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The Manipulation of Micro-organisms for the Production of Secondary Metabolites

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Introduction

The ability to manipulate a biological process can be of great importance in several respects. In the development of fermentations, manipulation often has an important role at all stages from screening to commercial production.

In seeking to manipulate a process, the main problem one encounters is the complexity of biological systems. Even for primary metabolites where the biosynthetic pathways are well studied, the results of manipulative efforts may not always be predicted with certainty, as they depend on the integration of the pathway of interest into the rest of the metabolism of the organism. Secondary metabolism is an area of biochemistry which is poorly understood. The effects of manipulation on secondary metabolism may be somewhat harder to predict. Although appreciable amounts of information have been gathered on the synthesis of particular (normally commercially important) secondary metabolites, this information may not always be relevant to the manipulation of other secondary metabolites, or even to manipulation of the same secondary metabolite in different strains.

At this point it is pertinent to consider the importance of secondary metabolites. Many microbial products classified as secondary metabolites have been, and will continue to be, of great economic, medical and scientific importance. Antibiotics are probably the best-known subdivision of this group of metabolites. It is likely that compounds with antimicrobial activity constitute only a small proportion of the total number of secondary metabolites which may exist. Most screens for secondary metabolites have been directed against compounds with such activity. Such screens are often relatively simple, and rely on the direct visual detection of the antibiotic or its effect.

Weinberg (1977) has recorded the variety of secondary metabolites in terms of their chemical structure. Since then many more classes of microbially derived organic compounds have been added to his original list. The devel-

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opment of screening methods for secondary metabolites with other biological activities, or with alternative (not necessarily biological) applications, is likely to result in more chemical types appearing in future lists. The desire to manipulate micro-organisms so that their full potential for secondary metabolite production may be exploited is likely to continue to be of interest for the foreseeable future.

Although many secondary metabolic processes have been studied for several decades, for example penicillin and gramicidin S biosynthesis, guidelines for manipulating both known and new systems are not always clear. This is largely a consequence of the extreme diversity of secondary metabolites. A huge range of metabolites with varied chemical structure and metabolic origins have been grouped together as secondary metabolites. It is hardly surprising that information gained for one system may not be of use in manipulating other, unrelated, systems.

A consideration of how compounds have come to be regarded as secondary metabolites is relevant. The metabolism of many micro-organisms leads to the biosynthesis of two apparently distinct types of products. It is from this observation that the definitions of a secondary metabolite have evolved (Rose, 1979). The first type of product includes metabolites such as glucose or glutamate which have clearly ascribed functions in the maintenance, growth, and reproduction of the microbial cell in which they are metabolized. Although under certain circumstances these metabolites can be excreted from the cells, they are frequently re-utilized by the producer organism. These products are termed primary metabolites as their synthesis or acquisition from the environment is often of primary importance for survival. The second type of product frequently has no clear function in the well-being of the producer cell and is in many instances not metabolized further. In addition, the apparent loss of ability of mutants to make this type of metabolite frequently results in no observable effect on the survival of the mutant strains compared with strains which produce the compound.

Because of the apparent secondary importance of these products, they are classified as secondary metabolites. To what extent this classification reflects a lack of knowledge of how the metabolism operates under the constraints of the natural environment or experimental conditions remains to be seen. The fact that over 1% of the DNA in a microbial cell may code for enzymes involved in the biosynthesis of secondary metabolites (Hopwood *et al.*, 1983) and that this information is passed on from generation to generation, suggests that their synthesis may fulfil important biochemical functions. There have been numerous attempts to find characteristics that may be shared by all systems classed as producing secondary metabolites. Some of those most frequently quoted are listed in *Table 1*. Many of the characteristics are shared by primary metabolic processes; others, such as the effect of growth rate, often require clarification (Demain, Aharonowitz and Martin, 1983). The effects of phosphate and trace metal concentrations form no discernible pattern (Weinberg, 1977).

The supposed limited taxonomic distribution of some systems may reflect the methodology used in screens and the extent to which other groups

Table 1. Characteristics frequently associated with secondary metabolic processes

1. In batch cultures employing nutritionally rich media, the production of a secondary metabolite is generally maximal only after most cell growth has occurred.
2. The synthesis of a secondary metabolite is often susceptible to repression by carbon and/or nitrogen sources present in the growth medium.
3. Synthesis of a secondary metabolite occurs within ranges of temperature, oxygen tension, specific metal ion and phosphate concentrations that are narrower than those over which growth occurs.
4. Production of a particular secondary metabolite has a limited taxonomic distribution.

of organisms have been screened for a compound: for example, β-lactam production was previously thought to be confined to certain fungi and streptomycetes but, with the development of better screening methods, β-lactam production by many bacteria is now recognized (de Lorenzo and Aguilar, 1984). Other secondary metabolites have a wider taxonomic distribution: examples are ethylene and hydrogen cyanide, which may be produced by a wide variety of fungi and bacteria (Primrose, 1979; Knowles and Bunch, 1986). Many factors can be involved in determining the type and amount of a secondary metabolite produced and excreted by a microbial cell (*Figure 1*). Although these can differ even in systems producing the same

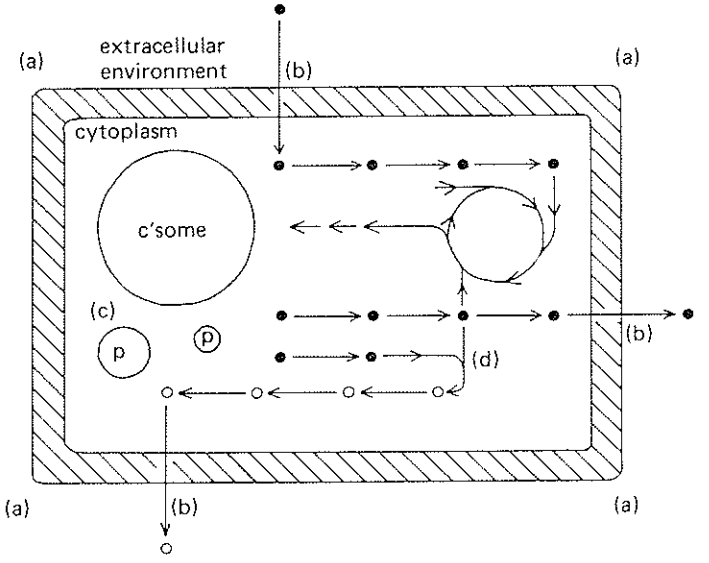

Figure 1. Factors within a secondary metabolic system that can determine the amount of product produced and detected. Primary metabolic pathways: ●→●→●; secondary metabolic pathways ○→○→○; (a) physiological factors, such as temperature, phosphate concentration, metal ion concentrations, oxygen tension, pH; (b) permeability barriers; (c) expression of genetic information, chromosomal (c'some) or plasmid-borne (P); (d) interrelationship of primary and secondary metabolic processes and end-products, and their regulation.

product (Knowles and Bunch, 1986), each of the factors provides opportunities for manipulation.

Many excellent reviews have been devoted to specific aspects of secondary metabolism. It would not be possible or desirable to compress this mass of available information into a review of this length. Instead we have chosen to give a broad overview of this area which we feel is opportune at this time.

This review discusses how a knowledge of biochemistry and genetics related to those factors determining the production or excretion of secondary metabolites may be employed to manipulate strains. The main thrust of attempts to manipulate strains is aimed at deregulation. Each important factor is considered in turn and manipulation of secondary metabolic processes related to the factor is discussed. Those areas which have in our opinion been underexploited or may prove more useful in the future, are highlighted.

Types of regulation found in secondary metabolism

Secondary metabolites are often produced only within a limited range of culture conditions. In order to observe their production, the essential requirements are: (a) the presence of the particular synthetase enzymes in an active state; (b) a supply of the appropriate precursors, and (c) export from the cell (for those compounds which accumulate extracellularly). The controls which regulate the production of secondary metabolites are essentially the same as the regulatory mechanisms found to operate in primary metabolism. These may be arbitrarily grouped under the following headings: (1) regulation by medium composition; (2) biochemical regulation; (3) genetic regulation.

REGULATION BY MEDIUM COMPOSITION

Physiological manipulations of this sort are involved in many aspects of the study and commercial exploitation of secondary metabolic systems. As with the production of other types of biochemicals, once a particular secondary metabolic process has been selected, optimization of the growth environment for maximum yields is a prerequisite before other types of manipulation are attempted. No change in the genotype of the producer organism is required and so the results obtained can frequently give valuable clues to how future research and development should proceed by revealing facets of the integration and operation of a secondary metabolic process within a cell. Many types of physiological manipulation have been attempted and the results collated in several excellent reviews (Vining, 1979, 1983; Malik, 1982; Aharonowitz, 1983; Demain, Aharonowitz and Martin, 1983; Kleinkauf *et al.*, 1986). In this section we concentrate on those observations which appear to be generally relevant to the majority of secondary metabolic processes.

The effects of environmental parameters such as temperature and pH are usually briefly evaluated during the early screening procedures or are initially

set at the values which give optimum growth of a secondary metabolite producer. Attention usually refocuses on them after more detailed studies involving the effect of growth medium composition on product formation have taken place. Manipulations involving media components are reviewed below.

Carbon source

Figure 1 illustrates the wide range of effects that alteration of the type and amount of a carbon source could have on the synthesis of a secondary metabolite. Thus, whether a process is stimulated or inhibited could be due, for example, to direct effects of the carbon source on the synthesis of the enzymes involved, or the way that its catabolism affects the concentrations of precursors for secondary metabolism, or the influence of the osmotic strength of the culture medium on the producer cell.

Not surprisingly it is very difficult to assess, even with well-studied processes, how a particular carbon source will affect a given system. It is well known that primary metabolic processes can often operate very differently in closely related micro-organisms (Umbarger, 1978), and similar observations have been made on a variety of secondary metabolic systems (Knowles and Bunch, 1986); choice of carbon source therefore remains largely undirected. Nevertheless, some useful observations have been made, as follows.

Growth rate. Many studies on secondary metabolite biosynthesis have noted a relationship between growth rate and product formation (Bu'lock *et al.*, 1975). Batch cultures employing media that can initially support rapid growth appear to synthesize secondary metabolites only after most of the biomass has been produced. On media where the initial growth rate is slower, production of the same secondary metabolite can become growth associated (Bunch and Knowles, 1980). Where such changes result from alterations in the nature of the carbon source, a variety of explanations have been put forward: some of these refer to the direct influence of the carbon source, such as carbon catabolite repression which is discussed below; others are more general and refer to the 'balance' of metabolism (Martin, 1977). The growth rate has been shown to affect a number of metabolic parameters in the cell, such as size of intracellular pools of primary metabolites, and gene dosage, as well as morphological characteristics such as cell size (Hütter, 1982). Of these, it appears that the most important effect is the 'balance' of metabolism which determines the production of precursors for secondary metabolite synthesis. Many observations have indicated that this is the case and two processes—the production of hydrogen cyanide and gramicidin S—will serve to illustrate this, although many similar results have been obtained from other systems (Aharonowitz and Demain, 1980).

Hydrogen cyanide is made by a wide variety of micro-organisms (Knowles and Bunch, 1986). In both fungi and bacteria the direct precursor is the amino acid glycine. If cyanogenic micro-organisms are grown in media which support rapid growth, cyanide is first detected during the early stationary

phase of batch cultures (Bunch and Knowles, 1982). However, it can easily be demonstrated that earlier in the culture, during the period of active growth, cells have the ability to synthesize cyanide at the same specific activity in cell extracts, although cyanide production by the whole cells is not seen. The time at which cyanide production can be observed reflects the composition of the culture medium. Growth of the cyanogenic snow mould basidiomycete in media with glucose as the major source of carbon results in cyanide being produced only during the stationary phase. Supplementation of the media with glycine results in cyanide production during late lag and early stationary phase. If betaine or *N,N'*-dimethylglycine are added with the glycine, cyanide production becomes growth associated (Bunch and Knowles, 1981).

Gramicidin S is first detected in many media during late log and early stationary phase cultures, yet, once again, the synthases can be detected and are active before gramicidin S activity appears. In this system, antibiotic production in continuous culture under a variety of limitations demonstrated that the synthases were present, but did not appear to generate gramicidin S (Vandamme, 1981).

This illustrates clearly that it is the integration of several areas of metabolism that determines, in this case, when a particular metabolite is synthesized. Practical use of this observation has been made in the commercial production of a variety of secondary metabolites such as the β-lactam antibiotics. In penicillin production, rapid growth on one carbon source, to generate biomass, may be followed by a period of secondary metabolite synthesis utilizing another carbon source. This type of manipulation will become of particular use during the development of immobilized microbial systems where division of growth and product formation is often crucial (Vick-Roy, Blanch and Wilke, 1983).

Carbon catabolite repression. There are many examples where carbon sources apparently exert a repressive effect on the production of a secondary metabolite. For example, Demain, Aharonowitz and Martin (1983) have collated observations on several antibiotic synthetic processes. However, it is still not clear whether the action of carbon sources such as glucose is via mechanisms similar to those observed in the bacterium *Escherichia coli* (Jacob and Monod, 1961), or whether the observations can be otherwise explained. Once again, the problem of grouping a diversity of metabolic processes together has led to no useful pattern becoming available to allow guidelines for the general manipulation of secondary metabolism. Thus there are examples where classical catabolite repression is postulated, such as in actinomycin production (Gallo and Katz, 1972) or cephalosporin biosynthesis (Demain, 1982). However, many other systems, where catabolite repression is indicated, are unaffected by cyclic AMP on its dibutyryl derivative; this is the case in penicillin biosynthesis in *Penicillium chrysogenum* (Demain, Aharonowitz and Martin, 1983). In other systems, catabolite repression and inhibition of enzyme synthesis by the carbon source appears to be implicated,

for example in the production of the antibiotics neomycin and siomycin (Kimura, 967; Majumdar and Majumdar, 1971). However, quite often the observations made can be explained by less direct involvement of the carbon source in a secondary metabolic process: for example, the inhibition of bacitracin biosynthesis by glucose has been attributed to low culture pH due to organic acid production (Haavik, 1974). It is therefore apparent that manipulations involving the carbon source, although somewhat unpredictable in their effect, will reveal whether, in a given medium, a process is regulated by the synthesis of the necessary enzymes or by the availability of primary metabolite precursors (Aharonowitz, 1983). This information can then be used to improve production via alterations of the carbon source at the start of the fermentation or during production, by changing the genotype via genetic manipulation to enhance the flow of metabolites down a given pathway or to relieve regulatory control.

Nitrogen source

For optimal growth the carbon–nitrogen balance in a culture medium must be correct. In addition, a large number of secondary metabolites contain nitrogen atoms, frequently more than one. Once again, the relationship between the nitrogen source and a secondary metabolic process is, more often than not, extremely complex. For example, it is postulated that many nitrogen-containing compounds have stimulatory effects on certain secondary metabolic processes related to morphological changes (Gräfe, 1982). In other cases, such compounds stimulate or inhibit production by unknown mechanisms, as in the role of methionine in cyanogenesis (Bunch and Knowles, 1982). However, it also appears likely that in many systems there is a direct link between nitrogen assimilation and secondary metabolism; this is to be expected, especially where nitrogen is incorporated into a product. There have been several reports of fluctuation of antibiotic synthesis in concert with the switch of a cell from one nitrogen-assimilatory mechanism to another: for example, β-lactam production by *Streptomyces clavuligenes* (Aharonowitz, 1979, 1980) declines when the glutamine synthetase activity is lowered; chloramphenicol production by *Streptomyces venezuelae* is strongly influenced by the type of nitrogen source employed (Vining and Chatterjee, 1982); finally, ethylene biosynthesis from methionine by *Escherichia coli* has been implicated in nitrogen salvage by the producer cells (Ince and Knowles, 1985). Gräfe (1982) has summarized some of the interrelationships between nitrogen catabolite repression and microbial secondary metabolism. It is interesting to note that he highlights the wide variety of factors which influence the level of product formation. He emphasizes that it is not only direct effects on the activity of the relevant enzymes which are important, but also effects on supply of precursors due, for example, to alterations in transport processes. Once again, results from investigations of nitrogen-source manipulations can indicate which type of genetic changes might contribute to increased production of a secondary metabolite.

Phosphate and trace elements

All the components of a culture medium could, in one way or another, affect secondary metabolite production. Of the many types of inorganic and organic chemicals that are frequently included, special note has been taken of the effect of inorganic phosphate and a variety of transition metals (Martin, 1977; Weinberg, 1970, 1977, 1978, 1982). One of the characteristics of microbial secondary metabolism often reported in the literature is that 'the synthesis of secondary metabolites has a much narrower tolerance for ranges of environmental inorganic phosphate and specific trace metals than does the growth of producer cells' (Weinberg, 1982).

Not all secondary metabolic processes behave in this way, and even in those that do, there is no easily discernible pattern (Weinberg, 1978). Given that phosphate and trace elements (e.g. Fe, Zn, Mn) are involved in so many areas of metabolism, the results from alterations of the concentration of these compounds in the culture media give few useful guidelines to specific manipulations which may be of benefit. Phosphate, for example, is involved in such systems as DNA, RNA and protein biosynthesis, carbohydrate metabolism, respiration, energy charge, and transport in the producer cell as well as acting, in some cases, as a co-substrate for a secondary metabolite (Martin, 1977). Similarly, trace elements are involved in many of the above processes. One suggestive observation, however, is that the three most commonly reported trace elements (Fe, Zn, Mn) generally reputed to exert an influence on secondary metabolism are found in forms of the enzyme superoxide dismutase (SOD; EC 1.15.1.1) (Fridovitch, 1974). The importance of iron, manganese and zinc to micro-organisms is reflected in the fact that they possess specific transport systems for these nutrients (Failla, 1977; Silver and Jasper, 1977; Neilands, 1984). Because the most prevalent form of iron in aerobic environments, iron III, is essentially insoluble, micro-organisms have evolved highly specific compounds, the siderophores, which chelate the iron III (Neilands, 1981a,b, 1984). The organisms take up iron as the complex, rather than in the free state, via specific uptake systems in the membrane. However, zinc and manganese uptake does not appear to involve compounds analogous to siderophores.

Several systems, including hydrogen cyanide production by *Pseudomonas aeruginosa*, are affected by the oxygen concentration in the culture medium. Maintaining a low oxygen concentration at the time of maximal cyanogenesis prevents the loss of cyanogenic activity observed in cultures when the oxygen concentration otherwise increases at the end of growth (Castric, Castric and Meganathan, 1981). A possible link between inactivation by oxygen or its metabolites and protection by SOD could be postulated.

Other factors

As mentioned above, many of the components of a medium can affect secondary metabolism and it is not possible or desirable to list the many manipulations of the culture media that could be attempted. Some guidelines

as to what may be beneficial in a particular system may be available in the literature.

Similarly, it is frequently found that temperature and pH can affect the production of a secondary metabolite. Once again, these can influence a very wide range of metabolic processes but, as with variations in the concentration of inorganic phosphate, it has often been found that secondary metabolite production occurs maximally over a small range of temperature (Weinberg, 1982). Also, itaconic acid production by *Aspergillus terreus* is highly dependent on the pH of the culture medium (Lockwood and Nelson, 1946). Using the information gained from many studies, rough guidelines have been reported for the formulation of culture media (Hütter, 1982). However, for better guidelines in the manipulation of culture media for optimal production of a secondary metabolite, more details of the process are required from biochemical and genetic studies. Using this information, it is then possible to direct metabolism to produce more of the desired product: for example, precursors fed at specific stages of a fermentation can result in elevated levels of the secondary metabolite; this is also useful for producing variants of the end-product. Advantage has been taken of the technique to produce a variety of penicillins (Sebek, 1984). It can be seen that the manipulations highlighted in this section are those that would be performed in the initial optimization of any microbial metabolic process. What they reveal is often applicable only to the selected system and similar processes within closely related micro-organisms. Care has to be taken when differentiating between a primary and a secondary metabolic process, but it may be better not to make the distinction.

BIOCHEMICAL REGULATION

Many mechanisms of biochemical regulation found in primary metabolism also occur in secondary metabolism. The induction or derepression of enzymes involved in the biosynthesis of secondary metabolites is of obvious importance but there is often some difficulty in determining whether a stimulatory response is due to true induction or to an increased availability of precursors (Martin and Demain, 1980; Okanishi and Hotta, 1980). The most clear-cut example of induction is the role of A-factor in streptomycin biosynthesis (Khoklov, 1982). Streptomycin-producing strains are normally capable of synthesizing A-factor: loss of the ability to synthesize A-factor results in strains which do not produce streptomycin; the subsequent addition of A-factor to certain streptomycin non-producing mutants results in restoration of the ability to synthesize streptomycin. The A-factor is also important for differentiation processes.

An interesting point which may provide a clue to the mechanism of action of A-factor is the fact that this molecule is a β-diketone (Khoklov, 1982). Such compounds are highly efficient metal-ion-chelating compounds (De, Khopkar and Chalmers, 1970) and it may be that A-factor acts by competing with other binding sites for metals within the organism. This could result in activation or inactivation of enzymes or may be of importance in the induction

or repression of metal-containing enzymes: for example, iron-chelating compounds have been shown to induce manganese-containing superoxide dismutase in *Escherichia coli* (Hassan and Moody, 1984).

Carbon catabolite and nitrogen catabolite repression are often encountered in secondary metabolite fermentations (Martin and Demain, 1980; Okanishi and Hotta, 1980; Demain, 1982; Aharonowitz, 1983; Demain, Aharonowitz and Martin, 1983).

Regulation by end-product inhibition or repression also appears to be a fairly common control mechanism in secondary metabolism (Martin and Demain, 1980; Okanishi and Hotta, 1980; Malik, 1982; Demain, Aharonowitz and Martin, 1983). Primary metabolites which share the same biosynthetic origins as certain secondary metabolites may limit production of the secondary metabolite by feedback inhibition and repression at earlier stages in the pathway. Alternatively, the secondary metabolites themselves may regulate their own synthesis (Drew and Demain, 1977; Martin, 1978; Martin and Demain, 1980).

GENETIC REGULATION OF SECONDARY METABOLITE PRODUCTION

Malik (1979) groups the genes controlling antibiotic production into five classes. These are:

1. Structural genes coding for enzymes involved in the biosynthesis of the secondary metabolite;
2. Regulatory genes that determine the onset and extent of repression (or induction) of the structural genes for biosynthesis;
3. Genes that determine the resistance of the producing organism to the product (where the product is toxic);
4. Genes controlling the permeability to the compound (entry, exclusion and excretion); and
5. Regulatory genes that control primary pathways. These genes influence the level of precursors and cofactors needed for antibiotic biogenesis.

The genetic regulation of secondary metabolite production is obviously highly complicated. Because so many factors affect production it may be very difficult to effect specific alterations, and the effects of mutation may not be readily predicted.

Other possible points of regulation which have been relatively neglected include the transport of compounds into and out of the cell. It is possible that the rate of influx of precursors and the rate of efflux of the products may limit the extent of secondary metabolite production (*see* the discussion of the importance of permeability: pages 135–137). On the basis of what is known about regulatory mechanisms encountered in secondary metabolism, strategies have been developed to manipulate the metabolism of organisms to desired ends.

Approaches to the manipulation of secondary metabolites—why manipulate?

Newly isolated micro-organisms often produce a secondary metabolite of interest in low concentrations. Once a compound with interesting activity is found, it is generally desirable to produce more of this compound for further testing and chemical characterization. This may be achieved most simply by growing larger volumes of culture, but for a process to be commercially viable a yield of several grams per litre may be required and more substantial productivity gains must be sought. Normally it is possible to enhance the yield to some degree by altering the composition of the growth medium and the culture conditions. However, there may be practical constraints to both approaches, such as the fermentation capacity and downstream processing facilities available, or the desire to use particular low-cost fermentation substrates.

Beyond changing the growth conditions, it is normally necessary to generate altered genotypes to obtain improvements in the yield or in other characteristics, as the maximum potential of a strain to produce a metabolite, either primary or secondary, is determined by the genome. The process of generating altered genotypes followed by the examination of the resulting strains for an improved titre of product or another desired characteristic may be termed strain improvement.

STRAIN IMPROVEMENT

Strain improvement may be divided into three phases (Rowlands and Normansell, 1983). These phases are: (1) the generation of altered genotypes; (2) the recognition and isolation of desirable genotypes; and (3) their acceptance in commercial processes.

It is important to recognize that what industrial microbiologists perceive as an improvement in a strain may not necessarily be advantageous to the organism. Indeed, in most cases such genetic alterations will reduce the biological efficiency, and selection pressure will act against them. The stability of highly productive industrial strains is often a problem.

The generation of altered genotypes

This is of primary importance in strain improvement. Altered genotypes can arise as a consequence of the following:

1. Natural mutation;
2. The use of mutagenic agents;
3. Recombination.

Natural mutation. The frequency of spontaneous mutations is normally so low that the screening of the large numbers of strains which would be

necessary is not feasible. There may be exceptions to this general case, such as if a useful mutation is associated with a particular morphological characteristic which allows it to be recognized more readily. To increase the frequency of mutation it is normally necessary to resort to the use of mutagenic agents. The use of mutagens also frequently generates mutations not often found among spontaneous mutations.

The use of mutagenic agents. A variety of mutagens may be employed, including radiation (ultraviolet, gamma- and X-irradiation) and chemical mutagens such as ethyl methane sulphonate (EMS) nitro-nitroso-methylguanidine (NTG) and the mustards. The type of mutation produced will be dependent on the type of DNA damage caused by the mutagen and the action of the cellular DNA repair pathways on the damage. A detailed consideration of these topics is outside the scope of this review, and for consideration of the use of mutagens in the context of strain improvement the reader is referred to Sermonti (1969, 1979), Bridges (1976) and Rowlands (1984a). Practical considerations in strain-improvement programmes include the choice of mutagen and the optimization of the mutagen dose. The possibility that mutagen specificity effects may occur suggests that several mutagenic agents should be used during a strain-improvement programme. It is preferable that mutagens causing damage subject to repair by different pathways are used (Rowlands and Normansell, 1983).

Most induced mutations are deleterious to the yield of the desired product. However, a minority are more productive than the parent strain. This may be illustrated by the results of Dulaney and Dulaney (1967), who compared the range in productivity of chlortetracycline of natural variants of *Streptomyces viridiofaciens* with that of the survivors of an ultraviolet treatment. Although a majority of survivors of ultraviolet treatment were inferior producers, there were also strains which produced more than twice the amount of chlortetracycline produced by the parent strain; this was significantly more than was produced by the best of the natural isolates. Similar results were obtained by Alikhanian (1962) in a study of albomycin production by mutagenized *Actinomyces subtropicus*. These results also illustrate the importance of optimizing mutagen dose: beyond a certain dose, the number of 'minus variants' increased markedly.

Recombination. Natural methods of DNA transfer between cells which may lead to recombination include transformation, conjugation and transduction, which are important in bacteria (Sherratt, 1981) and heterokaryosis and the sexual cycle in fungi (Sermonti, 1969; Fincham, 1983). Although these may all find some application in strain improvement, the most widely used methods of gene transfer for strain improvement are now those which are unlikely to occur in nature: parasexual breeding (in fungi), protoplast fusion and genetic engineering (Rowlands, 1984b).

Because of the absence of the sexual cycle in most fungal strains of commercial importance, the parasexual cycle has been of great importance in the development of these strains (Sermonti, 1969; Macdonald, 1983;

Rowlands, 1984b). Recombination is achieved by forcing two haploid strains with complementary auxotrophic markers to form a heterokaryon by growth of both on a minimal medium. Rare nuclear fusions may occur and recombination may take place by mitotic crossing-over between homologous chromosomes in the diploid and during the subsequent breakdown of the diploid to the haploid state (haploidization).

The powerful technique of protoplast fusion has developed rapidly in recent years. The method allows incompatibility barriers between different mating types, species and even genera of micro-organisms to be overcome (Peberdy, 1979; Ferenczy, 1981; Hopwood, 1981; Ball, 1983). An extension of this technique involves fusing liposomes containing DNA to protoplasts.

Genetic engineering, employing *in vivo* and *in vitro* DNA manipulation, allows the isolation and manipulation of specific sequences of DNA in a controlled way. Unlike techniques such as protoplast fusion or the parasexual cycle, which employ whole genomes, only small regions of a genome are involved. This means that the screening for the required recombinant may be greatly simplified (*see also* Chapters 2 and 4 of this volume).

The structure of individual genes, the gene dosage and the degree of expression can all be altered. The application of genetic engineering techniques to organisms, including those of current industrial interest (bacilli, lactic acid bacteria, pseudomonads, streptomycetes, yeasts and filamentous fungi), has been extensively covered (Sherratt, 1981; Timmis, 1981; Davies, 1983; Ball, 1984; Mishra, 1985; Old and Primrose, 1985).

Recognition and isolation of desired genotypes: screening

Once novel genotypes produced by mutation or recombination have been generated, these must be examined in such a way that desirable alterations will be recognized. The empirical approach involves the direct titre testing of large numbers of isolates in a random screening procedure. If anything is known about the pathways of biosynthesis of the compound of interest, and their regulation, more rational screening procedures may be employed and, even if detailed knowledge is not available, educated guesses may be made concerning the biosynthetic origins of a compound, based on its structure and any knowledge of related compounds or organisms (Rowlands, 1984b). In the case of the most commonly sought strain improvement, an increase in yield, the aim is to identify strains in which deregulation may have occurred. Rational screening techniques have been developed which exploit what is known about the different types of regulation encountered in secondary metabolism. As there is an incomplete knowledge of most systems, the use of such rational screening procedures is normally intended as a prescreen to reduce the numbers of strains which have to be examined (Rowlands, 1984b).

In seeking to obtain deregulated strains, the precise point at which deregulation occurs cannot always be readily predicted and only exhaustive examination of mutant or recombinant organisms may reveal it. For example, a strain selected for resistance to a toxic analogue may show increased pro-

ductivity, but the resistance may be a consequence of several possible alternative mutations or alterations, such as relief of end-product inhibition by an alteration in the biosynthetic enzymes; or it may be due to reduced permeability, with this or another mutation being responsible for overproduction. In the case of heavily mutagenized cultures, it should be recognized that there may be more than one mutation in any one isolate.

The approaches which are available in the rational screening for desirable alterations are considered below, and the reviews of Rowlands (1984b), Elander (1982) and Stanbury and Whitaker (1984) are commended to the reader.

Isolation of resistant mutants. The use of analogue-resistant mutants in the development of overproducers of primary metabolites, amino acids in particular, is well documented (Malik, 1979; Reed, 1982; Stanbury and Whitaker, 1984). It is supposed that one likely mechanism by which such mutants become resistant is by deregulation, leading to overproduction of the natural metabolites which can then effectively relieve the toxicity of the analogues by competition (Malik, 1979). The same principle can be applied in the development of strains which overproduce secondary metabolites.

Such mutants are of particular value in two cases: first, where the supply of primary metabolic precursors for the secondary metabolite limits the production of the secondary metabolite; secondly, where there is end-product inhibition or repression of enzymes in the pathway by primary or secondary metabolites. Where a primary metabolite is required for the synthesis of a secondary metabolite but is limiting in the fermentation, either it may be added to the fermentation, or enhanced production by the organism may be sought. The use of analogue-resistant mutants can remove feedback inhibition and result in enhanced endogenous production of the primary metabolite and thus an elevated titre of product (Elander *et al.*, 1971; Godfrey, 1973; Chang and Elander, 1979; Mendelowitz and Aharonowitz, 1983). The use of analogues of secondary metabolites can lead to isolation of analogue-resistant strains which have gained relief from feedback inhibition or repression by the secondary metabolite. However, as pointed out by Stanbury and Whitaker (1984) it is unlikely that a toxic analogue of the secondary metabolite could be found where the toxicity lay in the mimicking of the feedback control of the secondary metabolite—a compound which would not be necessary for growth. As an alternative to the use of analogues, a mutagenized culture could be plated out on a medium containing repressing levels of the secondary metabolite (Stanbury and Whitaker, 1984). The plates could then be examined for production of the metabolite in excess of this threshold level. In the case of antibiotic production, an overlay of an appropriate indicator organism could be used. If the end-product is toxic to the producing organism, more resistant mutants may need to be sought before proceeding to obtain significant improvements in the yield.

Selective detoxification. If growth-inhibiting chemicals can react with a product of interest in such a way as to become detoxified, this may be used in a

screen. For example, the fact that β-lactam antibiotics complex with Hg^{2+} or Cu^{2+} ions was used in the rational selection of improved cephalosporin C producers in strains of *Acremonium chrysogenum* (Chang and Elander, 1979). Incorporation of the toxic agent at a concentration which just inhibits the growth of the parent strain will permit the detection of overproducing mutants as a consequence of their increased ability to neutralize the toxic compound and their growth on plates; low producers and standard producers do not grow. False positives may arise as a result of cell-wall or membrane-permeability changes, or non-selective detoxification mechanisms.

Phosphate deregulation. Similarly, the use of the toxic phosphate analogues, arsenate or vanadate, may result in the isolation of control mutants (Martin *et al.*, 1979; Bowman, 1983; Bowman, Allen and Slayman, 1983). An alternative approach is to select for normal productivity in the presence of repressing levels of phosphate (Martin, 1977).

Catabolite derepression. The isolation of derepressed strains may be achieved by selecting for strains with the ability to utilize non-repressive substrates in the presence of toxic analogues of repressing substrates. The best example is growth on non-repressing carbon sources in the presence of 2-deoxyglucose (Ghose, Ghose and Gopalkrishnan, 1982; Hodgson, 1982). An alternative to seeking derepressed strains is to use fed-batch culture where the repressing nutrient is limiting (Malik, 1982).

Reversion of auxotrophs. Mutation to auxotrophy, a requirement for a primary metabolite to be supplied for growth, generally results in a reduced yield of a secondary metabolite if the primary metabolite is a precursor of a secondary metabolite; however, reversion of auxotrophs to prototrophy may lead to an enhanced yield of secondary metabolite (Godfrey, 1973). This is presumably due to secondary mutations which deregulate the pathway for production of primary metabolic precursors, leading to overproduction of these precursors in the revertants. An improved yield of secondary metabolite is sometimes encountered in auxotrophs grown in medium containing the appropriate growth supplements (Stanbury and Whitaker, 1984), but the reasons for this are not clear.

Methods involving titre depression. In the search for an improved titre of product it may be desirable to depress the normal yield such that improvements may be observed using relatively coarse but rapid techniques such as plate bioassays to screen for improved mutants (Rowlands, 1984b). The titre may be depressed using analogues of the end-product or biosynthetic intermediates or the compound itself: this is phenotypic titre depression (Rowlands, 1984a). Another approach is the genetic depression of the titre, employing auxotrophs (Rowlands and Normansell, 1983); reversion of auxotrophy or crossing into a prototrophic strain can then be performed.

Reversion of non-producers. Where titre-depressing mutations occur, the genetic suppression (reversion) of these mutations may lead to enhanced production (Dulaney and Dulaney, 1967; Dhar and Bose, 1968; Unowsky and Hoppe, 1978). Because of the low titre of the 'non-producing' strains, rapid plate-screening techniques may be used to identify the revertants. Again, improved strains will have undergone at least two mutations associated with the production of the secondary metabolite; the suppressing mutation(s) may result in a substantial improvement in yield. Non-producing strains may be analysed at the genetic or biochemical level to provide information on the biosynthetic pathway of the product. These strains are also useful in complementation tests with cloned biosynthetic genes.

Other approaches to the manipulation of secondary metabolite production

DIRECTED BIOSYNTHESIS AND THE PRODUCTION OF NOVEL SECONDARY METABOLITES BY MUTATIONAL BIOSYNTHESIS

The yield of a secondary metabolite can often be enhanced by the addition to the medium of precursors for desired products, or of inhibitors to channel intermediates away from unwanted products (Demain, 1981; Sebek, 1984). Similarly, the feeding of analogues of these precursors may, if the compound enters the cell, result in the synthesis of analogues of the secondary metabolite; some of these may possess useful properties. A result of analogue feeding may be that if endogenous production of the normal precursor is still operative, a mixture of products may result, and the often close chemical similarity may cause problems in downstream processing: this problem may be overcome by isolating mutants which are not able to synthesize the normal precursor but which still possess the appropriate enzymes which can act on the precursor analogue. Feeding of the analogue may therefore result in synthesis of novel secondary metabolites. The non-producing mutants are termed 'idiotrophs' and the production of novel compounds from idiotrophs by analogue feeding is termed mutational biosynthesis or mutasynthesis (Elander, 1980; Demain, 1981). Mutational biosynthesis may be considered to be a special case of biotransformation (Sebek, 1984). Although this technique has much promise, it also has its difficulties; these have been summarized by Davies (1983). As this author points out, a more detailed genetic and biochemical analysis of the mutants would allow a more rational approach to mutasynthesis which would undoubtedly provide the means of obtaining a wide variety of novel compounds.

BIOTRANSFORMATIONS OF SECONDARY METABOLITES

Manipulations leading to improvements in processes for secondary metabolite production need not be limited to the producer organisms. The preparation of semi-synthetic secondary metabolites—antibiotics in particular—by biotransformation is an area where there is also much scope for manipulation.

Biotransformations may require the presence of only one enzyme: the development of such a process is thus relatively simple compared with the often complex situations in organisms which synthesize secondary metabolites. Improvements may be sought in such factors as specific activity, specificity, kinetic parameters or stability. The transformations may be carried out in batch culture or using immobilized cells or enzymes. Eleven types of reaction are recognized (Sebek, 1984). The production of semi-synthetic penicillins, cephalosporins, aminoglycosides and rifamycins is of particular commercial importance.

VARIATION OF SECONDARY METABOLITE TYPE BY MUTATION

Mutation of an organism may lead to an alteration of the secondary metabolite produced. The lesion may occur at many levels, from the supply of precursors to the later stages in biosynthesis. A knowledge of the biochemistry and regulation of production may allow a specific alteration to be obtained.

IMPROVEMENTS IN CHARACTERISTICS OTHER THAN YIELD

In addition to improvements in yield, a variety of other types of improvement may be sought; these have been well summarized by Stanbury and Whitaker (1984). Desirable characteristics such as improved genetic stability, elimination or reduction of foaming, altered flocculating ability, improved ability to grow on cheaper substrates, resistance to toxic media components, elimination of undesirable products from production strains and the selection of strains with altered morphology and hence improved fermentation characteristics may be sought.

Rational strain development may be possible in many cases. For example, flocculating ability may be altered substantially by mutations in only a few genes (Stewart and Russell, 1977; Stewart, 1981).

Which areas of study may pay dividends in increasing our understanding of secondary metabolism?

We believe that in order to gain a better understanding of microbial secondary metabolism we must address a number of basic questions. Research in secondary metabolism has been very much biased towards ways in which the yield of economically important compounds, especially of antibiotics, may be improved. This research has obviously been conducted on strains which already produce the metabolite of interest. In addition, much research has been conducted on strains which have already been manipulated, sometimes over prolonged periods and many generations. To understand the reasons why secondary metabolites are produced we feel that it is necessary to concentrate on an analysis of wild-type isolates.

What is the molecular basis of regulation by inorganic nutrients?

The physiological basis for the regulation of secondary metabolism by inorganic nutrient regulation remains unknown. If the identity of the regulatory factors involved could be established, our understanding of the process of secondary metabolism would benefit enormously. In some cases it is recognized that there are changes—often gross changes—in cell composition associated with limitations for phosphate and metals (Tempest, 1969; Holme, 1972): for example, manganese limitation enhances citric acid production in *Aspergillus niger* (Kubicek and Röhr, 1982). The manganese limitation greatly affects the composition of the plasma membrane, and citrate production ensues. Whether citrate production requires the membrane to be altered or whether citrate production and an altered membrane are both consequences of manganese limitation is not clear. Secondary metabolite production, which may be linked to deficiencies in certain inorganic nutrients, is often associated with changes in composition of the cell membrane. If the functional basis for regulation by metals, or the regulatory genes involved in making a cell a producer rather than a non-producer, could be identified, our understanding of the process of secondary metabolism would be greatly increased. The door would be opened to specific manipulations of wild-type or near wild-type organisms, even of non-producers, in order to exploit their full genetic potential for secondary metabolite production. This could be done in the absence of undesirable mutations in other parts of the metabolism which would otherwise accumulate in strains derived during 'improvement' programmes involving many cycles of mutation and screening.

How do producers differ from non-producing organisms?

Relatively few data are available on the incidence of antibiotic producers in populations of wild-type organisms in genera of current industrial importance. Difficulties are, of course, presented by the often stringent nutritional and environmental factors necessary to observe antibiotic production. Nevertheless, it is possible, perhaps even likely, that some primary trigger for antibiotic production in the form of a metabolic lesion may exist; comparison of non-producers with antibiotic producers may reveal the basis for production. In this context, the observation that non-producing organisms can be mutated to antibiotic production is extremely interesting.

Mutation of non-producing wild-type isolates to antibiotic production—which genes are responsible?

The genetic potential of a strain to produce an antibiotic may not be expressed under particular culture conditions; indeed, the culture conditions required to observe production may be quite stringent. Other strains may possess the genetic potential to synthesize antibiotics but this may be hidden by particular genetic conditions (Sermonti, 1969). Kelner (1949) mutated seven streptomycetes and found that five out of the seven non-producing

strains gave rise to antibiotically active mutants at a frequency ranging from 0·01% to 1·9%. It was interesting that active mutants differing in their antibiotic spectrum could be obtained from the same parent culture. This appearance of antibiotic productivity in naturally inactive strains of streptomycetes has been attributed by Sermonti and Lanfaloni (1982) to as small a change as a single gene mutation.

The possibility that antibiotic genes are present but cryptic in strains which do not naturally show antibiotic activity is perhaps worthy of more attention. The implication is that one or perhaps only a few mutations can reveal the antibiotic-producing potential of a cell. The identification of the genes involved would be extremely interesting. Certainly, alterations in only one gene can determine whether an antibiotic is produced or not. Actinorhodin biosynthesis by *Streptomyces coelicolor* requires six biosynthetic genes: however, synthesis is observed only with a functional *act* II gene, which appears to be regulatory (Rudd and Hopwood, 1979); this gene is implicated in resistance to the antibiotic. The frequency of this supposed regulatory mutation was high (26 out of 76 mutants). A similar situation pertains in the synthesis of a red pigment, probably undecylprodigiosin (Hopwood and Chater, 1984) by *Streptomyces coelicolor*. In cosynthesis experiments one of five classes of mutants appeared to be regulatory (Rudd and Hopwood, 1980). Further evidence that relatively few genes may control the switching on or off of secondary metabolite synthesis is provided by a study of cosynthesis by inactive mutants of antibiotic-producing strains (Delić, Pigac and Sermonti, 1969). A complementation analysis revealed that the majority of mutants were control mutants rather than mutants in the biosynthetic pathway. A corollary to the suggestion that mutation in only one or few genes may be sufficient to result in antibiotic production, is the intriguing possibility that one might get the same result by inactivating the gene product(s) physiologically; this may explain why there is often a marked dependence on highly specific culture conditions. The use of either genetic or physiological manipulation of strains to achieve the same end is illustrated in systems for the overproduction of amino acids: these are described in the next section.

It should be remembered that the production of any secondary metabolite is under polygenic control (Hopwood and Merrick, 1977). Strains of micro-organisms with extremely high titres of secondary metabolic product, such as those used in the more highly developed antibiotic fermentations, are therefore likely to have accumulated large numbers of mutations compared with the original wild-type isolates. Understanding of the differences between a producer of an antibiotic and a non-producer may facilitate the development of highly productive strains at a faster rate than has previously been the case.

How important is permeability as a limiting factor in secondary-metabolite production?

When the secondary metabolite accumulates in the medium surrounding the organism it is possible that productivity is limited by the net rate of efflux

efflux across the wall and membrane. The primary barrier to the passage of solutes into and out of the cell is the plasma membrane (Rose, 1979).

A common mechanism of resistance to toxic chemicals is alteration of the permeability of the cell wall and/or membrane. Such resistance can result in increased productivity for metabolites, probably because of increased rates of export from the cell. Improved productivity has been obtained from polyene antibiotic-resistant mutants of fungi (Queener, Wilkerson and Nash, 1982; Rowlands and Normansell, 1983; Rowlands, 1984b) and phage-resistant mutants of Actinomycetes and Streptomycetes (Alikhanian, 1962; Rowlands and Normansell, 1983; Rowlands, 1984b).

If membrane permeability is a limiting factor in the production of a secondary metabolite it is obviously desirable to try to enhance the permeability. A specific enhancement of the permeability for the metabolite of interest is to be preferred: a generalized increase in the permeability would allow the loss of essential compounds, probably leading to reduced viability and a lower yield of the desired secondary metabolite.

The possibility that genetic or physiological manipulation of membrane permeability can be employed to enhance metabolite excretion and to increase the productivity of the cell, is a concept worthy of more detailed investigation.

There is evidence to suggest that the onset of excretion of a primary metabolite, glutamate, by *Corynebacterium glutamicum* is a consequence of an uncoupling of the active uptake system for this compound (Clement *et al.*, 1984); these authors suggest that the glutamate uptake system can then act as a specific excretion system. Overproduction of glutamate by *Corynebacterium glutamicum* is one of the best-developed primary metabolic fermentations. Glutamate excretion may be triggered by treatments such as biotin limitation, addition of acylated surfactants, or penicillin (Clement *et al.*, 1984; Stanbury and Whitaker, 1984); major changes in the composition of the cell membrane are induced by such treatments. Although these changes are obviously important in the onset of glutamate excretion, the particular mechanism by which this becomes possible is not clear. Clement *et al.* (1984) considered that the treatments led to an altered membrane composition which in turn resulted in a specific uncoupling of the glutamate uptake system: in fact, cells of the glutamate producer lost 30% of their membrane phospholipids during the transition to glutamate production, and loss of phospholipids was accompanied by a progressive decrease in the rate of glutamate uptake (Clement and Lanéelle, 1986); serine uptake was not similarly affected. Altered membrane permeability is a commonly suggested cause of increased production of both primary and secondary metabolites. Mutants of *Streptomyces fradiae* have been obtained which require oleic acid for neomycin production (Arima *et al.*, 1973): these mutants have an altered membrane composition which is restored to parental-type composition by addition of exogenous fatty acid.

If a physiological uncoupling of a transport system allows efflux of solutes normally carried by that transport system, one might expect to see the same effect in cells in which the transport system has been altered by mutation. This has been investigated in the context of proline excretion by *E. coli*

(Rancourt *et al.*, 1984). A plasmid causing proline overproduction was introduced into a series of otherwise isogenic strains bearing well-defined lesions affecting the active uptake and catabolism of L-proline: proline was found to be excreted *only* if transport and/or catabolism were impaired; no proline was detectable in supernatants from strains with normal transport and catabolic activities. However, strains defective for proline catabolism (*put* A^-) and for proline porter I (*put* P^-) or II (*pro* P^-) excreted up to 1 g proline per litre. The authors suggested that genetic lesions eliminating active uptake can be used specifically to enhance metabolite secretion. The most interesting aspect of this genetic study of proline excretion was that, even in strains capable of overproduction, excretion was observed only in strains which had defects in uptake. Similarly, an overproduction of lysine has been observed in strains of *E. coli* with defective lysine transport systems (Halsall, 1975).

If physiological or genetic inactivation of active uptake systems are required in order to observe the excretion of a primary metabolite, it is possible that similar requirements are needed for the excretion of secondary metabolites; the membrane may thus be of great importance in secondary metabolite production. This is extremely interesting in the context of antibiotic production: antibiotic producers are often sensitive to their own antibiotics under growth conditions where production is not observed (Drew and Demain, 1977; Vining, 1979; Martin and Demain, 1980; Demain, Aharonowitz and Martin, 1983); as production starts, the organisms generally become resistant. In several cases this has been shown to correlate with a reduced ability to take up the compound (Vining, 1979), although there may also be protection as a result of target-site modification. It is tempting to speculate that the onset of resistance due to reduced uptake reflects a transition from a functional active uptake system for the antibiotic, to an uncoupled transport system. Indeed, just as an uncoupled transport system is a prerequisite for observing proline excretion, it may be that the transition to an uncoupled state is necessary for antibiotic excretion.

It is now clear that many hydrophilic antibiotics with intracellular targets enter cells by active accumulation via solute transport systems (Chopra and Ball, 1982); this is exploited in the design of antimicrobial agents (Gilvarg, 1981; Ringrose, 1985). The uptake and excretion of antibiotics and other secondary metabolites by producer organisms remains a relatively neglected field.

If one considers a situation where a change from active uptake of a compound to facilitated diffusion occurs, one would predict that the immediate consequence of such a change would be a substantial relief of any end-product inhibition or repression. It is interesting to note that toxic analogues used in attempts to relieve end-product inhibition and increase yield may also produce resistant mutants with altered permeability.

Conclusions

In order to exploit fully the potential of microbial systems for secondary metabolite production, a deeper understanding of the reasons why micro-organisms make secondary metabolites is required. This review has attempted

to highlight areas where a more detailed knowledge of the biochemistry, physiology and genetics of the producer organisms should allow the exploitation of microbial systems on a more rational basis. In particular, we feel that the role of transport systems in the efflux of secondary metabolites should be systematically explored, in the light of the evidence that this is of great importance in the excretion and overproduction of primary metabolites. Although considerable problems remain to be overcome, we believe that the areas to which we have drawn attention are potentially fruitful. A better understanding of secondary metabolism, in conjunction with the extremely powerful genetic techniques now available, may allow the development of a 'modular' approach to the production of existing and novel secondary metabolites. The aim of such an approach would be to put biosynthetic genes into genetic backgrounds which would allow the gene products to operate in an active state, with an adequate supply of primary metabolite precursors and efficient excretion of secondary metabolite products. A detailed appreciation both of the circumstances required to obtain optimal secondary metabolite synthesis and of those factors acting against their synthesis or reducing yield is necessary to achieve this end. We are already at a stage where whole biosynthetic pathways of *Streptomyces* antibiotics have been cloned (Malpartida and Hopwood, 1984; Stanzak *et al.*, 1986). When we perceive how biosynthesis is integrated into the rest of the metabolism of the organism, this will allow the optimization of the genetic background into which such biosynthetic genes are introduced.

A consequence of a deeper understanding of secondary metabolic processes will doubtless be improvements in the screening of wild-type isolates and their progeny. The nature of such improvements will depend on precisely what is established.

An unravelling of the intricacies of secondary metabolism will allow the full potential of microbes for secondary metabolite production to be developed: a greater variety of compounds, many of which have been previously unknown, will undoubtedly ensue. The search for novel applications, outside the pharmaceutical industry, for existing and new compounds from micro-organisms, is likely to stimulate further interest in secondary metabolism and its products. Continued attack at the physiological, biochemical and genetic levels must eventually repay our efforts, not only by satisfying scientific curiosity, but also by resulting in economically important products.

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