recent study on *S. coelicolor* A3, producer of actinorhodin, suggests that while ppGpp levels increase slightly at the end of the exponential growth, this increase alone is not sufficient for initiation of antibiotic production. Moreover, *relC* mutants of this strain are not deficient in either actinorhodin or undecylprodigiosin biosynthesis.

5.3. Autoregulators and Pleiotropic Effectors

The existence of intracellular effectors controlling essential steps of the life cycle, like sporulation or aerial mycelium formation, as well as the biosynthesis of secondary metabolites is widespread among microorganisms. Pleiotropic effectors of secondary metabolism acting at very low concentrations have been identified in many species.

The best studied is A-factor which is essential for induction of both sporulation and antibiotic biosynthesis in *S. griseus.* The discovery of this effector was originally based on the observation that addition of small amounts of the fermentation broth from a streptomycin-producing strain of *S. griseus* to cultures of a nonproducing strain restored the production ability. The active substance, named A-factor, was isolated and identified as 2-isocapryloyl-3-*R*-hydroxymethyl-γ-butyrolactone (Fig. 5.1). In *S. griseus* and *S. bikiniensis,* A-factor exerts a positive control over production of and resistance to streptomycin by binding to a protein negatively acting on the transcription of several genes.

A-factor was subsequently isolated from a number of actinomycete species. It appears to have different functions in different organisms,

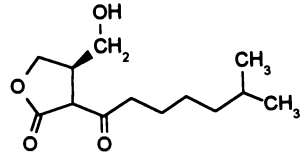

Figure 5.1. A-factor.

since it is not essential for antibiotic production or sporulation in some species that produce it. A gene governing A-factor formation, *afsA,* has been cloned from *S. bikiniensis.* In *S. coelicolor,* a different gene, *afsB,* has been isolated, which codes for a positive regulatory protein for production of the A-factor and several pigments; the presence of this gene is able to complement an A-factor-deficient mutant strain of *S. lividans.* A cascade of expression initiated by the protein product of *afsB* has been proposed to explain the control of secondary metabolism and differentiation stimulated by the gene.

Several other metabolites structurally related to A-factor have been isolated from actinomycetes, such as *S. viridochromogenes, S. cyaneofuscus,* and *S. virginiae.* All have in common a 2,3-disubstituted butyrolactone moiety and are thus usually referred to as butanolide factors. Some of them do not demonstrate any evident activity, whereas others, similar to A-factor, can induce differentiation at nanogram concentrations.

A structurally different regulator is B-factor (butyl-phosphoryl-adenosine), a substance originally isolated from yeast extract that restores rifamycin B production (but not aerial mycelium formation) in an antibiotic- and sporulation-negative mutant of *Nocardia mediterranei.* The product can be considered an autoregulator because it was later also isolated from a producing strain of the same *Nocardia* species.

Factor C is a regulatory secreted protein of about 34.5 kDa, widely distributed among streptomyces and possibly present also in eukaryotic cells. Factor C is a cytodifferentiation inducer but its action appears to be more related to formation of conidia than to antibiotic production.

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6

Genetics of Antibiotic Production

6.1. Introduction

Notwithstanding the industrial relevance of antibiotic-producing microorganisms, the development of their genetics remained for a long time at a rather primitive stage. A genetic system has been developed for only a small number of microorganisms. Since the actinomycetes and the lower eukaryotes filamentous fungi are the most representative antibiotic-producing microorganisms, we will restrict our description on the genetics of antibiotic production mainly to these two classes.

The genus *Bacillus,* among which *B. subtilis* is the most studied species, is also worth a separate discussion. Though it has a long history of economic importance, its commercial value lies mainly in the area of enzyme production. In the context of this chapter our interest in the genus *Bacillus* is essentially because of studies done on the correlation between antibiotic production and sporulation.

6.1.1. Streptomyces

Among the antibiotic-producing actinomycetes, the best known are the *Streptomyces.* These microorganisms possess a circular genome with a base composition of about 70–74 mol % G + C and with an estimated size in the range of 6 to 9 million bases*. This is more than

* Recent results suggest that the *Streptomyces* chromosome may be in a linear physical form.

twice that of the genomes of *E. coli* and *B. subtilis.* The most studied species in terms of genetics is *Streptomyces coelicolor.* Its genetic map, consisting of approximately 150 loci, is shown in a simplified form in Fig. 6.1. As already noted, a peculiarity which immediately shows up is the presence of two physically large regions of the genome which seem to be genetically silent. These two regions separate two other quadrants which contain the vast majority of the mapped genes. These two quadrants possess another interesting peculiarity in that they show a circular symmetry such that the order of the genes present in one region mirrors that of the other. This peculiarity might reflect a duplication event of an ancestral genome. In *S. coelicolor* the genes on the chromosome are organized in clusters and operons in a fashion very similar to that found in eubacteria such as *E. coli* and *B. subtilis.*

Streptomycetes lack a natural system of competence, i.e., they

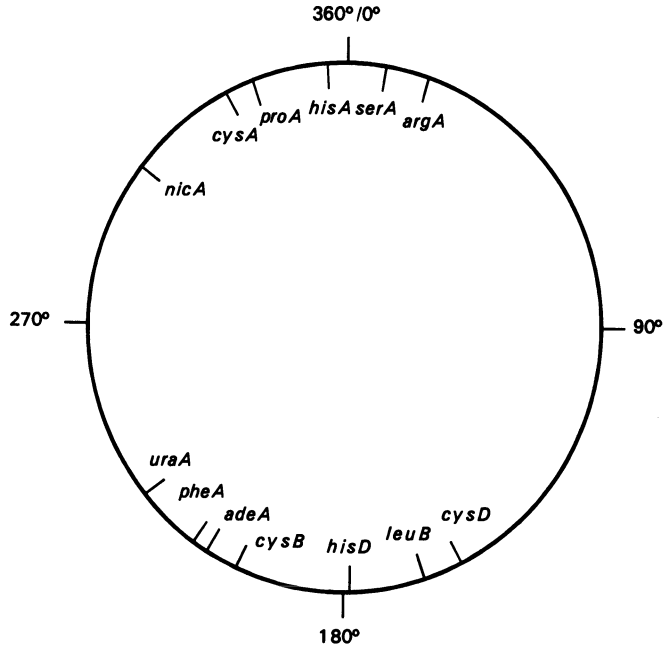

Figure 6.1. Simplified linkage map of *Streptomyces coelicolor.* Only a few genetic loci are indicated, to show the presence of two long, genetically silent, regions separating two other regions where the vast majority of mapped genes has been localized.

cannot be transformed by exogenous DNA. However, an artificially promoted uptake of DNA molecules by the use of protoplasts has been developed and is very efficient. *Streptomyces* also have a variety of extrachromosomal genetic elements similar to those previously found in other groups of bacteria. In addition, the presence of giant linear plasmids has been demonstrated in some species. Autonomously replicating and integrative plasmids, with well-developed natural systems of gene exchange through conjugation, as well as hetero-immune temperate bacteriophages (able to replicate in a fairly wide range of *Streptomyces* species), have been studied and developed into powerful cloning vectors. The vast majority of plasmid vectors, analogously to what happens in *E. coli,* are selectable for the presence of resistance markers such as to thiostrepton, neomycin, and viomycin. The plasmids routinely employed in *S. lividans,* the host of choice for many cloning experiments, derive essentially from three groups of plasmids: SCP2* (from *S. coelicolor*), and SLP1 and pIJ101 (from *S. lividans*). Plasmid vectors have been developed with the most diverse characteristics: some are able to replicate at high (pIJ702) or at low copy number (pIJ941), some can be used to isolate promoters (pIJ486/7) or allow positive selection of inserted DNA (pIJ699). Among bacteriophages the most useful has been the temperate phage ΦC31, reminiscent of the coliphage λ both in morphology and in general genetics.

Another previously mentioned peculiar feature of streptomycetes is a high degree of genetic instability which is often the result of large genome rearrangements, such as deletions or amplifications. These rearrangements occur at high frequency in unstable regions of the chromosome, and are normally localized close to one or two *a*mplifiable *u*nits of *D*NA (AUD). As shown by chromosome walking experiments and by the analysis of large chromosomal restriction fragments, using pulsed-field gel electrophoresis (PFGE), up to 10^5 base pairs of DNA can be lost in various *Streptomyces* species. In *S. lividans* deletable and amplifiable regions are part of the silent regions of the genome. And in *S. ambofaciens* the involvement of an SOS-like response in these phenomena of genetic instability has been proposed.

6.1.2. Filamentous Fungi

In comparison with genetically well-known species such as *Neurospora crassa* and *Aspergillus nidulans,* our knowledge of the genetic

and molecular biology of industrially relevant fungi is also at an early stage. Filamentous fungi are eukaryotic organisms possessing a genome with a complexity of about 20–40 million bases. Their genome, like that of other eukaryotes, is organized in true chromosomes, which can be as many as 13 per haploid genome. These are large linear DNA molecules located in a nucleus, and organized similarly to the chromosome of higher eukaryotes in that they are associated with histones in typical nucleosomal units. They differ in that their genome seems to be more compact than that of higher eukaryotes; to some extent more similar to that of bacteria.

In fungi, structural genes with related functions tend to be somehow distributed through the genome, and the multicistronic mRNA species typical of prokaryotes are seldom observed. An exception to the scattered gene distribution may be considered the so-called "cluster genes." These are huge DNA regions coding for large polypeptides which have multifunctional activities. The molecular analysis of these genes has shed some light on their evolutionary origin. For example, in the case of *aromA* of *A. nidulans,* the considerable homology present between the fungal gene and the corresponding genes of *E. coli* suggests that *aromA* may have evolved from the fusion of five unlinked ancestral units to form a single gene coding for five independent catalytic domains.

Molecular studies have revealed that, similarly to higher eukaryotes, the fungal genes contain introns, which are, however, smaller and less frequent. Indeed, only about 60% of the known genes possess introns (another well-known lower eukaryote, *Saccharomyces cerevisiae,* has only about 10% of its genes interrupted). The introns of filamentous fungi are usually short, on average less than 100 bases, and may be present up to eight times in one gene; their boundaries are similar to those present in yeasts or higher eukaryotes.

Finally, although filamentous fungi terminate transcripts with a poly(A) tail, the presence of a poly-adenylation site consensus sequence does not seem to be a necessary feature for translation.

Following the development of transformation systems via the formation of protoplasts, molecular cloning methods have been developed for many filamentous fungi. Despite this, gene cloning systems presently available lag far behind those available for prokaryotes. As a consequence of the almost total lack of plasmid vectors, most of the molecular

biology on fungal genes is done after transfer into *E. coli.* The recombinant plasmids are then used for transformation of the fungal cell, and integration of the cloned gene in the chromosome takes place. The most common selection systems used are based on complementation of nutritional mutations, though a number of positive selection systems have been developed based on resistance to antimetabolites such as oligomycin or hygromycin B. An alternative system makes use of the *amdS* gene of *A. nidulans;* this gene encodes for an acetamidase which allows the growth of the expressing cells in the presence of acetamide as the sole carbon source.

Strategies for the recovery of cloned genes from the fungal cells are generally based either on the fact that only those DNA segments containing the integrated *E. coli* plasmid are able to replicate in the prokaryotic host or, were a cosmid or lambda vector used, only those sequences containing the correct *cos* sites are able to be packaged efficiently into the phage heads. Another method of clone selection, which does not need the recovery of the cloned sequences from the fungal cells, is the so-called "sib selection." This strategy is based on the analysis of pools of clones; the candidate pools are, via a stepwise process, subdivided into other pools containing a progressively smaller number of individual clones until a single specific clone is identified.

6.1.3. Bacillus

B. subtilis has been for many years the prototype species for gram-positive genetics, and is the most widely studied and thoroughly mapped gram-positive microorganism. The large amount of genetic and molecular data available for this species, second only to *E. coli,* has been made possible by the utilization of virtually all of the genetic and molecular tools. The size of its circular chromosome has been estimated at around 4–5 million base pairs and more than 700 different loci have been identified; for most of these the relative position on the chromosome has also been genetically determined.

The regulation of genetic expression during the various growth phases has been studied in considerable depth, and the sequence of the various sigma factors determined. These act in a cascade during the sporulation phase and this process has been used as a model to study the regulation of expression of secondary metabolites; the production

of which generally occurs, as in other organisms, during the differentiation process.

Besides the availability of a variety of tools for classical genetic analysis (such as the possibility to transform competent cells and the presence of generalized transducing phages), the molecular biology tools available for *B. subtilis* are perhaps second only to *E. coli*. Because of the almost complete lack of endogenous plasmids, several plasmids from other sources have been stably transformed into *B. subtilis*. Most of the cloning vectors available derive from one of the following *Staphylococcus aureus* plasmids: pUB110, pC194, or pE194. The most commonly selected marker is antibiotic resistance, using such antibiotics as kanamycin, erythromycin, and chloramphenicol. Plasmid vectors have been developed for various purposes; there are shuttle vectors that can also replicate in *E. coli* (pHV14), promoter probe vectors (pPL703), expression vectors (pLIQ-1), etc. Almost all *B. subtilis* plasmids are maintained at a high copy number (ten or above). Therefore, to maintain a cloned gene at a low copy number, strategies involving its insertion into the bacterial chromosome have been developed. The use of transposons (Tn917) and of bacteriophage vectors (ρ11) has been described.

6.1.4. Secondary Metabolite Molecular Genetics

A great impulse in the understanding of the biosynthesis, regulation, and resistance mechanisms of secondary metabolite production has been given by the new techniques of molecular genetics; that is, by cloning and identifying the genes involved in these processes.

In many cases it has been found that the regulatory and biosynthetic genes are clustered together. These clusters can contain a single transcriptional unit (a single operon) or several units, but in any case the genes are physically linked to each other. Where antibiotics are concerned, it has been observed that often the genes responsible for self-resistance are also localized in these biosynthetic clusters. This has the obvious consequence that it is sometimes sufficient to identify and isolate one of the genes involved in the biosynthetic pathway, and then use this as a probe to "walk" on the chromosome and isolate other physically linked genes.

Several strategies have been adopted for the initial gene cloning and identification:

1. Transfer of a DNA library of an antibiotic-producing *Streptomyces* to another *Streptomyces* sensitive to the antibiotic, and selection of the resistant clones. From these the gene coding for self-resistance can be isolated.
2. Complementation of mutants blocked in the biosynthetic pathway. The transfer, in such strains, of the appropriate biosynthetic gene in its active form is revealed by a restored ability to produce the final compound.
3. Hybridization with DNA probes from genes isolated from similar biosynthetic pathways. This is possible because the genes coding for analogous functions in similar biosynthetic pathways, usually show a high degree of homology.
4. Hybridization with synthetic probes constructed on the basis of the amino acid sequence of biosynthetic enzymes.
5. Production of the metabolite in a host organism which is not a natural producer of the substance. In this case, all, or almost all, of the biosynthetic cluster has to be transferred in the host organism.

6.2. *Genes for Self-resistance*

Many microorganisms are sensitive to the antibiotics they produce. In fact, the addition of an antibiotic to a culture medium inoculated with the producer microorganism often results in growth inhibition. We thus have the paradox of the strain's ability to produce a fair amount of a substance that at low concentrations inhibits its growth.

Antibiotic-producing microorganisms have developed a variety of mechanisms to resolve this paradox. Broadly speaking, the adopted survival strategies can be grouped into three main classes:

1. Resistance by chemical modification or physical sequestration of the antibiotic molecule
2. Resistance by modification of the antibiotic target site in the producer
3. Permeability and efflux mechanisms of resistance

The type of resistance presented by any particular strain may depend on the antibiotic biosynthetic pathway, according to whether the

antibiotic is synthesized as an active molecule or as an inactive intermediate, activated during export. In the first case the cell will have to deal with intracellular drug–target interactions, in the other it will have to prevent the reentry of the extracellular antibiotic.

Another important consideration concerns the inducible or constitutive expression of these genes. Many secondary metabolites are synthesized specifically during an idiophase clearly distinguishable from a trophophase. Thus, the expression of the resistance genes is normally required only at a definite time in the life cycle. However, in some cases the production of the antibiotic is detectable during all growth phases. Therefore, it is obvious that, in this case, such microorganisms must have developed a resistance mechanism which is expressed constitutively along with the production of the active drug.

Let us now consider some specific examples.

6.2.1. *Resistance by Chemical Modifications or Sequestration of the Antibiotic Molecule*

Notwithstanding the large variety of chemical structures presented by antibiotic molecules, the most widespread modification systems are the *N*-acetylation of amino groups via acetyl CoA and the *O*-phosphorylation of the hydroxyl groups via ATP. It is interesting to note here that other mechanisms of inactivation typical of resistant clinical isolates are not found, or are only rarely found, in producer strains. For example, *O*-adenylation and even the *O*-acetylation of chloramphenicol has never been observed in chloramphenicol-producing microorganisms.

Genes encoding these *N*-acylases and *O*-phosphorylases have been detected in the producers of the aminoglycosides hygromycin, puromycin, viomycin, capreomycin, streptothricin, nurseothricin, and others. In most cases, only one of these activities can be found in a single producer strain, but there are occasions, such as the production of neomycins (*S. fradiae, M. chalcea, S. albogriseolus*), in which both activities are present. It seems that a combination of both inactivating enzymes may be required to get full resistance of the producer strain to the produced molecule. Moreover, some of these enzymes can act as intracellular detoxifiers during the biosynthetic process. Such is the case, for instance, with *S. alboniger,* a producer of puromycin. The biosyn-

thesis of this antibiotic seems to involve, via the action of the gene *pac,* some *N*-acetylated intermediates which are inactive on the ribosomes of the producer strain.

It is worth noting here that certain resistance genes have been so classified only because, when cloned and expressed in heterologous hosts, they were able to confer resistance to a drug, whereas in the original species they may have other functions. As an example, the gene *aac* isolated from *S. kanamyceticus,* producer of kanamycin, codes for an aminoglycoside acetyltransferase. When expressed in the sensitive host *S. lividans,* it is able to function as a resistance determinant. It seems, however, that in *S. kanamyceticus* its main function is the conversion of intermediates in kanamycin biosynthesis.

A large class of antibiotic-inactivating enzymes are the β-lactamases. *Streptomyces* species are known that produce β-lactams and they have evolved three different resistance methods for this class of compounds: production of β-lactamases, production of altered penicillin-binding proteins, and permeability barriers. It seems that in *Streptomyces,* in contrast to pathogenic bacteria, the main mechanism of resistance to β-lactam compounds is the low affinity of penicillin-binding proteins for these drugs (to be discussed later), whereas the production of β-lactamases is only of minor importance. However, the production of these enzymes is very common among *Streptomyces,* irrespective of whether they are β-lactam producers or not.

Finally, there are resistance mechanisms involving the action of drug-binding proteins that are able to sequester the drug, thus avoiding its contact with the molecular target. Although not the only resistance mechanism present in the oxytetracycline producer strain, *S. rimosus,* it appears that the product of the *otrA* gene has the function of sequestering the drug inside the cells while another resistance mechanism is actively exporting the drug out of the cells. Another example of resistance relating to a drug-binding protein is that to bleomycin. This is caused by the production of a small acidic protein able to sequester the drug. The gene coding for this protein was first identified in *Streptoalloteichus hindustanus,* a producer of the related antibiotic tallysomycin.

6.2.2. Resistance by Modification of the Antibiotic Target

This mode of self-defense consists in the production of a modified version of the specific target for the antibiotic action so that it becomes

insensitive or less sensitive to the interaction with the antibiotic molecule. It is possible to distinguish three types of this resistance mechanism.

The first is when the target of the drug action, in the producing strain, is refractory to the endogenous antibiotic. For example, *S. lactamdurans* and *S. cinnamoneus* possess a modified EF-Tu molecule (the elongation factor Tu is the target molecule in the sensitive strains) resistant to the antibiotics they produce, efrotomycin and kirrothricin, respectively. Some cases have been reported in which the resistance of the target molecule is higher in high producing mutants than in the wild-type strain.

The second type is when the expression of the refractory target is "induced" at the same time as that of antibiotic biosynthesis, whereas during the other phases of the life cycle a sensitive molecule is produced. This is the case with *S. sphaeroides,* producer of the antibiotic novobiocin. The strain possesses two distinct DNA gyrases: one, normally produced during vegetative growth, which is sensitive to the antibiotic, and a second, expressed during antibiotic production, which is markedly more resistant to the action of the antibiotic.

The third case is when the target of the drug action, normally sensitive, is modified by an appropriate enzyme in such a way that it becomes resistant to the drug. An example is the case of *S. azureus,* which produces thiostrepton, a molecule able to inhibit protein synthesis by binding to the large ribosomal subunit (50 S). The producer strain constitutively expresses a methylase which introduces a single methyl group into the ribose moiety of a specific adenine residue of the 23 S rRNA, thus hindering the binding of the drug to the ribosome. This was the first case of ribosomal resistance mechanism characterized in detail. Further studies revealed that posttranscriptional methylation of rRNAs is a common resistance mechanism, although in different producer strains there are differences in the methylation site. Either 23 or 16 S rRNA can be modified on different residues.

Finally, we come to the case of the strains producing β-lactam antibiotics. As previously stated, the main mechanism of resistance to this class of antibiotics, at least in *Streptomyces,* is the presence of low-affinity penicillin-binding proteins (PBPs). Examples of this mechanism are the β-lactam-producing strains *S. olivaceus* and *S. clavuligerus.* However, there are indications that the ability of a producer strain to

become resistant to β-lactam antibiotics is limited. Alteration of its PBPs beyond a certain degree may cause these enzymes to fail to perform functions essential for the survival of the cell.

6.2.3. *Resistance Resulting from Alteration of Permeability or Efflux Mechanisms*

A third system that can help the antibiotic-producing cells to resist their own products relies on the efficiency with which the drug is exported out of the cell and remains outside. The involvement of cell impermeability in the resistance to macrolide antibiotics has been postulated for a few strains, such as *S. ambofaciens,* producer of spiramycin, *S. halstedii* and *S. tendae,* producers of carbomycin, and *S. fradiae,* producer of tylosin.

In many producing strains a mechanism for secreting the toxic product out of the cell can also work as a resistance mechanism, allowing the cell to reexport any antibiotic molecule that might have reentered the cell.

Resistance mechanisms involving the efflux of the drug out of the cell have also been proposed for some macrolide-producing strains, i.e., *S. fradiae, S. ambofaciens,* and *S. thermotolerans.* Genes coding for macrolide resistance have been isolated from these strains which show significant homologies to ATP-dependent transport proteins, suggesting that their mechanism of action consists of the export of the antibiotic molecule.

The efflux of the drug from the producer cells is also one of the resistance mechanisms that *S. rimosus* evolved to resist its product, oxytetracycline. A gene has been detected which codes for a membrane-associated protein that promotes the efflux of the drug via an energy-dependent mechanism, similarly to the tetracycline resistance characterized in various clinical isolates. A similar mechanism has also been described in another tetracycline producer, *S. aureofaciens.*

6.3. *Regulatory Genes*

As discussed in Section 6.1, the differences in the genetic structure between bacteria and lower eukaryotes are considerable, and obviously

involve features of both the structural biosynthetic genes and the regulatory genes of secondary metabolism. For example, in bacteria the genes of coordinated pathways are usually linked, while in lower eukaryotes they are mostly scattered along the genome. This is not only a "geographical" curiosity, but has important consequences with regard to the mechanisms of their regulation. We will give a quick overview of what is known about the regulation of the biosynthetic genes involved in antibiotic production, looking first at actinomycetes (essentially *Streptomyces*), then bacilli (*B. subtilis*), and finally filamentous fungi.

6.3.1. Actinomycetes

It is conceivable that the antibiotic biosynthetic genes, and in many cases their resistance genes as well, are finely coordinated in both their temporal and quantitative expression. This can be achieved by controlling the expression of the genes of interest at both the transcriptional and translational level.

Transcription. One of the key factors for the regulation of expression of these genes is their location along the bacterial genome. This can be achieved by two common methods: (1) by organizing the genes into large operons, controlled by a single promoter, producing a polycistronic mRNA; (2) by physically linking, in a large multifunctional unit, the various polypeptides involved in one set of reactions, whose spatial proximity is crucial for efficiency of the biosynthesis. Another possibility is to have separate transcriptional units coordinated by *trans*-acting regulatory genes. It is obvious that these different possibilities are not mutually exclusive. The spatial organization of the genes along the genome will be discussed in more details in the section on structural biosynthetic genes.

The study of the DNA sequences involved in the regulation of transcription in Actinomycetales is still at an early stage; only a few promoters have been characterized. These studies have revealed functional homologies between streptomycetes promoter regions and those described in *E. coli.* For example, *S. lividans* can recognize promoters derived from *E. coli* and other eubacteria such as *Serratia marcescens* and *Bacillus brevis.* However, the reverse seems not to be true and indeed very few streptomycetes promoters are recognized by the *E. coli* transcriptional machinery.

A widespread mechanism of regulation of gene expression has been found in *B. subtilis, E. coli,* and other bacteria. This is the production of alternative sigma factors, or other proteins that can alter the specificity of the RNA polymerase, resulting in the selective expression of different sets of genes according to the new specificities acquired by the holoenzyme. The extent to which this molecular strategy is employed to control antibiotic biosynthesis and resistance, usually coupled with morphological differentiation, needs further studies, requiring comparisons of regulatory sequences. It is likely that promoter polymorphism, coupled with the RNA polymerase heterogeneity, contributes significantly to the control of gene expression in streptomycetes as it does in *B. subtilis.* The occurrence of multiple sigma factors in *S. coelicolor,* where as many as seven or more are present, is well documented, as is the presence of multiple putative promoter regions upstream of many of the structural genes sequenced.

The production of specific sigma factors, able to direct the selective expression of certain sets of genes during growth, and of other sets during secondary metabolism, is a possible regulatory system developed by these microorganisms. In addition, several putative *trans*-acting regulatory genes have been identified. For example, in *S. coelicolor* at least two defined loci, *absA* and *absB,* have recently been identified. These control the biosynthesis of the four antibiotics produced by the strain; these genes apparently function within a global regulatory pathway (or network). Two other *S. coelicolor* genes, which have been described to be involved in a global regulatory network for secondary metabolism, are *asfR* and *asfK.* The product of *asfR* is a large (993 amino acid) protein that when phosphorylated by a specific phosphokinase, produced by *asfK,* is able to stimulate transcription of antibiotic production genes. It is very likely that these two genes comprise a two-component regulatory system.

Another system acting on the regulation of secondary metabolism and cell differentiation at the transcriptional level is the production of autoregulatory substances, such as A-factor, discussed in Chapter 5. The final effect of A-factor is the binding of a positive regulatory protein to a DNA region which lies about 330–430 bases upstream of the transcription start point of the *strR* promoter. This activates the transcription of the gene, the product of which, in turn, is able to activate the other streptomycin biosynthetic genes.

Translation. The existence of a high bias in the codon usage, caused by the high G + C content of the actinomycete genome, raises the possibility that rarely used tRNA species might also serve to regulate gene expression. The leucine tRNA codon UUA is among the least frequently used, and seems to be confined to the transcripts of genes expressed during the shift from the vegetative growth phase and the differentiation phase. In *S. coelicolor,* the gene *bldA* codes for the tRNA having the UUA codon and thus appear to have a regulatory function at the translational level.

6.3.2. Bacillus

A variety of secondary metabolite genes in *Bacillus,* such as those associated with the production of antibiotics or competence development, are activated at the transition from the vegetative growth phase to the stationary phase. Under the same conditions, the formation of the endospore is also induced. These observations support the hypothesis of a possible common regulation. In *B. subtilis* the transcription of the sporulation genes, as well as that of the genes involved in the secondary metabolism, is totally dependent on the *spoOA* gene product which seems to be the receiver/regulator module of a two-component signal-transducing system. These systems are generally composed of a sensor/transmitter module which senses physiological signals and reacts by phosphorylating the receiver/regulator module. In this case, it is not yet clear which gene product might function as the sensor/transmitter module, and which, sensing signals such as a depletion of the guanine nucleotide pool, might activate its respective receiver/regulator module, *spoOA.* A similar hypothesis can be made for *spoOF,* another gene involved in the regulation of sporification.

As far as sporulation is concerned, the activated forms of *spoOA* and *spoOF* set off the sporulation-specific regulatory cascade; in this cascade, one of the actions is the inactivation by *spoOA* of the *abrB* gene product. This is a negative regulator of the expression of the cascade of five sigma factors which act as specific coordinators of the sporulation process. The *abrB* protein also regulates a variety of genes associated with the end of vegetative growth.

A first example of secondary metabolites, regulated by the same mechanism, could be that of the cyclic decapeptide antibiotic tyrocidine.

Its biosynthetic genes, *tycA* and *tycB,* are organized in an operon developmentally regulated. During the vegetative growth, *abrB* interacts directly with the promoter region repressing its transcription. The repression of *abrB* by the *spoOA* product relieves the inhibition, thus allowing the transcription of the operon and the synthesis of the antibiotic.

Another example is the regulatory pathway common to both the biosynthesis of the lipopeptide antibiotic surfactin and the regulation of genetic competence. Both pathways are indeed regulated by *comA,* which is in turn under the control of *abrB* and *spoOA.*

6.3.3. Filamentous Fungi

Although several fungal control genes have been isolated, there is not such a wealth of information about the control of the synthesis of fungal antibiotics. As mentioned in the introduction, the best studied species are *Aspergillus nidulans* and *Neurospora crassa.* The basic structure of their gene regulation systems has in part been established. The majority of the control genes so far identified seem to produce activator proteins that bind to the DNA molecule in regions located upstream of the regulated structural genes (UAS). As an example, we can consider the case of *amdS* of *A. nidulans,* a gene involved in acetamide utilization. Considerable progress has been made in understanding its regulatory mechanisms. This gene possesses a complex positive regulation system: at least four different regulatory genes have been identified, and for some of these, the putative "binding regions" of the control proteins have been identified. Interestingly, it seems that at any one time only one protein is bound and interacts with the transcriptional apparatus.

Less evidence is available for the mechanisms of action of negative regulators. They may bind to upstream regulatory regions as do the positive regulators, but may also act by interfering with the positive regulator proteins inhibiting their action. It seems, for example, that the mode of action of the control gene *qa-1S* is of this second type. This gene is implicated in the control of the *N. crassa qa* gene cluster, involved in quinic acid utilization. The *qa* structural genes are positively regulated by the product of *qa-IF* which binds to a conserved region located upstream of the coding regions of the structural genes. The

product of *qa-1S* seems to interact with that of *qa-IF,* thus rendering the final complex inactive. Another example of negative regulation is the carbon catabolite repression (CCR) of the syntheses of enzymes and permeases involved in the utilization of carbon and provision of energy for the cells. CCR imposes a double "lock" on the controlled genes, at least on the ethanol regulon and on the proline utilization gene cluster. This mechanism implies that both the structural and the regulatory genes are repressed concomitantly and independently.

creA is the negative regulatory gene which mediates CCR in *A. nidulans.* This gene also exerts its influence on penicillin biosynthesis, which is, at least in *A. nidulans,* a highly controlled process under a multifactorial control system. Indeed, culture media containing CCR-inducing carbon sources support lower antibiotic yields than media with nonrepressing carbon sources. Under repressing conditions the steady-state levels of the *ipnA* gene transcript, coding for the isopenicillin N synthetase (IPNS), are significantly reduced relative to those observed when the microorganism is grown in nonrepressing media. The data indicate that other steps of penicillin biosynthesis are regulated by CCR through transcriptional control of structural genes. It is interesting to note that *acvA,* coding for ACV synthase, which is transcribed divergently from *ipnA,* does not seem to be affected by CCR, while *acyA,* which follows *ipnA* in the *npaA* locus, and has the same orientation of transcription, also shows a reduced specific activity in glucose-grown cultures.

Recently, improvements in molecular biology techniques have allowed the identification of the biosynthetic genes in other penicillin-producing fungi such as *Penicillium chrysogenum.* In contrast to what we have seen in *A. nidulans,* the presence of transcriptional control has been demonstrated in this species, simultaneously affecting the expression of three biosynthetic genes (*pcbAB, pcbC,* and *penDE*). The highest levels of expression are reached during the exponential growth phase, whereas transcription of the three genes appears to cease as the growth rate decreases. Moreover, the expression of these biosynthetic genes is not repressed by high concentrations of glucose (i.e., is not under CCR control).

6.4. *Structural Biosynthetic Genes*

In the last few years, with the flourishing of molecular biology techniques, an increasing number of genes involved in the biosynthesis

of antibiotics, or other secondary metabolites, have been cloned. A complete list of all of the known genes is almost impossible, and its usefulness would be limited, in that new genes and sequences are published almost every week. A comprehensive picture of the general organization in the various genomes is emerging from the plethora of information derived from different organisms and for different antibiotics. Three observations seem to be of quite general applicability:

1. Production genes tend to be clustered together (often spanning several tens of thousands of bases).
2. Resistance genes are often part of the biosynthetic cluster.
3. Regulatory genes are also often part of these linkage groups.

The phenomenon of clustering of the biosynthetic genes is not only found in bacterial species. In eukaryotic organisms, secondary metabolites are also encoded by genes organized in clusters, although separated into subclusters located on different chromosomes. The evolutionary significance of this general phenomenon of the clustering of biosynthetic, resistance, and specific regulatory genes is still being debated. It is possible to speculate that the coordination of their temporal and quantitative expression is thus much easier than if they were dispersed along the chromosomes.

Another feature, which is emerging from the available data, is that antibiotics having a similar backbone structure are synthesized by similar biosynthetic apparatuses, notwithstanding major differences in the general biology of the producing species. Typical examples are the polyketide and the polypeptide antibiotics.

6.4.1. The Polyketides

In Chapter 4 we have seen that many antibiotics are synthesized through the reiterated condensation of short-chain carbon units. The mechanism is similar to that of fatty acid synthesis, catalyzed by the fatty acid synthase (FAS). The final molecule is made from a starter unit (typically an acetate molecule), with the addition of extender units (typically malonate), through several cycles of addition–reduction–dehydration–reduction. In the case of the polyketide antibiotics, these reactions (complicated by the fact that the reduction or dehydration steps are omitted in some cycles) are carried on by the so-called poly-

ketide synthases (PKSs). These share with their cousins (FASs) not only a functional similarity but also significant sequence homology, indicating that the PKSs might be derived from the FASs.

The enzymatic activities which are responsible for the different reaction steps can either be present in different domains of a single large multifunctional polypeptide (type I) or be organized in a multienzyme complex (type II).

6.4.1.1. Type I Polyketosynthetases

An example of this first type of PKS is that present in *Saccharopolyspora erythrea,* producer of the macrolide antibiotic erythromycin. The genes (*eryA*) coding for the polyketide portion of the antibiotic are organized in three large open reading frames (ORFs), which span about 35 kb, and in which it is possible to distinguish six repeated modules. The product of each module is able to perform a complete cycle of the six necessary to construct the final structure, 6-deoxyerythronolide B (6dEB). In the proposed model each module codes for the activities needed for one elongation step. These activities may be all of the following, or just some of them: acyltransferase (AT); β-ketoacyl carrier protein synthase (KS); acyl carrier protein (ACP); β-ketoreductase (KR); dehydratase (DH); enoyl reductase (ER); thioesterase (TE) for the final release and the lactonization of the product (Fig. 6.2). If the reactions of each elongation step were carried on by separate enzymes, as in type II PKS, a similar structure would imply the intervention of something like 28 different functional units. It is thus conceivable that the occurrence, at least in prokaryotes, of type I and type II PKS may be related to the complexity of the polyketide chain they make.

It is highly probable that other macrolides such as tylosin and spiramycin possess similar PKSs. However, these PKSs contribute only to the formation of the core of the final antibiotic; other important reactions have to occur to synthesize the final product. For the biosynthesis of tylosin, a cluster of genes has been isolated which transform the 16-membered lactone ring, tylactone, into the final antibiotic. These "late" *tyl* genes span about 35 kb and seem to be involved both in modifications of the tylactone ring and in the synthesis and addition of the three sugar moieties (Fig. 6.3). It is interesting to note that two

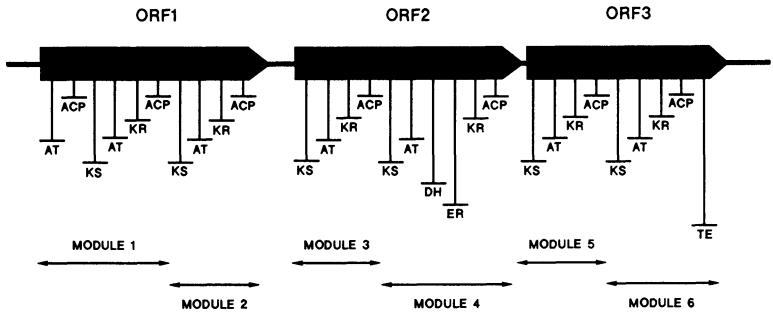

Figure 6.2. Organization of the locus *eryA* of *Saccharopolyspora erythrea.* ORF (open reading frame) indicates a region of DNA coding for a single polypeptide. Fatty acid synthase domains within ORFs are indicated by bars labeled as follows: ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; ER, enoyl reaductase; KR, ketoreductase; KS, keto-ACP synthase; TE, thioesterase. Each module encodes a functional synthase unit.

tylosin resistance genes, *tylB* and *tylC,* have been found in the same chromosomal region, spanning about 100 kb.

6.4.1.2. Type II PKSs

Genetics of this type of PKS was first described in *S. coelicolor,* for the pigment antibiotic actinorhodin. Its primary carbon skeleton is derived from eight acetate–malonate units and the physical and transcriptional organization of the seven genes involved in its production has been determined (Fig. 6.4). The cloned *act* genes are clustered on a 25-kb fragment where *actI* has been proposed to code for the major part of the polyketide synthase containing at least the condensing enzyme, β-ketoacyl synthase. *actIII* appears to code for a β-ketoreductase. The gene cluster contains also both the resistance conferring gene and a specific positive regulator *actII.* These genes (at least the two involved in the first biosynthetic steps *actI* and *III*) seem to be widespread among polyketide-producing streptomycetes. Indeed, biosynthetic genes sharing a high degree of homology with *actI* or *actIII* have also been isolated from other strains such as *S. violaceoruber,* producer of granaticin. It seems that the granaticin-producing PKS consists of at least six separate enzymes involved in the carbon chain assembly (Fig. 6.5). *S. glauces-*

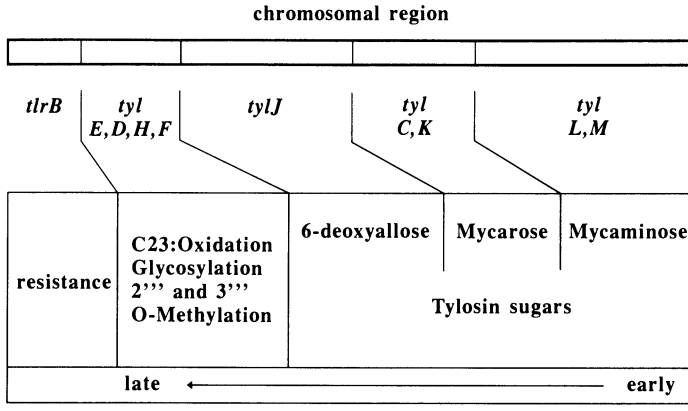

Figure 6.3. Organization of the final cascade of tylosin biosynthetic genes, clustered with one of the resistance genes. The biosynthetic reactions encoded by each gene are indicated at the bottom of the figure.

cens, producer of the anthracycline antibiotic tetracenomycin, also possesses a PKS similar to those of *S. coelicolor* and *S. violaceoruber.* A DNA fragment, detected by its homology with *actI,* has shown the presence of three ORFs which correspond to those present on the *gra* gene cluster (ORF1 to 3). It is also very interesting to note that the products of both *gra* and *tcm* ORF3 seem to be acyl carrier proteins (ACP). In *S. rimosus,* producer of oxytetracycline, a similar gene cluster has also been demonstrated.

In contrast, the same probes (*actI* and *III*) did not hybridize with other strains such as the already mentioned *Saccharopolyspora erythrea,* which have a type I PKS.

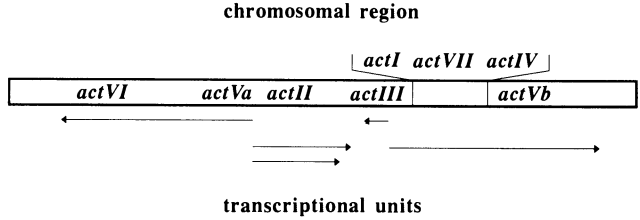

Figure 6.4. Organization of the cluster of actinorhodin biosynthesis genes (*act*) in *Streptomyces coelicolor.*

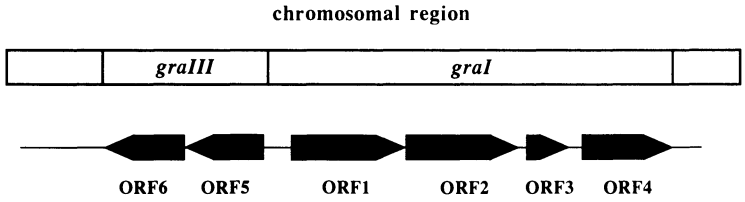

Figure 6.5. Organization of the loci *graI* and *III* of *Streptomyces violaceoruber*. These loci code for several peptides; *graI* contains four ORFs (1-4) all transcribed in the same direction, whereas *graIII* contains two ORFs (5-6) both transcribed in the reverse orientation of *graI*.

6.4.2. *The Polypeptide Class*

In this class a large variety of secondary metabolites can be included that share the common feature of being derived from amino acids (often nonprotein amino acids such as D-amino acids, amino hydroxy acids, and other unusual constituents). Their biosynthetic origin belongs to one of two categories: ribosomal and nonribosomal. As mentioned in Chapter 4, the nonribosomal synthesis is normally performed by a multifunctional enzyme complex usually referred to as the "thiotemplate multienzyme mechanism." A special subclass of these synthetases are those of the β-lactam antibiotics.

6.4.2.1. *The Thiotemplate Multifunctional Synthetases*

The basic steps of this machinery include the activation of the amino acids as adenylates, the acylation of specific template thiol groups, sometimes epimerization or *N*-methylation, and the polymerization in the specific sequence directed by the multifunctional structure; other reactions can include cyclization or other terminal modifications.

The biosynthetic genes are arranged in clusters—organized in large operons that code, in the case of synthetases activating more than one amino acid, for proteins containing repetitive domains (each of about 600 residues). Recently, many genes responsible for the biosynthesis of these peptide antibiotics have been identified, cloned, and sequenced. Sequence analysis derived from these (of either bacterial or fungal origin) has revealed a high degree of conservation among the peptide synthe-

tases (35–50% homology). The highly conserved motifs present in these genes seem to be the functional regions for the various biosynthetic steps, and a modular organization which is analogous to the model proposed for the type I PKSs.

We will take as an example the cyclic peptide antibiotic gramicidin S produced by *Bacillus brevis*. The antibiotic is derived by duplication of the peptide sequence D-Phe-Pro-Val-Orn-Leu. The biosynthetic genes are organized in an operon of about 18 kb, comprising *grsA*, *grsB*, and another gene *grsT*, of still uncertain function but which shares some homology with known thioesterase genes. It is tempting to speculate that the product of *grsT* might hydrolyze the thioester bond linking the completed peptide to the enzyme. The product of *grsA* is a protein of 1089 amino acids which epimerizes and activates the initiator amino acid phenylalanine; that of *grsB* contains 4453 residues and four repeated modules of about 600 amino acids. This multifunctional structure of the *grsB* protein allows the specific adenylation of four amino acids, the formation of enzyme-bound peptide intermediates, the condensation of the two pentapeptide chains, and the final release of the decapeptide molecule.

Similar arrangements of genes are also present in many other *Bacillus* strains such as *B. brevis* ATCC 8185, producer of tyrocidine, and *B. subtilis* ATCC 21332, producer of the lipopeptide antibiotic surfactin. They are also present in non-*Bacillus* species; the best characterized are those of the microorganisms producing β-lactam antibiotics.

6.4.2.2. The β-Lactam Family

A few genes coding for peptide antibiotics have been cloned from organisms other than *Bacillus* species. Most important are those of β-lactam antibiotics, penicillins, cephalosporins, and cephamycins, which have been isolated from different bacterial or fungal species. We shall consider separately the formation of isopenicillin N (IPN), which involves two steps common to the biosynthesis of all β-lactams, and the other biosynthetic steps which differ in penicillins and cephalosporins (see Figs. 4.15 and 4.16).

The common genes. The genes *pcbAB* and *pcbC* responsible for IPN synthesis are common to all β-lactam producers and share a high degree of conservation, both at the amino acid level and in their

chromosomal localization. *pcbAB* is the gene which codes for the aminoadipoyl-cysteinyl-valyl synthase (ACV synthase). This is a typical multifunctional peptide synthetase of about 3700 amino acids (depending on the species of origin). In this, three repeated domains can be distinguished, corresponding to each of the three amino acids to be condensed. These three modules have a high degree of homology with gramicidin S synthetase I and tyrocidine synthetase I. The *pcbC* gene encodes IPN synthase, a nonheme iron protein of about 330 residues, that cyclizes the tripeptide into the bicyclic structure of IPN.

These genes are highly conserved among both eukaryotic and prokaryotic species producing this class of antibiotics. Parallel interfungal domains share about 71% sequence homology. When fungal versus bacterial domains are considered, sequence homology remains quite high, about 48%. Perhaps the major difference rests in transcriptional control; *pcbC* is typically clustered near the *pcbAB* gene, but whereas in the fungal species the two genes are transcribed divergently (and thus belong to different transcription units), in prokaryotes they are transcribed in the same orientation and thus could be under the same control system (Fig. 6.6).

Penicillin biosynthesis genes. Following synthesis of IPN, the biosynthetic routes of the penicillins and cephalosporins diverge into two branches.

The last step of penicillin biosynthesis is catalyzed by a single gene product (encoded by *penDE*) IPN:acyl-CoA acyltransferase (IAT), which hydrolyzes IPN to 6-aminopenicillanic acid. In the presence of phenylacetic acid it also converts the latter into penicillin G. The gene has been isolated from *Penicillium chrysogenum;* a similar gene (*acyA*) has been isolated from *Aspergillus nidulans.* They share about 73% sequence identity and contain three introns (this being the first example of a fungal β-lactam gene containing introns). The gene product undergoes a posttranslational processing event—giving rise to two subunits of about 11 and 29 kDa that act as a heterodimeric enzyme.

Cephalosporin and cephamycin biosynthesis genes. The next step of cephalosporin biosynthesis is the epimerization of IPN to penicillin N (PN), coded for by *cefD.* This gene was first isolated from *S. clavuligerus* and consists of a single ORF coding for a protein of 398 amino acids. The gene is located upstream of the already mentioned *pcbAB* gene and is separated by only 81 base pairs from another gene, *cefE*

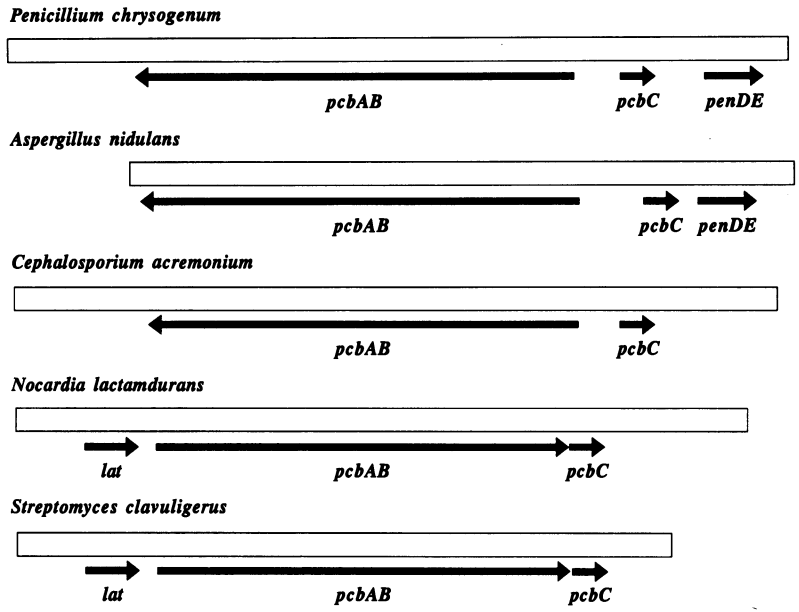

Figure 6.6. Organization of the cluster of genes involved in penicillin (*Penicillium chrysogenum, Aspergillus nidulans*), cephalosporin (*Cephalosporium acremonium*), and cephamycin (*Nocardia lactamdurans, Streptomyces clavuligerus*) biosynthesis. The arrows represent the orientation of the genes. Note the different orientation of *pcbAB* between prokaryotic and eukaryotic β-lactam producers.

with which it forms a polycistronic messenger. The *cefE* gene codes for the enzyme cephalosporin synthase (or expandase) that performs the next step in the cephalosporin biosynthesis: the expansion of the five-membered thiazolidine ring of PN to the six-membered dihydrothiazine ring of deacetoxycephalosporin C (DAOC). This intermediate is then hydroxylated, by a hydroxylase encoded by *cefF*, to form deacetylcephalosporin C (DAC). In contrast to the high degree of similarity observed between the bacterial and fungal genes coding for the first two enzymes of the β-lactam biosynthetic pathway, the expandase and the hydroxylase proteins of these two groups of organisms show important structural differences.

In the case of *S. clavuligerus, cefE* codes for a protein of 311 amino acids, whereas *cefF* codes for a protein of 318 amino acids. The two

genes are clearly separated and belong to different transcription units. However, they demonstrate a high degree of homology (72%), and recent studies indicate that the *cefE* gene product also possesses hydroxylase activity and vice versa. In *Cephalosporium acremonium,* only one gene is found, denoted *cefEF,* which possesses an ORF coding for a polypeptide of 36,462 Da demonstrating both activities. These observations lead to the fascinating hypothesis that, in actinomycetes, the two genes might derive from the duplication of an ancestral progenitor possessing both the expandase and the hydroxylase activity as in the filamentous fungi.

At this point in the divergent biosynthetic pathways, another important difference takes place between the β-lactam antibiotics of prokaryotic or eukaryotic origin. In filamentous fungi, such as *C. acremonium,* the biosynthetic route terminates with transfer of an acetyl group from acetyl CoA to DAC, leading to production of cephalosporin C. Although the gene (designated *cefG*) responsible for this reaction, DAC acetyltransferase, has not yet been isolated, mutants have been described.

In the case of bacterial biosynthesis, further modification leading to the formation of cephamycins has been described. In *S. clavuligerus,* three enzymatic steps are recognized but the corresponding genes have not been isolated.

Finally, it is worth mentioning another *Actinomyces* β-lactam biosynthetic gene, the L-lysine-ε-aminotransferase, which mediates the formation of α-aminoadipic acid, a precursor of ACV. This gene, designated *lat,* is found exclusively in *Streptomyces* and *Nocardia* producer strains. In *N. lactamdurans,* it is present as an ORF of 1353 bp (451 amino acids), located 64 bp from the 5′ end of the *pcbAB* gene. In these organisms the *lat* gene is strictly associated with β-lactam biosynthesis and is not a component of lysine primary metabolism. It seems that in *P. chrysogenum* and *C. acremonium* also an enzymatic activity is present similar to the one performed by the *lat* gene product. However, its relationships with the *lat* gene and moreover with the β-lactam biosynthetic pathway are unclear.

6.4.2.3. Ribosomally Synthesized Peptide Antibiotics

In recent years, several peptide antibiotics have been shown to be ribosomally synthesized. Among these are the lantibiotics produced by

gram-positive bacteria and actinomycetes. As for many other antibiotics, their synthesis is linked to the cessation of growth. Generally they are synthesized as a large peptide chain which undergoes various posttranslational modifications to give the final molecule. This is then exported from the producer cell by a dedicated export system. The genes involved in their biosynthesis (the structural gene and those involved in posttranslational modifications and export) are usually clustered together and are under the same regulation.

The lantibiotics can be divided into two subclasses, linear and circular. The structural genes for their biosynthesis can be located either on the bacterial chromosome or on plasmids. The precursor peptide typically presents an N-terminal leader sequence which is cleaved during the maturation process but does not share any similarity with the typical leader sequences of other exported proteins. It has other possible roles, such as that of chaperon for the posttranslational modifications.

Among the linear lantibiotics, subtilin, isolated from *B. subtilis* ATCC 6633, is one of the best studied and can be considered typical of this class. The structural gene for subtilin, *spaS,* encodes a precursor of 56 residues, whereas the mature product is composed of 32 amino acids. The gene *spaS* is expressed at very low concentrations during the exponential growth phase and its expression increases up to 200-fold in the stationary phase. The structural gene is expressed as a monocistronic transcriptional unit but is located in close proximity to three other adjacent genes, *spaB, spaY,* and *spaC,* that are essential for antibiotic maturation and secretion. Indeed, *spaB* and *spaC* play a role in posttranslational modifications whereas *spaY* appears to be involved in secretion. Genes encoding the subtilin-related lantibiotics epidermin and nisin have also been cloned from *Staphylococcus epidermis* and *Streptococcus lactis,* respectively. Notwithstanding the similarities of the gene organization in the three organisms, the differences in the amino acid sequences suggest that they may have different evolutionary origins.

6.4.2.4. Other Peptide Antibiotics

Finally it is worth mentioning other antibiotics which, although containing a peptide structure, are not synthesized through one of the above-described pathways. This is the case, for example, of the herbicide

antibiotic bialaphos, produced by *Streptomyces hygroscopicus.* Bialaphos is a tripeptide consisting of two L-alanine molecules and an L-glutamic acid analogue, phosphinothricin, which is the active moiety of the antibiotic. Of the 13 steps proposed for the biosynthetic pathway, 12 are involved in the synthesis of the phosphinothricin moiety, and one in the addition of the L-Ala-L-Ala dipeptide. Seven biosynthetic genes, identified by complementation of blocked mutants as well as by their proximity to the bialaphos resistance gene *bar* (an acetyl transferase, which is also involved in the biosynthetic pathway), are clustered together in a region spanning 16 kbp.

6.4.3. Other Antibiotic Families

An important family of antibiotics are the aminoglycosides, which are produced by several bacterial genera, such as *Streptomyces, Micromonospora,* and *Bacillus.*

Streptomycin is the first discovered antibiotic of this class, and perhaps the most extensively studied from the biosynthetic viewpoint. This antibiotic is produced by a number of *Streptomyces* species, including *S. griseus* and *S. glaucescens.* A number of genes have been identified in the biosynthetic cluster that also contains a resistance gene (*aphD*) and a putative regulatory gene (*strR*).

Finally it is worth mentioning the nucleoside antibiotics, which constitute a large group of microbial secondary metabolites. As an example of this class we will consider the antibiotic puromycin, produced by *S. alboniger.* Though the puromycin biosynthetic pathway is still not well understood, the complete set of biosynthetic genes has been located in a DNA fragment of 15 kbp. In this cluster of genes the following have been identified:

- The presumptive resistance gene (*pac*) which inactivates the molecule by *N*-acetylation, but which also appears to be involved in the biosynthesis pathway
- The biosynthetic gene *dmpM* which codes for the enzyme demethyl-puromycin-*o*-methyltransferase
- The *N*-acetylpuromycin hydrolase gene, *pacHY,* which seems to be involved in the export of the antibiotic

Obviously, many other antibiotics are known and for which the biosynthetic, regulatory, and resistance genes have been described. However, we think that those reported here give a sufficient background and a feel for the diversity and, at the same time, the overall analogies presented by the spatial organization and the regulation of expression of the genes involved in secondary metabolite production.

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7

Strain Improvement and Process Development

The productivity of strains isolated in a screening program is ordinarily very low; commonly a few milligrams of metabolite per liter of culture. Initial evaluation of the biological potential of a new metabolite and its chemical characterization requires grams of pure material. Kilograms and tens of kilograms are then needed for animal toxicology studies and later for clinical trials. Therefore, from the initial stages of the development of a new metabolite it is necessary to improve the productivity of the fermentation process. In later stages of development the increase of yields is of paramount importance to define an industrial process by which the substance can be produced at an acceptable cost.

The aims of a development program are not limited to increasing productivity. Genetic stability is also important, in order to avoid an extensive degeneration of the strain as a consequence of the high number of generations required to develop the large biomass for production-scale fermentations. Reduction of cost by development of strains growing on less expensive nutrients, or with a lower requirement for oxygen, is also frequently a target. In addition, there are problems related to the fermentation of specific metabolites. Examples are: (1) the morphology of the producing strain that greatly influences the viscosity of the medium and thus the aeration and power input requirement; (2) the elimination of pigments or other substances interfering with the recovery and purification process; (3) the increase of the percentage of a selected component when a complex of substances is produced.

Alteration of the producing strain's genetic characteristics is undoubtedly the most effective approach to attain these objectives, and

therefore this is the main subject of this chapter. However, the physiological aspects (such as medium composition and culture condition) should not be underestimated, because through these the maximum expression of the strain's genetic potential is attained. In addition, biochemical and physiological studies may suggest specific targets for the genetic program. It is therefore evident that the success of a fermentation development program depends on the interrelation of the genetic and the physiological approaches.

7.1. Strain Purification and Natural Variants

Initial improvement of the yields of a newly isolated strain is normally obtained by devising a suitable fermentation medium and adjusting the physical conditions of the culture such as aeration rate and temperature. This is done empirically, with previous experience being the only guide, and general information on the strain biology, as reported in Chapter 2, and nutrient regulation of antibiotic biosynthesis discussed in Chapter 5.

At this stage the contribution of the geneticist is ordinarily limited to strain purification and to the selection of natural variants. The purification of the strain is necessary because often the strain, although derived from a single colony, actually consists of a population of two or more genotypes. Identification of variants and purification of mixed cultures is accomplished by plating on several agar media, where different morphological characters can be observed. Isolation and subculture of the colonies can reveal an inherent genetic instability of the strain, as often is the case with actinomycetes. By testing different colonies for their productivity, high producing variants can be selected. The main objective of these processes is to reduce the variability normally observed in the flask fermentations of the soil isolates, and acquire a solid basis for the medium improvement studies and the subsequent genetic work.

7.2. Mutation and Selection

Experience shows that the most effective method for increasing the yield of a microbial metabolite consists of inducing mutations and

selecting higher producing strains. Although experimental evidence is lacking, it appears logical to hypothesize that the spectacular success of mutation/selection programs of productivity improvement (the yields of penicillin and tetracycline, for instance, have been improved more than 1000 times over those of the wild-type strains) is the result of the disruption of control mechanisms regulating antibiotic production. In fact, there is an extremely low probability that a random mutation results in an improved efficiency of a biosynthetic enzyme, whereas any of a number of different point mutations or deletions may inactivate a negatively controlling gene. A favorable mutation is, however, a rare event, and the main disadvantage of a random screening for high producing mutants is that, in the absence of a positive selection method, thousands of colonies must be tested for their productivity in order to identify an improved strain. Therefore, methods have been devised to increase, by indirect selection techniques, the fraction of high producers in the mutant population, an approach that is commonly referred to as "rational screening."

7.2.1. Mutagenesis

There are four aspects to be considered in the design of a mutagenesis program: preparation of the cells, choice of the mutagenic agent, efficiency of the mutation treatment, and mutant segregation.

1. Ideally the mutagenic treatment should be performed on mononucleated cells. When dealing with filamentous organisms this can be accomplished by treating dormant or germinating spores. However, with actinomycetes, general procedures to efficiently induce sporulation are not available, and high producing strains often lose the ability to sporulate. Thus, the mutagenic treatment is normally performed on hyphal fragments. These are prepared by submitting the mycelium of a vegetative culture to ultrasonic fragmentation. An appropriate procedure can give mononucleated fragments of hyphae but fragments containing a few nuclei are commonly obtained. Considering that the mutagenic treatment may inactivate about 90% of the nuclei, the probability that, after the treatment, a fragment contains more than one viable nucleus is reasonably low but must be taken into account, as discussed below in relation to mutant segregation.

2. There are few rational criteria for the choice of the mutagenic agent, provided that the microorganism is sensitive to its action. Past experience indicates that important results can be obtained by the use of nitrogen mustard or ultraviolet irradiation. Presently, however, the most frequently used agent is nitrosoguanidine (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine). Like ultraviolet irradiation and several chemical agents, nitrosoguanidine also induces mutations primarily by an error-prone repair mechanism but nitrosoguanidine treatment results in comparatively higher frequencies of mutants and lower lethality. Since different mutagens may induce different types of mutations (nitrosoguanidine, for instance, mainly induces GC → AT transitions, whereas ultraviolet irradiation induces transversions and deletions also), it may be advisable, in order to increase the variability, to change the mutagenic treatment when an organism has been subjected to a high number of mutation-selection runs.

3. The efficiency of mutagenic treatment is normally checked by determining the frequency of mutation toward an easily selectable marker, such as resistance to streptomycin or other antibiotics. The conditions of the treatment and the mutagen doses are generally adjusted to maximize the frequency of mutants. However, it has been pointed out that, since unfavorable mutations are much more frequent than favorable ones, the presence in a clone of two mutational events is not desirable. In fact, there is a high probability that one of the mutations has negative effects, offsetting the possible positive effects of the other. Therefore, optimization of the mutagenic treatment should be aimed at producing one mutation per nucleus.

4. Normally only one of the two DNA strands of a nucleus is altered by the mutagenic treatment, or different mutations may occur in the two strands. Moreover, often the mutagenic treatment is performed on multinucleate fragments of the microorganism and the same fragment may contain both mutated and nonmutated nuclei. It is therefore important to allow the cell to replicate a few times and subculture the clones in order to obtain colonies in which the mutants are segregated from the parental genotype. To obtain mutant segregation the cells are grown in a rich medium for a few generations, the mycelium is homogenized, sonicated, and plated on agar medium, from which the clones can be isolated.

7.2.2. *Selection by Random Screening*

The traditional approach of a development program, aimed at increasing the production yields of an industrial strain, consists of testing the productivity of clones randomly chosen among the survivors of mutagenic treatment. Culture of the clones is performed in flasks, and the antibiotic production is determined by analytical methods. Because of the extremely low frequencies at which desired mutations occur, a very large number of colonies must be tested, and therefore random screening is time-consuming and costly. On the other hand, it is often the only possible approach when little is known about the metabolite biosynthesis and the strain physiology and genetics; and, as already mentioned, it has given important results in the development of several antibiotics.

The main difficulty encountered with this method is the natural variability of the flask fermentations. In the initial stages of development, only substantial increases of yields are of interest and these can be easily detected. With improved, high producing strains, a yield increase of, say, 10% may be important, and can be masked by the phenotypic variability of the fermentation when this is of the same order of magnitude. Repetition in duplicate or triplicate of the fermentation of each colony could overcome this difficulty but this implies a substantial increase of work and cost. An alternative procedure, denoted "recycling screening" has been proposed and is presently frequently applied. This consists of pooling the colonies that in the first fermentation test gave the highest yields (for instance, 5% of the tested colonies), growing them in a mixed culture, and submitting the mycelium thus produced to a new run of mutagenesis. The operation is repeated several times and after the last run the best producers are individually retested. Both theoretical considerations and experimental verifications have demonstrated that this method is indeed effective and valuable.

In random screening programs, much attention should be given to the culture medium and conditions of test flask fermentations. These can in fact greatly influence the selection. A glucose-rich medium could, for instance, favor the selection of carbon catabolite derepressed mutants. A relatively poor medium could select clones improved in nutrient utilization. Testing the productivity at an earlier than usual time, may select for fast-growing strains, or for strains producing at a faster rate.

Thus, although a medium is often chosen similar to that already used for production, variations may be introduced for specific reasons.

A method frequently used to improve the efficiency of random screening is to prescreen the mutagenized colonies on solid medium. Cells are seeded on an agarized nutrient medium, and the developing colonies are overlaid with soft agar containing a microorganism sensitive to the antibiotic produced by the strain under development. After a suitable incubation time an inhibition zone can be observed around the producing colonies, the diameter of which is approximately proportional to the logarithm of the antibiotic concentration. Colonies showing higher than average inhibition diameters are then tested in flask fermentation.

The great advantage of this preselection method is the large number of colonies that can be screened at the same time. There are, however, several limitations that must be considered. One is that the apparent increase of colony productivity can simply be the result of a larger size colony. In this respect, consideration of the ratio of the diameter of the colony to that of the halo as the discriminating factor has been proposed. Another important limitation is that small but significant increments in yield may be overlooked, because these give only a minor variation in the size of large diameters. This difficulty can be sometimes overcome by the use of a test organism that gives, under the conditions adopted, very small zones of inhibition around the average producing colonies. In this case small differences can be detected. Probably the major drawback of the method is that in many cases, for reasons not clearly understood, there is no correlation between the inhibition zone diameter and the productivity of the colony when grown in submerged culture conditions. There are, in fact, examples (such as the isolation of cephalosporin C-overproducing mutants of *Cephalosporium acremonium* or of strains of *Streptomyces kasugaensis* overproducing kasugamycin) in which the method has been successfully used, and others in which it failed.

7.2.3. Rational Screening

The expressions *rational screening* or *enrichment procedure* indicate methods by which the fraction of overproducers in the population of mutagenized cells is increased by using positive selection techniques

potentially related to productivity. Some of these techniques, below described, can be very powerful and many cases have been reported of considerable yield increases obtained in this way, superseding the tedious work of random screening.

1. Selection of mutants resistant to the antibiotic produced. As discussed in Chapter 6, many antibiotic-producing strains are sensitive to the antibiotic they produce. Mutants resistant to high concentrations of antibiotic can normally be easily selected by a stepwise increase in the concentration of the antibiotic in the plates on which mutagenized colonies are growing. These may constitute a more favorable background than wild-type strains for yield improvement. Sometimes, the mutant strains themselves can be overproducers, because of an alteration of the antibiotic production regulation mechanism.

2. Selection of morphological variants. Although no rational explanation is generally available, it has been noted that, in several cases, the frequency of overproducers is higher among the colonies differing from the original strain in their morphology. The frequency of nonproducers is also high among colonies presenting morphological variations. This aspect can be exploited because nonproducers may be useful for biosynthesis studies (see use of blocked mutants in Chapter 4) and for the reversion method described below.

3. Reversion of nonproducing mutants. Mutants that have lost their producing ability are more frequent than overproducers, and can be easily detected in the plate assay prescreen (although it is always necessary to check in flask fermentation whether this is a permanent, genetically determined, loss). When the inability to produce is caused by an alteration of a regulatory mechanism, there is a fair probability that reversion to production, by a subsequent mutation, occurs along with an altered mechanism of regulation, thus resulting in increased productivity. This method has been successfully used with the tetracycline-producing microorganisms *Streptomyces rimosus* and *S. viridifaciens*. Revertants of a nonproducing mutant were obtained which produced more than twice as much chlortetracycline than the original strain.

4. Selective detoxification. Some chemical agents, primarily metal ions (such as copper, aluminum, mercury ions), that are toxic for the producing organisms can bind to antibiotics, giving stable complexes. When cells are seeded on a solid medium containing a toxic concen-

tration of a selected metal ion, the growth of most of the colonies is inhibited. However, if the ion forms a complex with the antibiotic produced, overproducing colonies will survive because the action of the metal is prevented by its binding to the antibiotic excreted in the medium. Positive results by the application of this technique have been obtained, for instance, in the isolation of cephamycin overproducers by selecting strains of *Streptomyces lipmanii* on phenylmercuric ions, and of cephalosporin C overproducers of *Cephalosporium acremonium* with the use of copper, manganese, and mercury.

5. Selection of overproducers of a biosynthetic precursor. There are cases in which the rate-limiting step of an antibiotic biosynthetic pathway is the production of a biosynthetic precursor, often a primary metabolite. The selection of overproducers of the precursor may provide strains producing higher yields of antibiotic. An example is the production of pyrrolnitrin by *Pseudomonas fluorescens.* This antibiotic derives from tryptophan, and an increased productivity was observed by addition of this amino acid to the cultures. The same result was obtained by selecting tryptophan-overproducing strains, on the basis of resistance to tryptophan toxic analogues. Similarly the limiting step in *Nocardia lactamdurans* efrotomycin biosynthesis is the availability of uracil, the precursor of the pyridone moiety of this antibiotic. Spontaneous mutants resistant to the toxic analogue 5-fluorouracil were isolated that were also high producers of efrotomycin in minimal medium cultures.

7.3. Gene Recombination

Gene recombination can bring desirable genes together in one strain. Positive characteristics can be acquired simultaneously by the producer strain, and negative characteristics can be bred out. It can become an important technique, in addition to mutant selection, once several lineages of mutants have been established. For well-characterized organisms, natural recombination systems may be used, such as conjugation, transduction, and transformation, whereas for those less characterized the protoplast fusion technique can be useful.

Genetic engineering provides the possibility of controlled gene manipulation, and allows the alteration of specific characteristics of a

producing strain. It can be used to relieve rate-limiting steps of the biosynthetic pathway by increasing, for instance, the gene dosage, or altering a regulation mechanism. Obviously this requires a good knowledge of antibiotic production biochemistry and genetics.

7.3.1. Natural Fertility Systems

These systems have been extensively used in the development of fungal strains but much less for the yield increase of antibiotics produced by bacteria. An example of strain improvement obtained through conjugation is the interspecies mating between auxotroph strains of *Streptomyces rimosus* and of *S. kanamyceticus.* The first is a producer of paromomycin and of a minor amount of neomycin, the latter of kanamycin. Among the prototroph recombinants, strains were found that produced kanamycin in high yields.

Recombination in secondary metabolite-producing fungi is usually obtained by the parasexual cycle. In the parasexual cycle, recombination is the result of mitotic processes, in which the basic phenomenon is the formation of a heterokaryon through an anastomosis formed between hyphae of two different strains. Although this event is very rare, it can be selected by growing complementary auxotroph strains in mixed culture on a minimal medium, where only heterokaryons can develop.

Strain improvement programs based on the parasexual cycle can give either haploid segregants, or stable diploid hybrids. Both of these outcomes have been observed in programs for improvement of penicillin production by *Penicillium chrysogenum.* Enhanced penicillin yields were achieved by crossing parental strains differing only in markers required to select for heterokaryons, and thus obtaining diploid strains. However, haploid segregants, obtained by recombining two second-generation strains (derived from a common ancestor possessing two different alleles determining high production levels), also demonstrated higher productivity than the parental strains.

7.3.2. Protoplast Fusion

Protoplast fusion is probably the most applicable and versatile of the techniques for genetic recombination. This technique does not require an extensive knowledge of the biochemistry or genetics of anti-

biotic production. The basic methods generally employed have been briefly described in Chapter 2. However, the procedure for an efficient formation of protoplasts and for their regeneration must be optimized for each strain, a task not always easily performed.

Protoplast fusion has been applied to the improvement of both fungal and actinomycete antibiotic producers. It has been especially valuable with *C. acremonium,* an organism for which no convenient methods for sexual or parasexual exchange are available. Several cephalosporin C-overproducing strains have been obtained by intraspecies protoplast fusion of auxotrophic mutants of this microorganism. Strains efficiently utilizing inorganic sulfate for antibiotic production were also isolated.

Another well-known example is the development of fast-growing strains of *P. chrysogenum* producing only minor amounts of *p*-hydroxypenicillin V. This metabolite is a contaminant of penicillin V that interferes with the chemical transformation of the latter into cephalosporins.

A successful example of the use of protoplast fusion with actinomycetes is the strain improvement in the cephamycin C production by *N. lactamdurans.* Strains which consistently produced more antibiotic than the original one were obtained by testing the progeny of a cross of two nonmarked mutants, diverged from a common parental strain.

7.3.3. Genetic Engineering

The recently developed DNA recombinant techniques and the knowledge of the genetics of antibiotic production (an account of which has been given in Chapter 6) provide rational methods by which strain improvement can be attained by gene manipulation. There are several approaches that can be followed, according to the specific gene organization of the producing microorganism under development. Some relevant examples are reported below.

1. Structural gene amplification. It is often possible, in a biosynthetic pathway, to identify the rate-limiting step, that is, the enzymatic reaction conditioning the flow of the intermediates through the pathway. When this is the case, an increased dosage of the gene coding for the enzyme involved can result in

higher productivity. In a *C. acremonium* strain, for instance, it was determined that the rate-limiting steps in cephalosporin C production were the ring expansion of penicillin N and the subsequent hydroxylation of deacetoxycephalosporin C. The enzyme catalyzing both of these reactions is the product of the gene *cefEF*. This gene was cloned in a plasmid carrying a hygromycin-resistant marker and introduced into the producing organism. Among the transformants, strains were found producing higher levels of cephalosporin C, in which the presence of an additional copy of the *cefEF* gene was demonstrated.

2. Regulatory genes and resistance genes. There is evidence, as mentioned in Chapter 6, that some biosynthetic gene clusters also contain regulatory genes, which may act either negatively, repressing the structural genes' expression, or positively, as expression inducers. In *Streptomyces coelicolor* the production of methylenomycin is negatively regulated by a gene, situated in a DNA region at the end of the biosynthetic cluster, in which alterations or deletions result in an increased production of the antibiotic. In the same organism the gene *actII* functions as a positive regulator of the production of actinorhodin. Introduction in a producing strain of additional copies of this gene resulted in a remarkable increase in the level of antibiotic production.

Some genes, such as those contained in the $dnrR_1$ and $dnrR_2$ DNA segments of *Streptomyces peucetius,* producer of daunorubicin, demonstrate an ability to stimulate both secondary metabolite production and resistance. Insertion of these segments in a wild-type strain increased the production of the antibiotic and of a key biosynthetic intermediate. In addition, insertion of the $dnrR_2$ segment into a daunorubicin-sensitive mutant restored the normal levels of resistance to this antibiotic.

The mechanism by which sometimes extra copies of the resistance-conferring genes determine an increased antibiotic production is not well understood. It is possible that, in some cases, the increased resistance capacity allows a higher level of production otherwise toxic for the producer. Alternatively, it has been suggested that, since the mechanism of resistance may be an enhanced rate of antibiotic exported from the cell, the relief of feedback inhibition can result in higher antibiotic productivity.

7.4. *Process Development*

The aim of process development is to establish a procedure allowing the maximum expression of the microorganism biosynthetic potential. Three phases may be recognized in process development studies:

1. Flask fermentation studies, during which essentially the effect of medium composition on the growth of the cultures and on antibiotic production is examined
2. Studies in small fermenters, in which the effects of several parameters, such as aeration, agitation, temperature, and pH value, are determined
3. Scaling up studies, by which the conditions assuring high productivity in small-scale fermentations are adapted to industrial scale

7.4.1. *Flask Fermentation*

Nutritional requirements and media composition allowing the maximum expression of the genetic potential are normally studied in flask fermentations, because of the high number of variables involved. It has to be pointed out that for each higher producing strain obtained through genetic manipulation, it is necessary to adjust the medium composition, in order to fully exploit the strain potential.

With antibiotic-producing organisms, normally only complex media give high production levels. Since the results obtained in flask fermentation experiments must then be transferred to industrial scale, inexpensive and readily available raw material should be utilized (a brief account of the materials commonly used in industrial antibiotic production is given in Chapter 9). In a first round of experiments, different ingredients are usually tested. Then, in subsequent series of experiments, the medium is optimized, by introducing variations in the ingredient concentrations.

Because of the high number of interdependent variables affecting the overall outcome, the design of the medium optimization experiments is ordinarily based on statistical methods. One such design, frequently adopted, consists of 32 flask fermentations, in which the different combinations of two levels of five different variables are tested.

The results can be conveniently analyzed by computerized programs. It must be remembered that the successful use of such methods requires a low level of variability between fermentations run under identical conditions, and therefore a very accurate standardization of all relevant parameters.

7.4.2. Experiments in Small Fermenters

The determination of several important parameters that vary during the course of the fermentation cannot be easily performed in shaken flasks. Therefore, these are measured in small fermenters (usually of 30–50 liter capacity) that can be equipped with instruments for the continuous measurement of some parameters, and from which samples can be easily taken for laboratory analysis. Instrumentation is available for on-line determination of pH, dissolved oxygen, and CO_2 concentration in efflux gas. Aeration, stirring speed, and pressure can be regulated. Analysis of samples taken at regular intervals provides data on nutrient concentration, such as reducing sugars, and ammonium and phosphate ions, in addition to biomass volume and antibiotic production.

By plotting the time course of the different parameters, a graphic representation of the fermentation profile can be produced. By introducing appropriate variations the effect of the various parameters on productivity can be established, and suitable corrections can be introduced. For example, there may be a critical period in which the oxygen requirement by the growing culture is very high. This is revealed by a fall in the oxygen concentration in the broth, which can be corrected by increasing the aeration or the stirring speed. An optimum pH level for production can be identified, and pH correction can be introduced by the addition of mineral acid or base. Nutrient concentration can also be optimized on the basis of the rate of consumption and the temporal relationship of their concentration to the time course of the secondary metabolite production.

7.4.3. Scaling Up of the Process

The adaptation of processes, studied in flasks or small fermenters, up to industrial scale is a branch of the relatively recent discipline of

bioengineering. We discuss here the principal factors that are affected by the fermentation scale.

1. In batch fermentations, the number of generations required to reach the final biomass concentration is a function of the logarithm of the fermenter volume. A high number of generations is thus required to complete growth in an industrial fermenter. An indirect impact of this on antibiotic production is that, at each generation, a certain number (characteristic of each strain) of mutants is produced. The summation of the mutants produced in a generation with the progeny of those produced in previous generations results in a continuously increasing proportion of mutants (ordinarily nonproducers) in the cell population. Obviously this can significantly affect the final yields. There is little that can be done to avoid this inconvenience other than selecting a very genetically stable producing strain.

2. In small-volume fermentations the culture medium is usually sterilized in batches, by heating the entire volume to a predetermined temperature for a given period of time. The same procedure applied to very large volumes often results in extensive degradation of the nutrients, because of the long period of time required to bring the medium to the desired temperature, and afterwards to cool it. Therefore, the alternative technique of continuous sterilization (or high-temperature, short-time sterilization) is normally applied to large volumes of medium. This technique consists of rapidly heating the culture medium flowing through a pipe to a suitably high temperature and cooling it after a short time.

3. Aeration and agitation. As a first approximation, scaling up of the aeration rate is achieved by keeping the air influx volume per unit volume of culture broth constant. To obtain approximately the same mixing and turbulence on scaling up of the agitation, the power input to the driver per volume unit is kept constant. However, the actual efficiency of agitation is greatly influenced by several factors, such as the geometry of the fermenter and of the impeller, and thus the optimum stirring speed has, in part, to be determined empirically. It may happen that the maximum air influx or power input that can be provided by industrial equipment is insufficient to meet the oxygen demand in critical phases of the fermentation. In this case, a possible solution can be to decrease the growth rate by lowering the temperature or by using slowly utilized nutrients.

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8

Biological Transformations

Modifications of an antibiotic structure that do not interfere with its intrinsic activity, i.e., with the ability to interact with its target molecule in susceptible microorganisms, can be of fundamental importance with regard to *in vivo* biological activity and the practical usefulness of the antibiotic. Properties such as the membrane transport (and hence the penetration into bacterial cells), absorption and distribution throughout the body, and susceptibility to the action of inactivating enzymes can be drastically influenced by modifications of the original molecule.

Whereas most of the successful modifications have been carried out by chemical methods, since the beginning of the antibiotic era some have been obtained by biological approaches. In the early days it was observed that penicillins with different biological properties could be obtained by the addition to *Penicillium* cultures of different precursors of the antibiotic side chain. Subsequently, biological modifications, in addition to chemical modifications, were attempted for practically all of the microbial secondary metabolites of interest. Several approaches were successful. We briefly describe here the most interesting ones, choosing from the vast literature a few examples noteworthy for their originality or for their practical value.

8.1. Precursor-Directed Biosynthesis

8.1.1. Addition of Precursors to the Producing Strain

As mentioned in the chapter on antibiotic biosynthesis, the enzymes of secondary metabolism often display a broad substrate speci-

ficity. Hence, a fairly common characteristic of antibiotic-producing microorganisms is their ability to synthesize a complex of structurally related substances, rather than single compounds. The relative amounts of the components of a complex normally depend on the availability of specific precursors. Hence, the addition of a selected exogenous precursor to the culture can result in the preferential production of the component desired.

This important observation was first made in *Penicillium chrysogenum* fermentations, in which different penicillins (including penicillin G, X, K, and F; Fig. 8.1) were produced according to the composition of the growth medium. When the fermentation medium was supplemented with corn-steep liquor (which is a useful nitrogen source but also contains fair amounts of phenylacetic acid), only penicillin G was obtained in relatively high yields. This result not only made the addition of phenylacetic acid to the media standard practice for the

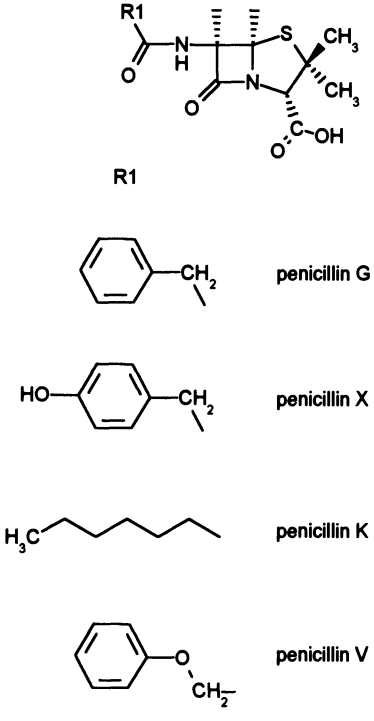

Figure 8.1. Some penicillins produced by *Penicillium chrysogenum,* and penicillin V, obtained by addition of phenoxyacetic acid to *P. chrysogenum* cultures.

production of penicillin G, but also suggested an important extension of precursor addition. It was in fact confirmed that nonnatural penicillins could be produced by adding synthetic precursors to the fermentation media. No less than 100 new penicillins were obtained in this way. Among these, phenoxymethylpenicillin (penicillin V, Fig. 8.1), the first penicillin therapeutically effective by oral administration, was biosynthesized by addition of phenoxyacetic acid.

The success demonstrated with penicillins, of altering a complex composition or of producing new compounds, prompted the application of the precursor addition technique to a number of secondary metabolite fermentations.

8.1.1.1. Alteration of the Composition of Antibiotic Complexes

Sometimes one of the members of an antibiotic complex demonstrates a biological activity superior, in some respect, to that of the other components. It is thus desirable to increase its relative abundance in the mixture of products. In other cases, when the final industrial product is a complex of several substances, it is important to have means of controlling the complex composition. This may be important, for example, in order to keep it within the specifications set up during the development stage and earlier clinical studies. As the following examples demonstrate, both of these results can be obtained by precursor addition.

Streptomyces verticillus produces 12 different bleomycins, glycopeptides differing in their C-terminal amine. The production of bleomycin A_2 (Fig. 2.8) was increased from 55% to 80% of the total antibiotic yield when a specific precursor, 3-aminopropyl-dimethylsulfonium chloride, was added to the fermentation medium.

Tolypocladium inflatum produces several cyclosporins, cyclic peptides differing in their amino acid at position 2. Addition of D, L-α-aminobutyric acid, the precursor of cyclosporin A (Fig. 1.8), to cultures of the producing strain suppressed the formation of all of the other components, and gave cyclosporin A as the only product of the fermentation.

The antibiotic teicoplanin is produced by *Actinoplanes teichomyceticus* as a mixture of five main components, denoted T-A2-1 to T-A2-5. They differ in the structure of an acyl chain linked by an amide

bond to a glucosamine moiety. Three of these substituents are branched fatty acids, namely isodecanoic acid (T-A2-2) and anteiso- and iso-undecanoic acid (T-A2-4 and T-A2-5, respectively). The biosynthesis of branched fatty acids depends on the initiator molecule. This is the coenzyme A ester of isobutyric acid for the even carbons, iso-fatty acids; and 2-methylbutyric acid or isovaleric acid for the odd carbons, anteiso- and iso-fatty acids. By addition of these acids (or of their natural precursors, valine, isoleucine, or leucine) to *A. teichomyceticus* cultures the production of the different teicoplanins can be controlled within large limits. For instance, upon valine addition, almost exclusive production of T-A2-2 is observed (Fig. 2.16).

8.1.1.2. New Product Biosynthesis

The addition of nonnatural precursors to antibiotic fermentations often results in the production of novel substances. However, because of the competition with the natural substrates, the yields of the new products are generally low, and a mixture of products is obtained.

We report here two examples, one old and a more recent one, in which this competition was practically eliminated by the so-called washed mycelium (or resting cells) technique.

Azomycin (2-nitroimidazole) is a product of *Streptomyces* and *Pseudomonas* strains. Studies on its biosynthesis indicated that it derives from the oxidation of 2-aminoimidazole. The synthesis of its 4(5)-alkyl derivatives was attempted by submitting a series of 4(5)-aminoimidazoles to the action of an azomycin-producing streptomycetes. To avoid competition with the natural substrate, the mycelium of grown cultures was filtered, washed, and resuspended in a buffer solution. Following this treatment, the production of azomycin was minimal, and the incubation of the synthetic precursor yielded the

Figure 8.2. Conversion of 5-ethyl-2-aminoimidazole into 5-ethyl-2-nitroimidazole by cells of *Streptomyces* LE 3342.

desired derivatives (Fig. 8.2). One of the compounds thus produced, 4-ethyl-2-nitroimidazole, was found much more effective than azomycin in curing experimental infections by *Trichomonas vaginalis.*

Asperlicin, an antagonist of the cholecystokinin receptor, produced by *Aspergillus alliaceus,* is a complex molecule derived from the condensation of two anthranilate, one tryptophan, and one leucine unit. Several derivatives could be prepared by the addition of tryptophan or leucine analogues to suspensions in buffer of the producing strain's washed mycelium. Again following this treatment, only traces of the natural metabolite were produced. Methyl-substituted tryptophan, for instance, gave rise to methyl-substituted asperlicins in both the tryptophan- and the anthranilate-derived moieties (Fig. 8.3). Since during normal biosynthesis anthranilate is a precursor of aromatic amino acids, this indicates that under the conditions used, anthranilate derives from tryptophan degradation. Both natural and nonnatural lipophilic amino acids could substitute for leucine, to give the corresponding asperlicin derivatives.

8.1.2. Addition of Nonnatural Precursors Coupled with Interrupted Biosynthesis

Two elegant methods have been proposed to definitively solve the problem of competition between the natural substrates and the exog-

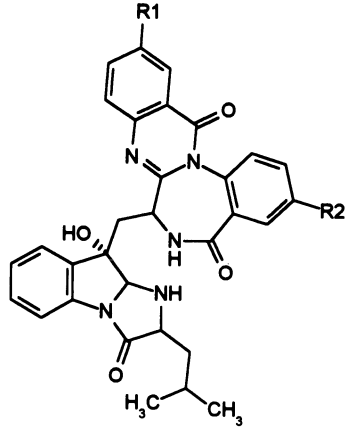

Figure 8.3. Asperlicin (R1 = R2 = H) and dimethylasperlicin (R1 = R2 = CH_3), a derivative obtained by addition of 6-methyltryptophan to cell suspensions of *Aspergillus alliaceus.*

enous analogues in the biosynthesis of novel metabolites. The first is termed mutational biosynthesis, or mutasynthesis, the second is the use of specific inhibitors.

8.1.2.1. Mutational Biosynthesis

This method is based on the isolation of mutant strains blocked in the biosynthesis of one of the moieties constituting the antibiotic. These mutants, called idiothrophs, can be easily identified since antibiotic production is restored upon addition of the nonproduced precursor to the cultures. When synthetic analogues of the precursor are added to fermentations of such strains, the biosynthesis of the corresponding analogue of the antibiotic can be exclusively obtained.

The first known example is the production of hybrimycins, analogues of the neomycins produced by *Streptomyces fradiae.* Hybrimycins differ from the natural products in the aminocyclitol moiety. Instead of the 2-deoxystreptamine present in neomycins, they contain either streptamine or epistreptamine, the nonnatural precursors added to the cultures (Fig. 8.4). Several other aminoglycosides with modified streptamines were similarly prepared, e.g., the analogues of ribosta-

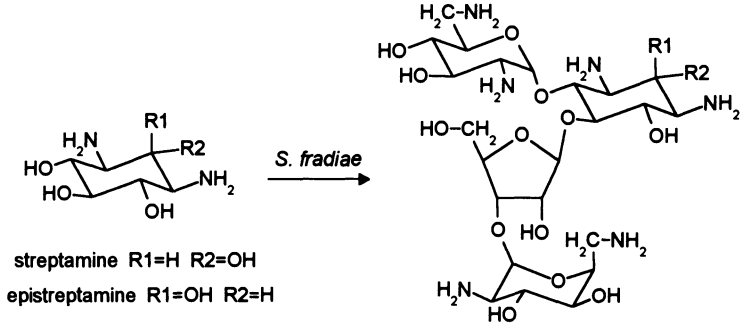

Figure 8.4. Hybrimycins, antibiotics produced by mutational biosynthesis, adding streptamine or epistreptamine to cultures of a mutant of *Streptomyces fradiae* blocked in deoxystreptamine biosynthesis.

mycin, butirosin, and sisomycin. Streptomutin, an analogue of streptomycin containing 2-deoxystreptidine, was obtained by the addition of this aminocyclitol to a *Streptomyces griseus* idiothroph.

This technique was applied to other families of antibiotics, particularly the macrolides. Among these, flurithromycin (8-fluoroerythromycin) was obtained by adding 8-fluoroerythronolide B (chemically synthesized from the erythromycin aglycone erythronolide B) to a mutant of *Saccharopolyspora erythrea* blocked in the synthesis of the aglycone (Fig. 8.5). Flurithromycin has an antibacterial activity comparable to that of erythromycin A, and an improved stability in acidic solutions.

Recently, new derivatives of the avermectins, the antiparasitic macrolides, have also been produced by mutasynthesis. In this case a mutant of *Streptomyces avermitilis* was isolated that lacks a functional enzyme for the conversion of valine and isoleucine into isobutyric and 2-methylbutyric acid, respectively. The latter are the initiator molecules in the biosynthesis of the polyketide chain from which the "a" and "b" series of avermectins originate. A large number of novel avermectins could be obtained by feeding carboxylic acids, or their precursors, to cultures of this mutant. Branched-chain acids, as well as acids with alicyclic or heterocyclic substituents, were accepted as substrate. The size limit appears to be eight carbon atoms but the presence of polar groups on the chain prevents incorporation.

8.1.2.2. Use of Specific Inhibitors of the Biosynthetic Process

As an alternative to the isolation of blocked mutants, the biosynthesis of the macrolide aglycones can be prevented by addition of cerulenin, an inhibitor of polyketide synthesis, to the cultures of a producing organism. Two new macrolide antibiotics, chimeramycin A and B, were produced by this technique.

Streptomyces ambofaciens, the producer of the antibiotic spiramycin, was cultivated in the presence of cerulenin. Protylonolide, the aglycone of tylosin (isolated from a blocked mutant of *S. fradiae*), was added to the grown culture and converted to the novel antibiotics constituted by the tylosin aglycone and the spiramycin sugars (Fig. 8.6).

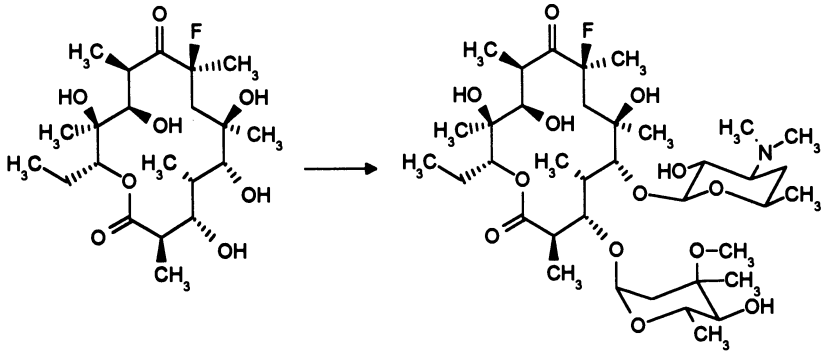

Figure 8.5. Conversion of 8-deoxyerythronolide B to 8-fluoroerythromycin A by cultures of a blocked mutant of *Saccharopolyspora erythrea.*

8.2. Genetic and Molecular Biology Methods

8.2.1. Mutation

The mutagenic treatment of a producing organism can result in the isolation of strains producing substantial amounts of minor components of antibiotic complexes, or sometimes of novel substances not detected in the cultures of the parent organism. This may be caused by the inactivation of an enzyme resulting in the diversion of the intermediates flow from the main biosynthetic pathway, or sometimes by the reactivation of silent enzyme expression. In some cases industrially relevant derivatives have been obtained from mutant strains of the microorganism producing the original antibiotic.

Streptomyces peucetius is the producer of the antitumor antibiotic daunomycin. This microorganism possesses the gene coding for an enzyme, 14-daunomycin hydroxylase, that is not, however, normally expressed. By mutagenic treatment, a variant was isolated—denoted *S. peucetius* var. *caesius* because of the bluish color of the aerial mycelium—in which the synthesis (or the functionality) of the hydroxylase was reactivated. Thus, the main fermentation product of this variant was 14-hydroxydaunomycin, or adriamycin (now renamed doxorubicin), considered the most clinically effective antitumor antibiotic.

A similar success of this approach was the production of the demethyltetracyclines. A mutant of the chlortetracycline-producing or-

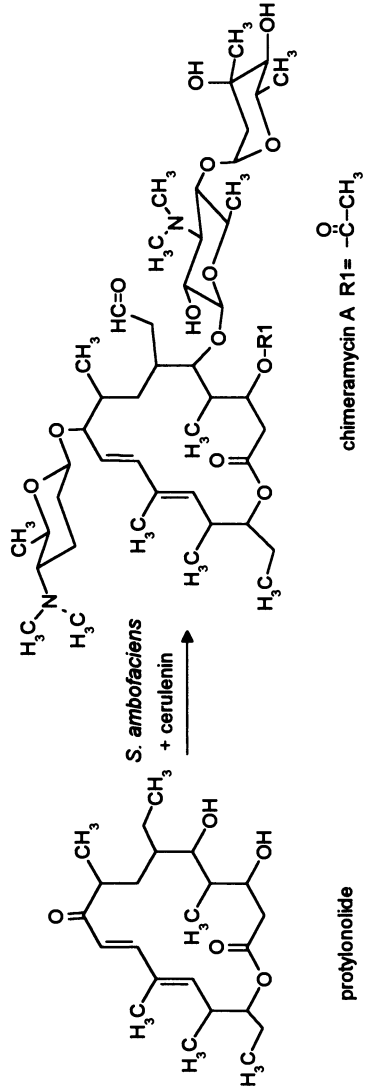

Figure 8.6. Conversion of protylonolide (the aglycon of tylosin) to chimeramycins by *Streptomyces ambofaciens*, in the presence of cerulenin, an inhibitor of polyketide synthase.

ganism, *Streptomyces aureofaciens,* was isolated which was blocked in the pretetramide methylation step—one of the first reactions in the tetracycline biosynthesis. The mutant is able to convert pretetramide, instead of methylpretetramide, into the final product. This can be either demethylchlortetracycline or demethyltetracycline (Fig. 8.7), depending on the availability of chloride ions in the fermentation medium.

The main product of *Nocardia mediterranea* (now reclassified as *Amycolatopsis mediterranei*) is rifamycin B. By mutagenic treatment a mutant strain of this organism was obtained that was blocked in the last steps of the antibiotic biosynthesis. This is the conversion of rifamycin SV to rifamycin B by condensation of a carboxy-methyl unit. The intermediate, rifamycin SV (Fig. 1.7), thus accumulates in the fermentation broth. This product, previously obtained only by chemical degradation of rifamycin B, is the essential starting material for the semisynthesis of the commercially important rifamycin derivatives, including rifampicin.

8.2.2. Conjugation

Both intra- and interspecies conjugation have been fairly easily obtained between antibiotic-producing actinomycetes. However, only in a few cases has the production of novel metabolites been observed. For instance, *S. aureofaciens* was crossed with *S. rimosus* in the hope of obtaining the hybrid molecule hydroxy-chloro-tetracycline, but none of the recombinant clones produced the desired compound.

A few novel rifamycins were isolated from recombinant strains obtained by the conjugation of *N. mediterranea* mutants blocked in different biosynthetic steps. However, the new active metabolites produced, 3-hydroxy- and 3,31-dihydroxy-rifamycin SV, appear to be the

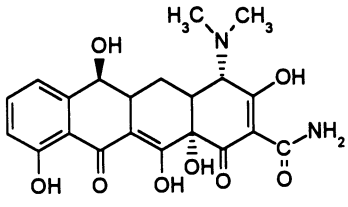

Figure 8.7. Demethyltetracycline, obtained from a mutant of *Streptomyces aureofaciens* unable to methylate pretetramide.

result of the activation of silent hydroxylases in the recombinant strain, rather than the result of a recombination of the biosynthetic genes.

A new anthracycline, iremycin, was produced by *Streptomyces violaceus* subsp. *iremyceticus.* This organism was derived by interspecific recombination of streptomycetes blocked in either anthracycline or macrolide biosynthesis. However, in this case also it is unclear whether the macrolide biosynthetic genes contribute to the new product formation or whether new product originates by a reshuffling in the host organism genome.

8.2.3. Genetic Engineering

The production of novel secondary metabolites by the recombinant DNA technique is a very promising approach, recently made possible by the isolation of the biosynthetic genes of an increasing number of antibiotics. "Shot-gun" experiments, consisting of the transfer of an antibiotic biosynthetic gene cluster into an organism producing a biosynthetically related metabolite, have indeed given rise to new molecules. Examples include the production of dihydrogranatirhodin by the introduction of the actinorhodin biosynthetic genes into the granaticin producer *Streptomyces violaceoruber,* and the production of noreryth-romycins by cloning DNA fragments from the oleandomycin producer *Streptomyces antibioticus* into a blocked mutant of *Saccharopolyspora erythrea.* However, using this "shot-gun" approach, the outcome of the gene recombination cannot be predicted. In some cases, such as the production of aloesaponarin by *Streptomyces galileus* transformed with four actinorhodin genes, the product can be interpreted only on the basis of further experiments.

Two different approaches are available for the rational synthesis of new molecules. One is based on site-directed mutagenesis, the other is the production of hybrid metabolites by the introduction of a specific gene into a compatible biosynthetic pathway.

The feasibility of the first approach has been demonstrated by the biosynthesis of modified erythromycins. The gene coding for the hydroxylase that converts 6-deoxyerythronolide B into erythronolide B was inactivated, and reintroduced at its original site in the gene cluster. Cultures of the recombinant strain thus constructed produced the

predicted erythromycin derivatives lacking the hydroxy group at position 6.

The first example of hybrid metabolites obtained by design is the production of mederrhodins A and B. These were formed when the *actVa* locus (a segment of the actinorhodin biosynthetic gene cluster of *S. coelicolor*) was introduced into the medermycin producer *Streptomyces* AM 7161. The *actVa* gene product includes a hydroxylase activity, and in fact mederrhodins differ from medermycin in the presence of a hydroxyl group in a position corresponding to that carrying a hydroxyl group in the actinorhodin structure (Fig. 8.8).

The above-mentioned examples have only an academic interest. However, attempts are in progress in several laboratories to apply these techniques to the formation of therapeutically or industrially relevant metabolites. An antibiotic possibly of practical interest obtained by rational design is, for instance, isovalerylspiramycin. This new active

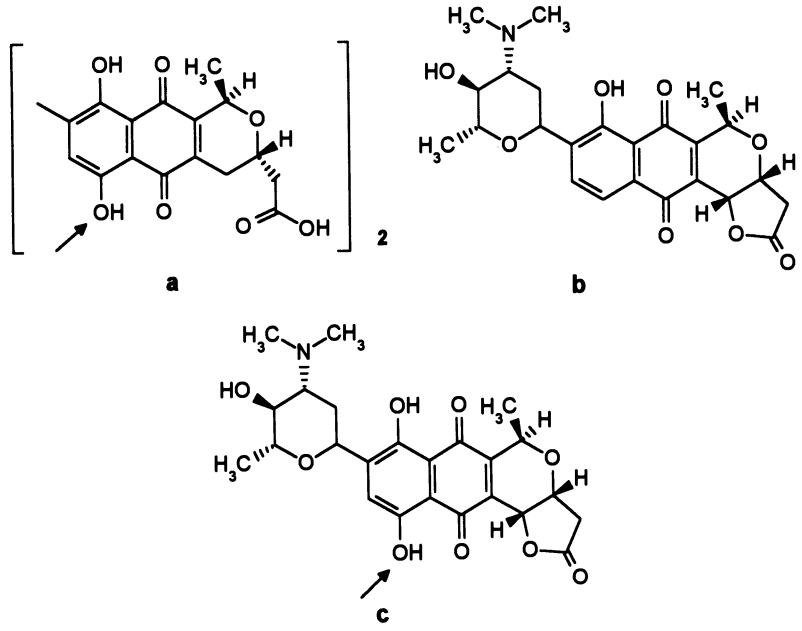

Figure 8.8. Mederrhodin (c), obtained by introducing the *actV* locus of *Streptomyces coelicolor*, producer of actinorhodin (a), into *Streptomyces* AM 7161, producer of medermycin (b). The arrows indicate the hydroxyl group introduced by the *actV* gene product.

spiramycin was obtained by the transfer of the *Streptomyces thermotolerans carE* gene, coding for the attachment of an isovaleryl moiety to carbomycin, into the spiramycin producer *S. ambofaciens*. Cultures of the recombinant strain produced the hybrid antibiotic (Fig. 8.9).

8.3. Screening of Microorganisms for Specific Transformation Reactions

The microbial transformation approach is normally attempted when a desired reaction, such as deacylation, demethylation, or hydroxylation, cannot be performed on an antibiotic by chemical means because of molecule instability or of the reactivity of other functions present in the structure. Most of the studies reported in the literature are relatively recent ones indicating that this method is now considered a very valuable approach. However, here too the first example also goes back to the early attempts to modify the penicillin molecule. No practical chemical method was available to hydrolyze the penicillin G side chain in order to produce 6-aminopenicillanic acid—the starting material for the preparation of all of the semisynthetic penicillins. In 1960 several groups reported that this reaction could be performed in high yields by incubation of the antibiotic in cell suspensions of different microorganisms, such as *Escherichia, Nocardia* species, *Alcaligenes faecalis*. The hydrolysis is the result of a widespread family of isoenzymes, the penicillin acylases, that demonstrate a certain degree of substrate specificity. Those of bacterial origin preferentially hydrolyze penicillin G (Fig. 8.10), whereas those produced by actinomycetes and fungi have a higher affinity for penicillin V. In fact, one of the industrial processes for the production of 6-aminopenicillanic acid is based on the hydrolysis of penicillin V by the immobilized enzyme from the fungus *Bovista plumbea*. The acylases from bacteria can perform the reverse reaction also, that is, the acylation of 6-aminopenicillanic acid. The direction in which the reaction proceeds is determined by the pH of the medium: pH values around 8 are optimal for hydrolysis, and around 5.5 for synthesis.

In contrast to penicillin, the deacylation of cephalosporin C to 7-aminocephalosporanic acid, a useful intermediate for semisynthetic

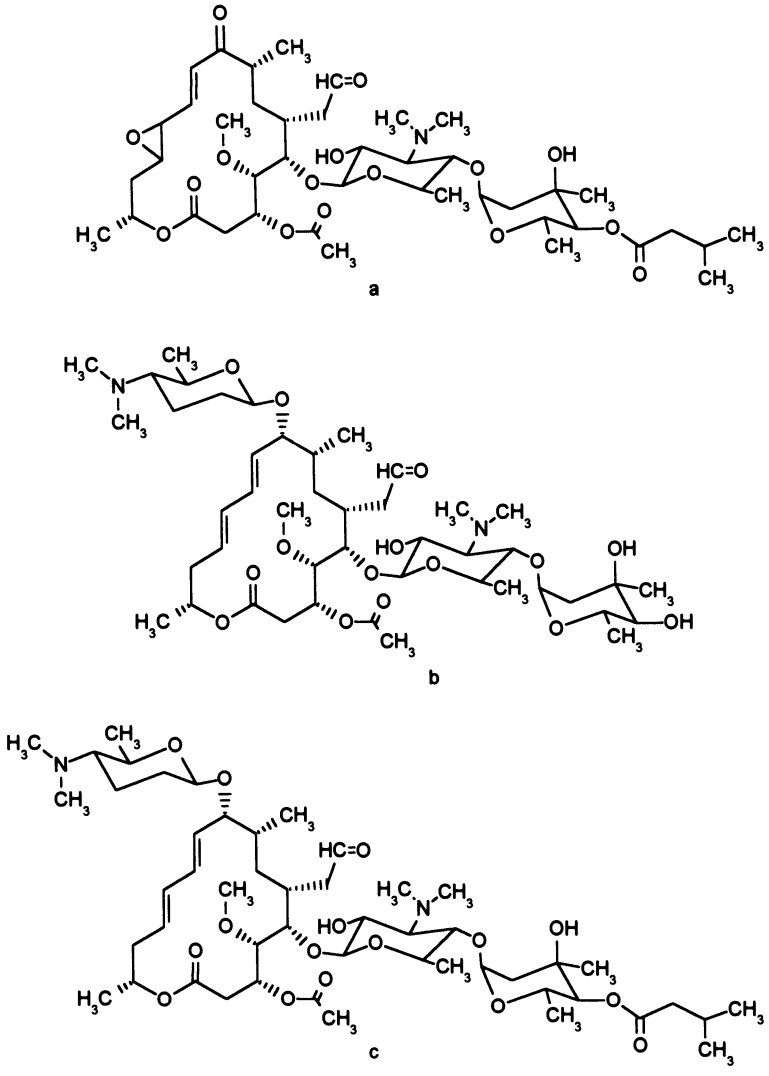

Figure 8.9. Isovalerylspiramycin (c), obtained by introducing the *carE* gene of *Streptomyces thermotolerans,* producer of carbomycin (a), into *Streptomyces ambofaciens* producer of spiramycin (b).

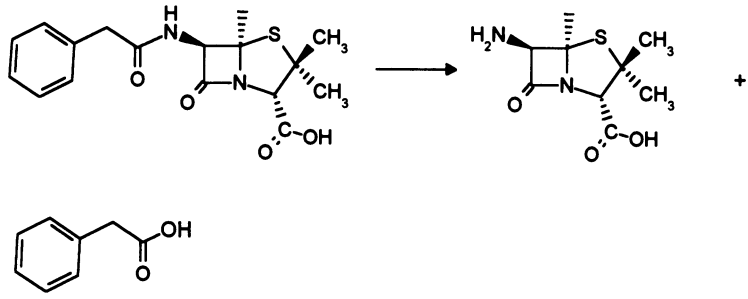

Figure 8.10. Enzymatic hydrolysis of penicillin G to 6-aminopenicillanic acid.

cephalosporins, cannot be easily accomplished by the biological route. The difficulty is not related to the cephalosporin nucleus, but to the structure of the D-aminoadipoyl side chain; this is not recognized as a substrate by the hydrolyzing enzymes. A method for circumventing this obstacle consists of converting the D-aminoadipoyl chain into a ketoadipoyl chain by the use of a D-amino acid oxidase; the latter is accepted as a substrate by several microbial acylases.

Among the many examples reported in the recent literature the following microbial transformations appear of potential practical interest.

Microorganisms of different genera were screened for their ability to hydrolyze the peptide bond linking *N*-methyl-D-leucine, the N-terminal amino acid of the glycopeptide vancomycin B. A strain identified as *Actinomadura citrea* was isolated that could perform the reaction with transformation yields of about 90%, thus providing material for the synthesis of vancomycins modified at the N-terminal residue (Fig. 8.11). It is also noteworthy that the screening of the organisms was performed by selecting strains able to grow on a model compound, the dipeptide D-leucyl-D-tyrosine, which approximates the N-terminal dipeptide of the antibiotic.

Daptomycin is a semisynthetic antibiotic of potential therapeutic interest. It is synthesized by deacylation of the lipopeptide antibiotic complex A21978C (constituted by three factors characterized by the presence of a branched side chain of 11 to 13 carbon atoms) and reacylation of the resulting peptide with a shorter linear fatty acid chain. A series of selected organisms, including several *Actinoplanes,* was screened

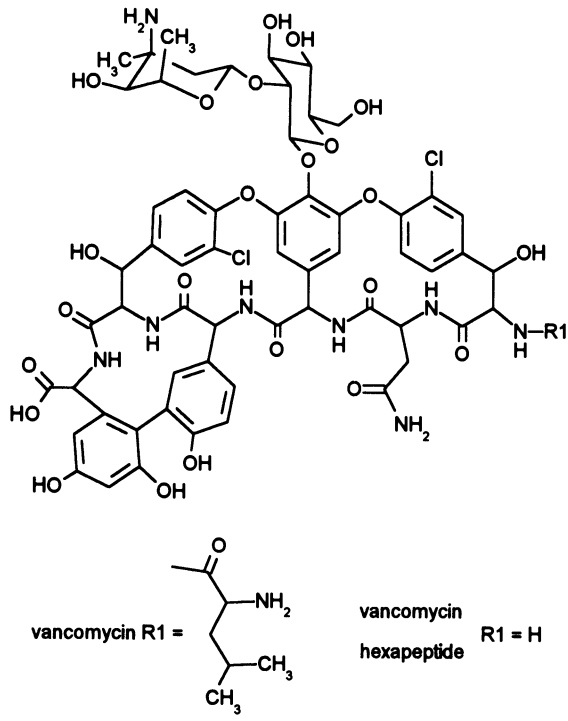

Figure 8.11. Vancomycin hexapeptide, obtained by the hydrolytic activity of *Actinomadura citrea.*

for their ability to deacylate A21978C. One very effective strain was found to be *Actinoplanes utahensis.* However, the chemical reacylation of the peptide presented some problems, because of the presence of an ornithine amine susceptible to the action of acylating reagents. The microbial deacylation was therefore performed on a *tert*-BOC A21978C derivative, in which the reactive function was protected (Fig. 8.12). The deacyl-peptide protected at the ornithine amino group was thus obtained. This could now be easily acylated chemically, deprotected, and transformed into the desired daptomycin molecule.

The same microorganism, *A. utahensis,* was utilized to enzymatically remove the linoleoyl group from the N-terminus of the hexapeptide antifungal antibiotic echinocandin. Reacylation with various acids gave a series of echinocandin analogues, among which cilofungin (an

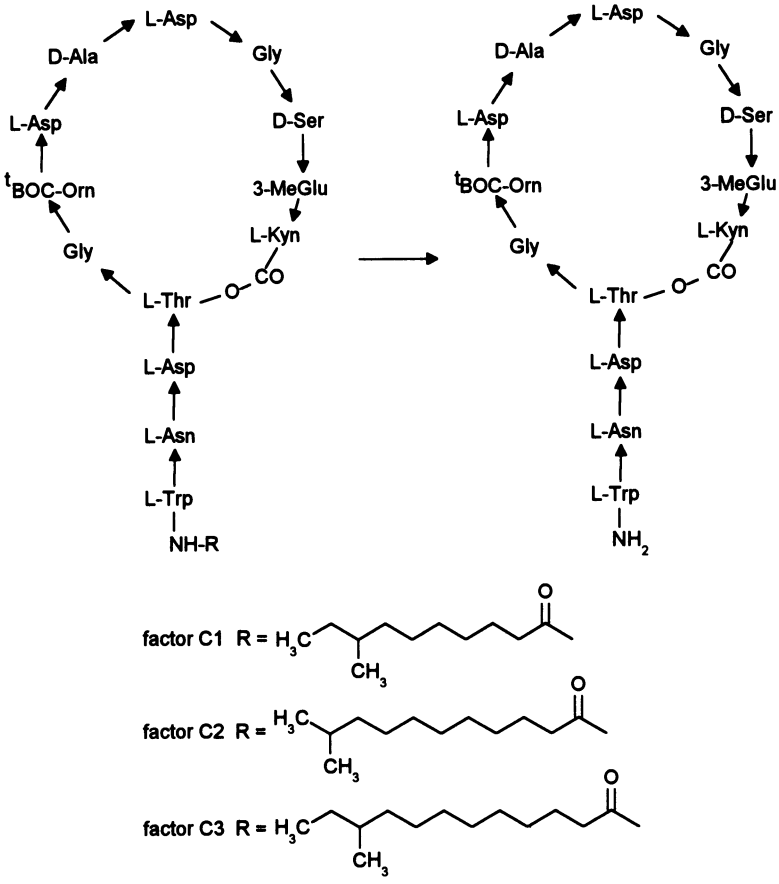

Figure 8.12. Deacylation of *tert*-BOC-A21978C by cultures of *Actinoplanes uthaensis*. 'BOC, *tert*-butoxycarbonyl.

analogue with a 4-*n*-octyloxybenzoyl chain) is currently in clinical trials with apparent success.

Among other bioactive metabolites, the antiparasitic milbemycins, structurally related to the avermectins, are the object of an intensive program of biological transformation. Hydroxylation of milbemycins in position 13, 29, and 30 were observed after incubation with cultures of actinomycetes or fungal strains, such as *Cunninghamella echinulata* or *Syncephalastrum racemosum*. The new compounds are considered

valuable starting materials for the preparation of further chemical derivatives.

The microbial modification of the immunosuppressive agents FK 506 and FR 900520 is also being actively studied. In both cases the objective is the demethylation of the various methoxy groups present in these metabolites. Either the concomitant demethylation of several groups, or the selective demethylation of the 13-methoxy group, was obtained by incubation of the compounds with actinomycetes cultures. The main value of these studies is to provide information about the structure–activity relationship in these molecules.

8.4. *Enzymatic Synthesis of New β-Lactams*

The broad substrate specificity of the peptide antibiotic synthetases has been exploited for the synthesis of several analogues of the natural products. Gramicidin S congeners, for instance, have been obtained by substituting in a cell-free incubation system, either valine or phenylalanine. These were omitted from the mixture of the amino acids constituting the molecule and replaced with leucine or phenylalanine derivatives, respectively. The formation of the corresponding leucine or substituted phenylalanine analogues of the natural antibiotic was observed.

Whereas some of these studies are of academic interest, the recent findings on the enzymatic synthesis of β-lactam antibiotics may lead to discoveries of practical importance. We therefore give next a brief account of the recent results obtained.

8.4.1. *Modified Tripeptides*

As we have seen in the previous chapters, the first enzyme in the β-lactam biosynthetic pathway is the ACV synthase. This catalyzes the condensation of L-α-aminoadipic acid, L-cysteine, and L-valine to form the tripeptide L-α-aminoadipoyl-L-cysteinyl-D-valine (L-L-D-ACV). A systematic analysis of the enzyme substrate specificity has revealed that a few dicarboxylic acids are able to substitute for aminoadipic acid, and that either leucine or α-aminobutyric acid can replace valine in an *in vitro* synthesis system. Whereas the replacement of aminoadipic acid

has no practical implications, tripeptides altered in the valine residue can give rise, as will be seen, to novel β-lactam molecules.

8.4.2. Synthesis Catalyzed by Isopenicillin N Synthase (IPNS)

Isopenicillin N synthase, commonly called cyclase, converts the L-L-D-ACV tripeptide into isopenicillin N. When tripeptides containing a valine analogue as the third residue were incubated with the enzyme, the corresponding penicillin derivatives were obtained. For example, the tripeptide ending with α-aminobutyric acid generated the demethylpenicillin N. However, together with that of the expected products, the formation of novel β-lactam structures was observed. In the case of α-aminobutyric acid a cepham molecule was produced; this is a β-lactam with a fused ring of six atoms, similar to a hydrogenated cephalosporin (Fig. 8.13). Similar mixtures of β-lactams (with either five- or six-atom rings) were produced when isoleucine substituted for valine in the tripeptide.

When unsaturated amino acids, such as allylglycine, replaced valine in the tripeptide, a complex mixture of products was obtained. These included not only the expected penicillin and cepham derivatives, but new bicyclic β-lactams with a seven-atom ring system. In addition, some derivatives were hydroxylated, revealing the ability of the enzyme to function as an oxygenase, as well as a dehydrogenase (Fig. 8.14).

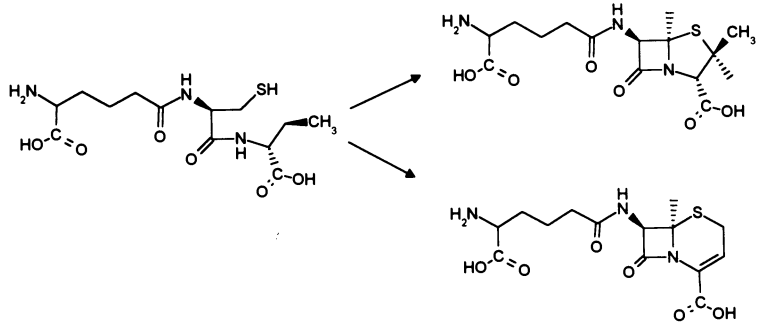

Figure 8.13. β-Lactams produced by the action of isopenicillin N synthase on aminoadipoyl-cysteinyl-α-aminobutyric acid.

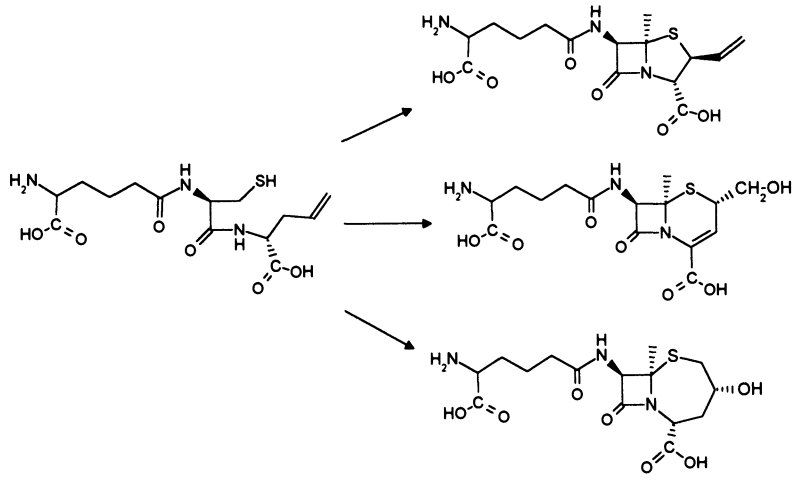

Figure 8.14. Some of the β-lactams produced by the action of isopenicillin N synthase on aminoadipoyl-cysteinyl-allylglycine.

Rather limited structural variations are possible in the cysteine structure. However, methylpenicillins (substituted in either position 5 or 6) were produced when 3- or 2-methylcysteine respectively replaced cysteine as the second residue in the tripeptide.

On the whole, these results demonstrate that β-lactams with a variety of novel structures can be synthesized by *in vitro* systems with the use of suitable tripeptides and IPNS. Some of the compounds obtained possess a fairly good antimicrobial activity and it is quite possible that new, therapeutically useful products will be synthesized in the near future.

8.4.3. Variations in the Penicillin Side Chain

The availability of purified enzymes has allowed, in the last few years, the study of the structural features that carboxylic acids must have in order to be accepted as substrates for the exchange with the aminoadipoyl chain of isopenicillin N. In *Penicillium chrysogenum* this exchange is catalyzed by the enzyme acyl-CoA:isopenicillin N synthetase. This is a bifunctional enzyme, composed of two subunits, one catalyzing the hydrolysis of the aminoadipoyl chain with formation of

the 6-aminopenicillanic acid (6-APA), and the other the transacylation step. This consists of the transfer of the acyl moiety from acyl-coenzyme A (acyl-CoA) to 6-APA. Incubation of chemically synthesized acyl-CoA derivatives with 6-APA and the latter subunit permitted the determination of the substrate specificity of the enzyme. Linear aliphatic acids are accepted as substrates only when composed of six to eight carbon atoms. Similarly, methyl-substituted phenylacetic acids are accepted only if the methyl group is at a distance of six or eight carbons from the carboxyl group. Keto acids with an even number of carbons are a substrate of the reaction, but only the ketohexanoic and the ketooctanoic acids were efficiently transformed.

This system is being proposed as an alternative to the chemical acylation of 6-APA, especially if coupled with an enzymatic synthesis of the various acyl-CoAs now available. Nevertheless, it is difficult to predict whether it will ever be really competitive with the very efficient chemical methods.

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9

Production of Secondary Metabolites

The application of present-day technology to the production of antibiotics and other secondary metabolites began with the common effort that academic and industrial institutions made, in the early 1940s, in response to the urgent need for large-scale production of the newly discovered penicillin.

The details of the industrial processes are considered a trade secret, and as such are not disclosed. However, the general outline of the processes are well known, and this chapter aims to give an account of the commonly applied procedures.

Whether the object is to produce a few grams in the laboratory of a new substance for biological testing, or to produce tons of a commercial product, two basic factors must be considered: the genetic potential of the producing strain and the conditions allowing for maximum expression of this capacity. The improvement of strains by genetic methods and the process development have been discussed in Chapter 7. We mainly deal here with the technology of the production process.

9.1. Strain Maintenance and Preservation

It is obviously of prime importance that a producing strain is preserved, for long periods of time, free from phenotypic or genetic alterations. Two methods are commonly adopted: lyophilization and storage in liquid nitrogen.

Lyophilization, or freeze-drying, consists of the removal of water from frozen cell suspensions by sublimation under reduced pressure.

Normally a small portion of a vegetative culture, or of sporulated mycelium, is placed in a vial that, after lyophilization, is sealed under vacuum. Cryoprotective agents are added to reduce the cell damage induced by freezing; skim milk powder or sucrose is frequently used for this purpose. Lyophilization ensures the viability of strains for more than 10 years, without any special maintenance procedure. In addition, vials of lyophilized strains can be easily transported with no need for refrigeration.

For preservation in liquid nitrogen, cells in the logarithmic growth phase are collected, suspended in a medium containing either glycerol or dimethylsulfoxide and distributed in ampoules that are slowly cooled to the final temperature. Commonly the ampoules are kept in the vapor phase of liquid nitrogen, rather than immersed in the liquid phase. This method of storage requires more maintenance work than lyophilization, but allows easier retrieval and handling of the strains.

For everyday work a common practice is to prepare a surface culture on a slant from which several other slant cultures can be derived. These can be kept in a refrigerator for several months, especially if covered with mineral oil to prevent desiccation. However, in the case of actinomycetes, slant-to-slant propagation often results in the loss of the original characters because of the genetic instability of these organisms.

A frequently used method to ensure a constancy of repeated fermentations consists of subdividing into many ampoules a submerged culture of a clone previously tested for its productivity, and of storing these frozen cultures at -5 to $-20°C$.

9.2. Fermentation Technology

9.2.1. Shake-Flask Fermentation

Small-scale fermentations of antibiotic-producing microorganisms are normally performed as submerged flask cultures. Shake-flask fermentations are used: (1) to culture the soil isolates in the screening programs, (2) to compare the production capacity of mutant strains of an organism, (3) to assess the effect of different medium components

on productivity, and (4) to provide an inoculum culture for larger-scale fermentations.

As we have seen, the vast majority of antibiotics are produced under aerobic conditions by filamentous microorganisms. The main requirements for a satisfactory production (in addition to fermentation medium composition, which will be discussed later) are a sufficient oxygen supply and a suitable temperature. The aeration needed to meet the oxygen requirement is provided by growing the cultures in a small volume of medium in relatively large flasks (for instance, 50 or 100 ml of medium in 500-ml flasks) that are placed on a rotary shaker, giving an orbital movement to the liquid. The flasks have usually one or more baffles to increase the turbulence and thus the oxygen transfer. The flasks must be sealed in such a way as to permit gaseous exchange, but prevent the entry of contaminating microorganisms. Cotton plugs were mainly used in the past; at present, loosely fitting metal caps, reusable plastic plugs, or one to two layers of a sterile, permeable synthetic material are more commonly adopted.

The desired temperature is maintained either by placing the shakers (which can carry from 50 to several hundred flasks) in a temperature-controlled room, or in an incubator box fitted with a temperature-control unit.

A variation of this basic technology is the use of reciprocating shakers instead of rotary shakers. In this case the cultures can be grown in large test tubes as an alternative to Erlenmeyer flasks.

9.2.2. Stirred Fermenters

Since the surface-to-volume ratio decreases with increasing volumes, air exchange becomes insufficient and shaken flask fermentations can be performed only on a laboratory scale. Therefore, fermentations from a few liters to many cubic meters in volume are carried out in stirred fermenters with forced aeration. The technology of stirred fermenters is substantially more complex than that of shaken cultures, mainly because of the problem of potential contamination. Basically, a fermenter is a stainless steel (although glass may be used for fermenters up to 10 liters in volume) closed vessel, fitted with a stirring device, forced aeration and a temperature control system. In addition, it is normally provided with inlets for feed lines, outlets for sampling, har-

vesting, or draining, and probe holders for controlling instrumentation (Fig. 9.1).

The height-to-diameter ratio of the fermenters varies, but for antibiotic production it is often from two to four. The internal surface must be carefully polished, especially where pipework or probe holders enter the vessel, to avoid contaminating microorganisms sticking. The agitation is performed by a series of impellers, whose design is relevant for both the efficiency of stirring and energy consumption. Either vertical flat-bladed, turbine-type or skew-bladed impellers are used. Great attention must be paid to the sealing system around the shaft which drives the impellers, to avoid the entry of contaminants in the case of a top drive system, or leakage of broth when the drive is at the bottom. Baffles are commonly installed to increase the turbulence.

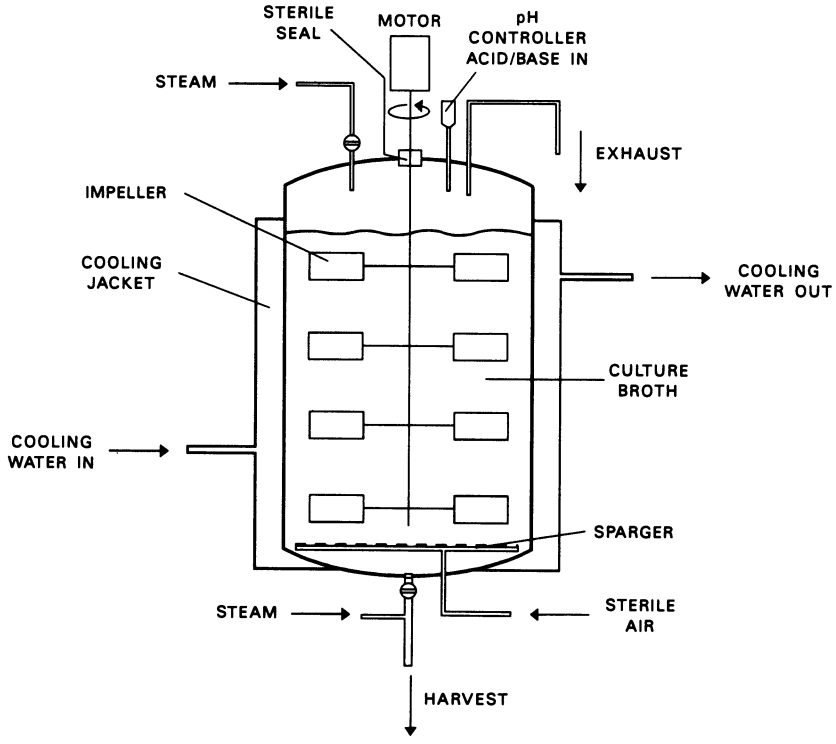

Figure 9.1. Simplified scheme of an industrial fermenter.

Compressed air is "sparged" immediately below the lowest impeller, to allow disruption of large bubbles into smaller ones and thereby enhancing oxygen transfer. Fermenters generally operate with aeration rates of 0.3 to 1 volume of air per liquid volume per min. This requires the sterilization of enormous volumes of air; on an industrial scale this is obtained by filtration systems. The traditional system uses depth filters—cylinders filled with glass wool in which microbial cells are trapped by a combination of physical means. Today, the depth filters are often replaced by membrane filter—cartridges with pores of about 0.2 μm. This pore size successfully prevents the entry of bacteria but is not sufficient to avoid contamination from bacteriophages. The air outlet is generally fitted with a control valve, permitting operation of the fermentation under pressure. Any condensate must be drained away from the vessel, to avoid contamination, and the exhausted air is often filtered for protection of the environment.

Temperature control is provided either by a jacket surrounding the vessel or by internal coils in which circulates pressurized steam for heating, or cold water for cooling.

All of the lines for feeding, sampling, harvesting, or draining must be fitted with appropriate valves, which are kept sterile by a continuous flow of steam.

Industrial fermenters are normally equipped with probes to continuously measure temperature, pH, oxygen concentration, and CO_2 concentration in the exhausted air.

In some rare cases, antibiotic production is performed in a different type of fermenter called the air-lift fermenter. This differs from the stirred type in that the mixing of the broth is not performed by impellers but by a flux of the air, which induces a cyclical flow in the liquid.

9.3. The Process

9.3.1. The Inoculum

Two phases of development are normally recognized in the cultures of microorganisms for the production of secondary metabolites: a vegetative phase, or trophophase, in which there is a vigorous growth and a negligible antibiotic production; and a fermentative phase, or idio-

phase, in which the culture is stationary and the antibiotic production is initiated. There are several reasons to carry out as much of the vegetative growth phase as possible in a vessel separate from that of the productive phase.

One stems from economic considerations. To run a fermentation in the tens of cubic meter scale is expensive and the cost of some factors, such as energy for aeration and agitation, is time dependent. In addition, the capital investment in a fermentation plant is very high and a large fermenter must be intensively utilized for the production phase. Therefore, it is convenient to develop the vegetative culture in smaller vessels.

A second reason is to minimize the risk of contamination. If the final stage is inoculated using a large biomass, the growth of a few contaminant cells that have survived the medium or air sterilization will have negligible effects.

A third very important reason is that experience has demonstrated that in antibiotic production the final yields are considerably influenced by the size and the metabolic state of the biomass used as inoculum to initiate the fermentation stage.

The optimum inoculum volume must be determined empirically for the different fermentations. In most cases it has been found to be in the range of 1% to over 10% of the fermentation volume. Thus, a fermentation carried on in fermenters of 150 cubic meters of capacity may require an inoculum of several thousand liters. This can be conveniently provided only by a multistage system. Shake-flask cultures are used to inoculate small fermenters, whose culture are used as the inoculum of larger ones, and so on. Up to four stages may be necessary to prepare the final inoculum.

Rich complex media are normally used for the growth of the vegetative cultures, in order to rapidly obtain a large biomass. Since antibiotic production at this stage is not desired (and in fact should be avoided to prevent the risk of autotoxicity or of feedback inhibition), relatively high concentrations of rapidly assimilated carbon and nitrogen sources (such as glucose, peptone, yeast extract, ammonium ions) and phosphates can be utilized.

The metabolic stage of the inoculum is also relevant; cultures in the late logarithmic phase of growth generally give the best performance. One aspect of the final fermentation that may be influenced by the quality of the vegetative culture is the physical form in which the my-

celium grows. Actinomycetes and filamentous fungi can grow either as diffused hyphae or as pellets up to a few millimeters in diameter. In several fermentations it has been observed that one of the two forms gives better results than the other, and that the size of the inoculum and the texture of the mycelium can in part determine the manner in which the final culture grows.

9.3.2. The Production Stage: Medium Composition

In the production stage, three phases of growth can normally be distinguished: a lag phase, in which the cells adapt to the new environment and no growth is observed; a logarithmic phase, in which there is rapid growth; and a stationary phase, in which there is no further net growth and a steady production of antibiotic.

It is desirable that the fermentation medium allows the formation of a large biomass in the shortest possible time, and sustains the productive stationary phase for as long as possible. The medium composition is therefore most relevant and certainly the most complicated of the factors influencing production yields, particularly with high producing industrial strains.

The complexity of medium composition studies derives from the fact that final yields are determined not only by the amount of basic nutrients, such as carbon and nitrogen sources, contained in the commercially available ingredients, but also by the proportions in which they are mixed and by their physical state.

Readily assimilated carbon and nitrogen sources, such as glucose and ammonia or amino acids, can be used to obtain a high growth rate and a large biomass. These can be provided economically as molasses or hydrolyzed low-cost proteins. However, as previously discussed, these nutrients often repress antibiotic production, and must be substituted by substances metabolized at a lower rate. Commonly used carbon sources are lactose, sucrose, maltose, starch or dextrins, and glycerol. Sometimes vegetable oils are used in continuous feeding to extend the antibiotic production phase.

The nitrogen sources commonly used fall in a few classes: (1) grain and bean meal or flour, such as soy meal, cottonseed flour, peanut meal, dried distiller's solubles, corn-steep liquor; (2) meat waste products, such as meat or blood hydrolysate and fish proteins, of which a

large variety are available; (3) dairy waste products which, however, with the exception of whey are rather expensive; (4) yeast extract or yeast autolysate, which is a source of vitamins and growth factors, is added in small amounts, being expensive.

Rapidly metabolized nutrients can be used in some cases, provided that their concentration is such that they are completely utilized at the end of the logarithmic phase. The great advantage of using meals in the fermentation is that they are solid, and the slow rate of their solubilization and hydrolysis provides a steady and low concentration of nutrients suitable for sustaining for a long time a high level of antibiotic production.

The components of the medium must be balanced—not only with respect to the major nutrients, but also taking into consideration the requirements of mineral salts. Minerals most often added to the media are the salts of calcium, potassium, sodium, magnesium, sulfates, and phosphates. Small amounts of manganese, iron, zinc, cobalt, and copper are also normally required. The amount of phosphates is often critical: the difference between the concentrations needed for a satisfactory growth rate and those inhibiting the antibiotic production is sometimes very small. Solid calcium carbonate is an ingredient that was often used; besides providing the calcium needed by the cells it had an important function in preventing excessive acidification of the medium by neutralizing acids produced through the catabolism of sugars. The pH of the broth is in fact an important parameter, and is now normally maintained at the desired values by intermittent addition of mineral acids or bases.

Excessive foam formation is a common problem in many fermentation processes and is largely dependent on the medium composition. Some fermenters are equipped with mechanical foam-breaking devices, but most often chemical antifoam agents are added to the medium. These act by lowering the surface tension at the liquid–air interface. Silicone-based agents are very effective but, since they are not metabolized, can create some problems during the filtration of the harvested broth. Animal or vegetable fats or oils, such as lard oil, sunflower, or soy oil, are effective both as antifoam agents and as carbon sources.

The process by which the medium is sterilized can have a considerable influence on the fermentation. In the past, sterilization was per-

formed in batch, by heating the whole broth to 120°C for half an hour. However, with the large volumes of broth used in industrial fermentations, the process of heating and cooling was necessarily slow and the broth remained at high temperatures for long periods of time, causing deleterious changes in the ingredients. Nowadays, sterilization is normally accomplished by allowing the medium to flow through a heating system so that it is brought to 140°C for a few minutes and is rapidly cooled to a suitable temperature.

9.3.3. The Production Stage: Aeration and Physical Parameters

Antibiotic-producing organism are all aerobic, and generally have a high oxygen requirement for optimal growth and production. In the logarithmic growth phase especially, the availability of oxygen may be critical and in some fermentations even a short interruption, or insufficient rate of aeration at this time, results in a dramatic decrease of product yield. Again, the optimal rate of aeration differs for different fermentations and must be established empirically. As stated previously the requirement may be as high as one volume of air per volume of broth per minute, and the air supply can represent a sizable fraction of the total cost of a fermentation. However, the requirement is not the same in all phases of fermentation and the supply can be modulated during the process to reduce costs without negative effects on the performance.

To increase dissolved oxygen concentration, fermentations are often carried out under pressures greater than atmospheric. However, this also increases the carbon dioxide concentration which has, in general, negative effects. One of the important functions of aeration is to remove carbon dioxide from the broth, and therefore a certain flux of air must be maintained even when it is not necessary for oxygen supply.

The stirring device of a fermenter must provide efficient mixing of the liquid, solid, and gaseous phases of which a fermentation system is composed. Air must be dispersed in the liquid phase to enhance the oxygen transfer. The solid nutrients and the mycelium must be kept in a homogeneous suspension, and the temperature and dissolved nutrient concentration must be equally distributed throughout the fermenter. The efficiency of mixing is to some extent proportional to the speed at which the stirrer revolves, but also depends on several other

factors, such as the diameter of the impeller, the presence and shape of the baffles, and most of all the viscosity of the fermentation broth. There are obvious limitations as to how fast a system can be stirred; some are economic, relating to energy consumption, others concern the shearing forces that can hinder the formation or physically damage the mycelium. As in the case of aeration, the appropriate stirring speed must be determined empirically, but may be varied during the course of the fermentation according to need.

Temperature is another parameter exerting a marked influence on secondary metabolite production. For each antibiotic-producing microorganism there is an optimal temperature for antibiotic production, which is normally somewhat lower than the optimal temperature for growth. This temperature may vary in actinomycetes from 28 to 32°C, and in fungi from 25 to 28°C. An efficient cooling system to disperse the heat produced by the metabolism and the agitation, and precise regulation of the temperature are essential for good performance. In fact, a difference of even 1°C can have a noticeable effect on the final yield. In several cases it has been observed that good results can be obtained by shifting slightly the temperature during the course of fermentation, adapting it to the culture development phases.

9.3.4. Continuous and Fed-Batch Fermentation

All antibiotics are produced by batch fermentation in which the cultures develop through the above-mentioned lag, logarithmic, and stationary phases. Since production occurs only in the last phase, many studies and attempts have been made to implement a system of continuous fermentation. This system involves keeping a culture in a balanced condition in which most of the cells are in a productive phase. The continuous removal of part of the broth and the mycelium is compensated by a slow growth sustained by a steady feeding of nutrients. This technique has never been developed on an industrial scale because of several drawbacks, the most important of which may be the accumulation of nonproducing mutants that can constitute, after many generations, a large part of the cell population.

In contrast, the fed-batch technique is widely applied. This process involves extending the duration of the stationary phase by an appropriate feeding of nutrients—either continuously or at suitable intervals

of time. The underlying concept is that one important cause of cessation of antibiotic biosynthesis is the exhaustion of nutrients, especially of carbon sources. On the other hand, concentration of sugars in the medium at the start of fermentation is limited by carbon catabolite repression effects and by other factors, such as the adverse effects of excessive osmotic pressure and a high medium viscosity. Addition of nonrepressive and noninhibitory levels of carbon sources in the late phase has been proven to be an effective method of extending the production time by several days or even weeks.

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