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Antimycins are an extended family of depsipeptides that are made by filamentous actinomycete bacteria and were first isolated more than 60 years ago. Recently, antimycins have attracted renewed interest because of their activities against the anti-apoptotic machineries inside human cells which could make them promising anti-cancer compounds. The biosynthetic pathway for antimycins was recently characterised but very little is known about the organisation and regulation of the antimycin (*ant*) gene cluster. Here we report that the *ant* gene cluster in *Streptomyces albus* is organized into four transcriptional units; the *antBA*, *antCDE*, *antGF* and *antHIJKLMNO* operons. Unusually for secondary metabolite clusters, the *antG* and *antH* promoters are regulated by an extracytoplasmic function (ECF) RNA polymerase sigma factor named σ^{AntA} which represents a new sub-family of ECF σ factors that is only found in antimycin producing strains. We show that σ^{AntA} controls production of the unusual precursor 3-aminosalicylate which is absolutely required for the production of antimycins. σ^{AntA} is highly conserved in antimycin producing strains and the -10 and -35 elements at the σ^{AntA} regulated *antG* and *antH* promoters are also highly conserved suggesting a common mechanism of regulation. We also demonstrate that altering the C-terminal Ala-Ala residues found in all σ^{AntA} proteins to Asp-Asp increases expression of the *antFG* and *antGHIJKLMNO* operons and we speculate that this Ala-Ala motif may be a signal for the protease ClpXP.

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Regulation of antimycin biosynthesis by the orphan ECF RNA polymerase sigma factor σ^{AntA}

Ryan F. Seipke[1,2]*, Elaine Patrick[1] and Matthew I. Hutchings[1]*

[1]School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom

[2]Current address: The Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT United Kingdom

*For correspondence E-mail: m.hutchings@uea.ac.uk or r.seipke@leeds.ac.uk

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INTRODUCTION

Approximately 60% of the antibiotics and anticancer compounds currently used in human medicine are derived from the secondary metabolites of soil-dwelling *Streptomyces* species and other filamentous actinomycetes. Although the vast majority of these natural products were discovered more than 40 years ago, the advent of genome mining and new tools to unlock so-called "silent" pathways mean that these bacteria still offer us the best hope of developing new antibiotics for clinical use. The antimycin family of natural products were discovered nearly 65 years ago and initially attracted interest because of their potent antifungal activity (Dunshee *et a*l., 1949). Antimycins are widely produced by *Streptomyces* species and they exhibit a range of bioactive properties, including antifungal, insecticidal and nematocidal activity. This is the result of their ability to inhibit cytochrome c reductase, an enzyme in the respiratory chain in bacteria and mitochondria. Antimycins are also used as piscicides (brand name Fintrol®) to kill off unwanted scaled fish in the farming of catfish, which are relatively insensitive to antimycins (Finlayson *et al.*, 2002). More recently antimycins have been shown to be potent and selective inhibitors of the mitochondrial Bcl-2/Bcl-x_L-related anti-apoptotic proteins which are over-produced by drug resistant cancer cells. Over-production of Bcl-2/Bcl-x_L proteins in cancer cells confers resistance to multiple chemotherapeutic agents whose primary mode of action is to trigger apoptosis. Antimycins bind to the hydrophobic groove of Bcl-2-type proteins and inhibit their activity in a mechanism of action that is independent of their activity against electron transport (Tzung *et al.*, 2001). A synthetic derivative of antimycin A_3, 2-methoxyantimycin A_3 (2-MeAA), no longer inhibits the respiratory chain, but retains potent antagonistic activity toward Bcl-2-related proteins and induces apoptosis (Tzung *et al*., 2001; Schwartz *et al.*, 2007). This has led to suggestions that antimycin derivatives such as 2-MeAA could be used alongside traditional apoptosis-inducing chemotherapeutics to block drug resistance and kill cancer cells. There is significant interest in bioengineering antimycins with improved pharmacological properties for the treatment of cancer and infectious diseases.

Despite their unique chemical structure and important biological properties, the antimycin biosynthetic pathway was only reported very recently (Seipke *et al*., 2011a,b) and rapid progress has been made in elucidating the biosynthetic steps in this pathway over the last two years (for a recent review see Seipke and Hutchings, 2013). Antimycins are produced by a hybrid non-ribosomal peptide synthetase (NRPS) / polyketide synthase (PKS) assembly line for which the complete biosynthetic pathway has been proposed (Sandy *et al.*, 2012; Yan *et al.*, 2012). The AntFGHIJKLN proteins encode the biosynthetic pathway for the unusual starter unit, 3-

aminosalicylate-CoA (Schoenian et al., 2012; Sandy et al., 2012). The AntCD proteins comprise the hybrid NRPS / PKS machinery, and AntE and AntM are crotonyl-CoA reductase and discrete ketoreductase homologues, respectively (Sandy et al., 2012). AntO and AntB are tailoring enzymes. AntO is predicted to install the *N*-formyl group (Yan et al., 2012; Sandy et al., 2012), and AntB is a promiscuous acyltransferase that catalyses a transesterification reaction of a hydroxl group at C-8 to result in the acyloxyl moiety and the chemical diversity observed at R1 (Sandy *et al.*, 2013). The *antA* gene encodes an extracytoplasmic function (ECF) RNA polymerase sigma (σ) factor named σ^{AntA} which, like all other ECF σ factors, contains only two of the four σ^{70} domains (Staron *et al.* 2009).

The resurgence of interest in the biosynthesis of antimycins and particularly in engineering new analogues with better pharmacological properties led us to investigate the transcriptional organisation and regulation of the antimycin gene cluster. The only regulator encoded by the *ant* gene cluster is σ^{AntA}, but regulation of secondary metabolite clusters by ECF σ factors is unusual and has not yet been reported in *Streptomyces* species. To our knowledge only two examples of ECF σ factor regulation of antibiotic biosynthesis have been described and both differ from σ^{AntA} because they are co-encoded with, and regulated by, anti-σ factors whereas σ^{AntA} is an orphan, i.e. it has no co-encoded anti-σ factor. The two known examples both control lantibiotic production in rare actinomycetes. In *Microbospora corallina*, the pathway specific regulator MibR and the ECF σ^{MibX} regulate microbisporicin biosynthesis and σ^{MibX} is regulated by MibW (Foulston and Bibb, 2010). In *Planomonospora alba* the pathway specific regulator PspR, the ECF sigma factor σ^{PspX} and its anti-σ factor PspW all regulate production of the lantibiotic planosporicin (Sherwood and Bibb, 2013). The closest homologues to σ^{MibX} and MibW are σ^{PspX} and its anti-σ factor PspW, suggesting a common mechanism of regulation for these lantibiotics.

Here we characterize the gene organization of the antimycin gene cluster and the role of σ^{AntA} in *Streptomyces albus* S4. We report that σ^{AntA} is regulated at the transcriptional level and controls production of the unusual precursor 3-aminosalicylate that is required for antimycin production. We also show that σ^{AntA} represents a new sub-family of ECF σ factors that are only found in the *ant* gene clusters of *Streptomyces* species and provide evidence that suggests σ^{AntA} regulation of the divergent *antGF* and *antHIJKLMNO* operons is conserved in all antimycin producing strains. Finally we provide preliminary evidence that the activity of σ^{AntA} is affected by the two C-terminal amino acid residues such that altering the natural Ala-Ala residues to Asp-Asp increases expression of the σ^{AntA} target genes. Since a C-terminal Ala-Ala motif is a well known signal for the serine protease ClpXP (Flynn *et al.* 2003) this may provide a novel post-

translational mechanism for controlling σ^{AntA} activity without the need for an anti-σ factor.

Materials and Methods

Growth media and strains. Streptomyces strains (Table 1) were grown on mannitol-soya flour (MS) agar and Lennox broth (LB) (Kieser *et al*., 2000), and *Escherichia coli* strains (Table 1) were grown on LB or LB agar. Growth media was supplemented with antibiotics as required at the following concentrations: apramycin (50 µg/ml), carbenicillin (100 µg/ml), hygromycin B (50 µg/ml), kanamycin (50 µg/ml), nalidixic acid (50 µg/ml). All *Streptomyces* strains were created using cross-genera conjugation in which DNA was transferred from *E. coli* ET12567/pUZ8007 (MacNeil *et al*., 1992) according to standard methods (Kieser *et a*l., 2000).

Cosmid library construction and screening. A Supercos1 cosmid library was constructed from *Streptomyces albus* S4 genomic DNA partially digested with Sau3AI and packaged into Gigapack III XL phage according to the manufacturer's instructions (Agilent Technologies). One thousand cosmid clones were screened by PCR using primers RFS172 and RFS173 (Table S2), which target an internal fragment of the *antC* gene. Cosmid 456 and cosmid 213 tested positive and were end-sequenced using primers RFS184 and RFS185 (Table S2) and mapped onto the *Streptomyces albus* S4 genome using BLAST 2.2.23+ (Altschul *et al*., 1990).

Construction of Streptomyces albus S4 *mutant strains.* Mutant strains were constructed using λ-RED based PCR-targeting mutagenesis (Gust *et al*., 2003). A disruption cassette consisting of a conjugal origin of transfer (*oriT*) and the apramycin resistance gene, *aac(3)IV* from pIJ773 (Gust *et al*., 2003), was generated by PCR using BioTaq polymerase (Bioline) and oligonucleotide primers (Table S2) containing 39 nt of homology that included the start and stop codons of each gene (with the exception of the STRS4_02213-02217 multi-mutant) and 36 nt upstream or downstream of the open reading frame. The resulting PCR products were gel purified and electroporated into *E. coli* BW25113/pIJ790 harboring either cosmid 456 (Δ*STRS4_02194*, Δ*STRS4_02195*, Δ*antA, ΔantC*) or cosmid 213 (Δ*STRS4_02222*, Δ*STRS4_02213-STRS4_02217*). Transformants were screened for the presence of mutagenised cosmid by *Not*I digestion. Mutagenised cosmids were moved to S. *albus* S4 by conjugation. Transconjugants were selected for apramycin resistance and kanamycin sensitivity. The integrity of mutant strains was verified by PCR using flanking primers for each deleted coding sequence together and in combination with the P1 and P2 primers which target the apramycin cassette (Gust *et al.*, 2003). Combinations RRF228 and 229, RRF278 and 279 and RRF329 and 330 were used to test the *02194*, *02195* and

111 *02212* knockouts, respectively (Table S2).

112 *Construction of plasmids.* In order to heterologously express and purify AntA, the *antA*
113 coding sequence was PCR-amplified from genomic DNA using oligonucleotide primers
114 engineered to possess NdeI and HindIII restriction sites (RFS230 and RFS 231, Table S2) using
115 Phusion polymerase (New England Biolabs). The resulting PCR product was gel purified and
116 digested with NdeI and HindIII (Roche) and ligated with pET28a (Novagene) cut with the same
117 enzymes using T4 DNA ligase (Promega) to create pET28a-*antAI.* DNA sequencing using the T7
118 promoter and T7 terminator primers (Novagene) verified the integrity of the cloned *antA* coding
119 sequence. In order to construct the *antA* over-expression / complementation plasmid, pIJ10257-
120 *antA*, the *antA* coding sequence was excised from pET28a-*antA* using NdeI and HindIII and
121 ligated to pIJ10257 (Hong *et al*., 2005) cut with the same enzymes. pIJ10257-*antA* was
122 introduced into *Streptomyces* strains by conjugation and transconjugants were selected for
123 resistance for hygromycin.

124 In order to generate complementation constructs in which transcription of wild-type and
125 mutated *antA* was initiated by its native promoter, we replaced the *antB* gene with an apramycin
126 resistance cassette using the REDIRECT system described above using oligos RFS188 and
127 RFS189 (Gust *et al*., 2003, Table S2). The apramycin cassette possesses two FRT sites recognised
128 by the FLP recombinase. The mutagenised cosmid was introduced into *E. coli* strain BT340,
129 which expresses a FLP recombinase when cultured at 42 ºC (Gust *et al*., 2003). FLP
130 recombinase-mediated excision of the apramycin resistance cassette leaves an 81 bp in-frame
131 "scar." Cosmid 213 Δ*antB-flp* was used as template for PCR with the forward primer RFS351
132 and the reverse primers RFS231 or RFS352 (Table S2). RFS351 targets 270 bp upstream of the
133 putative *antB* start codon, and RFS231 and RFS352 both target an identical sequence in the C-
134 terminus of *antA* , with the exception that RFS352 introduces two C->A point mutations, which
135 introduces A172D and A173D changes into the resulting AntA protein. These PCR products were
136 cloned into pGEMT-Easy (Promega) and verified by DNA sequencing using M13R and M13F
137 oligonucleotides. Next, the *antA*-containing inserts were excised from pGEMT-Easy by EcoRI
138 digestion and ligated with pAU3-45 (Bignell *et al*., 2005) digested with the same enzyme. pAU3-
139 45-*antA*-AA and pAU3-45-*antA*-DD were introduced into *Streptomyces* strains by conjugation
140 and transconjugants were selected for resistance to thiostrepton.

141

142 *Phylogenetic analysis.* Antimycin gene clusters were analysed from *S. ambofaciens* ATCC 23877

(AM238663, (Choulet *et al*., 2006)), *S. blastmyceticus* NBRC 12747 (AB727666, (Yan *et al*., 2012)), *S. gancidicus* BKS 13-15 [AOHP00000000, (Kumar *et al*., 2013), *S. griseoflavus* Tü4000 (ACFA00000000), *S. hygroscopicus* subsp. *jinggangensis* 5008 (NC_017765), *S. hygroscopicus* subsp. *jinggangensis* TL01 (NC_020895), *Streptomyces* sp. 303MFCol5.2 (ARTR00000000), *Streptomyces* sp. TOR3209 (AGNH00000000, (Hu *et al*., 2012), *S. albus* S4 (CADY00000000, (Seipke *et al*., 2011b)), *S. albus* J1074 (NC_020990), *Streptomyces* sp. SM8 (AMPN00000000), *Streptomyces* sp. NRRL2288 (JX131329), (Yan *et a*l., 2012)), *Streptomyces* sp. LaPpAH-202 (ARDM00000000), *Streptomyces* sp. CNY228 (ARIN01000033). σ^{AntA} proteins were aligned to five (when possible) random proteins from each ECF RNA polymerase σ factor subfamily defined by Staron *et al* (Staron *et a*l., 2009) by using ClustalΩ (Sievers *et a*l., 2011). The phylogenetic tree was created using PhyML 3.0 with the default settings (Guindon *et al*., 2010) and visualised using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

HPLC analysis. Wild-type and mutant strains were cultured atop a cellophane disc on MS agar. At the time of harvest, the cellophane disc containing mycelia was removed and either processed for RNA extraction (below) or discarded. Bacterial metabolites were extracted from the spent agar using 50 ml of ethyl acetate for 1 hour. 20 ml of ethyl acetate was evaporated to dryness under reduced pressure and the resulting residue was resuspended in 400 µl 100% methanol. In all cases, the methanolic extracts from at least two biological replicates were mixed and centrifuged at >16,000g in a microcentrifuge prior to analysis. Antimycin A_1–A_4 standards were purchased from Sigma-Aldrich. 35 microliters of methanolic extract was separated on a Phenomenex C18(2) 5 µm 4.6 x 150 mm using a Hitachi L-6200 HPLC system and the following gradient (solvent A: water, solvent B: methanol, flow rate 1 ml/min): 0-20 min, 10-100% B; 20-34 min 100% B; 34.1-44 min, 10% B. Samples were analysed with a Shimadzu M20A Photo Diode Array.

RNA analysis. For all experiments involving RNA, *S. albus* S4 strains were cultivated at 30 °C on MS agar atop a cellophane disc to facilitate the easy harvest of mycelia into microcentrifuge tubes. Transcription was arrested using a stop solution (95% ethanol, 5% acid phenol) diluted 1:4 with water. Total RNA was extracted using a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and included both an on-column and a post-column DNaseI treatment. The absence of DNA contamination was assessed by PCR. DNase-treated RNA was reverse transcribed using 250 µg of random hexamers and Superscript III reverse transcriptase (RT, Invitrogen) with an extension temperature of 55 °C.

For co-transcription analysis, twenty-nine cycles of PCR amplification with six primer sets (Table S2) were performed using cDNA originating from 5 µg of RNA with BioTaq Polymerase (Bioline). Primer sets were designed to span the intergenic regions of the antimycin cluster and targeted at least 300 bp upstream of putative start codons to account for promoters driving transcription from multiple sites within a transcriptional unit. RNA from the complemented *antA* mutant strain (Δ*antA*/pIJ10257-*antA*) was used, because transcript abundance was greater for operons involved in 3-aminosalicylate biosynthesis. The PCR products obtained were cloned into either pCRII-TOPO (Invitrogen) or pGEM-T Easy (Promega) and sequenced by either the Genome Analysis Centre (Norwich, UK), Source BioScience (Cambridge, UK), or Eurofins MWG Operon (Ebersberg, Germany) using oligonucleotide primer M13r (Integrated DNA Technologies).

For quantitative RT-PCR, gene-specific primers were designed to amplify ~100 bp from the first and last gene of each transcriptional unit in the antimycin cluster. cDNA was diluted (1 volume of cDNA to 2 volumes of water) and target genes were quantified using a Bio-Rad CFX96TM instrument and SensiFast™ SYBR No-ROX kit (Bioline). Each treatment consisted of three biological replicates and two technical replicates. The calculated C*t* (threshold cycle value) for each target gene was normalized to the C*t* obtained for the *hrdB* gene, which encodes the vegetative sigma factor and is routinely used as a reference gene for transcriptional analyses (Kelemen *et al*., 1996).

For mapping of transcriptional start sites, 10 µg of RNA from the Δ*antA*/pIJ10257-*antA* strain was processed using the FirstChoice® RLM-RACE Kit (Ambion) according to the manufacturer's instructions with the following modifications: for cDNA synthesis, Superscript III RT (Invitrogen) was used to according the manufacturer's instructions using an extension temperature of 55 °C. The gene-specific primers used for each transcriptional unit are listed in Table S2. The final PCR products were gel purified and cloned into pCRII-TOPO (Invitrogen) or pGEM-T Easy (Promega) and sequenced using oligonucleotide primers M13r (Integrated DNA Technologies) by either the Genome Analysis Centre (Norwich, UK), Source BioScience (Cambridge, UK) or Eurofins MWG Operon (Ebersberg, Germany). The transcriptional start site was determined to be the nucleotide immediately adjacent to the sequence of the 5'RLM-RACE RNA adapter.

Bacterial two-hybrid analysis. The full STRS4_02195, AntA, and STRS4_04339 (SigB orthologue) coding sequences were PCR-amplified from S4 genomic DNA using Phusion

Polymerase (New England Biolabs) and primers RFS280 and RFS281 (*STRS4_02195*), RFS282 and RFS283 (*antA*), and *sigB* (RFS284 and RFS285) (Table S1). The gel purified PCR products were digested with BamHI and KpnI (Roche) and cloned into bacterial two-hybrid plasmids pUT18C and pKT25 (Karimova *et al*., 1998) cut with the same enzymes. Cloned inserts were sequenced by The Genome Analysis Centre (Norwich, UK) using primers RFS286, RFS287 (pUT18C clones) and RFS288 and RFS289 (pKT25 clones) to ensure that no mutations had occurred. Plasmid combinations of interest were co-electroporated into *E. coli* DHM1 and processed for β-galactosidase activity as previously described (Hutchings *et al*., 2002).

Results and Discussion

Organisation and expression of the antimycin gene cluster. To facilitate mutagenesis of the antimycin gene cluster, we constructed a Supercos1 library of the *S. albus* S4 genome (Genbank accession CADY00000000.1) and screened the library by PCR against an internal fragment of *antC*. We identified two overlapping cosmids containing *antC* and confirmed that cosmid 213 contains the complete predicted *ant* gene cluster by deleting genes adjacent to the cluster using PCR-targeted mutagenesis (Fig. 1). To define the upstream border we deleted *STRS4_02194*, which encodes a separate NRPS and *STRS4_02195* which encodes a predicted membrane protein of unknown function. To determine the downstream border we deleted *STRS4_02212* and *STRS4_02214-STRS4_02217* which are predicted to encode a nitrate / nitrite assimilation protein and an ABC-transport system, respectively. To determine if these mutations affect antimycin production we performed bioassays against the human pathogen *Candida albicans* and observed no obvious difference in the ability of the *S. albus* S4 strains to inhibit the growth of *C. albicans* compared to wild-type (Fig. 2A). High performance liquid chromatography (HPLC) confirmed that antimycin production is not affected by any of these mutations showing that STRS4_02194, STRS4_02195, STRS4_02212, and STRS4_02214-02217 mark the boundaries of the *ant* gene cluster (Fig. 2B). The gene organization of the *ant* cluster suggests there is a minimum of four transcriptional units with the largest being the *antHIJKLMNO* operon (Fig 1). Almost all of these ORFs overlap, suggesting transcriptional and translational coupling, but as a proof of principle we confirmed that the *antGF* and *antHIJKLMNO* genes are co-transcribed by performing end-point RT-PCR. Six primer pairs were designed to span the intergenic (or overlapping gene) regions of the *antGF* and *antHIJKLMNO* operons and targeted at least 300 bp upstream of the putative start codons to detect transcriptional read-through. Six PCR products were obtained by

RT-PCR analysis and sequenced to confirm that *antGF* and *antHIJKLMNO* form two operons. No products were obtained when reverse transcriptase was omitted (Fig. S1). In addition to confirming that *antGF* and *antHIJKLMNO* are organized into operons, this also validates our approach to analysing their expression using qRT-PCR to measure mRNA levels of the first and last genes in each operon.

Streptomyces species have a complex life cycle that includes growth as a substrate mycelium that gives rise to aerial mycelia and sporulation. To determine at which stage of the life cycle the antimycin gene cluster is expressed we measured expression of the four *ant* operons after 18 and 42 hours growth on mannitol-soya flour (MS) agar. After 18 hours growth on MS agar *S. albus* S4 consists entirely of substrate mycelium but after 42 hours the substrate mycelium has differentiated to produce aerial mycelium and spores. All four *ant* operons are expressed at a significantly higher level at 18 hours (in substrate mycelium) compared to 42 hours which suggests that all four *ant* operons are switched off following differentiation (Fig. 3A). Conversely, HPLC analysis of mycelium and culture medium extracted at the same time points only detected antimycins in the 42 hour samples suggesting there is a lag between *ant* gene expression and antimycin production (Fig 3B) . This is probably due to the time it takes for the precursor to be produced and for the antimycin scaffold to be assembled and then accumulate to detectable levels. Most notably, these data suggest that specific regulatory mechanisms exist to activate *ant* gene expression in substrate mycelium and switch it off again following differentiation. Since *antA* is the only putative regulatory gene in the *ant* gene cluster we investigated the role of σ^{AntA} in regulating antimycin production.

Antimycin production is dependent on the orphan ECF sigma factor σ^{AntA}. To investigate the role of σ^{AntA} in regulating antimycin biosynthesis, we deleted the *antA* gene and tested the mutant strain against *C. albicans* in a bioassay. The *antA* mutant is significantly less active against *C. albicans* compared to wild-type and this is consistent with loss of antimycin production (Seipke *et al*., 2011a). Complementation of this mutant with the *antA* gene under the control of the strong constitutive *ermE** promoter restores bioactivity against *C. albicans* to wild-type levels (Fig. 4A) and HPLC analysis confirmed that antimycins are not produced by the *antA* mutant (Fig. 4B). We conclude that σ^{AntA} is required for antimycin production

To determine which of the four *ant* promoters are regulated by σ^{AntA} we used qRT-PCR to measure *ant* operon expression in the wild-type and *antA* strains grown for 18 hours on MS agar. Deletion of *antA* did not affect the level of transcription of either the *antBA* or *antCDE* operons,

but transcription of both the *antGF* and *antHIJKLMNO* operons was significantly reduced in the *antA* mutant (Fig. 5). This suggests that σ^{AntA} positively regulates the transcription of the *antFGHIJKLMNO* genes which encode biosynthesis of 3-aminosalicylate, the precursor used by the AntC NRPS. Furthermore, over-expression of σ^{AntA} in 42 hour cultures activates the expression of the *antGF* and *antHIJKLMNO* operons leading us to conclude that no additional regulators are required to activate the *antG* and *antH* promoters (Fig. 6). In addition the *antB* and *antC* promoters must be regulated by a transcription factor encoded outside of the *ant* gene cluster since they are upregulated at 18 hours relative to 42 hours growth. To confirm this we introduced cosmid 213 into *S. lividans*, *S. coelicolor* M145 and the *S. coelicolor* superhost strains M1146, M1152 and M1154 (Gomez-Escribano and Bibb, 2011) but failed to detect antimycin production, supporting the idea that at least one additional transcription activator is required.

σ^{AntA} and its putative binding site are highly conserved. Bioinformatic analysis failed to identify the common ECF σ factor promoter motifs upstream of the *antG* and *antH* genes, notably the "AAC" motif in the -35 region and the "CGT" motif in the -10 region (Staron *et al*., 2009). We therefore mapped the transcriptional start sites of the *antGF* and *antHIJKLMNO* operons using 5'-RLM RACE and identified -10 and -35 regions which share high nucleotide sequence identity with one another, but not with the σ^{AntA}-independent *antB* promoter (Fig. 7A). Six antimycin producing *Streptomyces* strains have been reported previously (Riclea *et al*., 2012; Seipke *et al*., 2011a; Yan *et a*l., 2012) and we identified eight more putative antimycin gene clusters whilst searching for σ^{AntA} orthologues in Genbank (Table S1 and Experimental Procedures). Since the 14 known σ^{AntA} orthologues share 66% sequence identity (Table S1, Fig. S2), we hypothesise that σ^{AntA} regulation of the *antG* and *antH* promoters will be common to all antimycin producing *Streptomyces* strains. To investigate this, we searched for the *antG* and *antH* promoter motifs in the 14 known or predicted antimycin gene clusters encoded by published *Streptomyces* genome sequences. All 14 *antG* promoters contain very high sequence identity in the -35 and -10 regions, although *S. ambofaciens* has an 18 nucleotide spacer between the -35 and -10 element compared to the typical 17 nucleotide spacer (Fig. 7B). High nucleotide conservation was also observed at the *antH* promoter and the -10 element contains a "CTC" motif that is 100% conserved across all promoters although again spacer regions between the -10 and -35 elements vary in length between 17 and 18 bp (Fig. 7B). The *in silico* data therefore suggests that σ^{AntA} has highly conserved -35 and -10 binding sites at the *antG* and *antH* promoters of all antimycin producing

Streptomyces strains. Scanning the complete published *S. albus* genome with the AntA -10 and -35 binding sites (using GLEME2 – part of MEME) returns only two significant hits, the *antGF* and *antHIJKLMNO* promoters suggesting there are no other σ^{AntA} targets (results not shown). This strongly suggests that σ^{AntA} is a pathway-specific regulator of antimycin biosynthesis.

σ^{AntA} represents a new sub-family of ECF sigma factors. σ^{AntA} contains only the σ_2 and σ_4 domains (Pfam families PF04542 and PF08281) which is characteristic of the ECF family of RNA polymerase σ factors (Staron et al., 2009). However, σ^{AntA} does not fit into any of the ECF sub-families listed in the well-maintained public database ECF *Finder* (Staron *et al*., 2009). Multiple sequence alignments of the 14 σ^{AntA} homologues in the database and representatives of all known ECF sub-families revealed that the σ^{AntA} proteins form a distinct clade and therefore represent a new sub-family of ECF σ factors (Table S1 and Fig. S3). ECF σ factors are rare in secondary metabolite gene clusters and to our knowledge this is the first example in *Streptomyces* species (Foulston and Bibb, 2010; Sherwood and Bibb, 2013). The only obvious candidate for an anti-σ^{AntA} factor in the antimycin gene cluster is the putative membrane protein STRS4_02195. However, it is absent from the *ant* clusters in other streptomycetes, its removal has no effect on antimycin biosynthesis (Fig. 2) and it does not interact with σ^{AntA} in bacterial two-hybrid analysis (Fig S4) which leads us to conclude that σ^{AntA} is an orphan ECF that is not subject to anti-σ factor control. However, since *antA* expression is activated in substrate mycelium (by an as yet unknown regulator) and switched off following differentiation we predict that a mechanism might exist to remove σ^{AntA} protein at this stage of growth. The only unusual feature in the primary sequence of the 14 σ^{AntA} homologues is the conserved C-terminal Ala-Ala (AA) motif (Fig. S2) which is a known signal for the serine protease ClpXP (Flynn *et al*., 2003). To test whether the C-terminal AA residues are required for σ^{AntA} activity we made two identical constructs expressed under the control of the native *antB* promoter. The first construct drives production of the wild-type protein (designated $\sigma^{AntA\text{-}AA}$) and the second drives production of an altered protein in which the AA has been replaced with DD (designated $\sigma^{AntA\text{-}DD}$). We introduced these constructs into the *antA* mutant and measured expression of the *antGF* and *antHIJKLMNO* operons in these strains. Both operons are significantly more highly expressed in the strain producing $\sigma^{AntA–DD}$ compared with the wild type $\sigma^{AntA–AA}$ protein (Fig. 8). These data suggest that the two C-terminal residues play an important role in the stability and / or activity of σ^{AntA} and may target σ^{AntA} for proteolysis by ClpXP. Unfortunately, all attempts to detect the σ^{AntA} protein by immunoblotting whole cell extracts with polyclonal anti-σ^{AntA} antibodies have been unsuccessful while tagging the protein at

the N-terminus inactivates the protein (not shown). Future work will therefore be required to determine the role of the C-terminal AA motif in σ^{AntA} however, we have provided preliminary evidence that suggests σ^{AntA} might be a direct target for ClpXP, thereby bypassing the requirement for the additional level of anti-σ regulation. This would also provide a rapid mechanism to shut down precursor biosynthesis when antimycins are no longer required.

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Figure 1

The antimycin biosynthetic gene cluster in *Streptomyces albus* S4.

Genes shaded grey indicate those that are required for antimycin biosynthesis. Genes shaded black were experimentally determined not to be required for antimycin biosynthesis. Narrow black arrows indicate the presence of four operons and the direction of their transcription. The locations of cosmid 213 and cosmid 456 are indicated by horizontal lines and the double vertical hash indicates that cosmid 456 is comprised of additional DNA that falls outside the boundaries of this schematic.

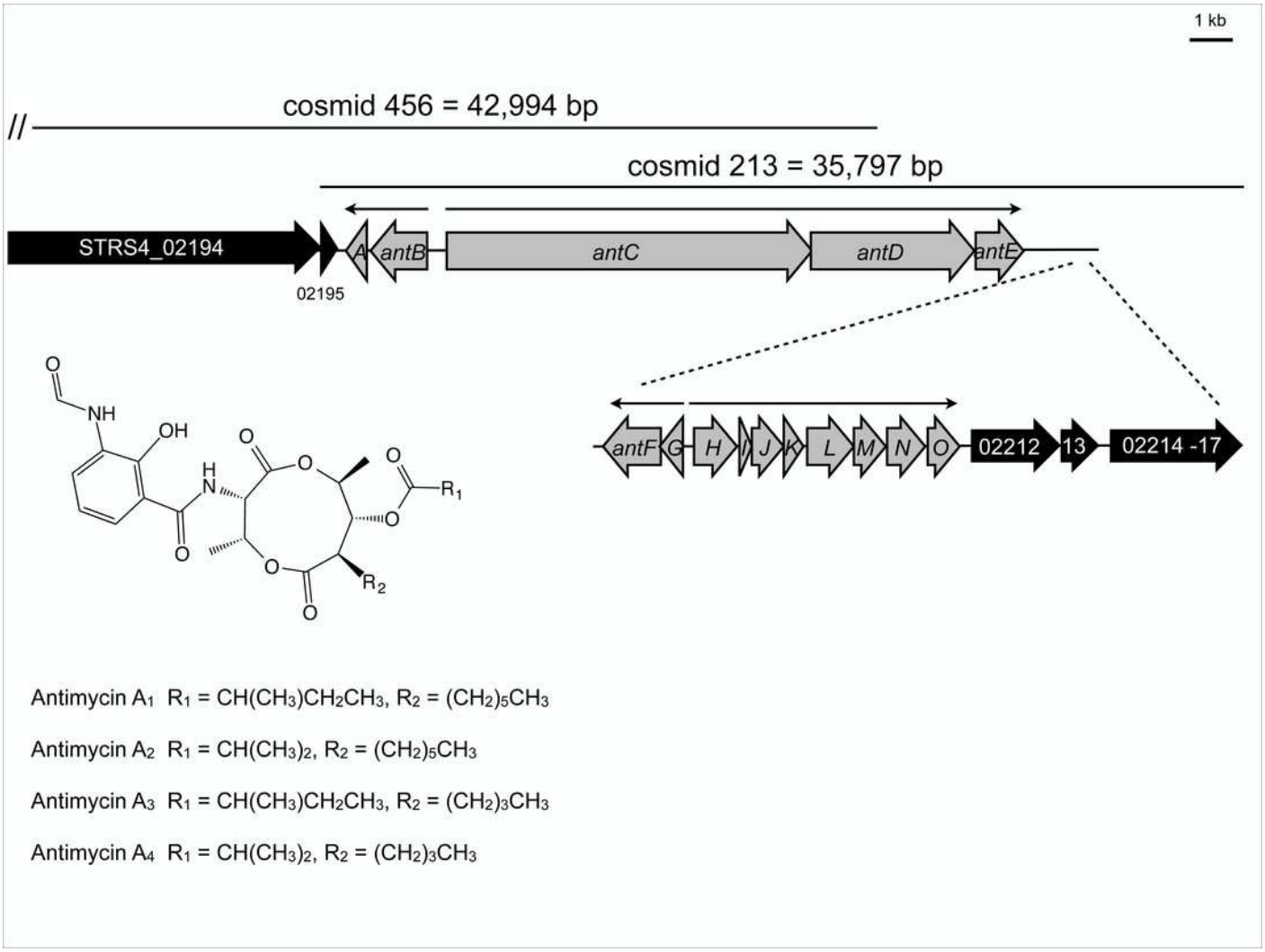

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Figure 2

Defining the boundaries of the antimycin gene cluster.

(A) *Streptomyces albus* S4 WT and mutant strains challenged with *Candida albicans.* Null mutants of genes adjacent to the gene cluster (coloured black in Fig. 1) produce an antimycin-positive phenotype, characterised by a large circular zone of cleared *C. albicans* growth. The Δ*antC* mutant strain displays an antimycin-negative phenotype, but retains residual antagonistic activity against *C. albicans* due to the production of candicidin, a second antifungal compound produced by this strain (Barke *et al.*, 2010; Seipke *et al.*, 2011a). (B) High-performance liquid chromatography (HPLC) of metabolites produced by *S. albus* S4 WT and mutant strains. The Δ*antC* mutant does not produce antimycins, while null mutations in genes adjacent to the antimycin cluster had no effect on antimycin production.

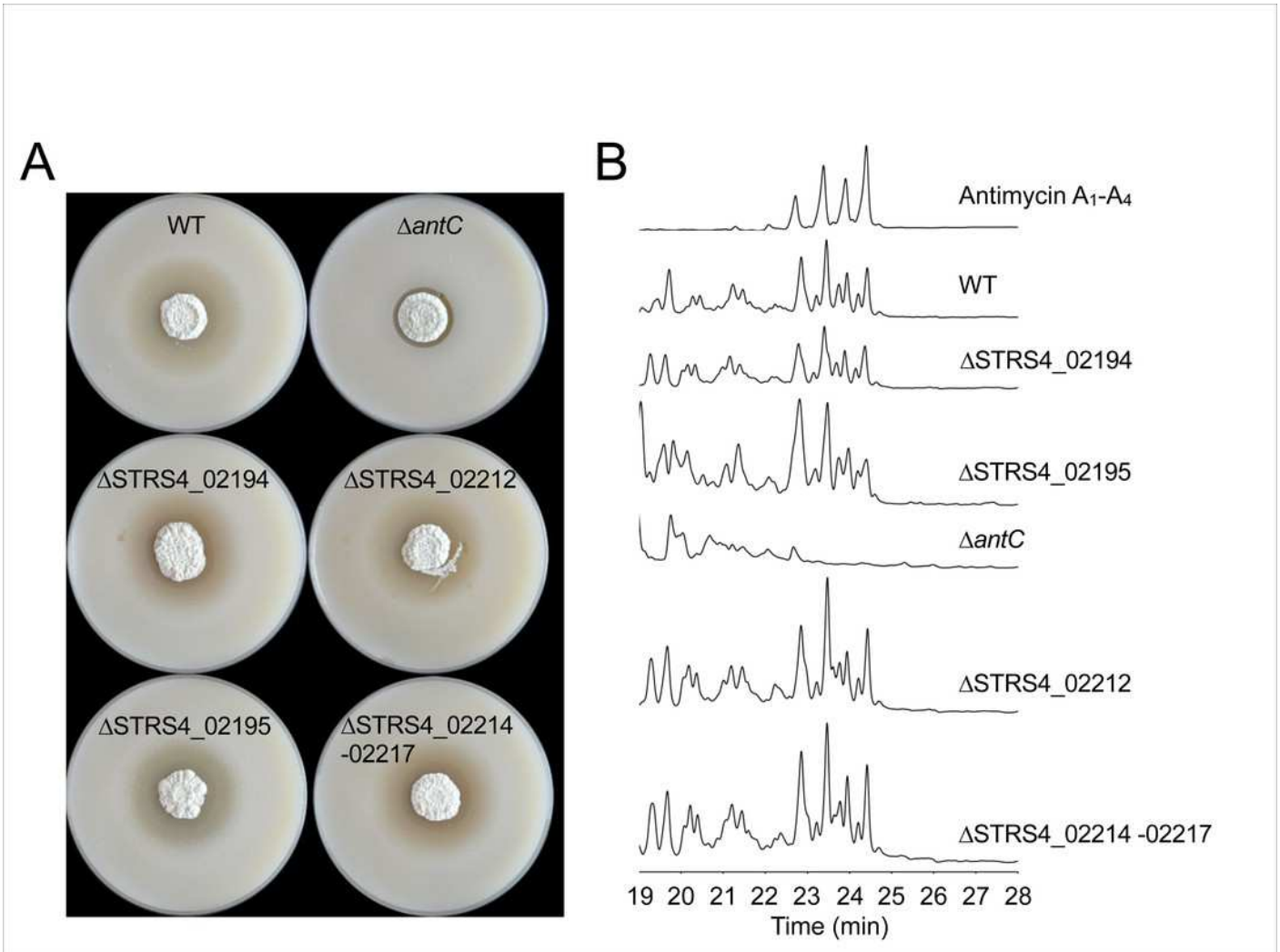

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Figure 3

There is a delay between expression of the antimycin biosynthetic genes and the production of antimycins.

(A) HPLC analysis of metabolites produced by *S. albus* S4 wild-type. Antimycins are detected in media extracts of 42 hour old but not 18 hour old cultures. (B) qRT-PCR analysis of the antimycin gene cluster in 18 and 42 hour old cultures shows that expression of the antimycin gene cluster is significantly down-regulated following differentiation. *** denote that values reported are statistically significantly different with a *P* value< 0.001 in a Student's T-test.

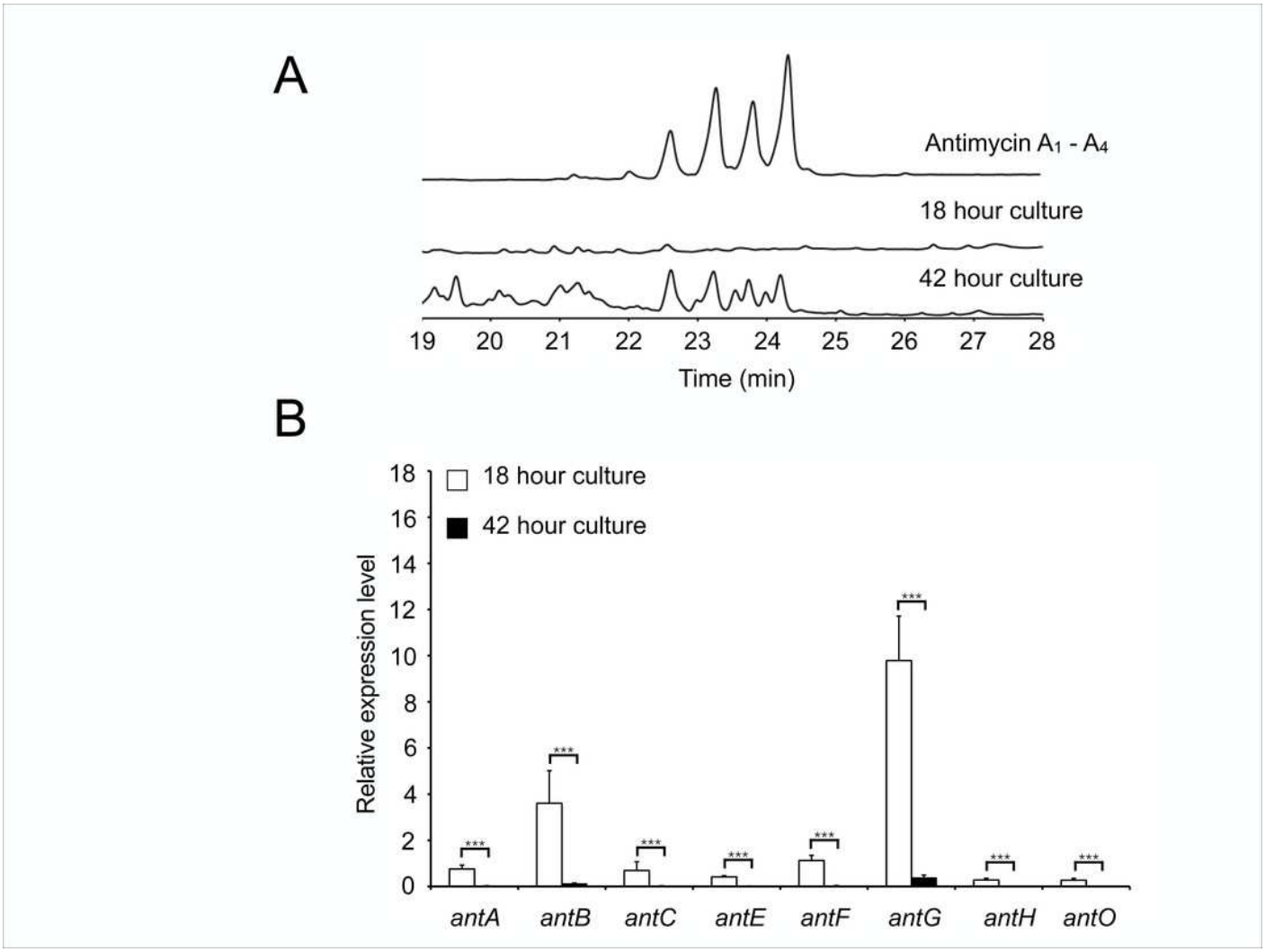

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Figure 4

σ^{AntA} is required for the biosynthesis of antimycins.

(A) *S. albus* S4 strains challenged with *Candida albicans.* The Δ*antA* null mutant shows dramatically reduced bioactivity compared to the wild-type strain and the complemented strain (Δ*antA*/pIJ10257-*antA*). The residual bioactivity of the Δ*antA* mutant is due to the continued production of candicidin, a second antifungal compound. (B) HPLC analysis of metabolites produced by *S. albus* S4 strains. Antimycins were only detected in extracts prepared from the wild-type and the Δ*antA*/pIJ10257-*antA* strains, and not Δ*antA* null mutant.

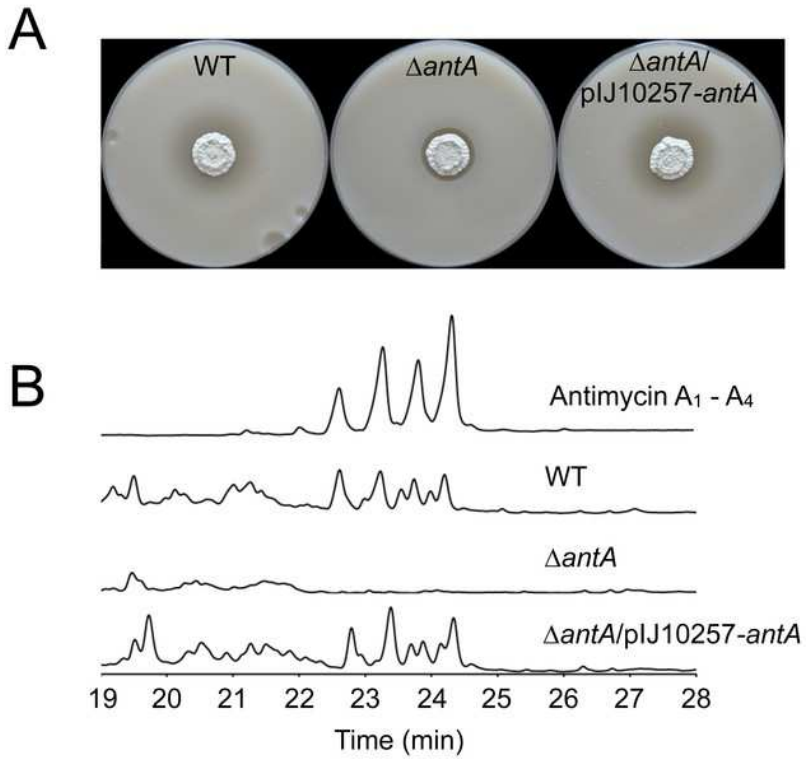

Figure 5

σ^{AntA} activates transcription of the *antFG* and *antHIJKLMNO* operons.

qRT-PCR analysis of antimycin genes in the wild-type and Δ*antA* strains after 18 hours growth. Transcription of *antFG* and *antHIJKLMNO* is significantly reduced in the Δ*antA* mutant strain, whereas transcription of *antBCDE* are unaffected. *** denote that values reported are statistically significantly different in a Student's *t* test with a *P* value< 0.001 in a Student's T-test.

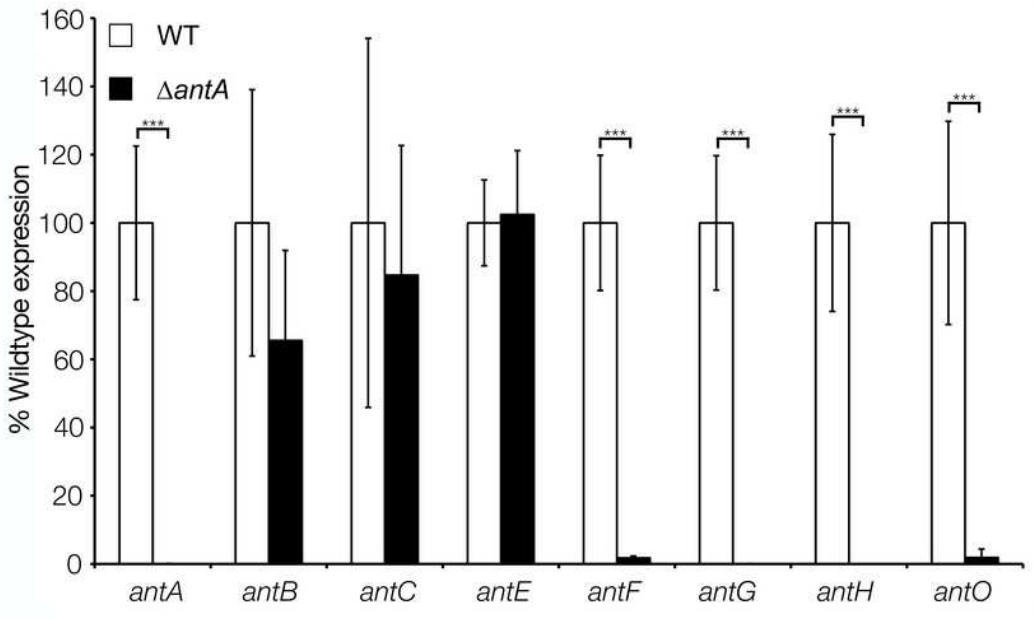

Figure 6

σ^{AntA} alone is sufficient to activate transcription of *antFG* and *antHIJKLMNO* in 42 hour old cultures.

qRT-PCR analysis of wild-type or Δ*antA*/pIJ10257-*antA* in 42 hour old cultures shows that repression of σ^{AntA}-regulated genes can be overcome by over-expressing *antA*.

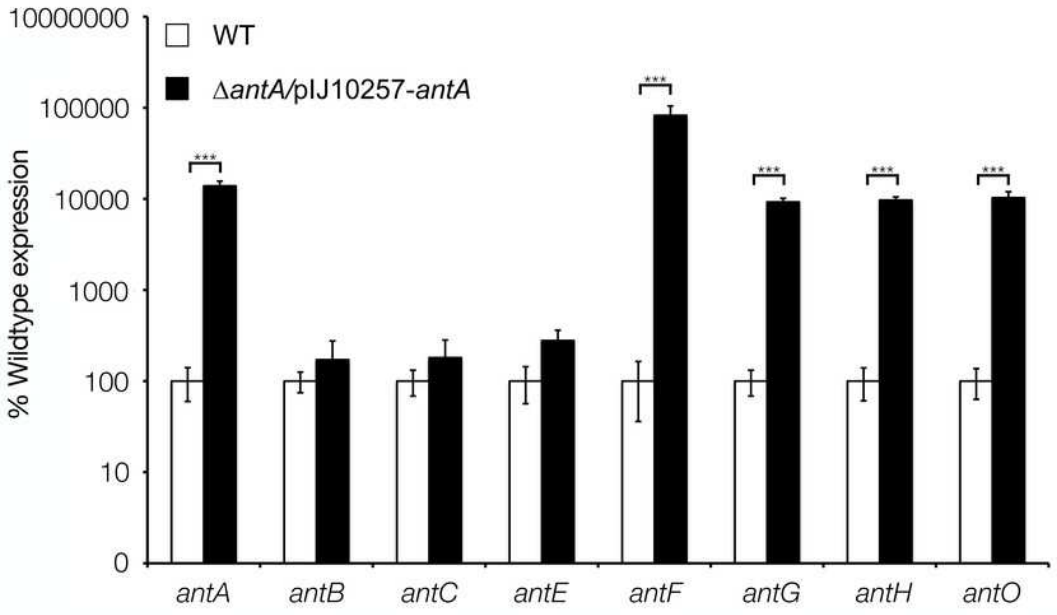

Figure 7

Identification of σ^{AntA} promoter motifs.

(A) The -10 and -35 motifs at the σ^{AntA}-target promoters of *antFG* and *antHIJKLMNO* are nearly 100% identical and display zero nucleotide identity with the promoter region of *antAB.* Shared identity is indicated by grey shading. The nucleotides mapped by 5'RLM-RACE is denoted by +1 and are shown in bold face (B) Conservation of the *S. albus* S4 *antFG* and *antHIJKLMNO* promoter elements in other antimycin-producing *Streptomyces* species. Conservation between the experimentally determined promoter region of *S. albus* S4 and the putative promoter regions of other antimycin producers is indicated by grey shading. (C) Consensus sequence for the -35 and -10 promoter elements recognised by σ^{AntA} displayed as a WebLogo (Crooks *et al.*, 2004). Below are the full strain names and accession numbers for antimycin-producing strains: *S. ambofaciens* ATCC 23877 (AM238663), *S. blastmyceticus* NBRC 12747 (AB727666), *S. gancidicus* BKS 13-15 (AOHP00000000), *S. griseoflavus* Tü4000 (ACFA00000000), *S. hygroscopicus* subsp. *jinggangensis* 5008 (NC_017765), *S. hygroscopicus* subsp. *jinggangensis* TL01 (NC_020895), *Streptomyces* sp. 303MFCol5.2 (ARTR00000000), *Streptomyces* sp. TOR3209 (AGNH00000000), *S. albus* S4 (CADY00000000), *S. albus* J1074 (NC_020990), *Streptomyces* sp. SM8 (AMPN00000000), *Streptomyces* sp. NRRL2288 (JX131329), *Streptomyces* sp. LaPpAH-202 (ARDM00000000), *Streptomyces* sp. CNY228 (ARIN00000000).

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A

S. albus S4 promoters mapped by 5'RLM-RACE

```
                 -35          N17          -10      +1
antGF:       CGGGGGATTGCCGCCGCCTCCTCGCGCCTCTTCCTCTGC--[61nt]-ATG
antHIJKLMNO: CGAGGGATAGCGGACCGGGGCGGACGACTCTTCCCGGGTA-[91nt]-GTG
antAB:       GGTGAATCCCTAAACGCGTGGCTCCTAGGGTGATGCCAA--[30nt]-GTG
```

B

Predicted σ^{AntA} binding sites in other antimycin producers

antFG promoter

antHIJKLMNO promoter

```
                      -35        N17/18        -10      +1
S. albus S4           CGGGGGATTGCCGCCGCCTCCTCGC_GCCTCTTCCTCTGC  CGAGGGATAGCGGACCGGGGCGGACGA_CTCTTCCCGGGTA
S. albus J1074        CGGGGGATTGCCGCCGCCTCCTCGC_GCCTCTTCCTCTGC  CGGGGGATAGCGGACCGGGCCGGACGA_CTCTTCCCGGGTA
S. SM8                CGGGGGATTGCCGCCGCCTCCTCGC_GCCTCTTCCTCTGC  CGGGGGATAGCGGACCGGGCCGGACGA_CTCTTCCCGGGTA
S. NRRL 2288          CGGGGGATTGCCGCCGCCTCCTCGC_GCCTCTTCCTCTGC  CGAGGGATAGCGGACCGGGGCGGACCA_CTCTTCCCGGGTA
S. LaPpAH-202         CGGGGGATTGCCGCCGCCTCCTCGC_GCCTCTTCCTCTGC  CGAGGGATAGCGGACCGGGGCGGACGA_CTCTTCCCGGGTA
S. CNY228             CGGGGGATTGCCGCCGCCTCCTCGC_GCCTCTTCCTCTGC  CGAGGGATAGCGGACCGGGGCGGACGA_CTCTTCCCGGGTA
S. ambofaciens        CCGGGGAGTGGAACGGCGCCGTGTCCGCCTCTTGAGGGGG  ACGGGGATAGCCGGGCCGCCCGCCCGGCCTCTTACCGCAAC
S. 303MFCol3.2        GCGGGGATTGTGGTTCGGCGCGGGG_ACCTCTTGTTCGGG  CGGGGGATTGCGGGTCTCCCGCACGGC_CTCTTTCCCAGAC
S. gancidicus         CGGGGGAGTGCCGGCCGCCCTCGCC_GCCTCTTCTTCTGG  GCGGGGATAGCGGGCGCGGTGCGCGGC_CTCTGTTCTCGGT
S. TOR3209            CCGGGGAGTGGGAGCCGCGGGCGCCCGCCTCTTGTGGGGG  GCGGGGATAGCCGGACCGTCCGTCCGGCCTCTTACCGCAAC
S. hygroscopicus TL01 CCGGGGAGTGCGGGCGGCCGCGACC_GCCTCTTGAGGGGG  GCAGGGATAGCCGGGCCGCCCGCCCGGCCTCCTTACCGGAA
S. hygroscopicus 5008 CCGGGGAGTGCGGGCGGCCGCGACC_GCCTCTTGAGGGGG  GCAGGGATAGCCGGGCCGCCCGCCCGGCCTCCTTACCGGAA
S. griseoflavus       CCGGGGAGTGCGGCCGGACGCGGCG_GCCTCTTGAGGAGG  CCGGGGATAGCCGGGCCGCACGCCCGGCCTCTTACCGCAAC
S. blastmyceticus     GGAGGGAGAGCGGAGCCGCCCGGCC_GACTCTTCTCTCAG  GCGGGGATGGAACTCCCCTGCCCCGTA_CTCCTTATGTCTG
```

C

-35 consensus

-10 consensus

Figure 8

Altering the terminal Ala-Ala motif of σ^{AntA} results in higher expression of σ^{AntA} targets.

The Δ*antA* null mutant was complemented with either a wild-type copy of *antA* or a variant of *antA* encoding A172D and A173D point mutations. After 42 hours of growth, transcription of σ^{AntA}-targets in the Δ*antA*/*antA*-DD strain were significantly greater than both the wild-type and Δ*antA*/*antA*-AA strains, suggesting the terminal Ala-Ala motif modulates stability and/or activity of σ^{AntA}. ** and *** denote that values reported are statistically significantly different with a *P* value< 0.01 and 0.001 in a Student's T-test, respectively.