Noncoding RNA of Glutamine Synthetase I Modulates Antibiotic Production in *Streptomyces coelicolor* A3(2)\(\uparrow\)\‡

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Overexpression of antisense chromosomal cis-encoded noncoding RNA(s) (ncRNAs) in glutamine synthetase I resulted in a decrease in growth, protein synthesis, and antibiotic production in *Streptomyces coelicolor*. In addition, we predicted 3,597 cis-encoded ncRNAs and validated 13 of them experimentally, including several ncRNAs that are differentially expressed in bacterial hormone-defective mutants.

In many diverse organisms, noncoding RNA (ncRNAs) have been identified as major regulators of translation efficiency and message stability (9, 23). Recent screens for ncRNAs in human predict approximately 30,000 structured RNAs (22), and many have been characterized as having a variety of regulatory functions and thus are not simply transcriptional-by-products or “transcriptional noise” (2, 5). However, the number of ncRNAs identified in bacteria is lagging behind those found in eukaryotes. Since the first publication of a fully sequenced bacterial genome, a number of genomewide computational screens for ncRNAs in microorganisms have been conducted, predicting 50 to 2,000 ncRNAs (11). ncRNAs are grouped into cis-encoded or trans-encoded classifications, depending on whether the ncRNA structure is located in the transcribed sequence of its target or found at a distance, for instance, in an intergenic region. The former type of ncRNA is reported mainly in bacterial plasmids, whereas the latter class accounts for most of the ncRNAs reported in bacterial chromosomes. These trans-encoded ncRNAs require helper proteins such as Hfq to bind to the target mRNA. They can either degrade or stabilize the target mRNA, and they are supposed to be involved in the regulation of metabolic pathways due to environmental change or stress responses (23). Recently, cis-and trans-encoded ncRNAs have been identified in mycobacteria, and the function of a trans-encoded ncRNA has been demonstrated (1). Several groups or researchers (12, 18, 20) have predicted and partially validated the existence of ncRNAs in *Streptomyces*, a genus of Gram-positive soil bacteria with high biotechnological relevance due to their diverse secondary metabolite production, including the production of a large variety of antibiotics of commercial importance. However, in all of these cases where trans-encoded ncRNAs were identified, their function (if any) or mode of action remained completely unknown.

In the model streptomycete *Streptomyces coelicolor*, no functional analysis of ncRNA has been conducted, nor are there any homologues to the main components of the RNA silencing system in the eukaryote, or the main ncRNA processing enzymes in prokaryotes, apart from homologues to enzymes, which are able to degrade double-stranded RNA. To explore a potential role of ncRNA in *S. coelicolor*, we first focused on the *glnA* gene (SCO2198), which encodes for glutamine synthase I (GSI) (6), a key regulatory target of central nitrogen metabolism and major player in the link of nitrogen assimilation to antibiotic production. An intragenic ncRNA in *glnA* called “cnc2198.1” was predicted (Fig. 1). The *cnc2198.1* ncRNA was detected in early exponential phase for the wild-type and two signaling molecule-impaired strains tested by reverse transcription-PCR (RT-PCR) and tagged-primer-specific quantitative RT-PCR (qRT-PCR; data not shown) (14). Primer-specific cDNA synthesis was performed with tagged primer to retrotranscribe the antisense transcript and then used as a template for qRT-PCR analysis (see Table S2 and other supplementary material). We went on to test whether this ncRNA would also be functional, despite the absence of any previously characterized RNA-binding proteins involved in the processing of

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ncRNA identified in eukaryotes or prokaryotes. An antisense 88-bp fragment that overlaps with cnc2198.1 (cnc2198.1as; 120 bp) was cloned into the multicopy plasmid pIJ8781 under the control of the thiostrepton-inducible promoter tipAp (see the supplemental material). The resulting plasmid pTE313, as well as the vector pIJ8781, was introduced into S. coelicolor M145. The two strains were grown to an optical density at 450 nm (OD\textsubscript{450}) of 0.7 in SMM (19), and the expression of cnc2189.1as was induced with thiostrepton in dimethyl sulfoxide (DMSO; 5 μg/ml). Pure DMSO was used as a negative control. When cnc2198.1as expression was induced, GSI signal detected by

FIG. 2. Western blot analysis of GSI. Protein levels were analyzed by Western hybridization with anti-GSI specific antibodies at 1 and 5 h after induction of cnc2198.1as (M145/pTE313) expression (+) or addition of DMSO (−) in M145/pIJ8781 or M145/pTE313.

FIG. 3. Predicted secondary structure of \textit{glnA} mRNA and cnc2198.1as RNA. The web-based RNAfold software was used to predict the secondary structure of the \textit{glnA} and cnc2198.1as transcripts. The single-stranded regions in the structures are indicated as the most likely nucleotides to be involved in the interaction between the two RNA molecules; however, the interaction between ncRNA and mRNA could also occur before the secondary structure formation or involve rearrangements in the secondary structure, for instance, by RNA helicases.
Western analysis with a GSI antibody (dilution, 3:10,000) using 100 μg of total protein extract, decreased to 72% ± 9% and 63% ± 6% at 1 and 5 h, respectively, in comparison to M145 harboring the vector alone (Fig. 2). (The values are averages of three independent growth curves.) This shows that cnc2198.1as indeed affects protein expression levels of its target gene glnA by perhaps interacting with the few nucleotides of glnA mRNA (Fig. 3) inducing the unfolding and the complementary annealing of the two transcripts that would lead to a stall of translation and/or degradation, possibly through a mechanism independent of the classical ncRNA processing enzymes.

This molecular effect also had important and wide-ranging regulatory consequences at the phenotypic level. Not only did growth decrease (Fig. 4), but there was also a substantial effect on antibiotic production, the major regulated physiological process of Streptomyces. The red pigmented antibiotic undecylprodigiosin production of M145/pTE313 decreased 4-fold after induction of cnc2198.1as expression compared to the negative control and 3.5-fold compared to the level seen in M145/pIJ8781 when measured as reported (19) (Fig. 5).

To explore the general relevance of ncRNAs in Streptomyces, we also conducted a computational genomewide prediction of ncRNAs by using RNAz v1.01 on the 54% of the genome of S. coelicolor that aligned with S. avermitilis as reported previously in yeast (17; see also the supplemental material). The presence of ncRNA was predicted where the RNA classification confidence value was ≥0.5. Of the 3,895 ncRNA elements predicted, 3,597 overlapped with 2,965 annotated coding sequences, and the remaining 298 were located in the intergenic regions. The predicted ncRNAs included all tRNAs and rRNAs annotated in RFAM and TIGR (see Table S1 in the supplemental material). Our predictions also overlapped to a large extent with those found in previous studies: 28 out of 32 predictions by Pánek et al. (12) and 55 of 114 predictions by Swierzcz et al. (18) were confirmed.

Total RNA was extracted as reported previously (19), and ncRNA transcripts were analyzed by primer-specific qRT-PCR (see Table S2 and other supplemental material). For each transcript, the signal was normalized to the specific transcriptional background. Signals above the negative control cnc2966 (no ncRNA predicted) were considered validated. Of the 18 predicted cis-encoded ncRNA elements that were analyzed, 13 were shown to have antisense ncRNA present either in the wild type or in two signaling molecule (-butyrolactone) mutants (Table 1). These 18 ncRNAs correspond to those genes which were differentially expressed in a -butyrolactone mutant (E. Takano et al., unpublished data). Interestingly, two ncRNAs (cnc3669.2 and cnc3993.2) were detected only in the two signaling molecule impaired mutants and were not present in the wild type. This, for the first time, suggests that these signaling molecules are involved in the regulation of ncRNA expression in S. coelicolor.

This is the first report to elucidate a cis-encoded antisense...
TABLE 1. Confirmation of predicted antisense ncRNAs

<table>
<thead>
<tr>
<th>ncRNAa</th>
<th>Predicted antisense ncRNAa</th>
<th>M145</th>
<th>M751</th>
<th>M752</th>
</tr>
</thead>
<tbody>
<tr>
<td>cnc2966</td>
<td>(− (3.06 ± 1.02))</td>
<td>− (5.09 ± 0.37)</td>
<td>− (1.04 ± 0.13)</td>
<td></td>
</tr>
<tr>
<td>cnc0902</td>
<td>+ (18.21)</td>
<td>+ (66.04)</td>
<td>+ (12.53)</td>
<td></td>
</tr>
<tr>
<td>cnc2819</td>
<td>+ (230.86)</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td></td>
</tr>
<tr>
<td>cnc3005(5-7)</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td></td>
</tr>
<tr>
<td>cnc3005.5</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td></td>
</tr>
<tr>
<td>cnc3005(1-2)</td>
<td>− (NA)</td>
<td>− (NA)</td>
<td>− (NA)</td>
<td></td>
</tr>
<tr>
<td>cnc3609.1</td>
<td>+ (56.95)</td>
<td>+ (692.54)</td>
<td>+ (93.13)</td>
<td></td>
</tr>
<tr>
<td>cnc3609.2</td>
<td>− (3.70)</td>
<td>+ (150.26)</td>
<td>+ (17.27)</td>
<td></td>
</tr>
<tr>
<td>cnc3893.2</td>
<td>− (1.54)</td>
<td>+ (7.11)</td>
<td>+ (2.11)</td>
<td></td>
</tr>
<tr>
<td>cnc4296</td>
<td>− (0.61)</td>
<td>− (2.71)</td>
<td>− (1.08)</td>
<td></td>
</tr>
<tr>
<td>cnc4425</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td>+ (NA)</td>
<td></td>
</tr>
<tr>
<td>cnc4727</td>
<td>− (0.90)</td>
<td>− (5.02)</td>
<td>− (0.81)</td>
<td></td>
</tr>
<tr>
<td>cnc5368</td>
<td>− (0.95)</td>
<td>− (3.17)</td>
<td>− (1.16)</td>
<td></td>
</tr>
<tr>
<td>cnc5998.1</td>
<td>+ (−1.10)</td>
<td>− (NA)</td>
<td>− (1.04)</td>
<td></td>
</tr>
<tr>
<td>cnc5998.2</td>
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<td>+ (NA)</td>
<td>+ (NA)</td>
<td></td>
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<tr>
<td>cnc6624</td>
<td>(−1.13)</td>
<td>− (1.07)</td>
<td>+ (1.45)</td>
<td></td>
</tr>
<tr>
<td>cnc7476</td>
<td>+ (26.40)</td>
<td>+ (19.21)</td>
<td>+ (17.98)</td>
<td></td>
</tr>
</tbody>
</table>

a  cnc3005(5-7) (spanning three predicted ncRNAs in SCO3005, cnc3005.5, cnc4425, and cnc5998.2 could not be compared to the background since unspliced products were observed, but a clear peak was detected in the real sample, indicating the presence of a transcript. cnc4425 and cnc6624 may have been a result from a readthrough of a convergent mRNA corresponding to SCO4424 and SCO6624, respectively. All other ncRNAs that were confirmed are most likely from individual transcripts.

b qRT-PCR was used to detect the antisense RNAs in three strains of S. coelicolor (M145 [wild type], M751, and M752). A plus sign (+) indicates confirmed antisense ncRNAs, and a minus sign (−) indicates those not confirmed. NA, not available due to a noisy dissociation curve. The differences of qRT-PCR signal strengths compared to background are indicated in parentheses. The ncRNA numbers correspond to the SCO numbers of the target gene. cnc2966 was the negative control.

ncRNA in S. coelicolor and to show that it has a regulatory effect on cellular physiology: the overexpression of the ncRNA of the GSI gene yielded surprising growth and antibiotic production defects. This suggests that the abundant ncRNAs we identified may also play a crucial biological role in Streptomyces. A decrease in growth by overexpression of artificial GSI antisense ncRNAs has been observed in Mycobacterium tuberculosis (8). The decrease in glutamine synthase activity led to the reduced poly-L-glutamate/glutamine in the cell wall, which resulted in impaired antibiotic production and altered morphological differentiation (7, 15). Another possible mechanism for the effect of the overexpressed antisense ncRNA could be that the ncRNA inhibits transcription or translation (23). S. coelicolor (and probably related bacteria as well) may use these specific machinery of cis-encoded ncRNA as a novel and general regulatory mechanism, far beyond the limited plasmid-encoded examples reported earlier.

ncRNAs in eukaryotes are far more common than in bacteria and may reflect the complex regulation needed in multicellular organisms (24). The apparent abundance of functional ncRNAs we found in S. coelicolor could reflect its increased regulatory needs, given its unusually large genome (similar to yeast), an ability to adapt to a wide variety of environments and nutritional sources, and a highly dynamic secondary metabolism, including the finely tuned sequence of antibiotic production pathways. These complex regulatory networks may be mediated by signaling molecules influencing ncRNA levels. Lenz et al. (10) have shown that the expression of the trans-encoded sRNAs Qrr1 to Qrr5 in Vibrio cholerae is mediated by Hfq and dependent on LuxO (the regulator of the lux operon), whose activity is controlled by the quorum-sensing autoinducers CAI-1, Hal-1, and AI-2. Qrr1 to Qrr5 in turn repress the expression of LuxR, the main transcriptional regulator of the quorum-sensing regulon (21). ncRNA therefore seems to play a much larger role in this group of bacteria than previously suspected.

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