Biochemistry and Genetic Regulation of Commercially Important Antibiotics

Edited by

Leo C. Vining

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PREFACE

Commercial production of antibiotics has been an established area of biotechnology for about 40 years. Over this period, it has undergone intensive development, and recent progress in the manipulation of genetic material promises even more striking advances in the years ahead. Genetic engineering in the antibiotic field depends on a sound knowledge of the way in which the drugs are biosynthesized. The genetic systems in producing microorganisms must also be understood. We need to know their scope and complexity, but we also require information about the location and function of genes that specify or control the enzymes for antibiotic biosynthesis. As secondary metabolites, antibiotics share a wide literature on biochemical and physiological aspects of the fermentation process. It, too, is a rich source of information, pointing the way to future development of antibiotic biotechnology.

This book surveys current knowledge in these areas. The first three chapters deal broadly with the genetics of the most important antibiotic producers and with the range of metabolic controls that affect antibiotic production. In each subsequent chapter, the biosynthesis of a commercially important group of antibiotics is related to genetic and metabolic control systems in the producing organisms. For some antibiotic groups, our knowledge extends little further than an appreciation of biosynthetic relationships, and, in general, much remains to be done before the genetic control of antibiotic production is well understood.

The aim of this book, in bringing the information together for the first

time, is to provide an authoritative reference for those in industry, universities, and other institutions concerned with microbial biosynthesis of useful products. The book should be of particular value to those seeking to apply the new techniques of genetic engineering in this field. Because it not only considers the most recent advances but also provides the background for an overall appreciation of the subject, the book should also meet the needs of advanced students in applied microbiology and biochemistry classes.

LEO C. VINING

CONTENTS

CHAPTER

1

Actinomycete Genetics and Antibiotic Production

David A. Hopwood

I. INTRODUCTION

There are several recent reviews that deal with the genetics of antibiotic production by actinomycetes (Hopwood and Merrick, 1977; Hopwood, 1979) and the possible involvement of plasmid-borne genes in some cases (Hopwood, 1978; Okanishi, 1979). In the few years since these summaries were compiled, the fragmentary knowledge of the number and organization of the genes concerned with the synthesis of even the most important antibiotics has not grown very greatly. However, there has been a significant increase in the versatility and scope of genetic analysis in antibiotic-producing actinomycetes (Chater and Hopwood, 1982), so a much more complete and incisive study of the subject is now possible. Most of this chapter is concerned with these methodological advances. In later sections, I review the progress that has been made, largely by use of the older genetic techniques, in understanding genetic control of antibiotic biosynthesis by certain actinomycetes. From this, we see that progress has been made in analyzing some examples where antibiotic biosynthesis is determined by clusters of chromosomal genes. As for plasmid involvement, methylenomycin A in *Streptomyces coelicolor* A3(2) (and now also in a related strain of *S. violaceus-*

ruber) remains the only well-established example of an antibiotic whose biosynthesis is determined by plasmid-borne structural genes, although tylosin in *Streptomyces fradiae* may belong in this category. The last couple of years have seen some further preliminary suggestions of plasmid involvement in antibiotic production but a larger number of cases in which such suggestions have later been questioned by the authors of the original claims. The subject is still open. The genetic and molecular tools are available for decisive analysis of specific examples of antibiotic synthesis; where they have not been used, conclusions should be viewed with caution.

This chapter is not primarily concerned with industrial applications of actinomycete genetics; its main preoccupation is with the *analysis* of genetic control of antibiotic biosynthesis rather than with strain construction. However, in the final section, some examples of antibiotic discovery through genetic recombination are discussed, partly to emphasize the latent potential of this approach and to show how understanding genetic control of antibiotic biosynthesis would enable such work to proceed in a much more certain fashion. There is no doubt that genetics has a considerable part to play in the rational or semirational development of higher-yielding strains of industrial antibiotic producers. This topic has been covered elsewhere (Hopwood and Chater, 1980) and I will not discuss it here, except to point out that empirical gene amplification by shotgun self-cloning on multicopy plasmid vectors, made possible by recent advances in recombinant DNA technology, is a realistic additional approach to this problem (Chater et al., 1982).

II. ROUTES TO GENETIC ANALYSIS IN ACTINOMYCETES

The actinomycetes, and particularly certain members of the genus *Streptomyces*, might now be regarded as the most versatile group of microorganisms in terms of the range of tools available for use in genetic analysis. In the following sections, some of the features of these genetic tools are outlined.

A. Natural Systems of Genetic Recombination

1. Plasmid-mediated Conjugation. Intrastrain genetic recombination in mixed cultures of auxotrophic mutants has been reported in many streptomycetes and in *Nocardia* (formerly *Streptomyces*) *mediterranea* and *Micromonospora* spp. (reviewed by Hopwood and Merrick, 1977). Recombination between strains of other *Nocardia* spp., now classified as *Rhodococcus*, has also been studied (Adams and Brownell 1976; Brownell et al., 1981), but these organisms, like the related strains of *Mycobacterium* in which recombination has also been reported, are not known as antibiotic producers. Evidence that such recombination in *Streptomyces* and *N. mediterranea* is due to conjugation between hyphae rests primarily on genetic analysis, which showed

that groups of distantly linked genes from each parent are frequently inherited together by recombinants; this result is expected for a conjugation process but not for recombination brought about by transduction or transformation. That this conjugation is (at least in part) plasmid-mediated has been demonstrated in *S. coelicolor* A3(2), *Streptomyces lividans* 66, and *Streptomyces rimosus*.

The wild-type *S. coelicolor* A3(2) harbors two autonomous sex plasmids, SCP1 and SCP2, which are responsible for yielding recombinants at frequencies up to about 10^{-5} of the total progeny of a mixed culture of two genetically marked strains (Bibb and Hopwood, 1981), since recombination between pairs of SCP1$^-$ SCP2$^-$ strains occurs only at frequencies of 10^{-7} or lower. Direct interaction of SCP1 with the chromosome, leading to chromosomal transfer, is shown by the behavior of strains carrying SCP1 integrated into the chromosome (NF strains and other donor types), which give extremely high levels (greater than 10^{-1}) of recombinants in crosses with SCP1$^-$ strains (Hopwood et al., 1973) as well as by the isolation of SCP1′ strains (Hopwood and Wright, 1976). SCP2 has been directly implicated in gene exchange through the discovery of SCP2* variants, which, though still present autonomously, enhance chromosomal recombination by a factor of at least 10^3 (Bibb et al., 1977; Bibb and Hopwood, 1981). It is possible that the "residual" recombination manifested in crosses between pairs of SCP1$^-$ SCP2$^-$ strains is also plasmid-mediated, since there are at least two further plasmids in *S. coelicolor* A3(2). SLP1 occurs as a DNA sequence integrated into the chromosome close to the *strA* locus; it becomes excised from the chromosome, together with contiguous chromosomal segments of variable length, and can be found as autonomous ccc DNA molecules of a series of sizes (SLP1.1, SLP1.2, etc.) after interspecific matings (or by in vitro DNA transfer) into *S. lividans* 66 (Bibb et al., 1981). SLP4 was also detected in *S. lividans* after matings between *S. coelicolor* A3(2) and *S. lividans* 66 derivatives. In this case, plasmid DNA was not detected; the plasmid was revealed by the fact that putative SLP4$^+$ *S. lividans* transconjugants inhibited the parent *S. lividans* culture by the so-called "lethal zygosis" reaction, a phenotype (see later) characteristic of conjugative *Streptomyces* plasmids (Bibb et al., 1977). Both SLP1 and SLP4 promote recombination of chromosomal genes in *S. lividans*. Whether or not one or both of these sex plasmids is responsible for the low-level recombination seen in SCP1$^-$ SCP2$^-$ matings of *S. coelicolor* A3(2) is not yet known, since strains lacking SLP1 or SLP4 have not been isolated.

In *S. lividans* 66 itself, the picture with respect to plasmid involvement in chromosomal recombination is somewhat simpler (T. Kieser, D. A. Hopwood, and H. M. Wright, unpublished results). Marked derivatives of the wild-type undergo recombination at a level of about 10^{-6}. This has been shown to be due to the presence of one, or perhaps two, plasmids. These were detected by the isolation, following protoplast formation and regeneration (see later), of strains sensitive to lethal zygosis by the parent strain. Two

independent plasmids, SLP2 and SLP3, were revealed by the isolation of $SLP2^-$ $SLP3^+$ strains (sensitive to lethal zygosis by $SLP2^+$ but not by $SLP3^+$ strains), $SLP2^+$ $SLP3^-$ strains (sensitive to lethal zygosis by $SLP3^+$ but not $SLP2^+$ strains), and $SLP2^-$ $SLP3^-$ strains (sensitive to lethal zygosis by both plasmids). SLP2 certainly promotes chromosomal recombination, which occurs at a level of around 5×10^{-5} in $SLP2^+ \times SLP2^-$ crosses. However, it is not certain that SLP3 can do so. In any event, the important finding is that pairs of $SLP2^-$ $SLP3^-$ strains yield no detectable recombinants (only occasional nonparental genotypes of classes explicable by reversion of single markers have been detected, rather than the more complex genotypes expected for most recombinants). It follows that all detectable recombination in *S. lividans* 66 is plasmid-mediated. $SLP2^-$ $SLP3^-$ strains therefore provide a useful background in which the effects of other plasmids can be studied (Kieser et al., 1982).

In *S. rimosus*, part of the recombination observed in pairwise crosses was shown to be mediated by a plasmid (SRP1), but a residual low level of recombination was observed in crosses between pairs of $SRP1^-$ strains (Friend et al., 1978). Recently, a second sex plasmid, SRP2, has been identified in these strains (E. J. Friend, personal communication); this is responsible for part of the recombination observed in $SRP1^- \times SRP1^-$ crosses.

Apart from plasmids native to *S. coelicolor* A3(2) and *S. lividans* 66, some further plasmids, originally detected in other strains, have been transferred to these two strains and found to promote chromosomal recombination. Notable is pIJ101, a multicopy, broad-host-range plasmid from *S. lividans* ISP5434, which is a very efficient sex plasmid, giving 1% or more of non-selected recombinants when present in either *S. coelicolor* A3(2) or *S. lividans* 66 (Kieser et al., 1982). This finding may be particularly significant because it suggests that this, or other broad-host-range plasmids, might be used to introduce efficient chromosomal recombination, and so a versatile genetic mapping capability, into strains that are originally found to give a few or no detectable recombinants.

The nature of the conjugation process in *Streptomyces* is almost unknown, although it is clear that it involves few plasmid-coded functions (many fewer than in gram-negative bacteria; Willetts, 1980) since small plasmids are conjugative. SLP2, SLP3, SLP4, SRP1, and SRP2 have not been detected physically and so are of unknown size; SCP1 is large, probably in excess of 170 kb (Westpheling, 1980), like a related sex plasmid, pSV1, which resembles SCP1 in coding for methylenomycin production and resistance (Aguilar and Hopwood, 1982). SCP2 is about 30 kb in size (Schrempf et al., 1975; Bibb et al., 1977; Schrempf and Goebel, 1977), but no more than half of it is required for conjugation (Bibb et al., 1980a). The size of the smallest SLP1 plasmid so far isolated, SLP1.6, is only 9.4 kb (Bibb et al., 1981), and pIJ101 is only 8.9 kb in size (Kieser et al., 1982). Moreover, derivatives of pIJ101 that are nonconjugative have deletions or insertions

of DNA affecting only about 2 kb of the plasmid, suggesting that very few plasmid-borne genes are needed for conjugation.

2. Generalized Transduction. This is well documented in *Streptomyces venezuelae*, in which a phage called ϕSV1 has been found to promote transduction of a number of auxotrophic mutations to prototrophy (Stuttard, 1979).

3. Transformation of Competent Mycelia. Strains of *Streptomyces virginiae* and *Streptomyces kasugaensis*, were found to be unusual in going through a stage of competence for the uptake of linear fragments of DNA (Roelants et al., 1976), but these experiments involved radioactively labeled DNA, not genetic markers, and no system of genetic transformation was developed. In *Thermoactinomyces vulgaris*, a classical competence for transformation was found, and generalized transformation of chromosomal markers was studied (Hopwood and Wright, 1972). This thermophilic strain is probably not closely related to the *Streptomyces*, *Micromonospora*, and *Nocardia* spp. (conceivably, it represents a parallel development of the mycelial habit from a different eubacterial stock), and so does not provide a model for genetic manipulation of these mesophilic organisms, although it could perhaps be harnessed to analyze antibiotic production by *Thermoactinomyces* itself (Pirali et al., 1974).

B. Artificial Systems of Genetic Analysis

1. Generalized Recombination through Protoplast Fusion. Protoplast fusion is a means of bringing about intrastrain chromosomal recombination at very high frequency (Hopwood et al., 1977; Baltz, 1978; Ochi and Katz, 1978). Up to 20% of the total progeny arising from nonselective regeneration of fused protoplasts can be recombinant genotypes when fusion is carried out under optimal conditions in the presence of about 50% polyethyleneglycol (PEG) (reviewed by Hopwood, 1981a). In fusions between multiply marked strains, when crossing-over around the whole chromosome could be monitored, it was deduced that crossing-over can occur in widely separated map intervals, indicating a probable complete diploidy of the fusion bodies (Hopwood and Wright, 1978; Hopwood, 1981a); this contrasts with the situation in the mero-diploids generated by conjugation (Hopwood, 1967). Also, several rounds of recombination occur between fusion and recovery of the haploid progeny of regeneration (Hopwood and Wright, 1978). Both these factors lead to a loosening of linkage, making the determination of map locations for distant markers more difficult, though not impossible (Baltz, 1980).

2. Plasmid "Curing" by Protoplasting and Regeneration. As in *Staphylococcus aureus* (Novick et al., 1980), so in *Streptomyces* (Hopwood, 1981b; Aguilar and Hopwood, 1982), with some plasmids the simple expedient of forming and regenerating protoplasts can lead to a significant frequency of plasmid-free individuals. This effect has been suggested to occur at protoplast regeneration in *S. aureus* (Novick et al., 1980); in *Streptomyces*, loss at protoplast formation and regeneration have not been discriminated (Hopwood, 1981b).

3. Transformation/Transfection of Protoplasts by Plasmid or Phage DNA. When *Streptomyces* protoplasts are treated with plasmid or phage DNA in the presence of 20–25% PEG and are subsequently allowed to regenerate, transformants or transfectants are obtained (Bibb et al., 1978; Kruegel et al., 1980; Suarez and Chater, 1980a). At saturating DNA concentrations, up to 80% of regenerating protoplast colonies can be transformed with plasmid DNA (Bibb et al., 1980b). Phage/host systems vary in this respect; in some, over 1% of protoplasts are transfected, while in others a "competent" fraction, representing only about 10^{-4} of protoplasts, become transfected (Suarez and Chater, 1980a). At nonsaturating DNA concentrations, up to 10^7 transformants can be achieved per microgram of plasmid DNA (Thompson et al., 1982a), but only about 5×10^5 transfectants can be achieved per microgram of phage DNA. However, if positively charged DNA-free liposomes are added to the mixture of phage DNA and protoplasts and the PEG concentration is raised to that optimal for protoplast fusion (about 50%), at least 10^7 transfectants can be obtained per microgram of phage DNA (Rodicio and Chater, 1982). This treatment appears also to aid reproducible recovery of the maximum frequency of plasmid transformants, or may even raise it.

4. Gene Cloning on Plasmid or Phage Vectors. Use of the above transformation/transfection system allowed the development of DNA cloning procedures on endogenous *Streptomyces* plasmid and phage vectors (Bibb et al., 1980a; Suarez and Chater, 1980b; Thompson et al., 1980). SLP1.2 has provided the best low-copy-number plasmid-cloning vectors so far developed—namely, pIJ41 and pIJ61, which carry two antibiotic resistance markers, one for vector selection and the other allowing clone recognition by insertional inactivation (Thompson et al., 1982b). However, the possibly rather restricted host range of the SLP1 plasmids (*S. lividans* 66 and *Streptomyces reticuli* to date) may limit their utility as vectors for streptomycetes in general. SCP2 may have a slightly wider host range and has been used for cloning in *S. coelicolor* A3(2) (Bibb et al., 1980a; Thompson et al., 1982a), where SLP1-based vectors cannot be used because of incompatibility with the resident chromosomal SLP1 sequences, as well as in *S. lividans* 66 and *Streptomyces parvulus* ATCC 12434. The development of SCP2-based vectors