

1 **Direct proteolytic control of an extracytoplasmic function RNA polymerase sigma factor**

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13 **Keywords:** *Streptomyces*; regulation of secondary metabolism; antimycin; ECF sigma factors;

14 **proteolysis; ClpXP**

15 **Abstract**

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

29

30 **Importance**

31 Most antibiotics are derived from secondary metabolites produced by *Streptomyces* species.
32 The recent rise in the number of bacterial infections resistant to antibiotics has led to renewed interest
33 in discovery of new secondary metabolites produced by these microbes. An average species of
34 *Streptomyces* harbours ~30 biosynthetic pathways, but the majority of them are not in the laboratory.
35 A key approach is therefore activation of these “silent” pathways, but new insights into how their
36 expression is regulated are required. Our findings reveal that the ECF σ factor (σ^{AntA}) that regulates
37 antimycin biosynthesis lacks an anti- σ partner and instead is controlled by the Clp-protease system.
38 These data establish direct proteolysis as a novel strategy for the control of ECF RNA polymerase σ
39 factors and will aid the pursue of silent biosynthetic pathways.

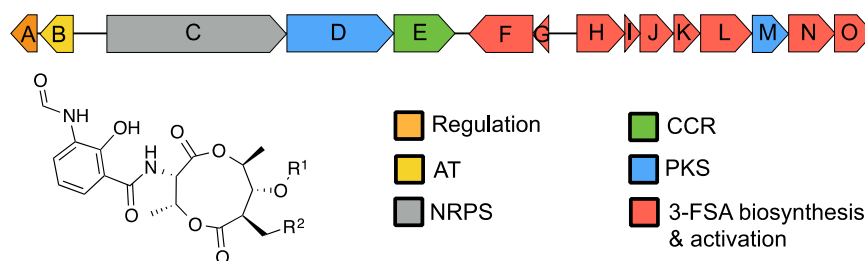
40 **Introduction**

41 The survival of any organism relies upon its ability to respond to environmental change. This
42 feature is especially true of bacteria, which often live in hostile and fluctuating environments.
43 *Streptomyces* bacteria thrive in soils. The success of this genus of filamentous, sporulating bacteria
44 is linked to their complex lifecycle and keen ability to sense and respond to its surroundings. Notably,
45 a multitude of bioactive secondary or specialised metabolites are produced in response to
46 environmental cues¹. More than half of all small molecule therapeutics critical for human health and
47 wellbeing are derived from or inspired by *Streptomyces* natural products².

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

58 Antimycins have been known for 70 years and are the founding member of a large class of
59 natural products widely produced by *Streptomyces* species^{5,6}. Recently, antimycins were shown to be
60 potent and selective inhibitors of the mitochondrial Bcl-2/Bcl-X_L-related antiapoptotic proteins that
61 are overproduced by cancer cells and confer resistance to chemotherapeutic agents whose mode of
62 action is activation of apoptosis⁷. The ~25 kb antimycin (*ant*) BGC harboured by *S. albus* is composed
63 of 15 genes organised into four polycistronic operons *antAB*, *antCDE*, *antFG* and *antHIJKLMNO*
64 (Fig. 1)^{8,9}. The regulation of this *ant* BGC is unusual compared to other secondary metabolites. Its
65 expression is regulated by FscRI, a cluster-situated LuxR-family regulator of candicidin biosynthesis;

66 FscRI activates expression of *antAB* and *antCDE*¹⁰. Importantly, *antA* is a cluster-situated regulator
 67 that encodes an Extra Cytoplasmic Function (ECF) RNA polymerase σ factor (σ^{AntA}) that activates
 68 expression of the remaining operons: *antGF* and *antHIJKLMNO* (Fig. 1)⁹.



69

70 **Fig. 1** | Schematic representation of the antimycin (*ant*) biosynthetic gene cluster. AT,
 71 acyltransferase; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; CCR, crotonyl-
 72 CoA carboxylase/reductase, 3-FSA, 3-formamidosalicylate. Antimycins: Antimycin A₁, R¹=
 73 COCH(CH₃)CH₂CH₃, R²=(CH₂)₄CH₃; Antimycin A₂, R¹=COCH(CH₃)₂, R²=(CH₂)₄CH₃; Antimycin
 74 A₃, R¹=COCH₂CH(CH₃)₂, R²=(CH₂)₂CH₃; Antimycin A₄, R¹=COCH(CH₃)₂, R²=(CH₂)₂CH₃.

75

76

77 σ^{AntA} , like all ECF σ factors, is similar to the housekeeping σ^{70} family, but only possesses two
 78 of the four highly characteristic sigma domains: domains σ_2 and σ_4 ; these regions of sigma bind the
 79 -10 and -35 promoter elements, respectively and are sufficient for recruitment of RNA polymerase¹¹.
 80 Genes encoding ECF σ factors are almost always co-transcribed with their cognate anti- σ factor¹².
 81 This class of anti- σ factors are transmembrane proteins that selectively bind to and inactivate a partner
 82 σ factor until its release is stimulated, usually by an exogenous signal^{12,13}. After the σ factor is released
 83 it recruits RNA polymerase to express a defined regulon that usually includes the σ factor-anti- σ
 84 factor operon itself, which thus establishes a positive auto-feedback loop in the presence of the
 85 inducing stimulus. *Streptomyces* species encode a large number of ECF σ factors (>30 per strain) and
 86 nearly all of these regulate genes required for morphological differentiation and/or response to
 87 environmental stress and, in contrast to σ^{AntA} , are not dedicated regulators of one biosynthetic
 88 pathway⁹. In addition, unlike the canonical ECF σ factors, whose activities are controlled by cognate
 89 anti- σ factors, σ^{AntA} appears to be an “orphan”, lacking such a regulatory partner protein and thus has
 90 created curiosity about how its activity is controlled.

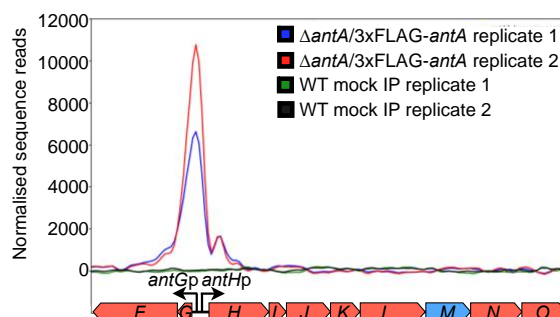
91 The Clp-protease system is essential for normal bacterial proteostasis and is best characterised
92 in *Escherichia coli*^{14,15}. The Clp protease is a multi-enzyme complex composed of a barrel-shaped
93 peptidase, ClpP and a regulatory enzyme, either ClpA or ClpX (or ClpC in some organisms). ClpA
94 and ClpX (and ClpC) are all AAA+-family protein unfoldases that recognise an N- and/or C-terminal
95 recognition signal (degron) and utilise ATP to unfold and translocate proteins to the peptidase
96 chamber where they are degraded into short peptides¹⁶. In *Streptomyces* species, the peptidase is
97 specified by two genes instead of one and is redundantly encoded¹⁷. The primary peptidase is encoded
98 by *clpPIP2*, whose corresponding proteins form a complex with ClpX or ClpA to facilitate normal
99 proteostasis; the second peptidase is encoded by *clpP3P4*, but its expression only occurs when the
100 primary system is compromised^{18,19}. The best understood degron is the SsrA tag from *E. coli*
101 (AANDENYALAA), which is added co-translationally to polypeptides stalled on ribosomes^{20,21}. The
102 *E. coli* SsrA tag has been comprehensively studied and the C-terminal Ala-Ala-COO⁻ of this motif is
103 essential for proteolysis by ClpXP²². Intriguingly, the C-terminus of σ^{AntA} harbours the sequence Ala-
104 Ala-COO⁻, which previously led us to speculate that ClpXP may modulate its level/activity⁹.

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

111 Results

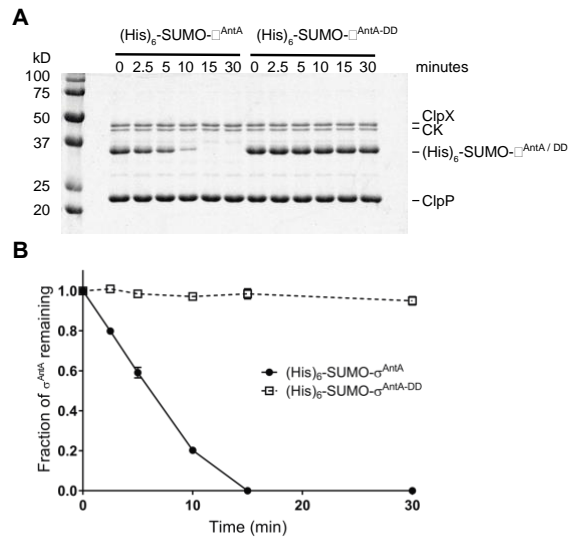
112 **σ^{AntA} orthologues are a new subfamily of ECF σ factor that regulate production of the**
113 **antimycin biosynthetic starter unit.** Since its initial discovery six years ago, more than 70 *ant* BGCs
114 have been identified within actinomycete genera, including in *Actinobacteria*, *Actinospica*,
115 *Saccharopolyspora*, *Streptacidiphilus* and *Streptomyces*⁵. Each of these BGCs harbours a single
116 regulator, σ^{AntA} (53-100% shared amino acid identity across all orthologues), which lacks a cognate
117 anti- σ factor partner^{5,9}. Our previous work with *S. albus* S4 established that σ^{AntA} orthologues
118 comprise a new subfamily of ECF σ factors^{9,23}. We demonstrated σ^{AntA} is required for expression of
119 *antFG* and *antHIJKLMNO*, which encode a standalone ketoreductase (AntM) and proteins required
120 for the production/activation of the starter unit, 3-formamidosalicylate (3-FSA) (Fig. 1). We also
121 mapped the transcriptional start sites and identified conserved promoter sequences for these operons
122 in all known antimycin BGCs at the time⁹. The conservation of σ^{AntA} and target promoters within *ant*
123 BGCs from taxonomically diverse species, suggests that σ^{AntA} -mediated regulation of these genes is
124 direct. To verify this hypothesis, we performed ChIP-seq with a *S. albus* S4 $\Delta antA$ mutant
125 complemented with an N-terminal 3xFLAG-tagged version of σ^{AntA} . The number of reads that
126 mapped to the promoters of *antGF* and *antHIJKLMNO* was enriched for both biological replicates of
127 $\Delta antA/3x\text{FLAG-}antA$ compared to that of the wild-type mock-immunoprecipitated control, indicating
128 that σ^{AntA} directly activates the production of the 3-FSA starter unit during antimycin biosynthesis
129 (Fig. 2).

130



131 **Fig. 2 | 3xFLAG- σ^{AntA} binds to the *antGF* and *antHIJKLMNO* promoters *in vivo*.** Shown is a
132 graphical representation of normalised sequence reads mapped to the intergenic region of *antG-antH*
133 (shown at bottom). The genomic coordinates depicted are nucleotides 43,148 to 51,448 of contig
134 CADY01000091.1 of the *S. albus* S4 genome⁴⁹. WT, wild-type; IP, immunoprecipitation.

135
136 **σ^{AntA} is degraded by the ClpXP protease *in vitro*.** The activity of almost all characterised
137 ECF σ factors are modulated by a cognate anti- σ factor, which is typically a small transmembrane
138 protein co-encoded within the same operon, so the absence of an anti- σ factor partner to control σ^{AntA}
139 is particularly intriguing, and makes σ^{AntA} be considered an orphan regulatory protein. An inspection
140 of σ^{AntA} amino acid sequences revealed a C-terminal Ala-Ala in 67 out of the 71 orthologues
141 (Supplementary Fig. 1). A C-terminal Ala-Ala is an important component of a common class of
142 degrons for the ClpXP protease²². This observation led us to hypothesise that the activity of σ^{AntA}
143 could be modulated by proteolysis instead of by an anti- σ factor. To test this hypothesis, we
144 performed *in vitro* proteolysis. Previous work indicated that *S. albus* S4 σ^{AntA} was insoluble when
145 overproduced by *E. coli*, so we pursued the overproduction and purification of the orthologue from
146 *Streptomyces ambofaciens* ATCC 23877, which is an experimentally demonstrated producer of
147 antimycins²⁴. *S. ambofaciens* σ^{AntA} (75% shared amino acid identity with *S. albus* S4 σ^{AntA}) was
148 purified as an N-terminal (His)₆-SUMO-fusion protein. The (His)₆-SUMO tag increases solubility
149 and eases purification of putative substrates, without altering recognition of C-terminal degrons by
150 ClpXP. ClpX orthologues from *E. coli* and *S. ambofaciens* possess 60% shared amino acid identity
151 and therefore likely recognise similar substrates for degradation. Thus, ClpXP from *E. coli* was
152 purified and its ability to degrade (His)₆-SUMO- σ^{AntA} was assessed. Degradation of (His)₆-SUMO-
153 σ^{AntA} was apparent as early as 2.5 min after addition of ATP and all of the sample was degraded by
154 15 min (Fig. 3). Substrates of ClpXP become resistant to proteolysis by specific alterations of the C-
155 terminal Ala-Ala²². Therefore, to investigate degradation specificity in the above experiment we
156 constructed and tested a variant of *S. ambofaciens* σ^{AntA} in which the C-terminal Ala-Ala was mutated
157 to Asp-Asp ((His)₆-SUMO- $\sigma^{\text{AntA-DD}}$). Strikingly, the Asp-Asp variant was stable against ClpXP
158 degradation over the lifetime of the assay (Fig. 3). Thus, the degradation of (His)₆-SUMO- σ^{AntA} and
159 the characteristic resistance afforded by the Ala-Ala-to-Asp-Asp mutation demonstrates that σ^{AntA} is
160 a direct substrate of ClpXP *in vitro*.



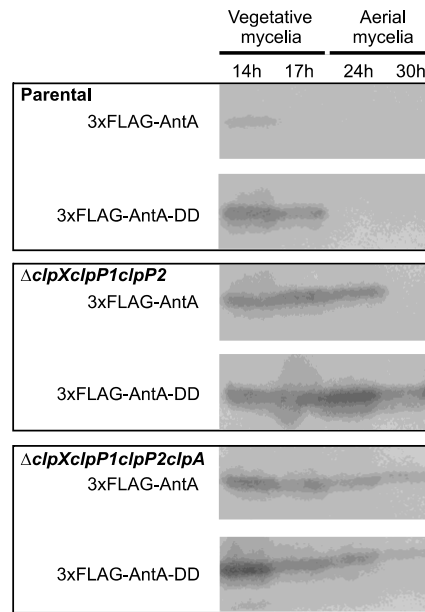
161

162 **Fig. 3 | Proteolysis of *S. ambifaciens* σ^{AntA} by ClpXP *in vitro*.** (A) SDS-PAGE analysis of
 163 proteolysis reactions containing 37 pmols (His)₆SUMO- σ^{AntA} or (His)₆SUMO- $\sigma^{\text{AntA-DD}}$. (B)
 164 Densitometry analysis SDS-PAGE images for three independent proteolysis experiments. The mean
 165 is plotted and error bars illustrate the standard error of the mean (± 1 SEM).
 166

167 **σ^{AntA} is degraded by ClpXP protease *in vivo*.** To investigate if the *in vitro* degradation σ^{AntA}
 168 demonstrated above is relevant to its regulation *in vivo* we adopted a genetic strategy to assess the
 169 abundance of σ^{AntA} in mutant strains with defects in Clp-proteolysis. First, we deleted the *clpX*, *clpP1*,
 170 and *clpP2* genes from *S. albus* S4. The resulting mutant underwent a normal developmental cycle,
 171 albeit sporulation was less robust on ISP2 and MS medium (Supplemental Fig. 3). Next genes
 172 encoding the 3xFLAG- σ^{AntA} or 3xFLAG- $\sigma^{\text{AntA-DD}}$ fusion proteins were generated and introduced into
 173 the parental strain and the $\Delta clpXclpP1clpP2$ mutant so the abundance of these proteins could be
 174 assessed over a developmental time course by Western blotting with anti-FLAG antisera. This
 175 experiment was initially performed with the σ^{AntA} fusions integrated on the chromosome under
 176 control of the native protein. However, a reliable signal could not be detected for 3xFLAG- σ^{AntA} and
 177 only a trace amount of the Asp-Asp variant was observed, presumably indicating that the cellular
 178 level of σ^{AntA} is normally low because the native promoter is relatively weak. The experiment was
 179 therefore repeated with 3xFLAG- σ^{AntA} and 3xFLAG- $\sigma^{\text{AntA-DD}}$ expression driven by a stronger,
 180 constitutive promoter, *ermE**²⁵. Analysis of the resulting immunoblot revealed that 3xFLAG- $\sigma^{\text{AntA-DD}}$
 181 ^{DD} was more abundant than 3xFLAG- σ^{AntA} in extracts prepared from vegetative mycelia (14h and

182 17h) of the parent and $\Delta clpXclpP1clpP2$ strains (Fig. 4). Strikingly, 3xFLAG- σ^{AntA} and 3xFLAG-
183 $\sigma^{\text{AntA-DD}}$ could only be detected in extracts from aerial mycelia (24h and 30h) of the $\Delta clpXclpP1clpP2$
184 strain and not the parent; the Asp-Asp variant was also present in greater relative abundance (Fig. 4).
185 These data support the hypothesis that σ^{AntA} levels, and thus its ability to activate gene expression is
186 modulated by the ClpXP protease, however the conspicuous absence of 3xFLAG- σ^{AntA} and the
187 presence 3xFLAG- $\sigma^{\text{AntA-DD}}$ in protein extracts prepared from the latest time point suggests the
188 involvement of degradative factor(s) in addition to ClpXP.

189 **σ^{AntA} is degraded by ClpAP protease *in vivo*.** Taken together, the data presented above
190 establishes that ClpXP likely acts degrades σ^{AntA} *in vivo*, but also suggested the existence of other
191 factor(s) that affect σ^{AntA} levels, especially later in the morphological development cycle. ClpA is an
192 alternative targeting protein that forms a proteolytic complex with ClpP capable of degrading SsrA-
193 tagged proteins²¹. Indeed, an overlap in proteins comprising the ClpAP and ClpXP degradomes has
194 been observed for *E. coli*²⁶. Thus, we hypothesised that ClpAP may also be able to degrade σ^{AntA} . We
195 therefore generated a $\Delta clpXclpP1clpP2clpA$ mutant and re-assessed the abundance of the 3xFLAG-
196 σ^{AntA} and 3xFLAG- $\sigma^{\text{AntA-DD}}$ by immunoblotting as above. Analysis of the resulting immunoblot
197 revealed that 3xFLAG- σ^{AntA} and 3xFLAG- $\sigma^{\text{AntA-DD}}$ were present in equal relative abundance within
198 $\Delta clpXclpP1clpP2$ and $\Delta clpXclpP1clpP2clpA$ lysate prepared after 14, 17 and 24hrs of growth (Fig.
199 4). Strikingly, 3xFLAG- σ^{AntA} was observed in lysate prepared after 30hrs of incubation only for the
200 $\Delta clpXclpP1clpP2clpA$ strain. Taken together, these *in vivo* data indicate that σ^{AntA} is degraded by both
201 the ClpXP and ClpAP proteases.



202
203 **Fig. 4 | The abundance of σ^{AntA} is enhanced in the absence of the Clp protease *in vivo*.** Cells from
204 the indicated strains were cultivated over a developmental time course on agar media. Protein extracts
205 were generated from 100mg of either vegetative mycelia (14 and 17 hours) or aerial mycelia (24 and
206 30hours) was harvested and lysed. Thirty micrograms of total protein were analysed by Western
207 blotting with anti-FLAG antisera. The images shown are derived from uncropped original images
208 shown in Supplementary Fig. 4.

209 Discussion

210 ECF σ factors are a major component of bacterial signal transduction, are typically involved
211 in responding to external stimuli and their activity is canonically understood to be controlled via a
212 cognate anti- σ factor protein; the anti- σ is usually membrane bound and almost always encoded at
213 the same locus¹². In this study, we characterised *in vitro* and *in vivo*, an ECF σ factor named σ^{AntA}
214 that does not possess any identifiable anti- σ factor partner and as a consequence has evolved a
215 different mechanism of regulation.

216 We established that σ^{AntA} is a cluster-situated regulator of antimycin biosynthesis and showed
217 by CHIP-sequencing that it directly binds upstream of genes required for 3-FSA production. Although
218 abundant within *Streptomyces* species, the activity of ECF σ factors that have been characterised are
219 involved in responding to environmental stress and/or regulating morphological differentiation. To
220 our knowledge, σ^{AntA} is the only ECF σ factor that is a cluster-situated regulator in the genus
221 *Streptomyces*. Indeed, cluster-situated ECF σ factors have only thus far been observed within BGCs
222 for lantibiotics produced by so-called rare actinomycetes and these are controlled by anti- σ factors.
223 In *Microbospora corallina*, MibR and σ^{MibX} regulate microbisporicin biosynthesis and σ^{MibX} is
224 controlled by the anti- σ factor, MibW²⁷; in *Planomonospora alba*, PspR and σ^{PspX} regulate
225 planosporicin production and σ^{PspX} is controlled by the anti- σ factor, PspW²⁸.

226 The C-terminal Ala-Ala present within σ^{AntA} orthologues served as a clue that instead of an
227 anti- σ factor that ClpXP may regulate σ^{AntA} activity. We unambiguously demonstrated that ClpXP
228 degraded σ^{AntA} *in vitro*, but not an altered σ^{AntA} variant in which Ala-Ala was changed to Asp-Asp.
229 We also assessed the level of σ^{AntA} *in vivo* and showed that it was more abundant within vegetative
230 mycelia than in aerial mycelia and was partially stabilised by the Asp-Asp mutation, which was
231 consistent with our previous experiments that showed the *ant* BGC is downregulated at the level of
232 transcription upon the onset of aerial growth⁹. We demonstrated that the abundance of σ^{AntA} and the
233 Asp-Asp variant was higher *in vivo* in a $\Delta clpXclpP1clpP2$ mutant strain and further so when *clpA*
234 (orthologous to SCO7532 (*clpC2*)) was deleted. It was surprising that the Asp-Asp mutation did not

235 fully protect σ^{AntA} from proteolysis *in vivo*, however enhanced abundance of $\sigma^{\text{AntA-DD}}$ in
236 $\Delta\text{clpXclpP1clpP2}$ and $\Delta\text{clpXclpP1clpP2clpA}$ genetic backgrounds relative to the parent strain is
237 consistent with previous studies indicating N-terminal and internal motifs can also be important for
238 substrate recognition by Clp-proteases^{26,29}. However, involvement of another protease, such as Lon,
239 in the degradation σ^{AntA} cannot be excluded.

240 Direct ClpXP or ClpAP proteolysis of an ECF σ factor, as shown here, has not been reported
241 previously. However, it has been linked to ECF σ factors in the past, where proteolysis of σ^{S} in *E.*
242 *coli* and σ^{T} in *S. coelicolor* occurs via their association with an adapter protein or peptide,
243 respectively^{30,31}. In addition, ClpXP proteolysis of the anti- σ factors RseA and RsiW enables
244 expression the σ^{E} and σ^{W} regulons in *E. coli* and *Bacillus subtilis*, respectively³²⁻³⁵. ClpXP has also
245 been linked to the turnover of other transcription factor families. For instance, the λ repressor-like
246 proteins (InterPro ID=IPR010982) PopR and its paralogue ClgR, which participate in a feedback loop
247 regulating expression of the *clp* genes in *S. lividans*^{36,37}, and the global oxygen-sensing regulator,
248 FNR in *E. coli*³⁸.

249 Expression of the *ant* BGC is atypical compared to other BGCs in that it is expressed during
250 vegetative growth, but downregulated upon the onset of aerial growth. Its expression is cross-
251 activated by FscRI, a LuxR-family regulator, from the candicidin BGC, which activates expression
252 of *antBA* and *antCDE*¹⁰. This regulation in turn enables direct activation of the 3-FSA biosynthetic
253 operons (*antGF* and *antHIJKLMNO*) by σ^{AntA} . The cellular level of σ^{AntA} is antagonised by the Clp-
254 protease system, for which it is a direct target and is ultimately responsible for clearing residual σ^{AntA}
255 when FscRI is inactivated following the onset of morphological differentiation¹⁰. The above model
256 (Supplementary Fig. 5) is intriguing and begs the question why is it important for σ^{AntA} to be actively
257 cleared from cell? One possibility is that aberrant/excess production of 3-FSA is cytotoxic, however
258 previous experiments in which the *antA* gene was artificially overexpressed did not adversely impact
259 growth of the organism⁹. An alternative hypothesis for why σ^{AntA} must be rapidly removed from the
260 cell is to prevent unnecessary consumption of L-Trp. Biosynthesis of L-Trp is biologically expensive

261 and it is the most chemically complex and least abundant of the 20 common proteinogenic amino
262 acids³⁹. It is tempting to speculate that the evolutionary rationale underpinning this regulatory strategy
263 is owed to the cell needing to dedicate more of this amino acid to production of proteins or metabolites
264 involved in development. This is consistent with recent data showing that deletion of *trpM*, which
265 controls precursor availability for L-Trp biosynthesis in *S. coelicolor* and presumably all
266 streptomycetes, fails to undergo normal morphological development⁴⁰.

267 In conclusion, here we establish direct proteolysis by the Clp-protease system as an alternative
268 control strategy for ECF σ factors, which provides a new lens through which to examine microbial
269 signal transduction and the regulation of natural product biosynthesis in *Streptomyces* species.
270 Understanding the diversity of regulatory strategies controlling the expression of these pathways is
271 critical for the development of new tools for exploiting the ‘silent majority’ of biosynthetic pathways
272 harboured by these organisms.

273 **Materials and methods**

274 **Growth media, strains, cosmids, plasmids, and other reagents.** *Escherichia coli* strains
275 were propagated on Lennox agar (LA) or broth (LB)^{41,42} and *Streptomyces albus* S4 strains were
276 cultivated using LA, LB, and mannitol-soya flour (MS) agar or broth⁴¹. Development of *clp* mutants
277 was assessed on MS and ISP2 medium⁴¹. Culture medium was supplemented with antibiotics as
278 required at the following concentrations: apramycin, 50 µg/ml; carbenicillin, 100 µg/ml;
279 chloramphenicol, 25 µg/ml; hygromycin, 50 µg/ml; kanamycin, 50 µg/ml; nalidixic acid, 25 µg/ml.
280 *Streptomyces* strains were constructed by conjugal mating with *E. coli* ET12567 as previously
281 described⁴¹. Enzymes were purchased from New England BioLabs unless otherwise stated, and
282 oligonucleotides were purchased from Integrated DNA Technologies, Inc. All of the strains, cosmids,
283 and plasmids used in this study are described in Supplementary Table 1, and all of the
284 oligonucleotides used are provided in Supplementary Table 2.

285 **Construction of plasmids.** The insert for each plasmid generated in this study was prepared
286 by PCR amplification with Q5 High-Fidelity DNA polymerase and oligonucleotides containing
287 restriction sites. PCR-amplified inserts were restricted and cloned into the relevant plasmids cut with
288 the same enzymes by standard molecular biology procedures. All clones were sequenced to verify
289 the integrity of insert DNA. The restriction sites used for cloning are provided with the plasmid
290 descriptions in Supplementary Table 1.

291 **ChIP-sequencing and bioinformatics analyses.** The *antA* coding sequence was amplified
292 with RFS629 and RFS630, which contain KpnI and EcoRI restriction sites, respectively. The
293 restricted PCR product was cloned into pSETNFLAG digested with the same enzymes. The resulting
294 plasmid was then restricted with NotI and EcoRI to release *ermE**p-3xFLAG-*antA*, which was
295 subsequently cloned into pAU3-45 digested with the same enzymes. pAU3-45-3xFLAG-*antA* was
296 mobilised to an apramycin-marked $\Delta antA$ strain⁹. Cultivation of the wild-type and
297 $\Delta antA$ /pAUNFLAG-*antA* strains for ChIP-sequencing were performed exactly as described
298 previously¹⁰. The pure DNA resulting from immunoprecipitates from two biological replicates of

299 wild-type and $\Delta antA$ /pAUNFLAG-*antA*, as well non-immunoprecipitated chromosomal DNA, were
300 sequenced with the Illumina HiSeq3000 platform with 150-nucleotide paired-end reads by the
301 University of Leeds Next Generation Sequencing Facility at the St. James Teaching Hospital NHS
302 Trust. The resulting reads were analysed exactly as described previously¹⁰. The graphic in Figure 2
303 was generated using DeepTools computeMatrix and plotProfile functions⁴³.

304 **Construction of *S. albus* S4 *clp* mutant strains.** All deletions were performed by
305 mutagenising cosmids using RecET recombineering in *E. coli* followed by their subsequent
306 mobilisation to *S. albus* strains via conjugal transfer. The *clpXclpP1clpP2*-containing cosmid, cos117
307 and *clpA*-containing cosmid, cos251 were obtained by screening a previously constructed *S. albus* S4
308 Supercos1 cosmid library⁸ by PCR using oligonucleotides PBB001 and PBB002 (*clpX*) and PBB067
309 and PBB068 (*clpA*). Cos117 and cos251 were mutagenised as required using *E. coli* recombineering
310 with strain GB05-red⁴⁴ and a deletion cassette. Deletion cassettes were generated by PCR from *paac*-
311 *apr-oriT*⁴⁵ and consisted of the apramycin resistance gene, *aac(3)IV* and a conjugal origin of transfer
312 (*oriT*), which was flanked by Φ C31-*attL* and -*attR* sites for excision of the cassette. Oligonucleotides
313 used to generate deletion cassettes included 39 nt of homology upstream or downstream of the target
314 open reading frame(s) and are listed in Supplementary Table 2. The resulting PCR products were
315 digested with DpnI, gel purified and electroporated into arabinose-induced *E. coli* GB05-red
316 harbouring cos117 or cos251. Transformants were screened for the presence of mutagenised cosmid
317 by PCR using oligonucleotides listed in Supplementary Table 2 and the integrity of the locus was
318 verified by DNA sequencing. Mutagenised cosmids were electroporated into *E. coli*
319 ET12567/pUZ8002 and mobilised to a strain of *S. albus* S4 harbouring an entire antimycin BGC
320 deletion (Δant) by conjugation as described⁴¹. Transconjugants were screened for apramycin
321 resistance and kanamycin sensitivity. The integrity of apramycin-marked mutants was verified by
322 PCR using the oligonucleotides listed in Supplementary Table 2. The apramycin deletion cassette
323 was subsequently excised from the chromosome by conjugal introduction of pUWLint31, which is a
324 replicative plasmid with a temperature sensitive origin of replication that expresses the Φ C31

325 integrase required for removal of the cassette⁴⁵. Transconjugants were screened for loss of apramycin
326 resistance and excision of the cassette was verified by polymorphic shift PCR and DNA sequencing
327 of the product.

328 **Immunoblot analysis.** Spores of parental strain, *S. albus* Δ antall, Δ clpXclpP1clpP2 and
329 Δ clpXclpP1clpP2clpA mutants carrying pPDA or pPDD were grown on SFM agar (buffered with
330 50mM TES, pH 7.2) covered with cellophane discs. Protein extracts were prepared from mycelia
331 collected at regular intervals during growth (14h, 17h, 24h and 30h) as follows: 100 mg of cells were
332 resuspended in 200 μ l lysis buffer (50 mM sodium phosphate buffer, pH 7.0, 150 mM sodium
333 chloride, 10 mg/ml lysozyme, cOmplete, Mini, EDTA-free protease inhibitors (Roche) and 100 mg
334 of 0.1 mm glass beads (PowerLyzer®)) and lysed by vortexing for 30 min at 2000 rpm, 37°C, with a
335 subsequent incubation for another 30 min at 37°C. The obtained suspension was centrifuged for 20
336 min at 20,000g at 18°C. Thirty micrograms of the clarified protein extract were subjected to SDS-
337 PAGE and then transferred to nitrocellulose membrane (pore size 0.2 μ m) for Western blot analysis.
338 The membrane was probed with mouse monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody
339 (Sigma), 1:10 000, and the signals were detected using Pierce™ 1-Step Ultra TMB Blotting Solution
340 (Thermo Scientific).

341 **Protein purification and *in vitro* ClpXP proteolysis assays.** The wild-type *antA* gene was
342 PCR amplified and cloned into the AgeI and HindIII sites of the pET23b-SUMO vector, which
343 harbours an N-terminal (His)₆-SUMO tag⁴⁶. The plasmid for production of (His)₆-SUMO- $\sigma^{\text{AntA-DD}}$
344 was generated by site-directed mutagenesis (Agilent QuikChange) using primers listed in
345 Supplementary Table 2. (His)₆-SUMO- σ^{AntA} and (His)₆-SUMO- $\sigma^{\text{AntA-DD}}$ were produced by *E. coli*
346 Rosetta(DE3) (Novagen) grown in LB at 37 °C until OD₆₀₀ 0.5, followed by induction with 0.4 mM
347 IPTG and growth at 18 °C for 16 hours. Cells were resuspended in 50 mM sodium phosphate, pH 8,
348 1M NaCl, 20 mM imidazole, 10% glycerol, and 1 mM DTT and lysed by french press at 28 kpsi,
349 followed by treatment with protease inhibitor cocktail set III, EDTA-free (Calbiochem) and
350 benzonase (Millipore Sigma). (His)₆-SUMO- σ^{AntA} and (His)₆-SUMO- $\sigma^{\text{AntA-DD}}$ proteins were purified

351 by Ni-NTA affinity chromatography and Superdex-75 gel filtration and stored in 50 mM potassium
352 phosphate, pH 6.8, 850 mM KCl, 10% glycerol, and 1 mM DTT. *E. coli* ClpX and ClpP proteins
353 were purified as described previously^{46,47}.

354 *In vitro* ClpXP proteolysis assays were performed at 30 °C by preincubating 0.3 μM ClpX₆
355 and 0.8 μM ClpP₁₄ with ATP regeneration system (4 mM ATP, 50 μg/mL creatine kinase, 5 mM
356 creatine phosphate) in 25 mM HEPES-KOH, pH 7.5, 20 mM KCl, 5 mM MgCl₂, 10% glycerol,
357 0.032% NP40, and 0.2 mM DTT and adding substrate to initiate the reactions. Samples of each
358 reaction were taken at specific time points and stopped by addition of SDS-PAGE loading dye and
359 boiling at 100 °C before loading on Tris-Glycine-SDS gels. Bands were visualized by staining with
360 colloidal Coomassie G-250 and quantified by ImageQuant (GE Healthcare) after scanning by
361 Typhoon FLA 9500 (GE Healthcare). The fraction (His)₆-SUMO-σ^{AntA} remaining was calculated by
362 dividing the (His)₆-SUMO-σ^{AntA} density at a given time point by the density at time zero and
363 normalized by ClpX density.

364 **Data availability**

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

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