Regulation of antimycin biosynthesis by the orphan ECF RNA polymerase sigma factor σAntA

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**INTRODUCTION**

 Approximately60% of the antibiotics and anticancer compounds currently used in human medicineare 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 al*., 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-xL-related anti-apoptotic proteins which are over-produced by drug resistant cancer cells. Over-production of Bcl-2/Bcl-xL 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 A3, 2-methoxyantimycin A3 (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 proteinsencode the biosynthetic pathway for the unusual starter unit, 3-aminosalicylate-CoA (Schoenian et al., 2012; Sandy et al., 2012). The AntCDproteins comprise the hybrid NRPS / PKS machinery, and AntEand 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 σAntA which, like all other ECF σ factors, contains only two of the four σ70 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 σAntA, 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 σAntA because they are co-encoded with, and regulated by, anti-σ factors whereas σAntA 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 σMibX regulate microbisporicin biosynthesis and σMibX is regulated by MibW (Foulston and Bibb, 2010)*.* In *Planomonospora alba* the pathway specific regulator PspR, the ECF sigma factor σPspX and its anti-σ factor PspW all regulate production of the lantibiotic planosporicin (Sherwood and Bibb, 2013). The closest homologues to σMibX and MibW are σPspX 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 σAntA in *Streptomyces albus* S4. We report that σAntA 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 σAntA represents a new sub-family of ECF σfactors that are only found in the *ant* gene clusters of *Streptomyces* species and provide evidence that suggests σAntA regulation of the divergent *antGF* and *antHIJKLMNO* operons is conserved in all antimycin producing strains. Finally we provide preliminary evidence that the activity of σAntA 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 σAntA 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 σAntA 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 al*., 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*, ∆*antA, ΔantC*) or cosmid 213 (∆*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 *02212* knockouts, respectively (Table S2).

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

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

*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 al*., 2012)), *Streptomyces* sp. LaPpAH-202 (ARDM00000000), *Streptomyces* sp. CNY228 (ARIN01000033). σAntA proteins were aligned to five (when possible) random proteins from each ECF RNA polymerase σ factor subfamily defined by Staron *et al* (Staron *et al*., 2009) by using ClustalΩ (Sievers *et al*., 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 A1–A4 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 σAntAin regulating antimycin production.

***Antimycin production is dependent on the orphan ECF sigma factor σAntA.*** To investigate the role of σAntA in regulating antimycin biosynthesis, we deleted the *antA* gene and tested the mutant strain against *C. albicans* in a bioassay*.* The *antA* mutantis 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* geneunder the control of the strong constitutive *ermE\** promoterrestores 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 σAntA is required for antimycin production

 To determine which of the four *ant* promoters are regulated by σAntA 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 σAntA 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 σAntA 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* promotersmust 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.

***σAntA 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 σAntA-independent*antB* promoter (Fig. 7A). Six antimycin producing *Streptomyces* strains have been reported previously (Riclea *et al.*, 2012; Seipke *et al.*, 2011a; Yan *et al*., 2012) and we identified eight more putative antimycin gene clusters whilst searching for σAntAorthologues in Genbank (Table S1 and Experimental Procedures). Since the 14 known σAntA orthologues share 66% sequence identity (Table S1, Fig. S2), we hypothesise that σAntA 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 σAntA 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 σAntA targets (results not shown). This strongly suggests that σAntA is a pathway-specific regulator of antimycin biosynthesis.

***σAntA represents a new sub-family of ECF sigma factors.*** σAntAcontains only the σ2 and σ4 domains (Pfam families PF04542 and PF08281) which is characteristic of the ECF family of RNA polymerase σ factors (Staron et al., 2009). However, σAntA 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 σAntA homologues in the database and representatives of all known ECF sub-families revealed that the σAntA 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-σAntA factor in the antimycingene 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 σAntA in bacterial two-hybrid analysis (Fig S4) which leads us to conclude that σAntA is an orphan ECF that is not subject to anti-σ factor control. However, since *antA* expressionis activated in substrate mycelium (by an as yet unknown regulator) and switched off following differentiation we predict that a mechanism might exist to remove σAntA protein at this stage of growth. The only unusual feature in the primary sequence of the 14 σAntA 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 σAntA 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 σAntA-AA) and the second drives production of an altered protein in which the AA has been replaced with DD (designated σAntA-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 σAntA–DD compared with the wild type σAntA–AA protein (Fig. 8). These data suggest that the two C-terminal residues play an important role in the stability and / or activity of σAntA and may target σAntA for proteolysis by ClpXP. Unfortunately, all attempts to detect the σAntA protein by immunoblotting whole cell extracts with polyclonal anti-σAntA 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 σAntA  however, we have provided preliminary evidence that suggests σAntA might be a direct target for ClpXP, thereby bypassing the requirement for the additional level of anti-σregulation. This would also provide a rapid mechanism to shut down precursor biosynthesis when antimycins are no longer required.

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