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REVIEW

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Antibiotic production by actinomycetes: the Janus faces of regulation

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Abstract This manuscript reviews some of the common regulatory mechanisms that control antibiotic production in actinomycetes. These ubiquitous bacteria, collectively responsible for the earthy smell of soil, are prolific producers of antibiotics and other secondary metabolites. The content of this review is biased towards the author's current research interests, concerning the action of regulatory gene products that control transcription of antibiotic-biosynthetic genes and the associated involvement of low molecular weight signalling molecules of the gamma-butyrolactone family. As a result, much fertile ground remains unturned particularly in the area of environmental monitoring and responses of actinomycetes to stimuli so perceived. Reviews casting a broader net are cited in the text.

Keywords Actinomycetes · Antibiotic production · SARPs · Gamma-butyrolactones · Pathway-specific regulation · Pleiotropic regulation

Introduction

With his reputation for causing springs suddenly to gush from hillsides to the consternation of uninvited guests, Janus was a favourite deity in Ancient Rome, which provided no fewer than seven hills on which this act might be rehearsed. In addition to 'God of Beginnings and Endings' (creatively taken to include most civic celebrations) the dual-faced Janus held various other portfolios, including 'Guardian of Gateways, Doorways and Bridges'. Janus was also Keeper of the Gateway to his own temple, which was kept open in times of war (presumably to encourage performance of his party trick) and closed only during peacetime, i.e., rarely. There he stood in the doorway with one face illuminated and the other dark. Hence, Janus imagery has been widely used to symbolise opposites or to place intellectual concepts in apposition (as, powerfully, in [10]). The present article discusses interplay between positive and negative control mechanisms that operate during antibiotic production.

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Antibiotic-biosynthetic gene clusters

Genes encoding the biosynthesis of antibiotics and other secondary metabolites are typically clustered within the respective actinomycete genomes or, in a few known cases, on giant linear plasmids. In genetical terms, low molecular weight antibiotics are quite complex; commonly 10–50 genes might be required to encode synthesis of a molecule of Mr $<1{,}000$. In contrast, a single gene can readily encode a 100 KDa protein. Each antibiotic-biosynthetic gene cluster occupies up to 1% of the actinomycete genome and organisms that produce multiple antibiotics (probably the norm; see [8]) harbour multiple discrete gene clusters dedicated to that end. In addition to genes that encode enzymes required for antibiotic production ('biosynthetic genes' hereinafter), such clusters also typically include one or more antibiotic-resistance determinants [9] and usually, but not always, one or more regulatory genes. The latter commonly regulate the gene clusters in which they are found. As a deceptively simple example, the streptomycin-biosynthetic (*str-sts*) cluster in *Streptomyces griseus* includes a single regulator (*strR*) plus a single resistance determinant. ('Regulator' is used here interchangeably to mean either a gene or its product). In contrast, the *tyl* gene cluster of *S. fradiae*, producer of tylosin, is decidedly extravagant with three resistance genes plus at least five regulators (Fig. 1). Nevertheless, cohabitation of regulatory and biosynthetic genes is not an invariant

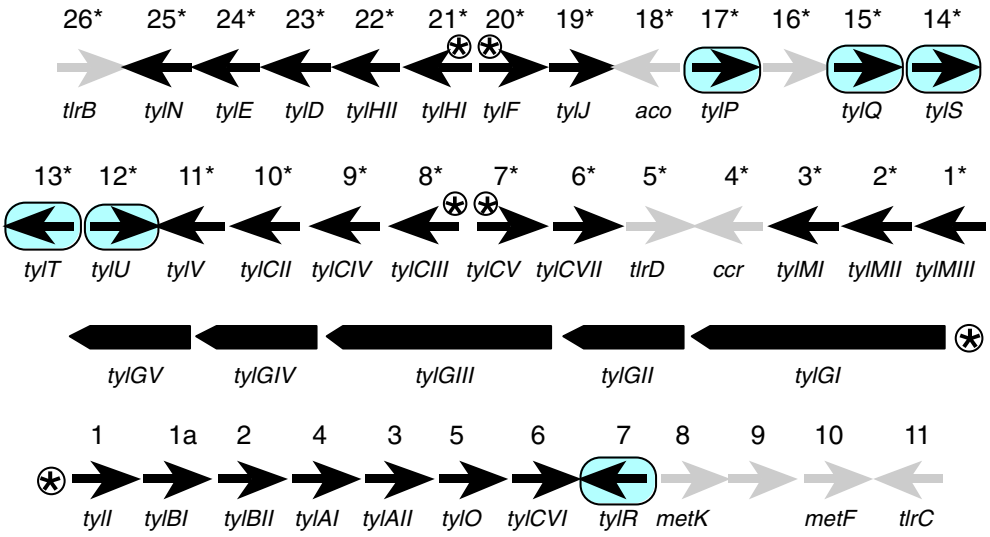

Fig. 1 The tylosin-biosynthetic gene cluster of *S. fradiae*. Not drawn to scale. The cluster occupies a contiguous portion of the genome (approximately 85 kb). Regulatory genes are outlined in *boxes*. Tylosin-biosynthetic genes are represented by *black arrows*. Resistance determinants (designated *'tlr'*), ancillary genes and others that are unassigned are represented as *grey arrows*. The full complement of biosynthetic genes could, in principle, be expressed from three pairs of divergent promoters (*stars*) via operon control

feature of these clusters. There is no regulator associated with the much-studied *ery* genes of *Saccharopolyspora erythraea* and no other gene in that organism has yet been shown to control erythromycin production. The *tyl* cluster also includes 'ancillary' genes that are non-essential for tylosin production [7]. Orthologues of these are commonly associated with primary metabolism in other organisms; paralogues are presumably present elsewhere in the *S. fradiae* genome.

Positive control mechanisms

Antibiotic-biosynthetic gene clusters are subject to cascade regulation that ultimately (i.e., at the 'lowest' hierarchical level) involves positive control (for a review, see [6]). Wherever negative control is encountered, there is typically a lower level of positive control, involving direct activation of otherwise cryptic promoters that mediate transcription of antibiotic-biosynthetic genes or operons. At that basal level, control is *pathway-specific* with no associated effect(s) on other aspects of metabolism.

Pathway-specific transcriptional activators

Following the advent of DNA sequencing, a widespread family of pathway-specific regulatory proteins, later designated 'SARPs' (*S*treptomyces *A*ntibiotic *R*egulatory *P*roteins; [33]), was soon revealed by comparison of their deduced aminoacid sequences. Founder members of the SARP club included ActII-ORF4 and RedD, transcriptional activators of the *act* and *red* gene clusters that, respectively, encode production of actinorhodin and undecylprodigiosin in *S. coelicolor*, and DnrI that controls daunorubicin-biosynthetic genes in *S. peucetius*. However, although many antibiotic-biosynthetic clusters among *Streptomyces* spp. harbour SARP-encoding genes (commonly, one per cluster), this is not a golden rule. The activator StrR is not a SARP and regulation of streptomycin biosynthesis in *S. griseus* is SARP-independent.

Positive control by SARPs and other pathway-specific regulatory proteins is consistent with the observation that deletion or inactivation of the respective regulatory gene usually abolishes antibiotic production. Interestingly, however, such proteins are not normally present in saturating amounts. This became evident when 'self-cloning' of DNA fragments (later shown to increase the copy number of specific regulator genes) caused elevated levels of antibiotic production, ostensibly reflecting elevated levels of regulatory proteins (for a review, see [5]). This strategy remains a panacea for enhancement of antibiotic production and even applies to organisms already subjected to empirical strain improvement. Thus [24], an advanced production strain of *S. fradiae* generated significantly more tylosin following self-cloning of the pathway-specific regulator, *tylR*, or the SARP-encoding gene (*tylS*) that activates expression of *tylR*.

Following transcriptional analysis of (a few) gene clusters to locate the respective promoters, allied with genetical analysis to identify candidate regulators, and complemented by DNA-binding studies with purified regulatory proteins, it now appears plausible that entire antibiotic-biosynthetic gene clusters can be activated by single regulatory proteins (one per cluster). For example, the *act*II-ORF4 gene is proposed to control transcription from all of the *act* biosynthetic genes [11] and the ActII-ORF4 protein binds to two pairs of divergently oriented promoters located at key intergenic sites within the *act* cluster [1]. Similarly, the *dnrI* gene of *S. peucetius* controls transcription from the (daunorubicin-biosynthetic) *dnr* cluster [17] which, from gene organisation alone, must involve at least ten promoters. Within that cluster, the DnrI protein binds to at least two sites, again involving pairs of divergent promoters [31]. Finally for

now, transcription from the whole of the streptomycin-biosynthetic gene cluster of *S. griseus* is controlled by *strR* with extreme parsimony in promoter usage [32]. Binding of the StrR protein has been demonstrated at three out of eight postulated control sites, with sequence-based predictions indicating at least one further target [22]. With so many genes to account for in any given cluster it is not surprising that most analyses are incomplete but, nevertheless, the aforementioned generalization seems plausible. A notable feature of this model is extensive usage of operon control to minimise the number of promoters, as exemplified by control of streptomycin production, viz., turn on the *strR* promoter and the resultant StrR protein turns on 25 *str-sts* biosynthetic genes via activation of just 8 promoters. All blocks of codirectional genes are controlled as operons. Nothing could be simpler!

Higher levels of control

Before all of that, of course, industrial microbiologists were already familiar with *pleiotropic* control of antibiotic production, having routinely encountered non-producing mutants that had also lost the ability to sporulate. Such phenomena pointed to proteins that function within multiple pathways, most likely as control elements linking the regulatory cascades that govern secondary metabolism and morphological differentiation. In the hierarchy of regulation, such linkage operates at higher levels than pathway-specific control. At even higher levels, extracellular stimuli elicit intracellular responses so that to understand fully the regulation of antibiotic production would be to understand in detail how an organism responds to changes in its environment. Progress there is hard made and classic reviews of microbial physiology, written a quarter of a century ago, are still essential reading [18]. In comparison, the manipulation of regulatory genes, conveniently encountered in readily identifiable clusters (as typified by studies in the present author's laboratory), is much more straightforward.

Positive control of tylosin production

When the *tyl* gene cluster of *S. fradiae* was first sequenced, three genes immediately presented themselves as candidate positive regulators. Two of them (*tylS* and *tylT*) were deduced to encode SARPs whereas the other, *tylR*, was clearly related to *acyB2* from *S. thermotolerans*, producer of carbomycin. The latter gene had already been described as a positive regulator [2], the first to be associated with macrolide antibiotic production. The novelty of finding two SARPs apparently associated with a single biosynthetic pathway was diminished somewhat when tylosin production survived insertional inactivation of *tylT*, leaving the role(s) of its product yet to be determined [4]. However, knockouts (KOs) of *tylS* or, earlier, *tylR* [3] totally abolished production of tylosin or any of its chemical components. (Tylosin is a 'macrolide' antibiotic, consisting of a polyketide lactone substituted with three deoxyhexose sugars.) Loss of the *tylR* transcript in *tylS*-KO strains (but not *vice versa*) revealed that TylS controls expression of *tylR*, and restoration of tylosin production in *tylS*-KO strains, due to forced expression of *tylR* from a heterologous promoter, revealed that TylR is a global activator of the *tyl* cluster. At least in such engineered strains, TylR can apparently activate all of the tylosin-biosynthetic genes with no essential role for TylS [4]; N. Bate et al., manuscript in preparation, author's laboratory). Hypothetically, TylR might do that from pairs of divergent promoters at just three intergenic target sites (see Fig. 1). This was the first demonstration that some SARPs (here, TylS) can act above the basal level in regulatory cascades that govern antibiotic production.

Activation of *tylR* is not the only direct role of TylS. Immediately adjacent to [*tylS-tylT*] in the regulatory 'nerve centre' of the *tyl* cluster lie two genes (formerly designated *orf12** and *orf11*;* see Fig. 1) whose transcription, it now turns out, does not survive disruption of *tylS* [24]. When first encountered [3], *orf12** was unlike any entry in the database although a few orthologues (with no assigned or even speculative functions) have since been reported. It now transpires that *orf12** (*tylU*) is another *tyl* regulatory gene. Although there is no obvious phenotype associated with overexpression of *tylU* in *S. fradiae* wild type, disruption of *tylU* reduces tylosin production by about 80%. Normal production can be restored in such strains by introducing an intact copy of *tylU*, or by overexpressing an extra copy of *tylR*, but not at all by *tylS* (N. Bate et al., manuscript in preparation, author's laboratory). Bearing in mind that overexpression of *tylR* similarly rescues the tylosin-production phenotype of *tylS*-KO strains (which also lack TylU) these data suggest that TylS and TylU combine (mechanism unspecified) in the activation of *tylR*. Western analysis with 'anti HIS-tag' antibody, aimed at detecting endogenously produced TylR in engineered strains of *S. fradiae*, has confirmed that model. The level of HIS-tagged TylR produced in *tylU*-KO strains is much lower than in isogenic $tylU^+$ strains (D. Bignell, unpublished data, author's laboratory). In summary, TylS drives expression of *tylU* and the TylU protein acts in combination with TylS to facilitate TylR production, for which TylS (but not TylU) is indispensable.

One size does not fit all

By now it is clear that different SARPs act differently in different organisms. During daunorubicin production by *S. peucetius*, or synthesis of actinorhodin and undecylprodigiosin by *S. coelicolor*, SARPs control directly the promoters of antibiotic-biosynthetic genes and seem to operate exclusively at that lowest regulatory level. In

contrast, TylS might not act at that level at all. In a further variation on this theme, a SARP (CcaR) encoded in the 'cephamycin C cluster' of *S. clavuligerus* controls (at least some) cephamycin C-biosynthetic genes by activating multiple promoters while also controlling, directly or indirectly, another regulatory gene (*claR*). The latter is located in, and is specific for, the 'clavulanic acid cluster' (the two sets of genes are immediately adjacent in the *S. clavuligerus* genome where they form a 'supercluster'). As *claR* seems to control late, but not early, 'clavulanic acid genes', it is not clear in this context whether ''one activator per cluster'' remains intact. That aphorism, together with ''one cluster per activator'', would again be violated in the (perhaps, likely?) event that CcaR were shown to activate directly any of the 'clavulanic acid genes' (for discussion and references; see [23, 28]). In these various ways, what you see depends on where you look. Janus might well concur with that!

Negative control mechanisms

Gamma-butyrolactone autoregulators

Almost 40 years ago, it was shown by Kokhlov and colleagues that a small, diffusible molecule of the gamma-butyrolactone (GB) family could influence secondary metabolism and morphological differentiation in *S. griseus* [for a review of GBs, see [34]). Non-sporulating mutants that also failed to make streptomycin were restored to wild type behaviour if supplied exogenously with a GB designated 'A-factor' (Fig. 2). Those effects of A-factor were elicited with nanomolar concentrations of the GB and were observed only in mutants, which later proved to be defective in A-factor biosynthesis. Supplementation of *S.griseus* wild type with A-factor did not enhance streptomycin production. Since then, involvement of GBs in regulation of antibiotic production by *Streptomyces* spp. has been studied quite widely, but with greatest impact in two laboratories.

From the work of T. Beppu, S. Horinouchi and colleagues in Tokyo over the past 20 years, A-factor mediated control of streptomycin biosynthesis in *S. griseus* has been shown to involve a regulatory cascade triggered by classical induction (i.e., de-repression) of a single activator gene, *adpA*. De-repression of *adpA* involves binding of A-factor to a repressor protein, ArpA, with consequent dissociation of the latter from the *adpA* promoter [21]. That is the sole binding target for ArpA which, in turn, is the only known receptor for A-factor in *S. griseus*. The AdpA protein activates a regulon of multiple regulatory genes whose products separately trigger secondary metabolism and various aspects of morphological differentiation, including sporulation [35]. In the context of streptomycin production, AdpA activates the *strR* promoter, thereby triggering production of the StrR protein which activates the streptomycin-biosynthetic gene cluster. Again, nothing could be simpler! As it happens, other systems are typically much less simple.

Incisive studies by Y. Yamada, T. Nihira and colleagues in Osaka, mainly focused on virginiamycin production in *S. virginiae* and the involvement of GBs known as 'virginiae butanolides' (VBs; Fig. 2), have revealed significant differences from ArpA-mediated events in *S.griseus*. For example, a VB-binding protein, BarA, from *S. virginiae* represses multiple target promoters and thereby blocks virginiamycin production, without affecting sporulation [14, 20]. Such repression is relieved by binding of butanolides to BarA which, unlike ArpA, also regulates synthesis of the cognate GB. Again unlike ArpA, but similar to FarA (a GB-binding protein from *S. lavendulae* [16]), BarA targets the promoter of its 'own' gene, *barA* (i.e., BarA is autoregulatory).

Related studies in Leicester were strongly influenced by the secure demonstration in Osaka that BarA binds to, and blocks expression of, its downstream neighbour, *barB*, and that VBs could reverse such repression by binding to BarA and causing its dissociation from the *barB* promoter [14]. When aminoacid sequences of the *tylP* and *tylQ* products were first deduced [3], they were clearly similar to BarA and BarB, respectively, suggesting that TylP (a candidate GB-binding protein) might regulate expression of *tylQ*, although at that time no function could be suggested for the TylQ protein or, indeed, for BarB. The function of TylQ was resolved experimentally before that of TylP.

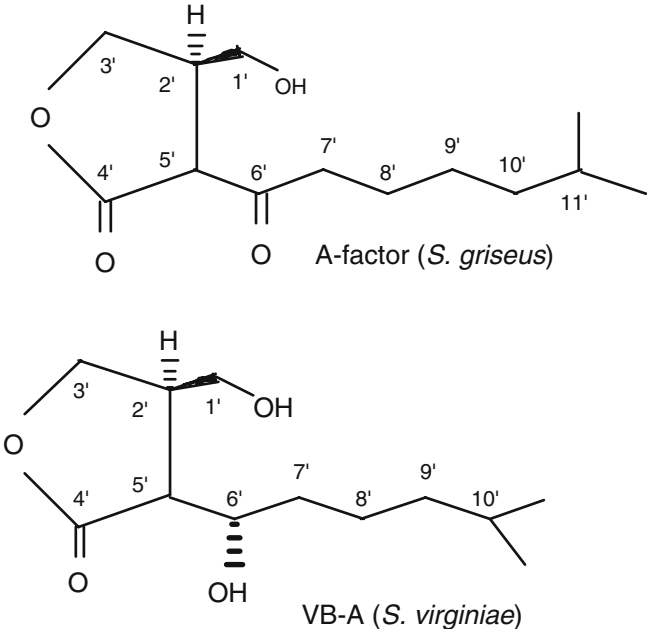

Fig. 2 Structures of gamma-butyrolactone autoregulators. There are three chemical classes that differ in substitution at the 6-position (-keto as in A-factor from *S. griseus*; -alpha hydroxyl, as in virginiae butanolides e.g., VB-A from *S. virginiae*; -beta hydroxyl, as in IM-2 from *S. lavendulae*). The length of the hydrophobic tail can also vary

Negative control of tylosin production

TylQ is a repressor that is proposed to target the *tylR* promoter. This model was derived from two observations. When the *tyl* gene cluster of *S. fradiae* wild type was subjected to transcript analysis via reverse transcription-polymerase chain reaction, *tylQ* was found to be active at early stages of fermentation but was the only *tyl* gene to be silent following the onset of tylosin production [25]. In parallel, similar analysis was applied to a strain ('*tylQ*-KO') within which *tylQ* had been disrupted and to another engineered strain ('*tylQ*-OE') in which *tylQ* was overexpressed under control of a strong, constitutive promoter. The *tylQ*-OE strain failed to express many of the *tyl* genes and produced no tylosin, even after extended fermentation. In contrast, tylosin production began early in the *tylQ*-KO strain and, significantly, transcripts from *tylQ* and *tylR* were never detected concurrently.

Enhanced tylosin production by *tylQ*-KO strains has industrial consequences. When *tyl* DNA was obtained from an advanced *S. fradiae* strain of the Lilly industrial production lineage, no changes were found in the coding sequences of any of the regulatory genes (*tylP,Q,R,S,T,U*) except for a single T–A transversion within *tylQ* [26]. This would have changed a single aminoacid residue, histidine to glutamine, within the downstream helix (significantly, the DNA recognition moiety) of an N-terminal helix–turn–helix motif that is characteristic of many repressors. None of the *tyl* regulatory genes had suffered promoter mutations during decades of empirical strain improvement involving random mutagenesis nor had any changes occurred in other obvious promoter regions (for example, between divergent biosynthetic genes). That single base change in *tylQ* occurred quite early in the Lilly strain lineage (perhaps very early) and it clearly 'killed' the TylQ repressor. Thus, tylosin production by an engineered derivative of *S. fradiae* wild type was abolished by constitutive expression of a wild type copy of *tylQ* but was unaffected by overexpression of the mutant allele derived from the production strain [26]. And that's not all. Disruption of *tylQ*, followed by overexpression of *tylR*, in *S. fradiae* resulted in sequential and significant enhancements of tylosin production (N. Bate, unpublished data, author's laboratory).

Confirmation that TylP does indeed control *tylQ* was preceded by the observation of 'PARE' sequences (see below), characteristic of target sites for GB-binding proteins, located upstream of *tylP*, *tylQ* and *tylS* [27]. Again, this development was predicated on work from the Osaka group [15] who derived consensus recognition sequences from multiple authentic target sites for BarA ('BARE' sequences) and the autoregulatory binding site of FarA ('FARE' sequence). In engineered strains of *S. lividans*, TylP powerfully inhibited expression of a reporter gene fused to *tylP* or *tylQ* promoter DNA containing the respective 'PARE' sequences and also, but less strongly, inhibited expression from the *tylS* promoter [27]. The clear inference was that TylP controls not only *tylQ* (a repressor represses a repressor) but also *tylS* and, in autoregulatory fashion, *tylP*. That model has recently been confirmed directly. His-tagged TylP was shown to bind specifically to the three respective 'PARE' sequences *in vitro* and such binding was reversed by extracts, ostensibly containing GB(s), from *S. fradiae* fermentation broths (D. Bignell et al., manuscript in preparation, author's laboratory).

The current model for regulation of tylosin production, summarized in Fig. 3, is clearly not yet complete. The model says nothing about positive control of *tylS* and, in its present form, it probably cannot adequately account for enhanced production of tylosin in *tylP*-KO strains [27] although it does predict an increase in mycelial TylS content under such conditions. Presumably, in the absence of TylP, there must be an another way of turning *tylQ* down or off.

Questions for the future

Like BarA, TylP controls antibiotic production by regulating multiple genes, including its own determinant.

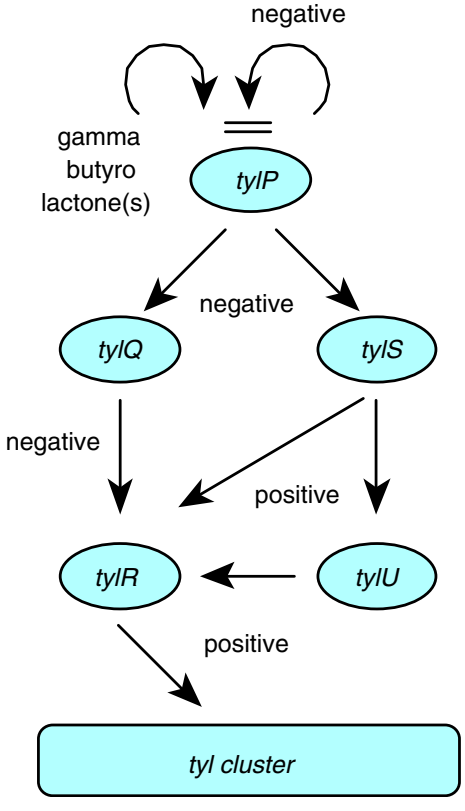

Fig. 3 Current model for regulation of tylosin production in *S. fradiae*. At early stages of fermentation, in the presumed absence of gamma-butyrolactone(s), *tylP* is subject to autorepression, TylQ represses *tylR*, and the *tyl* gene cluster is not expressed, although TylS activates expression of *tylU* at this time. Subsequently, following presumed derepression of *tylP* by uncharacterized gamma-butyrolactone(s), *tylQ* is silenced allowing TylS and TylU to activate expression of *tylR*. The TylR protein activates the cluster of tylosin-biosynthetic genes

Whether, like BarA and ScbR (a GB-binding protein from *S. coelicolor* [29]), TylP also influences cognate GB production remains to be established. In addition to TylP, a growing list of GB-receptors, including ScbR [30] and SpbR from *S. pristinaespiralis* [12], each regulate a 'SARP gene'. How common will this turn out to be? There is also a SARP gene (*vmsR*), associated with virginiamycin production, that functions downstream of *barB* in the regulatory cascade [13]. BarB now appears to be a repressor [19]; does it control *vmsR* directly? ArpA in *S. griseus* has only a single target promoter [see 32]. How common is that among GB-binding proteins? And to what extent might other low Mr compounds, including intermediates or end products of the respective biosynthetic pathways, also influence antibiotic production in addition, or as alternatives, to GBs?

Conclusions

Observed interactions between regulatory elements during antibiotic biosynthesis are becoming ever more dissimilar and not suggestive of a common pattern, which brings us to the final keynote conclusion. In the theatre of antibiotic production, genomics might well set the regulatory stage by listing the actors. But their entrances and exits, and what goes on in between, will differ from play to play. To understand how any specific plot is thickened will still require detailed experimentation — that means good old-fashioned biochemistry and genetics. Best of all, throw in some good old-fashioned microbial physiology and one day we might *really* understand the regulation of antibiotic production by actinomycetes.

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