Regulation of antimycin biosynthesis is controlled by the ClpXP protease

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Abstract

The survival of any microbe relies upon its ability to respond to environmental change. Use of Extra Cytoplasmic Function (ECF) RNA polymerase sigma (σ) factors is a major strategy enabling dynamic responses to extracellular signals. *Streptomyces* species harbor a large number of ECF σ factors; nearly all of which regulate genes required for morphological differentiation and/or response to environmental stress, except for σ^{AntA}, which regulates starter-unit biosynthesis in the production of antimycin, an anticancer compound. Unlike a canonical ECF σ factor, whose activity is regulated by a cognate anti-σ factor, σ^{AntA} is an orphan, raising intriguing questions about how its activity may be controlled. Here, we reconstitute *in vitro* ClpXP proteolysis of σ^{AntA}, but not a variant lacking a C-terminal di-alanine motif. Furthermore, we show that the abundance of σ^{AntA} *in vivo* is enhanced by removal of the ClpXP recognition sequence, and that levels of the protein rise when cellular ClpXP protease activity is abolished. These data establish direct proteolysis as an alternative and thus far unique control strategy for an ECF RNA polymerase σ factor and expands the paradigmatic understanding of microbial signal transduction regulation.

Importance

Natural products produced by *Streptomyces* species underpin many industrially- and medically-important compounds. However, the majority of the ~30 biosynthetic pathways harboured by an average species are not expressed in the laboratory. This undiscovered biochemical diversity is believed to comprise an untapped resource for natural products drug discovery. A major roadblock preventing the exploitation of unexpressed biosynthetic pathways is a lack of insight into their regulation and limited technology for activating their expression. Our findings reveal that the abundance of σ^{AntA}, which is the cluster-situated regulator of antimycin biosynthesis, is controlled by the ClpXP protease. These data link proteolysis to the regulation of natural product biosynthesis for the first time and we anticipate that this will emerge as a major strategy by which actinobacteria regulate production of their natural products. Further study of this process will advance understanding

43 of how expression of secondary metabolism is controlled and will aid pursuit of activating
44 unexpressed biosynthetic pathways.

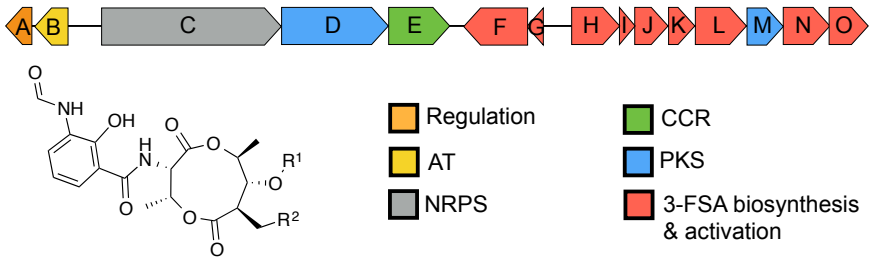
45 **Introduction**

46 The survival of any organism relies upon its ability to respond to environmental change. This
47 feature is especially true of bacteria, which often live in hostile and fluctuating environments.
48 *Streptomyces* bacteria thrive in soils. The success of this genus of filamentous, sporulating bacteria
49 is linked to their complex lifecycle and keen ability to sense and respond to its surroundings. Notably,
50 a multitude of bioactive secondary or specialized metabolites are produced in response to
51 environmental cues (1). More than half of all small molecule therapeutics critical for human health
52 and wellbeing are derived from or inspired by *Streptomyces* natural products (2).

53 *Streptomyces* species typically harbour a large number of biosynthetic pathways, but only a
54 few of them are expressed under common laboratory conditions. The biochemical diversity encoded
55 by these silent pathways is a tremendous untapped resource for discovery of new antibacterial agents
56 and other therapeutics. All available data indicates that the production of natural products is
57 controlled predominantly at the level of transcription. Although there are complex regulatory
58 cascades that tightly control expression of biosynthetic genes, they are ultimately activated, repressed
59 or de-repressed by so-called cluster-situated regulators—regulatory protein(s) encoded within the
60 biosynthetic gene cluster (BGC) (3, 4). Major roadblocks preventing the exploitation of silent
61 biosynthetic pathways are a lack of insight into their regulation and limited technology for activating
62 their expression.

63 Antimycins have been known for 70 years and are the founding member of a large class of
64 natural products widely produced by *Streptomyces* species (5-6). Recently, antimycins were shown
65 to be potent and selective inhibitors of the mitochondrial Bcl-2/Bcl-X_L-related antiapoptotic proteins
66 that are overproduced by cancer cells and confer resistance to chemotherapeutic agents whose mode
67 of action is activation of apoptosis (7). The ~25 kb antimycin (*ant*) BGC harboured by *S. albus* is
68 composed of 15 genes organised into four polycistronic operons: *antBA, antCDE*, *antGF* and
69 *antHIJKLMNO* (Fig. 1) (8, 9). The regulation of the *ant* BGC is unusual compared to other secondary
70 metabolites. Its expression is regulated by FscRI, a cluster-situated LuxR-family regulator of

71 candicidin biosynthesis; FscRI activates expression of *antBA* and *antCDE* (10). Importantly, *antA* is
72 a cluster-situated regulator that encodes an Extra Cytoplasmic Function (ECF) RNA polymerase σ
73 factor (σ^{AntA}) that activates expression of the remaining operons: *antGF* and *antHIJKLMNO* (Fig. 1)
74 (9).



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76 **Fig. 1.** Schematic representation of the antimycin (*ant*) biosynthetic gene cluster. AT,
77 acyltransferase; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; CCR, crotonyl-
78 CoA carboxylase/reductase, 3-FSA, 3-formamidosalicylate. Antimycins: Antimycin A_1, R^1=
79 $COCH(CH_3)CH_2CH_3$, R^2= $(CH_2)_4CH_3$; Antimycin A_2, R^1=$COCH(CH_3)_2$, R^2= $(CH_2)_4CH_3$; Antimycin
80 A_3, R^1= $COCH_2CH(CH_3)_2$, R^2= $(CH_2)_2CH_3$; Antimycin A_4, R^1= $COCH(CH_3)_2$, R^2= $(CH_2)_2CH_3$.
81

82 σ^{AntA}, like all ECF σ factors, is similar to the housekeeping σ^{70} family, but only possesses two
83 of the four highly characteristic sigma domains: domains σ2 and σ4; these regions of sigma factors
84 bind the -10 and -35 promoter elements respectively, and are sufficient for recruitment of RNA
85 polymerase (11). Genes encoding ECF σ factors are almost always co-transcribed with their cognate
86 anti-σ factor (12). This class of anti-σ factors are transmembrane proteins that selectively bind to and
87 inactivate a partner σ factor until its release is stimulated, usually by an exogenous signal (12, 13).
88 After the σ factor is released, it recruits RNA polymerase to express a defined regulon that usually
89 includes the σ factor-anti-σ factor operon itself, which thus establishes a positive auto-feedback loop
90 in the presence of the inducing stimulus. *Streptomyces* species encode a large number of ECF σ
91 factors (>30 per strain) and nearly all of these regulate genes required for morphological
92 differentiation and/or response to environmental stress and are not dedicated regulators of one
93 biosynthetic pathway. Interestingly, unlike the canonical ECF σ factors, whose activities are
94 controlled by cognate anti-σ factors, σ^{AntA} lacks an identifiable anti-σ factor partner and as a
95 consequence has created curiosity about how its activity is controlled.

96 The Clp-protease system is essential for normal bacterial proteostasis and is best characterized
97 in *Escherichia coli* (14, 15). The Clp protease is a multi-enzyme complex composed of a barrel-
98 shaped peptidase, ClpP and a regulatory enzyme, either ClpA or ClpX (or ClpC in some organisms).
99 ClpA and ClpX (and ClpC) are all AAA+-family protein unfoldases that recognise an N- and/or C-
100 terminal recognition signal (degron) and utilise ATP to unfold and translocate proteins to the
101 peptidase chamber where they are degraded into short peptides (16). In *Streptomyces* species, the
102 peptidase is specified by two genes instead of one and is redundantly encoded (17). The primary
103 peptidase is encoded by *clpP1P2*, whose corresponding proteins form a complex with ClpX or ClpA
104 to facilitate normal proteostasis; the second peptidase is encoded by *clpP3P4,* but its expression only
105 occurs when the primary system is compromised (18, 19). The best understood degron is the SsrA
106 tag from *E. coli* (AANDENYALAA), which is added co-translationally to polypeptides stalled on
107 ribosomes (20, 21). The *E. coli* SsrA tag has been comprehensively studied and the C-terminal Ala-
108 Ala-COO^- of this motif is essential for proteolysis by ClpXP (22). Intriguingly, the C-terminus of
109 σ^{AntA} harbours the sequence Ala-Ala-COO^-, which previously led us to speculate that ClpXP may
110 modulate its level/activity (9).

111 Here, we reconstitute ClpXP proteolysis of σ^{AntA} *in vitro* and show that it is dependent upon
112 the C-terminal Ala-Ala. We also show that the abundance of σ^{AntA} *in vivo* is higher when Ala-Ala is
113 changed to Asp-Asp and that abundance σ^{AntA} is elevated in the absence of genes encoding the
114 primary peptidase, ClpP and its unfoldase, ClpX. These data establish direct proteolysis as an
115 alternative, and thus far unique, control strategy of ECF RNA polymerase σ factors, expanding the
116 paradigmatic understanding of microbial signal transduction regulation.

117 **Results and discussion**

118 **σ^{AntA} orthologues are a new subfamily of ECF σ factor that regulate production of the**
119 **antimycin biosynthetic starter unit.** Since its initial discovery six years ago, more than 70 *ant* BGCs
120 have been identified within the Actinobacteria including *Actinospica*, *Saccharopolyspora*,
121 *Streptacidiphilus* and *Streptomyces* (5). Each of these BGCs harbours a single regulator, σ^{AntA} (53-
122 100% shared amino acid identity across all orthologues), which lacks a cognate anti-σ factor partner
123 (5, 9). Our previous work with *S. albus* S4 established that σ^{AntA} orthologues comprise a new
124 subfamily of ECF σ factors (9, 23). We demonstrated σ^{AntA} is required for expression of *antGF* and
125 *antHIJKLMNO*, which encode a standalone ketoreductase (AntM) and proteins required for the
126 production/activation of the starter unit, 3-formamidosalicylate (3-FSA) (Fig. 1). We also mapped
127 the transcriptional start sites and identified conserved promoter sequences for these operons in all
128 known antimycin BGCs at the time (9). The conservation of σ^{AntA} and target promoters within *ant*
129 BGCs from taxonomically diverse species, suggests that σ^{AntA}-mediated regulation of these genes is
130 direct. To verify this hypothesis, we performed ChIP-sequencing with a *S. albus* S4 Δ*antA* mutant
131 complemented with an N-terminal 3xFLAG-tagged version of σ^{AntA}. The number of reads that
132 mapped to the promoters of *antGF and antHIJKLMNO* was enriched for both biological replicates of
133 Δ*antA*/3xFLAG-*antA* compared to that of the wild-type mock-immunoprecipitated control, indicating
134 that σ^{AntA} presumably directly activates the production of the 3-FSA starter unit during antimycin
135 biosynthesis (Fig. 2).

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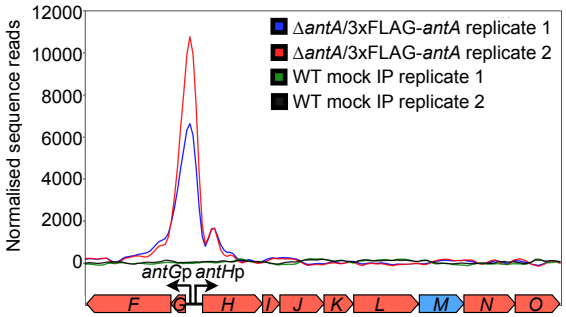


137 **Fig. 2. 3xFLAG-σ^{AntA} binds to the *antGF* and *antHIJKLMNO* promoters *in vivo*.** Shown is a
138 graphical representation of normalised sequence reads mapped to the intergenic region of *antG-antH*
139 (shown at bottom). The genomic coordinates depicted are nucleotides 43,148 to 51,448 of contig
140 CADY01000091.1 of the *S. albus* S4 genome[49]. WT, wild-type; IP, immunoprecipitation.

141 **σ^{AntA} is degraded by the ClpXP protease *in vitro.*** The activities of almost all characterized
142 ECF σ factors are modulated by a cognate anti-σ factor, which is typically a small transmembrane
143 protein co-encoded within the same operon. Intriguingly, σ^{AntA} lacks an anti-σ factor and is therefore
144 an orphan, indicating a unique mechanism is likely at work to control σ^{AntA} activity. An inspection of
145 σ^{AntA} amino acid sequences revealed a C-terminal Ala-Ala in 67 out of the 71 orthologues (Fig. S1).
146 A C-terminal Ala-Ala is an important component of a common class of degrons for the ClpXP
147 protease (22). This observation led us to hypothesize that the activity of σ^{AntA} could be modulated by
148 proteolysis instead of by an anti-σ factor. To test this hypothesis, we performed *in vitro* proteolysis.
149 Previous work indicated that *S. albus* S4 σ^{AntA} was insoluble when overproduced by *E. coli*, so we
150 pursued the overproduction and purification of the orthologue from *Streptomyces ambofaciens* ATCC
151 23877, which is an experimentally demonstrated producer of antimycins (24). *S. ambofaciens* σ^{AntA}
152 (75% shared amino acid identity with *S. albus* S4 σ^{AntA}) was purified as an N-terminal $(His)_6$-SUMO-
153 fusion protein. The $(His)_6$-SUMO tag increases solubility and eases purification of putative substrates,
154 without altering recognition of C-terminal degrons by ClpXP. ClpX orthologues from *E. coli* and *S.*
155 *ambofaciens* possess 60% shared amino acid identity and therefore likely recognise similar substrates
156 for degradation. Thus, ClpXP from *E. coli* was purified (Fig. S2) and its ability to degrade $(His)_6$-
157 SUMO-σ^{AntA} was assessed. Degradation of $(His)_6$-SUMO-σ^{AntA} was apparent as early as 2.5 min after
158 addition of ATP and all of the sample was degraded by 15 min (Fig. 3). Substrates of ClpXP become
159 resistant to proteolysis by specific alterations of the C-terminal Ala-Ala (22). Therefore, to investigate
160 degradation specificity in the above experiment we constructed and tested a variant of *S. ambofaciens*
161 σ^{AntA} in which the C-terminal Ala-Ala was mutated to Asp-Asp ($(His)_6$-SUMO-$\sigma^{AntA\text{-}DD}$). Strikingly,
162 the Asp-Asp variant was stable against ClpXP degradation over the lifetime of the assay (Fig. 3).
163 Thus, the degradation of $(His)_6$-SUMO-σ^{AntA} and the characteristic resistance afforded by the Ala-
164 Ala-to-Asp-Asp mutation demonstrates that σ^{AntA} is a substrate of ClpXP *in vitro*.

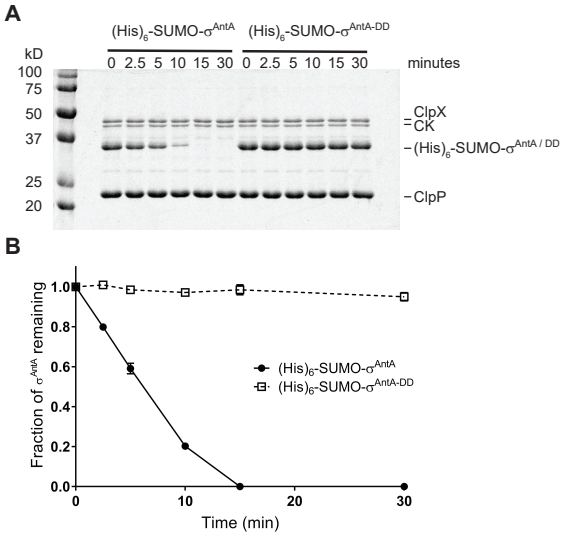
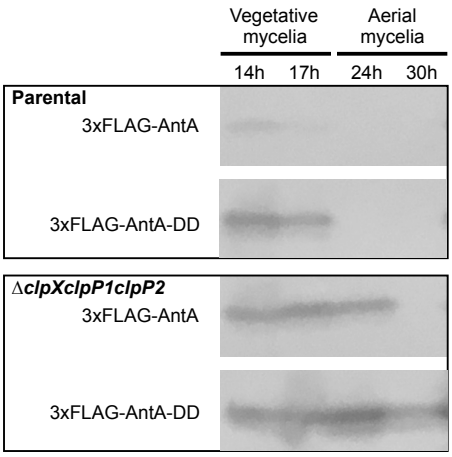


Fig. 3. Proteolysis of *S. ambofaciens* σ^{AntA} by ClpXP in vitro. (A) SDS-PAGE analysis of proteolysis reactions containing 37 pmols $(His)_6$SUMO-σ^{AntA} or $(His)_6$SUMO-$\sigma^{AntA\text{-}DD}$. (B) Densitometry analysis SDS-PAGE images for three independent proteolysis experiments. The mean is plotted and error bars illustrate the standard error of the mean (±1 SEM).

σ^{AntA} is degraded by the ClpXP protease *in vivo.* To investigate if the *in vitro* degradation of σ^{AntA} demonstrated above is relevant to its regulation *in vivo*, we deleted the operon encoding the *clpX, clpP1, and clpP2* genes from *S. albus* S4. The resulting Δ*clpXclpP1clpP2* mutant underwent a normal developmental cycle, albeit sporulation was less robust, which is consistent with growth characteristics reported for mutation of equivalent genes in *S. coelicolor* (Fig. S3) (25). Next, genes encoding the 3xFLAG-σ^{AntA} or 3xFLAG-$\sigma^{AntA\text{-}DD}$ fusion proteins were generated and introduced into the parental strain and the Δ*clpXclpP1clpP2* mutant so the abundance of these proteins could be assessed over a developmental time course by Western blotting with anti-FLAG antisera. This experiment was initially performed with the σ^{AntA} fusions integrated on the chromosome under control of the native promoter. However, a reliable signal could not be detected for 3xFLAG-σ^{AntA} and only a trace amount of the Asp-Asp variant was observed, presumably indicating that the cellular level of σ^{AntA} is normally low because the native promoter is relatively weak. The experiment was therefore repeated with 3xFLAG-σ^{AntA} and 3xFLAG-$\sigma^{AntA\text{-}DD}$ expression driven by a stronger, constitutive promoter, *ermE** (26). Analysis of the resulting immunoblot revealed that 3xFLAG-$\sigma^{AntA\text{-}DD}$ was more abundant than 3xFLAG-σ^{AntA} in extracts prepared from vegetative mycelia of the

186 parent and Δ*clpXclpP1clpP2* strains (Fig. 4). Strikingly, 3xFLAG-σ^{AntA} and 3xFLAG-$\sigma^{AntA\text{-}DD}$ could
187 only be detected in extracts from aerial mycelia of the Δ*clpXclpP1clpP2* strain and not the parent; the
188 Asp-Asp variant was also present in greater relative abundance (Fig. 4), which was consistent with
189 our previous experiments that showed the *ant* BGC is downregulated at the level of transcription upon
190 the onset of aerial growth (9). Interestingly, the conspicuous absence of 3xFLAG-σ^{AntA} and the
191 presence 3xFLAG-$\sigma^{AntA\text{-}DD}$ in protein extracts prepared from the latest time point sampled suggests
192 the potential involvement of an additional degradative factor(s). Taken together, these data support
193 the hypothesis that σ^{AntA} levels, and thus its ability to activate gene expression of *antFGHIJKLMNO*
194 is modulated by the ClpXP protease.



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196 **Fig. 4. The abundance of σ^{AntA} is enhanced in the absence of the ClpXP *in vivo.*** Cells from the
197 indicated strains were cultivated over a developmental time course atop cellophane discs on agar
198 media. Protein extracts were generated from 100 mg of either vegetative mycelia (14 and 17 hours)
199 or aerial mycelia (24 and 30 hours). Thirty micrograms of total protein were analysed by Western
200 blotting with anti-FLAG antisera. The images shown are derived from uncropped original images
201 shown in Fig. S4.

202 **Antimycins are not overproduced in the absence of ClpXP.** The above experiments
203 indicate that the cellular level of σ^{AntA} is more abundant in the absence of the ClpXP protease. In
204 order to determine if an increased level of this transcription factor ultimately influenced the final
205 production titre of antimycins, we used LC-HRMS to assess the abundance of antimycins in chemical
206 extracts generated from the Δ*clpXclpP1clpP2* and parental strains grown atop a cellophane disk on
207 MS agar in triplicate. The extracted ion chromatograms representing antimycin A_1, A_2 A_3 and A_4
208 were used to determine the peak area for each compound, which was subsequently normalised based
209 on the wet mycelial weight of the sample. Interestingly, the results indicated that total antimycin
210 production by the Δ*clpXclpP1clpP2* mutant (15.57 AU ± 2.86) and parental strain (16.59 AU ± 1.12)
211 was not statistically significantly different (*P* value 0.59) Table S1. This result is consistent with a
212 previous experiment where overexpression of *antA* did not increase the titre of antimycins, because
213 it only results in overexpression of *antGF* and *antHIJKLMNO* (genes encoding the production of the
214 AntG-*S*-3-formamidosalicylate starter unit) and not the remaining genes (*antABCDE*) in the BGC
215 (9). This also presumably indicates that starter unit biosynthesis is not rate limiting for antimycin
216 production.

217 **Model for the regulation of antimycin biosynthesis.** Our model for the regulation of
218 antimycin biosynthesis is depicted in Fig. 6. Expression of the *ant* BGC is cross-activated by FscRI,
219 a LuxR-family regulator, from the candicidin BGC, which activates expression of *antBA* and *antCDE*
220 (10). This regulation in turn enables direct activation of the 3-FSA biosynthetic operons (*antGF* and
221 *antHIJKLMNO*) by σ^{AntA}. The expression of *antBA* and *antCDE* is down regulated following the
222 onset of morphological differentiation, presumably because the ligand sensed by the FscRI PAS
223 domain is no longer available (9-10). The cellular level of σ^{AntA} is antagonised by the ClpXP protease,
224 for which it is a direct target, and is ultimately responsible for clearing residual σ^{AntA} when FscRI is
225 inactivated following the onset of morphological differentiation (10). While ClpXP proteolytic
226 control of transcription factor activity, and in particular that of ECF σ factor / anti- σ factors, has been
227 shown previously (27, 28), (29-32); (33, 34); (35) it has thus far not been directly linked to the control

228 of cluster-situated regulators of natural product biosynthesis. This finding provides a new lens
229 through which to examine microbial signal transduction and the regulation of natural product
230 biosynthesis in *Streptomyces* species. Understanding the diversity of regulatory strategies controlling
231 the expression of these pathways is critical for the development of new tools for exploiting the 'silent
232 majority' of biosynthetic pathways harbored by these organisms.

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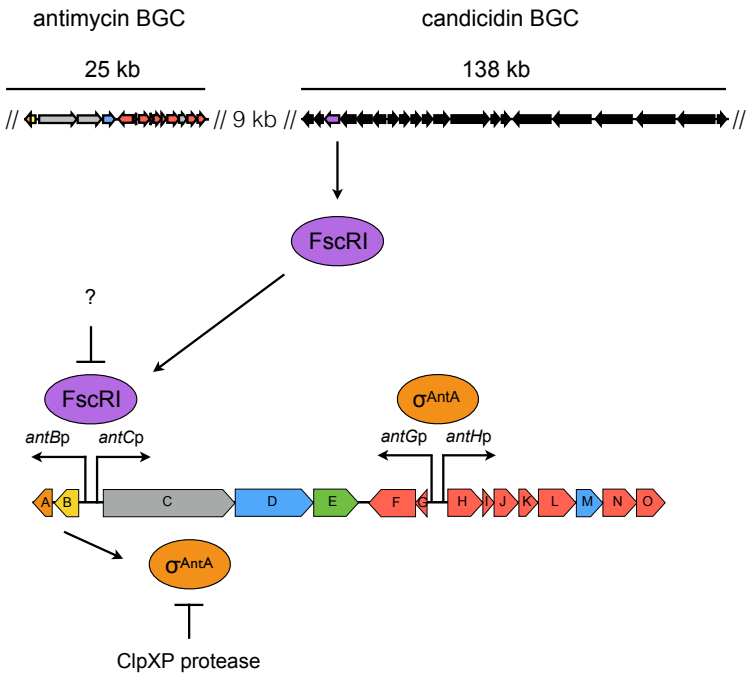

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235 **Fig. 5. Model for the regulation of antimycin biosynthesis.** The upper panel displays the relative
236 locations of the antimycin and candicidin BGCs in the *S. albus* S4 chromosome. In the lower panel,
237 FscRI, a LuxR-family regulator, from the candicidin BGC, activates expression of *antBA* and
238 *antCDE*. This in turn enables direct activation of the 3-FSA biosynthetic operons (*antGF* and
239 *antHIJKLMNO*) by σ^{AntA}. The cellular level of σ^{AntA} is antagonised by ClpXP-protease system, for
240 which it is a direct target and is ultimately responsible for clearing residual σ^{AntA} when FscRI is
241 inactivated following the onset of differentiation.

242 **Experimental procedures**

243 **Growth media, strains, cosmids, plasmids, and other reagents.** *Escherichia coli* strains
244 were propagated on Lennox agar (LA) or broth (LB) (36, 37) and *Streptomyces albus* S4 strains were
245 cultivated using LA, LB, and mannitol-soya flour (MS) agar or broth (36). Development of *clp*
246 mutants was assessed on MS and ISP2 medium (36). Culture medium was supplemented with
247 antibiotics as required at the following concentrations: apramycin, 50 μg ml^{-1}; carbenicillin, 100 μg
248 ml^{-1}; chloramphenicol, 25 μg ml^{-1}; hygromycin, 50 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; nalidixic acid, 25
249 μg ml^{-1}. *Streptomyces* strains were constructed by conjugal mating with *E. coli* ET12567 as
250 previously described (36). Enzymes were purchased from New England Biolabs unless otherwise
251 stated, and oligonucleotides were purchased from Integrated DNA Technologies, Inc. All of the
252 strains, cosmids, and plasmids used in this study are described in Table S2, and all of the
253 oligonucleotides used are provided in Table S3.

254 **Construction of plasmids.** The insert for each plasmid generated in this study was prepared
255 by PCR amplification with Q5 High-Fidelity DNA polymerase and oligonucleotides containing
256 restriction sites. PCR-amplified inserts were restricted and cloned into the relevant plasmids cut with
257 the same enzymes by standard molecular biology procedures. All clones were sequenced to verify
258 the integrity of insert DNA. The restriction sites used for cloning are provided with the plasmid
259 descriptions in Table S2.

260 **ChIP-sequencing and bioinformatics analyses**. The *antA* coding sequence was amplified
261 with RFS629 and RFS630, which contain KpnI and EcoRI restriction sites, respectively. The
262 restricted PCR product was cloned into pSETNFLAG (10) digested with the same enzymes. The
263 resulting plasmid was then restricted with NotI and EcoRI to release *ermE**p-3xFLAG-*antA*, which
264 was subsequently cloned into pAU3-45 (38) digested with the same enzymes. pAU3-45-3xFLAG-
265 *antA* was mobilised to an apramycin-marked Δ*antA* strain (9). Cultivation of the wild-type and
266 Δ*antA*/pAUNFLAG-*antA* strains for ChIP-sequencing were performed exactly as described
267 previously (10). The pure DNA resulting from immunoprecipitates from two biological replicates of

268 wild-type and Δ*antA*/pAUNFLAG-*antA*, as well non-immunoprecipitated chromosomal DNA, were
269 sequenced with the Illumina HiSeq3000 platform with 150-nucleotide paired-end reads by the
270 University of Leeds Next Generation Sequencing Facility at the St. James Teaching Hospital NHS
271 Trust. The resulting reads were analysed exactly as described previously (10). The graphic in Fig. 2
272 was generated using DeepTools computeMatrix and plotProfile functions (39).

273 **Construction of the *S. albus* S4 Δ*clpXclpP1clpP2* mutant strain.** Deletion of
274 *clpXclpP1clpP2* was carried out using RecET recombineering in *E. coli* as follows. The
275 *clpXclpP1clpP2*-containing cosmid, cos117 was obtained by screening a previously constructed *S.*
276 *albus* S4 Supercos1 cosmid library (8) by PCR using oligonucleotides PBB001 and PBB002. Cos117
277 was mutagenized as required using *E. coli* recombineering with strain GB05-red (40) and a deletion
278 cassette. The deletion cassette was generated by PCR from paac-apr-oriT (41) and consisted of the
279 apramycin resistance gene, *aac(3)IV* and a conjugal origin of transfer (*oriT*), which was flanked by
280 ΦC31-*attL* and -*attR* sites for excision of the cassette. Oligonucleotides used to generate deletion
281 cassettes included 39 nt of homology upstream or downstream of the target open reading frame(s)
282 and are listed in Table S3. The resulting PCR product was digested with DpnI, gel purified and
283 electroporated into arabinose-induced *E. coli* GB05-red harboring cos117. Transformants were
284 screened for the presence of mutagenized cosmid by PCR using oligonucleotides listed in Table S3
285 and the integrity of the locus was verified by DNA sequencing. The mutagenized cosmid was
286 electroporated into *E. coli* ET12567/pUZ8002 and mobilized to a strain of *S. albus* S4 harboring an
287 entire antimycin BGC deletion (Δantall) (42) by conjugation as described (36). Transconjugants were
288 screened for apramycin resistance and kanamycin sensitivity. The integrity of an apramycin-marked
289 mutant was verified by PCR using the oligonucleotides listed in Table S3. The apramycin deletion
290 cassette was subsequently excised from the chromosome by conjugal introduction of pUWLint31,
291 which is a replicative plasmid with a temperature sensitive origin of replication that expresses the
292 ΦC31 integrase required for removal of the cassette (41). Transconjugants were screened for loss of
293 apramycin resistance and excision of the cassette was verified by polymorphic shift PCR and DNA

294 sequencing of the product.

295 **Immunoblot analysis.** Spores of the parental strain, *S. albus* Δantall and Δ*clpXclpP1clpP2*
296 mutant harboring pPDA or pPDD were grown on MS agar (buffered with 50mM TES, pH 7.2)
297 covered with cellophane discs. Protein extracts were prepared from mycelia collected at regular
298 intervals during growth: 14h, 17h, 24h and 30h for Δantall and Δ*clpXclpP1clpP2* harboring 3xFLAG-
299 AntA constructs; 17h, 20h, 23h and 30h for Δantall and Δ*clpXclpP1clpP2* harboring the 3xFLAG-
300 FscRI construct. Protein extracts were generated as follows: 100 mg of cells were resuspended in 200
301 µl lysis buffer (50 mM sodium phosphate buffer, pH 7.0, 150 mM sodium chloride, 10 mg ml^{-1}
302 lysozyme, cOmplete, Mini, EDTA-free protease inhibitors (Roche) and 100 mg of 0.1 mm glass beads
303 (PowerLyzer®)) and lysed by vortexing for 30 min at 2000 pm, 37°C, with a subsequent incubation
304 for another 30 min at 37°C. The obtained suspension was centrifuged for 20 min at 20,000 x *g* at
305 18°C. Thirty micrograms of the clarified protein extract were subjected to SDS-PAGE and then
306 transferred to nitrocellulose membrane (pore size 0.2 µm) for Western blot analysis. The membrane
307 was probed with mouse monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody (Sigma), 1:10
308 000, and the signals were detected using Pierce™ 1-Step Ultra TMB Blotting Solution (Thermo
309 Scientific).

310 **Protein purification and *in vitro* ClpXP proteolysis assays.** The wild-type *antA* gene was
311 PCR amplified and cloned into the AgeI and HindIII sites of the pET23b-SUMO vector, which
312 harbors an N-terminal $(His)_6$-SUMO tag (Wang *et al.* 2007). The plasmid for production of $(His)_6$-
313 SUMO-$\sigma^{AntA\text{-}DD}$ was generated by site-directed mutagenesis (Agilent QuikChange) using primers
314 listed in Table S3. $(His)_6$-SUMO-σ^{AntA} and $(His)_6$-SUMO-$\sigma^{AntA\text{-}DD}$ were produced by *E. coli*
315 Rosetta(DE3) (Novagen) grown in LB at 37 °C until OD_{600} 0.5, followed by induction with 0.4 mM
316 IPTG and growth at 18 °C for 16 hours. Cells were resuspended in 50 mM sodium phosphate, pH 8,
317 1M NaCl, 20 mM imidazole, 10% glycerol, and 1 mM DTT and lysed by french press at 28 kpsi,
318 followed by treatment with protease inhibitor cocktail set III, EDTA-free (Calbiochem) and
319 benzonase (Millipore Sigma). $(His)_6$-SUMO-σ^{AntA} and $(His)_6$-SUMO-$\sigma^{AntA\text{-}DD}$ proteins were purified

320 by Ni-NTA affinity chromatography and Superdex-75 gel filtration and stored in 50 mM potassium
321 phosphate, pH 6.8, 850 mM KCl, 10% glycerol, and 1 mM DTT. *E. coli* ClpX and ClpP proteins
322 were purified as described previously (43, 44).

323 *In vitro* ClpXP proteolysis assays were performed at 30 °C by preincubating 0.3 μM $ClpX_6$
324 and 0.8 μM $ClpP_{14}$ with ATP regeneration system (4 mM ATP, 50 μg ml^{-1} creatine kinase, 5 mM
325 creatine phosphate) in 25 mM HEPES-KOH, pH 7.5, 20 mM KCl, 5 mM MgCl2, 10% glycerol,
326 0.032% NP40, and 0.2 mM DTT and adding substrate to initiate the reactions. Samples of each
327 reaction were taken at specific time points and stopped by addition of SDS-PAGE loading dye and
328 boiling at 100 °C before loading on Tris-Glycine-SDS gels. Bands were visualized by staining with
329 colloidal Coomassie G-250 and quantified by ImageQuant (GE Healthcare) after scanning by
330 Typhoon FLA 9500 (GE Healthcare). The fraction $(His)_6$-SUMO-σ^{AntA} remaining was calculated by
331 dividing the $(His)_6$-SUMO-σ^{AntA} density at a given time point by the density at time zero and
332 normalized by ClpX density.

333 **Chemical analysis.** *S. albus* S4 strains were cultivated atop a cellophane disc on MS agar at
334 30 °C for 7 days in triplicate. At the time of harvest, the cellophane disc containing mycelia was
335 removed and the quantity of biomass was determined. Bacterial metabolites were extracted from both
336 the mycelia and the 'spent' agar for 1 hr using 50 ml of ethyl acetate. Thirty millilitres of ethyl acetate
337 were evaporated to dryness under reduced pressure and the resulting residue was resuspended in
338 100% methanol (300 μl). Immediately prior to LC-HRMS analysis, methanolic extracts were
339 centrifuged at 16,000 × *g* in a microcentrifuge tube for 5 min to remove insoluble material. Only the
340 supernatant (3 μl) was injected into a Bruker Maxis Impact TOF mass spectrometer equipped with a
341 Dionext Ultimate 3000 HPLC as previously described (45). The peak area associated with the
342 extracted ion chromatograms for antimycin A_1, A_2, A_3 and A_4 present in agar and mycelial extracts
343 was determined and used to calculate the total antimycins produced for each replicate. These values
344 were subsequently used to determine the arithmetic mean for total antimycin production for each

345 strain. Statistical significance was assessed in MS Excel by a homoscedastic Student's t-test with a
346 two-tailed distribution.

347 **Data availability**

348 The next-generation sequencing data obtained in this study are available under
349 ArrayExpress accessions E-MTAB-7700 and E-MTAB-5122.

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359 **Author contributions**

360 BB, SK and AF performed experiments, interpreted data and wrote sections of the manuscript;
361 TAB wrote sections of the manuscript; RFS designed the study, performed experiments and wrote
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