cis-Antisense RNAs in _Streptomyces_

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cis-Antisense RNAs (asRNAs) provide very simple and effective gene expression control due to the perfect homology between regulated and regulatory transcripts. In cases where the antisense transcript covers the ribosome binding site of the target mRNA, the messenger cannot enter a ribosome, and it is often degraded. In some bacteria, the degradation system includes the double strand-specific endoribonuclease RNase III. In the antibiotic-producing clade of *Streptomyces*, the antisense control system is not yet understood, although it might direct the organism's complex development. Initial studies in *Streptomyces* have found a number of asRNAs. Apart from this, hundreds of mRNAs have been shown to bind RNase III. In this study, to show whether this binding is mediated by an antisense transcript, we tested 17 RNase III-binding mRNAs for antisense expression. Our RACE mapping revealed that all of these mRNAs possess cognate asRNA. A control group of 10 different mRNAs (encoding selected transcriptional regulators) exhibited three asRNAs. Northern blots revealed the expression profiles of 17 novel transcripts in both wild type M145 and *rnc* mutant strains. Additionally, a transcript antisense to the transcriptional regulator AdpA was found. Our findings suggest that the mRNA-asRNA-RNase III degradation system is functional in *Streptomyces*.

Introduction

sRNAs in bacteria

Bacterial small RNAs are important post-transcriptional regulators that control a variety of cell processes. A large majority of these RNAs acts on target mRNAs via base pairing, an antisense mechanism that leads to positive or negative regulation of the target protein, as reviewed in Thomason et al. [1]. Such antisense RNAs fall into two groups: *cis*- (asRNAs) and *trans*-encoded (sRNAs)[2].

The *trans*-acting sRNAs are encoded distinctly from their target mRNA(s), which is also the reason why the sRNA-mRNA pair shares reduced complementarity. However, due to the limited complementarity, the sRNAs are usually able to act on multiple targets. The *trans*-encoded sRNAs have been extensively characterized and discussed in many reviews [3, 4]. The limited base pairing requires the RNA chaperone protein Hfq in a number of bacteria. However, Hfq is either missing or its homolog has not yet been found in several bacterial clades, including Actinomycetes [5].

Although the reported proportions of *cis*-antisense expression vary from 13% in *Bacillus subtilis* [6], 27% in *Synechocystis* PCC6803 [7], 30% in *Anabaena* [8], 46% in *Helicobacter pylori* [9], and up to 49% in *Staphylococcus aureus* [10], much less attention has been paid to bacterial asRNAs. These transcripts are encoded on the DNA strand opposite to their specific targets. Both asRNA and mRNA are produced by an overlapping transcription, thus sharing perfect complementarity (summarized in [11]). The ability of asRNAs to modulate mRNA level is not only by a post-transcriptional mechanism but may they function directly on a transcriptional level causing a collision of RNA polymerases traveling in an opposite direction (transcription interference, for review see Thomason et al. [1]). The mechanisms of post-transcriptional action of asRNAs may be divided into two groups: (i) asRNA influences the stability of the target mRNA by either tagging for degradation or stabilizing its structure and/or (ii) asRNA affects translation either by blocking or promoting the ribosome binding site [10, 12].

In the cases of negative antisense control, the sense-antisense RNA complex formation results in its rapid cleavage. In many bacteria, two dominant endoribonucleases, RNase E and RNase III, are

involved in the degradation process. The RNase E enzyme, as a member of the degradosome complex with Hfq in *E. coli*, cleaves the single-stranded RNA parts (reviewed in Carpousis et al. [13]). The RNase III enzyme cleaves the double-stranded RNAs [14]. Although RNase III was initially shown to be associated with the processing of ribosomal RNAs [15], [13], its involvement in the degradation of sense/antisense pairs is recently being reported in an increasing number of publications [16, 17].

In *Staphylococcus aureus,* deep sequencing of the short RNA fraction revealed a massive accumulation of 22-nucleotide RNA fragments generated by the RNase III cleavage of paired transcripts [10]. More than 75% of the fragments corresponded to regions with overlapping transcription from all over the chromosome. The number of short RNA fragments was significantly decreased in the RNase III-deletion strain. In contrast, such a collection of short RNA fragments was not found when using a similar transcriptome analysis for the Gram-negative bacterium *Salmonella enterica* [18].

Bacteria of the genus *Streptomyces* undergo a complex mycelial life cycle. Their growth starts with the germination of spores that develop into a vegetative mycelium of branching hyphae. Subsequent development of aerial hyphae is considered to be a cell response to nutrient depletion [19]. While part of the vegetative mycelium can lyse and be used as a nutrient source, the synthesis of antibiotics reaches its maximum to avoid competitive organisms. Eventually, the aerial hyphae are dissected into spores by sporulation septa, producing chains of connected uninucleoid spores.

The complexity of the morphological and physiological differentiation in *Streptomyces* can be documented by the existence of more than 900 transcriptional protein regulators that control the metabolic and developmental transitions. Among them, over 60 sigma factors have been identified thus far. Confirmations of the expected employment of small RNAs in the regulation of cell processes, including primary metabolism, developmental transitions, antibiotic production, and various stress responses is being increasingly reported [20-24]. Hundreds of *cis*-acting asRNAs were identified using RNA sequencing in two recent studies [25, 26].

RNase III from the *Streptomyces* genus is thought to be a global regulator of antibiotic biosynthesis [27-32]. In *Streptomyces coelicolor*, its gene, termed *rnc*, lies in the *absB* locus [27]. In *rnc* mutant strains, the production of all four antibiotics (actinorhodin, undecylprodigiosin, CDA, and methylenomycin) was severely reduced [33-35]. Microarray analysis was used to compare the levels of gene expression in the *S. coelicolor* parental strain and the RNase III mutant strain [29]. A wide effect of the ribonuclease was found, mainly on genes connected with sporulation and antibiotic production. In the *rnc* mutant strain, sporulation genes were up-regulated, whereas activators of the biosynthetic pathways (e.g., *actII-ORF4, redD, redZ*, and *cdaR*) were down-regulated, which is consistent with defects in antibiotic production. However, Strakova et al. [36] showed that *rnc* expression is activated from the first hour of germination, suggesting a more general role for RNase III, either in ribosomal RNA or in asRNA processing. Subsequent microarray and co-immunoprecipitation analyses, performed by Gatewood and co-workers, revealed at least 777 mRNAs bound by the RNase III enzyme. The authors also showed that the absence of the enzyme directly or indirectly affected the levels of hundreds of mRNAs and at least two small RNAs. These very valuable results greatly inspired the work described in this paper. Here, we further exploited their results to find connections between asRNAs and RNase III, which have not yet been reported in Streptomyces. We subtracted 17 mRNAs that are bound by the RNase III enzyme [30] to show if they possess an antisense transcript. The search for asRNAs within the selected group of mRNAs was 100% successful. Moreover, 4 transcripts, antisense to independently selected mRNAs that encode transcription regulators (SigB, SigH, SigR, and AdpA) have also been revealed. These findings suggest

that the mRNA/asRNA/RNase III degradation pathway is fully functional in *Streptomyces* and can also direct antibiotic production.

Materials and methods

Bacterial strain, cultivation

In this study, the *Streptomyces coelicolor* wild-type strain M145 [37] and its RNase III-deletion strain derivative (rnc, M145 *rnc::aac*(3)IV [34]) were used. 10^8 spores were inoculated on solid R2YE medium [37] covered by cellophane at 29°C. Bacterial samples were collected after 24, 48 and 72 hours of cultivation, where each time point represented a different developmental stage, i.e., vegetative mycelium, aerial mycelium, and spores.

RNA isolation

Total RNA was isolated using a TRIzol method [38]. Harvested cells were immediately submerged in TRIzol reagent (Ambion) on ice (1 ml of TRIzol per 50 cm^2 of culture dish surface area). Five glass beads (3 mm in diameter) were added to the cell suspension. The cells were disrupted using a Minilys homogenizer (Precellys) twice for 2 mins at 3000 rpm and twice for 2 mins at 4000 rpm, cooled on ice between the cycles. The samples were subsequently centrifuged for 2 mins at 10.000 g and purified in TRIzol/chloroform (5:1) and chloroform. For RNA precipitation, the samples were incubated in isopropanol at -20°C overnight and centrifuged for 30 mins at 10.000 g. RNA samples were washed in ethanol and resuspended in 30 µl of RNase-free water. Residual DNA in the RNA samples was removed by DNase I treatment (Ambion). Typically, a concentration between 1-3.5 µg/µl was obtained. RNA quality was checked on a 1% agarose gel.

5´ and 3´RACE

As a proof of antisense RNA expression and for determination of both their 5´ and 3´ ends, the FirstChoice RLM-RACE Kit (Ambion) was used following the manufacturer's protocol with the following exceptions:

1. CIP enzyme treatment was not performed in the 5'RACE procedure.
2. A gene-specific primer (see Fig. 1) was used instead of random decamers in the 5'RACE. The 5'RACE primers as well as the probes used for Northern blot hybridizations were designed to cover the ribosome binding site and start codon of a cognate mRNA. All of the primers were arranged using the Primer3 software (http://sourceforge.net/projects/primer3/) [39].
3. The PrimeScript (Takara, 100 units per 10 µl of reaction mixture) was used as a reverse transcriptase. Negative controls lacking the enzyme were also used.
4. The reverse transcription proceeded at 42°C for 45 mins and 48°C for 10 mins.
5. Preceding the 3´RACE, total RNA samples were polyadenylated by 5 units of Poly(A) Polymerase I (New England Biolabs), according the manufacturer's protocol.

Final PCR products were separated on a 1.2% agarose gel. Products that were found in samples but absent in negative controls were excised and purified using the Qiagen MinElute PCR purification kit. The purified products were cloned into the TOPO vector using the TOPO TA Cloning (Invitrogen) and transformed into One Shot TOP10F' competent cells (Invitrogen). Plasmids containing the cDNA inserts were extracted using the QIAprep Miniprep kit. Eventually, the cloned inserts were sequenced.

Northern blot analysis

RNA samples (30 µg) were denatured for 10 mins at 70°C in RNA loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanole, 10 mM EDTA) and separated in a 1% agarose gel containing formaldehyde, provided by the NorthernMax Kit (Ambion). Separated samples were transferred onto positively charged nylon membranes (ZetaProbe, BioRAD) by electroblotting at 240 mA for 45 mins. The nylon membrane was UV-crosslinked.

Oligonucleotides were radioactively labeled on their 5' ends by γ-^{32}P-ATP using T4 polynucleotide kinase (Thermo Scientific) and purified (QIAquick Nucleotide Removal Kit, Qiagen). Hybridization was performed in ULTRAhyb hybridization buffer (Ambion) overnight at 37-42°C. The membranes were then washed twice with 2xSSC, 0.1% SDS (NorthernMax kit) at room temperature and once with 0.1x SSC, 0.1% SDS (NorthernMax kit) at 42°C. The membranes were dried and exposed in a BAS cassette (Fuji-Film) after 4 days. The signals were visualized using a Phosphorimager FX (Bio-Rad) and quantified using QuantityOne analysis software (Bio-Rad).

Results and discussion

Using the RNA-seq approach, Gatewood et al. (2012) compared gene expression between the *S. coelicolor* M145 wild type strain and the JSE1880 *rnc*-mutant strain. The authors found that approximately 10% of all mRNAs from the vegetative state of growth were directly or indirectly affected by RNase III. In addition, they applied RNA immunoprecipitation to detect mRNAs targeted by the enzyme (referred to as BARD). However, the necessity of involvement of other transcripts for the binding of the double-strand-specific enzyme to the mRNAs is still unknown. *In vitro* assays showed that the RNase III digests several messengers, such as SCO3982 to SCO3988, and SCO5737, unattended [30] (see below), but other lone transcripts, such as SCO0762, were not cleaved [40]. Another study reported that the RNase III enzyme requires a minimum of approximately 20 bp of RNA for binding *in vivo,* whereas naturally occurring stem-loop structures of most mRNAs are too short to be bound by the enzyme [41].

***In silico* search**

To determine if an antisense regulatory mechanism mediates RNase III-mRNA binding, we compared SCO numbers of the messengers increased by more than two-fold in JSE1880 or detected in BARD (both listed in Table S2 [30]), with scr numbers of *S. coelicolor* small RNAs (sRNAs) found or predicted up-to-date [21-23, 25, 26]. As the genome coordinates were not always easy to trace, it was sufficient to compare gene numbers for our requirements. The SCO and scr numbers would either match absolutely or their difference was allowed to be 1 (SCO# - scr# ≤ abs1; meaning that the genes are adjacent and possibly antisense).

Between 153 mRNAs (79 found in the BARD) and 1713 sRNAs (known or predicted), 74 matches were found, i.e., 48.4% (28 in the BARD, i.e., 35.4%). From these 74 matches, 14 mRNAs were matched to two or three adjacent scr# (as the possible difference was -1, 0, or 1). These data, summarized in Table 1, strongly suggest that many mRNAs are not able to bind RNase III by themselves, but rather need an antisense transcript to form a double strand that is recognized by the enzyme.

Predictions and experimental verifications of new *cis*-antisense transcripts

Based on the hypothesis, we selected 17 mRNAs known to be targeted by RNase III (enriched in the BARD in [30], mRNAs selected from tables 2 and S3 in the paper). We rationalized that many asRNAs

overlap the ribosome binding site and the start codon of their target, leading to negative translational control. Following this, we designed all the DNA primers that have been used here to find antisense transcripts (Fig. 1). To demonstrate expression of the antisense transcript, 5' RACE analyses were performed using the primers. Sequence(s) extended from the primer was/were PCR amplified and sequenced. From the 17 samples tested, 17 revealed 5'end(s) of new *cis*-antisense transcripts, signifying a 100% confirmation of the selection (Fig. 1A). The 3' RACE revealed 16 3' ends (*scr0168* unsuccessful, northern blot was not performed).

Using the RACE method, we did not identify any *trans*-acting sRNAs. Because we were able to find only the transcripts encoded in *cis*, it could be rationalized that the involvement of RNase III in the antisense control possibly requires 100% complementarity in formed duplexes.

To confirm the existence of the *cis*-antisense transcripts and to compare their expression during *Streptomyces* cell development (24 h, 48 h, and 72 h at standard growth conditions, see methods), northern blot analyses were performed. From the 17 analyses, 13 revealed an apparent signal (Fig. 2A). The signals of the remaining four were too weak or undetectable. The ability of RNase III to specifically degrade double-stranded RNA complexes makes it an ideal candidate to process the antisense RNAs that are base-paired with their targets. Therefore, differential northern blot analysis was performed to compare asRNA expression between M145 wild type and *rnc* mutant. Surprisingly, from these 13 northern blot positive results, only four were increased in the *rnc* strain when compared to the expression in the wild type strain. These include *scr0494, scr0864, scr2198,* and *scr3983*.

Antisense transcripts positively affected by the absence of RNase III

scr2198 is an asRNA to the *glnA* gene (SCO2198, encoding a glutamine synthase) and was previously independently shown to be expressed, termed "cnc2198.1" [23]. The authors performed a detailed functional analysis and showed that overexpression of cnc2198.1 affects growth rate and antibiotic production. In the overexpression strain, the intracellular level of the targeted GlnA protein was decreased by 40%. The authors speculated that the glnA-cnc2198.1 RNA complex blocks glnA translation, which may lead to its subsequent degradation. We further hypothesize that the complex is degraded by the RNase III enzyme, as our northern blot revealed that the asRNA level is increased in the *rnc* mutant. Our RACE mapping estimated the size of the antisense transcript to 432 nt, nearly four times longer than the 121 nt transcript described by D'Alia. Moreover, the detected 3' end overlapped the adjacent SCO2197, revealing that *scr2198* is in fact the 5'UTR of the SCO2197 mRNA, which is further processed, as our northern blot showed a second, weaker fragment of approximately 400 nt in size. Both of the detected fragments, as well as the cognate SCO2198 mRNA, had the strongest expression signals in samples from vegetative 24 h old cells, even strengthened in the rnc strain.

The *scr0494* is antisense to an iron-siderophore binding lipoprotein. Two forms of the *scr0494* asRNA, 289 nt and 242 nt in length, were detected by both RACE and northern analyses. In the *rnc* mutant, this RNA has the highest level at 24 h, whereas in the wild type, the higher level was at 48 h. Its target gene is annotated to encode an iron-siderophore binding lipoprotein.

The 152 nt scr0864 asRNAs had a stronger signal at 24 h in the wild type, but at 48 and 72 h, the signal was stronger in the rnc mutant strain. Corresponding expression of the SCO0864 mRNA, which encodes an ECF sigma factor, dominated in wt in younger cells (24 and 48 h). Other sigma factors that were revealed to possess an antisense transcript are mentioned below.

Antisense expression opposite to mRNAs that are targeted by RNase III in vitro

Stem-loop mRNA structures are thought to be too short to be digested by the RNase III enzyme. However, some authors suggest that the stem-loop structures on mRNAs are the only possible targets for RNase III activity. Indeed, the enzyme is involved in pre-rRNA, tRNA, and polycistronic RNA processing, where such stem-loop structures are present [42, 43] and possibly long enough to be cleaved by the enzyme. Gatewood clearly showed that some unattended mRNAs are targeted and cleaved by RNase III in vitro (SCO3982 to SCO3988 mRNA region, or SCO5737). On the other hand, we show that at least one member of the SCO3982-SCO3988 region – SCO3983, a hypothetical protein with TTA codons – possesses an antisense gene. Its transcript, scr3983, is 103 nt long and exhibited a strongest expression signal in vegetative cells (24 h) of the rnc strain. The mRNA also had a strong signal at 24 h and 48 h, even elevated in the rnc strain. Moreover, we observed that the scr3983 sequence is nearly identical to the region adjacent to SCO3268.

In addition to the identified scr3983 RNA, another example of an mRNA targeted by RNase III *in vitro* is SCO5737, which encodes a polynucleotide phosphorylase. We also tested this gene for potential antisense expression. Based on northern blots, however, no antisense transcript was revealed. The mRNA transcript is long enough (2220 nt) to form several longer double-stranded regions observed by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi; fig. not shown), which are most likely targeted by the RNase III enzyme *in vitro*.

AdpA was initially found in *Streptomyces griseus* as a pleiotropic transcription regulator of sporulation and antibiotic production [44]. In *Streptomyces coelicolor*, not only could AdpA-mRNA bind purified RNase III *in vitro*, but, as also shown, AdpA and the RNase III coordinated the expression of each other in a posttranscriptional feedback loop [45]. This finding may rationally explain the *rnc* phenotype that affects expression of proteins involved in sporulation and antibiotic production. According to Gatewood's results, the *adpA* RNA was enriched 22-fold in the BARD. Based the mentioned results, we expected that AdpA expression is controlled by an antisense mechanism. Indeed, our northern blot has shown one antisense transcript (Fig. 2A). Although the transcript, termed *as-adpA*, was weakly detected (faint signal of more than 800 nt in size at the 48 h and 72 h stage of growth) in the wt strain, it was clearly found in the rnc strain at later stages, peaking at the 48 h old mycelium. This finding was confirmed by primer extension, where a radioactively labeled probe (TTAGCAGTATGAGCCACGACTC) was used for reverse transcription followed by PCR (for reaction conditions see 5'RACE in Material and methods, fig. not shown). This suggests that *adpA-mRNA* and the *as-adpA* may form a double stranded complex that is recognized and cleaved by RNase III (in the wt strain).

Antisense transcripts that are not positively affected by the absence of RNase III

Although we speculated that the RNase III enzyme is an ideal candidate for the paired transcript degradations, our comparative expression analyses of several asRNAs in wt and rnc mutant strains did not clearly support this hypothesis, even though these antisense transcripts were originally found due to the observation that their sense mRNAs are targeted by RNase III.

In two cases, our experiments showed a positive effect of the RNase III enzyme on the cellular level of asRNAs (scr0323 and scr5112). Furthermore, Gatewood et al. showed that the majority of known sRNAs, detected in their RNA-seq analysis, do not exhibit significant expression differences between the wild-type and the rnc-deletion mutant (between others, the AdpA-RNA, contrary to our northern

blot result, see below). An undistinguishable or even a positive effect of the enzyme on the stability of scr4677-SCO4676 complex, as an example of sense-antisense RNA pair in *Streptomyces*, was also observed in [46]. The possibility that the RNase III enzyme does not always post-transcriptionally degrade sense-antisense complexes is also inferred from a study on sRNA degradation by three different RNases (RNase Y, J1, and III) in another Gram-positive bacteria model, *Bacillus subtilis* [47]. In *Bacillus*, RNase III depletion has little effect on antisense RNAs observed by high-resolution tiling arrays. Although several RNAs showed increased abundance in the RNase III mutant, their half-lives were not affected by the enzyme, as observed by northern blot analysis. The authors concluded that the role of RNase III in *Bacillus subtilis* lies more likely in indirect transcriptional control rather than post-transcriptional RNA turnover. One may argue that the function of the enzyme might be substituted by other ribonucleases in Δ*rnc* strains or the genome contains another RNase III homolog.

The antisense transcripts, whose expression was undistinguishable between wt and rnc, or even up-regulated in the wt strain, included the following:

- scr0198, antisense to a hypothetical protein – A faint signal was observed during the time course, with a peak at 48 h in wt cells. However, its messenger had a stronger signal in 24 h and 48 h old rnc cells.
- scr0219, antisense to a nitrate reductase delta chain – Although two forms were detected by the RACE analysis, northern blot revealed only one 102 nt long transcript that was highly expressed at 24 h and then reduced or undetectable at 48 h in the wild type or rnc strain, respectively. Corresponding expression of its cognate mRNA was also strong at 24 h in both strains, but in the rnc strain, the expression peaked at 48 h, suggesting increased stability of the transcript.
- scr0323, antisense to a hypothetical protein – Using RACE, two transcripts, 291 nt and 210 nt, were detected. The northern blot showed a faint 210 nt product but also a stronger 291 nt transcript peaking at 24 h in the wt, which was much higher than the rnc strain, where the highest expression peaked at 48 h. Corresponding expression of its cognate mRNA was maximal at 24 h and declined during the course in both wt and rnc strains. However, the total expression level was higher in the rnc mutant, suggesting increased stability of the messenger transcript.
- scr0703, antisense to a putative regulatory protein – Two apparent forms were detected by both RACE and northern analyses. Similarly to scr2198, the RACE approach revealed an overlap of the scr0703 into the adjacent SCO0702, suggesting that this transcript is a 5' UTR of the SCO0702 mRNA. According to the northern analysis result, a 334 nt transcript had a similar expression profile, peaking at 48 h in both wt and rnc strains. Similar to the SCO0323 mRNA, the SCO0703 mRNA level peaked at 24 h and decreased in older cells in both strains, and the transcript was also up-regulated in the rnc strain.
- scr0772, antisense to a putative regulatory protein – Although only one product was detected using RACE, the northern analysis revealed two forms, 125 nt and ~100 nt. The levels of both were weak but their constitutive profile was undistinguishable between wt and rnc strains.
- scr1626, antisense to cytochrome P450 – Both, a stronger 237 nt and a weaker 179 nt transcripts were detected by RACE and northern blot. The latter analysis showed the expression profile of both products to be undistinguishable between wt and rnc samples dominating in 24 h.
- scr4077, antisense to a hypothetical protein – Three or even four distinguishable products were detected by both RACE and northern analyses. Similar levels of the primary 300 nt transcript was visible on blots in wt and rnc at 24 h; older samples only contained the shorter (processed) fragments with one 232 nt peak at 48 h and dominating in wt against rnc. Corresponding expression of its

cognate mRNA at 24 h was maximal in the wt strain, but at 48 h the strongest signal was revealed in the *rnc* strain.
- scr5112, antisense to an integral membrane protein – RACE analysis revealed and northern blots confirmed expression of two forms – 170 nt and 143 nt, dominating in the wt at 24 h.
- scr5123, antisense to a small membrane protein – Both RACE and northern analyses detected one 339 nt transcript, expressed weakly but constitutively; there was also a shorter processed form detected only by RACE. In addition to scr0703 and scr2198, scr5123 was revealed to be the 5'UTR of SCO5122.

Small RNAs found in BARD [30]

In Gatewood's work, expression of two sRNAs, *scr6925* and *scr2101*, was enriched in the *rnc* strain. These data imply their antisense role and subsequent processing by the RNase III enzyme. The expression of *scr2101* was previously revealed by Swiercz [22]. Gatewood and colleagues showed that the molecule is up-regulated in the JSE 1880 strain (lacking the RNase III), where its level was 7-fold higher compared to the wild type strain. *scr2101* was also shown to be bound by the enzyme (presented in the BARD, although its enrichment was only 0.57). As the RNA's gene lies on the opposite strand between SCO2100 and SCO2101, we raised the question whether these two protein encoding genes form an operon, producing one common transcript targeted by the *scr2101* antisense RNA. The potential SCO2100-SCO2101 common transcript was used as a template for reverse transcription using a DNA probe complementary to the 5' end of the SCO2101 mRNA (TGTCCCGGCTGCTCCAGGGA). The second DNA primer, used for the following PCR amplification, was identical to the 3' end of the SCO2100 mRNA sequence (CGTAGGTCCCCGCCCGCT), thus forming a 635 nt product, which was indeed produced. Usage of a negative control PCR reaction, where the original RNA was used instead of a template, eliminated the risk of false positive results (Fig. not shown). This finding suggests that the antisense function of *scr2101* targets the region between two open reading frames. The resulting double strand transcript is subsequently bound by the RNase III enzyme either to process or to degrade the transcripts. These data clearly show that not only mRNAs but also their cognate asRNAs are possibly bound by the RNase III enzyme. Although the binding experiments are outside the scope of this paper, our findings raise demand to expand the RNase III-binding analyses to the unannotated sRNA transcriptome.

Antisense transcripts to sigma factor genes

To determine whether the existence of antisense transcripts involves only those messengers bound by RNase III according to Gatewood's results or is even more widespread, we decided to test the antisense expression in a control group of genes whose transcripts have not been shown to bind RNase III. For these tests, we selected 10 sigma factors (HrdA, HrdB, HrdC, HrdD, SigB, SigD, SigE, SigF, SigH, SigI, SigR, and WhiG), important transcriptional regulators that were previously shown to govern gene expression, controlling cell development and/or responses to various stresses [48]. As mentioned above, to detect antisense transcripts, probes used in both the RACE and northern blot analyses were designed to cover the RBS and START codon of the target mRNA. Interestingly, the results show that, out of the 10 sigma factor genes, three (*sigB, sigH, sigR*) possess antisense expression (Fig. 1B, 2B). Their antisense transcripts scr0600, scr5243, and scr5216 are 210, 244, and 296 nt long, respectively.

The transcript **scr0600** is antisense to the sigma factor SigB-mRNA (SCO0600). Its expression level increased with time in the *rnc* strain, from the lowest in vegetative 24 h old cells to a maximum at 72

h, whereas the expression in the wt strain peaked at 24 h and 72 h. Corresponding expression of the cognate mRNA was continuous in the wt, but lower in the rnc strain at 24 h.
On the other hand, the level of **scr5243** RNA, an antisense transcript to the SigH-mRNA (SCO5243) was faint but detectable during the whole time course in both wt and rnc strains. Corresponding expression of its cognate mRNA reached its maximum at 48 h in both wt and rnc strains.
Expression of the last transcript, the **scr5216** RNA antisense to the sigma factor SigR (SCO5216), was continuous during the course with a little fainter signal at 48 h in both wt and rnc strains. On the other hand, its cognate mRNA transcript reached its maximum at 48 h in both wt and rnc strains.

Summary

The genus *Streptomyces* can be presented as a model bacterial group lying on the top of prokaryotic cellular complexity. Their 8-10 Mbp long genome encodes the phenotype of all developmental stages, including morphological changes (spore formation and germination, vegetative branching hyphae, aerial twisting mycelium), secondary metabolite production (antibiotics and a variety of other bioactive compounds, siderophores, pigments, etc.) and a capacity to respond to all possible environmental changes (diverse stresses). Developmental transitions and environmental intricacy evolved an advanced regulatory network that requires a concerted action of more than 900 transcription regulatory proteins known thus far. The results obtained suggest an equivalent role and possibly even bigger number of non-protein-coding RNA regulators. The *cis*-antisense transcripts are efficient gene expression modulators with minimal space requirements on their genome [49], with a theoretical capability to act on nearly all genes. Moreover, the mode of their action (whether based on co-transcriptional collision or post-transcriptional 100% complementarity) is simple and effective. Our work suggests that antisense transcription is widespread in *Streptomyces* and somehow connected with the function of RNase III, although the absence of the *rnc* gene did not greatly influence the majority of the transcripts. It is noteworthy that the majority of antisense transcripts found here escaped previous sRNA predictions and/or whole genome searches, suggesting that their expression level is often low and may be lost during statistical background subtraction. Nevertheless, the results in this work unveil the extensiveness of the antisense transcripts and their role in gene expression control in *Streptomyces*.

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Figure legends

Fig. 1. Novel asRNAs (scr####) revealed by 5‘ and 3‘ RACE method, and their genome locations. Full green line represents 5’RACE inner primers, dotted green line represents 5’RACE outer primers, full orange line represents 3’RACE inner primers, dotted orange line represents 3’RACE outer primers.
Fig. 2. Differential expression analyses of novel asRNAs and their target mRNAs in WT and rnc strains. Three black lines (from long to short) represent RNA samples from vegetative mycelium (24 h), aerial mycelium (48 h), and spores (72 h), respectively. Sizes of the products well corresponded to those obtained by RACE. Primers used are shown on the right. The 5S expression control is included below.

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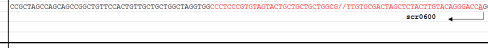
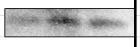
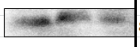
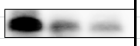
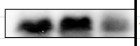
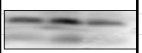
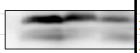
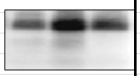
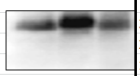
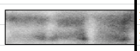
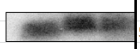
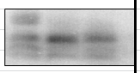
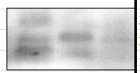
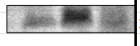
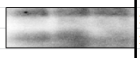
A

asRNA	Enrichment of sense mRNA in BARD (Gatewood et al.)		RACE	genome position	
	gene	BARD enrichment		start	stop
scr0168	SCO0168	100		158865	?
scr0198	SCO0198	47		188834	189100
scr0219	SCO0219	30		212563	212462
scr0323	SCO0323	138		324930	325220
scr0494	SCO0494	19		527749	528037
scr0703	SCO0703	3		743622	743289
scr0772	SCO0772	120		821384	821260
scr0864	SCO0864	234		911888	912039
scr1626	SCO1626	23; match - deep seq. Moody		1741529	1741765
scr2081	SCO2081	86		2234376	2234528
scr2198	SCO2198	12; match and confirmed - D'Alia		2365058	2364627
scr3983	SCO3983	1,5		4384779	4384677
scr4077	SCO4077	184		4470986	4470687
scr4878	SCO4878	1,6		5310225	5310692
scr5040	SCO5040	122		5479488	5479758
scr5112	SCO5112	1,4; match - predicted		5557360	5557191
scr5123	SCO5123	247		5571912	5571574

B

GENE NAME	RACE	genome position	
		start	stop
scr0600		640055	639846
SCO0600			
scr5243		5704948	5705187
SCO5243			
scr5216		5675507	5675212
SCO5216			

A

GENE NAME	Northern blots of asRNAs: WT (24 48 72)	Northern blots of asRNAs: rnc (24 48 72)	size (nt)	primer used
scr0198			267	CGGGAGGCTGAGAACGATGT
SCO0198			866	GGTCACGACGACCGGACAG
scr0219			102	CCGGAAGGCCCCTGATGA
SCO0219			770	TCGTGTGGTACGTGTGCTCG
scr0323			291 210	GGACATCACGTCGAGGTGGA
SCO0323			602	ATGACGCCGGAGCTTGTACG
scr0494			289 242	AAAGGGATGCCCGGGATGCT
scr0703			334 258 133	ACAGCAAGGAGACCGCGGTGC
SCO0703			227	GTGCCGACTCTTCTTCCAGCA
scr0772			125 115	AAGGAAGAGGTCCCCATGGA
scr0864			152	AGGAAGGACCACAGCACCATGA
SCO0864			596	AACGTGTCCTGGGTCAGATCGT
scr1626			237 179	TTGCGGAGCGAGACTGTGA
scr2198			432 425	GGAGTAGGAGGAGCTGGATGT
SCO2198			1409	CCAGGCGGTGGTACGAGTTC
scr3983			103	ATAGGAGCTGAGCAGCGTGCT
SCO3983			341	TCGCCCAGGAGTAGGTGCAG
scr4077			300 232 184	ACACACCGGGAGTGCCAGT
SCO4077			272	ACGGTGAAGTCCTCGATCACG
scr5112			170 143	GTGAAATGGACGAATCATGACG
scr5123			339	CCGACTGCGACCGACC
scr2792 (as-adpA)			800	TTAGCAGTATGAGCCACGACTC
5S				GCTGTAAGGCTTAGCTTCCGGGT

B

GENE NAME	Northern blots of asRNAs to sigma factor mRNAs: WT (24 48 72)	Northern blots of asRNAs to sigma factor mRNAs: rnc (24 48 72)	size (nt)	primer used
scr0600			210	CAGCTCACCGTGCTGGAGGAG
SCO0600 sigB mRNA			846	GTCCCGGAAGAACCGCTTGATCTC
scr5243			244	GAGGGCACACGTTCTCCGAAC
SCO5243 sigH mRNA			1086	GAAGTGCCGCTTGATCTCAC
scr5216			296	TCGACCAGATGTACTCGGCCG
SCO5216 sigR mRNA			684	TACGAGTTGATGAAGGTGTTGG
5S				GCTGTAAGGCTTAGCTTCCGGGT

Table 1. ***In silico*** **search for sRNA genes adjacent to mRNAs that are up-regulate**

Selected genes whose expression increased in JSE1880 (blue indicate transcripts present in the BARD)[30]		Antisence/adjacent small RNAs		Reference
SCO	0499	scr	0500	Moody [26]
SCO	0500	scr	0500	Moody [26]
SCO	0955	scr	0954	Moody [26]
SCO	1150	scr	1150	Moody [26]
SCO	1565	scr	1566	Swiercz [22]
SCO	1626	scr	1625	Moody [26]
SCO	1630	scr	1631	Swiercz [22]
SCO	1659	scr	1658	Swiercz [22]
SCO	1659	scr	1659	Swiercz [22]
SCO	1660	scr	1659	Swiercz [22]
SCO	1700	scr	1700	D'Alia [23]
SCO	1906	scr	1906	Swiercz [22]
SCO	1906	scr	1907	Swiercz [22]
SCO	2197	scr	2198	Swiercz [22]
SCO	2198	scr	2198	D'Alia [23]
SCO	3003	scr	3004	Swiercz [22]
SCO	3113	scr	3112	Swiercz [22]
SCO	3113	scr	3114	Swiercz [22]
SCO	3132	scr	3133	Moody [26]
SCO	3132	scr	3133	D'Alia [23]
SCO	3216	scr	3216	Swiercz [22]
SCO	3217	scr	3217	Swiercz [22]
SCO	3783	scr	3782	Swiercz [22]
SCO	4095	scr	4096	Swiercz [22]
SCO	4142	scr	4143	Swiercz [22]
SCO	4144	scr	4143	Swiercz [22]
SCO	4145	scr	4145	Swiercz [22]
SCO	4145	scr	4146	Swiercz [22]
SCO	4229	scr	4229	Swiercz [22]
SCO	4249	scr	4249	Swiercz [22]
SCO	4283	scr	4283	Moody [26]
SCO	4698	scr	4699	Moody [26]
SCO	4698	scr	4699	Vockenhuber [25]
SCO	4748	scr	4749	Moody [26]
SCO	4875	scr	4874	Swiercz [22]
SCO	4882	scr	4883	Swiercz [22]
SCO	4947	scr	4947	Swiercz [22]
SCO	4948	scr	4947	Swiercz [22]
SCO	5106	scr	5106	Moody [26]
SCO	5112	scr	5112	Swiercz [22]

SCO	5113	scr	5112	Swiercz [22]
SCO	5142	scr	5141	Swiercz [22]
SCO	5142	scr	5143	Moody [26]
SCO	5145	scr	5144	Swiercz [22]
SCO	5145	scr	5145	Swiercz [22]
SCO	5145	scr	5146	Moody [26]
SCO	5163	scr	5164	Moody [26]
SCO	5163	scr	5164	Swiercz [22]
SCO	5464	scr	5465	Moody [26]
SCO	5476	scr	5476	Swiercz [22]
SCO	5477	scr	5476	Swiercz [22]
SCO	5519	scr	5518	Moody [26]
SCO	5520	scr	5521	Swiercz [22]
SCO	5521	scr	5521	Swiercz [22]
SCO	5536	scr	5536	Panek [21]
SCO	5537	scr	5537	Swiercz [22]
SCO	5578	scr	5577	Swiercz [22]
SCO	5757	scr	5756	Swiercz [22]
SCO	6276	scr	6277	Moody [26]
SCO	6277	scr	6277	Moody [26]
SCO	6278	scr	6277	Moody [26]
SCO	6279	scr	6280	Vockenhuber [25]
SCO	6280	scr	6280	Vockenhuber [25]
SCO	6282	scr	6281	Moody [26]
SCO	6283	scr	6281	Moody [26]
SCO	6283	scr	6284	Moody [26]
SCO	6284	scr	6284	Moody [26]
SCO	6284	scr	6285	Moody [26]
SCO	6396	scr	6396	Moody [26]
SCO	6716	scr	6715	Moody [26]
SCO	6716	scr	6716	Moody [26]
SCO	6716	scr	6717	Moody [26]
SCO	6728	scr	6729	Moody [26]

ɛd in rnc mutant

Comment
mRNA up-regulated in JSE1880 [30]
mRNA up-regulated in JSE1880 [30]
cis asRNA
mRNA up-regulated in JSE1880 [30]
predicted
predicted
predicted
predicted
predicted
predicted RNAz
confirmed
predicted
predicted
confirmed
predicted
predicted
predicted
predicted RNAz
predicted
predicted
predicted
predicted
predicted
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predicted
predicted
predicted
predicted
mRNA up-regulated in JSE1880 [30]
]
mRNA up-regulated in JSE1880 [30]
predicted
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mRNA up-regulated in JSE1880 [30]
predicted

predicted
predicted

predicted
predicted
mRNA up-regulated in JSE1880 [30]

predicted

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predicted
#234
predicted
predicted
predicted

mRNA up-regulated in JSE1880 [30]	cis asRNA

mRNA up-regulated in JSE1880 [30]	cis asRNA

mRNA up-regulated in JSE1880 [30]	cis asRNA
]
]
cis asRNA
cis asRNA

cis asRNA
mRNA up-regulated in JSE1880 [30]

mRNA up-regulated in JSE1880 [30]