

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
18 enabling such signal transduction. *Streptomyces* species harbour a large number of ECF σ factors;
19 nearly all of which regulate genes required for morphological differentiation and/or response to
20 environmental stress, except for σ^{AntA} , which regulates starter-unit biosynthesis in the production of
21 antimycin, an anticancer compound. Unlike a canonical ECF σ factor, whose activity is regulated by
22 a cognate anti- σ factor, σ^{AntA} is an orphan, raising intriguing questions about how its activity may
23 be controlled. Here, we reconstitute *in vitro* ClpXP proteolysis of σ^{AntA} , but not a variant lacking a
24 C-terminal di-alanine motif. Furthermore, we show that the abundance of σ^{AntA} *in vivo* is enhanced
25 by removal of the ClpXP recognition sequence, and that levels of the protein rise when cellular
26 ClpP-protease activity is abolished. These data establish direct proteolysis as an alternative and thus
27 far unique control strategy for an ECF RNA polymerase σ factor and expands the paradigmatic
28 understanding 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
33 interest in discovery of new secondary metabolites produced by these microbes. An average species
34 of *Streptomyces* harbours ~30 biosynthetic pathways, but the majority of them are not in the
35 laboratory. A key approach is therefore activation of these “silent” pathways, but new insights into
36 how their expression is regulated are required. Our findings reveal that the ECF σ factor (σ^{AntA}) that
37 regulates antimycin biosynthesis lacks an anti- σ partner and instead is controlled by the Clp-
38 protease system. These data establish direct proteolysis as a novel strategy for the control of ECF
39 RNA polymerase σ factors and will aid the pursue of silent biosynthetic pathways.

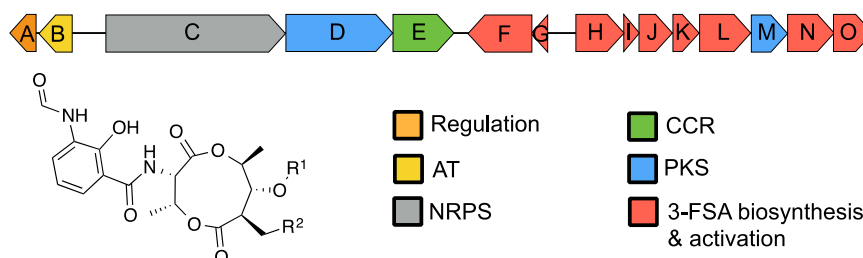
40 **Introduction**

41 The survival of any organism relies upon its ability to respond to environmental change.
42 This 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.
45 Notably, 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
51 agents 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,
54 repressed or de-repressed by so-called cluster-situated regulators—regulatory protein(s) encoded
55 within the 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
57 activating 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
60 be potent and selective inhibitors of the mitochondrial Bcl-2/Bcl-X_L-related antiapoptotic proteins
61 that are overproduced by cancer cells and confer resistance to chemotherapeutic agents whose mode
62 of action is activation of apoptosis⁷. The ~25 kb antimycin (*ant*) BGC harboured by *S. albus* is
63 composed of 15 genes organised into four polycistronic operons *antAB*, *antCDE*, *antFG* and
64 *antHIJKLMNO* (Fig. 1)^{8,9}. The regulation of this *ant* BGC is unusual compared to other secondary
65 metabolites. Its expression is regulated by FscRI, a cluster-situated LuxR-family regulator of

66 candidin biosynthesis; FscRI activates expression of *antAB* and *antCDE*¹⁰. Importantly, *antA* is a
67 cluster-situated regulator that encodes an Extra Cytoplasmic Function (ECF) RNA polymerase σ
68 factor (σ^{AntA}) that activates expression of the remaining operons: *antGF* and *antHIJKLMNO* (Fig.
69 1)⁹.



70

71 **Fig. 1** | Schematic representation of the antimycin (*ant*) biosynthetic gene cluster. AT, acyltransferase; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; CCR, crotonyl-CoA carboxylase/reductase, 3-FSA, 3-formamidosalicylate. Antimycins: Antimycin A₁,
72 R¹= COCH(CH₃)CH₂CH₃, R²= (CH₂)₄CH₃; Antimycin A₂, R¹=COCH(CH₃)₂, R²= (CH₂)₄CH₃;
73 Antimycin A₃, R¹= COCH₂CH(CH₃)₂, R²= (CH₂)₂CH₃; Antimycin A₄, R¹= COCH(CH₃)₂, R²=
74 (CH₂)₂CH₃.
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79 σ^{AntA} , like all ECF σ factors, is similar to the housekeeping σ ⁷⁰ family, but only possesses
80 two of the four highly characteristic sigma domains: domains σ 2 and σ 4; these regions of sigma
81 bind the -10 and -35 promoter elements, respectively and are sufficient for recruitment of RNA
82 polymerase¹¹. Genes encoding ECF σ factors are almost always co-transcribed with their cognate
83 anti- σ factor¹². This class of anti- σ factors are transmembrane proteins that selectively bind to and
84 inactivate a partner σ factor until its release is stimulated, usually by an exogenous signal^{12,13}. After
85 the σ factor is released it recruits RNA polymerase to express a defined regulon that usually
86 includes the σ factor-anti- σ factor operon itself, which thus establishes a positive auto-feedback
87 loop in the presence of the inducing stimulus. *Streptomyces* species encode a large number of ECF
88 σ factors (>30 per strain) and nearly all of these regulate genes required for morphological
89 differentiation and/or response to environmental stress and, in contrast to σ^{AntA} , are not dedicated
90 regulators of one biosynthetic pathway⁹. In addition, unlike the canonical ECF σ factors, whose

91 activities are controlled by cognate anti- σ factors, σ^{AntA} appears to be an “orphan”, lacking such a
92 regulatory partner protein and thus has created curiosity about how its activity is controlled.

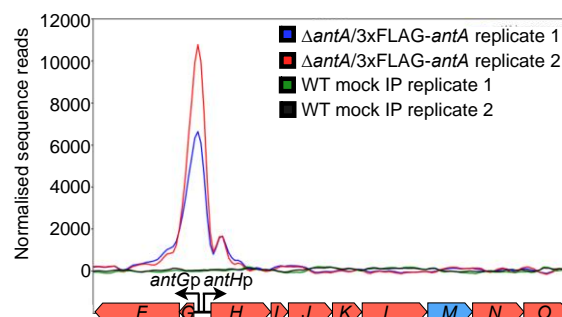
93 The Clp-protease system is essential for normal bacterial proteostasis and is best
94 characterised in *Escherichia coli*^{14,15}. The Clp protease is a multi-enzyme complex composed of a
95 barrel-shaped peptidase, ClpP and a regulatory enzyme, either ClpA or ClpX (or ClpC in some
96 organisms).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

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

114 Results

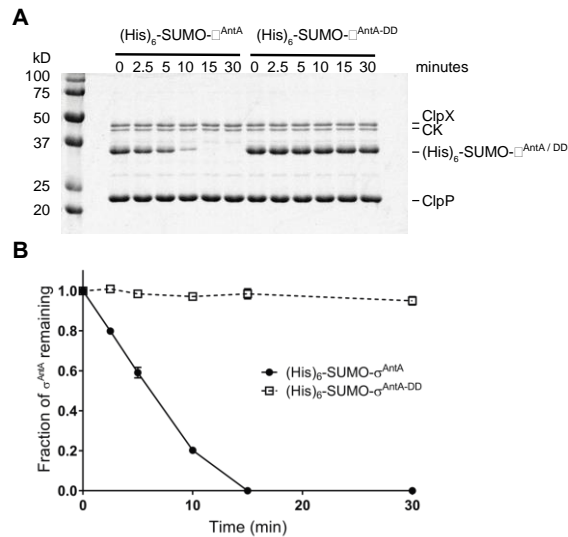
115 σ^{AntA} orthologues are a new subfamily of ECF σ factor that regulate production of the
116 antimycin biosynthetic starter unit. Since its initial discovery six years ago, more than 70 *ant*
117 BGCs have been identified within actinomycete genera, including in *Actinobacteria*, *Actinospica*,
118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

133



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

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



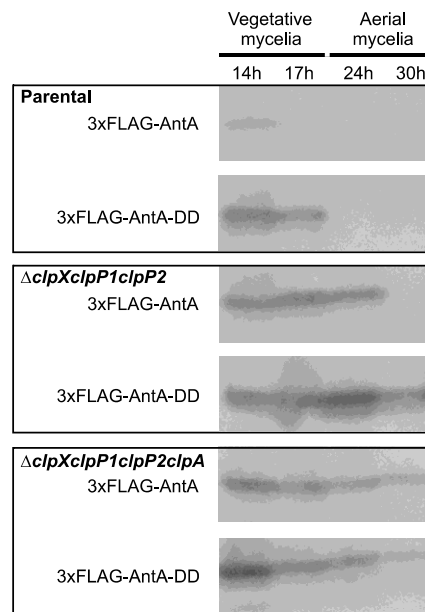
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165 **Fig. 3 | Proteolysis of *S. ambifaciens* σ^{AntA} by ClpXP *in vitro*.** (A) SDS-PAGE analysis of
166 proteolysis reactions containing 37 pmols (His)₆SUMO- σ^{AntA} or (His)₆SUMO- $\sigma^{\text{AntA-DD}}$. (B)
167 Densitometry analysis SDS-PAGE images for three independent proteolysis experiments. The mean
168 is plotted and error bars illustrate the standard error of the mean (± 1 SEM).
169

170 σ^{AntA} is degraded by ClpXP protease *in vivo*. To investigate if the *in vitro* degradation
171 σ^{AntA} demonstrated above is relevant to its regulation *in vivo* we adopted a genetic strategy to assess
172 the abundance of σ^{AntA} in mutant strains with defects in Clp-proteolysis. First, we deleted the *clpX*,
173 *clpP1*, and *clpP2* genes from *S. albus* S4. The resulting mutant underwent a normal developmental
174 cycle, albeit sporulation was less robust on ISP2 and MS medium (Supplemental Fig. 3). Next
175 genes encoding the 3xFLAG- σ^{AntA} or 3xFLAG- $\sigma^{\text{AntA-DD}}$ fusion proteins were generated and
176 introduced into the parental strain and the $\Delta clpXclpP1clpP2$ mutant so the abundance of these
177 proteins could be assessed over a developmental time course by Western blotting with anti-FLAG
178 antisera. This experiment was initially performed with the σ^{AntA} fusions integrated on the
179 chromosome under control of the native protein. However, a reliable signal could not be detected
180 for 3xFLAG- σ^{AntA} and only a trace amount of the Asp-Asp variant was observed, presumably
181 indicating that the cellular level of σ^{AntA} is normally low because the native promoter is relatively
182 weak. The experiment was therefore repeated with 3xFLAG- σ^{AntA} and 3xFLAG- $\sigma^{\text{AntA-DD}}$ expression
183 driven by a stronger, constitutive promoter, *ermE*³²⁵. Analysis of the resulting immunoblot revealed
184 that 3xFLAG- $\sigma^{\text{AntA-DD}}$ was more abundant than 3xFLAG- σ^{AntA} in extracts prepared from vegetative

185 mycelia (14h and 17h) of the parent and $\Delta clpXclpP1clpP2$ strains (Fig. 4). Strikingly, 3xFLAG-
186 σ^{AntA} and 3xFLAG- $\sigma^{\text{AntA-DD}}$ could only be detected in extracts from aerial mycelia (24h and 30h) of
187 the $\Delta clpXclpP1clpP2$ strain and not the parent; the Asp-Asp variant was also present in greater
188 relative abundance (Fig. 4). These data support the hypothesis that σ^{AntA} levels, and thus its ability
189 to activate gene expression is modulated by the ClpXP protease, however the conspicuous absence
190 of 3xFLAG- σ^{AntA} and the presence 3xFLAG- $\sigma^{\text{AntA-DD}}$ in protein extracts prepared from the latest
191 time point suggests the involvement of degradative factor(s) in addition to ClpXP.

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



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

212 Discussion

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

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

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

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

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

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

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

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

276 **Materials and methods**

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

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

294 **ChIP-seq and bioinformatics analyses.** The *antA* coding sequence was amplified
295 with RFS629 and RFS630, which contain KpnI and EcoRI restriction sites, respectively. The
296 restricted PCR product was cloned into pSETNFLAG digested with the same enzymes. The
297 resulting plasmid was then restricted with NotI and EcoRI to release *ermE**p-3xFLAG-*antA*, which
298 was subsequently cloned into pAU3-45 digested with the same enzymes. pAU3-45-3xFLAG-*antA*
299 was mobilised to an apramycin-marked $\Delta antA$ strain⁹. Cultivation of the wild-type and
300 $\Delta antA$ /pAUNFLAG-*antA* strains for ChIP-seq were performed exactly as described
301 previously¹⁰. The pure DNA resulting from immunoprecipitates from two biological replicates of

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

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

328 replication that expresses the Φ C31 integrase required for removal of the cassette⁴⁵.
329 Transconjugants were screened for loss of apramycin resistance and excision of the cassette was
330 verified by polymorphic shift PCR and DNA sequencing of the product.

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

344 **Protein purification and *in vitro* ClpXP proteolysis assays.** The wild-type *antA* gene was
345 PCR amplified and cloned into the AgeI and HindIII sites of the pET23b-SUMO vector, which
346 harbours an N-terminal (His)₆-SUMO tag⁴⁶. The plasmid for production of (His)₆-SUMO- $\sigma^{\text{AntA-DD}}$
347 was generated by site-directed mutagenesis (Agilent QuikChange) using primers listed in
348 Supplementary Table 2. (His)₆-SUMO- σ^{AntA} and (His)₆-SUMO- $\sigma^{\text{AntA-DD}}$ were produced by *E. coli*
349 Rosetta(DE3) (Novagen) grown in LB at 37 °C until OD₆₀₀ 0.5, followed by induction with 0.4 mM
350 IPTG and growth at 18 °C for 16 hours. Cells were resuspended in 50 mM sodium phosphate, pH 8,
351 1M NaCl, 20 mM imidazole, 10% glycerol, and 1 mM DTT and lysed by french press at 28 kpsi,
352 followed by treatment with protease inhibitor cocktail set III, EDTA-free (Calbiochem) and
353 benzonase (Millipore Sigma). (His)₆-SUMO- σ^{AntA} and (His)₆-SUMO- $\sigma^{\text{AntA-DD}}$ proteins were

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

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

367 **Data availability**

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

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378 **References**

- 379 1. Zhu, H., Sandiford, S. K. & van Wezel, G. P. Triggers and cues that activate antibiotic
380 production by actinomycetes. *J. Ind. Microbiol. Biotechnol.* **41**, 371–386 (2013).
- 381 2. Newman, D. J. & Cragg, G. M. Natural products as sources of new drugs over the 30 years
382 from 1981 to 2010. *J. Nat. Prod.* **75**, 311–335 (2012).
- 383 3. van Wezel, G. P. & McDowall, K. J. The regulation of the secondary metabolism of
384 *Streptomyces*: new links and experimental advances. *Nat. Prod. Rep.* **28**, 1311–23 (2011).
- 385 4. van der Heul, H. U., Bilyk, B. L., McDowall, K. J., Seipke, R. F. & van Wezel, G. P.
386 Regulation of antibiotic production in Actinobacteria: new perspectives from the post-
387 genomic era. *Nat. Prod. Rep.* **35**, 575–604 (2018).
- 388 5. Joynt, R. & Seipke, R. F. A phylogenetic and evolutionary analysis of antimycin
389 biosynthesis. *Microbiology* **164**, 28–39 (2018).
- 390 6. Dunshee, B. R., Leben, C., Keitt, G. W. & Strong, F. M. The isolation and properties of
391 antimycin A. *J. Am. Chem. Soc.* **71**, 2436–2437 (1949).
- 392 7. Tzung, S. P. *et al.* Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3.
393 *Nat. Cell. Biol.* **3**, 183–191 (2001).
- 394 8. Seipke, R. F. *et al.* A Single *Streptomyces* symbiont makes multiple antifungals to support
395 the fungus farming ant *Acromyrmex octospinosus*. *PLoS ONE* **6**, e22028–8 (2011).
- 396 9. Seipke, R. F., Patrick, E. & Hutchings, M. I. Regulation of antimycin biosynthesis by the
397 orphan ECF RNA polymerase sigma factor σ^{AntA} . *PeerJ* **2**, e253 (2014).
- 398 10. McLean, T. C., Hoskisson, P. A. & Seipke, R. F. Coordinate regulation of antimycin and
399 candicidin biosynthesis. *mSphere* **1**, e00305–16 (2016).
- 400 11. Helmann, J. D. The extracytoplasmic function (ECF) sigma factors. *Adv. Microb. Physiol.*
401 **46**, 47–110 (2002).
- 402 12. Staroń, A. *et al.* The third pillar of bacterial signal transduction: classification of the
403 extracytoplasmic function (ECF) σ factor protein family. *Mol. Microbiol.* **74**, 557–581
404 (2009).
- 405 13. Paget, M. Bacterial Sigma Factors and Anti-Sigma Factors: Structure, Function and
406 Distribution. *Biomolecules* **5**, 1245–1265 (2015).
- 407 14. Gur, E., Biran, D. & Ron, E. Z. Regulated proteolysis in Gram-negative bacteria—how and
408 when? *Nat. Rev. Microbiol.* **9**, 839–848 (2011).
- 409 15. Baker, T. A. & Sauer, R. T. ClpXP, an ATP-powered unfolding and protein-degradation
410 machine. *Biochim. Biophys. Acta.* **1823**, 15–28 (2012).
- 411 16. Olivares, A. O., Baker, T. A. & Sauer, R. T. Mechanistic insights into bacterial AAA+
412 proteases and protein-remodelling machines. *Nat. Rev. Microbiol.* **14**, 33–44 (2015).
- 413 17. de Crécy-Lagard, V., Servant-Moisson, P., Viala, J., Grandvalet, C. & Mazodier, P.
414 Alteration of the synthesis of the Clp ATP-dependent protease affects morphological and
415 physiological differentiation in *Streptomyces*. *Mol. Microbiol.* **32**, 505–517 (1999).
- 416 18. Viala, J., Rapoport, G. & Mazodier, P. The *clpP* multigenic family in *Streptomyces lividans*:
417 conditional expression of the *clpP3 clpP4* operon is controlled by PopR, a novel
418 transcriptional activator. *Mol. Microbiol.* **38**, 602–612 (2000).
- 419 19. Viala, J. & Mazodier, P. ClpP-dependent degradation of PopR allows tightly regulated
420 expression of the *clpP3 clpP4* operon in *Streptomyces lividans*. *Mol. Microbiol.* **44**, 633–643
421 (2002).
- 422 20. Keiler, K. C., Waller, P. R. & Sauer, R. T. Role of a peptide tagging system in degradation of
423 proteins synthesized from damaged messenger RNA. *Science* **271**, 990–993 (1996).
- 424 21. Gottesman, S., Roche, E., Zhou, Y. & Sauer, R. T. The ClpXP and ClpAP proteases degrade
425 proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev.*
426 **12**, 1338–1347 (1998).
- 427 22. Flynn, J. M. *et al.* Overlapping recognition determinants within the *ssrA* degradation tag
428 allow modulation of proteolysis. *Proc. Natl. Acad. Sci. USA.* **98**, 10584–10589 (2001).

- 429 23. Seipke, R. F., Hutchings, M. I. & Hutchings, M. I. The regulation and biosynthesis of
430 antimycins. *Beilstein J. Org. Chem.* **9**, 2556–2563 (2013).
- 431 24. Schoenian, I. *et al.* An unprecedented 1,2-shift in the biosynthesis of the 3-aminosalicylate
432 moiety of antimycins. *ChemBioChem* **13**, 769–773 (2012).
- 433 25. Luo, Y., Zhang, L., Barton, K. W. & Zhao, H. Systematic Identification of a Panel of Strong
434 Constitutive Promoters from *Streptomyces albus*. *ACS Synth. Biol.* **4**, 1001–1010 (2015).
- 435 26. Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T. & Baker, T. A. Proteomic Discovery of
436 Cellular Substrates of the ClpXP Protease Reveals Five Classes of ClpX-Recognition
437 Signals. *Mol. Cell.* **11**, 671–683 (2003).
- 438 27. Foulston, L. C. & Bibb, M. J. Microbisporicin gene cluster reveals unusual features of
439 lantibiotic biosynthesis in actinomycetes. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 13461–13466
440 (2010).
- 441 28. Sherwood, E. J. & Bibb, M. J. The antibiotic planosporicin coordinates its own production in
442 the actinomycete *Planomonospora alba*. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E2500–E2509
443 (2013)
- 444 29. Hoskins, J. R., Yanagihara, K., Mizuuchi, K. & Wickner, S. ClpAP and ClpXP degrade
445 proteins with tags located in the interior of the primary sequence. *Proc. Natl. Acad. Sci. USA.*
446 **99**, 11037–11042 (2002).
- 447 30. Mika, F. & Hengge, R. A two-component phosphotransfer network involving ArcB, ArcA,
448 and RssB coordinates synthesis and proteolysis of sigmaS (RpoS) in *E. coli*. *Genes Dev.* **19**,
449 2770–2781 (2005).
- 450 31. Mao, X-M. *et al.* Dual positive feedback regulation of protein degradation of an extra-
451 cytoplasmic function σ factor for cell differentiation in *Streptomyces coelicolor*. *J. Biol.*
452 *Chem.* **288**, 31217–31228 (2013).
- 453 32. Ades, S. E., Connolly, L. E., Alba, B. M. & Gross, C. A. The *Escherichia coli* sigma(E)-
454 dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an
455 anti-sigma factor. *Genes Dev.* **13**, 2449–2461 (1999).
- 456 33. Alba, B. M., Leeds, J. A., Onufryk, C., Lu, C. Z. & Gross, C. A. DegS and YaeL participate
457 sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic
458 stress response. *Genes Dev.* **16**, 2156–2168 (2002).
- 459 34. Flynn, J. M., Levchenko, I., Sauer, R. T. & Baker, T. A. Modulating substrate choice: the
460 SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+
461 protease ClpXP for degradation. *Genes Dev.* **18**, 2292–2301 (2004).
- 462 35. Zellmeier, S., Schumann, W. & Wiegert, T. Involvement of Clp protease activity in
463 modulating the *Bacillus subtilis* sigma W stress response. *Mol. Microbiol.* **61**, 1569–1582
464 (2006).
- 465 36. Bellier, A. & Mazodier, P. ClgR, a Novel Regulator of *clp* and *lon* expression in
466 *Streptomyces*. *J. Bacteriol.* **186**, 3238–3248 (2004).
- 467 37. Bellier, A., Gominet, M. & Mazodier, P. Post-translational control of the *Streptomyces*
468 *lividans* ClgR regulon by ClpP. *Microbiology* **152**, 1021–1027 (2006).
- 469 38. Mettert, E. L. & Kiley, P. J. ClpXP-dependent Proteolysis of FNR upon Loss of its O₂-
470 sensing [4Fe–4S] Cluster. *J. Mol. Biol.* **354**, 220–232 (2005).
- 471 39. Alkhalaf, L. M. & Ryan, K. S. Biosynthetic manipulation of tryptophan in bacteria: pathways
472 and mechanisms. *Chem. Biol.* **22**, 317–328 (2015).
- 473 40. Palazzotto, E. *et al.* TrpM, a Small Protein Modulating Tryptophan Biosynthesis and
474 Morpho-Physiological Differentiation in *Streptomyces coelicolor* A3(2). *PLoS ONE* **11**,
475 e0163422–17 (2016).
- 476 41. Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. Practical
477 *Streptomyces* Genetics. John Innes Foundation (2000)
- 478 42. Seipke, R. F., Grünschow, S., Goss, R. J. M. & Hutchings, M. I. *Methods Enzymol.* **517**, 47–
479 70 (2012).

- 480 43. Ramirez, F., Dundar, F., Diehl, S., Gruning, B. A. & Manke, T. deepTools: a flexible
481 platform for exploring deep-sequencing data. *Nucleic Acids Res.* **42**, W187–W191 (2014).
482 44. Fu, J. *et al.* Full-length RecE enhances linear-linear homologous recombination and
483 facilitates direct cloning for bioprospecting. *Nat. Biotechnol.* **30**, 440–446 (2012).
484 45. Myronovskyi, M. *et al.* Iterative marker excision system. *Appl. Microbiol. Biotechnol.* **98**,
485 4557–4570 (2014).
486 46. Wang, K. H., Sauer, R. T. & Baker, T. A. ClpS modulates but is not essential for bacterial N-
487 end rule degradation. *Genes Dev.* **21**, 403–408.
488 47. Neher, S. B., Sauer, R. T. & Baker, T. A. Distinct peptide signals in the UmuD and UmuD'
489 subunits of UmuD/D' mediate tethering and substrate processing by the ClpXP protease.
490 *Proc. Natl. Acad. Sci. USA.* **100**, 13219–13224 (2003).
491 48. Kim, Y. I., Burton, R. E., Burton, B. M., Sauer, R. T. & Baker, T. A. Dynamics of substrate
492 denaturation and translocation by the ClpXP degradation machine. *Mol. Cell* **5**, 639–648
493 (2000).
494 49. Seipke, R. F. *et al.* Draft genome sequence of *Streptomyces* strain S4, a symbiont of the leaf-
495 cutting ant *Acromyrmex octospinosus*. *J. Bacteriol.* **193**, 4270–4271.