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# Regulation of secondary metabolism in streptomycetes

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While the biological functions of most of the secondary metabolites made by streptomycetes are not known, it is inconceivable that they do not play an adaptive ecological role. The biosynthesis of secondary metabolites under laboratory conditions usually occurs in a growth phase or developmentally controlled manner, but is also influenced by a wide variety of environmental and physiological signals, presumably reflecting the range of conditions that trigger their production in nature. The expression of secondary metabolic gene clusters is controlled by many different families of regulatory proteins, some of which are found only in actinomycetes, and is elicited by both extracellular and intracellular signalling molecules. The application of a variety of genetic and molecular approaches is now beginning to reveal fascinating insights into the complex regulatory cascades that govern this process.

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## Introduction

Streptomycetes and related actinomycetes continue to be prolific sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti-infectives, anti-cancer agents or other pharmaceutically useful compounds. The production of secondary metabolites by the Gram-positive mycelial streptomycetes generally coincides with, or slightly precedes, the development of aerial hyphae in surface-grown cultures. In liquid-grown cultures, it is generally confined to stationary phase, and it is frequently assumed to result from nutrient limitation. With only one known exception [1], genes for the production of individual secondary metabolites are arranged in clusters that vary in size from a few to over 100 kb [2<sup>••</sup>,3,4<sup>•</sup>,5]. Most but not all of these clusters contain pathway-specific regulatory genes whose expression frequently depends on genes that are required for the production of several secondary metabolites made by the strain. Some of these genes, most notably the *bld*

genes [6,7] are also needed for the formation of aerial hyphae and spores.

In this short review, I focus primarily on the regulation of secondary metabolism, and particularly antibiotic production, in streptomycetes, and on recent work that provides insight into the physiological signals and molecular mechanisms involved in its activation. Space limitations preclude discussion of most of the genes that are also required for morphological differentiation.

## Growth rate, nutrient limitation and the onset of secondary metabolism

### A role for ppGpp in initiating secondary metabolism under conditions of nitrogen-limitation

The notion that a reduction in growth rate, if not growth cessation, is an important signal for triggering secondary metabolism is consistent with much of the published literature. Given its likely participation in the growth rate control of gene expression in unicellular bacteria [8], the role of the highly phosphorylated guanosine nucleotide (p)ppGpp in triggering antibiotic production in streptomycetes has received considerable attention. The ribosome-associated ppGpp synthetase (RelA) is required for antibiotic production under conditions of nitrogen limitation in *Streptomyces coelicolor* A3(2) [9] and for cephamycin C production in *Streptomyces clavuligerus* [10,11]. Whether ppGpp was directly involved in promoting transcription of antibiotic biosynthetic genes or whether the latter was an indirect consequence of a reduction in growth rate prompted by ppGpp-mediated inhibition of rRNA synthesis was unclear. However, when a modified *relA* gene was used to induce ppGpp synthesis in *S. coelicolor* without eliciting a detectable reduction in growth rate, transcription of *actII-orf4*, the pathway-specific regulatory gene for actinorhodin (Act) production, occurred [12<sup>••</sup>]. This provides the most convincing evidence yet for a direct role for ppGpp in activating the transcription of antibiotic biosynthetic genes. The mechanism by which this occurs is not known, but it is interesting to note that mutations that confer resistance to rifampicin and that by-pass the requirement for ppGpp synthesis for activation of antibiotic production occur in the  $\beta$  subunit of RNA polymerase (RNAP; [13]). It is conceivable that these mutations mimic the effect of ppGpp binding to RNAP [14] and lock it in a conformation that permits, or favours, transcription of secondary metabolic gene clusters.

### A role for phosphate in repressing antibiotic production

While RelA is absolutely required for antibiotic production in *S. coelicolor* upon nitrogen starvation, it is

dispensable under conditions of phosphate limitation, where a ppGpp-independent signalling mechanism must operate to initiate secondary metabolism [9]. An excessive level of inorganic phosphate in the culture medium prevents the production of many structurally diverse secondary metabolites [15], and in at least some cases this reflects repression of transcription of biosynthetic gene clusters [16,17]. Mutation of the two-component regulatory system PhoR-PhoP of *Streptomyces lividans* resulted in reduced levels of alkaline phosphatase activity and phosphate transport at low phosphate concentrations, and in a marked increase in the level of Act and undecylprodigiosin (Red) production [18\*\*]. While this might indicate a direct or indirect role for phosphorylated PhoP in repressing transcription of antibiotic biosynthetic genes (pleiotropic or pathway-specific), phosphate inhibition of antibiotic production is still observed at high phosphate levels in a *phoPR* deletion strain, perhaps reflecting a role for intracellular phosphate itself in the inhibition of secondary metabolism.

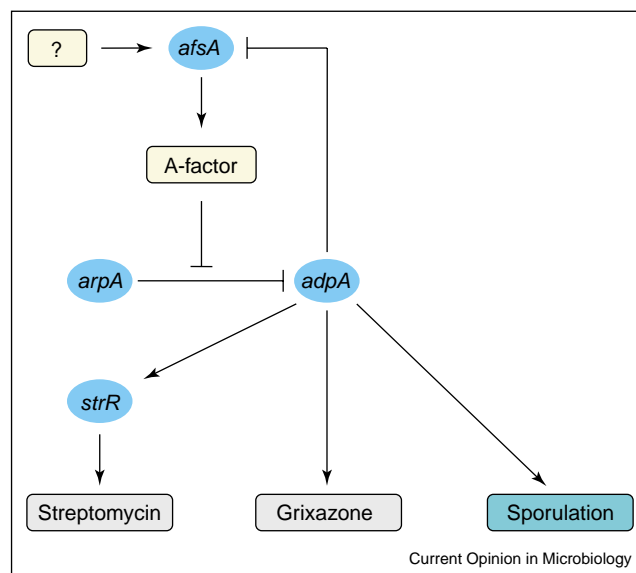
Consistent with this idea, inactivation of the polyphosphate kinase (PPK) of *S. lividans*, which produces polyphosphate during conditions of phosphate sufficiency, also resulted in a marked increase in Act production, and increased levels of transcription of pathway-specific regulatory genes for Act, Red and a calcium-dependent antibiotic (CDA) [19]. Under conditions of phosphate limitation, polyphosphate would normally be broken down into inorganic phosphate by exopolyphosphatases. Since this presumably cannot occur in a *ppk* mutant, intracellular levels of phosphate would be expected to be lower upon depletion of extracellular phosphate than in the wild-type strain. How elevated levels of intracellular phosphate might exert an inhibitory effect on secondary metabolism remains speculative.

## Extracellular signals for secondary metabolism

### A role for A-factor, a $\gamma$ -butyrolactone, in both secondary metabolism and morphological differentiation

$\gamma$ -butyrolactones are produced by many, if not all streptomycetes [20,21], and by several other genera of actinomycetes [22,23], and are implicated in the onset of secondary metabolism in several species. The most characterized  $\gamma$ -butyrolactone is A-factor (2-isocapryloyl-3R-hydroxymethyl- $\gamma$ -butyrolactone) of *Streptomyces griseus*. Unusually, A-factor is required for both secondary metabolism (streptomycin and grinoxone production) and morphological differentiation. The role of A-factor, which requires *afsA* for its synthesis, in regulating the onset of streptomycin production has been largely resolved [24,25,26\*]. Binding of A-factor to its cytoplasmic binding protein ArpA releases the latter from the *adpA* promoter, allowing *adpA* transcription. AdpA is required for activation of transcription of *strR*, the pathway-specific regula-

Figure 1



The A-factor regulatory cascade of *Streptomyces griseus*. A-factor is detectable in the culture medium just before the onset of streptomycin production. The signal(s) (shown as '?') that trigger its synthesis, mediated in some manner by AfsA, are not known.

tory gene for streptomycin production (Figure 1), and for expression of other members of the *adpA* regulon, some of which are required for morphological differentiation. *adpA* appears to be the only ArpA-dependent gene for both secondary metabolism and morphological differentiation, and to play a role in modulating A-factor synthesis once the  $\gamma$ -butyrolactone has fulfilled its function in triggering both processes [26\*]. Although attempts to crystallize ArpA have persistently failed, the structure of the homologous CprB from *S. coelicolor* has provided insights into the likely conformational changes that occur upon ligand binding that result in dissociation of ArpA from the *adpA* promoter [24]. The function of CprB, however, is unknown.

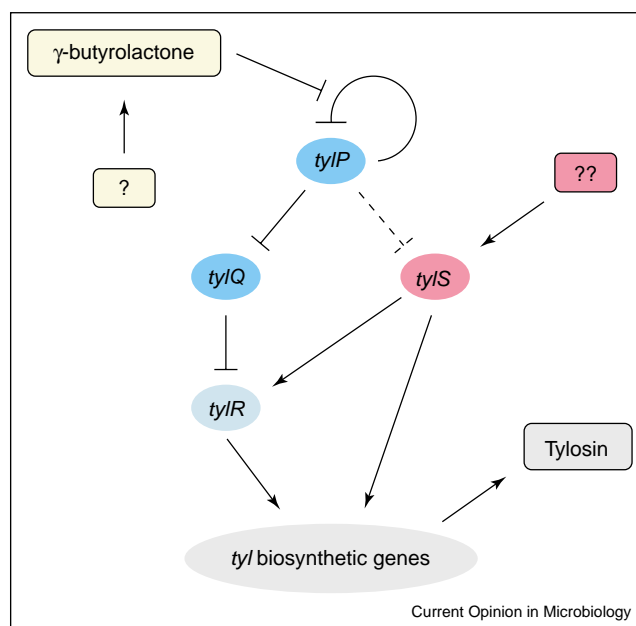
### Are $\gamma$ -butyrolactones generally pathway-specific regulators?

In contrast to A-factor, most  $\gamma$ -butyrolactones appear to be devoted to the regulation of secondary metabolism. Thus the virginiae butanolides (VB) control virginiamycin production in *Streptomyces virginiae* [27\*], IM-2 [(2R,3R,1'R)-2-1'-hydroxybutyl-3-hydroxymethyl  $\gamma$ -butanolide] elicits the biosynthesis of the nucleoside antibiotics showdomycin and minimycin in *Streptomyces lavendulae* [28], and SCB1 [(2R,3R,1'R)-2-(1'-hydroxy-6-methylheptyl)-3-hydroxymethylbutanolide] evokes the precocious production of the pigmented antibiotics Act and Red in *S. coelicolor* [29]. None of these  $\gamma$ -butyrolactones are required for or influence morphological differentiation. Unlike ArpA, which plays no role in A-factor synthesis [26\*], the VB, IM-2 and SCB1 binding proteins

are all required for normal levels of production of their cognate  $\gamma$ -butyrolactone [28–30]. While *afsA* and *arpA* are separated by over 100 kb of DNA in *S. griseus* [31], their homologues in *S. virginiae* and *S. lavendulae* are juxtaposed, and in *S. virginiae* they lie between the two secondary metabolic gene clusters that they regulate (those for virginiamycin M and virginiamycin S [27]). Similarly, although initially recognized by their role in regulating Act and Red production, the corresponding genes of *S. coelicolor*, *scbA* and *scbR*, are adjacent to, and directly regulate, a cluster of genes that encode a Type I polyketide of unknown function [32]. In the absence, but not presence, of SCB1, ScbR binds to the promoter of the pathway-specific regulatory gene *kasO* repressing its transcription. By contrast, ScbR does not bind to the promoter regions of pathway-specific regulatory genes for Act and Red production [29]. Moreover, deletion of *scbR* and *scbA* resulted in reduced and elevated levels of pigmented antibiotic production, respectively, contrasting with the mutant phenotypes of their homologues in the other three species. Thus it is conceivable that SCB1 and *scbAR* do not normally play a role in regulating Act and Red synthesis. Perhaps persistent repression of the Type I polyketide gene cluster, which would be predicted in a *scbA* mutant, accentuates Act and Red production through enhanced precursor provision (all three pathways utilize malonyl-CoA). Alternatively, it may indicate a greater degree of regulatory cross-talk between individual secondary metabolic pathways than is currently assumed (also see AbsA1A2 and CDA below).

The occurrence of genes encoding homologues of  $\gamma$ -butyrolactone-binding proteins in several other secondary metabolic gene clusters [33,34] is consistent with the idea that the usual role of these compounds is to regulate the production of a specific secondary metabolite. A particularly interesting example is the tylosin biosynthetic gene cluster of *Streptomyces fradiae* which contains, remarkably, no fewer than five putative regulatory genes [35]: *tyIP* and *tyIQ* (encoding  $\gamma$ -butyrolactone-binding protein homologues), *tyIT* and *tyIS* (encoding *Streptomyces* antibiotic regulatory protein (SARP) homologues [36]; see below), and *tyIR*. Four of these genes (*tyIT* is not essential for tylosin production) appear to encode a regulatory cascade (Figure 2) in which TyIP, in the absence of a presumed but currently unidentified  $\gamma$ -butyrolactone, represses *tyIS* and *tyIQ* transcription. *tyIQ*, in turn, represses transcription of *tyIR*, which functions as an activator of tylosin biosynthetic genes [37,38,39,40]. *tyIS* is also required for full activation of *tyIR*, and may also play a role, directly or indirectly, in the activation of other tylosin biosynthetic genes [35]. TyIQ shows most amino acid sequence similarity to the more distantly related homologues of *bona fide*  $\gamma$ -butyrolactone-binding proteins BarB [41] and JadR<sub>2</sub> [42]. Interestingly all three of these proteins, which have yet to be shown to bind any ligand, act as negative regulators of antibiotic production in path-

Figure 2



Model of the pathway-specific regulatory cascade for tylosin biosynthesis in *Streptomyces fradiae*. In the absence of its cognate  $\gamma$ -butyrolactone, TyIP represses its own synthesis, permitting expression of *tyIQ*. TyIQ represses transcription of *tyIR*, which is required for expression of the *tyI* biosynthetic genes. When  $\gamma$ -butyrolactone concentrations (influenced by unknown factors, shown as '?') reach an appropriate level, *tyIP* is expressed, repressing expression of *tyIQ* and hence allowing expression of *tyIR*. TyIS is also required for expression of *tyIR* and, independently of *tyIR*, for expression of some of the *tyI* biosynthetic genes. Partial inhibition of *tyIS* transcription by TyIP is shown by the dotted line. Since both TyIP and TyIS are positive regulators of tylosin production, the inhibitory effect of TyIP on *tyIS* transcription is presumed to be offset by additional, currently unknown regulatory inputs (shown as '??'). Homologues of  $\gamma$ -butyrolactone binding proteins are shown in blue, and the SARP homologue in red.

way-specific regulatory cascades. However, the recent discovery of an *afsA* homologue, *jadW<sub>1</sub>*, in the jadomycin gene cluster [42], and the acidic character of JadR<sub>2</sub>, which is reminiscent of other established  $\gamma$ -butyrolactone-binding proteins, suggest that JadR<sub>2</sub> does indeed bind a cognate  $\gamma$ -butyrolactone despite its lower level of amino acid sequence identity to ArpA orthologues.

In contrast to the inhibitory role played by most of the  $\gamma$ -butyrolactone-binding proteins in regulating the transcription of secondary metabolic gene clusters, SpbR appears to play a positive role in pristnamycin production in *Streptomyces pristinaespiralis*. SpbR binds to the promoter of the SARP-encoding gene *papR1*, deletion of which results in a reduction, but not abolition, of pristnamycin biosynthesis [43]. A putative  $\gamma$ -butyrolactone capable of inhibiting the DNA-binding activity of SpbR was identified but not structurally characterised. In addition to abolishing antibiotic production, deletion of *spbR* also

severely impaired growth of agar-grown cultures, presumably reflecting a broader role for *spbR* in regulating metabolism.

#### **$\gamma$ -butyrolactone synthesis and quorum sensing**

Currently, little is known about the biosynthesis of  $\gamma$ -butyrolactones. AfsA was initially thought to play a direct role in A-factor synthesis, but more recent studies of BarX [44] and ScbA [29] suggest that the AfsA family of proteins may play complex regulatory roles rather than function as catalytic enzymes. The only gene identified to date with a proven role in  $\gamma$ -butyrolactone synthesis is *barS1*, which encodes the last catalytic step in VB biosynthesis and which lies adjacent to *barA*, the VB-specific binding protein, between the virginiamycin M1 and S gene clusters [27,45].

The exogenous addition of a  $\gamma$ -butyrolactone to a streptomycete culture often results in precocious antibiotic production (e.g. [29]). While this has prompted speculation that these compounds act as quorum sensors analogous to the homoserine lactones of Gram-negative bacteria [46], it seems just as likely that their synthesis occurs in response to an unknown physiological signal, perhaps some aspect of nutrient limitation, and that they do not simply function as indicators of population density. Perhaps their role is to co-ordinate both secondary metabolism and morphological differentiation throughout the developing mycelial colony, rather than to effect communication between dispersed members of the same species.

#### **PI factor – a novel extracellular elicitor of antibiotic production**

Although there were earlier reports of extracellular signalling molecules other than  $\gamma$ -butyrolactones that could elicit secondary metabolism in actinomycetes [47], the only recent example is the identification of PI factor as an elicitor of the anti-fungal glycosylated polyene pimaricin in *Streptomyces natalensis* [48]. PI factor (2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol) elicited pimaricin production in a mutant (*npi287*) deficient in PI and pimaricin synthesis, and stimulated pimaricin production in the wild-type strain. Remarkably, addition of A-factor also restored pimaricin production in the *npi287* mutant, although PI could not complement an A-factor deficient mutant of *S. griseus*. Presumably *S. natalensis* possesses a  $\gamma$ -butyrolactone signalling system that can elicit pimaricin production and that shares some functional similarity to the A-factor cascade of *S. griseus*. It will be interesting to see whether PI exerts its influence on pimaricin production through a recently identified novel pathway-specific regulatory protein PimR. PimR contains an N-terminal domain corresponding to the SARP family of transcriptional activators, a central domain with similarity to the nucleotide triphosphate binding motif characteristic of the LuxR family of DNA-binding proteins, and a C-terminal domain that resembles guanlylate cyclases [49].

### **The SARP family of regulatory proteins**

#### **Pathway-specific regulators**

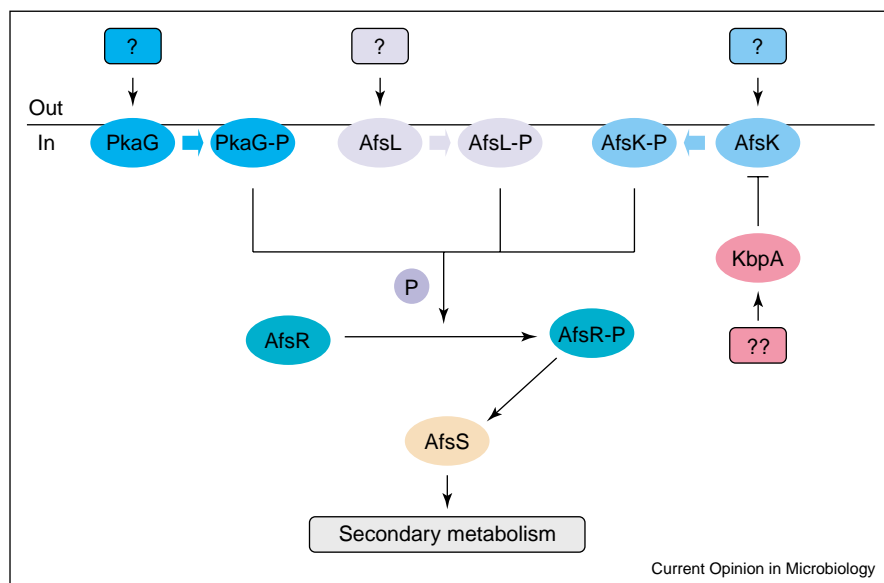
Many of the pathway-specific regulatory proteins that control secondary metabolism in streptomycetes belong to the SARP family [36]. These transcriptional activators contain a winged helix-turn-helix motif towards their N-termini that is also found in the OmpR family of proteins, and at least some of the SARPs appear to recognize heptameric repeats within the promoter regions of genes that they regulate [36,50–52]. They have been found associated with secondary metabolic gene clusters that encode aromatic polyketides (e.g. [33,52,53]), ribosomally and non-ribosomally synthesized peptides [50,54], undecylprodiginines [55], Type I polyketides [32,56,57],  $\beta$ -lactams [58] and azoxy compounds [59]. While genes encoding phylogenetically diverse classes of bacterial regulatory proteins occur in many secondary metabolic gene clusters, the SARP family of proteins have only been found in actinomycetes, and most of them within the streptomycetes (other genera include *Mycobacterium*, *Nocardia*, *Thermobifida* and *Lechevalieria*).

One member of the SARP family, CcaR, regulates the biosynthesis of both cephamycin C and clavulanic acid in *Streptomyces clavuligerus* [60]. CcaR binds several promoter regions within the cephamycin C gene cluster [61,62], presumably activating their transcription, as well as positively regulating its own synthesis. Disruption of *ccaR* also abolishes expression of *claR*, which encodes a LysR-type regulatory protein that is required specifically for clavulanic acid production. However, CcaR does not appear to interact directly with the *claR* promoter [61].

#### **Pleiotropic regulators of secondary metabolism**

While SARPs generally appear to function as pathway-specific regulatory proteins, at least one pleiotropic regulator, AfsR of *S. coelicolor*, incorporates a SARP domain. The N-terminal region of the 993 amino acid AfsR shows significant amino acid sequence identity to the SARP family of proteins, while the central region contains both A- and B-type ATP-binding consensus sequences. AfsR appears to play a key role as an integrator of multiple physiological and environmental signals that are transduced by phosphorylation cascades (Figure 3; [63\*]). The genome sequence of *S. coelicolor* A3(2) encodes at least 34 eukaryotic-like serine/threonine or tyrosine protein kinases [64]. One of these, the membrane-associated AfsK, autophosphorylates on threonine and serine residues, presumably on sensing a particular environmental signal, thus enhancing its kinase activity. The activated AfsK then phosphorylates threonine and serine residues of the cytoplasmic AfsR, greatly enhancing its DNA-binding activity. AfsR-P then serves to activate transcription of *afsS*, which encodes a 63-amino acid protein that functions in an unknown manner to enhance production of Act, Red and CDA. While binding of ATP to AfsR is not required for binding of the protein to the *afsS*

Figure 3



Model of the serine-threonine protein kinase cascade of *Streptomyces coelicolor*. Unknown and presumably extracellular signals (shown as '?') activate the autophosphorylation of the membrane associated protein kinases, which then phosphorylate the pleiotropic regulatory protein AfsR, permitting synthesis of AfsS, which enhances secondary metabolite production. Unknown factors that influence the synthesis and/or activity of KbpA are shown as '??'.

promoter, it is required for transcriptional activation, suggesting that the energy obtained from ATP hydrolysis may be required for isomerisation of a closed complex consisting of RNA polymerase and AfsR into a transcriptionally competent open complex. In addition to AfsK, other kinases — including PkaG and AfsL — are capable of phosphorylating AfsR [63], consistent with a role for AfsR in integrating a variety of environmental signals. Interestingly, the kinase activity of AfsK is inhibited by binding of KbpA to its N-terminal region [65]. The delayed transcription of *kbpA* is consistent with a role in restoring the signal cascade to its pre-stimulatory condition.

### The LAL family of transcriptional regulators

While the SARP family of proteins gained early recognition by virtue of their role as pathway-specific regulators of antibiotic production in the well-studied *S. coelicolor*, at least 13 Type I polyketide gene clusters and two glycopeptide gene clusters contain genes that encode members of the LAL family (large ATP-binding regulators of the LuxR family) of transcriptional regulators. These proteins contain an N-terminally located nucleotide triphosphate (NTP) binding motif and a C-terminal helix-turn-helix motif characteristic of the LuxR family of DNA-binding proteins. Like the SARP family, homologues with end-to-end similarity appear to be confined to the actinomycetes. While little is understood of the overall role of these proteins in regulating the onset of antibiotic production, deletion of, or mutations in, the NTP-binding domain of

PikD abolished pikromycin production in *Streptomyces venezuelae*, although PikD was not required for transcription of all of the pikromycin biosynthetic genes [66]. Three LAL homologues are encoded in the nystatin gene cluster of *Streptomyces noursei* [67], where mutational analysis suggests a complex regulatory cascade for the production of the polyene macrolide. Multiple LAL homologues also occur in the amphotericin (three; [68]), candicidin (three; [69]) and geldanamycin (two; [70]) gene clusters.

### *absA1A2* – pleiotropic or pathway-specific regulatory genes?

The *absA1A2* locus, which encodes a two-component histidine kinase-response regulator pair, was one of the first to be identified in *S. coelicolor* that appeared to play a pleiotropic role in regulating secondary metabolism, influencing the production of all four of the antibiotics known to be made by the strain [71]. Genetic analysis indicated that the phosphorylated form of the response regulator AbsA2 acted as a negative regulator of antibiotic biosynthesis [72]. Further studies demonstrated that mutations in the *absA1A2* locus that enhance antibiotic production impair AbsA2 kinase activity, while mutations that repress antibiotic synthesis impair AbsA2-P phosphatase activity [73]. Intriguingly, the genome sequence of *S. coelicolor* revealed that the *absA1A2* locus lies within the cluster of genes that encode CDA. Furthermore, a particular mutant allele of *absA1* that has a negative effect on antibiotic production had a more severe effect on

transcription of the *cda* genes than on those of the *act* and *red* gene clusters [73], although it did so apparently without affecting transcription of *cdaR*, a member of the SARP family and a putative pathway-specific regulatory gene for CDA synthesis. Thus, the primary role of AbsA1A2 may be to function as pathway-specific regulators for CDA production that act independently of CdaR. Their apparent role in Act and Red production may reflect an indirect physiological consequence of their inactivation. Alternatively, it may reflect, as suggested above, hitherto unrecognized regulatory cross-talk between different secondary metabolic gene clusters.

## Conclusions

One of the most striking characteristics of the regulation of secondary metabolism in streptomycetes is its diversity and complexity. But perhaps this is not surprising. While secondary metabolites must confer an adaptive advantage, the roles of most are not fully understood. This is true even for antibiotics, where anti-microbial activity in the laboratory may not reflect the sole or primary function of the compound in nature. Consequently, different secondary metabolic gene clusters are likely to respond to disparate environmental and physiological signals and stresses mediated by an array of signal transduction systems. Moreover, secondary metabolism is usually coordinated with the onset of morphological development in surface-grown cultures, a process that is subject to its own regulatory checkpoints and cascades, adding additional complexity. There are tremendous challenges ahead in understanding the regulatory cascades that link environmental and developmental signals to pleiotropic and ultimately pathway-specific regulatory genes for secondary metabolism. How are all of these different signals integrated, ultimately at the level of pathway-specific regulators? Nevertheless, the availability of entire genome sequences, whole genome microarrays, and the development of technologies for proteome analysis and for the temporal-spatial analysis of gene expression have undoubtedly provided the means to tease apart these complex interactions. The value in doing so to fully exploit the biosynthetic potential of these organisms should not be underestimated.

## Acknowledgements

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