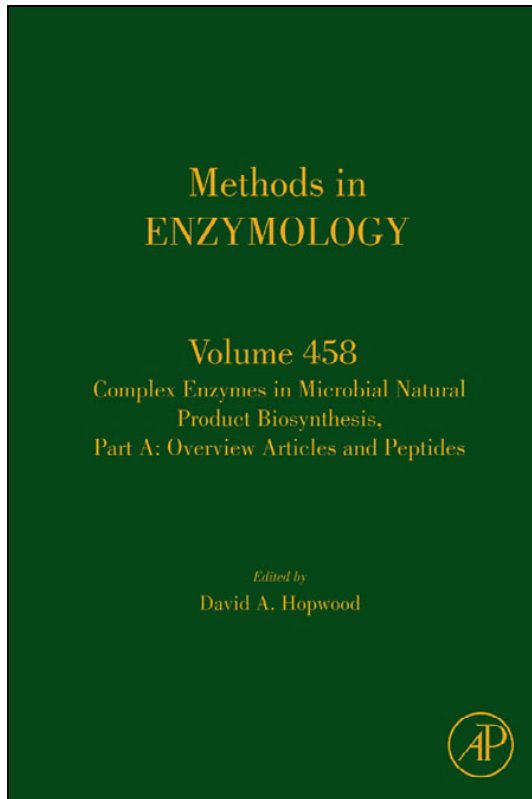


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ANALYZING THE REGULATION OF ANTIBIOTIC PRODUCTION IN STREPTOMYCETES

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Abstract

This chapter outlines the approaches and techniques that can be used to analyze the regulation of antibiotic production in streptomyces. It describes how to isolate antibiotic nonproducing and overproducing mutants by UV, nitrosoguanidine (NTG), transposon, and insertion mutagenesis, and then how to use those mutants to identify regulatory genes. Other approaches to identify both pathway-specific and pleiotropic regulatory genes include overexpression and genome scanning. A variety of methods used to characterize pathway-specific regulatory genes for antibiotic biosynthesis are then covered,

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including transcriptional analysis and techniques that can be used to distinguish between direct and indirect regulation. Finally, genome-wide approaches that can be taken to characterize pleiotropic regulatory genes, including microarray and ChIP-on-Chip technologies, are described.

1. INTRODUCTION

This chapter will focus on experimental procedures for analyzing the transcriptional regulation of antibiotic production in streptomycetes, although in general the same procedures can be used to study the control of expression of any gene in any microorganism. The actinomycetes, the family to which the streptomycetes belong, are responsible for the production of over two-thirds of known antibiotics (defined as compounds produced by one microorganism that inhibit the growth of another), and the biosynthesis of many of these compounds is discussed elsewhere in this series. While of immense fundamental interest, insights into the regulation of antibiotic production provide new opportunities for knowledge-based approaches for strain improvement to complement the classical and undoubtedly successful strategy of mutation and screening for improved productivity. Many of the approaches described here are also theoretically applicable to other actinomycetes, but since many of the tools used are available only for streptomycetes, we have confined our considerations to this genus. This contribution is not intended to review our current knowledge of the regulation of antibiotic production in these organisms (see Chapter 5 in this volume for a more complete coverage), but instead to provide a primer for those interested in developing an understanding of the regulation of synthesis of a particular compound. To assist in this endeavor, we have quoted references that demonstrate the use of many of the techniques described. Frequently, these stem from our own studies, and we apologize for not providing a comprehensive list of publications that utilize these technologies.

2. THE REGULATION OF ANTIBIOTIC PRODUCTION IN STREPTOMYCETES

Antibiotics are the products of complex biosynthetic pathways that utilize primary metabolites as building blocks. Apparently without exception, all of the genes that are required for the production of a particular compound are clustered together in the chromosome, or sometimes on a plasmid, of the producing organism, markedly facilitating the isolation and

analysis of entire antibiotic biosynthetic gene clusters. In addition to containing the genes encoding the biosynthetic enzymes, such clusters (which can vary in size from around 15 kb to over 100 kb) frequently contain pathway-specific regulatory genes that control the onset of biosynthesis, as well as genes involved in antibiotic export and self-resistance.

Antibiotic production in streptomyces usually starts at the onset of stationary phase in liquid grown cultures, and coincides with the beginning of morphological differentiation in agar-grown cultures (see [Bibb, 2005](#), for a review). Where they exist, pathway-specific regulatory genes play a key role in triggering stationary-phase expression, and indeed there is evidence to suggest that for at least some gene clusters the level of the pathway-specific regulatory protein is the only limiting factor in determining the onset of antibiotic biosynthesis. Constitutive expression of such regulatory genes may lead to precocious antibiotic synthesis during rapid growth, and their overexpression can result in marked increases in the level of antibiotic production ([Gramajo et al., 1993](#); [Takano et al., 1992](#)).

Many streptomyces produce several compounds with antimicrobial activity, and often the onset of production of some or all of these antibiotics is coordinately controlled by pleiotropic regulatory genes. Consequently, mutations in these genes abolish or impair the synthesis of several of the antibiotics made by a particular strain. It is striking that while pathway-specific regulatory genes, such as the *Streptomyces* Antibiotic Regulatory Proteins (SARPs) ([Bibb, 2005](#)), are absolutely required for antibiotic production, most of the pleiotropic regulatory genes characterized thus far are only required under particular growth conditions. This suggests that they serve to sense different environmental parameters, such as various nutritional limitations, and as a consequence mutants deficient in a particular pleiotropic regulatory gene often have wild-type phenotypes when grown on some growth media and mutant phenotypes on others. An example of such a pleiotropic regulatory gene is *relA* of *Streptomyces coelicolor* A3(2), which encodes synthesis of the intracellular signaling molecule (p)ppGpp. This highly phosphorylated guanine nucleotide plays a key role in regulating stationary phase gene expression in *Escherichia coli* (reviewed in [Magnusson et al., 2005](#)), and so it is perhaps not too surprising that it has also been shown to play a crucial role in triggering antibiotic biosynthesis in *S. coelicolor* ([Chakraborty and Bibb, 1997](#); [Sun et al., 2001](#)). However, it appears to be required only under conditions of nitrogen limitation; growth of a *relA* mutant under conditions of phosphate limitation results in a wild-type phenotype. Interestingly, induction of (p)ppGpp synthesis in the wild-type strain under conditions of nutritional sufficiency appears to directly activate the transcription of pivotal pathway-specific regulatory genes, suggesting that there are no intermediary steps in the regulatory cascade ([Hesketh et al., 2001, 2007a](#)). Binding of (p)ppGpp to RNA polymerase may allow it to adopt a configuration that favors transcription initiation at

the promoters of these genes, or to select a particular σ factor that preferentially recognizes their promoter sequences. DasR, a DNA binding protein of *S. coelicolor*, is another example of a pleiotropic regulatory protein that links antibiotic production to the nutritional status of the cell (Rigali *et al.*, 2008). DasR inhibits the production of two different antibiotics by binding to operator sequences upstream of the pathway-specific regulatory genes of each of the biosynthetic gene clusters, thus repressing their transcription. Repression is relieved in the presence of the metabolite glucosamine-6-phosphate, the abundance of which indicates levels of extracellular *N*-acetylglucosamine, an important environmental source of carbon and nitrogen.

Among the many genes that have been identified as having a pleiotropic influence on antibiotic biosynthesis, there are a subset, exemplified by the *bld* (for bald) genes of *S. coelicolor*, which in addition to being pleiotropically deficient in antibiotic production are also unable to erect aerial hyphae (hence the term bald) and to undergo normal morphological development. While the effect on antibiotic biosynthesis for some of these mutants may be indirect and reflect gross changes in physiology, it is apparent that some (e.g., *bldA* of *S. coelicolor*) play a direct regulatory role in controlling both morphological development and antibiotic biosynthesis. *bldA* encodes the only tRNA in *S. coelicolor* that can translate the rare leucine codon UUA; the presence of such UUA codons in many pathway-specific regulatory genes (Chater and Chandra, 2008) provides a means for implementing translational control over antibiotic biosynthesis, the physiological basis of which is not currently understood.

Many antibiotics are also regulated by small diffusible extracellular signaling molecules, the γ -butyrolactones (see Takano, 2006, for a review and Chapter 6 in this volume) and 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (Corre *et al.*, 2008). While most of these compounds appear to regulate the production of specific antibiotics, at least one, the γ -butyrolactone A-factor made by *Streptomyces griseus*, regulates the production of several secondary metabolites and the onset of morphological differentiation (Ohnishi *et al.*, 2005). Although these signaling molecules are reminiscent of the *N*-acyl homoserine lactones involved in quorum sensing in many Gram-negative bacteria, it is not clear that they always play a similar role in streptomycetes, where their synthesis may instead be triggered at least in part by environmental or nutritional signals.

In the following sections, we have attempted to describe the methodologies that can be used to identify and analyze genes that are involved in regulating antibiotic biosynthesis in streptomycetes. Where detailed protocols have been reported previously, we have directed the reader to specific references.

3. IDENTIFYING REGULATORY GENES FOR ANTIBIOTIC BIOSYNTHESIS

3.1. Random generation of antibiotic nonproducing or overproducing mutants by UV, NTG, transposon, and insertion mutagenesis

The classical approach to identifying genes, and consequently regulatory genes, involved in antibiotic biosynthesis is to carry out UV and/or NTG mutagenesis (see [Kieser *et al.*, 2000](#), for detailed protocols). If UV mutagenesis is carried out, care must be taken to avoid photoreactivation which compromises the efficacy of the mutagenic treatment in at least some streptomycetes. Mutagenesis is then followed by screening for loss of or overproduction of the compound of interest. If the compound is pigmented (e.g., actinorhodin of *S. coelicolor*), then visual screening for changes in production can be readily accomplished. If not, then an activity screen is generally conducted using a sensitive microorganism to detect antibiotic nonproducing or overproducing mutants. This can be accomplished in a number of ways, just two of which are described below:

1. The survivors of mutagenesis, which have been grown on a medium that promotes antibiotic production, are replicated to agar to yield a set of master plates; the original survivors of mutagenesis are then overlaid generally in soft nutrient agar with a sensitive assay microorganism and screened for loss of inhibition of the background lawn of growth. Any candidate nonproducers can then be retrieved from the set of master plates, and loss of antibiotic production confirmed by bioassay and physical techniques, such as High Performance Liquid Chromatography (HPLC) and/or mass spectrometry. Alternatively, although more laborious, small cylinders of agar containing the survivors of mutagenesis can be extracted using a suitable cork borer and arrayed on a 20 cm x 20 cm assay plate and embedded (but not submerged) in a thin layer of soft nutrient agar containing the assay organism. Potential nonproducing mutants can then be isolated directly from the top of the agar cylinders and analyzed further (e.g., see [Wright and Hopwood, 1976](#)).
2. The survivors of mutagenesis are picked to 96-well agar plates, preferably with the use of robotics. Once grown, the arrayed survivors can be inoculated (using robotics or pin-tools) into 96-well or 32-deep-well plates containing a suitable liquid growth medium, and samples of the culture supernatants used in standard assays for bioactivity.

Antibiotic overproducing mutants can be identified using the same procedures by screening for enhanced levels of inhibition of the assay organism.

Since many streptomycetes make more than one antibiotic, such simple activity screens may not suffice. While it may be possible to choose a growth medium that favors the production of one antibiotic over others, or to use an indicator organism that is sensitive only to the compound of interest among those made by the streptomycete, it may nevertheless be necessary to resort to physical means (e.g., high throughput HPLC or Matrix Assisted Laser Desorption Ionisation–Time of Flight (MALDI–ToF) mass spectrometry analysis to detect the loss or overproduction of a particular compound.

Another approach that can be used to obtain antibiotic nonproducing and overproducing mutants is mutational cloning (Chater and Bruton, 1983; Kieser *et al.*, 2000). In its initial form, this involved the use of derivatives of the temperate phage Φ C31 from which the attachment site *attP* had been deleted and into which had been inserted an antibiotic resistance gene selectable in the streptomycete of interest. Relatively small fragments (about 1–2 kb) of the latter's genomic DNA are inserted into the phage vector, and the phage library introduced into the antibiotic producing streptomycete by protoplast transfection (if this technology is not available for the streptomycete of interest, the initial phage library can be made in, e.g., *Streptomyces lividans*, and introduced into the antibiotic producer by phage infection; about two-thirds of streptomycetes are sensitive to Φ C31). Stable antibiotic resistant lysogens can only be obtained by homologous recombination between the insert cloned in the phage vector and homologous sequences present in the host's genome. If the cloned insert is internal to a transcription unit, then this will generally result in loss of function of the disrupted gene. Screening of lysogens for loss of antibiotic production thus serves to identify insertional mutants in the biosynthetic gene cluster of interest. In principle, a similar procedure could now be adopted using a plasmid vector. For example, a small-insert library of genomic DNA from the strain of interest could be generated in *E. coli* in a conjugative plasmid (such as pSET151; Bierman *et al.*, 1992) that lacks any site-specific integration system and that is selectable in the *Streptomyces* strain of interest. Stable ex-conjugants can only be obtained by homologous recombination between the cloned inserts and homologous sequences present in the host. Screening for loss of antibiotic production should again reveal the required insertional mutants.

More recently, it has been possible to contemplate the use of transposon mutagenesis to identify genes involved in antibiotic production. Although several transposon systems have been described for use in streptomycetes, the resulting phenotypes have often been found not to be linked to the transposon insertion, suggesting that introduction of the transposon and/or its delivery vector into the host may itself promote random mutagenesis. Nevertheless, the pKay1 system developed by Fowler (personal communication, for further information contact Bibb), which is based on Tn4560, has been used to identify genes for antibiotic production in *S. coelicolor*,

although again, not all of the non-producing mutants are transposon-linked (Fowler, Hesketh, and Bibb, unpublished results).

3.2. Identifying regulatory genes from nonproducing mutants

While antibiotic overproducing mutations are likely to act by derepressing expression of the cluster or increasing precursor or cofactor availability, the majority of mutations that result in loss of antibiotic production will lie in genes encoding biosynthetic enzymes rather than regulatory proteins. Here, we consider ways in which these nonproducing mutants can be used to identify the corresponding regulatory genes.

3.2.1. Inability to undergo cosynthesis

Since antibiotics are generally the products of stepwise linear biosynthetic pathways, it is often possible to carry out cosynthesis tests between mutants blocked at different points in the pathway. This assumes that any intermediates that accumulate in the blocked mutants are freely diffusible and capable of being taken up by other mutants grown in close proximity. In principle then, if we take two mutants A and B, where mutant B is blocked later in the pathway than mutant A, mutant B will accumulate an intermediate that may be able to enter mutant A and provide a missing intermediate for continued synthesis of the antibiotic in the latter strain. Mutant B would thus be a “secretor” and mutant A a “converter.” Mutants that are unable to act as “secretors” or “convertors” are candidates for regulatory mutants, since they are likely not to express any of the biosynthetic enzymes. (However, note that mutants unable to produce an extracellular signaling molecule are also likely to be classified as convertors in such tests, and potentially eliminated as regulatory candidates; see O’Rourke *et al.* (2009), who described such a system involved in methylenomycin biosynthesis.)

3.2.2. Transposon mutants

The advantage of transposon mutagenesis is that it may be possible to rapidly identify the gene into which the transposon has inserted, which by definition is involved in antibiotic production. This can be accomplished either by cloning the transposon out into a tractable host such as *E. coli*, by selecting for an antibiotic resistance gene carried by the transposon and then sequencing flanking regions, or (more efficiently) by Ligation-Mediated-Polymerase Chain Reaction (LM-PCR; Guilfoyle *et al.*, 1997) of DNA isolated from the mutant followed by nucleotide sequencing. In either case, if a genome sequence for the organism of interest is available, the precise point of transposon insertion can be determined and the putative antibiotic biosynthetic gene identified. The transposon insertion may have disrupted a pleiotropic or a pathway-specific regulatory gene, but in a nonproducing mutant it is more likely to have occurred in one of the more numerous

genes encoding biosynthetic enzymes. In the latter case, the sequences adjacent to the point of transposon insertion can be used as hybridization probes with cosmid or BAC libraries of wild-type streptomycete DNA to identify clones that singly or collectively contain the entire biosynthetic gene cluster by “genome walking,” and hence any pathway-specific regulatory genes contained therein. Alternatively, LM-PCR combined with nucleotide sequencing could be used to “walk” towards the required regulatory gene(s).

3.2.3. Insertional mutants

It is possible to rapidly identify antibiotic biosynthetic genes from insertional mutants in a similar fashion. If phage-based mutational cloning has been used to create an antibiotic nonproducing mutant, phages released from the antibiotic nonproducing lysogen can be amplified in *S. lividans*, their DNA isolated (Kieser *et al.*, 2000) and the mutagenic DNA fragment that lies internal to a transcription unit identified. If a plasmid-based approach has been used, growth in liquid culture in the absence of selection should lead to the excision of some of the integrated plasmids. Transformation of *E. coli* with a crude cell lysate from such a culture may be sufficient to recover the mutagenic plasmid. Alternatively, restriction endonuclease digestion of genomic DNA obtained from the mutant with an enzyme that does not cut within the plasmid vector, followed by ligation and transformation of *E. coli*, should lead to isolation of the required DNA sequence. For both phage- and plasmid-based mutational cloning, the nucleotide sequence of part or all of the mutagenic insert can be determined and used to isolate the remainder of the biosynthetic gene cluster by “genome walking.”

3.2.4. Genetic complementation

Once antibiotic nonproducing mutants have been isolated, be they regulatory mutants or not, then the corresponding wild-type genes can be identified by genetic complementation. This requires methodology to introduce wild-type DNA efficiently back into the mutant host, either by protoplast transformation or by conjugation from *E. coli* (see Kieser *et al.*, 2000, for both procedures). If the latter approach is adopted, it may be necessary to use a methylation-deficient *E. coli* donor strain to avoid the methyl-specific restriction systems possessed by some streptomycetes (e.g., *S. coelicolor* and *Streptomyces avermitilis*). ComPLEMENTING clones can be obtained by cloning relatively short fragments (around 5 kb) of wild-type DNA into the mutant strain on autonomously replicating plasmid vectors (that may be transferred by conjugation from *E. coli*) and screening for restoration of antibiotic production. Plasmid DNA can then be isolated from the complementing clones and analyzed by nucleotide sequencing.

Should the complementing clone not contain a pathway-specific regulatory gene, but one of the genes encoding a biosynthetic enzyme, the former can be obtained using the procedure of “genome walking” outlined above for transposon mutagenesis. However, given that the mutation (if not in a pleiotropic regulatory gene) will almost certainly lie within a large biosynthetic gene cluster, maximal information will be gained by cloning (and subsequently sequencing) large segments of wild-type DNA that might conceivably contain the entire biosynthetic gene cluster, including the desired regulatory gene(s). This could be achieved by using a low copy number *Streptomyces* plasmid vector based on SCP2 that may be conjugable from *E. coli* (Kieser *et al.*, 2000). Although, to our knowledge, not demonstrated thus far in streptomyces, an alternative approach would be to make a library of wild-type DNA in a cosmid vector containing an antibiotic resistance gene that can be selected in streptomyces and that has been modified to allow conjugation from *E. coli* into the nonproducing mutant. Selection for the resistance gene carried on the cosmid vector will result in integration of the cosmid clones into the streptomyces genome by homologous recombination. Screening for restoration of antibiotic biosynthesis will identify cosmid clones containing the required wild-type gene. Since the streptomyces containing the complementing clones now possesses two duplicated segments of genomic DNA of ~40 kb separated only by the cosmid vector, relaxation of antibiotic selection should lead to a second crossover that liberates the integrated cosmid vector together with part or all of either the wild-type or mutant biosynthetic gene cluster. Transformation of *E. coli* with a cell lysate of the streptomyces culture after such nonselective growth and selection for the antibiotic resistance gene carried on the cosmid vector should permit recovery of the required cosmid clone, which can then be characterized by nucleotide sequencing. Ultimately this approach, perhaps combined with additional “genome walking,” should lead to the identification of putative pathway-specific regulatory genes.

3.3. Identifying antibiotic regulatory genes by overexpression

As indicated previously, the enhanced or constitutive expression of pathway-specific regulatory genes can increase markedly the level of antibiotic biosynthesis, and the same is true for at least some pleiotropic regulatory genes. Consequently, in principle members of both classes of regulatory gene can be isolated by cloning wild-type DNA in a high copy number plasmid vector and screening for elevated levels of antibiotic production. Alternatively, such wild-type DNA could be cloned in an expression vector with a strong constitutive promoter with the aim of achieving high levels of expression of the regulatory gene throughout growth without the requirement of any additional potentially developmentally controlled regulatory elements. While high copy number vectors would appear to be

the obvious choice, integrative expression vectors may achieve the same end; indeed there is evidence that just a single additional copy of a pathway-specific regulatory gene is sufficient to cause a marked increase in antibiotic production (Hopwood *et al.*, 1985). DNA sequencing of the cloned inserts should then reveal the required regulatory gene(s). (Note that if the cloned regulatory gene is present in a stably integrated vector that cannot readily be recovered from the streptomycete genome, then it will be necessary to first clone it out into *E. coli* using a selectable marker present in the integrated element, as outlined earlier in this chapter.)

3.4. Genome scanning for regulatory genes for antibiotic biosynthesis

Given the high-throughput nature and decreasing costs of nucleotide sequencing delivered by the next-generation sequencing technologies, such as Solexa, 454, and Solid, it is now feasible to consider identifying antibiotic biosynthetic gene clusters, and their corresponding pathway-specific and indeed pleiotropic regulatory genes, by a procedure known as genome scanning. In this approach, genomic DNA of the streptomycete of interest is subject to high-throughput sequencing to yield around fivefold coverage of the genome of interest. While this is not sufficient to yield a single genomic contig, it provides a database of contigs that can then be interrogated *in silico* to identify genes that are potentially components of the gene cluster of interest. These sequences then provide a means to generate high-fidelity PCR probes from genomic DNA that can be used to probe cosmid or BAC libraries of the antibiotic producing streptomycete of interest. Sequencing of the corresponding cosmid or BAC clones, perhaps with additional rounds of library screening, should ultimately reveal putative pathway-specific regulatory genes, the nature of which can then be confirmed by targeted mutagenesis. Similarly, the contig database can also be interrogated *in silico* to identify potential pleiotropic regulatory genes, which can be further analyzed by mutagenesis and overexpression.

3.5. Confirming the nature of antibiotic regulatory genes

Regardless of how a putative antibiotic regulatory gene has been identified, but particularly in the case of transposon-induced mutations and genes identified by overexpression, further mutational analysis is generally required to show conclusively that the gene is a true regulator of antibiotic biosynthesis. In the case of transposon mutagenesis, given the frequent lack of linkage of the mutant phenotype to the transposon insertion, it is crucial to either complement the mutant with the wild-type gene or to inactivate the identified gene by targeted mutagenesis to confirm its role in antibiotic production. Similarly, for genes identified by apparent genetic complementation or overexpression,

it is important to show for the former that restoration of antibiotic production does not reflect suppression (rather than genetic complementation) of the original mutation, and for the latter that it reflects a true regulatory role for the cloned gene(s) rather than a perturbation of physiology that is able to restore antibiotic biosynthesis.

For UV and NTG mutagenesis, and potentially for some transposon-induced mutations, it may be important to determine the effect of a null mutation in the gene of interest. The original mutation may not have completely abolished gene function, giving a partially deficient antibiotic phenotype and suggesting a nonessential role in antibiotic biosynthesis, whereas the corresponding null-mutation may reveal complete dependency. Indeed, in the case of one antibiotic regulatory gene, point and null mutations gave completely opposite phenotypes (see McKenzie and Nodwell, 2007, and references therein).

There are several ways in which targeted mutations can be made in actinomycetes:

3.5.1. PCR targeting

This technology relies on the use of the lambda *red* recombination system to rapidly create defined mutations in cloned DNA present in *E. coli* that can then be introduced into the wild-type strain of interest and integrated into the host genome, at the same time replacing the wild-type copy of the gene with a null mutant allele (Gust *et al.*, 2004 and Chapter 7 in this volume). This is most efficiently carried out on a sequenced cosmid clone containing the gene of interest. The mutated cosmid is then further modified to allow transfer by conjugation into the pertinent streptomycete, where double-crossover recombination mediated through long flanking sequences is used to replace the wild-type gene with the mutant allele.

3.5.2. Insertion and deletion mutagenesis by homologous recombination

If appropriate clones and the lambda *red* recombination system are not available, then targeted mutations (either insertions or deletions) can be made by creating a mutant allele in *E. coli*, transferring it by conjugation or transformation into the wild-type streptomycete, and selecting or screening for the desired mutant, which can then be characterized for antibiotic production. This can be achieved by simply inserting an antibiotic resistance gene into the streptomycete gene of interest at a position likely to result in a null allele, by replacing part or all of the gene with an antibiotic resistance cassette, or by creating a precise and preferably in-frame deletion (to avoid possible polar effects on downstream genes). All of these modifications can be achieved by routine cloning and/or by using PCR.

4. CHARACTERIZING REGULATORY GENES FOR ANTIBIOTIC BIOSYNTHESIS

4.1. Pathway-specific regulatory genes

If the gene that has been identified is a pathway-specific regulatory gene, it will be necessary to determine which of the genes in the cluster it regulates (it may not regulate all of them), and ultimately whether it does so directly or indirectly. This can be accomplished in a number of ways.

4.1.1. Northern analysis

In principle, Northern analysis (Kieser *et al.*, 2000) remains the simplest procedure to monitor the effects on transcription of a gene set of interest of a mutation in a putative pathway-specific regulatory gene. By using radioactively labeled probes corresponding to the entire biosynthetic gene cluster (made, e.g., by nick-translation (Sambrook and Russell, 2001) of a cosmid clone containing the entire biosynthetic gene cluster), it is possible in one experiment to assess the role of a particular pathway-specific regulatory gene. RNA is isolated from the wild-type and mutant strains, size fractionated by denaturing gel electrophoresis, transferred to a suitable membrane (often positively charged nylon), and hybridized with labeled DNA corresponding to the entire biosynthetic gene cluster. Autoradiography is then used to determine the number and size of transcripts present in the wild-type strain, and to identify any that are missing or changed in abundance in the mutant. Given that antibiotic production very often occurs in a growth phase-dependent manner, ideally Northern analysis should be carried out using RNA samples obtained at different stages of growth (or at stages of growth shown previously to be associated with production).

4.1.2. Low-resolution S1 nuclease protection analysis

Unfortunately, given the relatively short half life of bacterial mRNA compared to eukaryotic transcripts, autoradiographs from Northern analyzes often reveal smears of degraded mRNA that can be difficult to interpret. As a consequence, low resolution S1 nuclease protection analysis is often used instead to assess the effects of mutation of a putative pathway-specific regulatory gene on transcription of an antibiotic biosynthetic gene cluster (Kieser *et al.*, 2000). In this procedure, DNA segments internal to or overlapping the predicted termini of individual transcripts derived from the cluster are hybridized to total RNA isolated from the wild-type and mutant strains, and the resulting DNA-RNA hybrids treated with S1 nuclease to remove unhybridized single strands of DNA and RNA. The protected double-stranded DNA fragments are resolved by agarose

gel electrophoresis, blotted onto a suitable membrane (traditionally nitrocellulose, more recently nylon), and hybridized with a labeled probe. The resulting pattern of bands serves to identify any transcripts that are altered in the mutant and to localize the approximate start- and end-points of individual transcripts.

4.1.3. High-resolution S1 nuclease protection analysis

S1 nuclease protection analysis can be used not only to determine whether or not a particular gene in the cluster is transcribed in the mutant strain, but when used in high resolution mode it can identify, at the nucleotide level, the precise site of transcription initiation. This may provide information that can be used subsequently to identify consensus binding sites for a pathway-specific transcriptional activator. In this procedure, a DNA fragment is labeled on the 5'-end of the strand that is complementary to the transcript of interest and which extends beyond the likely transcriptional start site. The labeled probe (now generally made by PCR using an oligonucleotide primer that has been labeled on its 5'-end with ^{32}P using polynucleotide kinase) is hybridized to total RNA and the resulting DNA-RNA hybrids are treated with S1 nuclease and resolved on a sequencing gel adjacent to a sequencing ladder derived from either the same labeled PCR product or by using the oligonucleotide used to generate the PCR product as a sequencing primer. Consequently, it is generally possible to locate, within 1–3 nucleotides, the precise transcriptional start site of a particular gene. The addition of sequences at the 3'-end of the labeled DNA strand of the probe that are not homologous to genomic DNA (accomplished when designing the unlabeled primer used in the PCR generation of the probe) enables the detection of read-through transcription (as opposed to reannealing of the DNA probe; [Kieser *et al.*, 2000](#)), and thus provides additional information about overall transcript organization.

4.1.4. Primer extension

Primer extension is an alternative to high resolution S1 nuclease mapping ([Kieser *et al.*, 2000](#)). It uses a 5'-end-labeled synthetic oligonucleotide and reverse transcriptase to make a cDNA copy of the 5'-end of a transcript of interest, the length of which localizes the transcriptional start site to within a couple of nucleotides when resolved on a denaturing acrylamide gel adjacent to a sequence ladder derived from the same synthetic oligonucleotide. This procedure is often used in streptomyces instead of high resolution S1 nuclease mapping and is less labor-intensive, but it can be less reliable (premature termination, which appears to occur more frequently with GC-rich DNA, can lead to incorrectly predicted transcriptional start sites). While primer extension is used to localize transcriptional start sites, it is not generally used to detect the presence or absence of a particular mRNA.

4.1.5. *In vitro* transcription and dinucleotide priming

Although not used on a routine basis, should there be a need, it is possible to confirm the results of S1 nuclease mapping or primer extension by carrying out *in vitro* run-off transcription analysis (Kieser *et al.*, 2000). Ideally this should be done using RNA polymerase isolated from the streptomycete of interest. However, should an alternative σ factor be implicated in the transcription of an antibiotic biosynthetic gene, and if that σ factor can be purified, then it is possible to carry out *in vitro* reconstitution experiments using commercially available *E. coli* core RNA polymerase (Fujii *et al.*, 1996). In this procedure, labeled transcripts are produced *in vitro* from a purified DNA template that terminates before the predicted end of the transcript (hence the term “run-off transcript”). The sizes of the run-off transcripts are determined by denaturing gel electrophoresis using labeled size markers and autoradiography. In an extension of the procedure, *in vitro* transcription can be initiated using a dinucleotide (Janssen *et al.*, 1989). The ability of just 1 of the 16 possible dinucleotides to prime *in vitro* transcription suggests a truly unique start site, whereas the ability of two or three dinucleotides to prime could be indicative of staggered sites (which should be corroborated by high-resolution S1 nuclease mapping).

4.1.6. Reverse transcription PCR and quantitative real time RT-PCR

More recently, the presence or absence of transcripts (but not their start sites) is often determined by Reverse Transcription-PCR (RT-PCR). Although only semiquantitative, it is a rapid technique that employs synthetic oligonucleotides, reverse transcriptase, and DNA polymerase to amplify, as DNA, an internal segment of the transcript of interest. The amplified product can then be analyzed by agarose gel electrophoresis (e.g., see Stratigopoulos *et al.*, 2004). Where the transcription of a gene is not completely abolished, if the number of cycles is varied, it may be possible to gain some semiquantitative insight into the level of transcription. Preferably, quantitative real time RT-PCR (qRT-PCR) is carried out (for a review, see Kubista *et al.*, 2006). This requires fluorescently labeled oligonucleotide primers, or the use of a dye that fluoresces only in the presence of double-stranded DNA, which can be used to monitor the kinetics of formation of the amplified product and provide accurate estimates of transcript abundance over a wide dynamic range. Given the availability of different fluorescent labels, it is now possible to multiplex the qRT-PCR procedure to permit the analysis of several genes simultaneously.

The absence of a transcript, as deduced by any of the above procedures, does not prove direct regulation by a pathway-specific regulatory protein, but with the localization of the transcriptional start site in the wild-type strain, it provides the necessary information to carry out additional experiments that

address this possibility in a direct manner (see [Electrophoretic Mobility Shift Assay and Surface Plasmon Resonance](#)).

4.1.7. Electrophoretic mobility shift assay (EMSA)

Once the transcriptional start site of a gene whose transcript is absent in a putative regulatory mutant has been determined, for example by S1 nuclease mapping or primer extension, it is then possible to address whether the gene is directly or indirectly regulated by the putative pathway-specific regulatory protein. The most frequently used procedure thus far is EMSA ([Fried and Crothers, 1981](#); [Garner and Revzin, 1981](#)). In this approach, a labeled (generally by PCR) DNA fragment that contains sequences that encompass or lie upstream of the transcriptional start site is incubated with increasing amounts of the putative regulatory protein. The latter may be purified (e.g., after His-tagging), or be present in a crude cell extract. After incubation, the potential protein–DNA complexes are subjected to non-denaturing gel electrophoresis in parallel with an aliquot of the DNA fragment that has not been incubated with protein. A reduction in the mobility of the labeled DNA fragment (a “band-shift”) is indicative of a protein–DNA complex. The proportion of the DNA fragment retarded should increase with increasing amounts of protein. Moreover, to ensure that a specific interaction is occurring, it should be possible to abolish the band-shift by adding excess unlabeled fragment, while an excess of a nonspecific competitor should not compete out the band-shift. The most suitable nonspecific competitor is an unrelated DNA fragment of the same size and base composition.

To locate the site of interaction more precisely, it may be possible to use shorter and/or nonoverlapping fragments derived from the DNA sequence used in the original EMSA. Beyond that, DNaseI foot-printing ([Galas and Schmitz, 1978](#)) and site-directed mutagenesis can be used to define the interaction more precisely, and to determine the specific nucleotides that play a crucial role in protein binding.

4.1.8. Surface plasmon resonance

While it may be possible to obtain some semiquantitative data about DNA–protein interactions from EMSA, Surface Plasmon Resonance (SPR, commercialized initially by Biacore, now GE Healthcare (<http://www.biacore.com/lifesciences/company/presentation/introduction/index.html>)) allows for much more stringent kinetic analysis. SPR enables real-time detection and monitoring of DNA–protein interactions, and provides quantitative information on rates of association and dissociation, and hence rate and equilibrium constants. In principle, either the DNA fragment containing the binding site or the regulatory protein can be attached to the Biacore chip, and the other interacting partner applied through the flow channel. SPR can also

be used to quantify the effects of mutations in either the DNA-binding site or the regulatory protein on the interaction.

4.1.9. Reporter genes

Reporter genes encode protein products that can be detected and quantified readily, and include those conferring fluorescence, luminescence, antibiotic resistance, or a conveniently assayable enzyme activity (reviewed in [Dauert *et al.*, 2000](#)). Reporters are, therefore, useful tools for quantitatively assaying the transcriptional activity of promoters of interest, and could be recruited for measuring changes in the level of transcription of different operons present in a particular antibiotic biosynthetic gene cluster that occur as a result of mutation or overexpression of a putative regulatory gene. In practice, however, few examples of this particular application exist in the literature, which perhaps reflects the fact that a convenient, reliable reporter system to rival that based on *lacZ* in *E. coli* has yet to be developed for streptomycetes (discussed in [Kieser *et al.*, 2000](#)). Early attempts to apply the *lacZ*-based system to *S. lividans* or *S. coelicolor* met with only limited success due to issues with plasmid instability, suboptimal codon usage, and interference by native enzymes with β -galactosidase activity. Since then, systems based on green fluorescent protein (GFP), the kanamycin resistance gene *neo*, luciferase (*lux*), the *xylE* gene from the *Pseudomonas* TOL plasmid, and the *redD* gene from the *S. coelicolor* undecylprodigiosin antibiotic cluster have all been tried with varying degrees of success (reviewed in [Kieser *et al.*, 2000](#)). Endogenously produced compounds that fluoresce at a similar wavelength to GFP interfere with the use of this reporter in certain *Streptomyces* species, while both the *neo* and *redD* systems are at best only semi-quantitative. *xylE* and *lux* show the most promise as transcriptional reporters, and in recent work the *lux* system has been specifically optimized for use in high-GC bacteria, including streptomycetes ([Craney *et al.*, 2007](#)).

However, because of likely differences in transcript stability between a native mRNA and that produced from a reporter gene, regardless of the potential ease of use of reporter systems, analysis of wild-type and mutant strains at the RNA level, using the methods described earlier, will ultimately be required to confirm any quantitative changes observed using a reporter gene.

4.2. Pleiotropic regulatory genes

If the gene that has been identified is a pleiotropic regulatory gene, approaches that more globally capture patterns of gene expression will be required to help dissect the influence it exerts on the antibiotic gene clusters present in the strain. This is possible for organisms for which the genome sequence has been determined, allowing the application of DNA microarray and proteomics technologies ([Hesketh *et al.*, 2007a,b](#);

Huang *et al.*, 2001, 2005; Lian *et al.*, 2008; Takano *et al.*, 2005), but may also be achievable to a more limited extent in strains where genome sequence data exists only for a closely related species. The goal of any such functional genomics study in this context is to define the series of cellular events that link the identified regulatory gene with the antibiotic production phenotype observed. This may include both transcriptional and posttranscriptional effects, necessitating the use of both transcriptome and proteome analysis to view the complete picture. In reality however, costs and the local availability of equipment or expertise is likely to limit the approach chosen to only one or other of these methods, and in this chapter we confine our considerations to microarray analyzes (although the principles of experimental design and data analysis will be similar in each case).

DNA-microarrays were developed to detect the presence and abundance of labeled nucleic acids in a biological sample, and consist of a solid surface onto which many thousands of different DNA molecules have been chemically bonded (Bier *et al.* (2008) present a recent useful overview). For gene expression analysis, the labeled nucleic acids from the experimental samples are derived from mRNA and the DNA immobilized on the array has been designed so that every gene in the genome of interest is represented. Quantification of the amount of label hybridized, via Watson–Crick duplex formation, to each different DNA molecule on the array can therefore produce a simultaneous measurement of the expression levels of many thousands of genes. DNA microarrays can differ in a number of ways (the length of the DNA molecules immobilized; whether the array has been manufactured by robotic spotting or *in situ* synthesis; the number of different DNA molecules used to represent each gene; whether or not intergenic regions of the genome are also represented; cost) and will also differ in their sensitivity and accuracy of performance. Different array technologies can also require different methods for labeling of the sample to be hybridized, which can impact on how the samples are quantified. The Affymetrix platform, for instance, uses biotin-labeled cDNA or cRNA which dictates that only one sample can be hybridized to each array, whereas for many other technologies the labeled samples can be prepared using Cy3 or Cy5 fluorescent dyes which, since they emit fluorescence at significantly different wavelengths, allows two samples to be hybridized and detected per array. The former method thus yields an estimation of the absolute value of gene expression, while the latter produces a relative ratio-based measurement. Careful consideration to the choice of the array platform to be used in any transcriptomics experiment should be given, and is helped by clearly defining the desired outcomes of the study within the framework of the budget available.

Having decided on the technology to be used for the analysis, the next important consideration is the design of the experiment to produce the samples to be labeled and hybridized. Many pleiotropic regulatory genes are

only required for antibiotic production under particular nutritional conditions, and the culture medium used therefore needs to be carefully selected to suit not only production of the antibiotics but also the requirement for the regulator under study. Using the optimal growth conditions, three experimental designs that can be used for investigating the function of a pleiotropic regulatory gene are described below. In practice, and if resources permit, it may be desirable to combine at least two of these to reliably identify candidate genes that play intermediary roles in the regulation of antibiotic biosynthesis. In any event, the putative involvement of candidates identified using any of these approaches will need to be confirmed experimentally.

4.2.1. The effect of gene deletion

The analysis of differences in patterns of gene expression between the wild-type strain and a mutant in which the gene of interest has been cleanly deleted (e.g., by using one of the methods described above) is usually the first step in identifying sets of genes that are likely to be controlled by the pleiotropic regulatory gene. Since antibiotic production in the wild-type strain is normally growth phase-dependent rather than constitutive, this is usually achieved by sampling (in at least triplicate) both wild-type and mutant strains at several comparable time points during growth in liquid culture, such that at least one sample from the exponential, transition, and stationary phases of growth is present. Statistical analysis is then used to identify genes that are significantly up- or down-regulated as a result of the mutation, and clustering algorithms can be applied to find groups of genes that have similar expression profiles, and thus may be coregulated (Boutros and Okey (2005), D'haeseleer (2005)). If the pleiotropic regulator in question is predicted to be a DNA-binding protein, the upstream regions of the putatively coregulated sets of genes can be searched for common promoter sequences (using tools such as MEME (<http://meme.sdsc.edu/meme>) and ProDoric (<http://prodoric.tu-bs.de>)) to provide evidence for coregulation, and any putative protein binding site identified can be assessed experimentally using the *in vitro* techniques described earlier for the analysis of pathway-specific regulatory genes. In this way, it may be possible to build up a list of the genes that are direct targets for regulation by the mutated gene, and these can be assessed bioinformatically, looking for sequence homology to known regulatory factors or enzymes that may be involved in precursor supply or cofactor biosynthesis, for those most likely to be involved in influencing production of the antibiotics. Construction of targeted mutations in the candidate genes will ultimately be required to confirm any role in antibiotic biosynthesis, and the resultant mutant strains may subsequently form the subject of additional rounds of transcriptome/proteome analysis to further characterize the links to antibiotic production.

4.2.2. The effect of gene overexpression

The enhanced expression of a regulatory gene carried on a high copy number plasmid vector, or overexpressed from a strong constitutive promoter, is expected to produce the opposite effect to gene deletion: genes found to be up-regulated as a result of gene mutation should be down-regulated in the overexpression strain (and *vice versa*). Analysis of overexpression strains along the lines outlined for the deletion mutant above will therefore provide complementary data helping to identify sets of genes that are subject to control by the pleiotropic regulator, and which may therefore also be involved in the control of antibiotic production.

4.2.3. The effect of controlled induction of gene expression

The disadvantage of using the gene deletion or overexpression approaches described above is that in both cases steady-state conditions are being analyzed in which the cells have physiologically adapted to the change in expression of the pleiotropic regulatory gene. This means that the expression of numerous genes that are not directly controlled by the gene under study will also be significantly different between the strains analyzed. These indirect effects can potentially arise via additional regulatory genes which are *bona fide* members of the regulon of the pleiotropic regulatory gene (and thus will also be of interest), or be the result of compensatory changes occurring in response to deleterious effects on cellular metabolism caused by the mutation/overexpression. One approach that helps to distinguish between the direct and indirect effects of a regulatory gene is to induce a controlled change in its expression and analyze the response dynamically over a short period of time. This is most conveniently done by expressing a copy of the gene of interest under the control of a tightly regulated promoter, and is therefore limited to strains where such systems are available (e.g., streptomycete vectors carrying the thiostrepton-inducible promoter of *tipA* (as used in Hesketh *et al.*, 2007a; Huang *et al.*, 2005), and potentially the epsilon-caprolactam-inducible promoter present in pSH19 (Herai *et al.*, 2004). Genes that are directly under the control of the induced regulatory gene should exhibit instant changes in gene expression, whereas those further down the sequence of events will take a longer time to show a response. Compensatory changes should similarly not be evident in the data until after the more direct effects have been observed.

4.2.4. Defining genome-wide DNA–protein interactions by chromatin immunoprecipitation and microarray analysis (ChIP–on–Chip)

Once genes that are likely to be controlled by a pleiotropic regulatory gene have been identified (e.g., by microarray analysis), it may be necessary to determine whether regulation occurs in a direct or indirect manner.

While in principle this can be accomplished by EMSA or SPR analyzes on individual promoter regions, a more labor-efficient and global approach is accomplished by carrying out ChIP on Chip analysis. This generally requires a genome sequence and suitable microarrays for the organism of interest, purified regulatory protein and a corresponding antibody (although it may be possible to use commercially available antibodies made to, e.g., a FLAG tag that has been engineered into the protein prior to overexpression and purification). In brief, cultures at an appropriate stage of growth are treated with formaldehyde to cross-link regulatory proteins to their cognate and occupied (*in vivo*) binding sites. DNA, with any covalently attached proteins, is isolated from the treated cultures and sheared to give fragments that are 500–1000 bp in length. The required DNA–protein complexes are then isolated using antibody to the protein or its tag. The protein is removed from the DNA fragment by heat treatment, and the DNA amplified by PCR using a fluorescent dye, such as Cy5, and used to probe a microarray that contains all of the intergenic regions present in the genome (it may also be possible to use arrays confined to protein-coding sequences, but there would need to be some confidence that sequences existed on the array that were located close enough to the 5′-end of the coding sequences to be available for hybridization to the labeled probe). Development of the hybridized array followed by bioinformatic analysis will then reveal genes that are candidates for direct binding by the pleiotropic regulatory protein and therefore likely to be direct regulatory targets.

This in itself is not sufficient to establish direct regulation—hybridization to the array may be nonspecific or not accurately reflect the *in vivo* situation. Lack of, or altered, transcription of the newly identified genes in the pleiotropic mutant should first be confirmed by, for example, microarray or qRT-PCR analysis, and a direct interaction should ultimately be demonstrated by EMSA or SPR.

4.2.5. Systematic evolution of ligands by exponential enrichment (SELEX)

Although apparently not used thus far for streptomycetes, an alternative approach to identifying the binding sites (and hence directly controlled genes) of a pleiotropic regulatory protein is SELEX (Stoltenburg *et al.*, 2007; Tsai and Reed, 1998). In this approach, a random nucleotide sequence of ~20 bp is synthesized that is flanked by opposing PCR primer sites. This highly degenerate mixture of nucleotide sequences is then incubated with the purified regulatory protein and subjected to EMSA analysis. Although a retarded band is unlikely to be apparent upon ethidium bromide staining after the first EMSA, material is extracted from the region of the gel where a shifted band would be expected to migrate to, and subjected to PCR using primers corresponding to the flanking sites. The subsequent PCR product is then incubated with the purified regulatory protein and the

EMSA, gel extraction and PCR repeated until a band becomes visible by ethidium bromide staining. Subsequent sequencing of the PCR products should then reveal *in vitro* binding sites for the regulatory protein of interest. Bioinformatic analysis is then required to identify putative binding sites present in the genome sequence of the organism of interest. Confirmation that the protein does indeed bind to such sites *in vivo* and regulates the corresponding genes then needs to be obtained by transcriptional analysis of the regulatory mutant (e.g., by qRT-PCR). Note that while repeated rounds of SELEX should enrich for the most tightly binding nucleotide sequences *in vitro*, these are not necessarily representative of the majority of physiologically relevant binding sites present in the genome.

In a variation on this approach, Elliot *et al.* (2001), Horinouchi *et al.* (2000), and Yamazaki *et al.* (2000) used purified regulatory proteins with fragmented genomic DNA to which PCR primers had been attached as the target sequences. Multiple rounds of PCR and EMSA assays yielded a number of candidate genes for further analysis.

4.3. Concluding remarks

The regulation of antibiotic production is a highly complex process, but a plethora of technologies can now be used to gain a greater understanding of how the transcription of the often large gene clusters that encode these compounds is triggered. We hope that this article, which is not intended to be a comprehensive description of all of the approaches that can be used, will provide the reader with the initial understanding required to embark on such studies.

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