Coordinate Regulation of Antimycin and Candididin Biosynthesis

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ABSTRACT Streptomyces species produce an incredible array of high-value specialty chemicals and medicinal therapeutics. A single species typically harbors ~30 biosynthetic pathways, but only a few them are expressed in the laboratory; thus, poor understanding of how natural-product biosynthesis is regulated is a major bottleneck in drug discovery. Antimycins are a large family of anticancer compounds widely produced by Streptomyces species, and their regulation is atypical compared to that of most other natural products. Here we demonstrate that antimycin production by Streptomyces albus S4 is regulated by FscRI, a PAS-LuxR family cluster-situated regulator of the polyene antifungal agent candididin. We report that heterologous production of antimycins by Streptomyces coelicolor is dependent on FscRI and show that FscRI activates the transcription of key biosynthetic genes. We also demonstrate through chromatin immunoprecipitation sequencing that FscRI regulation is direct, and we provide evidence that this regulation strategy is conserved and unique to short-form antimycin gene clusters. Our study provides direct in vivo evidence of the cross-regulation of disparate biosynthetic gene clusters specifying unrelated natural products and expands the paradigmatic understanding of the regulation of secondary metabolism.

IMPORTANCE Natural products produced by members of the phylum Actinobacteria underpin many industrially and medically important compounds; however, the majority of the ~30 biosynthetic pathways harbored by an average species are not expressed in the laboratory. Understanding the diversity of regulatory strategies controlling the expression of these pathways is therefore critical if their biosynthetic potential is to be explored for new drug leads. Our findings reveal that the candididin cluster-situated regulator FscRI coordinately controls the biosynthesis of both candididin and antimycin, which is the first observation of cross-regulation of disparate biosynthetic gene clusters specifying unrelated natural products. We anticipate that this will emerge as a major strategy by which members of the phylum Actinobacteria coordinately produce natural products, which will advance our understanding of how the expression of secondary metabolism is controlled and will aid the pursuit of “silent” biosynthetic pathway activation.

KEYWORDS: natural products, regulation of secondary metabolism, secondary metabolism, Streptomyces

Microbial natural products underpin most of the pharmaceuticals in clinical use (1), and filamentous members of the phylum Actinobacteria, such as Streptomyces species, are prolific producers of these diverse small molecules. Streptomyces species typically harbor between 20 and 50 biosynthetic pathways, but only a few them are expressed under common laboratory conditions (2). The biochemical diversity encoded by these silent or unproductive biosynthetic pathways is widely believed to be a tremendous untapped source of new antibacterial agents and other therapeutics. The
regulation of natural-product biosynthesis is complex and typically involves pleiotropic global regulators that either directly activate or repress biosynthetic genes or do so via cluster-situated activators or repressors (3). Major roadblocks preventing the exploitation of silent biosynthetic pathways are a lack of insight into their regulation and limited technology for activating their expression. Advances in this area have significant potential to unlock the diversity of natural products for drug discovery.

Antimycin-type depsipeptides are a large class of natural products widely produced by *Streptomyces* species (see references 4 and 5 for recent reviews). Antimycins are the archetypal members of this family and have been known for more than 65 years (6). They possess a myriad of biological properties, including antifungal, insecticidal, and nematocidal activities, owing to their ability to inhibit mitochondrial cytochrome c reductase (7), and are used commercially as a fish pesticide (brand name, Fintrol) (8). Recently, antimycins were found to be potent and selective inhibitors of the mitochondrial Bcl-2/Bcl-XL-related antiapoptotic proteins that are overproduced by cancer cells and confer resistance to chemotherapeutic agents whose mode of action is activation of apoptosis (9).

The hybrid nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) pathway encoding the biosynthesis of antimycins remained enigmatic until it was elucidated recently in *Streptomyces albus* S4 (10, 11). The ~25-kb antimycin (ant) biosynthetic gene cluster is composed of 15 genes organized into four polycistronic transcription units, *antBA*, *antCDE*, *antGF*, and *antHIJKLMNO* (Fig. 1) (12). The *antFGHIJKLNO* genes specify the biosynthesis of the unusual starter unit 3-formamidosalicyl coenzyme A (CoA) (13–15). AntCD make up the hybrid NRPS/PKS assembly line, while AntE and AntM are crotonyl-CoA reductase and discrete ketoreductase homologs, respectively, and AntB is an acyltransferase responsible for the acyloxyl moiety and the chemical diversity observed at R1 (Fig. 1) (14, 16, 17).

The *ant* genes are expressed during vegetative growth and are significantly downregulated during aerial growth, such that the gene cluster is not constitutively active and suggesting that its expression is tightly regulated (12). The *ant* gene cluster harbors a single cluster-situated regulator, an extracytoplasmic function RNA polymerase sigma (σ) factor named σ^antA^, which only activates the transcription of operons *antGF* and *antHIJKLMNO*, suggesting that the regulator(s) controlling the expression of *antBA* and *antCDE* must be encoded at another locus (12). We consistently have been unable to heterologously produce antimycins by using a variety of *Streptomyces* strains, including *S. coelicolor*, which had previously been reported to be a suitable host for the expression of this pathway (18). We presumed that this anomaly related to the unknown regulator(s) controlling the expression of *antBA* and *antCDE* and sought to identify and characterize the transcription factor(s) in this study.
Here we demonstrate that antimycin production in *S. albus* S4 is regulated by FscRI, a LuxR family cluster-situated regulator of the polyene antifungal agent candicidin. We report that heterologous production of antimycins by *S. coelicolor* is dependent on FscRI and show that FscRI activates the expression of *antBA* and *antCDE*. We also demonstrate through chromatin immunoprecipitation (ChIP) and sequencing that FscRI regulation is direct and provide evidence that this regulation strategy is conserved and unique to short-form *ant* gene clusters. Our findings reveal coordinate control of antimycin and candicidin biosynthesis, thereby providing direct in vivo evidence of the cross-regulation of disparate biosynthetic gene clusters specifying unrelated natural products, and expand our paradigmatic understanding of the regulation of secondary metabolism.

**RESULTS AND DISCUSSION**

**Identification of FscRI binding sites associated with antimycin biosynthesis.** We previously characterized α*AntA* as an activator of *antGF* and *antHIJKLMNO* expression and postulated that the regulator(s) governing the expression of the remaining operons (*antBA* and *antCDE*) must be encoded elsewhere in the *S. albus* S4 genome (Fig. 1) (12). Recently, increased antimycin production was observed in a strain of *S. albus* J1074 engineered to heterologously overproduce PimM (19). PimM is a cluster-situated activator of pimaricin (or natamycin) biosynthesis and belongs to the PAS-LuxR family of transcriptional regulators, which harbor an N-terminal PER-ARNT-SIM) domain that recognizes stimuli such as light, oxygen, redox potential, or other ligands to modulate the activity of a C-terminal helix-turn-helix DNA-binding motif (20–22). Orthologs of PimM also control the production of related polyene antifungal agents, amphotericin (AmphDIV), nystatin (NysRIV), filipin (PteF), and candicidin (FscRI) and a coronafacic-acid-like phytotoxin (CfaR) (23–27). The amino acid sequences of polyene PAS-LuxR regulators are 65 to 94% identical and show functional cross complementarity, a consequence of their nonperfect inverted repeat binding sequence (5'-CTVGGGAWWTCCCBAG-3') (28). *S. albus* S4 also produces candicidin (29) and harbors an FscRI ortholog (11); thus, we hypothesized that FscRI was the missing regulator of antimycin biosynthesis.

FscRI was recently characterized in the candicidin producer *Streptomyces* sp. strain FR-008 and is required for the expression of 16 out of 21 genes within the gene cluster (26). DNA motifs consistent with those recognized by PimM-type regulators were identified upstream of *fscA*, *fscB*, and *fscD*, which each encode a type I PKS, and *fscRIV*, which is a LAL regulator (a large ATP-binding regulator of the LuxR type) (26). The amino acid sequences of FscRI FR-008 and FscRI S4 are 100% identical, and inspection of the *S. albus* S4 genome sequence revealed the presence of DNA motifs identical to those upstream of *fscA*, *fscB*, *fscD*, and *fscMI* in *Streptomyces* sp. strain FR-008 (Fig. 2). Thus, we used these DNA sequences with the MEME suite (30) to search for similar motifs within the antimycin gene cluster, which resulted in the identification of two putative FscRI binding sites upstream of *antBA* and one upstream of *antCDE* (Fig. 2). Taken together, these findings suggest that the cluster-situated regulator of candicidin biosynthesis, FscRI, may directly activate the expression of both *antBA* and *antCDE*.

**FscRI is required for antimycin production.** Our bioinformatic analyses led us to hypothesize that FscRI S4 activates the transcription of *antBA* and *antCDE* and is thus likely to be required for the production of antimycins. To investigate this possibility, we deleted the *fscRI* gene by CRISPR/Cas9 editing and tested the resulting mutant (Δ*fscRI*) against *Candida albicans* in a bioassay. As predicted, the Δ*fscRI* mutant strain no longer inhibited the growth of *C. albicans*, which is consistent with loss of antimycin and candicidin production (Fig. 3) (12). Complementation of this mutant with pJ10257- *fscRI*, which contains the *fscRI* gene under the control of the constitutive *ermE* promoter, restored bioactivity against *C. albicans* to wild-type levels and verified that loss of bioactivity was not due to other mutational events (Fig. 3). Ultrahigh-performance liquid chromatography–high-resolution electrospray ionization–mass spectrometry (LC-HRESI-MS) confirmed that compounds with molecular formulae con-
Sustained with antimycins A₁ to A₄ were only present in chemical extracts prepared from wild-type and ΔfscRI mutant S. albus S4 strains harboring pIJ10257-fscRI but not in those from the ΔfscRI mutant (Fig. 3). Taking these findings together, we conclude that FscRI is required for the production of antimycins and candicidin by S. albus. Given the rather flexible and conserved binding site of PAS-LuxR regulators, it is conceivable that orthologs of FscRI could also cross-regulate a gene cluster(s) other than the one in which they are encoded. This is an intriguing possibility that has not been rigorously explored.

To our knowledge, cross-regulation of disparate natural-product biosynthetic gene clusters by a cluster-situated regulator has only been demonstrated once previously. In Streptomyces clavuligerus, the cephamycin and clavulanic acid gene clusters comprise a contiguous “supercluster” (31). The biosynthesis of both cephamycin and clavulanic acid is coordinately controlled by CcaR, a SARP (Streptomyces antibiotic regulatory protein)-type activator harbored within the cephamycin gene cluster (32–34). It is interesting that not only are these gene clusters contiguous, but unlike antimycin and candicidin, both molecules are structurally similar and possess complementary biological activities (cephamycin is a β-lactam antibiotic, and clavulanic acid is a β-lactamase inhibitor).

**Heterologous production of antimycins by S. coelicolor requires FscRI.** Yan et al. cloned the ant gene cluster from Streptomyces sp. strain NRRL 2288 and hetero-

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**Promoter Sequence (5′-3′)**

<table>
<thead>
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<th>Promoter</th>
<th>Sequence</th>
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</tr>
<tr>
<td>fscB1</td>
<td>CGAGGGAAATTCACCAG</td>
</tr>
<tr>
<td>fscB2</td>
<td>TTAGGGAACCGCAG</td>
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<tr>
<td>fscD</td>
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<tr>
<td>antB2</td>
<td>TTAGGTGAATCCCTAA</td>
</tr>
<tr>
<td>antC</td>
<td>CTAGGTATTTCCCTGG</td>
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**FIG 2** FscRI binding sites within the S. albus S4 antimycin gene cluster. The top panel shows experimentally verified FscRI binding sites upstream of genes within the candidicidin biosynthetic gene cluster (fscA, fscB1, fscB2, fscD, and fscRIV) and putative FscRI binding sites upstream of antB and antC within the ant biosynthetic gene cluster. The middle panel displays the WebLogo (61) for the verified and putative FscRI binding sites above, and the bottom panel shows the relative locations of FscRI binding sites (as red boxes) upstream of antB and antC.
ologously produced antimycins with \textit{S. lividans} and \textit{S. coelicolor} M145 (18). To our surprise, even though the ant gene cluster nucleotide sequences of NRRL 2288 and S4 are >97% identical (18), we have consistently been unable to repeat these findings by using both gene clusters and multiple genetic backgrounds, including \textit{Streptomyces} sp. strain S3, \textit{S. lividans} 66, and \textit{S. coelicolor} M145, M1146, M1152, and M1153 (29, 35, 36; R. F. Seipke and M. I. Hutchings, unpublished data). Previously, we presumed that poor availability of one of more biosynthetic precursors (i.e., tryptophan, threonine, pyruvate, and acyl-CoA) precluded the production of antimycins and/or that the pathway was simply not expressed by the heterologous hosts under our growth conditions. But given our observations described above, we hypothesized that \textit{S. coelicolor} did not produce antimycins because of a lack of FscRI rather than as a result of culture conditions. To test this hypothesis, we introduced cosmid 213, containing the entire S4 ant gene cluster (10), into \textit{S. coelicolor} M1146 (36) and also introduced pIJ10257-fscRI. The resulting strains were then tested for the ability to inhibit the growth of \textit{C. albicans} by bioassay. Consistent with our hypothesis, M1146 harboring solely cosmid 213 or pIJ10257-fscRI did not inhibit the growth of \textit{C. albicans}; however, the cointegrate harboring both cosmid 213 and pIJ10257-fscRI inhibited \textit{C. albicans} growth (Fig. 4; see Fig. S1 in the supplemental material). We recapitulated this experiment with the ant gene cluster from NRRL 2288 and the pAL2602 cosmid clone generated by Yan et al. (18) and obtained identical results (see Fig. S1). The data set for the heterologous

\textbf{FIG 3} FscRI is required for the biosynthesis of antimycins by \textit{S. albus} S4. (A) \textit{S. albus} S4 strains challenged with \textit{C. albicans}. The ΔfscRI mutant does not show detectable bioactivity against \textit{C. albicans} compared to the wild-type (WT) and complemented (ΔfscRI/pIJ10257-fscRI) strains. (B) LC-HRESI-MS analysis of chemical extracts prepared from strains shown in panel A; the extracted ion chromatograms (M+H)+ for antimycins A1 to A4 are shown for each strain.
expression of the S4 ant gene cluster was corroborated by LC-HRESI-MS detection of antimycins A1 to A4 in chemical extracts prepared from M1146 harboring cosmid 213 and pIJ10257-fscRI and their absence from M1146 alone or harboring only cosmid 213 (Fig. 4). These results demonstrate that FscRI is required by S. coelicolor for heterologous production of antimycins with two different ant cluster cosmid clones. More importantly, however, our findings suggest that integral components of biosynthetic pathways may be encoded by disparate loci, which is consistent with recent observations that the Pseudonocardia metabolite gerumycin is encoded by two loci separated by over 90 kb (37).

**FscRI activates the expression of antBA and antCDE.** The observation that FscRI is required for heterologous production of antimycins by S. coelicolor suggests that it activates the expression of both antBA and antCDE. To evaluate this hypothesis, we first engineered cosmid 213 such that antBA and antCDE were expressed from the rpsL(XC) promoter (38) and the ermE* promoter (39), respectively. As we expected, M1146 harboring solely this engineered cosmid displayed an FscRI-independent ability to produce antimycins, which is consistent with bioinformatic data showing the absence of FscRI binding sites elsewhere within the ant gene cluster (Fig. 5). Next, we engineered two more variants of cosmid 213 such that the expression of only antBA or only antCDE was driven by ermEp2, leaving the native FscRI-dependent promoters of antCDE and antBA intact, respectively. The engineered cosmids and either pIJ10257 or pIJ10257-fscRI were mobilized to M1146, and the ability of the resulting strains to produce antimycins was assessed by LC-HRESI-MS. As anticipated, antimycins A1 to A4 were detected only in chemical extracts prepared from M1146 harboring pIJ10257-fscRI.

FIG 4 Heterologous production of antimycins by S. coelicolor is FscRI dependent. (A) S. coelicolor M1146 strains challenged with C. albicans. Only M1146 harboring both cosmid 213 and pIJ10257-fscRI inhibits the growth of C. albicans compared to M1146 and M1146 harboring cosmid 213. (B) LC-HRESI-MS analysis of chemical extracts prepared from strains shown in panel A; the extracted ion chromatograms (M+H)^+ for antimycins A1 to A4 are shown for each strain.
and cosmid 213 with either \textit{ermE}p2-driven \textit{antBA} or \textit{antCDE} and not those generated from M1146 harboring just pIJ10257 and cosmid 213 with \textit{ermE}p2-driven \textit{antBA} or \textit{antCDE} (Fig. 5). These data provide \textit{in vivo} evidence that FscRI may activate the expression of both \textit{antBA} and \textit{antCDE} and are consistent with our hypothesis that FscRI acts on these promoters.

**FscRI directly activates the transcription of \textit{antBA} and \textit{antCDE}**. The simplest interpretation of our bioinformatic analysis and heterologous expression data is that FscRI activation of \textit{antBA} and \textit{antCDE} is direct. We initially sought to verify this hypothesis by performing electrophoretic mobility shift assays with purified FscRI protein; however, FscRI harboring either an N-terminal or a C-terminal hexahistidine tag was insoluble when overproduced by \textit{Escherichia coli} (data not shown), which was surprising given than His$_6$-FscRI$_{FR-008}$ was reportedly soluble (26). Nevertheless, we adopted a ChIP-sequencing approach to determine if the \textit{antB} and \textit{antC} promoters were bound by FscRI \textit{in vivo}. We complemented the \Delta fscRI mutant with an N-terminal 3×FLAG-tagged version of FscRI expressed from the \Phi C31 integration site. The resulting strain (\Delta fscRI/pSETNFLAG-fscRI mutant) inhibited the growth of \textit{C. albicans} and that of the wild-type strain equally (see Fig. S2 in the supplemental material). ChIP-
sequencing was carried out with anti-FLAG antibodies and lysate from the ΔfscRI/pSETNFLAG-fscRI mutant and wild-type strains cultivated in LB, which facilitates the production of both antimycin and candicidin. Immunoprecipitated DNA from two biological replicates of S4 wild-type and ΔfscRI/pSETNFLAG-fscRI and nonimmunoprecipitated chromosomal DNA were sequenced with the Illumina HiSeq3000 platform and processed as described in Materials and Methods. As we anticipated, the numbers of sequencing reads that mapped to the antB and antC promoter regions were enriched for both biological replicates of ΔfscRI/pSETNFLAG-fscRI compared to that of the wild-type mock-immunoprecipitated control (Fig. 6). These data provide definitive evidence that FscRI binds to the antB and antC promoters and likely promotes the transcription of antBA and antCDE (Fig. 6).

FscRI regulation of antimycin biosynthesis is conserved for S-form antimycin gene clusters. We and others previously identified 14 ant gene clusters that were classified as short-form (S-form, 15 genes), intermediate-form (I-form, 16 genes), and long-form (L-form, 17 genes) clusters (4, 18). There are six taxa, all related to S. albus S4, that encode S-form ant gene clusters: S. albus S4, S. albus J1074, Streptomyces sp. strain SM8, Streptomyces sp. strain NRRL2288, Streptomyces sp. strain LaPpAH-202, and Streptomyces sp. strain CNY2288 (40). I-form ant gene clusters are encoded by two species, Streptomyces sp. strain 303MFCol5.2 and Streptomyces sp. strain TOR3209, which lack either antQ or antP, respectively. L-form ant gene clusters are encoded by six taxa, S. ambofaciens ATCC 23877, S. blastmyceticus NRBC 12747, S. gancidicus BKS 13-15, S. griseoflavus Tü4000, S. hygroscopicus subsp. jinggangensis 5008, and S. hygroscopicus subsp. jinggangensis TL01. To determine if FscRI cross-regulation of antimycin biosynthesis is likely to be widespread, we first looked for orthologs of FscRI in genomes of antimycin producers. S. blastmyceticus and Streptomyces sp. strain NRRL2288 were omitted from this analysis because their genome sequences are not available. A tBLASTn search of a local blast database for the deduced amino acid sequence of FscRI S4 revealed that organisms harboring an S-form ant gene cluster also harbor an FscRI ortholog (>99% amino acid sequence identity), whereas the top tBLASTn hits for taxa harboring either an I- or an L-form ant gene cluster displayed a rather low level of shared amino acid identity (36 to 46%), with the exception of one organism, Streptomyces sp. strain TOR3209, which possesses an ortholog of FscRI S4 (79% identical; see Table S3 in the supplemental material). Next, we closely inspected all 14 ant gene clusters for the presence of FscRI DNA-binding motifs, which revealed that only S-form ant gene clusters harbor a motif consistent with that identified in this study, which was somewhat surprising, as we expected Streptomyces sp. strain TOR3209 to also harbor this motif, given that it encodes what appears to be an FscRI.
ortholog (see Fig. S4 in the supplemental material). Taking these findings together, we conclude that cross-regulation of antimycin biosynthesis by FscRI is likely a conserved regulatory strategy for bacteria that harbor an S-form ant gene cluster but was not a strategy adopted by taxa possessing I-form or L-form variants. The regulation of I-form and L-form ant gene clusters has not yet been investigated, so the regulatory mechanism(s) controlling the expression of antBA and antCDE is unknown; however, bioinformatic analyses suggest that, like S-form gene clusters, the genes encoding the biosynthesis and activation of 3-formamidosalicylate (antGF and antHIJKLMNO) are regulated by σ^{AntA} (12).

Antimycin and candicidin do not act synergistically. It is reasonable to assume that coordinate production of antimycin and candicidin may confer a competitive advantage upon the producer, akin to coordinate control of the β-lactam antibiotic cephamycin and the β-lactam inhibitor clavulanic acid described above (31–34). One intriguing explanation for this could be that the compounds act synergistically to inhibit the growth of nearby fungi. We therefore used C. albicans to measure the MICs of antimycin (0.125 μg/ml) and candicidin (2 μg/ml) alone, as well as the MICs of pairwise mixtures of these agents, which allowed us to determine the fractional inhibitory concentration (FIC) index (see Materials and Methods). We calculated an FIC index of 2, which indicates that antimycin and candicidin do not interact synergistically or additively but also do not act antagonistically. This was surprising to us, because a slight synergistic effect against the fungus Escovopsis weberi was recently reported (41); however, an FIC index was not calculated, which limits interpretation and comparison of the data. An alternative possibility is that coordinate production of antimycin and candicidin serves to limit the development of resistance to either agent. The target of antimycin is cytochrome c reductase, and resistance can be conferred by a single point mutation (42), whereas development of resistance to candicidin and other polyene antifungal agents relies upon alteration of sterol biosynthesis, which incurs a significant fitness cost (43). It is also possible that coordinate production of these compounds relates to the monomeric precursors utilized by each pathway. For instance, the candicidin gene cluster harbors three genes (pabABC) responsible for the production of p-aminobenzoic acid (PABA) (44). In vitro studies of purified PabC revealed that its PABA synthase activity is inhibited by the aromatic amino acids tyrosine, phenylalanine, and tryptophan (45). It is conceivable that repression of PabC can be alleviated by AntF-GHIJKLMNO, which utilize tryptophan to generate the 3-formamidosalicylate starter unit and may underpin the rationale for the coordinate production of antimycin and candicidin.

Model of the regulation of antimycin biosynthesis. Our model of the regulation of antimycin biosynthesis is depicted in Fig. 7. FscRI activates the expression of antBA and antCDE, which in turn results in the σ^{AntA}-mediated expression of antGF and antHIJKLMNO (12). FscRI does not activate its own production; however, the expression of fscRI is regulated by a positive feedback loop where FscRI activates FscRIV, which in turn activates the transcription of fscRI (26). This observation, combined with our findings here and the fact that the ant gene cluster is expressed during vegetative growth and downregulated upon the onset of morphological differentiation (12), suggests that the ligand(s) recognized by the FscRI PAS domain, and perhaps all PAS domains of polyene PAS-LuxR regulators, is available only during vegetative growth. Following the inactivation of FscRI, the cell must have a strategy in place to prevent σ^{AntA} from activating its targets. Indeed, in the absence of a cognate anti-sigma factor that would ordinarily perform this task, σ^{AntA} seems to have evolved to be a direct substrate for the ClpXP protease (12).

As our understanding of the regulation of microbial natural-product biosynthesis increases, we anticipate that cross-regulation by cluster-situated regulatory proteins will emerge as a major strategy by which members of the phylum Actinobacteria coordinately produce selected natural products.
MATERIALS AND METHODS

Growth media, strains, cosmids, plasmids, and other reagents. *E. coli* strains were propagated on Lennox agar (LA) or broth (LB) (35), and *Streptomyces* strains were cultivated with LA, LB, and mannitol-soya flour (MS) agar or broth (35). Culture medium was supplemented with antibiotics as required at the following concentrations: apramycin, 50 μg/ml; carbenicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; hygromycin, 50 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 25 μg/ml. *Streptomyces* strains were constructed by cross-genus conjugation with *E. coli* as previously described (35). Enzymes were purchased from New England BioLabs unless otherwise stated, and oligonucleotides were purchased from Integrated DNA Technologies, Inc. All of the strains, cosmids, and plasmids used in this study are described in Table S1 in the supplemental material, and all of the oligonucleotides and other synthetic DNAs used are provided in Table S2 in the supplemental material.

Construction of plasmids. The insert for each plasmid generated in this study was prepared by PCR amplification with Q5 High-Fidelity DNA polymerase and oligonucleotides containing restriction sites. PCR-amplified inserts were restricted and cloned into the relevant plasmids cut with the same enzymes by standard molecular biology procedures. All clones were sequenced to verify the integrity of insert DNA. The restriction sites used for cloning are provided with the plasmid descriptions in Table S1 in the supplemental material.

Design of the apramycin theophylline riboswitch cassette. A Red recombineering template (pUC57-AprTheo) was designed and synthesized by MWG Biotech. The PCR template for recombineering was identical to that of pIJ773 (46), except that one end contained *ermE* p2 (39) repressed by a theophylline-controlled riboswitch (47). The synthesized cassette also contains an optional hexahistidine tag for knocking in a nickel affinity purification tag at the native locus. A schematic of the AprTheo PCR template is shown in Fig. S4 in the supplemental material, and further details concerning its design, including its DNA sequence, are available from FigShare at https://dx.doi.org/10.6084/m9.figshare.3838032.v1.

Construction of pUC19-promKanprom. To construct pUC19-promKanprom, the neomycin/kanamycin resistance marker from Supercos1 was PCR amplified with RFS444 and RFS445 and the product was used to replace the apramycin resistance gene and oriT of pIJ773 (46) by recombineering with *E. coli* GB05-red (48) to result in pIJ773KnFRT. Next, four PCR fragments were produced. (i) RFS406 and RFS407 were used to PCR amplify the kanamycin resistance from pIJ773KnFRT, (ii) RFS406 and RFS407 were used to PCR amplify the kanamycin resistance from pIJ773KnFRT, (iii) RFS658 and RFS659 were used to PCR amplify the ketoreductase gene *antC*. The DNA sequence was provided in Table S1 in the supplemental material.

FIG 7 Model of regulation of antimycin biosynthesis. The top panel displays the relative locations of the antimycin and candicidin gene clusters in the *S. albus* S4 chromosome. In the bottom panel, FscR activates the transcription of *antB* and *antCDE*, which results in production of the core *AntM/NRPS/PKS* megasynthase and production of the discrete acyltransferase *AntB* and *σ^AntA^*, which in turn activates the transcription of the ketoreductase gene *antM* and nine genes (*antFGHIJKLNO*) required for the biosynthesis and activation of the 3-formamidosalicylate precursor utilized by *AntC*. *σ^AntA^* does not possess a cognate anti-σ factor and instead appears to be inactivated by the ClpXP protease.
to PCR amplify the rpsL(XC) promoter from pCRISPomycyces-2 (38, 49), (iii) RF5667 and RF5668 were used to PCR amplify the erm* promoter from pSET152-ermEp, and (iv) RF5663 and RF5664 were used to linearize pUC19. The resulting PCR products were restricted with DpnI, gel purified, and assembled with the NEB HiFi DNA Assembly kit. The resulting plasmid, pUC19-promKanprom, contained a kanamycin resistance gene flanked by divergently firing rpsL(XC) and erm* promoters. A schematic of the prom-Kanprom PCR template is shown in Fig. S5 in the supplemental material, and its DNA sequence is available at http://www.ryanseipkelab.com/tools.html.

Cosmid manipulations. The ApriTheo PCR template described above was used to replace the antB or antC promoter harbored by cosmids 213 (12) by using RF5413 and RF5414 (antBp) or RF5415 and RF5416 (antCp) and the RedDirect PCR targeting system as previously described (46). The apramycin resistance gene and oriT were removed from modified cosmids with the FLP recombinase as previously described (46), resulting in cosmids 213-Aribio-FLP and 213-CDeRibo-FLP. Cosmids 213-Aribio-FLP and 213-CDeRibo-FLP were moved to E. coli GB05-red (48) and further engineered to harbor the ßC31 integrase, attP site, and apramycin resistance gene originating from pU10702 (also known as pMUC051) (50) by RecET recombining as previously described (48), resulting in cosmids 213-Aribio-FLP-BC31 and 213-CDeRibo-FLP-BC31. Cosmid 213-BC31-BC-prom was constructed by replacing the antB-antC intragenic region of cosmids 213-BC31 by recombining with GB05-red and a PCR product generated with pUC19-promKanprom and oligonucleotides RF5654 and RF5657. Thus, the ant gene cluster harbored by this final ßC31 integrative construct is entirely controlled by divergently firing rpsL(XC) and erm* promoters.

Deletion of fscRI. The fscRI gene was deleted by using the pCRISPomycyces-2 system described previously (49). First, a single-guide RNA protospacer was generated by annealing oligonucleotides RF5574 and RF5575 and the resulting DNA fragment was cloned into the BbsI site of pCRISPomycyces-2 by Golden Gate Assembly. Second, a homology-directed repair template consisting of ~3.8 kb of DNA homologous to the region adjacent to the Cas9-induced double-strand break was generated. The repair template was generated by sequentially cloning a HindIII-SpeI-restricted PCR fragment amplified with RF5521 and RF5522 into pU12738 (51) and then cloning a SpeI-KpnI-restricted PCR fragment generated with RF5523 and RF5524. The resulting plasmid (pU12738-fsCRI-UPON) was used as a PCR template with RF5572 and RF5573, and the resulting PCR product was restricted with XbaI and cloned into pCRISPomycyces-2 containing the protospacer targeting fscRI. The resulting CRISPR/Cas9 editing plasmid, pCRISPomycyces-2-fsCRI, was mobilized to S4 by conjugal transfer from E. coli ET12567/pUZ8002 as previously described (35). Temperature-sensitive pCRISPomycyces-2-fsCRI was cured from a single apramycin-resistant transconjugant by passage in LB at 37°C (two rounds) prior to the cultivation of a sporulated lawn on MS agar at 37°C. The resulting spores were serially diluted, and 11 single colonies were replica plated to assess apramycin sensitivity. Five apramycin-sensitive colonies were obtained and subsequently evaluated for the absence of fscRI by polymorphic shift PCR with RF5598 and RF5599. The integrity of the resulting ΔfsCRI null mutant was verified by DNA sequencing.

Chemical analysis. Streptomyces strains were cultured in M5 broth (50 ml in a 250-ml flask) while shaking (180 rpm) at 27°C for 7 days. M5 broth was supplemented with theophylline (4 mM) from the onset of culturing as required for M1146 strains. Bacterial cells were removed by centrifugation, and metabolites were extracted from the supernatant with a Phenomenex strata-XL C18 (100 µm, 30 mg, 1 ml) solid-phase extraction (SPE) column and a vacuum manifold. The column was first washed with 1 ml of 100% methanol followed by 1 ml of deionized water. The column was then loaded with supernatant (10 ml in total) prior to being washed with 1 ml of deionized water and then washed with 2 ml of 30% methanol. Metabolites were eluted from the SPE column in 100% methanol (0.3 ml). Equal amounts of methanolic extract from two independent replicates of each strain were mixed and centrifuged for 10 min at 16,000 × g just prior to injection in order to remove insoluble material. Only the supernatant (2 µl) was injected into a Bruker MaxIs Impact time of flight mass spectrometer and equipped with a Dionex UltiMate 3000 high-performance liquid chromatography apparatus with the same parameters as described previously (15).

Bioassays. Bioassays were performed essentially as described previously (52), except that 7 instead of 5 ml of soft nutrient agar was used and Streptomyces strains were cultivated at 30°C for 7 (instead of 10) days prior to a challenge with C. albicans CA6 (53). MS agar was supplemented with 2 mM theophylline for M1146 strains as required. Photographs of bioassay plates were taken ~48 h after the challenge.

MIC and FIC index determination. MIC assays were performed with 96-well flat-bottom microtiter plates in accordance with the Clinical and Laboratory Standards Institute guidelines adapted for C. albicans (54). The FIC index was determined according to reference 55 by evaluating the growth of C. albicans exposed to increasing pairwise concentrations of antimycin (0.0625 to 4 µg/ml) and candicidin (1 to 64 g/ml). The formula used was FIC index = FIC A + FIC B, where FIC A = MIC of combination/MIC of compound A and FIC B = MIC of combination/MIC of compound B.

Protein expression. Recombinant His6-FscRI and FscRI-His6 were produced with E. coli Rosetta BL21 (DE3) by using pET28a-fsCRI and pET30a-fsCRI, respectively. Production of His6-FscRI and FscRI-His6 was induced in mid-log phase by the addition of 1.5 mM isopropyl-β-d-thiogalactopyranoside. After 4 h of induction at 28°C, cells were harvested by centrifugation. The cell pellet was resuspended in 1× BugBuster Protein Extraction Reagent (Novagen) diluted with 50 mM Tris-Cl (pH 8.0), 200 mM NaCl, 20 mM imidazole, and 2 U of Dnase I and incubated room temperature for 20 min. Insoluble material was removed by the lysate by centrifugation (16,000 × g for 10 min). Soluble and insoluble fractions were visualized with InstantBlue (Expedeon) after 15% SDS-PAGE.
ChIP-sequencing and bioinformatic analyses. Wild-type or ΔfscRI/pSETNFLAG-fscRI mutant S4 bacteria were cultivated for 2 days in LB while shaking at 200 rpm at 28°C and processed for ChIP-sequencing exactly as described previously, with anti-FLAG M2 agarose beads (Sigma) (56), except that an Active Motif, Inc., EpiShear sonicator (30% amplitude, 30 s on and 30 s off for a total time of 13 min) was used to shear DNA to an average size of approximately 200 to 300 nucleotides. The pure DNA resulting from immunoprecipitates from two biological replicates of wild-type and ΔfscRI/pSETNFLAG-fscRI mutant S4 bacteria, as well as nonimmunoprecipitated chromosomal DNA, were sequenced with the Illumina HiSeq3000 platform with 150-nucleotide paired-end reads by the University of Leeds Next Generation Sequencing Facility at the St. James Teaching Hospital NHS Trust. The forward reads were mapped to the S4 genome with Bowtie2 version 2.1.0 (57), and the resulting alignments were converted from the .SAM to the .BAM format and sorted according to chromosomal position with SAMtools version 1.1 (58). The aligned and sorted .BAM files for immunoprecipitated samples were converted to the .bigWig format and normalized by read count compared to the DNA-only input control by using the default settings of deepTools bamCompare version 2.3.3 (59), with the exception that flag --ratio/H11005 subtract was used instead of the default --ratio/H11005 log2. This resulted in a single .bigWig file for each treatment, which was visualized with Integrated Genomics Viewer version 2.3.78 (60). Plots were generated with the deepTools programs computeMatrix and plotProfile (59) with a bin size of 50 and a custom .BED file specifying the region displayed.

Accession number(s). The next-generation sequencing data obtained in this study are available under ArrayExpress accession no. E-MTAB-5122. The DNA sequences of tools constructed during this study are available at http://www.ryanseipkelab.com/tools.html.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://dx.doi.org/10.1128/mSphere.00305-16.

Table S1, DOCX file, 0.03 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Figure S1, TIF file, 2.3 MB.
Figure S2, TIF file, 1.6 MB.
Figure S3, TIF file, 1.3 MB.
Figure S4, TIF file, 0.8 MB.
Figure S5, TIF file, 0.8 MB.

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