# RNase III-Binding-mRNAs Revealed Novel Complementary Transcripts in *Streptomyces*

*Dita Šetinová[1], Klára Šmídová[1], Pavel Pohl[2], Inesa Musić[2] and Jan Bobek[1,2,3*]*

[1] First Faculty of Medicine, Institute of Immunology and Microbiology, Charles University, Prague, Czechia, [2] Chemistry Department, Faculty of Science, J. E. Purkinje University, Ústí nad Labem, Czechia, [3] Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czechia

*cis*-Antisense RNAs (asRNAs) provide very simple and effective gene expression control due to the perfect complementarity between regulated and regulatory transcripts. In *Streptomyces*, the antibiotic-producing clade, 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, the double strand-specific endoribonuclease. In this study, we tested 17 mRNAs that have been previously co-precipitated with RNase III for antisense expression. Our RACE mapping showed that all of these mRNAs possess cognate asRNA. Additional tests for antisense expression uncovered *as-adpA*, *as-rnc*, *as3983*, *as-sigB*, *as-sigH*, and *as-sigR* RNAs. Northern blots detected the expression profiles of 18 novel transcripts. Noteworthy, we also found that only a minority of asRNAs respond to the absence of RNase III enzyme by increasing their cellular levels. Our findings suggest that antisense expression is widespread in *Streptomyces*, including genes of such important developmental regulators, as AdpA, RNase III, and sigma factors.

Keywords: *cis*-antisense RNA, RNase III, *Streptomyces*, antibiotics, gene expression control

## INTRODUCTION

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 gene, as reviewed in Thomason (Thomason and Storz, 2010). Such antisense RNAs fall into two groups: *cis*- (asRNAs) and *trans*-encoded (sRNAs) (Romby and Charpentier, 2010).

The *trans*-acting sRNAs are encoded distinctly from their target mRNA(s), which is also the reason why the sRNA-mRNA pair mostly shares reduced complementarity. On the other hand, the sRNAs are usually able to act on multiple targets. The *trans*-encoded sRNAs have been extensively characterized and discussed in many reviews (Papenfort and Vogel, 2009; Waters and Storz, 2009). 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 (Sun et al., 2002).

Reported proportions of *cis*-antisense expression in various bacteria vary from 13% in *Bacillus subtilis* (Nicolas et al., 2012), 27% in *Synechocystis* PCC6803 (Mitschke et al., 2011a), 30% in

*Anabaena* (Mitschke et al., 2011b), 46% in *Helicobacter pylori* (Sharma et al., 2010), and up to 49% in *Staphylococcus aureus* (Lasa et al., 2011). These transcripts are encoded on the DNA strand opposite to their specific targets. Both asRNA and mRNA are produced by overlapping transcription, thus sharing perfect complementarity (summarized in Lasa et al., 2012). The ability of asRNAs to modulate mRNA levels is not only mediated by post-transcriptional mechanisms but they may also directly impact transcription due to collisions between RNA polymerases traveling in opposite directions [transcription interference, for review see Thomason (Thomason and Storz, 2010)]. 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 ribosome access to the ribosome binding site (Geissmann et al., 2009; Lasa et al., 2011).

In many 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 single-stranded RNA (reviewed in Carpousis et al., 2009). The RNase III enzyme cleaves double-stranded RNA (MacRae and Doudna, 2007). Although RNase III was initially shown to be associated with the processing of ribosomal RNAs (Carpousis et al., 2009; Taverniti et al., 2011), its involvement in the degradation of sense/antisense pairs is being reported in an increasing number of publications (Blomberg et al., 1990; Gerdes et al., 1992; Lasa et al., 2011, 2012; Durand et al., 2012; Lioliou et al., 2012; Lybecker et al., 2014a,b; Le Rhun et al., 2016).

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 (Lasa et al., 2011). More than 75% of the fragments corresponded to the overlapping transcription from most regions of the chromosome. The number of short RNA fragments was significantly decreased in an 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* (Viegas et al., 2007).

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 (Chater and Losick, 1997). At this stage part of the vegetative mycelium is lysed and can be used as a nutrient source, while the synthesis of antibiotics reaches its maximum presumably to avoid competitive organisms. Eventually, the aerial hyphae are dissected into spores by sporulation septa, producing chains of 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

(Gruber and Gross, 2003). Besides sigma factors, one of the most pleiotropic transcription regulators is AdpA. AdpA is expressed in an A-factor-dependent manner in *Streptomyces griseus* and acts as a transcriptional repressor as well as an activator thus controlling expression of several hundred genes during *Streptomyces* development (Higo et al., 2012). In *Streptomyces coelicolor*, not only could AdpA-mRNA bind purified RNase III *in vitro*, but, as also shown, AdpA and RNase III coordinated the expression of each other in a posttranscriptional feedback loop (Xu et al., 2010). This finding may rationally explain the *rnc* (RNase III-deficient) mutant phenotype that affects expression of genes involved in sporulation and antibiotic production.

Although RNase III from the *Streptomyces* genus was recently shown to assist processing of ribosomal RNAs (Jones et al., 2014), it came to light as a global regulator of antibiotic biosynthesis (Adamidis and Champness, 1992; Aceti and Champness, 1998; Huang et al., 2005; Gatewood et al., 2012; Lee et al., 2013; Jones et al., 2014). In *Streptomyces coelicolor*, the deletion of the gene encoding RNase III [*rnc* gene, also termed as *absB* (Adamidis and Champness, 1992)] leads to a severely reduced production of at least four antibiotics (actinorhodin, undecylprodigiosin, CDA, and methylenomycin) (Price et al., 1999; Chang et al., 2005; Sello and Buttner, 2008). Microarray analysis was used to compare the levels of gene expression in the *S. coelicolor* parental strain and the RNase III mutant strain (Huang et al., 2005). 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 antibiotic biosynthetic pathways (e.g., *actII-ORF4, redD, redZ*, and *cdaR*) were down-regulated, which is consistent with defects in antibiotic production. However, Strakova (Strakova et al., 2013) revealed 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 (Gatewood et al., 2012), 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.

Confirmation 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 (Palecková et al., 2007; Pánek et al., 2008; Swiercz et al., 2008; D'Alia et al., 2010; Mikulík et al., 2014). Hundreds of *cis*-acting asRNAs were identified using RNA sequencing in two recent studies (Vockenhuber et al., 2011; Moody et al., 2013). Here, we further exploited Gatewood's results to see if there are connections between asRNAs and RNase III, which have not yet been reported in *Streptomyces*. We selected 17 mRNAs that are bound by the RNase III enzyme *in vivo* as stated in Gatewood et al. (2012) to check if they possessed an antisense transcript. Surprisingly, the search for asRNAs within the selected group of mRNAs was 100% successful. Moreover, additional analyses revealed antisense transcripts to selected mRNAs that encode RNase III

and several transcription regulators (AdpA, SigB, SigH, and SigR).

Although our data did not elucidate the exact role of newly found asRNAs in the RNase III-degradation pathway, the current findings further demonstrate that the antisense mechanism is widely present in *Streptomyces* and antisense RNAs are possibly involved in developmental and antibiotic synthesis control.

## MATERIALS AND METHODS

### Bacterial Strain, Cultivation

In this study, the *Streptomyces coelicolor* wild-type (wt) strain M145 (Kieser et al., 2000) and its RNase III-deletion strain derivative [*rnc*, M145 *rnc::aac*(3)IV (Sello and Buttner, 2008)] were used. $10^8$ spores were inoculated on solid R2YE medium (Kieser et al., 2000) covered by cellophane at 29°C. Bacterial samples were collected after 24, 48, and 72 h 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 (Van Dessel et al., 2004). Harvested cells were immediately submerged in TRIzol reagent (Ambion) on ice (1 ml of TRIzol per 50 cm² 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 min at 3,000 rpm and twice for 2 min at 4,000 rpm, cooled on ice between the cycles. The samples were subsequently centrifuged for 2 min 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 min 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 and 3.5 μg/μl was obtained. RNA quality was checked on a 1% agarose gel.

### 5′ and 3′ Race

RNA samples were isolated after 48 h of cultivation of both wt and *rnc* strains. Antisense RNA expressions were tested by means of the FirstChoice RLM-RACE Kit (Ambion) following the manufacturer's protocol with the several exceptions:

1. Because the uncapped 5′ ends of bacterial RNAs are sensitive to the CIP (calf intestinal phosphatase) enzyme dephosphorylation, the treatment was omitted from the 5′ RACE procedure.
2. A gene-specific primer (see **Figure 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 designed using the Primer3 software (http://sourceforge.net/projects/primer3/) (Untergasser et al., 2012).
3. The PrimeScript (Takara, 100 units per 10 μl of reaction mixture) reverse transcriptase was always included in the

experiment. Negative control lacking the enzyme was always attached to the experiment.
4. The reverse transcription proceeded at 42°C for 45 min and 48°C for 10 min.
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 *E. coli* One Shot TOP10F' competent cells (Invitrogen). Plasmids containing the cDNA inserts were extracted using the QIAprep Miniprep kit, and sequenced to map 5′ and 3′ ends of RNAs reverse-transcribed.

### Reverse Transcription and PCR

Experiments were performed according to the reverse transcription and PCR protocols described in the FirstChoice RLM-RACE Kit using gene specific DNA probes (details in Results and discussion). The PrimeScript (Takara, 100 units per 10 μl of reaction mixture) was used as a reverse transcriptase. A negative control PCR reaction used the original RNA sample as a template.

### Northern Blot Analysis

RNA samples (30 μg) were denatured for 10 min at 70°C in RNA loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 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, Bio-Rad) by electroblotting at 240 mA for 45 min. The nylon membrane was UV-crosslinked.

Oligonucleotides were radioactively labeled on their 5′ ends by $\gamma$-$^{32}$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 0.1xSSC, 0.1% SDS (NorthernMax kit) at 42°C. The membranes were dried and exposed in a BAS cassette on the imaging plate (Fuji-Film) for 4 days. The signals were visualized using a Phosphorimager FX (Bio-Rad) and quantified using QuantityOne analysis software (Bio-Rad), where the signals were standardized proportionally to the 5S RNA levels. Each northern blot was performed at least twice with samples from separate cultivations in the same conditions.

## RESULTS

Using the RNA-seq approach, Gatewood (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
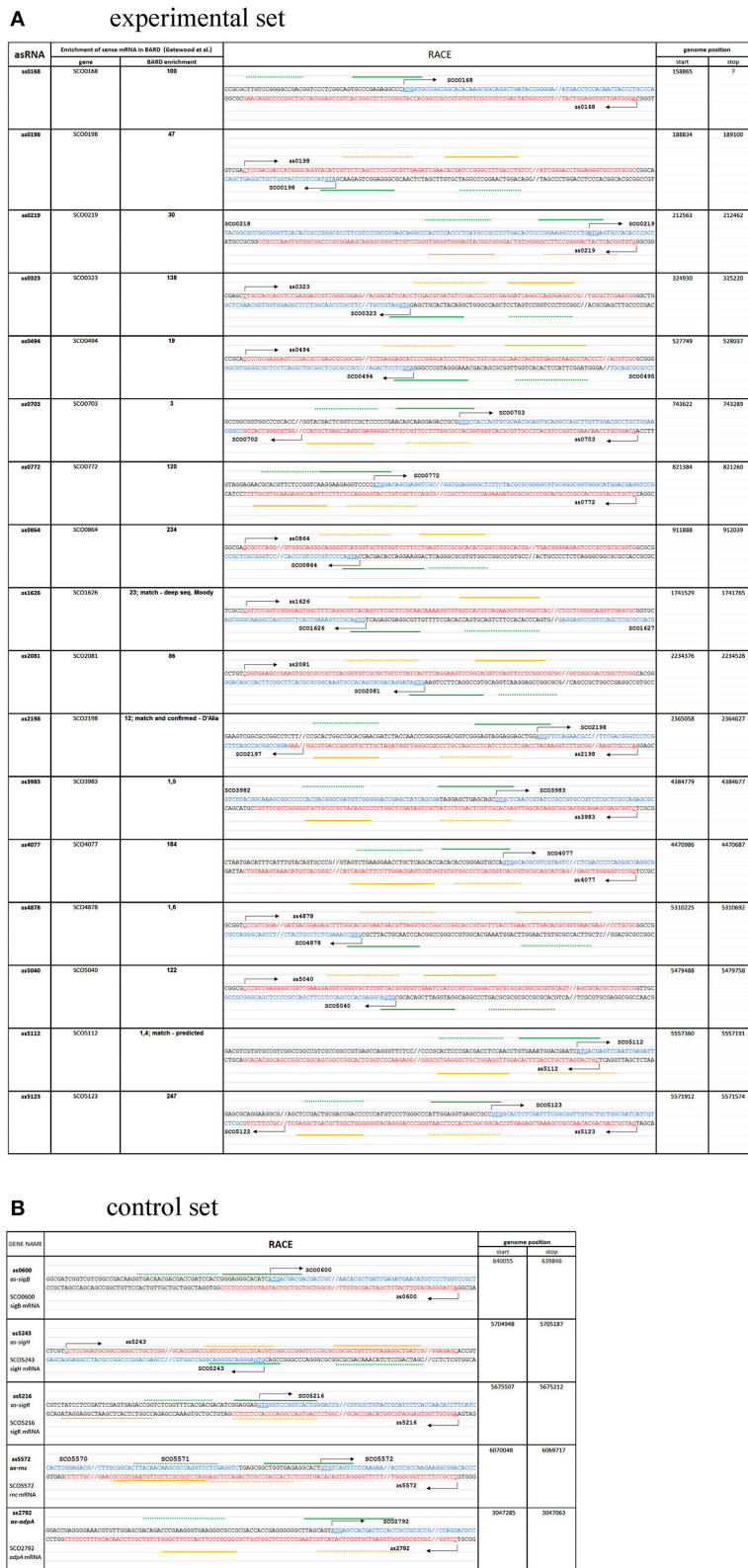
**FIGURE 1 |** Novel asRNAs revealed by 5′and 3′RACE method, and their genome locations (sequence of asRNA in red, sequence of mRNA in blue). Transcriptional start sites are indicated by arrows. Full green line represents 5′RACE inner primers, dotted green line represents 5′RACE outer primers, full orange line represents 3′RACE inner primers, dotted orange line represents 3′RACE outer primers. **(A)** Experimental set; **(B)** Control set; (see text for details).

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 in their paper as BARD, bead-antibody-RNA-D70A strain). However, the necessity of involvement of other transcripts for the binding of the double-strand-specific enzyme to the mRNAs is still unknown in the case of *Streptomyces*. *In vitro* assays showed that the RNase III digests mRNA transcripts SCO3982 to SCO3988 and SCO5737 unattended (i.e., without asRNA) (Gatewood et al., 2012), whereas another unattended transcript, SCO0762, was not cleaved (Xu et al., 2008). Although *Streptomyces* as GC rich bacteria form highly structured RNAs, naturally occurring stem-loop structures of most mRNAs are too short to be bound by the RNase III enzyme that requires a minimum of approximately 20 bp of double stranded RNA for binding *in vivo* (Robertson, 1982).

## RNase III-Binding mRNAs and Small RNAs Genome Vicinities

Here, to probe if the antisense RNA expression occurs in the vicinity of the RNase III-binding mRNAs (or alternatively, the binding is not complexed with asRNA), we firstly analyzed the genes whose transcripts are affected by RNase III (i.e., those mRNAs that are increased by more than two-fold in the JSE1880 *rnc*-mutant or detected in BARD as listed in the **Table S2** in Gatewood; Gatewood et al., 2012). We asked whether in the proximity of these genes lies a gene encoding for any of 1713 small RNAs found or predicted in *S. coelicolor* up-to-date (Pánek et al., 2008; Swiercz et al., 2008; D'Alia et al., 2010; Vockenhuber et al., 2011; Moody et al., 2013) that could act on the messenger by the antisense mechanism. We found out that from the 153 mRNAs increased in JSE1880 (at a single experimental time point), 45 neighbor with one of the 1713 small RNAs that could thus theoretically act as an asRNA. From these 45 mRNAs, 21 mRNAs were listed in the BARD, i.e., they bind RNase III. These data, summarized in **Table 1**, encouraged us to search for novel asRNAs expressed in the opposite direction to other RNase III-binding mRNAs.

## Experimental Search for Novel *cis*-Antisense Transcripts

Genes for the experimental analyses had been selected independently of the *in silico* search above. We altogether tested 30 genes for the possible novel antisense expression. The initial experimental set consisted of 17 exemplar genes which we selected out of a total of 37 genes whose mRNAs were enriched in the BARD, i.e., they co-precipitated with RNase III (listed in **Table 2**, see also Gatewood's Table 2 and Table S3 in Gatewood et al., 2012). Within this set, only *as1625*, *as2198*, and *as5112* genes have been predicted before (see **Table 1**). The control set consisted of three other genes—SCO5737, *adpA*, and *rnc* (the gene of RNase III) that had been previously shown to be directly targeted by RNase III *in vitro* (Xu et al., 2008, 2010; Gatewood et al., 2012). Additionally, to determine whether the existence of antisense transcripts involves only those messengers bound by RNase III or is even more widespread, we decided to add into the control set the genes whose transcripts have not been shown

to bind RNase III. For these tests, we selected 10 genes encoding sigma factors (HrdA, HrdB, HrdC, HrdD, SigB, SigD, SigE, SigH, SigR, and WhiG) as important transcriptional regulators that govern gene expression, controlling cell development and/or responses to various stresses (Bobek et al., 2014).

We reasoned that many asRNAs overlap the ribosome binding site and the start codon of their target, possibly leading to negative translational control. Following this, all of the DNA primers used here to find antisense transcripts have been designed accordingly (**Figure 1**). To demonstrate expression of antisense transcripts, 5′ RACE analyses were performed using the primers. Sequence(s) extended from the primer was/were PCR amplified and sequenced. To avoid false positive results, each reverse transcription was accompanied with a negative control sample lacking the reverse transcriptase in the reaction. The resulting electrophoretograms of both the experimental and the negative control samples were compared to exclude non-unspecific fragments in the experimental sample from sequencing.

From the total of 30 samples tested (involving both the experimental and the control set), 22 revealed a novel antisense transcript in *cis* (5′ end(s) detected; see **Table 2**).

From the 17 samples of the experimental set (i.e., our selection from those mRNAs enriched in the BARD), 17 cognate asRNAs were detected (three of them have been predicted previously as could be seen in **Table 1**), signifying a 100% outcome within this group (**Figure 1A**). The 3′ RACE revealed 16 3′ ends within the experimental set (*scr0168* unsuccessful, northern blot was not performed).

In the control set (**Figure 1B**), our RACE data detected transcripts antisense to *adpA* (*as-adpA*), *rnc* (*as-rnc*), and three sigma factor genes (*as-sigB, as-sigH, as-sigR*). Seven sigma factor genes and the transcript SCO5737, which is bound by RNase III *in vitro*, did not reveal antisense expression.

Due to our primer design, we did not detect such antisense transcripts acting on messenger's 3′ end [in Moody (Moody et al., 2013) termed as cutoRNAs]. Also the experimental approach did not allow identification of *trans*-encoded sRNAs, although similar complementary sequence on the genome could be found (as examples, novel RNAs as0772, as0600, as3983, and as5216 could theoretically act on other targets throughout the genome).

Sometimes the RACE analysis may produce false positive results due to the RNA self-priming during the reverse transcription which could be theoretically caused by stem-loop structures formation. In order to confirm the existence of the newly found *cis*-antisense transcripts and to compare their expression during *Streptomyces* cell development (24, 48, and 72 h at standard growth conditions, see methods), we performed northern blot analyses. From the total of 22 analyzed, 18 asRNAs revealed an apparent signal (**Figure 2**; for the raw northern blot images see **Presentation 1** in Supplementary Material). The signals of the remaining four were too weak or undetectable.

Details describing expression profiles of asRNAs are summarized in **Table S1** and signal quantification is presented in **Table S2** online, where the listed relative values proportional to the 5S RNA controls correspond with intracellular levels of the analyzed transcripts.

**TABLE 1 |** *In silico* search for sRNA genes adjacent to mRNAs that are up-regulated in rnc mutant.

| Selected genes whose expression increased in JSE1880 (blue indicate transcripts present in the BARD; Gatewood et al., 2012) | | Antisence/adjacent small RNAs (scr = small coelicolor RNA) | | References | Relative orientation of asRNA gene to its possible mRNA target (red arrow) and the second adjacent gene (black arrow), * unknown (ambiguous) strand of asRNA gene, => orientation of asRNA gene, cutoRNA—antisense transcripts acting on messenger's 3′ end | | Comment |
|---|---|---|---|---|---|---|---|
| SCO | 0499 | scr | 0500 | Moody et al., 2013 | Ambiguous | –> * <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 0500 | scr | 0500 | Moody et al., 2013 | Ambiguous | –> * <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 1150 | scr | 1150 | Moody et al., 2013 | cutoRNA | –> <= <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 1565 | scr | 1566 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 1626 | scr | 1625 | Moody et al., 2013 | cutoRNA | –> <= <– | as1625 analyzed here |
| SCO | 1630 | scr | 1631 | Swiercz et al., 2008 | Ambiguous | <– * <– | Predicted |
| SCO | 1659 | scr | 1659 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 1700 | scr | 1700 | D'Alia et al., 2010 | cis asRNA | <– => <– | Predicted by RNAz (D'Alia et al., 2010) |
| SCO | 1906 | scr | 1907 | Swiercz et al., 2008 | Ambiguous | <– * <– | Predicted |
| SCO | 2197 | scr | 2198 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 2198 | scr | 2198 | D'Alia et al., 2010 | cis asRNA | <– <= –> | Confirmed, *as2198* analyzed here |
| SCO | 3003 | scr | 3004 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 3113 | scr | 3114 | Swiercz et al., 2008 | Ambiguous | –> * <– | Predicted |
| SCO | 3132 | scr | 3133 | D'Alia et al., 2010 | cis asRNA | –> <= <– | Predicted by RNAz (D'Alia et al., 2010) |
| SCO | 3216 | scr | 3216 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 3217 | scr | 3217 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 4095 | scr | 4096 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 4142 | scr | 4143 | Swiercz et al., 2008 | Ambiguous | <– * <– | Predicted |
| SCO | 4144 | scr | 4145 | Swiercz et al., 2008 | Ambiguous | <– * <– | Predicted |
| SCO | 4145 | scr | 4145 | Swiercz et al., 2008 | Ambiguous | <– * <– | Predicted |
| SCO | 4145 | scr | 4146 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 4229 | scr | 4229 | Swiercz et al., 2008 | ambiguous | <– * –> | Predicted |
| SCO | 4249 | scr | 4249 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 4283 | scr | 4283 | Moody et al., 2013 | cutoRNA | –> <= <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 4698 | scr | 4699 | Moody et al., 2013 | Ambiguous | –> <= –> | Predicted |
| SCO | 4748 | scr | 4749 | Moody et al., 2013 | cutoRNA | –> => <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 4882 | scr | 4883 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 4947 | scr | 4947 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 5106 | scr | 5106 | Moody et al., 2013 | cutoRNA | –> => <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 5112 | scr | 5112 | Swiercz et al., 2008 | cis asRNA | –> * –> | Predicted, *as5112* analyzed here |
| SCO | 5142 | scr | 5143 | Moody et al., 2013 | Ambiguous | –> <= –> | Predicted |
| SCO | 5145 | scr | 5145 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 5145 | scr | 5146 | Moody et al., 2013 | cutoRNA | –> => <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 5163 | scr | 5164 | Swiercz et al., 2008 | Ambiguous | <– * –> | Predicted |
| SCO | 5476 | scr | 5476 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 5519 | scr | 5518 | Moody et al., 2013 | Ambiguous | <– * –> | Predicted |
| SCO | 5520 | scr | 5521 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 5521 | scr | 5521 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |

*(Continued)*

**TABLE 1 |** Continued

| Selected genes whose expression increased in JSE1880 (blue indicate transcripts present in the BARD; Gatewood et al., 2012) | | Antisence/adjacent small RNAs (scr = small coelicolor RNA) | | References | Relative orientation of asRNA gene to its possible mRNA target (red arrow) and the second adjacent gene (black arrow), * unknown (ambiguous) strand of asRNA gene, => orientation of asRNA gene, cutoRNA—antisense transcripts acting on messenger's 3′ end | | Comment |
|---|---|---|---|---|---|---|---|
| SCO | 5536 | scr | 5536 | Pánek et al., 2008 | Ambiguous | <– * –> | Termed #234 |
| SCO | 5537 | scr | 5537 | Swiercz et al., 2008 | Ambiguous | –> * <– | Predicted |
| SCO | 5757 | scr | 5756 | Swiercz et al., 2008 | Ambiguous | –> * –> | Predicted |
| SCO | 6277 | scr | 6277 | Moody et al., 2013 | cis asRNA | –> <= –> | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 6283 | scr | 6284 | Moody et al., 2013 | Ambiguous | –> * –> | Predicted |
| SCO | 6284 | scr | 6284 | Moody et al., 2013 | Ambiguous | –> * –> | Predicted |
| SCO | 6284 | scr | 6285 | Moody et al., 2013 | Ambiguous | –> <= –> | Predicted |
| SCO | 6396 | scr | 6396 | Moody et al., 2013 | Ambiguous | –> * –> | Predicted |
| SCO | 6716 | scr | 6716 | Moody et al., 2013 | cutoRNA | –> <= <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |
| SCO | 6716 | scr | 6717 | Moody et al., 2013 | cutoRNA | –> => <– | – |
| SCO | 6728 | scr | 6729 | Moody et al., 2013 | cutoRNA | –> => <– | mRNA up-regulated in JSE1880 (Gatewood et al., 2012) |

## Vice Versa: *scr2101* Small RNAs Found in BARD (Gatewood et al., 2012) Is Antisense to the SCO2100-SCO2101 Transcript

In Gatewood's work, expression of two small RNAs, *scr6925* and *scr2101*, was enriched in the *rnc* strain (scr = small *S. coelicolor* RNA). These data imply their antisense role and subsequent processing by the RNase III enzyme. The expression of *scr2101* was previously revealed by Swiercz (Swiercz et al., 2008). Gatewood et al. (2012) showed that the molecule is up-regulated in the JSE1880 strain (lacking the RNase III), where its level was 7-fold higher compared to that in 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-coding genes form an operon, producing one common transcript targeted by the *scr2101* antisense RNA. The potential SCO2100-SCO2101 common transcript was used here 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. Use of a negative control PCR reaction, where the original RNA was used instead of a template, eliminated the risk of false positive results (**Figure S1**). This finding suggests that the antisense function of *scr2101* (which thus should be termed as *as2101*) targets the region between two open reading frames. 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.

## DISCUSSION

### Novel asRNAs to mRNAs That Bind RNase III *in Vitro*

The double stranded stems of stem-loop mRNA structures are thought to be too short to be digested by the RNase III enzyme (Robertson, 1982). On the other hand, one may argue that the stem-loop structures on several mRNAs are the only possible targets for RNase III activity. Indeed, the enzyme is involved in pre-rRNA, tRNA, and polycistronic RNA processing (Conrad and Rauhut, 2002; Drider and Condon, 2004), where such stem-loop structures are present and possibly long enough to be cleaved by the enzyme. Gatewood clearly showed that some lone mRNAs are targeted and cleaved by RNase III *in vitro* (SCO5737, or the SCO3982 to SCO3988 mRNA region). In accordance to the first example, our experiments did not reveal any antisense transcript to the SCO5737 (which encodes a polynucleotide phosphorylase). Its mRNA transcript is 2,220 nt in length, and thus might be capable of forming several longer double-stranded regions observed by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi; fig. not shown), which are most likely targeted by the RNase III enzyme *in vitro*.

On the other hand, we show that at least one member of the SCO3982-SCO3988 operon – SCO3983 possesses an antisense gene. Its transcript, *as3983*, 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 *as3983* sequence is nearly identical to the region adjacent to SCO3268 and theoretically may thus also act on this transcript in *trans*.

**TABLE 2 |** The list of analyzed genes.

| | Genes selected for the analyses | Encoded protein | Relation of mRNA to RNase III | | RACE | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Ratio enrichment in BARD | Fold increase in JSE1880 | 5'/3' end detection result | Original strain from which the final RACE data were determined | Northern blot detection result | 5'end-antisense expression independently confirmed by RNA-seq data* |
| | | | Data taken from Gatewood et al. (2012) | | | | | |
| **Experimental set (mRNA co-precipitated with RNase III *in vivo*)** | SCO 0168 | Possible regulatory protein | 100 | 2.41 | +/− | WT | − | +/+ |
| | SCO 0198 | Hypothetical protein | 47.6 | 2.70 | +/+ | WT | + | −/− |
| | SCO 0219 | Putative nitrate reductase delta chain | 30 | 2.18 | +/+ | WT, RNC | + | −/− |
| | SCO 0323 | Hypothetical protein | 138 | 1.62 | +/+ | WT, RNC | + | +/+ |
| | SCO 0494 | Probable iron-siderophore binding lipoprotein | 19 | −2.14 | +/+ | RNC | + | −/− |
| | SCO 0703 | Putative regulatory protein | 119 | 1.36 | +/+ | RNC | + | −/− |
| | SCO 0772 | Putative regulatory protein | 3 | 2.46 | +/+ | WT, RNC | + | +/− |
| | SCO 0864 | Probable ECF-family sigma factor | 234 | 1.70 | +/+ | WT | + | −/− |
| | SCO 1626 | Cytochrome P450 | 22.8 | −2.6 | +/+ | WT, RNC | + | +/+ |
| | SCO 2081 | Hypothetical protein | 86 | 1.23 | +/+ | WT, RNC | − | −/− |
| | SCO 2198 | Glutamine synthetase I | 12.4 | 2.9 | +/+ | RNC | + | +/+ |
| | SCO 3983 | Hypothetical protein | 1.5 | 2.14 | +/+ | WT, RNC | + | +/− |
| | SCO 4077 | Hypothetical protein | 184 | −1.01 | +/+ | WT, RNC | + | +/− |
| | SCO 4878 | Glycosyltransferase | 1.6 | 2.00 | +/+ | RNC | − | +/− |
| | SCO 5040 | Hypothetical protein | 122 | 1.22 | +/+ | RNC | − | −/− |
| | SCO 5112 | Putative ABC transport systém integral membrane protein, BldKA | 1.4 | 2.56 | +/+ | WT, RNC | + | +/− |
| | SCO 5123 | Small membrane protein | 247 | 1.24 | +/+ | WT, RNC | + | +/− |
| **Control set** | SCO 2792 | AdpA | *In vitro* binding | | +/+ | RNC | + | +/+ |
| | SCO 5572 | RNase III | *In vitro* binding | | +/+ | WT | + | +/+ |
| | SCO 5737 | Guanosine pentaphosphate synthetase/polyribonucleotide nucleotidyltransferase | *In vitro* binding | | −/− | − | − | +/+ |
| | SCO 0600 | RNA polymerase sigma factor SigB | Unknown | | +/+ | WT | + | +/− |
| | SCO 0895 | RNA polymerase sigma factor HrdC | Unknown | | −/− | − | − | +/− |
| | SCO 2465 | RNA polymerase sigma factor HrdA | Unknown | | −/− | − | − | +/+ |
| | SCO 3202 | RNA polymerase sigma factor HrdD | Unknown | | −/− | − | − | +/+ |
| | SCO 3356 | RNA polymerase sigma factor SigE | Unknown | | −/− | − | − | +/− |
| | SCO 4769 | RNA polymerase sigma factor SigD | Unknown | | −/− | − | − | +/− |
| | SCO 5216 | RNA polymerase sigma factor SigR | Unknown | | +/+ | WT | + | +/− |
| | SCO 5243 | RNA polymerase sigma factor SigH | Unknown | | +/+ | WT | + | +/+ |
| | SCO 5621 | RNA polymerase sigma factor WhiG | Unknown | | −/− | − | − | −/− |
| | SCO 5820 | RNA polymerase sigma factor HrdB | Unknown | | −/− | − | − | +/− |

*The expression was confirmed from the RNA-Seq data (Vockenhuber et al., 2011)/(Moody et al., 2013).*

In addition to the identified as3983 RNA, we have found antisense transcripts to two other mRNAs targeted by RNase III *in vitro*. These two mRNAs code important developmental regulators that influence antibiotic production—AdpA and the RNase III itself.

According to Gatewood's results, the *adpA* mRNA was enriched 22-fold in the BARD. Therefore, we asked whether AdpA expression is controlled by an antisense mechanism. Indeed, our RACE analysis revealed a 223-nt long antisense transcript termed *as-adpA* (**Figure 1B**) and although subsequent

northern blot weakly detected the transcript in the wt strain (faint signal at 72 h stage of growth, **Figure 2B**), it was clearly found in the *rnc* strain at later stages, peaking at the 48 h old mycelium.

The *rnc* gene (SCO5572) that encodes the RNase III enzyme is the last member of a three gene operon, about 2,000 nt in length, that also encodes a hypothetical protein SCO5570 and a ribosomal protein L32 (SCO5571). A point mutation at amino acid position 120 of the RNase III protein causes deficiency in the ribonucleolytic activity of the enzyme, whereas its ability to bind double stranded RNAs remains intact (Huang et al., 2005). Observations that the *rnc* mRNA abundance is increased in these mutants led to discovery that RNase III cleaves among others its own transcript (Xu et al., 2008). Consistently, our northern blot analyses did not detect the *rnc* transcript in the wt strain, though we clearly confirmed the *as-rnc* RNA, which was detectable during the time course. Hence the antisense mechanism is probably involved in gene expression control of regulatory proteins such as the RNase III enzyme or the AdpA transcription regulator. Our future effort will be focused on more detailed characterization of the involvement of these two novel asRNAs in *Streptomyces* development.

## *as2198* RNA as an Example from the Experimental Set

*as2198* is an asRNA to the *glnA* gene (SCO2198, encoding a glutamine synthase) and was previously independently shown to be expressed, termed *cnc2198.1* (D'Alia et al., 2010). The authors performed a detailed functional analysis and revealed 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 be 432 nt, nearly four times longer than the 121 nt transcript described by D'Alia et al. (2010). Moreover, the detected 3′ end overlapped the adjacent SCO2197, revealing that *as2198* is in fact the 5′UTR of the SCO2197 mRNA. The fact that our northern blot showed a second, weaker fragment of 425 nt in size, could mean that the SCO2197 gene possesses another promoter or its transcript is further processed. 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.
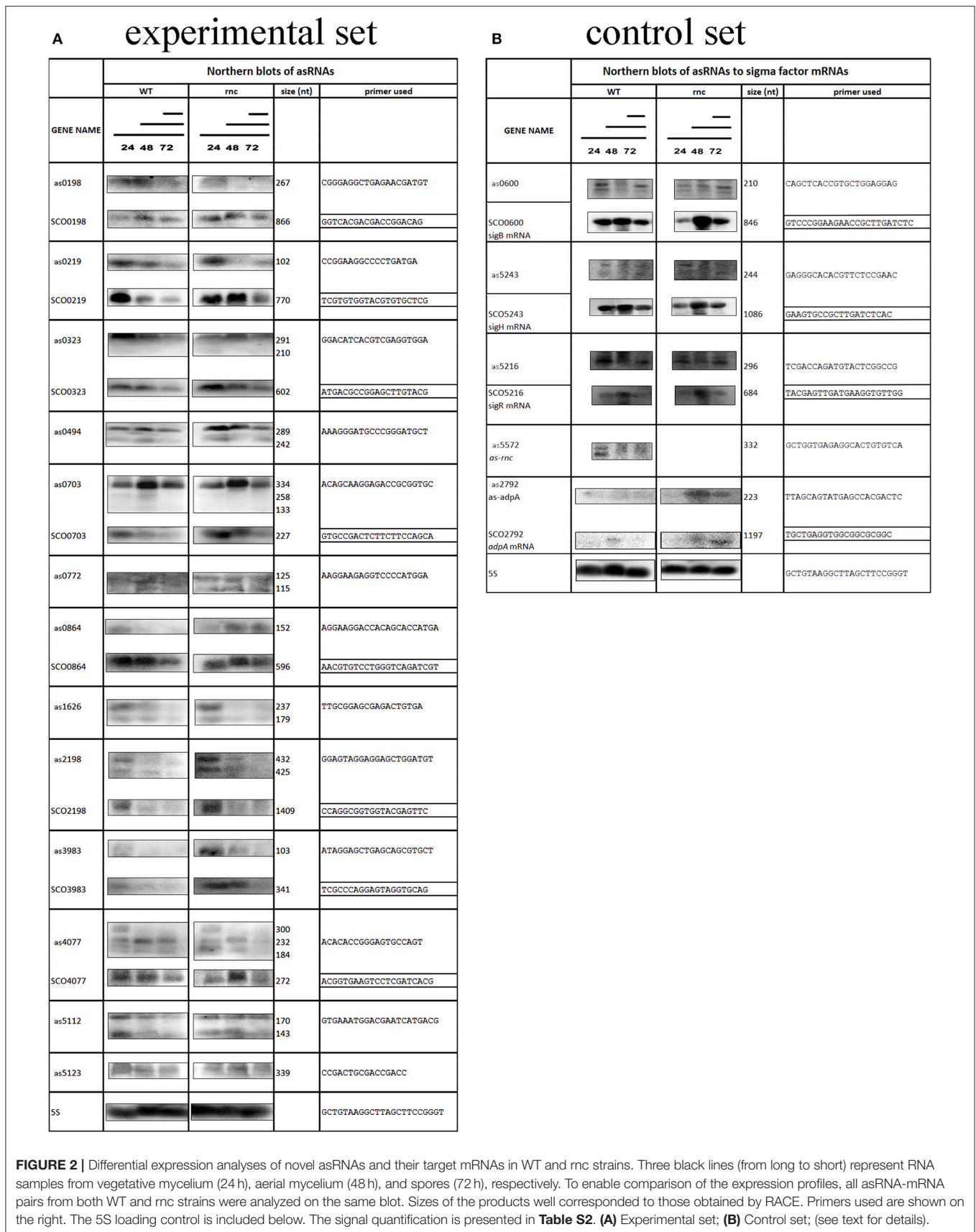
## Are the Sense-Antisense Transcripts Cleaved by RNase III?

Because the sense mRNAs of the antisense transcripts found here are targeted by RNase III (or at least, their expression was negatively affected in the presence of the enzyme), we speculated that the RNase III enzyme is the most likely candidate for the paired transcript degradations. The 18 northern blot positive

results proved the existence of novel asRNAs. However, only five of those asRNAs were increased in the *rnc* strain when compared to the expression in the wild type strain. These included *as0494, as0864, as2198, as3983*, and the *as-adpA*. On the other hand, in two other cases, our northern blots suggest even a positive effect of the presence of RNase III enzyme on the cellular level of asRNAs (*as0323* and *as5112*, see below). Consistently, Gatewood (Gatewood et al., 2012) 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. As another example of sense-antisense RNA pair with an undistinguishable or even a positive effect of the enzyme on its stability in *Streptomyces* is the *scr4677*-SCO4676 complex (Hindra et al., 2014). 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 bacterial model, *Bacillus subtilis* (Durand et al., 2012). 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 *rnc* mutants. As the RNase III-binding mRNAs may serve as a fruitful source for novel antisense transcript discoveries, we can assume that the mRNA-asRNA pairs are targeted by the enzyme but not always degraded. An involvement of some other RNA-binding proteins, such as RNA helicase or Hfq-like protein which has not been found in *Streptomyces* yet, could be expected (Gerhart Wagner, Uppsala University, Sweden, personal communication). On the other hand, the possibility that in other cases the antisense transcripts might protect their mRNA targets against the RNase III cleavage should be taken in mind. Clarification of the exact role of RNase III enzyme in *Streptomyces* remains to be established.

## asRNAs as a Potential Part of the Gene Expression Control System in *Streptomyces*

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 all the 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) that are encountered. Developmental transitions and environmental intricacy require advanced regulatory networks that involve a concerted action of more than 900 transcription regulatory proteins known thus far. Here we unveiled 22 novel antisense transcripts, out of which 18 were

**FIGURE 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. To enable comparison of the expression profiles, all asRNA-mRNA pairs from both WT and rnc strains were analyzed on the same blot. Sizes of the products well corresponded to those obtained by RACE. Primers used are shown on the right. The 5S loading control is included below. The signal quantification is presented in **Table S2**. **(A)** Experimental set; **(B)** Control set; (see text for details).

confirmed by northern blot analyses. These results 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 (Georg and Hess, 2011), 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 confirm the extensiveness of the antisense transcripts and raise the demand for elucidation of their role in gene expression control in *Streptomyces*.

## AUTHOR CONTRIBUTIONS

DŠ, PP, IM, and KŠ conceived the RACE and northern blot experiments, JB performed the *in silico* analysis and provided the project design, analyzed the results and wrote the manuscript. All authors reviewed the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02693/full#supplementary-material

**Figure S1 |** SCO2100-SCO2101 RT-PCR. RNA samples were isolated from both wt (lane 1) and rnc (lane 2) 48 h old cultures from the R2YE medium. The first probe TGTCCCGGCTGCTCCAGGGA primed the reverse transcription reaction and together with the second probe CGTAGGTCCCCGCCCGCT was used in the subsequent PCR. Additional samples originated from both strains but lacking reverse transcriptase in the reaction were used as negative controls (lanes 3 and 4).

**Table S1 |** Description of the expression profiles of detected asRNAs.

**Table S2 |** Quantification of the northern blot signals presented in Figure 2.

**Presentation 1 |** Raw northern blot images.

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