Molecular Regulation of Antibiotic Biosynthesis in *Streptomyces*

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SUMMARY

Streptomyces are the most abundant source of antibiotics. Typically, each species produces several antibiotics, with the profile being species specific. *Streptomyces coelicolor*, the model species, produces at least five different antibiotics. We review the regulation of antibiotic biosynthesis in *S. coelicolor* and other, nonmodel streptomyces in the light of recent studies. The biosynthesis of each antibiotic is specified by a large gene cluster, usually including regulatory genes (cluster-situated regulators [CSRs]). These are the main point of connection with a plethora of generally conserved regulatory systems that monitor the organism's physiology, developmental state, population density, and environment to determine the onset and level of production of each antibiotic. Some CSRs may also be sensitive to the levels of different kinds of...
ligands, including products of the pathway itself, products of other antibiotic pathways in the same organism, and specialized regulatory small molecules such as gamma-butyrolactones. These interactions can result in self-reinforcing feed-forward circuitry and complex cross talk between pathways. The physiological signals and regulatory mechanisms may be of practical importance for the activation of the many cryptic secondary metabolic gene cluster pathways revealed by recent sequencing of numerous *Streptomyces* genomes.

**INTRODUCTION**

Bacteria of the genus *Streptomyces* are a particularly abundant source of antibiotics and related compounds, providing more than half of medically important antimicrobial and antitumor agents. Early genetic mapping in the model organism *Streptomyces coelicolor* A3(2) provided the first evidence that the genes for biosynthesis of any particular antibiotic are clustered on the chromosome (1–3) or plasmids (4). Subsequently, molecular analyses revealed such clusters to be large (typically tens of kilobases) and usually to include several operons (5–7). This paradigm has held over the last 2 decades, during which hundreds more such clusters have been characterized. The sequenced gene sets provide an opportunity to study the regulation of antibiotic biosynthesis at the molecular level, illuminating the complex developmental interplay of antibiotic production with morphological differentiation in these mycelial, sporulating bacteria. Such studies may suggest ways of increasing production levels, both at the early stages of characterizing new products and at the level of large-scale industrial production. They may also provide routes to the activation of “silent” gene sets that are revealed by genome-level DNA sequencing.

Mining of genome sequences has revealed at least 29 clusters of likely biosynthetic genes for secondary metabolites in the *S. coelicolor* A3(2) model organism and 37 in the industrial organism *Streptomyces avermitilis*. Sequencing of other *Streptomyces* genomes showed that this was typical and that the majority of clusters are species specific among the genomes analyzed (8). Some of the conserved clusters determine the production of metabolites with roles in the physiology or development of the host, including metal-binding siderophores, spore pigments, and volatile odor compounds (notably geosmin, which is responsible for the earthy smell of streptomyces). We have generally confined this review to antibiotics—secondary metabolites whose main role appears to be to interfere with neighboring organisms (though we note that roles of many antibiotics in signaling have been proposed) (9–11).

Antibiotic production and morphological differentiation are generally activated when starvation or environmental changes bring about the end of rapid vegetative growth. Under these circumstances, a transient arrest of growth typically ensues, accompanied by a complex series of changes in global gene expression. Antibiotic production and morphological differentiation may then take place (12, 13). Antibiotic production at the industrial level is highly dependent on the fermentation conditions, with the need for a balance between, on one hand, providing nutrients for growth and as precursors for antibiotics and, on the other hand, the repressive effects of some of the most efficient sources of carbon, nitrogen, and phosphate. Numerous metals, such as zinc, iron, and manganese, are essential for bacterial growth, and some of them may also affect antibiotic production (14, 15). Apart from nutrient effects, the pH and dissolved oxygen level are also important for antibiotic production (16, 17). Although the optimization of industrial fermentations is highly specific for particular products and their producing organism, understanding the underlying responses of diverse pathways requires the study of a model organism. Our approach here has therefore been first to provide an up-to-date overview of the regulation of antibiotic production in *S. coelicolor* A3(2) and then to consider how the concepts developed in the model system are extended, reinforced, or challenged by information coming from biosynthetic gene sets in other streptomycetes. This article builds on three excellent broadly based reviews on the regulation of antibiotic production in *Streptomycetes* (18–20). Other wide-ranging reviews of the topic have also appeared recently (21, 22).

**CONTROL OF FIVE EXTENSIVELY STUDIED S. COELICOLOR ANTIBIOTIC PATHWAYS**

*Streptomyces coelicolor* was adopted early as a model streptomycete because of its amenability to genetic analysis, and its status as a model was reinforced by its production of two pigmented antibiotics (actinorhodin [ACT] and undecylprodigiosins [REDS]), as well as a structurally incompletely characterized polyketide (“cryptic polyketide” [CPK]), a calcium-dependent ionophore antibiotic (CDA), and an unusual cyclopentanone antibiotic (methyleneomycin [MM]) (Fig. 1). The main architect of *S. coelicolor* genetic studies, D. A. Hopwood, has written accounts of the organism with much valuable early history (23–25). For the present purposes, it is sufficient to point out that *S. coelicolor* is a complex mycelial prokaryote that reproduces by the formation of sporulating aerial hyphae. Aerial growth is fueled in part by nutrients from autolysis of the vegetative or substrate mycelium. On agar media, most antibiotic production also takes place at this stage, perhaps providing some protection for the nutrients being released. Under submerged liquid culture conditions (such as are used in industrial antibiotic production fermentations), such morphological differentiation does not usually take place. Nevertheless, antibiotic production is connected to the *S. coelicolor* life cycle, and this connection has preoccupied many researchers over several decades. With the application of genome-based technologies to *Streptomyces* biology, there are now signs that the disparate strands of different research programs are beginning to come together.

The first molecular analyses of biosynthetic gene clusters showed that they usually contain regulatory genes, often having major effects on the levels of production of the cognate antibiotic. These came to be termed “pathway-specific” regulators, in contrast with “global,” “higher-level,” or “pleiotropic” regulators. One of the first applications of microarray analysis to *S. coelicolor* revealed that some pathway-specific regulators also had wide-ranging effects on global transcription patterns, leading to the suggestion that the term “cluster-situated regulator” (CSR) would be a more accurate and objective description (26). The value of this term has been reinforced by recent evidence that some CSRs directly control the expression of genes in other clusters (see below). Of course, some of the wide-ranging effects may be indirect, ranging from the consequences of draining pools of precursors to physicochemical properties of the end products such as redox activity (27).

The first parts of this section focus on the five antibiotic biosynthetic pathways of *S. coelicolor* that have provided paradigms both for pathway-specific regulation and for its dependence on
Diverse antibiotics and autoregulator molecules produced by *Streptomyces coelicolor* A3(2) and some other streptomycetes. (A) The compounds from *S. coelicolor*. Actinorhodin (ACT) is a red/blue pH-indicating benzoisochromanequinone made by a type II polyketide synthase-based pathway involving a 22-gene cluster (291). Undecylprodiginines (REDs) are red hydrophobic tripyrroles made by a fatty acid synthase-like pathway involving a 22-gene cluster (292). Methylenomycin A (MM) is an epoxycyclopentenone made by an unusual pathway encoded by 11 genes located on the linear plasmid SCP1 (57). The calcium-dependent antibiotic (CDA) is a lipopeptide made by a route involving nonribosomal peptide synthases and specified by a 48-gene cluster (293). The autoregulator SCB1 (240) is one of several gamma-butyrolactone congeners whose synthesis involves three genes linked to the 19-gene cluster for biosynthesis of a structurally incompletely characterized yellow antibiotic (*S. coelicolor* polyketide [CPK]) (41,42, 294). Methylenomycin furans (MMFs) are autoregulators that control MM biosynthesis, and they are made by a pathway resembling that of gamma-butyrolactones that involves three biosynthetic and two regulatory genes next to the MM biosynthetic cluster (58). (B) Autoregulatory molecules from other streptomycetes. A-factor regulates streptomycin biosynthesis in *S. griseus* (295). Virginiae butanolides (VBs) induce the coordinated production of virginiamycin M and virginiamycin S, synergistically acting but biosynthetically distinct antibiotics in *S. virginiae* (235). IM-2 controls the production of showdomycin and miniminycin in *S. lavendulae* (237). PI factor enhances pimaricin production in *S. natalensis* (239). Avenolide is required at nanomolar concentrations for the onset of avermectin biosynthesis in *S. avermitilis* (238) (avenolide homologs are also found in *S. fradiae*, *Streptomyces ghanaensis*, and *S. griseoauranticus*).
global physiological influences. We particularly emphasize recent work on the complexity of regulation of the CSRs, the roles and diversity of autoregulators, the discovery that end products and late intermediates are sometimes ligands of CSRs, and molecular evidence of regulatory cross talk between apparently unrelated pathways. Global regulators controlling more than one of these pathways are dealt with in the subsequent subsections. In some cases we refer to *S. coelicolor* genes by their SCO numbers (for further information, see http://strepdb.streptomyces.org.uk).

**Activation of Actinorhodin Biosynthesis by Multiple Signal Inputs**

Actinorhodin (ACT), a polyketide-derived benzoisochromane-quinone (Fig. 1), is a weak antibiotic that is responsible for the pH-sensitive blue/red color from which *S. coelicolor* gets its name. Its biosynthesis is determined by five transcription units in the *act* gene cluster, four of which contain more than one gene. These five transcription units are completely dependent on the ActII-ORF4 protein, which binds to sequences in the target promoters via its N-terminal winged helix-turn-helix (HTH) domain and activates transcription through a C-terminal activation domain. Its target motif is repeats of the sequence TCGA at 11-bp intervals, which appear in five promoter regions in the *act* cluster (28, 29). ActII-ORF4 is a founder member of a protein family called *Streptomyces* antibiotic regulatory proteins (SARPs) (29). To avoid confusion, we emphasize here that the term SARP does not apply to the many other kinds of cluster-situated regulators of antibiotic biosynthesis, most of which have paralogues in diverse organisms and other physiological contexts. SARPs are a specific family of paralogous proteins that show a high specificity for antibiotic production in actinomycetes. The frequency and properties of SARPs are further surveyed later in this review.

The ActII-ORF4 determinant is embedded in the *act* cluster. Almost all information from global cellular physiology that affects the timing and level of ACT production is transmitted via ActII-ORF4 and influences either actII-ORF4 transcription or translation or (perhaps) the properties of the protein itself. The promoter region of *actII-ORF4* is a direct target for at least eight known regulatory proteins (Fig. 2): AdpA (a pleiotropic regulator of antibiotic production and development) (30), LexA (a global regulator of the DNA damage response) (28), AbsA2 (a global repressor of antibiotic synthesis [see below]) (31, 32), DasR (mediating the global response to N-acetylglucosamine [see below]) (33), DraR and AfsQ1 (activators responding to nitrogen excess) (34, 35), AtrA (a transcriptional activator, which also binds to targets associated with metabolism of acetyl coenzyme A [acetyl-CoA], an ACT precursor) (32, 36), and the xylene operon repressor ROK7B7 (SCO6008) (37) (Fig. 2). In addition, there are indications of binding of the nitrogen regulator GlnR (34) and proteins corresponding to SCO0310, -3932, and -5405 (38). There can be few documented cases of comparably complex regulation of a bacterial promoter, and it will be a challenge for the future to unravel the large number of possible interactions involving regulators binding at separate or overlapping sites. Furthermore, the presence of a particularly rare codon (UUU, one of six leucine codons) in actII-ORF4 mRNA makes its translation dependent on a developmentally significant trNA encoded by the *bldA* gene, which is further discussed in a later section (39). A second regulatory gene in the *act* cluster (*actR*) encodes a TetR-like protein that represses the adjacent *actA* operon, encoding the ACT export system (actA also contains a TTA codon) (39). Repression of export is relieved by interaction of ActR with late intermediates in ACT biosynthesis (40). This may ensure that the export system is functional before the potentially toxic end product begins to accumulate in the cytoplasm.

**Regulation of the “Cryptic Polyketide” Gene Cluster**

The *cpk* cluster directs production of a polyketide-derived antibiotic (“cryptic polyketide” [CPK]) and a (presumably related) yellow pigment (yCPK) whose structures are still undetermined (41, 42). Although the difficulty of characterizing CPK has impeded analysis of the regulation of the cluster, it clearly shows significant differences from *act* regulation: in particular, it involves a gamma-butyrolactone (GBL) signaling molecule, SCB1, and/or its congeners (SCBs) (Fig. 1) (43). Gamma-butyrolactone autoregulators are membrane diffusible and accumulate in cultures until, at above a certain concentration, they bind to a cytoplasmic protein and cause it to dissociate from, and thereby derepress, promoters of target genes (44, 45) (see below). It has been suggested that at least some *Streptomyces* autoregulators may coordinate physiological changes across the mycelium of a colony (18).

The key SCB biosynthetic gene *scbA* and a divergent regulatory gene *scbR* are located at one end of the *cpk* cluster. ScbR has a TetR-like N-terminal helix-turn-helix domain and belongs to a subfamily of such proteins whose founder member, ArpA, senses the gamma-butyrolactone A-factor in *Streptomyces griseus* (see below). ScbR binds to operator sites in the bidirectional *scbA/scbR* promoter and in the promoter for *cpkO*, a CSR gene essential for CPK biosynthesis (43) (Fig. 3). CpkO has an N-terminal SARP domain and an additional C-terminal domain of unknown function. Under suitable culture conditions, and by an undetermined mechanism, SCBs accumulate to a concentration high enough to bind to ScbR, causing ScbR to dissociate from the bidirectional *scbA/scbR* promoter. It was suggested that the resulting increase in ScbA and ScbR production has two consequences: a heterodimer of the two proteins is thought to form and then bind to a different site upstream of *scbA* to activate *scbA* transcription further, and SCB levels burgeon, sequester all free ScbR protein and releasing *cpkO* from repression (41) (Fig. 3). An alternative and perhaps simpler interpretation of the same data is possible, in which the only role of ScbA is to catalyze SCB biosynthesis and the regulatory effects are all mediated by the concentration-dependent interplay of SCBs with ScbR (20). Mathematical modeling of the *scbAR/cpk* system has shown that its autoamplifying nature may amount to a developmental commitment to CPK production, by acting as a bistable switch (46, 47). Recently, it was reported that a mutation in *scbR* leading to the amino acid change R120S had occurred independently in two lines of *S. coelicolor*, one of them being the fairly frequently used strain M600 (48). In some tests, but not others, the mutation appeared to affect the properties of ScbR and the details of timing and levels of expression of *cpkO*, *scbA*, and a regulatory gene for RED antibiotic biosynthesis (48). The phenotypic changes may be made difficult to interpret and reproduce if the mutation influences the bistable switch.

The initial discovery of SCBs was made possible by their stimulatory effects on pigmented antibiotic production when added exogenously to *S. coelicolor* cultures, though, paradoxically, eliminating endogenous SCBs by *scbA* deletion enhanced ACT and RED production (49). This enhanced production is mediated at least partially at the level of regulation of the relevant biosynthetic
genes and their CSR regulatory genes. However, since deletion of *scbA* also caused increased expression of *afsS* (50), a global activator of antibiotic biosynthesis discussed below, it is possible that the effects on ACT and RED production may be indirect, via AfsS. A further role in cross talk between the CPK, ACT, and RED pathways is played by ScbR2, the gene for which is located close to the SARP gene *cpkO*. ScbR2 directly represses *cpkO* and has 35%, near end-to-end, identity to ScbR, but it does not bind SCBs (51) (ScbR2 is therefore considered to be a “pseudo”-gamma-butyrolactone receptor, since there are no other gamma-butyrolactones in *S. coelicolor*). However, binding of ScbR2 to the *cpkO* promoter was unexpectedly relieved by adding ACT, leading to the derepression of *cpkO* and activation of CPK biosynthetic genes (51). Similar results were obtained by adding RED, but the poor solubility

FIG 2 Complexity of the promoter region of actII-ORF4. (A) Binding of regulatory proteins. The central line represents the actII-ORF3 to actII-ORF4 region, with the noncoding intergenic region given in white and coding sequences in green. Numbers refer to the transcription start site (+1) defined by Gramajo et al. (296). Binding to the diverse regulators (red lettering) was defined by different routes: footprinting results are given below the promoter, while gel-shifted fragments are indicated above as yellow boxes. Brackets indicate proteins defined only by affinity capture to the orange fragment shown. Also shown are binding sites inferred by coupling of experimental evidence of interaction with the promoter region and the presence of matches to known consensus binding sites. References are given in the text. (B) General nature of signal inputs influencing the expression of actII-ORF4. See the text for further details and references.
of RED and the possibility that it might interact with target DNA rather than the ScbR2 protein hamper unambiguous interpretation of the RED effect (52). Although RED and ACT are very different molecules, ScbR2 structural predictions revealed that its ligand-binding pocket had features reminiscent of QacR and TtgR, other members of the TetR superfamily that possess large helix-rich cavities capable of binding structurally diverse drugs (51, 53, 54). ScbR2 also represses the biosynthesis of SCBs by directly binding to the promoter region of \textit{scbA} (55). Thus, part of the complex cluster-situated regulatory system for CPK biosynthesis is implicated in cross talk with other antibiotic biosynthetic pathways, and CPK biosynthesis is subject to induction by at least two, and possibly three, entirely different secondary metabolites (SCBs, ACT, and RED) through the action of two ArpA-like repressors.

Other regulators also affect CPK biosynthesis. Thus, \textit{cpkO} is directly repressed by the phosphate response regulator (RR) PhoP (56), and recent work has shown that another global regulator, AfsQ1, directly activates the divergent promoter between the biosynthetic genes \textit{cpkO} and \textit{cpkD} (34).

**Cascade Regulation of Methylenomycin Biosynthesis**

The methylenomycin (MM) biosynthetic pathway of \textit{S. coelicolor} is encoded by the \textit{mmy} genes on a large linear plasmid, SCP1, which can remain autonomous or integrate into the chromosome (4). Like the \textit{cpk} cluster, the \textit{mmy} genes are regulated by a cascade involving the synthesis of a small autoregulator molecule (57) (Fig. 4). In this case, however, the autoregulator is not a gamma-butyrolactone; instead, it is a mixture of furans (methylenomycin furans [MMFs] such as that shown in Fig. 1), made by a pathway closely similar to that of GBLs (58). The regulatory cascade is complicated by the presence of genes for two ArpA-like proteins.

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**FIG 3** Regulation of CPK biosynthesis involves a gamma-butyrolactone and interplay with other biosynthetic pathways. Genes associated with the \textit{cpk} cluster are indicated by large open arrows. Regulatory interactions are indicated by bold arrows (activation steps) or bold lines ending with a bar (repressing or inhibitory steps). Small-molecule ligands are indicated in colored letters, with ACT and RED being the products of other pathways encoded by distant gene clusters. For further explanation and references, see the text.

**FIG 4** Regulation of methylenomycin biosynthesis, a cascade involving furan autoregulators. Large open arrows indicate genes associated with the gene cluster for MM biosynthesis. Regulatory interactions are indicated by bold arrows (activation steps) or bold lines ending with a bar (repressing or inhibitory steps). See the text for further details and references.
(MmyR and MmfR). The three furan biosynthetic genes (mmyfl, mmyfH, and mmyfP) and the two regulatory genes are directly adjacent to the mmy cluster, in the form of divergent transcription units (mmyfLHP–mmyR and mmyR). The mode of action of MmfR and MmyR has not been studied in vitro, but genetic evidence suggested that a putative MmyR/MMr complex represses two targets: the bidirectional promoter between the mmyfLHP–mmyR operon and mmyfr, to regulate MMF biosynthesis, and the promoter of mmyB, another regulatory gene in the mmy cluster, to regulate the MM biosynthetic genes (57). It is thought that when Mmfs reach a concentration that can release repression of the bidirectional promoter, furan biosynthesis accelerates to generate a concentration that relieves MmyR/MMr-mediated repression of mmyB. MmyB can then activate the MM biosynthetic genes. Early observations that MM production was induced by medium acidification raise the question of whether MmyR or MmfR is itself pH sensitive (59).

MmyB consists of an N-terminal DNA-binding domain of the Xre type and a C-terminal PAS-like domain such as involved in many sensory functions (60). The crystal structure of an MmyB-like protein from a thermophilic Gram-negative organism (purified from an Escherichia coli expression system) revealed that its PAS domain was complexed with a saturated fatty acid, prompting speculation that binding of a fatty acid ligand might be required for in vitro binding and transcriptional activity on mmyB (61). MmyB paralogues are widespread among actinomycetes and particularly among streptomycetes. For example, 13 proteins with near end-to-end similarity to MmyB (expected values of lower than 2e−7) are encoded in the S. coelicolor chromosome (61). About half of them are located very close to genes with homologues in secondary metabolism, and the others are next to diverging promoters for medium- or short-chain alcohol dehydrogenases. Only one of the paralogues, SCO4944, is widely conserved in other streptomycetes, and in S. griseus this gene is just one gene away from the biosynthetic gene for A-factor and is regulated by A-factor (61). Thus, MmyB-like proteins may be closely associated with secondary metabolism and fatty acid metabolism.

Like for ACT, MM production is dependent on the translation of regulatory UUA codons, in this case in the mRNAs of mmyB and mmyfl. As in the act cluster, there is also a specific regulatory system for MM export/self-resistance: an MM export gene (mmyr) is negatively regulated by an adjacent gene, mmyfj, encoding a member of the ArsR family of repressors, and MM or a late biosynthetic intermediate may release this repression, perhaps acting as an Mmyfj ligand (62, 63).

**Regulation of the Calcium-Dependent Antibiotic Gene Cluster**

Biosynthesis of the lipopeptide calcium-dependent antibiotic (CDA) (Fig. 1) involves huge modular nonribosomal peptide synthetases that generate peptide antibiotics, a class that, like polyketides, has received much attention from natural products scientists. The regulation of CDA biosynthesis has not been extensively studied, but, by analogy with other gene clusters, it is likely that the SARP CSR encoded by cdaU activates the cda biosynthetic genes. Several other regulatory genes are associated with the cluster, but the only ones to have been studied are the absA1/absA2 genes, which encode a classical two-component regulatory system of the type shown in Fig. 5. AbsA1 is a membrane-located sensor histidine protein kinase (64), the target of which is AbsA2, a response regulator whose phosphorylation increases its binding efficiency to, and repression of, the promoter of cdaR (31, 32, 65). Probably AbsA1 responds to an unknown signal by changing the ratio of its kinase/phosphatase activity in favor of the phosphatase, with the consequent dephosphorylation of phosphorylated AbsA2 (AbsA2–P) leading to derepression of cdaR. Remarkably, AbsA2–P also binds to and represses the promoters of pathway-specific regulatory genes in some other clusters (actII-ORF4 and redZ, but not cpkO) (31, 32, 65). This is a further example of cross talk between pathways to add to the case of ScbR2 (50, 51) (see above). At least one global regulator, AbsQ1, directly activates the cdaR promoter (34).

**A Minicascade Regulating Undecylprodigiosin Biosynthesis**

The pyrrole-based undecylprodigines (REDS) (Fig. 1) are made by a mixed pathway involving a fatty acid synthase-like part and a specialized nonmodular form of nonribosomal peptide synthase (66). The red pathway genes are regulated via a minicascade of two CRs, with RedZ activating the expression of redI, the direct activator gene for the biosynthetic genes (67). RedZ is an aberrant orphan response regulator that differs from the conventional response regulator component of two-component systems in two respects: its gene is not located next to a cognate histidine protein kinase gene, and its structure, though readily modeled onto known response regulator structures of the NarL-like class, does not have a complete set of conserved residues needed for phosphorylation (Fig. 5). Regulation of redZ has some features in common with that of actII-ORF4: both are direct targets for repression by AbsA2–P (31, 32), binding of DasR and AbsQ1 to the redZ promoter suggests that the promoter may respond to GlcNAc (33) and a glutamate-related signal (34), and redZ mRNA contains a bldA-dependent UUA codon, which appears to be the reason for the bldA dependence of RED production (67, 68).

In vitro binding of RedZ to the redD promoter is inhibited by the addition of RED antibiotic, implying that a feedback loop may modulate RED biosynthesis (69). RedD, like ActII-ORF4, is a SAP. It is presumed that RedD activates red biosynthetic genes directly, and a putative target site for RedD (repeats of the tetramer TGAG at 11-bp intervals) has been identified upstream of the large red operon that starts with redP (28). Surprisingly, this motif was also found upstream of some genes unconnected with RED biosynthesis (28).

**Phosphate Regulation of Antibiotic Production in S. coelicolor**

Under laboratory conditions, phosphate limitation of growing cultures activates phosphate scavenging and induces the growth transition that precedes stationary phase and secondary metabolism (70), (71). The two-component system PhoR-PhoP (which is widely distributed across prokaryotes) is the major signal transduction system for phosphate control in S. coelicolor and affects ACT and RED production by (directly or indirectly) influencing the transcription of act and red genes (72, 73). Similar effects on antibiotic production in other streptomycetes, including S. griseus (candidin) (74, 75), Streptomyces natalensis (pimaricin) (76), and Streptomyces rimosus (oxytetracycline) (75, 77), imply that this role of the PhoR-PhoP system is widespread. A recent extensive survey of the Pho regulon, using microarray analysis of chromatin immunoprecipitated with anti-PhoP antisera, concluded that, in addition to activating pathways for phosphate scavenging
and for cell wall polymer biosynthesis, PhoP mediates the transient shutdown of central metabolic pathways, some secondary metabolic pathways, and repressors of morphological differentiation (56) (Fig. 6).

PhoR is a membrane-bound sensor kinase that senses phosphate deprivation and then phosphorylates PhoP, its target response regulator, which binds to specific sequences in target promoters (Streptomyces PHO boxes, which are degenerate versions of consensus GTTCACC usually repeated at 11-bp intervals), to influence their expression (56, 78). In other bacteria, signal input to the PhoR sensor kinase is mediated by PhoU, a regulatory protein that is associated with a high-affinity phosphate uptake system (PstABCS) (79). In S. coelicolor, a PhoU-like protein is encoded by SCO4228, which is next to the phoRP genes, but it is not known whether this protein is implicated in phosphate signal transduction. However, elimination of the PstS transporter component of the phosphate uptake system caused actinorhodin overproduction (80), so it is likely that phosphate signal transduction is much the same in streptomycetes as in other bacteria (Fig. 6). In a homeostatic feedback loop, PhoP regulates levels of the PstS transporter, which, in turn, probably influences PhoP phosphorylation (80).

Phosphorylation of PhoP presumably enhances DNA binding. Although the overall picture of PhoP-mediated regulation is well established, understanding of the action of PhoP at the molecular level is still limited, apart from the finding that the positions of PHO boxes correlate with positive or negative action (81). Some reported observations are hard to explain. For example, PhoP binds to, and represses, the promoter of afsS, encoding a global activator of antibiotic production (82). This negative effect is counterintuitive since phosphate limitation activates production of ACT and RED, an effect mediated by enhanced transcription of the relevant biosynthetic genes. Likewise, deletion of phoP causes reduced ACT and RED production under phosphate limitation (82). Another PhoP-repressible gene encodes RpoZ, the omega variant of RNA polymerase.
subunit of RNA polymerase, yet RpoZ is required for normal levels of production of ACT and RED (72). It is notable that the effects of phoP deletion on Streptomyces lividans, a very close relative of S. coelicolor, were quite different: in that organism, in which ACT and RED production is turned off under most conditions, phoP deletion caused strongly increased production of the two antibiotics (73). These complications are probably partly caused by the complex constellation of other genes affected by PhoP, which include several pleiotropically acting developmental genes that themselves influence antibiotic production (56).

Although PhoP seems not to target CSR genes in the ACT or RED pathways directly, it does directly repress cdaR, the CSR gene for CDA biosynthesis (56). It also represses the bidirectional scbA-scbR promoter, presumably affecting the biosynthesis and signal transduction of SCB autoregulator molecules. The consequences of this regulation for the bistable switching of CPK biosynthesis mediated by the action of SCBs are not readily predictable (56). Surprisingly, PhoP also binds at exceptionally high levels to three sites internal to cpkB and cpkC, encoding two of the large CPK type I polyketide synthase (PKSs) (56). This binding appears to be necessary for transcription of the cpkBC genes.

PhoP also mediates cross talk between cellular responses to phosphate, carbon, and nitrogen availability (Fig. 6). Thus, PhoP-mediated regulation of ptsS was observed only at very high levels of extracellular carbon sources (80), and PhoP-repressible genes include several involved in nitrogen metabolism, notably glnR, whose product is the major nitrogen regulator of primary metabolism (83, 84), providing a means of adjusting nitrogen metabolism to phosphate availability.

**Regulation of Antibiotic Production by N-Acetylglucosamine**

Morphological differentiation and secondary metabolite biosynthesis are supported by nutrients released by autolysis of substrate mycelium (9, 85). N-Acetylglucosamine (GlcNAc) released from the cell wall during this process is reimported and phosphorylated by a phosphotransferase-based transport system (PTS) (36, 86). It is also likely that GlcNAc released by the breakdown of fungal chitin may help Streptomyces to sense the presence of fungi, triggering a “competition stress” mechanism to produce secondary metabolites (83), though it has been noted that chitin breakdown yields predominantly chitoooligosaccharides rather than GlcNAc.
(87). After uptake, the subsequent deacetylation of GlcNAc~P results in the cytoplasmic accumulation of GlcN~P. This serves as a signaling molecule, binding to the GntR-like regulator DasR and relieving repression of DasR target genes (Fig. 6). These include the genes for the PTS for GlcNAc uptake, forming a self-reinforcing feedback loop reminiscent of that involving PhoP and the phosphate uptake system (see above) (33, 36, 88), while downstream targets include the CSR genes for ACT (actII-ORF4) and RED (redD) (33, 36, 88). The addition of GlcNAc to solid minimal culture media stimulates antibiotic production and development, whereas an opposite, inhibitory effect is observed with rich medium, an observation that is difficult to explain in terms of the known activities of DasR (20). DasR can also induce transcription of the SCB gamma-butyrolactone signaling molecules (88).

Chitin is very abundant in soil and is likely to be a major source of carbon and nitrogen for streptomycetes (89). Consistent with this, large effects of DasR were observed when *S. coelicolor* and its *dasR* mutant were grown in the presence of added chitin on soil (or soil extract rich medium): the *dasR* mutant exhibited marked differences in expression of 679 genes, including many in the *act*, *red*, and *cpk* clusters (89). This illuminating study should encourage the use of similar conditions for the activation of antibiotic biosynthesis in other streptomycetes.

### Carbon Catabolite Repression of Antibiotic Production

It has long been known that extracellular glucose represses the production of many antibiotics, including ACT, but the mechanism has resisted molecular analysis, even though a role for glucose kinase in repression of the utilization of alternative carbon sources was indicated 30 years ago (90). Current evidence suggests that both catalytic activity and a specific interaction of the enzyme with glucose permease are involved in glucose repression (91). A thorough review of this topic is included in reference 20. A recent proteomic study examined the effects of added glucose on exponentially growing cultures of *S. coelicolor* and its glucose kinase (*gkA*) mutant in minimal medium supplied with mannitol or fructose (92). Significant effects of glucose on carbon source uptake, central carbon and nitrogen metabolic enzymes (and the nitrogen regulator GlnR), some developmental proteins, and proteins closely or directly involved in RED, CDA, and CPK antibiotic production were found, but there was no effect on DasR. Notably, GlkA was involved in the effects on RED and CDA production but did not affect glucose regulation of SCB1 and CPK biosynthetic enzymes, revealing an uncharacterized second mechanism (92). The central enzyme pyruvate phosphate dikinase, which was particularly highly repressed by glucose in a GlkA-independent manner, was suggested as a possible key player in this second mechanism (92). These complex data sets will be difficult to interpret until samples are also examined during the antibiotic production phase, and progress has been made on the molecular basis of glucose kinase effects.

Another major environmental carbon source is polymers containing xylose, and this is reflected in the ability of the xylose operon repressor (ROK7B7) to activate the DasR-repressible *nage2* gene for GlcNAc permease (20) and to bind to the promoters of the CSR genes *actII-ORF4* and *redD* (38, 54) (Fig. 6). Interestingly, *rok7B7* seems to be subject to GlkA-mediated glucose repression (92).

### Nitrogen Regulation of Antibiotic Biosynthesis

Mutations in the regulatory genes *draR* and *afsQ1* have effects on antibiotic production that are discernible only under nitrogen excess (35, 64, 65). Both genes are part of two-component regulatory systems (DraRK and AfsQ1Q2) that also affect morphological differentiation (35, 64, 65). In a comprehensive recent paper, AfsQ1 has been revealed as a direct repressor of primary nitrogen assimilation genes and as an activator of several antibiotic biosynthetic regulatory (*actII-ORF4*, *redZ*, and *cdaR*) and structural (*cpkA* and *cpkD*) genes, as well as regulating some developmental genes (*bldM*, *whiD*, and *ampF*) (34). It appears to function in the maintenance of C, N, and P balance, as judged by the finding that it also directly represses *pstS* (phosphate uptake) and binds to promoters of genes for a xylanase (*xyxA*) and a glyceraldehyde 3-phosphate dehydrogenase (*gap1*) (34) (Fig. 6). AfsQ1 recognizes a moderately conserved pair of 5-bp sequences separated by 6 bp, with the 5-bp sequences similar to that recognized by the nitrogen regulator GlnR. This results in cross-recognition of some sites (especially those in the promoters of nitrogen assimilation genes), leading to actual competition between GlnR activation and AfsQ1 repression (34). It is thought that a possibly glutamate-related signal activates the apparently membrane-associated AfsQ2 kinase and hence the phosphorylation-dependent activation of AfsQ1. This response is somehow enhanced by the AfsQ1-dependent expression of the *sigQ* gene, which diverges from the *afsQ1Q2Q3* operon (34). No such detailed analysis of the DraRK system has been published.

This is certainly not the whole story for nitrogen regulation. It was recently claimed that GlnR binds to the promoters of *actII-ORF4*, *redZ*, and *cdaR* (34), and one of its target genes, *glnK*, encoding the nitrogen-regulatory PII protein, influences the rates of antibiotic production and sporulation in a medium-dependent manner (93). Also, mutational inactivation of *NdgR*, an IclR-type regulator for nitrogen source-dependent growth in *S. coelicolor* (38), enhanced the production of ACT in minimal media containing certain amino acids (94). This effect seemed to be direct, since NdgR could bind to the promoters of some genes involved in antibiotic biosynthesis, including the *scha-scbR* intergenic region in *S. coelicolor* (see below) and promoters of doxorubicin biosynthetic genes in *Streptomyces peucetius* (94). Interestingly, the *ndgR* promoter is a target for the redox stress-induced sigma factor SigR (95), suggesting that redox stress might repress ACT production under certain nutritional conditions.

### Amino Acid Limitation and Ribosome-Mediated Effects

When the supply of amino acids becomes rate limiting for protein synthesis, bacteria produce the alarmones guanosine tetraphosphate (pppGpp) and pentaphosphate (ppppGpp), which inhibit the synthesis of tRNA and tRNA and activate expression of other genes by altering the RNA polymerase core-binding competitiveness of sigma factors (96). The (p)ppGpp synthase enzyme RelA is activated when a shortage of amino acids leads to an uncharged tRNA molecule entering the A site of the ribosome. In *S. coelicolor*, a *relA* disruption mutant did not produce ACT (97). This linking of (p)ppGpp to the onset of secondary metabolism was reinforced by the finding that (p)ppGpp overaccumulation increased ACT and RED production (98). Likewise, bialaphos production in *Streptomyces hygroscopicus* (99) and production of clavulanic acid and cephalomycin in *Streptomyces clavuligerus* (100) were re-
ported to be stimulated by (p)ppGpp accumulation. However, in a contradictory report, disruption of relA resulted in the enhanced production of clavulanic acid and cephalomycin C (101), so it is possible that not all antibiotic clusters respond in the same way to (p)ppGpp levels. In a further level of regulation of (p)ppGpp production, the transcription of relA itself responds to nitrogen limitation through the agency of an extracytoplasmic function (ECF) sigma factor, SigT (102). Mutations generating rifampin-resistant RNA polymerase can suppress the antibiotic deficiency of some relA or relC mutants (103,104). Thus, the available information is consistent with the idea that (p)ppGpp may increase the relative affinity of RNA polymerase for sigma factors that direct the enzyme to genes specific for antibiotic production.

During the transition of *S. coelicolor* from exponential growth to stationary phase, more than half of the ribosomes are degraded (105), and cleavage of a significant fraction of tRNAs leads to the accumulation of 30- to 35-nucleotide (nt) RNA species (106). Since the onset of secondary metabolite biosynthesis occurs during the transition, it is possible that ribosome and tRNA degradation are related to secondary metabolism, with the further possibility that the 30- to 35-nt RNA species could be signaling molecules for secondary metabolism and morphological differentiation.

Some ribosomal mutants show enhanced production of secondary metabolites. A point mutation in rpsL (encoding ribosomal protein S12) dramatically increased ACT production (107). Overexpression of ribosome recycling factor also causes the overproduction of antibiotics, perhaps reflecting enhanced protein synthesis during stationary phase, probably through increased ribosomal stability under amino acid starvation (108). Mutation of rsmG, which encodes a 16S rRNA methyltransferase, elevates protein synthesis and in turn enhances antibiotic production (109). Although there is not enough evidence to confirm that changes to ribosome structure contribute to the onset of antibiotic production, it has been reported that ribosome structure determines translation accuracy and speed (110). Slowing of translation speed when growth slows may enhance folding of nascent antibiotic biosynthetic proteins, thus enhancing antibiotic production (Y. Pan et al., unpublished data).

**Regulation at a Meeting Point of Primary and Secondary Metabolic Pathways**

One of the intermediates in CDA biosynthesis is 4-hydroxyphenylpyruvate (4HPP). 4HPP is also the substrate of the product of the *hppD* gene, which is involved in tyrosine catabolism. This gene is repressed both directly and indirectly by HpdR, an IclR-type protein encoded by a nearby gene (111) (Fig. 7). Repression is relieved by 4HPP (111). Remarkably, HpdR also binds to the promoter of the CDA biosynthetic gene *hmaS*, which encodes an enzyme converting 4HPP to 4-hydroxymandelate (112). Since *hmaS* transcription is reduced in an *hpdr* mutant, HpdR functions to activate *hmaS*. It seems that 4HPP accumulated from tyrosine catabolism during stationary phase binds to HpdR, relieving autorepression of *hpdr*. The overexpressed HpdR represses *hppD* as a repressor but enhances *hmaS* expression as an activator. As a result, 4HPP is redirected by HmaS to 4-hydroxymandelate, which is a precursor of CDA. Thus, 4HPP acts both as a metabolic intermediate and as a regulatory ligand in the partitioning of tyrosine catabolism between primary and secondary metabolism (Fig. 7) (112).

**AbsC and Zinc Dependency of Antibiotic Production**

About 5% of *S. coelicolor* proteins are predicted to bind zinc (113). It is therefore not surprising that the timing and levels of production of ACT and RED both depend on the amount of zinc supplied (114). A MarR-like repressor, AbsC, was implicated in this zinc response by virtue of the nonproduction of ACT and RED (and CDA) by an *absC* mutant on medium low in zinc (114). The effect

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**FIG 7** Model for the regulation of HpdR during tyrosine catabolism and CDA biosynthesis in *S. coelicolor*. As an autoregulator, HpdR regulates its own transcription (not shown). It also represses *hppD*, the product of which catalyzes the conversion of 4-hydroxyphenylpyruvate (4HPP) to homogentisate, but activates *hmaS*, which is involved in CDA biosynthesis. During vegetative growth, L-tyrosine in the medium is catabolized to 4HHP by TyrB. The accumulated 4HHP binds to HpdR and causes it to dissociate from the *hppD* and *hmaS* promoters, therefore initiating *hppD* expression and preventing *hmaS* expression. During stationary phase, L-tyrosine in the medium is limited, 4HHP is reduced, and expression of *hpdrD* is repressed, while *hmaS* expression is activated, directing 4HPP into CDA biosynthesis.
of AbsC on antibiotic production appears to be indirect. AbsC represses a gene cluster for a zinc-binding siderophore, coelbacbacin, and together with another repressor, Zur, which binds nearby in the same promoter, helps to maintain zinc homeostasis. The influence of AbsC on antibiotic production could possibly be mediated through coelbacbacin (coelbacbacin overproduction inhibits sporulation) (115), either in a signaling role or indirectly through changed intracellular zinc levels. Alternatively, some other AbsC-regulated gene(s) may be involved. The influence on ACT and RED biosynthesis is ultimately exerted via the CSRs actII-ORF4 and redD, both of which are underexpressed in the absC mutant (114). Indeed, there is DNA affinity capture evidence (disputed in reference 114) that AbsC may bind directly to the promoters of actII-ORF4 and redD (38).

The AfsK/AfsR/AfsS System and a Possible Interface with Hyphal Growth

Apart from the regulatory systems already described that link antibiotic production to major aspects of nutrition, many other global antibiotic production regulatory genes that are not closely linked to biosynthetic gene clusters have been identified in S. coelicolor (116). For example, AfsR positively controls the biosynthesis of pigmented antibiotics (ACT and RED) in S. coelicolor. The afsR gene is located far from the act and red gene clusters (117, 118), appears to be present in all streptomycetes, and positively regulates the production of diverse antibiotics (119–121). AfsR (993 amino acids [aa]) has an N-terminal SARP domain, a central NB-ARC-like ATPase domain that may act as a molecular switch (122), and a C-terminal possible tetratricopeptide repeat (TPR) domain suggesting protein-protein interaction (SMART database) (123). Similar ATPase domains are also present in CdaR and in two other SARPs of unknown function encoded in the S. coelicolor genome (see below).

In vitro, AfsR can be phosphorylated by AfsK, one of the 34 protein serine/threonine kinases (STKs) encoded in the S. coelicolor genome (124). The autokinase activity of AfsK, and hence its ability to phosphorylate AfsR, is inhibited by direct interaction with the product of the neighboring gene, kbpA (both genes are present in nearly all streptomycetes but absent from other actinomycetes) (125) (Fig. 8). It is interesting that six paralogues of KbpA are encoded in the S. coelicolor genome and that one paralogue, SgaA, in S. griseus has conditional effects on morphology (126) (interestingly, the AfsK-AfsR system is also conditionally required for development in S. griseus [127]). However, it is not known whether any of the KbpA paralogues interact with protein kinases.

Although the close linkage of afsK and afsR suggests that their interaction is meaningful (the two genes are rarely separated by more than 10 genes in Streptomyces genomes), mutation of afsK does not completely eliminate AfsR phosphorylation, and two other STKs (PkaG and AfsI) that can phosphorylate AfsR in vitro have been identified (128). It is therefore possible that AfsR integrates signals from more than one STK-dependent signal transduction system. Remarkably, S. coelicolor AfsK is also implicated in the phosphorylation of a key determinant of mycelial polar growth, DivIVA (129). It was found that AfsK is almost entirely located at hyphal tips, where DivIVA is concentrated, as part of a “polarisome” that controls tip extension (Fig. 8). The activity of AfsK on DivIVA is crucial for the splitting of polarisomes to initiate new hyphal branches and is also stimulated by the inhibition of cell wall synthesis, with AfsK perhaps being responsive to intermediates in peptidoglycan biosynthesis (129). It is not yet possible to relate the two quite different AfsK activities, because the AfsR and DivIVA experiments were done under different culture conditions. However, it may be profitable to investigate the possibility that antibiotic production is initiated mainly in compartments that do not have a tip (i.e., are not apical) and in which any AfsK present could therefore not be tip located and might be able to make contact with alternative substrates such as AfsR. This could provide a mechanism for switching between growth and antibiotic production (Fig. 8).

Nuclear magnetic resonance (NMR) analysis has suggested that AfsK binds S-adenosylmethionine (SAM), though the consequences of this interaction are not known; however, there have been several reports indicating that endogenously increased SAM synthetase or exogenously added SAM increases antibiotic production in different streptomycetes (130–133). There appears to be no information about whether endogenous SAM levels increase as growth comes to a stop (for example, if methionine is shunted to SAM biosynthesis when protein synthesis slows down). In the light of the discovery of the AfsK-DivIVA interaction, the question arises of whether binding of SAM might change the partner choice of AfsK.

AfsR recruits RNA polymerase to the promoter of the adjacent afsS gene (134). AfsS (also called AfsR2) has been studied as an apparent positive regulator of antibiotic production since its dis-
covery (135). It has some homology with the conserved linker domain 3 of sigma factors but is unlikely to function as a sigma factor on its own (136). A suggestion that AfsS might act cooperatively with sigma factors of the ECF subclass, which lack domain 3 (136), seems unlikely given that ECF sigma factors are ubiquitous while AfsS-like proteins are found in only some (not all) streptomyces. When present, afsS is seldom as closely linked to aFSR as in S. coelicolor (though quite often the two genes are within a few tens of genes of each other). AfsS may provide some species-specific beneficial modulation of AfsR function by virtue of its (probably mostly indirect) influences on the transcription of various stress response and stationary-phase genes (136). More than 117 genes were up- or downregulated in an afsS disruption mutant (136). Disruption of afsS also causes diminished actinorhodin production during phosphate starvation, an observation underpinned by the finding that AfsR and PhoP bind to overlapping sequences in the promoter region of afsS (82). Although AfsS is usually thought of as a regulator of antibiotic biosynthetic genes, transcriptome analysis showed that it also has major effects on nutritional starvation genes, which might possibly be responsible for the effects on antibiotic production (136).

AtrA, a TetR-Like Global Regulator of Antibiotic Production
A biochemical approach followed by reverse genetics led to the identification of AtrA, a TetR-like protein that binds to the promoter of the actII-ORF4 CSR gene, as an essential activator of actII-ORF4 expression. Neither RED nor CDA production was affected by an atrA mutation, but in S. griseus AtrA binds to the promoter of strR, the CSR gene for streptomycin production (32). AtrA orthologues are present in all Streptomyces genomes sequenced so far. atra is regulated by the PhoRP system, providing a link with phosphate availability, and a further nutritional link is provided by the activation by AtraA of nagE2, encoding the GlcNAc permease. nagE2 is also subject to repression by the GlcNAc-sensing pleiotropic regulator DasR (20, 36). Hence, the two regulators act synergistically on GlcNAc-mediated control of the DasR regulator (Fig. 6).

Other Two-Component Systems Influencing Antibiotic Production
We have already mentioned roles in antibiotic production for four two-component systems (AbsA1A2, PhoPR, DraRK, and AfsQ1Q2). These are among 67 two-component systems encoded in S. coelicolor chromosome (137). Other two-component systems influencing antibiotic production, but not closely linked to antibiotic biosynthetic genes, include the following: CutRS, affecting ACT production (138); EcrA1A2 (SCO2517 and -8), affecting only RED production (139); SCO0203/0204, which interplay with the orphan RR SCO3818 in exerting medium-dependent effects on ACT production (140, 141); and SCO5784 and -5, affecting the timing of ACT and RED production and sporulation, possibly through effects on ppGpp synthesis (142). In two surveys of several two-component systems, the AbrA1A2 system acted negatively on production of ACT, RED, and CDA and the formation of aerial mycelium, while the AbrC1C2C3 system, involving two histidine protein kinases, acted positively on all four aspects (143), and RapA1A2 was shown to stimulate ACT and CPK production (140). Several of the two-component systems implicated in regulating antibiotic production in S. coelicolor are not widely conserv
genes, so it has been assumed that no TTA-containing genes are essential. However, \textit{bldA} mutants are defective in development and in the production of many antibiotics, and, consistent with a role in regulating stationary-phase biology, the \textit{bldA}-specified tRNA increases in abundance when growth slows (158).

In \textit{S. coelicolor}, a TTA codon is present in actII-ORF4, redZ, and \textit{mmnB}, as well as in \textit{mmnL}, one of the structural genes for the MMF autoregulators. Changing the TTA codon of actII-ORF4, or of \textit{mmnB} and \textit{mmnL}, to an alternative leucine codon caused the specific restoration of production of the relevant antibiotic in a \textit{bldA} mutant (39, 57), providing a potentially useful route to the elimination of competing \textit{bldA}-dependent pathways. It is very common for a TTA codon to be present in cluster-situated regulatory genes: among 143 secondary metabolic biosynthetic gene clusters, 109 included TTA-containing genes, most of which encoded regulators (159). This, and the TTA codon in \textit{adpA} (see below), explains why disruption of \textit{bldA} eliminates biosynthesis of a variety of secondary metabolites in diverse streptomyces.

Unusually, the TTA-containing CSR gene \textit{ecar} for clavulanic acid/cephamycin C biosynthesis in \textit{S. clavuligerus} is fully expressed in a \textit{bldA} mutant (160, 161). This is thought to indicate a context effect on the extent to which UUA codons are translatable in a \textit{bldA} mutant, with UUA codons followed by \textit{G} or \textit{A} being more likely to be mistranslated in frame (160, 161). Further analysis of this hypothesis is needed in order that the effects of TTA codons in antibiotic gene clusters can be reliably predicted.

### The Master Regulator AdpA and Its Interaction with Developmental Genes

AdpA is a key transcriptional activator that has been studied biochemically mainly in \textit{S. griseus}, in which it was discovered (30). It is present in all streptomyces (orthologues are absent from most other actinomycetes), and \textit{adpA} mutants (originally called \textit{bldH} in \textit{S. coelicolor}) have pleiotropic deficiencies in aerial mycelium formation and antibiotic production, which may be medium dependent (162–166). Like most proteins of the AraC/XylS family, to which AdpA belongs, it contains two DNA-binding helix-turn-helix (HTH) motifs in its C-terminal region. Preliminary structural analysis of the DNA-binding domain of the \textit{S. griseus} protein complexed with DNA has been reported (167). A type 1 glutamine amidotransferase (GATase) 1-like domain in its N-terminal region suggests that AdpA may sense molecular signals.

In \textit{S. griseus}, the \textit{adpA} promoter is the sole target for the ArpA repressor, which is the sensor for the pleiotropic \textit{S. griseus}-specific regulatory gamma-butyrolactone molecule A-factor (30). When the concentration of A-factor reaches a threshold, ArpA is released from the \textit{adpA} promoter region. The pleiotropic effects of A-factor in \textit{S. griseus} are manifested entirely via AdpA. Recently, evidence has been obtained pointing to the possibility that even in \textit{S. coelicolor} \textit{adpA} may be regulated by the endogenous SCB gamma-butyrolactones (Fig. 9). In DNA affinity capture experiments with \textit{S. lividans}, which is very closely related to \textit{S. coelicolor}, proteins binding to the \textit{adpA} promoter included ScbR (the SCB-sensing parologue of ArpA) (168). Moreover, in independent DNA affinity capture experiments done with \textit{S. coelicolor} (169), a quite different protein, the SCO0608 protein (designated SlbR), was found to bind to both the \textit{adpA} promoter and the \textit{schA}-\textit{schB} promoter and to be sensitive to SCBs even though it did not resemble any known gamma-butyrolactone-binding protein. Mutation of SCP0608 caused early production of \textit{ACT} and \textit{RED} on solid medium and hypersporulation (169). Few other streptomycetes have candidate SlbR orthologues, but proteins with moderate end-to-end similarity are present in about half the species that have been subjected to genome sequencing.

Aside from these effects, regulation of \textit{adpA} is complex in both \textit{S. griseus} and \textit{S. coelicolor} (Fig. 9). It is repressed both by its own gene product (170) and, in \textit{S. coelicolor}, by another pleiotropic regulator, \textit{BldD} (171). In \textit{S. lividans}, and therefore probably in \textit{S. coelicolor}, another protein, ArfA, appears to activate \textit{adpA} expression (168). AdpA itself may sense adenine nucleotides: it was very recently shown that AdpA competes with the initiator protein DnaA for binding to the \textit{S. coelicolor} origin of chromosome replication and that the binding of AdpA to this region was relieved by ATP or ADP (172). It is therefore possible that depletion of adenine nucleotides may affect the autoregulation of \textit{adpA} expression, consistent with the observation that intracellular ATP levels are low at the onset of antibiotic production (173).

The \textit{S. coelicolor} \textit{adpA} mRNA is a target for processing by RNase III (152) and contains a \textit{bldA}-dependent UUA codon (\textit{adpA} is the only gene present in all streptomyces that always has a TTA codon). It is not known whether these two posttranscriptional processes interact. The UUA codon in \textit{adpA} mRNA always falls between the segments encoding the two major AdpA domains and accounts largely (but not entirely) for the aerial mycelium-defective phenotype of \textit{bldA} mutants of \textit{S. coelicolor} (164, 166). The abundance of \textit{bldA} tRNA is important in determining whether AdpA reaches levels sufficient to activate its target genes, which include \textit{bldA} itself, establishing a remarkable mutual feed-forward cascade (174). At least in \textit{S. lividans}, there is even a further level of \textit{adpA} regulation, since it appears that the antibiotic production-stimulatory effect of the addition of SAM (see above) is at least partly mediated through the UUA codon of \textit{adpA} mRNA, perhaps by modulating the maturation of the \textit{bldA} tRNA (175). We note in passing that the effects of SAM and adenosine nucleotides on AdpA activity might make their interconversion a pivot in AdpA-mediated activation of secondary metabolism and development.

The binding site for AdpA in DNA targets is rather degenerate (5’-TGGCSNGWWY-3’), and there are about 1,500 direct AdpA-binding sites in the \textit{S. griseus} chromosome (176). About 75% of these seem not to be involved in gene regulation, but probably ca. 500 of them have regulatory significance (176). In general, when bound to a target site, AdpA recruits RNA polymerase and activates transcription, though some targets (like \textit{adpA} itself) are repressed by AdpA (176). Targets in \textit{S. coelicolor} and \textit{S. griseus} include a considerable number of genes involved in secondary metabolism and morphological differentiation, though the spectrum of target genes is not completely congruent in the two species (30, 177). A notable target in \textit{S. griseus} is \textit{strR}, the CSR for streptomycin biosynthesis (the first AdpA target to be defined). The CSR gene (\textit{griR}) for the biosynthesis of the A-factor-dependent yellow pigment griaxzone in \textit{S. griseus}, on the other hand, is only indirectly dependent on AdpA (178).

AdpA-binding sites have also been found upstream of other CSR genes for antibiotic production, including \textit{actII}-ORF4 in \textit{S. coelicolor} (38) and \textit{sanG} for nikkomycin biosynthesis in \textit{Streptomyces ansochromogenes} (179). The role of AdpA at such target promoters can be complex. For example, of five AdpA-binding sites located upstream of \textit{sanG}, two (sites I and V) are used to activate transcription of \textit{sanG}, while three (sites II, III, and IV) lead to repression. It is speculated that a DNA loop formed via
protein-protein interactions between AdpA bound to sites II, III, and IV prevents RNA polymerase from chain elongation and represses the transcription of sanG. Interestingly, nikkomycin production in a site III mutant is much higher (3-fold) than that in the wild type (179).

**How Does BldD Regulate Development and Antibiotic Production?**

Mutation of bldD results in failure of S. coelicolor to develop aerial mycelium or to produce ACT, RED, MM, or CDA on most media (180, 181). BldD is structurally related to SinR, a protein of Bacillus subtilis, and to lambda repressor, both of which mediate transitions between different cellular states by acting as repressors of some genes and activators of others (182, 183). BldD is autorepressing (184–186). More than 150 other direct targets of BldD have been determined, leading to the delineation of a refined consensus target site, 5’-nTnCnC(A/T)nGnGTnAn-3’ (171). Among a relatively high representation of regulatory genes, the target genes include the following: adpA and bldA, bldC, encoding a small protein with a single MerR-like domain that is needed for development and antibiotic production (187); and nsdA, which encodes an apparent repressor of ACT, RED, and CDA production and of differentiation (188, 189). Orthologues of BldD and BldC are found in many actinobacteria, including some that are morphologically simple, have small genomes, and are not known as antibiotic producers, while NsdA orthologues are present in all streptomycetes and their closest relatives but are absent from other actinomycetes. NsdA is not obviously related to proteins of known structure or function, but streptomycetes usually have several paralogues. In Streptomyces bingchengensis, NsdA represses production of the macrolide milbemycin and a polyether, nangchangmycin (189). The effects of NsdA are thus applied to a wide range of antibiotic biosynthetic pathways. In the case of ACT, the effect of NsdA is exerted, directly or indirectly, via actII-ORF4 (188). It is possible that some of the pleiotropic defects of a bldD mutant are attributable to the derepressed expression of nsdA.

**Other Developmental Genes with Effects on Antibiotic Production: bldB, abaA and whiJ, and wblA**

The small protein BldB is required for antibiotic production and aerial growth, possibly through interaction with another protein, though little more is known about its mode of action (190). BldB appears to universal among, but peculiar to, streptomycetes. S. coelicolor has many small proteins giving end-to-end alignments with BldB, and one of these, SCO4542, has been further studied genetically. Its deletion caused apparent overproduction of ACT and/or RED and loss of aerial mycelium formation (191). Further analysis indicated a complex, medium-sensitive interplay of SCO4542 protein with the products of two neighboring genes,
SCO4543 (\textit{whiJ}) and SCO4544, both of which have predicted regulatory roles \cite{191}. It was suggested that the three proteins are involved in transducing an environmental signal that influences developmental progression and that SCO4542 mutants are abnormally rich in a cell type that produces pigmented antibiotics \cite{192}. Streptomyces all have multiple gene clusters comprising paralogues of SCO4542 to -4 \cite{191,193}. One such cluster is the \textit{abaA} cluster, discovered earlier through its presence in a DNA fragment that stimulated \textit{S. coelicolor} colony pigmentation at high copy number \cite{194}. Some of these clusters are widely conserved among different species, but others, including the \textit{whiJ} cluster, are species specific. It has been suggested that these clusters may generally determine responses of secondary metabolism and development to environmental signals associated with particular ecological niches \cite{191}. Interestingly, there are markedly fewer such clusters in \textit{S. venezuelae}, an organism that grows and sporulates rapidly and produces at least one antibiotic, chloramphenicol (Cm), during vegetative growth \cite{51}.

Another developmental gene, \textit{wblA}, encodes one of 11 \textit{S. coelicolor} proteins of the Wbl (WhiB-like) family \cite{195}. These small, redox- and nitric oxide-sensitive iron-sulfur proteins are widespread in actinobacteria and are absent from all other bacteria \cite{195}. Disruption of \textit{wblA} dramatically increased antibiotic production in \textit{S. coelicolor} \cite{195}, \textit{Streptomyces peucetius} \cite{196}, and \textit{Streptomyces sp. strain C4412} \cite{197}, prevented nearly all aerial hyphae from sporulating in \textit{S. coelicolor} \cite{195} and \textit{Streptomyces ghanensis} \cite{198}, affected the expression of hundreds of genes in \textit{S. coelicolor} \cite{195}, and slightly increased \textit{S. coelicolor} oxidative stress responses \cite{199}. On the other hand, a high copy number of \textit{wbla} depressed the production of ACT, RED, and CDA in \textit{S. coelicolor} (and of doxorubicin in \textit{S. peucetius}) \cite{200}. It was suggested that \textit{WblA} mediates a switch between antibiotic production and morphological development, such that (as suggested above for \textit{WhiJ} systems) mutation of \textit{wbla} causes increased representation in the colony of an antibiotic-producing cell type that is intermediate between growth and a developmentally committed state \cite{195}.

\section*{BROADENING FROM THE \textit{S. COELICOLOR} MODEL: EVOLUTION AND GENERALIZATION}

Among the hundreds of antibiotic biosynthetic clusters that have been sequenced, there is considerably greater diversity in the numbers, types, and linkage arrangements of CSR genes than has been revealed in \textit{S. coelicolor}. Comparatively few of the regulatory features of clusters from nonmodel organisms have been analyzed experimentally, but where they have been, new concepts usually arise, as we illustrate in this section with a few examples. Clearly, current knowledge is only the tip of the iceberg.

\section*{Antibiotic Biosynthesis Regulation: Cascades, Feedback Control, and Cross Talk}

Pathway-specific regulation can be very simple, yet idiosyncratic. For example, although the higher-level regulatory input into streptomycin production is quite complex, only one CSR, \textit{StrR}, is involved \cite{201}. \textit{StrR} does not resemble other characterized proteins, except in possessing a likely ATPase domain like those of ParA partitioning proteins. Few paralogues of \textit{StrR} have been found—none are present in \textit{S. coelicolor}, and we found only four in the seven genomes in StrepDB. Among 236 antibiotic biosynthesis clusters in the sequence database that we collected (Table 1, footnote a), one more \textit{strR}-like gene was found, at one end of the lankacidin cluster of \textit{Streptomyces rochei}, but disruption of this gene had no effect on antibiotic production \cite{202}.

In contrast to the case for streptomycin, the complex regulation of tylosin production in \textit{Streptomyces fradiae} involves at least five CSRs, including two SARPs and two \textit{ArpA-like} proteins \cite{203,204}. \textit{TylR}, a homologue of CSRs for carbomycin and spiramycin biosynthesis \cite{205}, is the direct activator of the tylosin biosynthetic genes. The expression of \textit{tylR} requires the combined action of two SARPs, \textit{TylS} and \textit{TylU} (which is described as a “SARP helper”). In a positive reinforcement circuit, \textit{TylS} also activates \textit{TylU}. Two \textit{ArpA-like} proteins, \textit{TylP} and \textit{TylQ}, provide overriding negative control of \textit{tylR} expression. Thus, \textit{TylQ} directly represses \textit{tylR}, and \textit{TylP} directly represses \textit{TylS}. Further subtlety is conferred by the ability of \textit{TylP} to repress both its own gene and that of \textit{TylQ}. Repression by \textit{TylP} can be lifted by a (still-unidentified) small-molecule ligand extractable from stationary-phase cultures. Thus, the accumulation of this ligand probably sets the regulatory system off. The logic of the two-step negatively acting part of the overall regulatory system is unclear, but in view of the evidence discussed elsewhere in this review, it seems possible that \textit{TylQ} might be sensitive to a different ligand, perhaps generated by the tylosin pathway, or possibly the product of another biosynthetic pathway in \textit{S. fradiae}.

Different regulatory strategies are found in different pathways for biosynthesis of polyether antibiotics, such as nanchangmycin and monensin, low-molecular weight compounds that are widely used in agriculture. The nanchangmycin (\textit{nann}) biosynthetic gene cluster includes at least six regulatory genes \cite{206}. \textit{NanR1} and \textit{NanR2} are SARPs essential for nanchangmycin biosynthesis, and \textit{NanR4} is AraC like and represses \textit{nanR1} and \textit{nanR2}, such that deletion of \textit{nanR4} resulted in a 3-fold increase of nanchangmycin \cite{207}. The roles of \textit{NanR3} (a putative LacI-like repressor) and the \textit{NanT5/NanT3} two-component system \cite{206} remain undetermined. Only three putative monensin CSRs have been recognized \cite{109}. Overexpression of the SARP \textit{MonR1} caused increased monensin production, but the roles of \textit{MonRII}, which resembles the negative regulator \textit{TcmR} from \textit{Streptomyces glaucescens}, and \textit{MonH}, which resembles CSRs with an N-terminal ATP-binding domain in other clusters, have not been studied \cite{208}. Thus, the pathways for different polyethers have different regulatory strategies.

Bearing in mind the binding of the RED antibiotic by RedZ, it was of interest to consider the occurrence and functions of atypical RRs (ARRs) in other antibiotic biosynthetic clusters. RedZ belongs to the NarL family ARRs, which include \textit{DnrN}, which is required for daunorubicin (DNR) biosynthesis in \textit{S. peucetius} \cite{209} (Fig. 5). Indeed, mutagenesis of residues in the position of the phosphorylation pocket of \textit{DnrN} had no effect on daunorubicin production levels \cite{209}, strongly suggesting that \textit{DnrN} is not regulated by conventional phosphorylation (similar conclusions were also reached for some orphan RRs with other physiological roles in \textit{Streptomyces} \cite{210–212}). Other NarL family ARRs associated with antibiotic clusters are shown in Fig. 5, as well as SCO1645, an \textit{S. coelicolor} protein with unknown function that is very similar to RedZ.

Other \textit{Streptomyces} ARRs belong to the OmpR family and are highly conserved (Fig. 5) \cite{213}. The most studied of these is \textit{JadR1} of \textit{S. venezuelae}. \textit{JadR1} has a winged HTH motif in its C-terminal output domain, and two important aspartic acid residues are re-

\footnote{Acknowledgments: We thank Peter Ram, Michael Kaposi, and Andrew Lederberg for helpful comments on an earlier draft of this chapter.}

\section*{References}

\begin{thebibliography}{100}
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\end{thebibliography}
**TABLE 1** SARPs encoded in antibiotic biosynthetic gene clusters from diverse streptomycetes

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<th>Pathway type</th>
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<th>Organism</th>
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<th>No. of TTA codons</th>
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(Continued on following page)
placed by Glu49 and Ser50 in its N-terminal receiver domain. The jadR1 gene is located upstream of jadJ, the first structural gene of the jadomycin biosynthetic cluster (69). As JadR1 could not be phosphorylated, it should sense some signaling molecules to start the antibiotic biosynthesis. Recent studies found that JadR1 activates the expression of jadomycin B (JdB) biosynthetic genes in the presence of a low concentration of JdB and its homologues, but high JdB concentrations led to the dissociation of JadR1 from its target promoters. Thus, JdB interacts with JadR1 directly in a dose-dependent manner (Fig. 10)(69). This was the first report that any ARR could be activated by binding to the end product or late biosynthetic intermediates of secondary metabolites. Clearly there is a need to investigate both the molecular basis of ARR-ligand interactions and their broader significance in relation to antibiotic production.

**TABLE 1** (Continued)

<table>
<thead>
<tr>
<th>Pathway type</th>
<th>Antibiotic</th>
<th>Organism</th>
<th>SARP length</th>
<th>No. of TTA codons</th>
<th>Gene name</th>
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<td>41</td>
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<td>Virginiamycin (two polyketide/peptide components)</td>
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<td>ymoS</td>
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<td>?</td>
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* Gene clusters were initially identified by searching the NCBI database for “Streptomyces” under the field “organism” and for “cluster” under the field “title.” Limits of 5,000 to 500,000 nucleotides were set. The exact query submitted to the database was, “Streptomyces [organism] AND cluster [title] AND 5000:5000000 [sequence length].” From 365 clusters initially listed, manual editing was used to eliminate clusters that were not for antibiotic biosynthesis. Further manual editing eliminated clusters that were SARP free but incomplete. From a final total of 236 clusters, 24% (57 clusters) encoded at least one SARP (homologues of ActII-ORF4 using BLASTP analysis). These are listed here in relation to the general type of biosynthetic pathway. “Small” SARPs are up to 400 aa long, “medium” are 401 to 799 bp, and “large” 800 bp or more. AHBA, 3-amino-5-hydroxybenzoic acid.

End product-mediated feedback regulation has also been found with CSRs of a different type. In the three-step CSR cascade controlling daunorubicin production in *S. peucetius*, the TetR-like product of *dnrO* activates the diverging gene *dnrN*, encoding an ARR which then initiates the transcription of *dnrI*, the SARP product of which turns on daunorubicin (DNR) biosynthesis. *dnrO* is repressed by its own product, DnrO, but once daunorubicin or its glycosylated late precursors are produced, they bind to DnrO, derepressing *dnrO* and apparently activating *dnrN* (214).

In *S. venezuelae*, the key jadomycin CSR JadR1 not only activates jadomycin biosynthesis directly but also represses the chloramphenicol biosynthetic genes (51). JadR1 binds directly to an extended sequence in the *cml* cluster, located downstream of the transcription start site of *cmlJ*. The mechanism by which binding at this site represses transcription has not been investigated.

**FIG 10** Cross-coordination of different antibiotic biosynthetic pathways by the pseudo-GBL receptors in *S. venezuelae*. The pseudo-gamma-butyrolactone receptor JadR2 directly represses the transcription of *jadR1* in the absence of ethanol and also binds chloramphenicol (Cm) and jadomycins. The cluster-situated regulator JadR1 activates the biosynthesis of jadomycin B by activating the transcription of biosynthetic structural genes. JadR1 also represses the production of Cm by binding to the promoters of the structural genes (51).
and it is also unclear how this binding sequence is related to those used in the jad cluster to activate jadomycin biosynthesis, but the discovery of this kind of CSR-mediated cross talk implies yet a further layer of complexity in the regulation of antibiotic biosynthesis.

The \textit{jadR1} gene is itself directly repressed by an ScbR2-like pseudo-gamma-butyrolactone receptor, JadR2, encoded by the adjacent diverging gene. Thus, during JadR2-mediated repression of \textit{jadR1}, Cm biosynthesis is derepressed. Repression of \textit{jadR1} is relieved by binding of either jadomycins or Cm to JadR2 (51) (Fig. 10). The resulting accumulation of JadR1 represses Cm biosynthesis and activates jadomycin biosynthesis. This resonates with similar observations with ScbR2 in \textit{S. coelicolor} (see “Regulation of the ‘Cryptic Polyketide’ Gene Cluster”). \textit{Streptomyces} genomes often encode several putative ArpA homologues, including many that group together with ScbR2 and JadR2 pseudo-gamma-butyrolactone receptors in the phylogenetic tree of ArpA-like proteins (215). Examples include Aur1R from butyrolactone receptors in the phylogenetic tree of ArpA-like proteins (215).

The N-terminal winged helix-turn-helix (HTH) DNA-binding motif of SARP binds repeated motifs (often heptamers) at an 11-nt spacing, and the adjacent transcriptional activation domain switches on the expression of target antibiotic production genes (29). The specific sequences recognized by SARP generally overlap the \(\sim 35\) regions of their targets, but sometimes the sequences are far from the transcriptional start point (tsp) of their target genes. Some SARP-binding sites contain three discernible heptamers (e.g., tylosin and nangchangmycin) (see above) (203, 206).

Like ActII-ORF4 and RedD, many other SARP are less than 300 residues long, and some of these have been shown, mainly by mutant phenotypes, to function as pathway-specific activators, including CcaR (cephemycin-clavulanic acid supercluster in \textit{S. clavuligerus}) (216), Dnr1 (daunorubicin biosynthesis in \textit{S. peucetius}) (218, 224), Aur1PR2 and Aur1PR3 (auricin biosynthesis in \textit{S. aureofaciens}) (225), TyfS and TyfT (which activate the transcription of the tylosin CSR gene \textit{tylR} in \textit{S. fradiae}) (226), FdmR1 (f derogamicin biosynthesis in \textit{S. griseus}) (219), ThnU (cephamycin C biosynthesis in \textit{Streptomyces cattleya}) (227), SrrY and SrrZ (lankamycin biosynthetic regulatory cascade in \textit{Streptomyces rochei}) (228), and VlmI (valanimycin biosynthesis in \textit{Streptomyces viridifaciens}) (217). These ActII-ORF4-like SARPs are referred to here as “small SARPs.” Members of a second class of SARP CSRs (“medium SARPs”) resemble CdaR in being around 600 aa long, and these all show end-to-end similarity to each other (229). Still other SARP CSRs are much larger, containing about 1,000 residues (“large SARPs”). All of these have the same three major functional domains: an N-terminal SARP domain, a central AAA domain (ATPase associated with diverse cellular activities) (230), and a conserved C-terminal domain of unknown function.

Several large SARPs have been shown to activate their cognate pathways. For example, SanG positively regulates nikkomycin biosynthesis in \textit{S. ansochromogenes} (221, 231), with its helix-turn-helix domain directly binding to the bidirectional sanN-sanO promoter region and activating the biosynthetic genes, and PolR controls polyoxin biosynthesis in \textit{S. cacaoi} subsp. \textit{asoensis} (222) through binding to the promoter regions of \textit{polC} and \textit{polB}. \textit{polR} itself is activated by another large SARP, PolY, the product of the adjacent gene (232). Binding of PolY at a typical SARP-binding sequence in the promoter region of \textit{polR} is enhanced by binding of ADP/ATP to the ATPase domain of PolY, which triggers PolY oligomerization (232) (Fig. 11). Since changes of ADP/ATP concentrations significantly affect the binding activity of PolY \textit{in vivo} (232).
Using our database of 236 antibiotic biosynthetic clusters from streptomycetes other than *S. coelicolor*, we found 57 with at least one SARP (Table 1). We evaluated the relative frequencies of the three SARP classes and their association with different classes of antibiotics (Table 1). Small SARPs were the most abundant (48 examples were found in 40 clusters), while medium (13 were present in 12 clusters) and large (11 were present in 10 clusters) SARPs were both moderately common. Although SARPs were associated with the biosynthetic genes for diverse classes of antibiotics, there was a strong association of all three classes with pathways involving polyketide biosynthesis (43 of 57 SARP-containing clusters were for polyketides). Perhaps SARPs sense some physiological parameter that is particularly relevant to polyketide biosynthesis, such as the availability of precursors or the balance between growth-associated fatty acid consumption and starvation-associated lipid degradation. Of the 72 SARP genes listed in Table 1, 28 contain TTA codons, about 10 times more often than the average for all Streptomyces genes, consistent with the notion that UUA codons are significant in regulating antibiotic biosynthesis (see above).

Although SARP genes are most often located within antibiotic production clusters, a BLAST survey of the *S. coelicolor* genome shows that in addition to AfsR, there are three others that are not CSRs: one medium and two large CSRs (Table 2). Unlike CSR SARPs, these are all present in a significant fraction of streptomycetes. It will be of considerable interest to investigate whether these SARPs influence production of different antibiotics in different species.

### Hormone-Like Autoregulators

Following the *S. griseus* A-factor paradigm (177), A-factor-like autoregulatory signal molecules have been found in many different *Streptomyces* species, with such autoregulatory systems often being associated with antibiotic biosynthetic gene clusters. For example, virginiae butanolides (VBs) (Fig. 1B) induce the coordinated production of virginiamycin M and virginiamicin S, synergistically acting but biosynthetically distinct antibiotics in *S. virginiae* (235), and IM-2 (Fig. 1B) controls the production of showdomycin and minycin in *Streptomyces lavendulae* (236, 237). Three further chemical classes of autoregulator have been identified (45) (Fig. 1): furans such as the MMFs; avernolide, which is required at nanomolar concentrations for the onset of avermec-tin biosynthesis in *S. avermitilis* (238) (avenolide homologues are also found in *S. fradiae*, *S. ghanaensis*, and *Streptomyces griseoauranti-cus*); and PI factor [2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol], which enhances pimaricin production in *S. natalensis* (239). Like A-factor, MMFs and avernolide appear to bind to specific ArpA-like binding proteins (240). The nature of the PI factor effect is not understood, since the PI factor requirement could be bypassed by adding A-factor, glycerol, ethylene glycol, or propanediol (76, 239).

Typically, the *afsA*-like biosynthetic gene (often with additional cotranscribed factor biosynthetic genes) is located next to a diverging, *arpA*-like gene, whose product regulates both itself and the biosynthetic operon. This autoregulatory organization presumably leads to a sudden accumulation of autoregulator. Unusually, the *A*-factor autoregulatory system in *S. griseus* is determined not by genes close to the streptomycin biosynthetic gene cluster but by scattered genes (*afsA* and *brpA* for *A*-factor biosynthesis and *arpA* for the *A*-factor receptor protein ArpA) (177). The separation of the *arpA* and *afsA* genes from each other may be responsible for the loss of the kind of gamma-butyrolactone autoinduction found in most other systems, so that *A*-factor is accumulated more gradually in a constitutive growth-dependent manner, eventually giving concentrations high enough to relieve repression of *adpA* by ArpA (177).

Knocking out *afsA*-like genes not only causes reduced production of particular antibiotics but also often impairs differentiation, while mutating ArpA-like binding proteins can accelerate it. For example, SabR of *S. ansochromogenes*, a regulatory protein encoded away from the *san* (nikkomycin biosynthesis) cluster, not only activates the CSR *sanG*, but also represses sporulation. No ligand for SabR has been found, but deletion of *sanG* causes morphological deficiency and loss of the brown pigment, raising the possibility that a nikkominycin-related metabolite might bind to SabR (231). Likewise, elimination of a similar protein, SpbR, caused defects in growth, pristinamycin biosynthesis, and morphological differentiation in *Streptomyces pristinaespiralis* (241). The possibility that these effects involve activity of the autoregulatory systems on the expression of *adpA* (see above) would be worth investigation.
The S. coelicolor studies have shown that numerous global regulators transmit various signal inputs, including nutrient availability, translation rates, developmental state, diverse stresses, and other environmental information, to the CSR genes. Such layered regulation has been called “pyramidal” or “cascade” regulation, though neither term seems apposite for systems in which multiple inputs drive a process forward (“confluent regulation” might be more accurate). Comparative genomic analysis of 14 sequenced Streptomyces genomes (G. Chandra and K. F. Chater, unpublished data) has shown that most of the global regulators described in the preceding sections are universal among streptomycetes, with an exception being the whiJ cluster and some of its paralogous clusters. Probably, therefore, the endogenous regulatory systems and their interplay are broadly the same in all streptomycetes, with an exception being the whiJ cluster and some of its paralogous clusters. Probably, the genes acquired earliest preceded the evolution of a complex secondary metabolism and have (or had) functions related to growth limitation. This fits with the broad range of attributes regulated by BldC, BldD, and WblA. From this point of view, relatively simple regulatory interfaces of gene clusters for antibiotic production with the ubiquitous global regulatory systems might be sufficient to ensure appropriate timing of antibiotic production in any host that the gene cluster enters through horizontal gene transfer, at least under some conditions in which production has a selective advantage. This would provide selective pressure for the retention of the gene cluster, which would presumably subsequently acquire regulatory adaptations increasing its value to the host.

**Global Regulators**

The S. coelicolor studies have shown that numerous global regulators transmit various signal inputs, including nutrient availability, translation rates, developmental state, diverse stresses, and other environmental information, to the CSR genes. Such layered regulation has been called “pyramidal” or “cascade” regulation, though neither term seems apposite for systems in which multiple inputs drive a process forward (“confluent regulation” might be more accurate). Comparative genomic analysis of 14 sequenced Streptomyces genomes (G. Chandra and K. F. Chater, unpublished data) has shown that most of the global regulators described in the preceding sections are universal among streptomycetes, with an exception being the whiJ cluster and some of its paralogous clusters. Probably, the genes acquired earliest preceded the evolution of a complex secondary metabolism and have (or had) functions related to growth limitation. This fits with the broad range of attributes regulated by BldC, BldD, and WblA. From this point of view, relatively simple regulatory interfaces of gene clusters for antibiotic production with the ubiquitous global regulatory systems might be sufficient to ensure appropriate timing of antibiotic production in any host that the gene cluster enters through horizontal gene transfer, at least under some conditions in which production has a selective advantage. This would provide selective pressure for the retention of the gene cluster, which would presumably subsequently acquire regulatory adaptations increasing its value to the host.

**ACTIVATION OF CRYPTIC SECONDARY METABOLITE GENE CLUSTERS**

Sequencing of several Streptomyces genomes revealed the presence of a large number of secondary metabolic gene clusters for previously unsuspected products and thus the potential to produce many more natural products than had previously been recognized (233, 243, 244). It has therefore become necessary to devise methods and strategies to identify physiological signals and regulatory mechanisms that can activate these “cryptic” pathways, thus unleashing the full biosynthetic potential of these prodigious producers of valuable natural products (245, 246). Currently used or proposed methods include manipulation of fermentation conditions, the use of ecologically based tricks, genome mining, ribosome engineering, genetic manipulation of the regulators controlling gene clusters, regulation of signaling molecules, and heterologous expression of gene clusters in different host strains (Fig. 13).
Fermentation Conditions and Antibiotic Production

Traditionally, modifying fermentation conditions has been crucial for industrial yield improvement. Some of the molecular information described in this review may help to make such explorations increasingly knowledge led and may permit greater interplay between fermentation development and targeted genetic manipulations. However, empirical observations may continue to be useful; for example, it was recently reported that addition of the rare earth scandium to the fermentation medium significantly stimulated the production of actinorhodin in *S. coelicolor*, of actinomycin in *Streptomyces antibioticus*, and of streptomycin in *S. griseus* (247). Genomic techniques may also be useful: based on transcriptome analysis, several novel compounds were isolated from *Streptomyces flavoelus* through screening six different media (248). This suggests that combining changes of fermentation conditions with -omic studies may identify ways of awakening cryptic gene clusters for novel compounds.

Coculture with Organisms Sharing Common Ecology

Specific environmental signals or nutritional components required for the activation of cryptic gene clusters may in some cases reflect the presence of interacting microorganisms in the natural environment. Coculture can be an effective method for activating the production of cryptic metabolites. For example, the coculture of a *Streptomyces* strain with mycotic acid-containing *Tsukamurella pulmonis* from soil samples induced the *Streptomyces* strain to produce a novel antibiotic (249). It is also noteworthy that *Streptomyces rapamycinicus* can specifically induce expression of silent biosynthetic gene clusters in *Aspergillus nidulans*, which shares the same habitat (250). The activation is achieved by *Streptomyces*-triggered fungal histone acetylation modifications (251). The growing understanding of cross talk between *Streptomyces* and other species may provide more opportunities for the discovery of novel natural products. However, even though this approach is effective, it is still laborious and obscure. Therefore, miniaturized high-throughput screening methods need to be used. A recent paper (252) describes how the small molecules in and around a living bacterial colony can be identified and quantified over time and space by nanoscale mass spectrometry. The method rapidly generated profiles of complex interorganism signal responses at the adjacent edges of pairs of different organisms, such as *B. subtilis* and *S. coelicolor*. The procedure involves establishing a dynamic droplet (liquid bridge) held between a solvent input capillary and an outlet capillary leading to a nanopore mass spectrometer (MS) system (nano-DESI). A colony is brought into contact with the droplet, and the solvent-extractable molecules are drawn off and subjected to MS-MS. Covarying fragments are used to identify particular molecular species. The same colony can be analyzed repeatedly to generate a time lapse sequence. This approach to the discovery of new bioactive molecules through molecular ecology is exceptionally promising.

Ribosome Engineering and Related Strategies

On the basis of findings that certain mutations of ribosomal components activated antibiotic biosynthesis, Ochi proposed ribosome engineering as a means to activate *Streptomyces* cryptic gene clusters (253). With this strategy, strains were cultured on sublethal concentrations of antiribosomal (and some other) antibiotics to select antibiotic-resistant mutants, which were tested for secondary metabolite profiles. The feasibility of this concept was first exemplified by the activation of ACT production in *S. lividans*, in which the act genes are normally not expressed (107). Other examples are summarized elsewhere (253). This strategy was also successful with unknown compounds. Hosaka et al. (254) subjected 1,068 nonproducing actinomycetes to sublethal concentrations of rifampin, gentamicin, and streptomycin. Antibacterial activity against *Staphylococcus aureus* 209P was detected in 43% of *Streptomyces* strains. Eight novel piperidamycins were identified alone in derivatives of one strain carrying mutations in *rpsL* and *rpoB*. Mutations in *rpoB* increased affinities of mutant RNA polymerase for promoters of certain genes involved in piperidamycin biosynthesis, which in turn enhanced the transcription of these genes. Mutations in *rpsL* enhanced protein synthesis during late growth phase (254). Further understanding of the mechanisms will facilitate the discovery of novel bioactive compounds. It has proved useful to introduce *rpsL* and *rpoB* mutations into strains of *S. coelicolor* designed to maximize the expression of introduced heterologous sets of biosynthetic genes (255, 256).

Discovery of New Signaling Molecules

A lack of specific signaling molecules such as gamma-butyrolactones (see above) may be a factor causing the silence of gene clusters. Other substances promote secondary metabolism and morphogenesis at low concentrations in various actinomycetes. Examples include goadsporin, a 19-aa peptide containing four oxazole and two thiazole residues produced by *Streptomyces* sp. strain TP-A0584 (257, 258), and desferrioxamine E, a siderophore secreted by *S. griseus* (257, 258). The discovery of more signaling molecules and their use against genome-sequenced *Streptomyces* strains will speed up the process of cryptic gene cluster activation and deepen our understanding of the roles of signaling molecules (45).

Genetic Manipulation of CSR Genes

It is possible to activate the expression of cryptic gene cluster by genetic manipulation of CSR genes (activators and repressors) (192). A straightforward strategy, simply to overexpress activators or delete repressors (259), has proved effective. For example, amplification of *sanG* significantly increased nikkomycin production in *S. ansochromogenes* (222, 231), overexpression of *polr* resulted in a 2-fold increase of polyoxin in *S. cacaoi* subsp. *asoensis* (222, 231), and a giant type I modular PKS gene cluster of *Streptomyces ambofaciens* ATCC 23877, spanning almost 150 kb, was activated by the overexpression of a LAL family regulatory gene, leading to the discovery of stambomycins, unusual glycosylated macrodilides with unique chemical structures and promising antiangiogenic activity against human cancer cell lines (260). Similarly, the activation of the cryptic *cpk* gene cluster and production of a novel antibiotic followed the deletion of *scbR2* in *S. coelicolor* (41), and transcription of *cpk* genes was also markedly increased in a mutant lacking the master regulator DasR (33). Deletion of the *scbR2* homologue *jadR2* in *S. venezuelae* activated jadomycin B production in the absense of the normally required stress challenges (51). The inactivation of a putative repressor gene, *pgaY*, in *Streptomyces* sp. strain PGA64 resulted in the production of two major angucycline metabolites, UWM6 and rabelomycin, which had not been detected in the parental wild-type strain, and many other metabolites were also produced in the mutatnt (261). Deletion of the neg-
Expression of heterologous clusters (268).

S. coelicolor and other species (52, 269–271), and what little is known about the functions points to a boost in the perceived importance of sRNA-mediated regulation of antibiotic production in the near future. Thus, the overexpression of scr5239, an sRNA recently identified in S. coelicolor, decreased ACT production, whereas depletion of scr5239 increased production (272), and overexpression of cnv2198.1as significantly decreased RED production (273). The mechanisms of these effects remain to be understood.

Protein Degradation Machinery

Transcriptional regulators or response proteins in antibiotic regulatory cascades may have to be removed after the fulfillment of their responsibilities, by target-specific or nonspecific proteases. Specificity of intracellular proteases is important for the efficient degradation of targeted proteins and the avoidance of undesired destruction of essential cellular proteins. Bacteria have different types of ATP-dependent proteases, including ClpAP, ClpC, ClpE, or ClpX. In contrast to only one clpP gene being present in most bacteria, five clpP-like (clpP1, clpP2, clpP3, clpP4, and clpP5) genes were identified in S. lividans (276), and at least three are present in all the streptomycetes represented in StrepDB. The Clp complex is a regulatory component of Streptomyces secondary metabolism, as demonstrated by increased ACT production in S. coelicolor and the activation of ACT production in S. lividans in clpX overexpression strains. Activation of ACT production in S. lividans is clpP1 dependent (276). It would be interesting to identify the ClpX targets implicated in antibiotic production. Involvement of other proteases in antibiotic production in other bacteria has been reported (277).

Proteasomes are large self-compartmentalized proteases found in archaea, eukaryotes, and certain actinobacteria, including Streptomyces, Frankia, and Mycobacterium (275, 278–280). The structure of proteasomes and their roles in intracellular protein degradation were summarized recently (281). The functions of the proteasome and its individual components have been investigated in Mycobacterium tuberculosis. The targeting of proteins to proteasomes requires the conjugation of a small “Pup” (prokaryotic ubiquitin-like protein) tag to substrates (275). The Streptomyces 20S proteasome was first identified in 1998, and its functions were initially investigated in 2007 (278, 282). A different protein profile was noticed in proteasome mutants of S. coelicolor, though no specific proteasome target was identified. Our preliminary data indicate that a Pup-like protein is a regulator of antibiotic production in S. coelicolor (G. Niu et al., unpublished data).

Epigenetic Regulation

Bacterial chromosomes are organized as nucleoids that, unlike the eukaryotic nucleus, are not surrounded by a nuclear membrane. Both the nucleoid and nucleus are compacted in ways that are compatible with DNA replication, chromosome segregation, and gene expression. It is well known that histones play important roles in nucleoid packing, chromatin structure, and gene transcription. It is not known whether histones play similar roles in regulation in eukaryotes, and analogous roles are played by bacterial nucleoid-associated proteins (NAPs), which are significant regulators of nucleoid physical structure and gene transcription (283). Manipulations of genes encoding heterochromatin protein 1 (HepA), histone methyltransferase (ClrD), and histone deacetylase (HDAC) resulted in modified chemical diversity in A. nidulans (284, 285). Application of DNA methyltransferase and HDAC inhibitors with different fungal genera resulted in enhanced chemical diversity of natural product profiles (285–287). These studies demonstrated that small-molecule epigenetic modifiers are effective tools for modifying biosynthetic pathways and for generating new compounds in fungi. In Streptomyces, both nucleoid-associated HU and Dps proteins affect nucleoid structure in spores of S. coelicolor (288), but little is known about the functions of NAPs in the regulation of gene transcription, especially in antibiotic biosynthesis. A more-detailed understanding of NAPs and their modifications is needed to know whether epigenetic modification exists in Streptomyces as prokaryotes. MacArthur and Bibb developed an in vivo DNase I sensitivity method to monitor the correlation between the physical nucleoid and the transcriptional profile of Streptomyces. This approach provides an opportunity to characterize and compare the NAPs associated with the active and repressive portions of the nucleoid (289). If coupled with the decay oligonucleotide technique developed by...
the same group (290), this approach can be used to identify histone-like proteins associated with antibiotic regulatory genes and to examine the role of these proteins in antibiotic production.

CONCLUDING REMARKS

The importance of understanding the regulation of antibiotic production has encouraged intensive international research, and the emerging picture is extremely complex. Basic cellular physiology (including nutrition, stress, developmental stage, and population density) plays a leading part in determining whether antibiotic production can go ahead, and the transmission of signals to specific sets of pathway genes involves conserved positively and negatively acting regulators, some of which are subject to regulation by small-molecule ligands or by phosphorylation. An emerging concept is that of cross talk between pathways, often mediated by unexpected promiscuity in the ligands and targets of regulatory proteins. Signal input and cross talk are coordinated in part by regulatory cascades and in part by the convergence of regulators upon common target promoters, especially those of cluster-situated regulators. Very often, there are multiple pathway-associated genes concerned with regulation, permitting signal input at more than one regulatory step. Both the nature of the regulators and the “wiring” of the cascades are diverse, but some features recur, such as the frequent involvement of SARP proteins, autoregulatory molecules such as gamma-butyrolactones and their receptor proteins, and TTA codons in regulatory genes. Other nascent areas expected to generate rapid advances in the near future include chromatin immunoprecipitation (ChIP)-Seq for the analysis of the targets of regulators and nano-DESI analysis of small molecules at the level of individual colonies. The complexity of these data will mean that system-level approaches will be needed to maximize information retrieval. These will greatly increase our understanding of the biology surrounding the production of antibiotics.

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