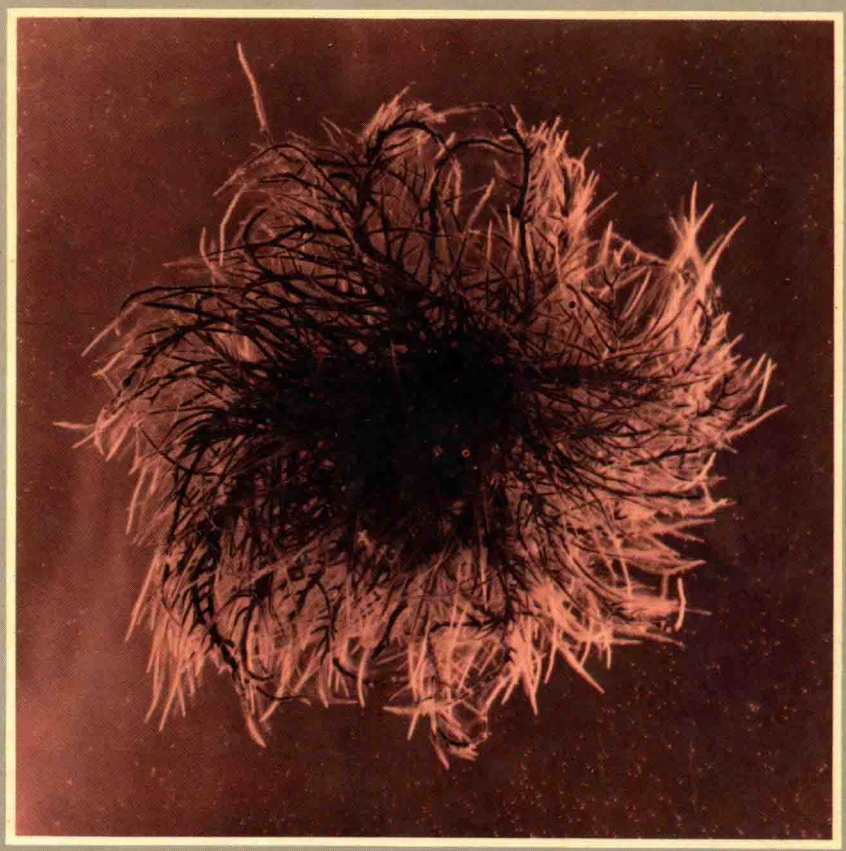
# MANIPULATING SECONDARY METABOLISM IN CULTURE



R.J. Robins and M.J.C. Rhodes

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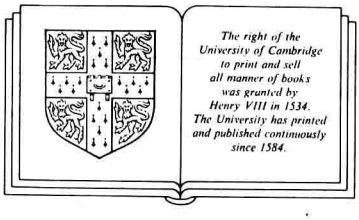


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Manipulating secondary metabolism in culture

# PREFACE

This volume contains the presentations made to the second meeting of the UK Section of the International Association for Plant Tissue Culture specifically devoted to secondary metabolites. The symposium was held at the AFRC Institute of Food Research, Norwich on the 16th and 17th September 1987. The previous meeting in this series was held at the Wolfson Institute of Biotechnology, Sheffield, in June 1985 and is also published by Cambridge University Press under the title of "Secondary metabolism in plant cell cultures" (edited by Morris, P., Scragg, A. H., Stafford, A. and Fowler, M. W.).

The title of the present volume reflects the changes in approach to plant cell cultures that have occurred over the intervening period. By 1985 a large body of descriptive information on the accumulation, or in many cases lack of accumulation, of secondary products by cells *in vitro* had been established. Much of this work involved empirical approaches, such as changes in the hormonal regimes, media constituents and other physiological factors. While in a number of cases such methods have been, at least in the short-term, successful, it was becoming increasingly apparent by the Sheffield meeting that a more fundamental understanding of why expression was poor was required. Evidence has been accumulating that the inherent heterogeneity of dispersed cultures, due to their observed genetic and biochemical instability, limits the usefulness of cell cultures for the production of secondary metabolites. In most of the successful cases, continual selection is needed to maintain high-producing strains. Yet a critical requirement for a commercially useful culture must be stability linked to high productivity. This need has instigated a number of investigations into the underlying inter-relationship between morphological and biochemical development. One rapidly expanding way in which such problems may be overcome is by the use of organ cultures, either generated by transformation with *Agrobacterium* species or maintained by a carefully balanced hormonal regime. Such systems readily lend themselves to studies of the biochemistry of secondary products and to manipulation at the genomic level.

The Norwich meeting was therefore designed to set the recent developments in context and to present an overview of the current status of research. The

underlying theme was the ways in which variation may be induced, exploited to increase the formation of valuable products, and stabilised. Particular emphasis was given to the potential role of genetic manipulation and to set the scene we were pleased to welcome Dr. M. J. Bibb of the AFRC Institute of Plant Sciences, John Innes Laboratory, Norwich to review the ways in which the formation of antibiotics by streptomycetes has been elucidated and is now being manipulated as a result of intensive genetic analysis. The success of their work is an example which those in the plant field would be only too willing to emulate!

Richard J. Robins

Michael J. C. Rhodes 16 February 1988

# ACKNOWLEDGEMENTS

A meeting of this type calls on the services of many people. We have been most generously supported by our colleagues at the Institute of Food Research. The use of the Laboratory facilities was kindly made available by Professor Peter Richmond, Head of Laboratory. Catherine Reynolds of the Liaison Office gave us much invaluable help and advice based on her wide experience of organising meetings and it is substantially due to her efforts that meals, rooms and coaches were all there when required. Gretchen Mason provided expert secretarial assistance with the preparation of abstracts, lists of contents etc, for which we are most grateful.

During the conference many helpers from our Group were on hand. John Payne, Martin Hilton and Andrew Spencer ran the slide projector; Judy Furze, Lindsay Aird, Abbi Peerless and Liz Bent ran the front of house and numerous errands; Nick Walton, John Hamill, Chris Waspe and Adrian Parr were all on hand to deal with the inevitable minor problems as they arose.

We should also like to thank all the speakers for making their contributions to the meeting and the chairmen for, mostly, keeping them to time. Having, as we did, a substantial number of conferees making an oral or poster communication prompted considerable constructive interaction – the raison d'être of such gatherings.

We are very pleased to acknowledge the financial support for the meeting most generously provided by:

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**Part VI: Techniques**

PLENARY LECTURE

# THE GENETIC MANIPULATION OF ANTIBIOTIC PRODUCTION IN *STREPTOMYCES*

M.J. Bibb, John Innes Institute and AFRC Institute of Plant Science Research, Colney Lane, Norwich, NR4 7UH, U.K.

*Abstract*. *Streptomyces* species produce the vast majority of antibiotics. Many of these secondary metabolites have important applications in medicine or agriculture. In addition to providing much-needed information on the biochemistry and regulation of antibiotic biosynthesis, gene cloning in *Streptomyces* can be used to effect improvements in yield and to produce antibiotics with novel structures. The rational manipulation of antibiotic production will generally require the isolation of antibiotic biosynthetic genes. The approaches that have been used to isolate these genes are described; they often utilise the clustered nature of antibiotic biosynthetic and resistance genes. Using genetic complementation, all of the genes required for the production of actinorhodin, a polyketide antibiotic made by *Streptomyces coelicolor* A3(2), were isolated. These genes were used to provide the first example of the production of novel antibiotics by genetic engineering and to effect a considerable increase in actinorhodin production.

## INTRODUCTION

Streptomycetes are Gram-positive mycelial soil bacteria that undergo a complex process of morphological differentiation (Chater 1984). At the onset of sporulation on solid media, or co-incident with the end of exponential growth in liquid culture, most *Streptomyces* species produce antibiotics. Indeed over 70 % of all known antibiotics are produced by members of this genus (Berdy 1980) and include anti-infectives (tetracycline, erythromycin), anti-cancer agents (daunomycin, adriamycin), animal growth promoters (monensin, tylosin), anti-helminthics (avermectins), herbicides (bialaphos), and agents for plant protection (kasugamycin, polyoxin). The commercial value of these compounds currently exceeds £ 4,000,000,000 per annum. In the search for new antibiotics the pharmaceutical industry has used a variety of approaches. These have included the development of extremely sensitive and frequently novel assay procedures to screen for organisms that produce new activities; the feeding of unnatural precursors to fermentations, using either the wild-type strain (directed biosynthesis) or strains prevented from producing their usual antibiotics, either by mutational damage (mutasynthesis) or by enyzyme inhibition (hybrid biosynthesis); and the chemical modification of known compounds, which has proved particularly successful for the beta-lactam antibiotics.

However, until recently no serious attempt had been made to utilise genetic recombination as a tool to increase genetic diversity and potentially to produce novel structures. Several recent advances in the field of Streptomyces genetics now permit such an approach. The ability to fuse protoplasts of different strains and to generate recombinants at a high frequency demonstrated one potential means whereby genetic recombination might be achieved in strain improvement programmes (Hopwood et al. 1977). However, the effectiveness of this approach in developing new antibiotics is likely to be limited by the degree of DNA sequence homology required for efficient recombination. This limitation has since been overcome by the development of an extremely efficient transformation system for Streptomyces protoplasts (Bibb et al. 1978) and by the development of a wide variety of cloning vectors, derived from both plasmids and phages indigenous to these organisms (see Chater 1986; Hopwood et al. 1986 b; Hopwood et al. 1987 for reviews). In this article the current status of the application of gene cloning to the understanding and manipulation of antibiotic production by streptomycetes is reviewed and possibilities for the future discussed. For a similar review see Hopwood (1986).

## ORGANISATION OF ANTIBIOTIC BIOSYNTHETIC GENES

The production of most antibiotics results from the sequential action of several enzymes that constitute a biosynthetic pathway; consequently many genes are generally required for the synthesis of any one compound. The tendency for the biosynthetic genes for a particular antibiotic to be clustered within the genome has been apparent for some time. These genes are generally located on the chromosome, with the plasmid-borne genes responsible for methylenomycin production by Streptomyces coelicolor A3(2) (Hopwood 1983) and Streptomyces violaceus-ruber SANK 95570 (Aguilar & Hopwood 1982) providing the only proven exceptions. However, it is only recently that the degree of clustering could be demonstrated physically. DNA cloning experiments have shown that all of the genes required for the production of actinorhodin (Malpartida & Hopwood, 1984), undecylprodigiosin (Feitelson et al. 1985; F. Malpartida & D.A. Hopwood, personal communication) and methylenomycin (L.J. Woodburn, N.K. Davis & K.F Chater, personal communication) by S. coelicolor, of tetracenomycin C by Streptomyces glaucescens (Motamedi & Hutchinson 1987), of cephamycin by Streptomyces cattleya (Chen et al. 1986) and of erythromycin by Streptomyces erythraeus (Stanzak et al. 1985) can be isolated on contiguous segments of DNA of, at most, a few tens of kilobases. Furthermore, while yet to yield definitively the entire biosynthetic pathways, cloning experiments have also demonstrated the clustering of genes involved in the biosynthesis of streptomycin in Streptomyces griseus (Distler et al. 1985; Ohnuki et al. 1985), of tylosin in Streptomyces fradiae (Cox et al. 1987), of bialaphos in Streptomyces hygroscopicus (Murakami et al. 1986), of granaticin in Streptomyces violaceoruber Tu22 (Malpartida et al. 1987), of milbemycin in Streptomyces hygroscopicus ssp. aureolacrimosus (Malpartida et al. 1987) and of oxytetracycline in Streptomyces rimosus (Butler et al. 1986) (in this case the biosynthetic genes are known to be in two distinct clusters; Rhodes et al. 1984). The clustering of antibiotic biosynthetic

genes thus appears to be a general feature of streptomycetes.

Although antibiotic biosynthesis generally occurs at the end of exponential growth, an antibiotic-producing streptomycete will usually need to be resistant to the compound that it is making. Unless the organism is intrinsically insensitive to the antibiotic, an active mechanism of resistance is required, e.g. inactivation of the intracellular form of the antibiotic, exclusion of the active form, or target site modification. There are now many examples of such mechanisms and in several cases the resistance determinants have been shown by DNA cloning to be closely linked to the corresponding biosynthetic genes (e.g. erythromycin resistance in S. erythraeus, actinorhodin and methylenomycin (Chater & Bruton 1985) resistance in S. coelicolor, bialaphos resistance in S. hygroscopicus, tetracenomycin C resistance in S. glaucescens, oxytetracycline resistance in S. rimosus, streptomycin resistance in S. griseus, tylosin resistance in S. fradiae and puromycin resistance in Streptomyces alboniger (J. Vara, personal communication) (see previous paragraph for references to the other quoted antibiotics). Thus, although streptomycetes may contain more than one resistance gene for the antibiotic that they produce (e.g. the neomycin-producing strain of S. fradiae, which is distinct from that producing tylosin, contains genes for the phosphorylation and acetylation of neomycin; Davies et al. 1979), at least one such determinant is generally closely linked to the corresponding biosynthetic genes.

The clustering of both biosynthetic and resistance genes presumably reflects the existence and evolution of mechanisms for the regulation of antibiotic production. It is thus not surprising to find several examples of genes involved in the regulation of antibiotic production closely linked to their corresponding biosynthetic genes. This has been demonstrated by DNA cloning for actinorhodin (Malpartida & Hopwood 1984), methylenomycin (Chater & Bruton 1985) and undecylprodigiosin (F. Malpartida & D.A. Hopwood, personal communication) production in S. coelicolor, for streptomycin biosynthesis in S. griseus (Ohnuki et al. 1985) and for bialaphos production in S. hygroscopicus (Anzai et al. 1987).

## APPROACHES TO CLONING ANTIBIOTIC BIOSYNTHETIC GENES

The rational manipulation of antibiotic production by gene cloning will generally require the isolation of the relevant biosynthetic genes. A variety of different approaches have been used to isolate antibiotic biosynthetic genes from streptomycetes, and many of these have taken advantage of the clustered nature of both biosynthetic and resistance determinants. Where the expression of a cloned gene was essential for its detection, an appropriate streptomycete strain has been used as recipient; this avoids the frequently observed failure of streptomycete genes to be expressed from their own regulatory sequences in potentially more convenient hosts such as Escherichia coli (Hopwood et al. 1986 a). Even if antibiotic biosynthetic genes could be readily expressed in E. coli it is by no means clear that such a host could provide the appropriate precursors to allow detection of the cloned genes. Although the systems for gene cloning in streptomycetes are

generally applicable across the genus, individual species differ in their ease of manipulation; protoplast regeneration may not have been optimised in some species and others may possess potent restriction systems. This is undoubtedly reflected in the choice of Streptomyces lividans 66 as a convenient host for many of the following cloning experiments. Each of the different approaches that have been used are described below.

Restoration of antibiotic production in blocked mutants of the producing strain

This approach requires the isolation or availability of antibiotic non-producing mutants that contain lesions in the biosynthetic pathway itself (rather than in some pleiotropic regulatory gene). Segments of DNA from a producing strain are inserted into a suitable streptomycete vector and transformants of the mutant derivative screened for the restoration of antibiotic production. This approach was used to isolate genes involved in the production of actinorhodin (Malpartida & Hopwood 1984) and undecylprodigiosin (Feitelson & Hopwood 1983) by S. coelicolor, of streptomycin by S. griseus (Distler et al. 1985; Ohnuki et al. 1985) and by Streptomyces bikiniensis (Kumada et al. 1986), of tetracenomycin C by S. glaucescens (Motamedi & Hutchinson 1987) and of clavulanic acid by Streptomyces clavuligerus (Bailey et al. 1984). This approach does not necessarily require that the entire gene corresponding to the mutant allele be present within the cloned segment, since recombination between the cloned segment and the mutant homologue in the chromosome may be sufficient to restore antibiotic production.

Cloning an entire biosynthetic pathway into a non-producing host

Given the general clustering of antibiotic biosynthetic genes, this one-step cloning procedure might appear to be the most efficient way in which to isolate all of the genes required for the production of a particular antibiotic. However, it is not without technical difficulties. It will generally require the cloning of large segments of DNA (of approximately 30 kilobases) from the producing organism into a convenient recipient and a suitable screening procedure for the required transformants. The cloning of such large segments may limit the choice of vector to one of the low copy number plasmid derivatives and the resulting low yield of recombinant DNA may hinder subsequent analysis. Nevertheless, such an approach has been used to isolate all of the genes required for cephamycin production by S. cattleya (in S. lividans; Chen et al. 1986) and for methylenomycin production by S. coelicolor (in a non-producing derivative of S. coelicolor that lacks the plasmid carrying these genes; L.J. Woodburn, N.K. Davis & K.F. Chater, personal communication).

Cloning an antibiotic resistance gene into a convenient recipient

The simplicity of selecting for antibiotic resistant clones makes this approach perhaps the easiest of all. The recipient may be a convenient cloning host, such as S. lividans, or a sensitive mutant of the producing strain. Once the resistance gene has been cloned, two

approaches may be taken, each of which assumes that the resistance determinant is closely linked to its corresponding biosynthetic genes. The ability of linked biosynthetic genes to restore antibiotic production to blocked mutants of the producing strain may be assessed directly, as for the isolation of genes involved in tetracenomycin C production by S. glaucescens (Motamedi & Hutchinson 1987). Alternatively, the cloned resistance gene may be used as a hybridisation probe to screen a genomic library for adjacent biosynthetic genes. This latter approach was used to isolate genes involved in bialaphos production by S. hygroscopicus (Murakami et al. 1986), in erythromycin production by S. erythraeus (Stanzak et al. 1986) and in oxytetracycline production by S. rimosus (Rhodes et al. 1984). In each case, with the exception of S. rimosus where an oxytetracycline-sensitive isolate of the producing strain was used, the resistance genes were initially cloned in S. lividans.

Detection of an individual antibiotic biosynthetic gene by cloning in a standard host

Two different versions of this approach have been used to isolate antibiotic biosynthetic genes, and both used S. lividans as cloning host. This method requires some detailed knowledge about one of the steps in the biosynthetic pathway and thus may not be of general applicability.

Phenoxazinone synthase is involved in the production of actinomycin D by Streptomyces antibioticus. Segments of DNA from the producing organism were cloned in S. lividans, which normally lacks this activity, and pools of transformants (approximately 5,000) were assayed for the acquisition of PHS using the ability of this enzyme to convert 3-hydroxyanthranilic acid into yellow cinnabarinic acid in vitro. Sib-selection was then used to isolate the appropriate clones (Jones & Hopwood 1984).

Two different detection procedures were used by Gil and Hopwood (1983) to isolate a gene encoding para-aminobenzoic acid (PABA) synthase. This enzyme, which is inhibited by the antibiotic sulphonamide, is responsible for the first step in the biosynthesis of candicidin by S. griseus, but is also required for the production of PABA for growth. In one approach, segments of DNA from a wild type S. griseus strain were cloned in a PABA auxotroph of S. lividans and selecton made for prototrophy. In the other approach, segments from a sulphonamide resistant mutant of S. griseus were cloned in S. lividans and selection was made for sulphonamide resistance. Both approaches yielded the same gene.

Mutational cloning in the producing strain

This approach depends on the use of derivatives of the temperate phage ØC31. This broad host range streptomycete phage can lysogenise a large number of Streptomyces species and normally does so by integrating into the chromosome at a specific location via its own phage attachment site (attP; Chater 1986). Derivatives of this phage have been developed that contain selectable antibiotic resistance

markers but that lack attP; although unable to lysogenise cells by the normal route, insertion of chromosomal fragments into these vectors permits the selection of stable antibiotic resistant lysogens by recombination between the cloned segment and its homologue present in the chromosome. Generally speaking, if the cloned segment is internal to a transcription unit, then integration of the recombinant phage results in disruption of the transcript to give a mutant phenotype. Hence it is possible to insert segments of chromosomal DNA from the producing strain into one of these vectors, to introduce the recombinant phage into the producing organism either by transfection or, after a round of lytic growth in a more convenient cloning host, by natural phage infection and to obtain lysogens by selecting for the antibiotic resistance marker carried by the phage. Screening these lysogens for the loss of antibiotic production should yield phage derivatives containing biosynthetic DNA. This DNA can be isolated directly from spontaneously released phages and used as a probe to screen genomic libraries for linked biosynthetic genes. Alternatively, it can be isolated by cloning DNA obtained from a non-producing lysogen into an appropriate host and selecting for the antibiotic resistance marker of the phage vector; the choice of an appropriate restriction enzyme should ensure the isolation of flanking antibiotic biosynthetic DNA.

The mutational cloning procedure was used to isolate genes involved in methylenomycin production from S. coelicolor (Chater & Bruton 1983) and offers the potential benefit that conditions for cloning in the species of interest need not be developed provided that the eventual host is sensitive to ØC31 infection; the primary cloning experiments can all be carried out in a host such as S. lividans prior to introduction into the producing organism by natural infection. The frequency of blocked mutants generated by mutational cloning should be no lower than that observed for conventional mutagenesis (Chater & Bruton 1985; Chater et al. 1985) and does not constitute a severe disadvantage.

Cloning for over-production

Provided a convenient and semi-quantitative screening procedure is available for the antibiotic of interest then random cloning of fragments of DNA from the producing organism into itself, or into a strain that makes the same antibiotic, may be used to clone antibiotic biosynthetic genes by assaying for increases in antibiotic production. There are two obvious ways in which this may occur. Increased gene dosage may enhance metabolic flow through a pathway, either by affecting particular rate-limiting steps or by increasing the general level of pathway enzymes (this may result from the use of a high copy number vector or from efficient transcriptional readthrough from a vector promoter). Alternatively, the cloning of a regulatory gene may disturb the normal regulation of the pathway and cause an increase in production. This approach was used to isolate genes involved in the biosynthesis of undecylprodigiosin by cloning DNA from S. coelicolor into the closely related strain S. lividans using a multi-copy plasmid vector; clones over-producing the red-pigmented antibiotic could be readily identified (J. Niemi, J.M. Ward, F. Malpartida and D.A. Hopwood, personal communication). Although the precise mechanism is not known,