Streptomyces as producers of diverse antibiotics

Members of the genus *Streptomyces* belong to the family *Actinomycetales*, a division of the Gram-positive bacteria characterized by a high genomic G+C content (mean 74 mol%) (Stackebrandt & Woese, 1981; Goodfellow & Cross, 1984; Fox & Stackebrandt, 1987; Goodfellow *et al.*, 1992; Stackebrandt *et al.*, 1992). The branching hyphae of these saprophytic soil bacteria obtain nutrients and energy largely by degrading insoluble organic material through the production of a variety of extracellular hydrolytic enzymes (McCarthy & Williams, 1992). In response to appropriate signals, generally believed, but not proven, to include nutrient limitation, the substrate mycelium gives rise to aerial hyphae, which yield chains of uninucleoidal spores to provide a mechanism for dispersal and the colonization of new environments (Chater & Losick, 1996). Streptomyces also exhibit a remarkable capacity for biochemical differentiation, producing a wide variety of secondary metabolites that include about half of all known microbial antibiotics (Bérczy, 1984; Miyadoh, 1993). Many of these compounds have important applications in human medicine as antibacterial, antitumour and antifungal agents, and in agriculture as growth promoters, agents for plant protection (generally fungicides), antiparasitic agents and herbicides. Antibiotic production in streptomycetes generally coincides with the onset of morphological differentiation, and the isolation of mutants that are deficient in both processes suggests at least some common elements of genetic control (Champness & Chater, 1994).

Within the last few years considerable progress has been made towards understanding the genetic and molecular basis of the regulation of antibiotic production by streptomycetes. These studies have focused largely on *Streptomyces griseus*, the producer of streptomycin (reviewed by Horinouchi & Beppu, 1995, and Piepersberg, 1995), and on *Streptomyces coelicolor* A3(2) (reviewed by Chater & Hopwood, 1993; Hopwood *et al.*, 1995; Chater & Bibb, 1996). Analysis in *S. coelicolor* in particular has been assisted by the availability of natural and artificial genetic systems, and by the ability of the strain to produce at least four antibiotics, enabling studies of pathway-specific and pleiotropic regulation of antibiotic production to be made. In addition, studies of the physiology and developmental biology (Hodgson, 1992; Chater, 1993; Champness & Chater, 1994; Chater & Losick, 1996) of *S. coelicolor* are yielding information that will be essential for a thorough understanding of the interactions that occur between primary and secondary metabolism, and for elucidating the regulatory networks that influence both morphological differentiation and antibiotic production.

In addition to its fundamental interest, a thorough knowledge of how the complex antibiotic biosynthetic pathways are regulated will inevitably allow for the development of more rational approaches to strain improvement (Chater, 1990). Although there is much to learn, this article summarizes work carried out in the author’s laboratory that has contributed to our current understanding of the regulation of antibiotic production in *S. coelicolor*. Much of this information has arisen from the application of gene cloning to streptomycetes. This same technology has revealed some unusual features of gene expression in streptomycetes that will also be reviewed.

Antibiotic production in streptomycetes occurs in a growth-phase-dependent manner

Antibiotic production in streptomycetes generally occurs in a growth-phase-dependent manner (Demain & Fang, 1995). For example, production of the anti-fungal antibiotic candidin in liquid-grown cultures of *S. griseus* occurs after vegetative growth, estimated by monitoring DNA synthesis, has ceased [Fig. 1, from Martín & McDaniel, 1975; note that biomass continues to increase after the onset of candidin production, presumably through the accumulation of storage compounds such as glycogen (Braña *et al.*, 1986) or triacylglycerol (Olukoshi & Packter, 1994)]. Similarly, in the perhaps more natural conditions of agar surface-grown cultures of *Streptomyces antibioticus*, oleandomycin production again occurs only after vegetative growth has finished (Fig. 2, from Méndez...
**S. coelicolor as a model streptomycete**

A thorough understanding of how antibiotic production is regulated will require an integrated approach that combines genetics with biochemistry, physiology and molecular biology. The most genetically characterized and genetically manipulable streptomycete is *S. coelicolor* (Hopwood et al., 1985). This organism has a well-developed genetic and physical linkage map (Kieser et al., 1992; Redenbach et al., 1996), and, in contradiction to the paradigm of *Escherichia coli*, possesses a linear, not circular, chromosome of approximately 8 Mb (Lin et al., 1993).

In addition, *S. coelicolor* possesses a number of accessory genetic elements, including at least two insertion sequences, IS110 and IS117 (Chater & Hopwood, 1993). It contains SCP1, a large linear plasmid of 350 kb (Kinashi & Shimaji-Murayama, 1991) that carries genes for production of the cyclopentanone antibiotic methylenomycin (Kirby & Hopwood, 1977) (still the only proven example of plasmid-determined antibiotic biosynthesis), and an integrative element, SLP1, that normally resides in a stable form in the chromosome, but which is capable of excision, conjugation and autonomous replication in other streptomycetes (Bibb et al., 1981; Omer & Cohen, 1984; Brasch & Cohen, 1993). *S. coelicolor* also possesses a conventional circular plasmid, SCP2, which is self-transmissible and capable of mobilizing the chromosome (Bibb et al., 1977; Bibb & Hopwood, 1981). High-frequency transfer of the plasmid into a lawn of recipient bacteria that lack the element causes inhibition of growth and sporulation of the recipient lawn. The resulting 'pocks', which were shown to be a general property of...
self-transmissible streptomycete plasmids, provided the plasmid-determined phenotype that was used to develop the transformation system that is currently used worldwide (Bibb et al., 1978). This technique is based on the addition of DNA, in the presence of PEG1000, to protoplasts made by treating mycelium with lysozyme in an osmotically stabilizing medium.

Some unusual features of gene expression in streptomycetes

The availability of a transformation system, and of readily isolable streptomycete plasmids and phages, led to the rapid development of DNA cloning systems (Bibb et al., 1980; Suarez & Chater, 1980; Thompson et al., 1980; reviewed by Hopwood et al., 1987). The ability to clone streptomycete genes, and to examine their expression in vivo, revealed some of the basic features of gene expression in these organisms. It soon became apparent that streptomycetes possess a high degree of promoter sequence heterogeneity (Bibb et al., 1985a; Janssen et al., 1989; Janssen & Bibb, 1988, 1990; Strohl, 1992). Some of this variability reflects the occurrence of multiple σ factors, which confer on RNA polymerase core enzyme the ability to recognize different subsets of promoter sequences (Westpheling et al., 1985; Buttner et al., 1988). Thus far, at least eight different σ factors have been identified in S. coelicolor (Buttner, 1989; Chater et al., 1989; Lonetto et al., 1994; Potůcková et al., 1995), and presumably more remain to be discovered.

Many streptomycete genes were found to be transcribed from more than one promoter (Bibb et al., 1985b; Buttner et al., 1987; Janssen & Bibb, 1988, 1990; Janssen et al., 1989; Strohl, 1992), sometimes by more than one form of RNA polymerase holoenzyme (Buttner et al., 1988; Westpheling & Brawner, 1989). Although the significance of this for the regulation of gene expression is not known, it may reflect changes in the availability of σ factors and other regulatory proteins during development. It also became apparent that a number of streptomycete mRNAs contravened the dogma of the time by lacking untranslated leader sequences (Janssen et al., 1989; Bibb et al., 1994; Strohl, 1992). Instead, they commenced either at, or one nucleotide before, the translation start codon, and therefore lacked conventional ribosome-binding sites, which show a degree of complementarity to a sequence close to the end of 16S rRNA (Stormo et al., 1982). For example, the primary transcripts for the neomycin resistance gene (aph) of Streptomyces fradiae (Janssen et al., 1989) and for the erythromycin-resistance gene (ermE) of Saccharopolyspora erythraea (formerly Streptomyces erythraeus) (Bibb et al., 1994) start at the first nucleotide of the ATG start codon and one nucleotide before the GTG start codon, respectively. Presumably, sequence information internal to such mRNAs is responsible for ribosome recognition. Although the reason for this unusual situation is not clear, the majority of leaderless transcripts found so far in streptomycetes are derived from antibiotic-resistance genes that confer immunity to an antibiotic made by the strain, and it may be that these leaderless mRNAs play an important role in the co-ordinate expression of antibiotic biosynthesis and resistance in the producing organism (Jannsen, 1993).

The advent of DNA cloning in streptomycetes inevitably led to the rapid accumulation of nucleotide sequence data for a variety of genes, confirming the predictable consequences of the high G + C content of streptomycete DNA on codon usage (Wright & Bibb, 1992). With the G + C content of the third codon position averaging around 90 mol%, streptomycete genes possess a highly biased codon usage. Consequently, a number of codons ending with A or U occur very infrequently, and at least one of these, the leucine codon UUA, may have acquired a regulatory function (see below). Although the high G + C content of streptomycete DNA can make sequence determination difficult, it does offer one compensation—the highly biased codon usage results in a predictable and distinct distribution of G + C content across codons. This characteristic feature formed the basis of a simple and reliable computer program for the localization of protein coding sequences and for the identification of frameshift errors that is also applicable to other organisms with high G + C DNA (Bibb et al., 1984).

Antibiotic biosynthetic genes occur in clusters that generally contain pathway-specific regulatory genes

It was not long before DNA cloning was applied to the study of antibiotic production. It was well-established that some streptomycetes made several different classes of antibiotics and that these compounds were generally the products of complex biosynthetic pathways. Although there was genetic evidence to indicate clustering of the biosynthetic genes, the ability to clone DNA in streptomycetes provided the first direct physical evidence for the extent of this clustering, and also served to identify the presence within these clusters of resistance determinants and pathway-specific regulatory genes.

This tight clustering was first demonstrated by cloning a 35 kb fragment of S. coelicolor DNA in Streptomyces parvulus that conferred production of the blue-pigmented polyketide antibiotic actinorhodin on its new host, which is not known to make any structurally related compounds (Malpartida & Hopwood, 1984). These studies were followed shortly afterwards by the first example of the use of DNA cloning to produce a new antibiotic – introduction of the actV genes from S. coelicolor into a Streptomyces strain that produced medermycin resulted in the synthesis of a novel hybrid compound, mederrhodin (Hopwood et al., 1985b).

Gene cloning has since been used to study the regulation of antibiotic production, and it is this topic that is considered in the remainder of this article. Much of the following work, the aim of which is to decipher the physiological signals and underlying regulatory mechanisms that are responsible for the growth phase-dependence of antibiotic production in S. coelicolor, was
conducted in the author’s laboratory, and apologies are given to those whose work is not covered in similar detail (for additional information see Chater & Bruton, 1985; Adamidis et al., 1990; Adamidis & Champness, 1992; Champness et al., 1992; Fernández-Moreno et al., 1992; Hobbs et al., 1992; for reviews see Champness & Chater, 1994; Hopwood et al., 1995; Chater & Bibb, 1996). Many factors appear to influence the onset of antibiotic production in streptomycetes (Fig. 3; Demain et al., 1983; Demain, 1992; Demain & Fang, 1995; Chater & Bibb, 1996); some of these are considered below.

**Pathway-specific regulatory genes play a pivotal role in determining the onset of antibiotic production in S. coelicolor**

We have focused our attention on two of the four antibiotics made by *S. coelicolor* – the blue-pigmented polyketide actinorhodin (Act) and the chemically distinct red-pigmented tripyrrole undecylprodigiosin (Red). Under conditions of nitrogen limitation in liquid culture, Act and Red show classical production kinetics, with synthesis limited to stationary phase. By studying the regulation of both of these compounds, we can examine pathway-specific and pleiotropic regulation of antibiotic production. Many antibiotic-biosynthetic-gene clusters contain pathway-specific regulatory genes, most of which appear to act as transcriptional activators (Chater & Bibb, 1996); for the Act and Red pathways, the genes are *actII-ORF4* (Fernández-Moreno et al., 1991) and *redD* (Narva & Feitelson, 1990), respectively. Their products belong to an expanding family of regulatory proteins that includes the pathway-specific regulatory gene for daunorubicin production in *Streptomyces peucetius* (Stutzman-Engwall et al., 1992), and the putative pleiotropic regulatory gene *afsR* (Horinouchi et al., 1990). *ActII-ORF4* and *RedD* show 33% amino acid sequence identity, but as one would expect, they are unable to substitute for one another even when over-expressed, although *ActII-ORF4* and *DnrI* apparently can (Stutzman-Engwall et al., 1992). In liquid culture, transcription of *redD* (Takano et al., 1992) and of *actII-ORF4* (Gramajo et al., 1993) occurs only during transition and stationary phase, and is followed shortly afterwards by transcription of the corresponding biosynthetic structural genes. If expression of either pathway-specific regulatory gene is forced during exponential growth, premature transcription of the corresponding biosynthetic structural genes results, and antibiotic production occurs while rapid growth continues. Thus, the principal limitation to antibiotic production, at least in these defined laboratory conditions, appears to be the availability of enough of the pathway-specific regulatory genes.
specific activator protein. Over-expression of the pathway-specific regulatory genes increases markedly the amount of antibiotic made (Strauch et al., 1991; Gramajo et al., 1993).

**Pleiotropic regulatory genes also control antibiotic production**

In addition to pathway-specific regulatory genes, *S. coelicolor* possesses several genes with pleiotropic effects on antibiotic production. At least some of these are likely to play regulatory roles. The product of one, AfsR, is a homologue of ActII-ORF4 and RedD, with its N-terminal region showing 33% amino acid sequence identity to each of the pathway-specific regulatory proteins. AfsR was first identified through its ability to stimulate Act production in *Streptomyces lividans*, a close relative of *S. coelicolor* in which the act and red genes are normally poorly expressed (Horinouchi et al., 1983). Recent work identified a small gene (afsS) located immediately downstream of afsR that encodes a protein of 63 amino acids which alone is capable of stimulating antibiotic production (Matsumoto et al., 1995; Vogtli et al., 1994). Re-examination of the earlier data does not contradict the hypothesis that afsR, and not afsS, is responsible for Act overproduction; moreover, the reduction in Act production that followed disruption of afsR could have reflected a polar effect on afsS expression. So is afsR really a regulatory gene for antibiotic production?

To address this question, high-copy-number plasmids that contained either afsR or afsS were made and both were shown to be capable of stimulating Act production in both *S. coelicolor* and *S. lividans*; however, the degree of stimulation mediated by afsR was significantly greater than that produced by afsS (Floriano & Bibb, 1996). Since the N-terminal region of AfsR is homologous to ActII-ORF4 and RedD, this might simply reflect regulatory cross-talk between AfsR and the pathway-specific activators when AfsR is expressed at high levels. However, the inability of afsR to suppress in-frame deletion mutations in actII-ORF4 or redD even when cloned at high copy-number indicates that afsR cannot substitute for the pathway-specific regulatory genes. Consistent with this, a deletion in afsR that is in-frame and therefore unlikely to influence the expression of afsS results in the loss of Act and Red production on some media. Transcription of actII-ORF4 and redD was not affected by the afsR mutation (Floriano & Bibb, 1996). So either AfsR influences Act and Red production independently of the pathway-specific regulatory proteins, or it is required for their activity, perhaps through their post-translational modification, or through the formation of a heteromultimeric protein complex. However it mediates its effects, afsR is clearly a true pleiotropic regulatory gene.

A second pleiotropic regulatory gene for antibiotic production in *S. coelicolor* is afsB. afsB mutants are defective in Act and Red production (Hara et al., 1983), while synthesis of methylenomycin and the calcium-dependent antibiotic (CDA), the other two antibiotics known to be made by the strain, appears to be unaffected (Adamidis & Champness, 1992). Efforts to complement afsB using a genomic library made in a low-copy-number plasmid led to the interesting discovery that additional copies of brdB, which encodes the major σ factor of *S. coelicolor* (Brown et al., 1992), restored Red and Act production in afsB mutants (Wietzorrek, 1996). The suppressive effect of brdB resembles a recent report in *Pseudomonas fluorescens* (Schnider et al., 1995), in which production of the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol, which are made during stationary phase, was stimulated by the presence of additional copies of rpoD, which encodes the major and essential σ factor of that organism. Whether this reflects a role for the major σ factor of both organisms in the transcription of antibiotic biosynthetic genes, or results from an indirect effect (e.g. provision of precursors), remains to be determined (see below).

The third pleiotropic regulatory gene for antibiotic production that has been studied in our laboratory is bldA (see Champness & Chatzer, 1994, and references therein). On most laboratory media, bldA mutants have a very dramatic phenotype; not only are they deficient in aerial mycelium formation, giving rise to the term ‘bald’, but they are also pleiotropically deficient in antibiotic production, failing to produce any of the four *S. coelicolor* antibiotics. bldA encodes the only tRNA in *S. coelicolor* that can efficiently translate the rare leucine codon UUA (Lawlot et al., 1987; Leskiw et al., 1991a). The dependence of Act production on bldA was shown to be due to the presence of a single UUA codon in the actII-ORF4 transcript; substitution of this codon on a plasmid-borne copy of actII-ORF4 with the synonymous leucine codon UUG rendered Act production bldA-independent (Fernández-Moreno et al., 1991). While control of transcription of bldA might seem to be a likely means by which to regulate growth-phase-dependent Act production, bldA transcripts are observed in minimal and rich liquid media throughout growth (Gramajo et al., 1993). Furthermore, we were unable to demonstrate any limitation on the translation of UUA codons in rapidly growing liquid cultures (Gramajo et al., 1993). Thus, while Act production is clearly bldA-dependent, we have been unable to demonstrate any regulatory role for bldA. In contrast, in studies conducted largely, but not entirely, with surface-grown cultures, Leskiw and colleagues did detect an increase in the level of bldA transcripts during growth, and limitations on the translation of UUA codons in young cultures (Leskiw et al., 1991b, 1993); the differences in the two sets of results may reflect differences in the strains and media used. The precise role that bldA plays in regulating antibiotic production therefore remains to be determined.

In contrast to actII-ORF4, the transcript derived from redD, the pathway-specific activator gene for Red production, does not contain a UUA codon (Narva & Feitelson, 1990), so how do we explain the bldA-dependence of Red synthesis? The ability to relieve bldA-dependence of Act production simply by replacing the UUA codon of actII-ORF4 with a synonymous UUG codon (Fernández-Moreno et al., 1991) implied that
transcription of actII-ORF4 was not bldA-dependent, and this has since been demonstrated experimentally in a series of S1 nuclease protection experiments using RNA isolated from otherwise isogenic bldA + and bldA strains (J. White, personal communication). In contrast, transcription of redD was shown to be highly bldA-dependent (J. White, personal communication), a result that implied the existence of another gene that presumably contained a UUA codon and that was required for redD transcription. Recent studies (C. Flaxman, D. A. Hodgson & J. White, personal communication), which followed on from the isolation of second-site suppressor mutants of bldA in which Red production, but not Act synthesis or sporulation, was restored (E. Guthrie & K. F. Chater, personal communication), have identified a likely candidate for this additional pathway-specific regulatory gene, redZ, which encodes a homologue of the response regulator family of two-component regulatory systems (Msadek et al., 1993), and which is located approximately 4 kb downstream of redD, contains a single UUA codon. Disruption of redZ results in loss of Red production and a marked reduction in the level of redD transcripts, suggesting that RedZ is a transcriptional activator of redD (J. White, personal communication). What regulates redZ expression? While the availability of a functional bldA tRNA remains a possibility, the results obtained with actII-ORF4 suggest that other factors may play a determining role.

Small diffusible signalling molecules influence the onset of antibiotic production in S. coelicolor

Small diffusible signalling molecules play an important role in triggering antibiotic production in some streptomycetes (for reviews see Chater & Bibb, 1996, and Horinouchi & Beppu, 1992, 1995). For example, the γ-butyrolactone A-factor is required for streptomycin production and sporulation in S. griseus (Khokhlov et al., 1967; Horinouchi & Beppu, 1995). S. coelicolor is capable of cross-feeding S. griseus mutants that are deficient in A-factor synthesis (Hara et al., 1983), and makes a series of six related compounds (Anisova et al., 1984; Efremenkova et al., 1985), but not A-factor itself. In an attempt to identify a role for these compounds in antibiotic production, we have purified a compound detectable only in transition- and stationary-phase cultures of S. coelicolor that causes the precocious production of both Act and Red in the wild-type strain. The compound was purified using standard procedures for the isolation of γ-butyrolactones (E. Takano, personal communication), and it is likely that it is one of the six compounds identified by Efremenkova et al. (1985). It thus appears probable that γ-butyrolactones do indeed play a role in determining the onset of antibiotic production in S. coelicolor. Since the activity we detected was found only in transition- and stationary-phase cultures, it seems unlikely that these compounds act simply as quorum sensors, i.e. as indicators of cell density akin to the homoserine lactone derivatives made by V. alginolyticus (Fuqua et al., 1994; Williams, 1994). Similar observations were made for the accumulation of virginc acid butanolide C that stimulates virginiamycin production in Streptomyces virginiae (Yang et al., 1995), and for A-factor production by S. griseus (Hara & Beppu, 1982). It thus appears more likely that the γ-butyrolactones are produced in response to certain physiological or environmental signals.

The highly phosphorylated nucleotide ppGpp may act as an intracellular effector for antibiotic production

A consistent feature throughout the Streptomyces literature is the likely role of a slowing or cessation of growth as a signal for determining the onset of antibiotic production. In E. coli, the highly phosphorylated nucleotide ppGpp appears to play a central role in the growth rate control of gene expression (Sarubbi et al., 1988; Hernandez & Bremer, 1990, 1993; Schreiber et al., 1991), and in the regulation of at least some genes that are expressed in stationary phase (Gentry et al., 1993). Furthermore, relC mutants of a number of streptomycete strains that are defective in ppGpp synthesis are also impaired in antibiotic production, leading Ochi (1990) to suggest that ppGpp may play a key role in determining the onset of antibiotic biosynthesis. In our own studies, we generally see a positive correlation between ppGpp synthesis and transcription of redD (Takano et al., 1992) and actII-ORF4 (Strauch et al., 1991) during transition and stationary phase of liquid-grown S. coelicolor cultures (Takano & Bibb, 1994). Moreover, if an exponentially growing culture is subjected to nutritional shutdown by rapidly depleting it of amino acids, large amounts of ppGpp are produced, and transcription of actII-ORF4 follows within minutes (Strauch et al., 1991). Unfortunately, relC mutants deficient in ribosomal protein L11, which is apparently required for activation of the ribosome-bound ppGpp synthetase, are also impaired in growth and so it is difficult to know whether or not the reduction in antibiotic production reflects reduced levels of ppGpp or impaired protein synthesis. To assess more rigorously whether there might be a causal relationship between ppGpp synthesis and antibiotic production, we used a PCR-based approach to clone the ppGpp synthetase gene (relA) of S. coelicolor (Chakraburty et al., 1996). The cloned gene was used to create a null-mutant that is totally deficient in ppGpp synthesis upon amino acid starvation (R. Chakraburty, personal communication). The resulting mutant grows at the same rate as the relA + strain; it fails to make Act or Red on some media, but does so on others. We believe that this indicates a crucial role for ppGpp in antibiotic biosynthesis under some nutritional conditions.

The role of σ factor heterogeneity in antibiotic production in S. coelicolor

Although S. coelicolor contains at least eight σ factors (Buttner, 1989; Chater et al., 1989; Lonetto et al., 1994; Potücková et al., 1995), the functions of several are unknown. Given the role that alternative σ factors play in growth phase-dependent gene expression in other bacteria (Boylan et al., 1993; Errington, 1993; Hennege-Aronis, 1993; Loewen & Hennege-Aronis, 1994), we set out to determine which form of RNA polymerase holoenzyme transcribes redD and actII-ORF4, and whether the avail-
identified a role for ppGpp as an intracellular effector for the inhibition (reviewed by Demain et al., 1992). Perhaps reflect changing physiological conditions that trigger the expression of these genes, or that influence the activities of their products, and the corresponding signal-transduction pathways that are responsible for the activation of antibiotic biosynthesis remains a major goal for the future.

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