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Coordinate regulation of antimycin and candicidin 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 mere handful of them are expressed in the laboratory, thus poor understanding of how natural products 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, candicidin. We report that heterologous production of antimycins by *Streptomyces coelicolor* is dependent on FscRI and show that FscRI activates transcription of key biosynthetic genes. We also demonstrate through ChIP sequencing that FscRI regulation is direct and we provide evidence to suggest that this regulation strategy is conserved and unique to short form antimycin gene clusters. Our study provides direct in vivo evidence for 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 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 expression of these pathways is therefore critical if their biosynthetic potential is to be explored for new drug leads. Our findings reveal that the candicidin cluster-situated regulator, FscRI coordinately controls both candicidin and antimycin biosynthesis, 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 actinobacteria coordinately produce
natural products, which will advance understanding of how expression of secondary metabolism is controlled and will aid pursuit of ‘silent’ biosynthetic pathway activation.

**Introduction**

Microbial natural products underpin most pharmaceuticals in clinical use (1) and filamentous 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 handful of 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). A major roadblock preventing exploitation of silent biosynthetic pathways is 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 (4) and (5) for recent review articles). Antimycins are the archetypal member 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 activity, owed 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 anti-apoptotic proteins which are over-produced by cancer cells and confer resistance to chemotherapeutic agents whose mode of action is activation of apoptosis (9).

The hybrid non-ribosomal 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, \textit{antBA}, \textit{antCDE}, \textit{antGF} and \textit{antHIJKLMNO} (Fig. 1) (12). The genes \textit{antFGHIJKLNO} specify the biosynthesis of the unusual starter unit, 3-formamidosalicyl-CoA (13-15). AntCD comprise 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 R\textsuperscript{1} (Fig. 1) (14, 16, 17).

The \textit{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 its expression is tightly regulated (12). The \textit{ant} gene cluster harbors a single cluster-situated regulator, an extracytoplasmic function (ECF) RNA polymerase sigma (\(\sigma\)) factor named \(\sigma^{\text{AntA}}\), which only activates transcription of operons \textit{antGF} and \textit{antHIJKLMNO}, suggesting that the regulator(s) controlling expression of \textit{antBA} and \textit{antCDE} must be encoded at another locus (12). We consistently have been unable to heterologously produce antimycins using a variety of \textit{Streptomyces} strains, including \textit{S. coelicolor}, which had previously been reported as a suitable host for expression of this pathway (18). We presumed this anomaly related to the unknown regulator(s) controlling expression of \textit{antBA} and \textit{antCDE} and sought to identify and characterise the transcription factor(s) in this study.

Here we demonstrate that antimycin production in \textit{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 \textit{S. coelicolor} is dependent on FscRI and show that FscRI activates expression of \textit{antBA} and \textit{antCDE}. We also demonstrate through ChIP-sequencing that FscRI regulation is direct and provide evidence to suggest this regulation strategy is conserved and unique to short form \textit{ant} gene clusters. Our findings reveal coordinate control of antimycin and candicidin biosynthesis, thereby providing the direct \textit{in vivo} evidence for cross-regulation of disparate biosynthetic gene clusters specifying unrelated natural products and expands the paradigmatic understanding of the regulation of secondary metabolism.
**Results and discussion**

**Identification of FscRI binding sites associated with antimycin biosynthesis.** We previously characterised $\sigma^{AntA}$ as an activator of ant$GF$ and ant$HIJKLMNO$ expression and postulated that the regulator(s) governing expression of the remaining operons (ant$BA$ and ant$CDE$) must be encoded elsewhere in the S. albus S4 genome (Fig. 1) (12). Recently, increased antimycin production was observed by 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 candidin (FscRI) and a coronafacic acid-like phytotoxin (CfaR) (23-27). Polyene PAS-LuxR regulators share 65-94% amino acid identity and show functional cross complementarity, a consequence of their non-perfect inverted repeat binding sequence (5'-CTVGGGAWWTCCCBAG-3') (28). S. albus S4 also produces candididin (29) and harbors an FscRI ortholog (11), thus we hypothesized that FscRI was the missing regulator of antimycin biosynthesis.

FscRI was recently characterised in the candididin-producer, Streptomyces sp. FR-008 and is required for the expression of 16 out of 21 genes within the gene cluster (26). DNA motifs consistent with those recognised by PimM-type regulators were identified upstream of $fscA$, $fscB$ and $fscD$, which each encode a type I polyketide synthase, and $fscRIV$, which is a LAL regulator (large ATP-binding regulator of the LuxR type) (26). FscRI$^{FR-008}$ and FscRI$^{S4}$ share 100% amino acid identity 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 S. sp. 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 ant$BA$ and one upstream...
of antCDE (Fig. 2). Taken together, these findings suggest that the cluster-situated regulator of candidin biosynthesis, FscRI, may directly activate the expression of both antBA and antCDE.

**FscRI is required for antimycin production.** Our bioinformatics analyses led us to hypothesize that FscRI$^S_4$ activates 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 using CRISPR/Cas9 editing and tested the resulting mutant (∆fscRI) against Candida albicans in a bioassay. As predicted, the ∆fscRI strain no longer inhibited the growth of C. albicans, which is consistent with loss of antimycin and candidin production (Fig. 3) (12). Complementation of this mutant with pIJ10257-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 loss of bioactivity was not due to other mutational events (Fig. 3). Ultra-high performance liquid chromatography high resolution electrospray ionisation mass spectrometry (LC-HRESI-MS) confirmed that compounds with molecular formulae consistent with antimycin A$_1$, A$_2$ A$_3$ and A$_4$ were only present in chemical extracts prepared from S. albus S4 wild-type and ∆fscRI harboring pIJ10257-fscRI strains but not the ∆fscRI mutant (Fig. 3). Taken together, we conclude that FscRI is required for production of antimycins and candidin by S. albus S4. Given the rather flexible and conserved binding site of PAS-LuxR regulators, it is conceivable that orthologs of FscRI could also cross-regulate 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 (ceph) and clavulanic acid (clav) gene clusters comprise a contiguous ‘super cluster’ (31). The biosynthesis of both cephamycin and clavulanic acid is co-ordinately controlled by CcaR, a SARP-type (Streptomyces antibiotic regulatory protein) activator harbored within the ceph gene cluster (32-34). It is interesting to note that not only are these gene clusters contiguous, but unlike antimycin
and candididin, both molecules are structurally similar and possess complementary biological activities
(i.e. 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. NRRL 2288 and heterologously produced antimycins using
*S. lividans* and *S. coelicolor* M145 (18). To our surprise, even though the *ant* gene clusters from NRRL
2288 and S4 share > 97% nucleotide identity (18), we have consistently been unable to repeat these
findings with both gene clusters using multiple genetic backgrounds, including *Streptomyces* sp. S3, *S.
lividans* 66 and *S. coelicolor* strains: M145, M1146, M1152 and M1153 (29, 35, 36, Seipke and
Hutchings, unpublished data). Previously, we presumed that poor availability of one or more
biosynthetic precursors (i.e. tryptophan, threonine, pyruvate and acyl-CoA’s) precluded production of
antimycins and/or that the pathway was simply not expressed by the heterologous hosts under our
growth conditions. But given our observations above, we hypothesized that *S. coelicolor* did not
produce antimycins due to 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 *S. coelicolor*
M1146 (36) and also introduced pIJ10257-*fscRI*. The resulting strains were then tested for their ability
to inhibit the growth of *C. albicans* by bioassay. Consistent with our hypothesis, M1146 harboring
solely Cosmid 213 or pIJ10257-*fscRI* did not inhibit the growth of *C. albicans*, however the co-
 integrant harboring both Cosmid 213 and pIJ10257-*fscRI* inhibited *C. albicans* growth (Fig. 4 and Fig
S1). We recapitulated this experiment with the *ant* gene cluster from NRRL 2288 using the pAL2602
cosmid clone generated by Yan *et al.* (18) and obtained identical results (Fig. S1). The dataset for
heterologous expression of the S4 *ant* gene cluster was corroborated by LC-HRESI-MS detection of
antimycin A₁, A₂, A₃ and A₄ in chemical extracts prepared from M1146 harboring Cosmid 213 and
pIJ10257-*fscRI* and their absence in M1146 alone or harboring only Cosmid 213 (Fig. 4). These results
demonstrate that FscRI is required by *S. coelicolor* for heterologous production of antimycins using
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 expression of antBA and antCDE.** The observation that FscRI is required for
heterologous production of antimycins by *S. coelicolor* suggests that it activates expression of both
antBA and antCDE. To evaluate this hypothesis, we first engineered Cosmid 213 such that antBA and
antCDE were expressed from 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 bioinformatics 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 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 mobilised to M1146 and the ability of the resulting strains
to produce antimycins was assessed by LC-HRESI-MS. As anticipated, antimycin A₁, A₂, A₃ and A₄
were only detected in chemical extracts prepared from M1146 harboring pIJ10257-fscRI and Cosmid
213 with either *ermEp2*-driven antBA or antCDE and not those generated from M1146 harboring just
pIJ10257 and Cosmid 213 with *ermEp2*-driven antBA or antCDE (Fig. 5). These data provide *in vivo*
evidence suggesting that FscRI may activate the expression of both antBA and antCDE and is
consistent with our hypothesis that FscRI acts on these promoters.

**FscRI directly activates transcription of antBA and antCDE.** The simplest interpretation of
our bioinformatics analyses and heterologous expression data is that FscRI activation of antBA and
antCDE is direct. We initially sought to verify this hypothesis by performing electrophoretic mobility
shift assays (EMSAs) using purified FscRI protein, however FscRI harboring either an N-terminal or
C-terminal hexa-histidine tag was insoluble when overproduced by *Escherichia coli* (data not shown),
which was surprising given than (His)₆-FscRIFR-008 was reportedly soluble (26). Nevertheless, we
adopted a chromatin immunoprecipitation (ChIP)-sequencing approach to determine if antB and antC promoters were bound by FscRI in vivo. We complemented the ∆fscRI mutant with an N-terminal 3xFLAG-tagged version of FscRI expressed from the ΦC31 integration site. The resulting strain (∆fscRI/pSETNFLAG-fscRI) inhibited the growth of C. albicans equal to that of the wild-type strain (Fig. S2). ChIP-sequencing was carried out with anti-FLAG antibodies and lysate from the ∆fscRI/pSETNFLAG-fscRI and wild-type strains cultivated in LB, which facilitates both antimycin and candicidin production. Immunoprecipitated DNA from two biological replicates of S4 wild-type and ∆fscRI/pSETNFLAG-fscRI as well as non-immunoprecipitated chromosomal DNA were sequenced using the Illumina HiSeq3000 platform and processed as described in the materials and methods. As we anticipated, the number of sequencing reads that mapped to the antB and antC promoter regions were enriched for both biological replicates of ∆fscRI/pSETNFLAG-fscRI compared to that of the wild-type mock-IP control (Fig. 6). These data provide definitive evidence that FscRI binds to antB and antC promoters and likely promotes 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, which were classified as short-form (S-form, 15 genes), intermediate-form (I-form, 16 genes) and long-form (L-form, 17 genes) (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. SM8, Streptomyces sp. NRRL2288, Streptomyces sp. LaPpAH-202, and Streptomyces sp. CNY228 (40). I-form ant gene clusters are encoded by two species, Streptomyces sp. 303MFCol5.2 and Streptomyces sp. TOR3209, which lack either antQ or antP, respectively. L-form ant gene clusters are encoded by six taxa, S. ambofaciens ATCC 23877, S. blastmyceticus NBRC 12747, S. gancidicus BKS 13-15, S. griseoflavus Tü4000, S. hygrosopicus subsp. jinggangensis 5008 and S. hygrosopicus subsp. jinggangensis TL01. In order 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 S. sp. NRRL2288 were omitted from this analysis because
their genome sequences are not available. A tblastn search using a local blast database and the deduced amino acid sequence of FscRI₄ revealed that organisms harboring an S-form ant gene cluster also harbor a FscRI ortholog (> 99% shared amino acid identity) whereas the top tblastn hits for taxa harboring either an I- or L-form ant gene cluster displayed a rather low shared amino acid identity (36-46%), with the exception of one organism, *Streptomyces* sp. TOR320, which possesses an ortholog of FscRI₄ (79% identical; Table S3). 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 anticipated *S. sp.* TOR320 to also harbor this motif given that it encodes what appears to be an FscRI ortholog (Fig. S4). Taken 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 expression of *antBA* and *antCDE* are unknown, however bioinformatics 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 β-lactamase 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 minimum inhibitory concentration (MIC) of antimycin (0.125 μg/ml) and candicidin (2 μg/ml) alone as well as the MIC for pairwise mixtures of these agents, which allowed us to determine the fractional inhibitory concentration (FIC) index (see methods). We calculated an FIC index = 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 could be that coordinate production of antimycin and candidicidin 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 candidicidin and other polyene antifungal agents relies upon altering sterol biosynthesis which incurs a significant fitness cost (43). It is also possible that coordinate production of these compounds may relate to the monomeric precursors utilized by each pathway. For instance, the candidicidin 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 may be alleviated by AntFGHIJKLMNO which utilize tryptophan to generate the 3-formamidosalicylate starter unit and may underpin the rationale for coordinate production of antimycin and candidicidin.

**Model for the regulation of antimycin biosynthesis.** Our model for the regulation of antimycin biosynthesis is depicted in Fig. 7. FscRI activates expression of *antBA* and *antCDE*, which in turn results in $\sigma^{\text{AntA}}$-mediated expression of *antGF* and *antHIJKLMNO* (12). FscRI does not activate its own production, however expression of *fscRI* is regulated by a positive feedback loop where FscRI activates FscRIV which in turn activates 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 down regulated upon the onset of morphological differentiation (12) suggests that the ligand(s) recognised by the FscRI PAS domain, and perhaps all PAS domains of polyene PAS-LuxR regulators, is only available during vegetative growth. Following inactivation of FscRI, the cell must have a strategy in place to prevent $\sigma^{\text{AntA}}$ from activating its targets. Indeed, in the absence of a cognate anti-sigma factor that would ordinarily perform this task, $\sigma^{\text{AntA}}$ seems to have evolved to be a direct substrate for the
As our understanding of the regulation of microbial natural product biosynthesis increase, we anticipate cross-regulation by cluster-situated regulatory proteins will emerge as a major strategy by which actinobacteria coordinate selected natural products.
Materials and methods

**Growth media, strains, cosmids, plasmids and other reagents.** *Escherichia coli* strains were propagated on Lennox agar (LA) or broth (LB) (35) and *Streptomyces* strains were cultivated using LA, LB and mannitol-soya flour agar (MSA) or broth (MSB) (35). Culture media 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-genera 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. All strains, cosmids and plasmids are described in Table S1 and all oligonucleotides and other synthetic DNAs are provided in Table S2.

**Construction of plasmids.** The insert for each plasmid generated in this study was prepared by PCR amplification using 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 using 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 description in Table S1.

**Design of the apramycin theophylline riboswitch cassette.** A λ 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 one end contained *ermEp2* (39) repressed by a theophylline-controlled riboswitch (47). The synthesized cassette also contains an optional hexa-histidine 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 and further details concerning its design, including its DNA sequence, is available from FigShare at https://dx.doi.org/10.6084/m9.figshare.3838032.v1.
**Construction of pUC19-promKanprom.** In order to construct pUC19-promKanprom, the neomycin/kanamycin resistance marker from Supercos1 was PCR amplified using RFS444 and RFS445, which 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: (1) RFS406 and RFS407 were used to PCR amplify the kanamycin resistance from pIJ773KnFRT, (2) RFS658 and RFS659 were used to PCR amplify the rpsL(XC) promoter from pCRISPomyces-2 (38, 49), (3) RFS667 and RFS668 were used to PCR amplify the *ermE* promoter from pSET152-*ermEp*, and (4) RFS663 and RFS664 were used to linearize pUC19. The resulting PCR products were restricted with DpnI, gel purified and assembled using the NEB HiFi DNA Assembly kit. The resulting plasmid, pUC19-promKanprom, contained a kanamycin resistance gene flanked by divergently firing *rpsL*(XC) and *ermE* promoters. A schematic of the promKanprom PCR template is shown in Fig. S5 and its DNA sequence is available at [http://www.ryanseipkelab.com/tools.html](http://www.ryanseipkelab.com/tools.html).

**Cosmid manipulations.** The AprTheo from above was used to replace the *antB* or *antC* promoters harbored on Cosmid 213 (12) using RFS413 and RFS414 (*antB*p), and RFS415 and RFS416 (*antC*p) and the ReDirect PCR targeting system and previously described (46). The apramycin resistance gene and oriT were removed from modified cosmids by the FLP recombinase as previously described (46) resulting in Cosmid 213-ABribo-FLP and Cosmid 213-CDEMribo-FLP. Cosmid 213-ABribo-FLP and Cosmid 213-CDEMribo-FLP were moved to *E. coli* GB05-red (48) and further engineered to harbor the ΦC31 integrase, *attP* site and apramycin resistance gene originating from pIJ10702 (also known as pMJCOS1) (50) using RecET recombineering as previously described (48) to result in Cosmid 213-ABribo-FLP-ΦC31 and Cosmid 213-CDEMribo-FLP-ΦC31. Cosmid213-ΦC31-BC-prom was constructed by replacing the *antB-antC* intragenic region of Cosmid 213-ΦC31 by recombineering with GB05-red and a PCR product generated using pUC19-promKanprom and oligonucleotides RFS654 and RFS657. Thus, the *ant* gene cluster harbored by this final ΦC31 integrative construct is entirely controlled by divergently firing *rpsL*(XC) and *ermE* promoters.
Deletion of *fscRI*. The *fscRI* gene was deleted using the pCRISPomyces-2 system described previously (49). First, a sgRNA protospacer was generated by annealing oligonucleotides RFS574 and RFS575, the resulting DNA fragment was cloned into the BbsI site of pCRISPomyces-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 RFS521 and RFS522 into pIJ12738 (51) followed by cloning a SpeI-KpnI restricted PCR fragment generated with RFS523 and RFS524. The resulting plasmid (pIJ12738-*fscRI*-UPDN) was used as a PCR template with RFS572 and RFS573, and the resulting PCR product was restricted with XbaI and cloned into pCRISPomyces-2 containing the protospacer targeting *fscRI*. The resulting CRISPR/Cas9 editing plasmid, pCRISPomyces-2-*fscRI*, was mobilised to S4 by conjugal transfer from *E. coli* ET12567/pUZ8002 as previously described (35). The temperature sensitive pCRISPomyces-2-*fscRI* was cured from a single apramycin-resistant transconjugant by passage in LB at 37 ºC (two rounds) prior to cultivation of a sporulated lawn on MS agar at 37 ºC. The resulting spores were serially diluted and 11 single colonies replica plated to assess apramycin sensitivity. Five apramycin-sensitive colonies were obtained and subsequently evaluated for the absence of *fscRI* by polymorphic shift PCR using RFS598 and RFS599. The integrity of the resulting Δ*fscRI* null mutant was verified by DNA sequencing.

**Chemical analysis.** *Streptomyces* strains were cultured in MS broth (50 ml in a 250 ml flask) whilst shaking (180 rpm) at 27 ºC for 7 days. MS 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 supernatant using 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 100% methanol followed by 1 ml deionised water. The column was then loaded with supernatant (10 ml in total) prior to a 1 ml wash with deionised water and a 2 ml wash with 30%
methanol. Metabolites were eluted from the SPE column in 100% methanol (0.3 ml). Equal amounts of methanolic extract for two independent replicates for each strain were mixed and centrifuged for 10 minutes at 16,000 x g just prior to injection in order to remove insoluble material. Only the supernatant (2 μl) was injected into a Bruker MaXis Impact TOF mass spectrometer and equipped with a Dionex Ultimate 3000 HPLC using the same parameters as described previously (15).

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

**Minimum inhibitory concentration (MIC) and fractional inhibition concentration index (FICI) determination.** MIC assays were performed in 96-well flat-bottom microtitration plates following the Clinical & Laboratory Standards Institute guidelines adapted for *C. albicans* (54). The FIC index was determined according to (55) by evaluating growth of *C. albicans* exposed to increasing pair-wise concentrations antimycin (0.0625 - 4 μg/ml) and candicidin (1 - 64 μg/ml). 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 (His)$_6$-FscRI and FscRI-(His)$_6$ were produced using *E. coli* Rosetta BL21(DE3) using pET28a-fscRI and pET30a-fscRI, respectively. Production of (His)$_6$-FscRI and FscRI-(His)$_6$ was induced in mid-log phase by addition of 1.5 mM isopropyl β-D-1-thiogalactopyranoside. After four hours of induction at 28 ºC, cells were harvested by centrifugation. The cell pellet was resuspended in 1x BugBuster Protein Extraction Reagent (Novagen) diluted with 50 mM Tris-Cl (pH 8.0), 200 mM NaCl, 20 mM imidazole and two units of DNase I and allowed to incubate at room temperature for 20 mins. Insoluble material was removed from the lysate by
centrifugation (16,000 x g for 10 mins). Soluble and insoluble fractions were visualised with InstantBlue (Expedeon) after having been subjected to 15% SDS-PAGE.

**ChIP-sequencing and bioinformatics analyses.** S4 WT or ΔfscRI/pSETNFLAG-fscRI were cultivated for two days in LB whilst shaking at 200 rpm at 28 °C and processed for ChIP-sequencing exactly as described previously using anti-FLAG M2 agarose beads (Sigma) (56), except that an Active Motif EpiShear sonicatior (30% amplitude, 30s on, 30s off for a total time of 13 minutes) was used to shear DNA to an average size of approximately 200-300. The pure DNA resulting from immunoprecipitates from two biological replicates of S4 WT and ΔfscRI/pSETNFLAG-fscRI as well as non-immunoprecipitated chromosomal DNA were sequenced using the Illumina HiSeq3000 platform with 150 nt paired-end reads by the University of Leeds Next Generation Sequencing Facility at St. James Teaching Hospital NHS Trust. The forward reads were mapped to the S4 genome using Bowtie 2 version 2.1.0 (57) and the resulting alignments were converted from .SAM to .BAM format and sorted according to chromosomal position using SAMtools version 1.1 (58). The aligned and sorted .BAM files for immunoprecipitated samples were converted to bigWig format and normalized by read count compared to the DNA only input control using the default settings of deepTools bamCompare version 2.3.3 (59) with the exception that the flag --ratio=subtract was used instead of the default --ratio=log2. This resulted in a single bigWig file for each treatment, which was visualised with the Integrated Genomics Viewer version 2.3.78 (60). Plots were generated using the deepTools programs computeMatrix and plotProfile (59) using a bin size of 50 and a custom .BED file specifying the region displayed.

**Data availability**

Next generation sequencing data is available under ArrayExpress accession E-MTAB-5122.

The DNA sequence of tools constructed during this study are [www.ryanseipkelab.com/tools.html](http://www.ryanseipkelab.com/tools.html).
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Figure Legends

FIG 1. Schematic representation of the antimycin biosynthetic gene cluster encoded by *Streptomyces albus* S4. Genes are color coded to indicate their function. AT, acyltransferase; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; CCR, crotonyl-CoA carboxylase/reductase. Antimycins:

- Antimycin A₁, $R^1 = \text{COCH(CH}_3\text{)CH}_2\text{CH}_3$, $R^2 = \text{(CH}_2\text{)}_4\text{CH}_3$;
- Antimycin A₂, $R^1 = \text{COCH(CH}_3\text{)}_2$, $R^2 = \text{(CH}_2\text{)}_4\text{CH}_3$;
- Antimycin A₃, $R^1 = \text{COCH}_2\text{CH(CH}_3\text{)}_2$, $R^2 = \text{(CH}_2\text{)}_2\text{CH}_3$;
- Antimycin A₄, $R^1 = \text{COCH}_2\text{CH}_2\text{CH}_3$.

FIG 2. FscRI binding sites within the *S. albus* S4 antimycin gene cluster. The upper panel shows experimentally verified FscRI binding sites upstream of genes within the candicidin biosynthetic gene cluster (*fscA*, *fscB1*, *fscB2*, *fscD* and *fscMI*) and putative FscRI binding sites upstream of *antB* and *antC* within the *ant* biosynthetic gene cluster. The middle panel displays the WebLogo (61) for 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*.

FIG 3. FscRI is required for the biosynthesis of antimycins by *S. albus* S4. (A) *S. albus* S4 strains challenged with *Candida albicans*. The ΔfscRI mutant does not show detectable bioactivity against *C. albicans* compared to the wild-type 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 antimycin A₁-A₄ are shown for each strain.

FIG 4. Heterologous production of antimycins by *Streptomyces 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...
(B) LC-HRESI-MS analysis of chemical extracts prepared from strains shown in panel (A); the extracted ion chromatograms [M+H]+ for antimycin A₁-A₄ are shown for each strain.

**FIG 5.** FscRI activates *antBA* and *antCDE* expression. LC-HRESI-MS analysis of chemical extracts prepared from *S. coelicolor* M1146 harboring variants of Cosmid213 engineered as described in the figure and pIJ10257 or pIJ10257-*fscRI* as indicated. The extracted ion chromatograms [M+H]+ for antimycin A₁-A₄ are shown for each strain.

**FIG 6.** 3xFLAG-FscRI binds to *antBA* and *antCDE* promoters in vivo. Graphical representation of normalised sequence reads mapped to the intergenic region of *antB-antC*, which is shown below. The double leftward slash denotes the sequence window presented does not contain the entire *antC* coding sequence. The genomic coordinates depicted are nucleotides 16873-20973 of contig CADY01000091.1 of the *S. albus* S4 genome (11).

**FIG 7.** Model for the regulation of antimycin biosynthesis. The upper panel displays the relative locations of the antimycin and candicidin gene clusters in the *S. albus* S4 chromosome. In the lower panel, FscRI activates transcription of *antBA* and *antCDE*, which results in production of the core AntC/AntD NRPS/PKS megasynthase and production of the discrete acyltransferase AntB and σ^AntA^, which in turn activates transcription the ketoreductase *antM* and nine genes (*antFGHIKLO*) 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.
**Supplementary material:**

**Table S1.** Bacterial strains, cosmids, fosmids and plasmids used in this study

**Table S2.** Oligonucleotide primers and other synthetic DNAs used in this study

**Table S3.** FscRI<sup>S1</sup> and putative orthologs encoded by antimycin producers

**FIG S1.** Bioactivity of *Streptomyces coelicolor* M1146 harboring pAL2602 is FscRI dependent. *S. coelicolor* M1146 harboring both pAL2602 and pIJ10257-<em>fscRI</em> antagonizes the growth of *Candida albicans* while M1146 harboring only pAL2602 or pIJ10257-<em>fscRI</em> does not.

**FIG S2.** Schematic of the pSETNFLAG-<em>fscRI</em> plasmid (left) and antifungal bioactivity of ∆<em>fscRI</em> expressing 3xFLAG-FscRI against *Candida albicans*. <em>aac(3)IV</em>, apramycin resistance cassette; <em>oriT</em>, origin of transfer; <em>attP</em>, ΦC31 attachment site. The Genbank files of pSETNFLAG and its parent, pSET152-<em>ermE</em>p are available at: http://www.ryanseipkelab.com/tools.html.

**FIG S3.** Clustal Ω alignment of the <em>antB-antC</em> intergenic region for S-form <em>ant</em> gene clusters. The putative start codons for <em>antB</em> (bold, red, reverse orientation) and <em>antC</em> (bold, blue forward orientation) and the three conserved FscRI binding sites are shaded grey.

**FIG S4.** Schematic of the theophylline riboswitch cassette AprTheo. P1, prime site 1; P2, prime site 2; P3, prime site 3; <em>aprR</em>, apramycin resistance; 6xHis, hexa-histidine affinity purification tag; the riboswitch is represented by a hairpin; FRT sites are for excision of the resistance marker by the Flp recombinase. Genbank file of the plasmid harboring this cassette is available at: http://www.ryanseipkelab.com/tools.html.
**FIG S5.** Schematic of the promKanprom cassette. P1, prime site (taggtctccgtctcactc) 1; P2, prime site (catatgggctctgttct); kanR, kanamycin resistance; FRT sites for excision of the resistance marker by the Flp recombinase. The Genbank file of the plasmid harboring this cassette is available at: http://www.ryanseipkelab.com/tools.html.
| Promoter | Sequence (5′–3′) |
|----------|------------------|
| *fscA*   | CTAGGGAAACACGGGG |
| *fscB1*  | CGAGGGGATTCACCAG |
| *fscB2*  | TTAGGGAAACCCGCAG |
| *fscD*   | CTAGGGATTTCAAAAAG |
| *fscRIV* | CTAAGGGATTCCCCCGG |
| *antB1*  | CTAGGGGTGATGCCAAG |
| *antB2*  | TTAGGTGAATCCCTAA |
| *antC*   | CTAGGTATTTCCTGGG |

![Diagram showing the promoter regions and sequence analysis](image-url)
\textit{rpsL(\textit{XC})p-antBA}  
\textit{ermE*p-antCDE}  

\textit{ermEp2-antBA}  
\textit{native-antCDE}  
\textit{pIJ10257}  

\textit{ermEp2-antCDE}  
\textit{native-antAB}  
\textit{pIJ10257}  

\textit{ermEp2-antBA}  
\textit{native-antCDE}  
\textit{pIJ10257-fscRI}  

\textit{ermEp2-antCDE}  
\textit{native-antAB}  
\textit{pIJ10257-fscRI}  

\textbf{Time (minutes)}
