Ciba Foundation Symposium 171

SECONDARY METABOLITES: THEIR FUNCTION AND EVOLUTION

A Wiley-Interscience Publication

1992

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Introduction

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The number and the diversity of secondary metabolites are subjects that have intrigued scientists for many years. I mean diversity in many senses: there is the diversity of chemical molecules, and the diversity of sources of these metabolites. One can argue that every living organism on earth either makes secondary metabolites, or, at the very least, participates in some form of secondary metabolism. It is important also to recognize the diversity of the potential functions of these molecules.

I would suggest that at least ten biological functions of secondary metabolites can be proposed (Table 1). I am not implying that these are the only functions, or that known secondary metabolites necessarily have these particular functions. The point of this list is to illustrate the wide diversity of functions that have been proposed for secondary metabolites, and the fact that we will, I hope, probably have additional functions suggested at this meeting. Some secondary metabolites are likely to have more than one biological role.

An interesting point about secondary metabolism is that whereas primary metabolism (intermediate metabolism) is *linear*, in the sense that its products stay with the organism and it is responsible for guaranteeing that an organism has sufficient nutrients and all the means it needs to produce the next generation,

TABLE 1 Some suggested biological functions for secondary metabolites

1. Competitive weapons against other bacteria, fungi, plants, amoebae, insects, etc. (Self-protection/exclusion)
2. Metal-transporting agents
3. Involved in plant–microbe symbiosis
4. Nematode–microbe symbiosis
5. Insect–microbe symbiosis
6. Sexual hormones (pheromones)
7. Differentiation effectors, between and within cells
8. Excretion of unwanted products
9. Products of 'selfish' DNA
10. Reserve pool of new pathways

secondary metabolism is a kind of 'lateral thinking' of microorganisms, or of any multicellular organism that produces a secondary metabolite. It is responsible for interactions between the organism and its environment. A good example is a class of compounds that one doesn't often think of as secondary metabolites, but which are in fact representative of secondary metabolism. This concerns pathogenic organisms. It is interesting to note that in microbial pathogens such as *Listeria*, the enzymes and toxins required for pathogenicity (that is, the interactions of the organisms with their mammalian host cells) are produced in a phase when the organism is not growing. It is a late phase of of development of the particular organism. So one can think of many substances associated with pathogenicity as being representative of secondary metabolism. I don't want to extend this analogy too far, but want to emphasize the point that the concept of secondary metabolism and the production of secondary metabolites seems to be concerned with what is going on outside the producing organism, rather than events going on inside. I think this is an important distinction.

There is much controversy about secondary metabolites in Nature, and in particular the question of what a secondary metabolite may actually do for the organism producing it. Many views have been expressed on this subject. Some people believe that we don't really know what secondary metabolites do, or at least that we can't establish what secondary metabolites may do for the organism concerned. Others take a different view. Dudley Williams, the co-proposer of this symposium, believes that secondary metabolites do play an important role in the life of the producing organism. This is something which clearly is open to discussion. I would like to have your suggestions on the functions of secondary metabolites in the organisms that produce them. I would like also to encourage you to discuss the whole gamut of possibilities with respect to the origins of secondary metabolites, and the production of secondary metabolites, in addition to their functions. We will never be able to comprehend the enormous diversity of these products, and the general considerations of what this kind of diversity means. This is what is interesting about this topic; it's why we are here, and why I am looking forward to a very interesting three days talking about this subject. Secondary metabolism has been ignored; there is nothing 'secondary' about its importance in biology!

Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry*

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Abstract. Microbial secondary metabolites are the low molecular mass products of secondary metabolism. They include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumour agents and growth promoters of animals and plants. They have a major effect on the health, nutrition and economics of our society. They have unusual structures and their formation is regulated by nutrients, growth rate, feedback control, enzyme inactivation and induction. Regulation is influenced by unique low molecular mass compounds, transfer RNA, σ factors and gene products formed during post-exponential development. The synthases of secondary metabolism are often coded by clustered genes on chromosomal DNA and infrequently on plasmid DNA. The pathways of secondary metabolism are still not understood to a great degree and thus provide a new frontier for basic investigations of enzymology, control and differentiation. Cloning and expression of genes in industrial microorganisms offer new opportunities for strain improvement and discovery. Microbial metabolites have already established themselves as coccidiostats, immunosuppressants, antihelminthic agents, herbicides and cholesterol-reducing drugs. Great potential exists for the discovery of antiviral, antiparasitic, antitumour and pharmacological compounds and new agricultural products. The future for natural products is bright indeed.

1992 Secondary metabolites: their function and evolution. Wiley, Chichester (Ciba Foundation Symposium 171) p 3–23

Secondary metabolites, also known as idiolites, are special compounds, often possessing chemical structures quite different from the primary metabolites (such as sugars, amino acids and organic acids) from which they are produced.

*Because of space limitations I have had to eliminate all citations prior to 1987, as well as citations to the work of my group. I apologize to all whose citations have been omitted and to my own students and associates: specific citations will be supplied upon request.

Idiolites from microorganisms are not essential for the growth of the producing culture but serve diverse survival functions in Nature. These special metabolites, in contrast to the general nature of primary metabolites, are produced only by some species of a genus, and by some strains of a species. Their unusual chemical structures include β-lactam rings, cyclic peptides containing 'unnatural' and non-protein amino acids, unusual sugars and nucleosides, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings. Idiolites are typically produced as slightly differing components of a particular chemical family, as a result of the low specificity of some enzymes of secondary metabolism. The main types of biosynthetic pathways involved are those forming peptides, polyketides, isoprenes, oligosaccharides, aromatic compounds and β-lactam rings. Knowledge of the pathways varies from cases in which the amino acid sequences of the enzymes and nucleotide sequences of the genes are known (for example, for penicillins and cephalosporins), to those in which even the enzymic steps are still unknown. Although most secondary metabolites are small (less than 1500 Da) and are produced by non-ribosomal systems, there does exist a family of ribosomally derived antibiotics of higher molecular weight (3000–4000 Da, 32–34 residues) known as lantobiotics (Bannerjee & Hansen 1988, Schnell et al 1988). These include nisin (produced by *Streptococcus lactis*), subtilin (*Bacillus subtilis*) and epidermin (*Staphylococcus epidermidis*).

Regulation of secondary metabolism

The intensity of secondary metabolism can often be increased by the addition of limiting precursors. Examples are shown in Table 1. Secondary metabolism occurs best at submaximal growth rates after growth has slowed down. The distinction between the growth phase (trophophase) and production phase (idiophase) is sometimes very clear, but in many cases idiophase overlaps trophophase. The timing between the two phases can be manipulated—the two phases are often distinctly separated in a complex medium favouring rapid growth, but overlap partially or even completely in a chemically defined

TABLE 1 Increase in intensity of secondary metabolism resulting from the addition of limiting precursors

Group	*Species*	*Secondary metabolite*	*Precursor*
Unicellular bacteria	*Bacillus polymyxa*	Colistin	Diamino-butyric acid
	Bacillus brevis	Gramicidin S	L- or D-Phenylalanine
Filamentous bacteria	*Streptomyces clavuligerus*	Cephamycin C	Lysine
Fungi	*Penicillium chrysogenum*	Penicillin G	Phenylacetic acid

medium supporting slower growth. A secondary metabolite is not 'secondary' because it is produced after growth, but because it is not involved in the growth of the producing culture. Thus, elimination of the production of a secondary metabolite by mutation will not stop or slow down growth; indeed, it may increase the growth rate.

The factors controlling the onset of secondary metabolism are complex and not well understood. Growth rate is important, but we do not know the mechanism(s) involved. Deficiencies in certain nutritional factors are also important, but again we are ignorant of the basic mechanisms.

The delay often seen before the onset of secondary metabolism was probably established by evolutionary pressures. Many secondary metabolites have antibiotic activity and could kill the producing culture if produced too early. Of course, the resistance of antibiotic producers to their own metabolites is well known (Cundliffe 1989 and this volume: 1992). Antibiotic-producing species possess suicide-avoiding mechanisms which are often inducible, but in some cases are constitutive. In the case of inducible resistance, death could result if the antibiotic is produced too early and induction is slow. Delay in secondary metabolite production until the starvation phase makes sense if the product is being used as a competitive weapon or endogenously as an effector of differentiation. In nutritionally rich habitats such as the intestines of mammals, where enteric bacteria thrive, secondary metabolite production is not as important as in soil and water, where nutrients limit microbial growth. Thus, secondary metabolites tend not to be produced by enteric bacteria such as *Escherichia coli* but by soil and water inhabitants such as bacilli, actinomycetes and fungi. Nutrient deficiency in Nature often induces morphological and chemical differentiation—that is, sporulation and secondary metabolism, respectively; both are beneficial for survival in the wild. Thus the regulation of the two types of differentiation is often related.

Most secondary metabolites are formed via enzymic pathways. The enzymes occur as individual proteins, free or complexed, or as parts of large multifunctional polypeptides carrying out a multitude of enzymic steps, as in polyketide synthases and peptide synthetases. The genes encoding the enzymes of secondary metabolism are usually chromosomal, but a few have been shown to be plasmid-borne, such as methylenomycin A of *Streptomyces coelicolor*. Whether chromosomal or plasmid-borne, the genes are usually clustered, especially in prokaryotes, but not necessarily as single operons. Expression of these genes is under strong control by nutrients, inducers, products, metals and growth rate. In most cases, regulation is at the level of transcription, as revealed by the absence of mRNA encoding idiolite synthases until growth rate has decreased.

Regulation by the carbon source

Glucose, usually an excellent carbon source for growth, interferes with the formation of many secondary metabolites. Polysaccharides (e.g. starch),

TABLE 2 Carbon sources interfering with secondary metabolism

Idiolite	*Interfering carbon source*	*Non-interfering carbon source*
Actinomycin	Glucose, glycerol	Galactose, fructose
Bacilysin	Glucosamine, starch, maltose, glycerol, ribose, xylose	Glucose
Benzodiazepine alkaloids	Glucose	Sorbitol, mannitol
Cephalosporin	Glucose, glycerol, maltose	Sucrose, galactose
Chlortetracycline	Glucose	Sucrose
Cycloserine	Glycerol	
Enniatin	Glucose	Lactose
Ergot alkaloids	Glucose	Polyols, organic acids
Erythromycin	Glucose, sucrose, glycerol, mannose, 2-deoxyglucose	Lactose, sorbose
Kanamycin	Glucose	
Oleandomycin	Glucose	Sucrose
Penicillin	Glucose, fructose, galactose, sucrose	Lactose
Peptide K-582	Glycerol	Glucose, sucrose, fructose, sorbitol
Puromycin	Glucose	
Rebecamycin	Sugars	Trisaccharides, polysaccharides
Tetracycline	Glucose	
Tylosin	Glucose, 2-deoxyglucose	Fatty acids

oligosaccharides (e.g. lactose) and oils (e.g. soybean oil, methyloleate) are often preferable for fermentations where secondary metabolism is desired. Examples of interfering carbon sources are given in Table 2. It should be noted that in certain cases (e.g. bacilysin) glucose is not an interfering carbon source, but other carbon compounds are.

In many secondary metabolite pathways, the enzymes subject to control by the carbon source are known. One is phenoxazinone synthase, an enzyme of the actinomycin pathway in *Streptomyces antibioticus*. Repression by glucose is exerted at the level of transcription; specific mRNA is low in trophophase, high in idiophase, and much lower in a glucose than in a galactose medium.

Regulation by the nitrogen source

Many secondary metabolic pathways are negatively affected by nitrogen sources favourable for growth—for example, ammonium salts. As a result, complex fermentation media often include a protein source (such as soybean

TABLE 3 Nitrogen sources interfering with secondary metabolism

Idiolite	*Interfering nitrogen source*	*Non-interfering nitrogen source*
Actinomycin	L-Glutamate, L-alanine, L-phenylalanine, D-valine	L-Isoleucine
Aflatoxin	Nitrate	NH_4^+
Alternariol	Nitrate, L-glutamate, urea	
Bikaverin	Glycine	
Candicidin	L-Tryptophan, L-tyrosine, L-phenylalanine, *p*-amino-benzoate	
Cephalosporin	NH_4^+, L-lysine	L-Asparagine, L-arginine
Chloramphenicol	NH_4^+	D-Serine, L-proline, DL-phenylalanine, DL-leucine, L-isoleucine
Erythromycin	NH_4^+	
Leucomycin	NH_4^+	Uric acid
Macbecin	L-Tryptophan, *p*-amino-benzoate, anthranilate	
Penicillin	NH_4^+, L-lysine	L-Glutamate
Rifamycin	NH_4^+, L-tryptophan, *p*-amino-benzoate	Nitrate, L-phenylalanine
Streptomycin	NH_4^+	Proline
Streptothricin	NH_4^+	DL-Aspartate, L-glutamate, DL-alanine, glycine
Tetracycline	NH_4^+	
Trihydroxytoluene	NH_4^+	
Tylosin	NH_4^+	Valine, L-isoleucine, L-leucine, L-threonine

meal) and defined media a slowly assimilated amino acid (such as proline) as the nitrogen source to encourage high production of secondary metabolites. Processes subject to regulation by the nitrogen source are shown in Table 3. Little information is available on the mechanisms underlying the negative effects of NH_4^+ and certain amino acids. In the production of tylosin, the sensitive enzyme appears to be valine dehydrogenase, which is repressed and inhibited by NH_4^+. Because valine is the best source of the acetate, propionate and butyrate precursors supplying the carbon atoms of the macrolide ring system, protylonolide, interference in valine degradation suppresses tylosin synthesis. In *Cephalosporium acremonium* (syn. *Acremonium chrysogenum*; *A. stricta*), at least two enzymes of the cephalosporin biosynthetic pathway, ACV synthetase and expandase, are repressed.

TABLE 4 Secondary metabolite processes susceptible to interference by inorganic phosphate

Bikaverin	Colistin	Nourseothricin
Butirosin	Ergot alkaloids	Streptomycin
Candicidin	Gentamicin	Tetracycline
Cephalosporin	Levorin	Tylosin
Clavulanic acid	Nanaomycin	Vancomycin

Regulation by the phosphorus source

Regulation by phosphorus sources (Table 4) includes both specific and general controls. A rather specific negative effect of inorganic phosphate arises from its ability to inhibit and/or repress phosphatases. Because biosynthetic intermediates of certain idiolite pathways (e.g. aminoglycoside antibiotics) are phosphorylated whereas the ultimate product is not, phosphatases are required in biosynthesis. For example, streptomycin biosynthesis by *Streptomyces griseus* includes at least three phosphate-cleavage steps and the process is very sensitive to phosphate concentration.

Phosphate also has a more general effect than the inhibition or repression of biosynthetic phosphatases; it appears to interfere in many secondary metabolic pathways not known to have phosphorylated intermediates. Such fermentations have to be conducted at levels of free phosphate (usually below 10 μM) which are sub-optimal for growth.

Induction of secondary metabolite synthases

In a number of secondary metabolite pathways, primary metabolites induce synthases and thus increase production of the final product. These include the induction of dimethylallyltryptophan synthetase by tryptophan in ergot alkaloid biosynthesis, leucine induction of bacitracin synthetase, and methionine induction of ACV synthetase, cyclase and expandase in the cephalosporin pathway of *C. acremonium*.

Of great importance in actinomycete fermentations is the inducing effect by endogenous metabolites which are not primary metabolites—for example, A-factor (2*S*-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) and related γ-butyrolactones. A-factor induces both morphological and chemical differentiation in *S. griseus* and *S. bikiniensis*, bringing on the formation of aerial mycelia, conidia, streptomycin synthases and streptomycin. A-factor induces at least ten proteins in *S. griseus* at the transcriptional level. One of these is streptomycin 6-phosphotransferase, an enzyme which functions both in streptomycin biosynthesis and in resistance. In an A-factor-deficient mutant, there is a failure of transcription of the entire streptomycin gene cluster (Mansouri et al 1989).

TABLE 5 Feedback regulation in secondary metabolism

Idiolite	*Enzyme*	*Mechanism*
Bacitracin	Bacitracin synthetase	Inhibition
Chloramphenicol	Arylamine synthetase	Repression
Cycloheximide	Unknown	
Ergot alkaloids	Dimethylallyltryptophan synthetase	Inhibition
Erythromycin	S-Adenosylmethionine: erythromycin C *O*-methyltransferase	Inhibition
Gramicidin S	Gramicidin S synthetases	Inhibition
Indolmycin	Initial enzyme	Inhibition
Kanamycin	Acetyltransferase	Repression
Lankacidin	Unknown	
Mycophenolic acid	*O*-Methyltransferase	Inhibition
Puromycin	*O*-Methyltransferase	Inhibition
Rubradirin	Unknown	
Tetracycline	Anhydrotetracycline oxygenase	Inhibition
Tylosin	S-Adenosylmethionine: macrocin *O*-methyltransferase	Inhibition

Many other actinomycetes produce A-factor, or related factors which differ in the length of the side chain. In those strains which do not produce streptomycin, the γ-butyrolactones induce the formation of the particular antibiotics that are produced, as well as morphological differentiation. A group of another five γ-butyrolactones, termed virginiae butanolides, are produced by the virginiamycin producer, *Streptomyces virginiae* (Kondo et al 1989), and by other streptomycetes.

Feedback regulation

Many secondary metabolites inhibit or repress their own biosynthetic enzymes (Table 5).

Control by growth rate

Growth rate control appears to be important in secondary metabolism and may be the overriding factor in the cases where nutrient limitation is needed for the production of secondary metabolites. For example, glucose or glycerol plus NH_4^+ support rapid growth but no bacitracin production by *Bacillus licheniformis* during trophophase. However, other more slowly used carbon sources (such as pyruvate, citrate or lactate) in the presence of NH_4^+, or other more slowly assimilated nitrogen sources (such as nitrate, alanine or glutamate)

in the presence of glucose, allow bacitracin to be produced during trophophase. More importantly, the degree of bacitracin production varies inversely with the growth rate.

In contrast, both a low growth rate *and* a particular type of nutrient deficiency are needed to support secondary metabolism in other cases. For example, *Streptomyces cattleya*, in chemostat culture, requires both a reduced growth rate and a phosphate deficiency to produce thienamycin. However, to produce cephamycin C, the same strain needs only a low growth rate, which can be brought about by deficiency of carbon, nitrogen or phosphate.

The production of microcin B17, a large (3255 Da) ribosomally derived peptide antibiotic, by *E. coli* is derepressed when growth slows and thus it is produced temporally, in the stationary phase. Its single structural gene is plasmid-borne, carried on a 3.5 kb DNA cluster which also contains three biosynthetic genes (involved in processing the final translation product) and three resistance genes. Temporal production involves transcriptional control; that is, slow growth rates bring on β-galactosidase production in genetic fusions between the B17 structural gene and *lacZ* (Connell et al 1987). Even when the cells are growing exponentially, the level of production of β-galactosidase varies inversely with growth rate. Growth restriction could result from limitation of carbon, nitrogen or phosphate.

Enzyme decay

The production of secondary metabolites eventually stops, as a result of feedback regulation (see above) and decay of the synthase(s). For example, most of the synthases involved in the production of peptide antibiotics by bacilli disappear a few hours after they are formed. In the producer of gramicidin S, *Bacillus brevis*, the inactivation of the gramicidin S synthetases is oxygen dependent and independent of protease action. Decay is slowed by the presence of thiols, amino acid substrates and a utilizable energy source. The sites of inactivation appear to be the SH groups involved in binding the amino acids to the enzymes.

A new academic frontier

For many years, basic scientists (with the possible exception of organic chemists) were not interested in secondary metabolism. There were so many exciting discoveries to be made in the area of primary metabolism and its control that secondary metabolism was virtually ignored; study of this type of non-essential (luxury?) metabolism was left for industrial scientists and university pharmacognocists to dabble in. Today, the situation is different. The basic studies on *E. coli* and other microorganisms have elucidated virtually all of the primary metabolic pathways and most of the relevant regulatory mechanisms; many of the enzymes have been purified and the genes encoding them isolated, cloned

and sequenced. Primary metabolism is no longer a frontier—it is a domesticated farm. The frontier is now secondary metabolism—a mysterious area of technology that has recently become science and poses many questions of considerable interest to academic scientists. What are the functions of idiolites in Nature? How are these molecules made? How are the pathways controlled? What are the origins of secondary metabolism genes? How did it happen that virtually the same genes, enzymes and pathways exist in organisms so different as the eukaryote *C. acremonium* and the prokaryote, *Flavobacterium* sp.? What are the origins of the resistance genes which producing organisms use to protect themselves from suicide? And are these the same genes as those found in clinically resistant bacteria? Fortunately, molecular biology has produced tools with which to answer many of these questions. It is clear that basic mechanisms controlling secondary metabolism are now of great interest to many academic (and industrial) laboratories throughout the world. In the following sections, I shall describe some of the academic progress being made in the area of the regulation of secondary metabolite biosynthesis.

Mechanism of general phosphate control

Although little is known about the mechanism of general phosphate control of secondary metabolism, phosphate control of candicidin production by *S. griseus* is certainly exerted at the level of transcription. The most advanced data on phosphate repression come from the *p*-amino-benzoic acid (PABA) synthase involved in candicidin production in *S. griseus*. Close to the 5′ end of the structural gene (*pabS*) is a 114 bp sequence and a promoter whose expression is controlled by the level of phosphate in the medium. The phosphate control sequence shows 78% identity to the 'phosphate box' of *pho* regulation in *E. coli* (Liras et al 1990).

The mechanism of γ-butyrolactone regulation of secondary metabolism in actinomycetes

Genes encoding proteins involved in the synthesis of A-factor are present on a plasmid in streptomycin producers such as *S. griseus* and *S. bikiniensis* but are chromosomally borne in *S. coelicolor*. In the latter organism, the structural gene *afsA* appears to be regulated by genes *afsB* and *afsR*; *afsB* independently controls synthesis of A-factor and the antibiotics actinorhodin, undecylprodigiosin, methylenomycin A and a calcium-dependent antibiotic (CDA). It encodes a potential DNA-binding protein which acts positively at the transcriptional level on *afsA* and regulatory genes controlling each antibiotic biosynthesis pathway in *S. coelicolor* (Horinouchi et al 1989). The *afsB* gene product is a 993 residue protein of M_r 105 600 containing two ATP-binding sequences and two DNA-binding sequences with a helix–turn–helix motif in its N-terminus.

In *S. griseus*, an A-factor-binding protein has been isolated which is the receptor of A-factor and a repressor of differentiation (Miyake et al 1989). When A-factor binds to this receptor, it is presumably removed from DNA, allowing the formation of aerial mycelia, conidia and streptomycin synthases. In *S. virginiae*, a butanolide C-binding protein (presumably the receptor of butanolide C and the repressor of differentiation in that organism) has been isolated (Kim et al 1989). In *S. coelicolor*, secondary metabolism and morphological differentiation appear to be independent of A-factor (Horinouchi & Beppu 1990). Thus the role of A-factor is unclear in *S. coelicolor*.

In *S. coelicolor*, gene *bldA* is required for morphological differentiation and chemical differentiation (i.e. the production of antibiotics, regulation and resistance). Differentiation shows signs of being controlled by a cascade-type mechanism (Hopwood 1988). At a relatively high level is *bldA*, which could be a master gene controlling the formation of aerial mycelia, conidia and secondary metabolites. It encodes a tRNA molecule which is involved in the translation of a rare leucine codon, UUA (TTA in the DNA). TTA is evidently absent in vegetative genes but present in some genes involved in differentiation, i.e. in genes encoding the regulation of antibiotic biosynthesis and resistance.

At the next lower level is presumably a gene containing a TTA codon which controls *afsB*. Once the *afsB* gene product has been made, it would turn on transcription of each of the specific antibiotic regulatory genes; it would also lead to A-factor synthesis.

With respect to the morphological differentiation cascade, below *bldA* would be *bldC*, which controls morphogenesis in *S. coelicolor* but not chemical differentiation. Under *bldC*'s control would be the *whi* loci which regulate conidia formation but not that of aerial mycelia or antibiotics. These loci include *whiG* for coiling, *whiA*, *whiB* and *whiI* for septation, *whiD* and *whiF* for wall-thickening and the rounding-off of spore compartments and *whiE* for spore pigmentation. *whiE* contains at least seven genes (Davis & Chater 1990). Other mutations (*absA*, *absB*) have been found in *S. coelicolor* which eliminate the formation of all four antibiotics without affecting morphological differentiation (Adamidis et al 1990). Although some information about the action of some of these genes is emerging—for example, *afsR* seems to specify a phosphorylation-dependent regulator (Stein & Cohen 1989, Horinouchi et al 1989)—it is not clear how their products cooperate to ensure efficient control of secondary metabolism (see Chater 1992: this volume).

Mechanism of growth rate (or temporal) control of secondary metabolism in bacilli

One set of important sporulation genes is *spoIIJ*, *spoOA*, and *spoOF*. Their products are part of a two-component signal transduction system (Antoniewski et al 1990, Perego et al 1989). Protein SpoIIJ appears to be the sensor kinase

which phosphorylates proteins SpoOA and SpoOF. Protein SpoOA binds to a specific region of the promoter region of the *abrB* gene, downstream from the transcriptional start site, and represses its transcription (Strauch et al 1990). Protein AbrB represses sporulation, so protein SpoOA acts by negative control to turn on sporulation.

For sporulation to occur, a deficiency in the intracellular content of guanine nucleotides and/or a deficiency of the carbon, nitrogen or phosphorus source are required. Guanine nucleotide deficiency, which can be induced by the antimetabolite decoyinine, induces the expression of *spoOH*, a key early positively acting sporulation gene (Dubnau et al 1987).

An involvement of some sporulation genes in secondary metabolism is clear from the work of Marahiel et al (1987) on the cloning of the tyrocidin biosynthetic genes. Gene *tycA* encodes tyrocidin synthetase A in *B. brevis*. It was shown by *lac* fusions that expression of *tycA* in *B. subtilis* is temporally controlled at the level of transcription. Required for its expression are sporulation genes *spoOA*, *spoOB* and *spoOE*, but not *spoOC*, *spoOF*, *spoOH* or *spoOJ*. The *abrB* gene product represses expression of *tycA* by binding to the *tycA* promoter (Robertson et al 1989), and thus protein SpoOA prevents this repression of antibiotic synthesis by repressing transcription of the *abrB* gene (Perego et al 1988). These important studies show that control of morphological differentiation and control of chemical differentiation are not identical, but share certain common genes and their protein products.

Opportunities for industry

The pharmaceutical industry has been the home of commercial secondary metabolism for over 40 years. However, about 10 years ago, a number of companies became discouraged with the expense of finding new antibiotics and began to leave the field of natural product discovery. This was ill-timed, because it is now widely appreciated that secondary metabolites possess many activities unrelated to the killing of microorganisms and some have already been developed into major pharmaceutical compounds. Another opportunity for industry is the development of improved producing strains by the use of recombinant DNA technology.

New applications of secondary metabolites

The selective action that microbial secondary metabolites exert on pathogenic bacteria and fungi and on rapidly growing cells in general was responsible for ushering in the antibiotic era, and for over 40 years we have benefited from the remarkable properties of the 'wonder drugs', the antibiotics. Their success rate was so impressive that these molecules are the major ones used for antibacterial, antifungal and antitumour chemotherapy. As a result of these

early successes, the pharmaceutical industry screened secondary metabolites for years almost exclusively for antimicrobial and antitumour activities. This biased and narrow view severely limited the potential application of microbial metabolites. Fortunately, this situation has recently changed, and we have now entered a new era in which microbial metabolites are being applied to diseases previously treated only by synthetic compounds.

For years, the major drugs for non-infectious diseases such as hypotensive and anti-inflammatory agents were strictly synthetic products. Similarly, major therapeutic agents (for example, coccidiostats and antihelminthics) used against parasitic diseases in animals arose solely from the screening of chemically synthesized compounds followed by molecular modification. Today, however, fermentation products such as monensin and lasalocid dominate the coccidiostat market. The avermectins, another group of streptomycete products, have high activity against helminths and arthropods. Indeed, their activity appears to be an order of magnitude greater than that of the previously discovered synthetic antihelminthic agents. Another natural compound of great interest is mevinolin (lovastatin), a fungal product which acts as a cholesterol-lowering agent. Mevinolin is produced by *Aspergillus terreus* and in its hydroxyacid form (mevinolinic acid) is a potent competitive inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase from mammalian liver. Other successes from microorganisms include animal growth promoters, insecticides, herbicides, plant growth regulators, immunosuppressants, uterocontractants and oestrogenic agents for animals. A number of groups have isolated microbial secondary metabolites possessing important pharmacological activities through screening with simple enzyme or receptor assays. The future will feature further applications in important areas of medicine and agriculture, such as new antiviral, antiparasitic and antitumour agents, pharmacological compounds and pesticides. Furthermore, many of the protein products of the new biotechnology industry will eventually be replaced by low molecular mass idiolites from microorganisms.

Genetic manipulation to increase secondary metabolism

The clustering of biosynthetic, resistance and regulatory genes of secondary metabolism has facilitated the application of recombinant DNA technology. Such clustering is almost the rule in actinomycetes but also occurs in bacilli and sometimes even in fungi. Gene clusters generally contain more than a single operon plus individual genes and they have been detected in microorganisms producing the following metabolites: methylenomycin A, actinorhodin, streptomycin, erythromycin, tylosin, carbomycin, tetracycline, valinomycin, bialaphos, chloramphenicol, penicillin and cephalosporin. Clustering makes it possible to transfer operons or groups of operons from one organism to another by recombinant DNA technology, thus facilitating an increase in dosage of a gene encoding a limiting enzyme of a pathway. The presence of a resistance gene in

the cluster makes the selection of recombinants very easy. That gene dosage is important in strain improvement is supported by the observation that gene amplification has been detected in high producers of penicillin obtained by non-recombinant methods (Smith et al 1989).

The regulatory genes are of great importance because production may be limited by the presence or absence of such genes, rather than by the dosage of a structural gene. Thus, disruption of the negatively acting regulatory gene *mmy* of methylenomycin biosynthesis increased production 17-fold. The introduction of one extra copy of the positively acting regulatory gene *act*II raised the synthesis of actinorhodin 30- to 40-fold. Regulatory gene *redD* increased undecylprodigiosin synthesis 30-fold, and *strR* increased streptomycin synthesis five- to seven-fold. It is clear that the exploitation of regulatory genes will play a major part in strain improvement in future industrial practice.

References

Adamidis T, Riggle P, Champness W 1990 Mutations in a new *Streptomyces coelicolor* locus which globally block antibiotic biosynthesis but not sporulation. J Bacteriol 172:2962–2969

Antoniewski C, Savelli B, Stragier P 1990 The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J Bacteriol 172:86–93

Banerjee S, Hansen JN 1988 Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J Biol Chem 263:9508–9514

Chater KF 1992 Genetic regulation of secondary metabolic pathways in *Streptomyces*. In: Secondary metabolites: their function and evolution. Wiley, Chichester (Ciba Found Symp 171) p 144–162

Connell N, Han Z, Moreno F, Kolter R 1987 An *E. coli* promoter induced by the cessation of growth. Mol Microbiol 1:195–201

Cundliffe E 1989 How antibiotic-producing organisms avoid suicide. Annu Rev Microbiol 43:207–233

Cundliffe E 1992 Self-protection mechanisms in antibiotic producers. In: Secondary metabolites: their function and evolution. Wiley, Chichester (Ciba Found Symp 171) p 199–214

Davis NK, Chater KF 1990 Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. Mol Microbiol 4:1679–1691

Dubnau EJ, Cabane K, Smith I 1987 Regulation of *spoH*, an early sporulation gene in bacilli. J Bacteriol 169:1182–1191

Hopwood DA 1988 Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proc R Soc Lond B Biol Sci 235:121–138

Horinouchi S, Beppu T 1990 Autoregulatory factors of secondary metabolism and morphogenesis in actinomycetes. Crit Rev Biotechnol 10:191–204

Horinouchi S, Malpartida F, Hopwood DA, Beppu T 1989 *afsB* stimulates transcription of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. Mol & Gen Genet 215:355–357

Kim HS, Nihira T, Tada H, Yanagimoto M, Yamada Y 1989 Identification of binding protein of virginiae butanolide C, an autoregulator in virginiamycin production from *Streptomyces virginiae*. J Antibiot (Tokyo) 42:769–778

Kondo K, Higuchi Y, Sakuda S, Nihira T, Yamada Y 1989 New virginiae butanolides from *Streptomyces virginiae*. J Antibiot (Tokyo) 42:1873–1876

Liras P, Asturias JA, Martin JF 1990 Phosphate sequences involved in transcriptional regulation of antibiotic biosynthesis. Trends Biotechnol 8:184–189

Mansouri K, Pissowotzki K, Distler J et al 1989 Genetics of streptomycin production. In: Hershberger CL, Queener SW, Hegeman G (eds) Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, DC, p 61–67

Marahiel MA, Zuber P, Czekay G, Losick R 1987 Identification of the promoter for a peptide antibiotic biosynthesis gene from *Bacillus brevis* and its regulation in *Bacillus subtilis*. J Bacteriol 169:2215–2222

Miyake K, Horinouchi S, Yoshida M et al 1989 Detection and properties of A-factor—binding protein from *Streptomyces griseus*. J Bacteriol 171:4298–4302

Perego M, Spiegelman GB, Hoch JA 1988 Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spoOA* sporulation gene in *Bacillus subtilis*. Mol Microbiol 2:689–699

Perego M, Cole SP, Burbulys D, Trach K, Hoch JA 1989 Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins SpoOA and SpoOF of *Bacillus subtilis*. J Bacteriol 171:6187–1696

Robertson JB, Gocht M, Marahiel MA, Zuber P 1989 AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthetic gene. Proc Natl Acad Sci USA 86:8457–8461

Schnell N, Entian KD, Schneider U et al 1988 Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. Nature (Lond) 333:276–278

Smith DJ, Bull JH, Edwards J, Turner G 1989 Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. Mol & Gen Genet 216:492–497

Stein D, Cohen SN 1989 A cloned regulatory gene of *Streptomyces lividans* can suppress the pigment deficiency phenotype of different developmental mutants. J Bacteriol 171:2258–2261

Strauch M, Webb V, Spiegelman G, Hoch JA 1990 The SpoOA protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc Natl Acad Sci USA 87:1801–1805

DISCUSSION

Bu'Lock: What has changed in recent times is on one hand the capabilities of the biochemists, which have extended enormously; and, because they have become more fundamental, they have provided us with a much deeper insight into the way biochemistry works. On the other hand, as the molecular biologists have moved into areas such as antibiotic production, we have begun to get explanations in the sorts of molecular terms that morphologists are now using to explain morphological development. The work of David Hopwood is a clear example of how everything that has been learned by the study of molecular biology, and its developing techniques, is beginning to enlighten us about secondary metabolism.

So far as the excitement of natural product studies is concerned, all that has happened in the recent period is that the structures that structural chemists have

come up with have become even more crazy! If you consider the structures of some of the macrocyclic lactones from streptomycetes, or of the low molecular mass compounds from marine organisms, or of, say, the toad toxins, from the chemist's point of view these are very unusual indeed.

Cane: It has been much less explored than in microorganisms, but is there any evidence that the theme of the clustering of biosynthetic genes applies to higher *plant* natural products?

Hopwood: For the anthocyanin biosynthetic pathway, which has been studied in plants such as maize and *Antirrhinum*, there is no gene clustering, as far as I know. There is the typical eukaryotic situation where the genes for different biosynthetic steps are scattered over the chromosomes. The real surprise as far as eukaryotes are concerned is the penicillin pathway in the fungi, where there is extensive clustering, as we shall hear later from Geoff Turner. It may have a special explanation, whereby the genes could have come from a prokaryote by lateral transfer.

Turner: I wonder if the clustering is as much of a surprise as we used to think. The earlier view was that in prokaryotes, pathway genes are clustered, and in eukaryotes they are not, but it's not just the antibiotic biosynthesis genes which are clustered; for a number of other pathways in the fungi there are two, three or four genes grouped together; for example, the proline and the quinic acid catabolism clusters in *Aspergillus nidulans* (Hullet al 1989, Hawkins et al 1988).

Demain: The fact is that even though the biosynthetic genes are clustered, they are not necessarily composed of single operons. I don't know of any single operons, with the possible exception of the peptide antibiotics from *Bacillus*, where just two or three enzymes are involved. In most cases of secondary metabolic pathways, there are a number of transcripts. With regard to clustering in fungi, the three genes of penicillin synthesis are clustered in *Penicillium*. However, for the longer pathway (the cephalosporin pathway) in the fungus *Cephalosporium acremonium*, we are dealing with genes on different chromosomes which are participating in a biosynthetic pathway.

Davies: Is anything known about the genetics of the biosynthesis of secondary metabolites in plants, in terms of the actual structure of biosynthetic clusters?

Bu'Lock: The Mendelian genetics of features like flower colour are well known; beyond that, there's little in terms of molecular genetics.

Cane: There are examples in various plants of biosynthetic enzymes whose genes have been cloned, for example strictosidine synthase from *Rauvolfia serpentina* and berberine bridge enzyme from *Eschscholtzia californica* (Kutchan et al 1991). But if one wants to produce desirable plant products in culture, say, on the notion that one could take an entire cluster of genes and move it into a more effective environment, such as one where the culture is more easily fermented, one will run into the problem of having to find the genes one at a time or in small groups, rather than taking a large piece of DNA and cloning it into a suitable host.

Demain: Let's take taxol as an example. Right now, it would be very desirable to make it in a microorganism (because of its potential use in cancer), but the enzymes are not known, in the yew tree. Since neither the enzymes nor the pathway are known, the ability to use recombinant DNA technology to synthesize taxol in microorganisms is limited.

Bu'Lock: It's because the biochemistry and now the molecular biology have been so much better worked out in microorganisms that secondary metabolism has come to be thought of as a feature of microorganisms; whereas in 1950, say, it was thought of purely as a characteristic of higher plants. This change has come because the microbial biochemistry and molecular biology of microorganisms are now so much better known than those for the classic plant systems.

Davies: I have suggested that all organisms make secondary metabolites. Is this likely to be true?

Bu'Lock: The scope for secondary metabolism has become restricted in the higher animals. There are things like the bufenolide toxins and so on, from amphibians, but the more sophisticated and highly integrated the organism, the less scope there seems to be for excursions into secondary metabolism. Animals have on the one hand a high requirement for integration and, on the other, so many other ways in which to display and exploit their genetic diversity.

Demain: When you made that statement, Julian, did you mean that every strain of, say, *Escherichia coli* has to make a secondary metabolite, or that there are some strains of *E. coli* that make secondary metabolites? At what level are you talking? We don't generally think of *E. coli* as a producer of secondary metabolites, but it is, especially strains isolated from the infant intestine.

Davies: I think any *E. coli* strain could make a secondary metabolite; the synthetic functions are likely to be coded on plasmids. I think humans make secondary metabolites. This is perhaps an incorrect way of describing detoxification by the liver, but it's a form of secondary metabolism, in my view, in that it's not something which is necessary for the organism to grow.

Nisbet: Surely the problem with secondary metabolites is that we have become stuck in a rut of describing them by the process by which they are made, or attributing the term 'secondary metabolite' to something that's made through so-called secondary metabolism. We need to think more broadly about the functions of these molecules. If one is looking for small molecules that have important physiological functions for the host, for its predators, or for its symbionts or parasites, or for interactions with mating types, then I agree with Julian Davies that what we call secondary metabolites in microbes have parallels in all biological systems. One might suggest that the endorphins are secondary metabolites that have been highly conserved. We have examples of reptilian and amphibian secondary metabolites with very potent actions, as neurotoxins or as antibiotics.