Complex Intra-Operonic Dynamics Mediated by a Small RNA in *Streptomyces coelicolor*

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**Abstract**

*Streptomyces* are predominantly soil-dwelling bacteria that are best known for their multicellular life cycle and their prodigious metabolic capabilities. They are also renowned for their regulatory capacity and flexibility, with each species encoding >60 sigma factors, a multitude of transcription factors, and an increasing number of small regulatory RNAs. Here, we describe our characterization of a conserved small RNA (sRNA), scr4677. In the model species *Streptomyces coelicolor*, this sRNA is located in the intergenic region separating SCO4676 (an anti-sigma factor-encoding gene) and SCO4677 (a putative regulatory protein-encoding gene), close to the SCO4676 translation start site in an antisense orientation. There appears to be considerable genetic interplay between these different gene products, with wild type expression of scr4677 requiring function of the anti-sigma factor SCO4677, and scr4677 in turn influencing the abundance of SCO4676-associated transcripts. The scr4677-mediated effects were independent of RNase III (a double stranded RNA-specific nuclease), with RNase III having an unexpectedly positive influence on the level of SCO4676-associated transcripts. We have shown that both SCO4676 and SCO4677 affect the production of the blue-pigmented antibiotic actinorhodin under specific growth conditions, and that this activity appears to be independent of scr4677.

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**Introduction**

*Streptomyces* bacteria are best known for their complex developmental life cycle and their vast secondary metabolic capabilities, which include producing the majority of naturally synthesized antibiotics. The *Streptomyces* life cycle involves transitioning between several differentiated states (reviewed in [1], [2]), and initiates with the germination of a single spore. Germ tube elongation and subsequent hyphal tip extension and branching lead to the formation of a filamentous cellular network known as the vegetative or substrate mycelium. Reproductive aerial hyphae are raised from the vegetative mycelium, with these aerial structures ultimately being converted into chains of dormant exospores. Aerial hypha formation is coupled, both genetically and temporally, with the onset of secondary metabolism and antibiotic production [3]. In the model organism *Streptomyces coelicolor*, the different growth and metabolic stages are readily distinguishable. During growth on agar media, aerial hyphae formation results in a fuzzy white colony appearance, while sporulation culminates with the production of a grey polyketide pigment that turns colonies grey [4]. Equivalent visual cues accompany antibiotic production, courtesy of the fact that *S. coelicolor* produces both blue and red pigmented antibiotics (actinorhodin and undecylprodigiosin, respectively).

The streptomycetes are predominantly found in the soil and have large genomes, ranging in size from 7–12 Mbp [3], [6], [7]. This presumably provides them with the genetic flexibility to adapt and respond to diverse environmental stresses. Consistent with this proposal is the observation that bacterial species with large genomes (>6 Mbp) typically encode a greater proportion of transcription regulators than those with smaller genomes [8]. Notably, *S. coelicolor* devotes more than 12% of its protein-coding genes to regulation [5], and the number of annotated regulatory non-coding RNAs continues to increase [9], [10], [11], [12], [13].

Like their protein counterparts, non-coding RNAs can have positive or negative regulatory impacts on their cellular targets. In bacteria, small RNAs (sRNAs), which typically range in size from 40–500 nucleotides, are the best studied of the non-coding RNAs. Most sRNAs exert their regulatory effects by either modulating the activity of a target protein, or more commonly, by base-pairing with target mRNAs and affecting their stability or translatability (reviewed in [14]). mRNA-targeting sRNAs can be divided into two categories based on their genomic context: trans-encoded regulatory RNAs are expressed at sites distinct from those of their target genes and often share only partial sequence complementarity with their target mRNAs, while cis-encoded (antisense) regulatory RNAs are expressed where they act and share complete complementarity with their mRNA targets.

Base pairing between antisense regulatory RNAs and their cognate sense mRNAs frequently leads to the formation of structures that can be recognized by the double strand-specific ribonuclease RNase III (e.g. [15], [16], [17]). In *S. coelicolor*, RNase III is a global regulator of antibiotic biosynthesis [18], [19], and it...
affects the abundance of anywhere between 79 and 200 transcripts, including those of at least two regulatory RNAs [20], [21]. Although it is well-established that RNase III acts as a pleiotropic regulator in S. coelicolor, regulatory connections between RNase III and cis-encoded regulatory RNAs have not been explored in any depth.

Increasing numbers of chromosomally-encoded antisense RNAs are being identified as a result of genome-wide studies in many bacteria [12], [22], [23]. In the streptomycetes, antisense RNAs have now been reported by a number of research groups (e.g. [10], [11], [12], [13]). One non-coding RNA having cis-regulatory potential is scr4677, a small RNA transcribed immediately upstream of SCO4676, in the intergenic region between SCO4676 and SCO4677. We found there to be intriguing genetic connections between the different products expressed from this locus, with wild type expression of scr4677 requiring the activity of SCO4677, scr4677 affecting the levels of SCO4676-associated transcripts, and RNase III unexpectedly having a positive effect on SCO4676 and SCO4677 levels while having no effect on scr4677. We found that modulating the levels of scr4677 had no obvious phenotypic consequence, deleting SCO4676 led to enhanced actinorhodin production under defined growth conditions, and loss of SCO4676 influenced both sporulation and antibiotic production.

Materials and Methods

Bacterial Strains and Growth Conditions
Streptomyces and Escherichia coli strains used in this study are summarized in Table 1. S. coelicolor strains were grown at 30°C on R2YE (rich, glucose-containing), minimal medium (MM) supplemented with mannitol or glucose, MYM (maltose, yeast extract, malt extract), SMMS (supplemented minimal medium solid), SFM (soy flour with mannitol or glucose) agar or in liquid R5 (rich, glucose-containing), YEME-TSB (yeast extract, malt extract – tryptone soya broth), or NNMP (minimal) media, as described previously [24], [25]. Streptomyces avermitilis was also grown on MYM agar medium, while Streptomyces venezuelae was grown in liquid MYM medium. E. coli strains were grown at 37°C in Luria-Bertani (LB) or SOB medium [26], with the exception of E. coli BW25113 containing pJ790 (Table 1), which was grown at 30°C.

RNA Isolation and Transcript Analysis
RNA was isolated and northern blot analysis was conducted as described previously [10], [13]. Probes used for detection of scr4677 by northern blotting included 4677-1 and 4677-LNA (locked nucleic acid probe) (Table S1). Detection of the scr4677 homologues in S. avermitilis and S. venezuelae was achieved using oligonucleotide probes svs3556 and sav3140, respectively (Table S1). S1 nuclease mapping of the SCO4676 transcript start site was performed as outlined previously [27] with the probe generated by PCR amplification using one radiolabelled oligonucleotide (4676 HOT) and one unlabeled primer (465717) (Table S1), together with cosmid StD31 (Table 1) as template. Semi-quantitative RT-PCR was conducted as described by Hinder et al. [28], using the primers listed in Table S1. The number of amplification cycles was optimized for each primer combination to ensure that the reaction products were being generated during the linear phase of the amplification process (cycle numbers ranged from 13 for 16S rDNA, to 32 for the cDNA for the SCO4677-SCO4676 read-through transcript). Negative control reactions involved adding RNA (without prior reverse transcription) as template to ensure that any amplification observed was not due to DNA contamination of any reaction component. 16S rRNA- or rpoB-specific cDNAs were PCR amplified as controls for RNA levels and RNA integrity. PCR products were separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. All analyses were conducted in triplicate (at a minimum), using samples from at least two independent RNA time courses.

Construction of sRNA Overexpression Constructs
The scr4677 overexpression strain was constructed by cloning scr4677 into the high copy E. coli-Streptomyces plasmid pWHM3 (Table 1). scr4677 was PCR amplified using primers 4677-3E and 4676 HOT (Table S1) with cosmid StD31 as template DNA, to give a product that was expected to encompass all associated regulatory elements (extending 159 nt upstream and 188 nt downstream of scr4677). The resulting product was first cloned into the Smal site of pJ2923 to give pMC148, and construct integrity was confirmed by sequencing. scr4677, together with its flanking sequences, was then excised as a BglII fragment and cloned into the BamHI site of pWHM3, to give pMC149. This construct, alongside empty pWHM3, was then passaged through the non-methylating E. coli strain ET12567 (Table 1) before being introduced into S. coelicolor M145 using protoplast transformation [24].

Deletion Strain Construction and Mutant Complementation
SCO4676 and SCO4677 deletion strains were constructed using RedDirect technology [29]. Both genes were individually replaced with an apramycin resistance cassette (amplified using primer pairs KO4676-1/KO4676-2 and KO4677-1/KO4677-2; Table S1), first within cosmid StD31, and then in the S. coelicolor M145 chromosome through double-crossover homologous recombination. Mutations were confirmed using BamHI restriction enzyme digestions (cosmid) and by PCR (cosmid and chromosome) using diagnostic primer combinations (upstream and downstream of SCO4676 or SCO4677, where wild type and mutant product sizes differed; and upstream and within the deleted coding sequence, where a product would only be detected if a wild type copy of the gene remained; see Table S1 for primer details). For SCO4676, where gene replacement with an apramycin resistance cassette might exert polar effects on the downstream SCO4675 gene, the resistance cassette, which was flanked by FRT (Flipase recognition target) sites, was excised from the genome. A plasmid encoding the Flp recombinase enzyme (pUWLFLP) (Table 1) was conjugated into the SCO4676 mutant strain. Resulting exconjugants were screened for loss of apramycin resistance and were subsequently confirmed using PCR (KO 4676-1IN and 4675-2IN; Table S1).

Wild type SCO4676, together with 261 bp of upstream and 211 bp of downstream sequence, was PCR amplified using phosphorylated oligonucleotides 4677-3E and 4675-2IN (Table S1). The amplified fragment was introduced into pJ2925 digested with SmaI. The resulting plasmid clone (pMC145; Table 1) was sequenced to confirm SCO4676 integrity before it was excised using BglII and introduced into BamHI-digested pJJ82 (Table 1). The resulting construct (pMC146; Table 1) was introduced into E. coli ET12567/pUZ8002, and conjugated into the SCO4676 deletion mutant, followed by selection for plasmid integration into the chromosome.
**Table 1.** Bacterial strains and plasmids used in this study.

| Streptomyces strains | Genotype, description or use | Reference |
|----------------------|-----------------------------|-----------|
| S. coelicolor M145   | SCP1, SCP2                  | [24]      |
| S. coelicolor E320   | M145 ASCO4676-3::aac(3)IV   | This work |
| S. coelicolor E321   | M145 ASCO4676               | This work |
| S. coelicolor E322   | M145 ASCO4677-3::aac(3)IV   | This work |
| S. coelicolor Δrnc    | M145 rnc::aac(3)IV          | [17]      |
| S. avermitilis MA-4680 | Wild type                | [6]       |
| S. venezuelae ATCC 10712 | Wild type                |           |

**E. coli strains**

|                         |                             |           |
|-------------------------|-----------------------------|-----------|
| DH5α                    | Plasmid construction and general subcloning | Invitrogen |
| ET12567/pUZ8002         | Generation of methylation-free plasmid DNA and conjugation of oriT-containing plasmids | [45] [46] |
| BW25113                | Construction of cosmid-based knockouts | [29]      |

**Plasmids/Cosmids**

|                         |                             |           |
|-------------------------|-----------------------------|-----------|
| pU790                   | Temperature sensitive plasmid carrying I-RED genes | [29]      |
| pU2925                  | General cloning vector      | [47]      |
| pWHM3                   | High copy number E. coli/Streptomyces plasmid | [48]      |
| Std31                   | Cosmid for SCO4676 and SCO4677 knockouts and PCR amplification | [49]      |
| pUWLFLP                 | Plasmid carrying the Flp recombinase-encoding gene | [50]      |
| pU82                   | Complementation of mutant strains | Gift from H. Kieser |
| pMC148                  | pU2925+ scr4677             | This work |
| pMC149                  | pWHM3+ scr4677              | This work |
| pMC145                  | pU2925+ SCO4676             | This work |
| pMC146                  | pU82+ SCO4676               | This work |
| pRT801                  | Integrative cloning vector  | [51]      |
| pMC150                  | pRT801+ SCO4676-3×FLAG      | This work |

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Construction of FLAG-tagged SCO4676 and Detection by Immunoblotting

To determine whether SCO4676 protein levels were impacted by scr4677, we created a C-terminally 3×FLAG-tagged fusion protein (creating an N-terminal fusion was complicated by the existence of scr4677-associated regulatory sequences that extended up to ~150 nt into the SCO4676 coding sequence). Using pMC145 (pIJ2925 carrying the SCO4676 complementing sequence; **Table 1**), overlap extension PCR was used to introduce an XhoI site at the C-terminal end of the coding sequence (between the sequences encoding the fourth and fifth final residues of the protein). Following digestion with XhoI, the linearized plasmid was ligated with a 3×FLAG-cassette with XhoI-compatible overhangs. FLAG-tag orientation was initially confirmed by PCR using primers USFLAGXho and 4675-2IN (**Table S1**), and the sequence integrity of the entire construct was verified by sequencing. The SCO4676-3×FLAG fusion was excised using BglII, and was introduced into the integrating vector pRT801 digested with BamHI, to give pMC150 (**Table 1**). As described for the complementation construct above, the plasmid was passed through the methylation deficient E. coli ET12567/pUZ8002, conjugated into the unmarked SCO4676 deletion mutant (E321; **Table 1**), and selected for using apramycin. In parallel, untagged SCO4676 was cloned into an integrating plasmid vector and introduced into S. coelicolor E321 to serve as a negative control.

Mycelium from plate-grown cultures was harvested after 20, 64 or 72 h, and equivalent amounts of biomass were resuspended in lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 10% [v/v] glycerol, and 150 mM NaCl), together with lysozyme (750 μg/mL) and protease inhibitor (Roche), as per the manufacturer’s instructions. The suspensions were sonicated on ice, centrifuged, and the soluble proteins retained. Equal volumes (20 μL) of protein extract and loading dye were mixed, heated at 95°C for 5–15 min before the proteins were separated using SDS-PAGE. Alternatively, soluble proteins were precipitated prior to electrophoresis, using a trichloroacetic acid-acetone mixture (1:9), before being resuspended in Tris buffer (pH 7.5) such that proteins were concentrated four fold. Size-fractionated proteins were transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad), and were probed with anti-FLAG antibodies (1/1500 dilution; Cell Signaling Technology), and anti-rabbit secondary antibodies (1/3000 dilution; Cell Signaling Technology) as per Duong et al. [52].

Phenotypic Comparisons of Wild Type, Mutant and Overexpression Strains

For phenotypic analyses, approximately 10⁶ spores were streaked out on a variety of solid media (outlined above). Pigmented antibiotic production and morphological development were compared among strains over a week-long time course. Antibiotic production was visually assessed during plate growth, while morphological development was assessed both visually and using light microscopy. All experiments were conducted a...
minimum of three times, using at least two independent spore stocks of all strains.

Spores of different S. coelicolor strains were also subjected to various stress conditions. This included exposure to heat (60 °C, as per [30]) for up to 15 min, 1% (v/v) SDS, 50 mM EDTA, 30 mg/ml lysozyme, 16 mg/ml vancomycin, 0.1 M dipyridyl, 0.05 M diamide, and 0.1 and 0.25% (v/v) hydrogen peroxide. Briefly, soft nutrient agar (0.4 g nutrient broth and 1.15 g nutrient agar per 100 mL medium) was inoculated with approximately 10^9 spores. This mixture was overlaid onto nutrient agar plates. Paper disks saturated with 20 μL of each compound were placed onto the solidified soft agar, and the plates were then incubated at 30 °C for 1 and 2 days. The diameter of any growth inhibitory zones was measured on both days.

To compare levels of actinorhodin production in liquid-grown cultures, 10 mL YEME-TSB was inoculated with ~10^7 spores/mL and these cultures were grown overnight at 30 °C in a shaking incubator. Five hundred microliters of overnight culture was then used to inoculate 40 mL of liquid minimal medium (NMMP), and these cultures were grown for a further 72 h. All comparisons were conducted using at least three independently grown cultures. Every 24 hours, duplicate 0.5 mL aliquots were removed, and an equal volume of 2 M KOH was added. Samples were then subjected to absorbance measurement at 450 nm. These cultures were grown for a further 72 h. All comparisons were conducted using at least three independently grown cultures. Every 24 hours, duplicate 0.5 mL aliquots were removed, and an equal volume of 2 M KOH was added. Samples were then subjected to absorbance measurement at 450 nm. These cultures were grown for a further 72 h. All comparisons were conducted using at least three independently grown cultures. Every 24 hours, duplicate 0.5 mL aliquots were removed, and an equal volume of 2 M KOH was added. Samples were then subjected to absorbance measurement.

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**Results**

**Comparative and Bioinformatics Analysis of scr4677 and its Surrounding Genetic Region**

In a previous study investigating small RNAs in S. coelicolor, we identified a small RNA termed scr4677 (where ‘scr’ stands for S. coelicolor RNA) [10]. Comparative analyses using select Streptomyces genome sequences available through StrepDB, the Broad Institute and NCBI websites suggested that scr4677 was widely conserved in the streptomycetes, being found in ~60% of genomes surveyed (Table 2). In S. coelicolor, scr4677 was flanked by SCO4677 and SCO4676, the latter of which appeared to be the first gene of a two-gene operon (SCO4676-4675) (Figure 1A). We therefore investigated the frequency with which these three genes were associated with scr4677. The greatest correlation was observed for SCO4676, which was always located immediately adjacent to scr4677, whenever scr4677 was present (Figure 1A; Table 2); the rest of the intergenic region housing scr4677 did not exhibit the same level of sequence conservation. SCO4676 encodes a putative DNA binding protein with a predicted N-terminal helix-turn-helix motif. Full length homologues were found only in the streptomycetes, but similar predicted DNA binding domains were identified in hypothetical proteins from a wide variety of Firmicutes. In S. coelicolor, SCO4675 was positioned 18 bp downstream of SCO4676. Despite this apparent close coupling, these genes were co-localized in fewer than 10% of Streptomyces chromosomes (Table 2). The function of SCO4675 is unknown, but homologous proteins are encoded

| **Table 2. Conservation of scr4677 and its surrounding genes** |
|--------------------------------------------------------------|
| **S. avermitilis** | ✓ | ✓ | ✓ | ? |
| **S. bingchenguensis** | ✓ | ✓ | ? | ? |
| **S. coelicolor** | × | ✓ | ✓ | × |
| **S. griseaenuantiacicus** | × | ✓ | ? | ? |
| **S. griseoflavus** | ✓ | ✓ | ✓ | ? |
| **S. griseus** | ✓ | ✓ | ✓ | ? |
| **S. lividans** | ✓ | ✓ | ✓ | ✓ |
| **S. scabies** | ✓ | ✓ | ? | ? |
| **S. sp. roseospinors** | × | ✓ | ? | ✓ |
| **S. sp. C** | ✓ | ✓ | ? | × |
| **S. sp. e14** | × | × | ✓ | ? |
| **S. sp. S4** | × | ✓ | ? | × |
| **S. sp. W007** | × | ✓ | ✓ | ? |
| **S. sviceus** | ✓ | ✓ | ? | ? |
| **S. venezuelae** | ✓ | ✓ | ✓ | ? |
| **S. viridochromogenes** | ✓ | ✓ | ? | ? |
| **S. zinciresistens** | ✓ | ✓ | ✓ | ? |

Conservation is based on similarity scores of >50%, covering >80% of the corresponding protein sequence; conservation for scr4677 required >60% identity over the entire sRNA sequence (conservation in the remaining intergenic sequence was found to be only ~50%).

*Reflects the presence of the gene in the chromosome, but not adjacent to scr4677 or SCO4676.*

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**Cell Free Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)**

Cell free extracts were prepared from S. coelicolor grown on cellophane discs overlaying 250 or 350 mL R2YE agar medium in 1.9 or 3 L glass dishes, respectively. Between 12–20 g of biomass (per harvest) was mixed in lysis buffer (as above) such that 0.9 mL lysis buffer was added per 100 mg biomass. Protease inhibitor (Roche) was then added to the suspension as per the manufacturer’s instructions, followed by lysozyme at a concentration of 2.5 mg/mL. The resulting mixture was incubated on ice for 45 min before being homogenized using a glass tissue grinder, and passed through a French press (Thermo Scientific) three times. The soluble fraction was separated by centrifugation at 16,000 g for 4°C for 20 min.

Probes used for EMSAs comprised complementary oligonucleotides (Table S1) that were first annealed by incubating equimolar amounts of each oligonucleotide in annealing buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 60 mM NaCl), heating to 95°C for 10 min and then slow-cooling to room temperature. Thirty picomoles of the annealed oligonucleotides were then 5’ end-labelled using [γ-32P]ATP.

Each 20 μL-binding reaction consisted of 10 mM Tris-HCl pH 7.5, 1 mM DTT, 1 μg poly dI/dC, 10% (v/v) glycerol, 0.5 to 25 nM [γ-32P]-labelled DNA fragment, and varying amounts of fresh cell-free extract. Competition assays were conducted by adding 1 μM of either unlabeled probe DNA (specific competitor), or 1 μM of unlabeled non-specific competitor DNA (from within SCO3287; Table S1; [28]). After being incubated at 30°C for 25–30 min, samples were separated on a non-denaturing 8% polyacrylamide gel. Electrophoresis was performed in 1×TBE buffer at 100 V for 50–60 min. Gels were then transferred to filter paper, wrapped using plastic wrap, and exposed to Kodak Biomax XAR films for 1 h at room temperature.
throughout the actinobacteria and the fruiting body-forming myxobacteria.

Upstream of scr4677 is the best characterized gene in the region: SCO4677. Like SCO4676, SCO4677 is generally conserved in the streptomycetes, and while it was associated with SCO4676 and scr4677 more frequently than SCO4675, it co-localized with these genes only ~30% of the time (Table 2). SCO4677 encodes a predicted anti-sigma factor that, unusually, is not clustered together with a cognate sigma factor- or anti-anti sigma factor-encoding gene. Instead, SCO4677 is predicted to affect the activity of the sporulation-specific sigma factor \( \sigma^F \) (encoded by SCO4035), and to interact with putative anti-anti sigma factors encoded by SCO0781 and SCO0869 [32]. SCO4677 is a direct target of the developmental regulator BldD [33] and appears also to be controlled by the WD-40 domain-containing protein WdpB (SCO5953) [34]. Expression of SCO4676, but not SCO4675, is also affected by BldD activity, while the expression of both genes is impacted by the loss of wdpB [34].

**Figure 1.** Organization, conservation and expression of scr4677. A) Schematic illustration (not to scale) of the genetic region surrounding scr4677. scr4677 is located 14 nt upstream of SCO4676, in the 248 nt intergenic region between SCO4677 and SCO4676, encoded on the strand opposite these two protein-coding genes. B) and C) scr4677 is found upstream of SCO4676 orthologues in many Streptomyces species, including S. coelicolor (left, where SCO4676 is co-transcribed with both SCO4677 and SCO4675), S. avermitilis (middle) and S. venezuelae (right). In B), the green colour denotes gene expression from the negative (antisense) strand, while red indicates expression from the positive (sense) strand. In C) expression of scr4677 (left) and its orthologues sar3140 (middle) and svr3556 (right) was investigated using RNA harvested throughout the developmental cycle (first timepoint: vegetative growth; second timepoint: aerial hyphae/fragmentation; third timepoint: sporulation) using northern blot analysis. D) Northern blot analysis of scr4677 expression in S. coelicolor following growth on minimal medium (MM) or rich (R2YE) medium for the times indicated. E) Northern blot analysis of scr4677 expression in S. coelicolor following growth on soy flour medium supplemented either with mannitol (SFM; left) or glucose (SFG; right), for the times indicated. For each northern blot, 5S rRNA served as the control for RNA loading and RNA integrity.

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**scr4677 is Highly Expressed in Diverse Streptomyces Species, and Exhibits Glucose-dependent Changes in Expression Profiles**

To determine whether scr4677 sequence conservation in the streptomycetes reflected the existence of an equivalent sRNA gene in these other species, or whether this conservation was simply due to the close proximity of the scr4677 sequence to SCO4676, we took advantage of RNA-seq data that we had generated for three evolutionarily divergent Streptomyces species: S. coelicolor, S. avermitilis and S. venezuelae [13]. We found that scr4677 homologues were highly expressed in all three species (Figure 1B), strongly supporting a functional role for this RNA molecule. We further validated these findings using northern blotting (Figure 1C), and found the sRNA was expressed at different stages of development in each species during submerged or surface growth on MYM medium: in S. coelicolor, expression increased throughout development, while in S. avermitilis, two transcripts were detected, with highest levels for both seen during the transition from vegetative to aerial growth (48 h); in S. venezuelae, high levels of expression were seen throughout the developmental time course, with a smaller
transcript also readily detectable during the early stages of vegetative growth (Figure 1C).

We had previously confirmed the existence of scr4677 in S. coelicolor using northern blotting, with RNA samples isolated from strains grown on either rich (glucose-containing R2YE) medium or minimal medium (MM) supplemented with mannitol [10]. As seen in Figure 1D, scr4677 was constitutively expressed during growth on MM with mannitol, but showed more temporal accumulation during growth on rich medium. Smaller transcripts were reproducibly detected in the rich medium-grown samples, but not in those isolated from MM-grown cultures. We reasoned that the smaller-transcript accumulation might be a general response to differences in overall media composition (rich versus minimal media), as they were also observed during growth on MM (Figure 1C). Alternatively, the different transcript profiles may result from differences in carbon source, with the classically rich medium containing glucose (and MM containing maltose), in contrast to the MM which contained mannitol - a poor carbon source for S. coelicolor. To differentiate between these possibilities, scr4677 transcript levels were examined in S. coelicolor during growth on soy flour agar medium supplemented with either mannitol or glucose as a carbon source (termed ‘SFM’ or ‘SGF’, respectively). The smaller scr4677 transcripts were observed exclusively in RNA samples isolated from cells grown on SFG (Figure 1E), suggesting the appearance of the smaller scr4677 transcripts may be glucose- (or preferred carbon source) dependent. Whether these transcripts are independently expressed sRNAs or stable degradation products has yet to be definitively determined; however, previous investigations have shown that all transcripts appear to share the same 5’ end but differ at their 3’ ends [10], supporting the idea that the smaller transcripts arise as a result of a processing – or premature termination – event.

Intra-operonic Differences in Expression of SCO4677-4675 in Response to Nutrient Conditions

Given the conservation of the scr4677 sequence in the intergenic region between SCO4676 and SCO4677, we postulated that scr4677 may regulate the expression of SCO4676 and/or SCO4677. Given that previous 5’ RACE experiments had determined the scr4677 transcription start site to be a mere 14 nt upstream of the SCO4676 translation start site [10], we first set out to map the transcription start site for SCO4676, to determine whether this mRNA would overlap the scr4677 transcript. Using S1 nuclease mapping with a probe extending ~150 nt into the SCO4676 coding sequence, the major SCO4676 transcription start site was determined to be ~50 nt upstream of its start codon (Figure 2A), consistent with our RNA-seq data, which showed a peak of transcription within this region (Figure 1B). This transcript would therefore share 37 nt of sequence overlap with scr4677 (Figure 2B). Interestingly, this is roughly equivalent to the size of the smaller scr4677 transcripts, as seen in Figure 1D. We also used semi-quantitative RT-PCR to assess the expression of each of SCO4675, SCO4676 and SCO4677 during growth on rich medium and MM supplemented with mannitol. As had been seen for scr4677 (Figure 1D), all genes were constitutively expressed over a 72 hour time course on MM-mannitol, whereas they were expressed less highly on rich (R2YE) medium, and showed subtle but reproducible differences in their expression profiles (Figure 2C). In particular, SCO4675 and SCO4676 were expressed most highly at 40 h, whereas SCO4677 was expressed more constitutively (relative to the 16S rRNA control).

In silico predictions by Castro-Melchor and colleagues [35] had suggested that SCO4677 and SCO4676 were co-transcribed. This prediction was borne out by our RNA-seq data (Figure 1B), and was further confirmed using semi-quantitative RT-PCR, which showed both SCO4677-SCO4676 and SCO4676-SCO4675 were co-transcribed (Figure 2C). scr4677 Impacts Transcript Levels of SCO4676 and SCO4677-4676

Given that scr4677 would have complete complementary to the SCO4677-4676 transcript and would share ~37 nt of sequence overlap with the SCO4676 transcript, we wanted to determine whether modulating scr4677 expression would impact the abundance of either of these transcripts. We cloned scr4677, together with additional upstream and downstream sequence to ensure all regulatory elements were included, into the high-copy number plasmid pWHM3. We introduced this overexpression plasmid into wild type S. coelicolor M145 and used northern blot analysis to confirm sRNA overexpression (~3 fold) during growth on rich R2YE medium, relative to an empty plasmid-containing control strain (Figure 3A).

Levels of SCO4676, SCO4677 and SCO4677-4676 transcripts were then compared for rich media-grown scr4677 overexpression and control strains. We found SCO4677-4676 expression levels were reproducibly higher (~2-3 fold) in the overexpression strain relative to a plasmid-carrying control strain over the entire 72 h-time course (Figure 3B), suggesting that scr4677 may stabilize the SCO4677-4676 polycistronic transcript. Unexpectedly, this did not lead to greater transcript levels for SCO4676, which were similar –

Figure 2. Transcriptional analysis of the genes flanking scr4677. A) S1 nuclease mapping of the 5’ end of the SCO4676 transcript. RNA samples were harvested after 40 or 64 hours of growth on minimal medium supplemented with mannitol. The transcription start site was determined by comparing the size of the protected fragments, indicated with an arrow, to a labeled 10 bp ladder that was run adjacent to these samples. B) Nucleotide sequence upstream of SCO4676 depicting: the SCO4676 start codon, bolded and outlined at the extreme 3’ end; the SCO4676 transcription start site and 5’ UTR indicated in blue italicized text; the scr4677 transcription start site and sequence illustrated in red text. These two transcripts share 37 nt of sequence overlap. C) Expression profiles for SCO4676-4675 genes in wild-type S. coelicolor following growth on R2YE (glucose-containing rich medium) and MM (minimal medium with mannitol) agar media for the length of time indicated in hours. Transcript levels were assessed using semi-quantitative RT-PCR, with the number of amplification cycles optimized for each transcript (SCO4675, SCO4676-75, SCO4676, SCO4677-728 cycles; SCO4677-7632 cycles; and 16S rRNA: 15 cycles). 16S rRNA served as a positive control for both the RT-PCR and for overall RNA levels and integrity. These experiments were conducted in triplicate, using at least two independent RNA time-courses. doi:10.1371/journal.pone.0085856.g002
or even slightly reduced – relative to that of the control strain, suggesting that SCO4676-specific transcripts may be less stable when scr4677 levels are increased. We also tested whether the apparent stabilizing effect of scr4677 on the SCO4677-4676 read-through transcript impacted the overall levels of SCO4676; this did not appear to be the case, as these were also similar in both scr4677 overexpression and control strains (Figure 3B).

Effect of RNase III on Gene Expression in the scr4677 Locus

The location of scr4677 relative to SCO4676, and the extent of complementarity shared by their transcripts, suggested that SCO4676-specific transcripts could be targeted for degradation by RNase III upon base-pairing with scr4677. We hypothesized that a strain lacking RNase III (Δrnc) would exhibit increased levels of SCO4676 mRNA, relative to a wild type strain. To test this, we used semi-quantitative RT-PCR, and found – contrary to expectations - SCO4676 levels were significantly lower in the Δrnc mutant strain (Figure 4A). We also examined the levels of the SCO4677-4676 read-through transcript, and found that these too were reduced relative to wild type levels (Figure 4B). To determine whether these observations could be correlated with changes in scr4677 transcript levels in Δrnc and wild type strains (Figure 4C), we performed northern blotting to examine sRNA expression.

In Figure 3A, we detected expression of scr4677 from rich (R2YE) medium-grown wild type and Δrnc mutant strains. In place of the appropriate size that was never seen in our empty plasmid containing strain. While we were occasionally able to detect a protein from scr4677-overexpression strain relative to a control-plasmid containing strain, this was not consistently observed. We examined expression at different time points, under different growth conditions (e.g. minimal medium, SFM, rich R2YE medium), and both with and without precipitation and concentration of cell extracts, but were never able to reproducibly detect the SCO4676-3×FLAG fusion. Therefore we have been unable to draw any conclusions regarding the translational impacts of scr4677 on SCO4676 expression.

Typically seen during growth on rich medium (see Figure 1) were also present at equivalent levels in wild type and mutant strains, suggesting that these transcripts were not generated as a result of RNase III cleavage.

Investigating the Effect of scr4677 on the Translation of SCO4676

Many sRNAs exert their regulatory effects via a translational means. Given the location of scr4677, it was not expected to basepair with the ribosome binding site of SCO4676, but it was possible that scr4677 binding to the untranslated region of the SCO4676 transcript could promote conformational changes in the SCO4676 mRNA, altering ribosome binding site accessibility and thus SCO4676 translation. To examine this possibility, we fused a 3×FLAG tag to the C-terminus of SCO4676, and used immunoblotting, attempted to follow its expression in the scr4677-overexpression strain relative to a control-plasmid containing strain. While we were occasionally able to detect a protein of the appropriate size that was never seen in our empty plasmid control strain, this was not consistently observed. We examined expression at different time points, under different growth conditions (e.g. minimal medium, SFM, rich R2YE medium), and both with and without precipitation and concentration of cell extracts, but were never able to reproducibly detect the SCO4676-3×FLAG fusion. Therefore we have been unable to draw any conclusions regarding the translational impacts of scr4677 on SCO4676 expression.
Probing the Biological Role of scr4677 and its Associated Gene Products

Given the genetic correlation shared by scr4677 and its flanking protein coding genes, and the fact that these genes are found in many Streptomyces species, we were interested in evaluating the functional role of these gene products. We generated marked and unmarked ΔSCO4676 mutant strains, with the latter constructed so as to obviate any potential polar effects on the downstream SCO4675. We compared the phenotype of ΔSCO4676 (both marked and unmarked) and wild type strains following growth on a number of different solid media types (rich medium (R2YE+glucose/mannitol), minimal medium+glucose/mannitol, soy flour medium+glucose/mannitol, maltose-yeast extract-malt extract (MYM), supplemented minimal medium solid (SMM with casaminoacids and mannitol) and LB), and found that both mutant strains resembled their wild-type parent during growth on all types of solid media tested (e.g. Figure 5A). We also tested whether ΔSCO4676 had any effect on liquid grown-cultures. We consistently observed increased actinorhodin production for the ΔSCO4676 strain in NMMP liquid medium relative to wild type (the markerless mutant behaved similarly to the ΔSCO4676 strain; data not shown), and this phenotype was partially complemented by re-introducing wild type SCO4676 on an integrating plasmid vector (Figure 5B).

As scr4677 overexpression led to a slight reduction in levels of SCO4676, we were interested in determining whether the phenotype of the scr4677 overexpression strain was similar that of the ΔSCO4676 mutant, or whether it had additional phenotypic differences that could potentially be attributed to an effect on other target mRNAs. We found the overexpression strain did not exhibit any reproducible phenotypic change relative to an empty plasmid-target mRNAs. We found the overexpression strain did not exhibit any reproducible phenotypic change relative to an empty plasmid-carrying control strain under any growth conditions tested (data not shown).

As we had established that SCO4676 was co-transcribed with SCO4677, we also generated a deletion strain of SCO4677. When grown on SFM agar plates, the ΔSCO4677 mutant strain overproduced actinorhodin (Figure 5A). It also exhibited apparently accelerated sporulation (Figure 5A), although some of this darker coloration was due to precocious actinorhodin production. Similar phenotypic effects have been previously reported for a ΔSCO4677 deletion mutant, and these could be effectively complemented following re-introduction of the wild type gene on an integrating plasmid vector [32].

The altered sporulation profile observed for ΔSCO4677, both here and in previous studies [32], suggested that SCO4677 may function in modulating spore development, particularly in light of the fact that SCO4677 interacts with the sporulation sigma factor SigF (which control the production of the grey spore pigment; [36]). We therefore wondered whether genetic perturbation of either ΔSCO4676 or scr4677 might also impact sporulation. We used light microscopy to examine sporulating ΔSCO4676 and scr4677 overexpression strains, but could not detect any morphological defects or sporulation abnormalities. We also tested these same strains for altered resistance to heat and a variety of other environmental stresses including cell envelope stress (SDS, EDTA, lysozyme, and vancomycin), oxidative and thiol stresses (hydrogen peroxide and diamide; respectively), and iron starvation (dipiridyl). In all instances, both mutant and overexpression strains behaved similarly to their corresponding wild type control strains.

Thus in summary, our phenotypic analyses revealed that SCO4677 disruption affected both antibiotic production and sporulation on several media types, SCO4676 disruption impacted actinorhodin production under specific growth conditions, and modulating scr4677 levels had no reproducible phenotypic consequences.

Exploring the Transcriptional Regulation of scr4677

In addition to investigating the regulatory role of scr4677, we also asked whether scr4677 transcription was controlled by either of its flanking protein-encoding genes (SCO4676 and SCO4677). Given that SCO4676 is predicted to have a helix-turn-helix motif and may therefore function as a DNA-binding protein, scr4677 expression was first examined in a ΔSCO4676 mutant strain. The majority of the ΔSCO4676 coding sequence in this strain had been replaced with an apramycin resistance cassette (in-frame deletion), although the first 21 aa (63 bp) of the N-terminus had been retained to ensure that the scr4677 promoter region was not affected (Figure 6A). Interestingly, we found scr4677 expression was virtually abolished in the ΔSCO4676 mutant (Figure 6B). This could be due to either the requirement of a functional SCO4676 protein for scr4677 expression, or the disruption of a regulatory element required for scr4677 transcription in the ΔSCO4676 mutant.

To differentiate between these possibilities, we took advantage of our scr4677 overexpression strain. We tested whether a ΔSCO4676 mutant strain carrying the scr4677 overexpression plasmid (where scr4677 had all the necessary elements for effective transcription) was impaired in its expression of scr4677. In this strain, scr4677 was effectively transcribed (Figure 6C), indicating that its expression did not require SCO4676 activity. This instead implied that scr4677 expression required a regulatory element located ~80–160 bp upstream of scr4677 start site, reflecting the sequence that was present in the overexpression construct, but missing in the ΔSCO4676 mutant. We assessed this possibility by conducting electrophoretic mobility shift assays using a probe spanning this region, together with cell-free extracts from a 48 h plate-grown culture, and determined that there was indeed a protein within these extracts that could specifically interact with this sequence (Figure 6C). We also assessed scr4677 transcript levels in a ΔSCO4676 mutant, and found it to be reproducibly expressed at lower levels than in its wild-type parent strain (Figure 6D). This suggested that SCO4676 activity might contribute to the full activation of scr4677 transcription. We tested the ability of extracts harvested from both ΔSCO4676 and ΔSCO4677 mutant strains to shift the 83 bp probe described above, and detected shifts equivalent to those observed for wild type extracts (Figure 6C). Repeated attempts to isolate the binding protein using affinity chromatography were unsuccessful, due at least in part to low protein concentrations: in S. coelicolor, scr4677 was only expressed during growth on solid medium (not during liquid culture), which severely limited our ability to harvest sufficient biomass for such an isolation procedure.

Discussion

In this study, we sought to begin characterizing the conserved small RNA scr4677. We observed that scr4677 expression differed depending on nutritional status, in that there were smaller transcripts reproducibly detected in RNA samples isolated from S. coelicolor grown on glucose-containing medium. These may represent smaller primary transcripts that are only expressed during growth on optimal carbon sources, although our data suggest they are more likely to be processed products, possibly resulting from generally increased nuclease activity under these growth conditions.

The role of antisense RNAs in modulating the stability of their sense counterparts has been studied most extensively in E. coli;
Figure 5. Phenotypic effect of SCO4676 and SCO4677 deletion. A) Phenotypic comparison of strains grown on SFM (soy flour supplemented with mannitol) for 48 h. Left image: top of plate; right image: bottom of the same plate. Strain descriptions for the left plate, starting from the top left are as follows: wild type (M145); apramycin resistance-marker containing SCO4676 deletion mutant (Δ4676::apr); Δ4676 with the apramycin resistance cassette removed to create an in-frame, markerless mutant (Δ4676 unmarked); and an apramycin resistance-marker containing SCO4677 deletion mutant (Δ4677::apr). B) Enhanced actinorhodin production by the SCO4676 deletion mutant (carrying the empty integrating plasmid pIJ82), relative to the wild type empty plasmid-carrying strain (M145+ pIJ82), as determined by measuring the $A_{633}$/biomass and normalizing to the dry cell weight (in mg).
This enhanced antibiotic production could be partially complemented by introduction of wild type SCO4676 on the integrating plasmid vector pIJ82. The data presented represent the average of three independent cultures, with each analyzed in duplicate; error bars indicate standard error. Asterisks indicate statistically significant differences (* = P-value < 0.05; ** = P-value < 0.001).

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however, there have only been a handful of systems examined in which antisense RNAs are expressed within the intergenic sequences of polycistronic mRNAs e.g. [37, 38]. One of the best studied of these is RyhB, an antisense RNA expressed from the intergenic region between *iscR* and *iscS*. During growth under iron-limiting conditions, RyhB stabilizes and promotes the accumulation of *iscR* transcripts (where *iscR* is the first gene of the *iscRSUA* operon), while simultaneously stimulating the degradation of the *iscSUA* polycistronic mRNA [37]. It has been suggested that these dichotomous effects stem from the formation of a stable secondary structural element downstream of *iscR* following RyhB binding [37]; this serves to both stabilize *iscR* transcripts, and promote downstream degradation of *iscSUA* [39]. A converse phenomenon was observed here, where scr4677 enhanced the stability of SCO4676-77 polycistronic transcripts, and destabilized SCO4676-specific transcripts. While our transcription analyses suggested that scr4677 negatively affected SCO4676 transcript levels, this effect was subtle. This observation is consistent with the emerging view that in bacteria, non-coding RNAs often act to ‘fine-tune’ gene expression (reviewed in [40]).

The differential effects of scr4677 overexpression on the SCO4676-4677 polycistronic transcript and the SCO4676-specific transcript could result from differences in scr4677 binding: the entire 70 nt small RNA sequence has the capacity to base-pair with the polycistronic transcript, while it would share more limited complementarity (37 nt) with the transcript initiating immediately upstream of SCO4676. Interestingly, structural predictions (using mFold) for the SCO4676-4677 intergenic sequence revealed an extended single stranded region ~70–90 nucleotides upstream from the translation start site of SCO4676 (Figure S1). This region includes an ‘ACAU’ motif, which matches a typical RNase E cleavage site [41]. It is conceivable that scr4677 interaction with this single stranded region alters its conformation, thereby protecting the read-through transcript from RNase E-mediated degradation. The SCO4676-specific transcript lacks this region, and association with scr4677 may instead lead to decreased transcript stability.

We initially hypothesized that the destabilizing effect of scr4677 on SCO4676 could be mediated by the catalytic action of RNase III, since the double stranded RNA that would form as a result of sRNA-mRNA base-pairing would be a reasonable candidate for RNase III-mediated degradation [as reviewed in [42]]. We found RNase III had no discernible effect on the levels of scr4677, in line with a recent report showing that in *Bacillus subtilis*, RNase III had little effect on antisense transcript levels [43]. Furthermore, in the *S. coelicolor rnc* mutant, levels of both SCO4676 and SCO4677-4676 read-through transcripts were reduced relative to that observed in a wild type strain, suggesting that RNase III was instead required for the expression and/or stability of these transcripts. It has been proposed that RNase III may bind a subset of RNAs and modulate

Figure 6. Effect of SCO4676 and SCO4677 deletion on the expression of scr4677. A) Northern blot analysis of scr4677 expression in wild type and SCO4676 deletion (apramycin-containing) strains during development on rich R2YE medium. 5S rRNA was used as a control for RNA abundance and RNA integrity. B) Northern blot analysis of scr4677 as described in A), only wild type and mutant strains carried scr4677 on a high copy number plasmid (pWHM3). C) Electrophoretic mobility shift assay of a [γ-32P] radiolabelled 83 bp probe containing the scr4677 upstream region (extending from +77 to +159 nt relative to the scr4677 transcription start site), following incubation with cell free extracts from the strains indicated. Binding specificity was tested using excess amounts of specific (non-radiolabeled probe) and non-specific competitor (sequence within SCO3287) DNA. D) Northern blot analysis of scr4677 expression during growth on rich (R2YE) medium in a wild type and SCO4677 deletion mutant strain. As above, 5S rRNA was used as a loading control.
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their stability via a non-catalytic mechanism [42]; whether the effect of RNase III on SCO4676 and SCO4677-4676 transcript levels is direct or indirect remains to be determined.

In considering alternative modes of activity for scr4677, we questioned whether scr4677 could be exerting its regulatory effects on SCO4676 at a translational level, by altering the structure of the SCO4676 UTR such that ribosome binding site accessibility was altered. In a situation where ribosome binding to the SCO4676 mRNA was hindered, this would leave the mRNA transcript susceptible to nucleolytic attack, and could lead to reduced levels of SCO4676 transcripts. Our data do not exclude this possibility. Such a mechanism has been observed for a number of antisense RNAs (e.g. [44]), and as such, we actively pursued testing this possibility for scr4677 and SCO4676. We were, however, unable to routinely detect tagged variants of SCO4676 by immunoblotting following during growth for any length of time, on any media tested. We expect the reason for this is simply that the protein is expressed at low levels in the cell, as suggested by our transcriptional analyses (e.g. Figure 1B).

Both ΔSCO4676 and ΔSCO4677 strains produced elevated levels of actinorhodin relative to their wild-type parents, albeit under different conditions. The SCO4677 deletion has previously been observed to affect both morphological development and antibiotic production [32]. In this earlier study, SCO4676 was also shown to interact in vitro with the sporulation sigma factor SigF, as well as with the putative anti-sigma factor antagonist SCO00869, suggesting that SCO4677 may function as an anti-sigma factor. Mutants lacking sigF or SCO00869 do not, however, exhibit any defects in antibiotic production [32], suggesting that SCO4677 may have additional functions and/or binding partners in the cell.

Collectively, our results suggest the existence of an genetic network linking the scr4677 sRNA, the proposed anti-sigma factor-like protein SCO4677, and SCO4676, a conserved protein that impacts actinorhodin production under specific growth conditions.

Supporting Information

Figure S1 Predicted secondary structure of A) the untranslated sequence between SCO4677 and SCO4676, and B) the 5′ untranslated sequence of the SCO4676-specific transcript. In A) the red text corresponds to the SCO4676 untranslated sequence (shown in B), while the blue text indicates an extended single stranded region. The four nucleotides highlighted with the green box indicate a typical RNAe E cleavage site ([A/G]GNAU).

(TIF)

Table S1 Oligonucleotides used in this study.

(XLSX)

Author Contributions

Conceived and designed the experiments: HH MAE. Performed the experiments: HH MJM SEJ. Analyzed the data: HH MJM SEJ MAE. Contributed reagents/materials/analysis tools: HH MJM SEJ MAE. Wrote the paper: HH MAE. Provided editorial comments: MJM SEJ.

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