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SCO5745, a Bifunctional RNase J Ortholog, Affects Antibiotic Production in *Streptomyces coelicolor*

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The bacterial RNases J are considered bifunctional RNases possessing both endo- and exonucleolytic activities. We have isolated an RNase J ortholog from *Streptomyces coelicolor* encoded by the gene *sco5745*. We overexpressed a decahistidine-tagged version of SCO5745 and purified the overexpressed protein by immobilized metal ion affinity chromatography. We demonstrated the presence of both 5'-to-3' exonucleolytic and endonucleolytic activities on the *Bacillus subtilis* thrS transcript. Exonucleolytic activity predominated with 5'-monophosphorylated thrS, while endonucleolytic activity predominated with 5'-triphosphorylated thrS. While *sco5745* is the only RNase J allele in *S. coelicolor*, the gene is not essential. Its disruption resulted in delayed production of the antibiotic actinorhodin, overproduction of undecylprodigiosin, and diminished production of the calcium-dependent antibiotic, in comparison with the parental strain.

The decay of mRNA and the concomitant regulation of gene expression at this level are controlled by a number of key RNases present in a variety of combinations in both Gram-negative and Gram-positive bacteria. There is growing appreciation of how evolutionarily distant bacteria functionally combine quite different sets of proteins in the regulation of RNA metabolism (1, 2). RNase J was the first of two bacterial RNases discovered to possess both exo- and endonucleolytic activities. RNase BN acts as a 3'-to-5' distributive exonuclease releasing mononucleotides and producing a ladder of digestion products. Cleaving endonucleolytically, it produces short 3'-end fragments. While active on single-stranded substrates, RNase BN prefers duplexed substrates and is inhibited by the presence of a 3'-end phosphoryl group and 3'-CCA sequence (3).

Originally characterized by two alleles in *Bacillus subtilis* (4), RNase J is widely distributed in other Gram-positive species as a single allele, as in *Mycobacterium smegmatis* (5), and as two alleles in *Thermococcus kodakaraensis* (6). RNases J have also been characterized in the Gram-negative bacterium *Sinorhizobium meliloti* (7), in the hyperthermophilic archaea *Pyrococcus abyssi* and *Thermococcus kodakaraensis* (8), and in plant chloroplasts (9). *B. subtilis* expresses two paralogous proteins, RNase J1 and J2. The two proteins possess endo- and exonuclease activities in vitro but are not equally active. Recently, it has been argued that the major function of RNase J1 is as an exonuclease, and no specific function has been assigned to RNase J2. However, the two proteins form a heterotrimer in vivo that cleaves a model substrate at a different site than the J1 homodimer (10). The single-stranded exonuclease activity of RNases J appears to be strongly dependent upon the phosphorylation state of the 5' end of transcripts, usually showing a distinct preference for the monophosphorylated state (4, 9, 11, 12).

*Streptomyces* are Gram-positive actinomycetes noted for their production of antibiotics, antifungals, and chemotherapeutic drugs (13). *Streptomyces coelicolor* is a model for the study of the regulation of antibiotic biosynthesis. Regulation occurs at multiple levels, from specific antibiotic pathways to the global control of multiple antibiotics (14). Because of our ongoing interest in mechanisms of RNA decay in *Streptomyces*, and their relationship to antibiotic production, we have undertaken the identification, isolation, and characterization of the *S. coelicolor* RNase J ortholog, SCO5745. We examined the activity of the purified enzyme by utilizing as a substrate the upstream leader region of the thrS gene from *B. subtilis*, a well characterized RNase J substrate (15, 16). We show that SCO5745 is active both as an exonuclease and as an endonuclease. Cleaving thrS internally at many sites, SCO5745 produces a ladder of short products located proximal to the 5' end, lending support to the hypothesis proposed by Tavernetti et al. (5) that RNase J uses a sliding mechanism for endonucleolytic cleavage of triphosphorylated substrates. We also demonstrate that *sco5745* is not essential and that disruption of the gene affects antibiotic production in the disrupted strain.

**MATERIALS AND METHODS**

**Bacterial strains.** *Escherichia coli* strains DH5α and Rosetta 2(DE3)pLysS (Novagen) were used as hosts for plasmid manipulation and protein overexpression, respectively. *E. coli* strains BW25113 and ET12567 (*dam* dam) were used for gene disruption and conjugation purposes (17). *E. coli* strains were grown on Difco nutrient agar or Luria-Bertani medium supplemented with carbenicillin (100 μg ml⁻¹) and/or kanamycin (50 μg ml⁻¹), as necessary. *S. coelicolor* M145 and JSE2301 were grown as necessary on soy flour-mannitol (SFM) agar to obtain spores, on R2YE agar, or in liquid medium (18) with viomycin (30 μg ml⁻¹), nalidixic acid (25 μg ml⁻¹), or apramycin (50 μg ml⁻¹). *Streptomyces* minimal medium containing carboxymethyl cellulose as a dispersant (18, 19) was used for liquid cultures. Spores were pregerminated as described previously (20), and growth of cultures was monitored by measuring the absorbance at 450 nm (A₄₅₀).

**Construction of the SCO5745 disruptant JSE2301.** To disrupt *sco5745*, the coding sequence was replaced by a viomycin resistance cassette using the REDIRECT Λ Red-mediated recombination system for *Streptomyces* (21). Thus, cosmid SC9A10, which bears *sco5745*, was introduced by electroporation into *E. coli* strain BW25113 containing the Λ Red cassette. The disruption of the SCO5745 gene was confirmed by sequencing the PCR-amplified fragment from the disrupted strain JSE2301.
TABLE 1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sco5745RD-F1</td>
<td>5'-CCA CGG GTA GTG GCC GCC TGT GAG GAG AGT CTT TGG AGG CTT AGG CTT CTC</td>
</tr>
<tr>
<td>Sco5745RD-R1</td>
<td>5'-GCT CCA GTG TGG GCC GTG CCG GCC GAC GCC CGG TCA ATT CCG GGG ATC CTT CTA CCA CC</td>
</tr>
<tr>
<td>5745OutF1</td>
<td>5'-CAT AGC ACA ACT GCT TCT GCA</td>
</tr>
<tr>
<td>5745OutR1</td>
<td>5'-AGG GGA GAT GTA AAC GTA CTC GCG</td>
</tr>
<tr>
<td>SCRNJF1</td>
<td>5'-AGC GGA GAT GTA AAC GTA CTC GCG</td>
</tr>
<tr>
<td>SCRNJF1</td>
<td>5'-CGG TGG GCC GCC GAC GCC TCA GTC CTC</td>
</tr>
<tr>
<td>PBHP128</td>
<td>5'-AGA ATT CTA ATA CGA CTC ACT ATA AGG AGA TTG AGA AAG ACA CAC G</td>
</tr>
<tr>
<td>PBHP129</td>
<td>5'-TAT CTA GAT ATC TTC TTG TGT TGC</td>
</tr>
</tbody>
</table>

\*Underlined sequences indicate restriction enzyme cleavage sites NdeI, BamHI, EcoRI, and XbaI in SCRNJF1, SCRNJR1, PBHP128, and PBHP129, respectively.

plasmid. The viomycin resistance cassette was excised from plj780 (18, 21) with EcoRI and HindIII. The cassette was used as a template for PCR and amplified using primers Sco5745RD-F1 and Sco5745RD-R1 (Table 1). The strain carrying both cosmids SC9A10 and the \( \lambda \) Red plasmid was electrotransformed with the extended viomycin resistance cassette. Transformants were plated on Difco nutrient agar containing carbenicillin (100 \( \mu \)g ml\(^{-1}\)) and kanamycin (50 \( \mu \)g ml\(^{-1}\)). Larger colonies were retested on plates with selective antibiotics (carbenicillin, kanamycin, viomycin) to enrich for cells bearing the mutant cosmids. Colonies were screened by PCR using primers 5745OutF1 and 5745OutR1 (Table 1). These primers are situated approximately 100 bp upstream and downstream, respectively, of the deleted SCO5745 open reading frame (ORF) and produced a 1,670-bp fragment if SCO5745 had been replaced by the viomycin resistance cassette. COSmid DNA from disrupted transformants was electroporated into the methylation-deficient \( E. \) coli host strain ET12567 containing the nontransmissible plasmid pUZ8002 (18, 21). The marked cosmids were transferred to \( S. \) coelicolor M145 by conjugation with the \( E. \) coli strain. Desired \( S. \) coelicolor exconjugants (sco5745 negative; \( \text{Vio}^+ \) Kan\(^+\)) were selected by restreaking single colonies on R2YE plates with and without kanamycin, and the identity of positive exconjugants was verified by PCR using primers 5745OutF1 and 5745OutR1 (Table 1). The strain bearing the disrupted sco5745 was designated JSE2301.

Two strains (JSE2301/pJSE2255 and JSE2301/pJSE2200) were constructed for antibiotic production studies. The plasmids pJSE2255 and plj8600 (see “Plasmid construction” below) were electroporated into \( E. \) coli ET12567/pUZ8002, and transformants were conjugated with JSE2301. Integration of either plasmid into the JSE2301 chromosome was confirmed by selecting for \( S. \) coelicolor- and apramycin-resistant exconjugants and via PCR using primers SCRNJF1 and SCRNJR1 (Table 1).

**Plasmid construction.** The sco5745 gene was PCR amplified using the Expand high-fidelity PCR system (Roche) and 50 ng of \( S. \) coelicolor M145 chromosomal DNA as the template. A forward primer, SCRNJF1 (Table 1), introduced an NdeI site overlapping the translation start site. A reverse primer, SCRNJR1 (Table 1), introduced a BamHI site immediately downstream of the translation stop codon. The PCR product was cloned into plasmid pCR2.1-TOPO in accordance with the manufacturer's protocol and incorporated [\( \alpha ^{-32}\)]P-CTP (3,000 Ci mmol\(^{-1}\); GE Healthcare) and eluted stepwise in lysis buffer containing 50, 100, 150, and 200 mM imidazole. Fractions were analyzed by 12.5% lithium dodecyl sulfate (LDS)-PAGE analysis, and those containing SCO5745, free of contaminating proteins, were pooled. Pooled fractions were dialyzed against storage buffer, i.e., 300 mM NaCl, 50 mM NaPO\(_4\) [pH 8], 5 mM MgCl\(_2\), and 10% glycerol, and stored at \(-80^\circ C\).

**Preparation of RNA substrates.** Templates for in vitro transcription were created by linearizing plasmid pJSE2250 with XbaI to produce a ca. 384-base \( \text{thrS} \) transcript. Unlabeled, large-scale transcription reactions were performed using the Megascript T7 high-yield transcription kit (Ambion) according to the manufacturer’s protocol, and transcripts were subsequently either 5'- or 3'-end labeled. For 5'-end labeling, transcripts were treated with calf intestinal alkaline phosphatase (CIP), followed by T4 polynucleotide kinase (PNK; Promega) and [\( \gamma ^{-32}\)]P-ATP (3,000 Ci mmol\(^{-1}\); GE Healthcare). For synthesizing 3'-end-labeled substrates, the 5'-end was first converted to 5'-P by treatment with CIP and T4 PNK. Subsequently, 5'-3'-labeled cytidine 5'-bis(phosphate) (cPc) was ligated to the 3'-end with T4 RNA ligase (Promega) and incubated overnight at 14°C. For 5'-end triphosphate labeling, the Megascript T7 high-yield reaction mixture was modified by reducing the GTP concentration from 75 mM to 15 mM and including 70 \( \mu \)Ci of [\( \gamma ^{-32}\)]P-GTP (6,000 Ci mmol\(^{-1}\); PerkinElmer). The specific activities of the 5'-monophosphorylated and 5'-triphosphorylated substrates were equalized (ca. 4,000 cpm/pmol) by mixing them with unlabeled transcript. Internally labeled substrates were transcribed in vitro using T7 RNA polymerase (Promega) in accordance with the manufacturer’s protocol and incorporated [\( \alpha ^{-32}\)]P-CTP (3,000 Ci mmol\(^{-1}\); PerkinElmer). Following in vitro transcription, unincorporated label was removed either by use of a NucAway spin column (Ambion) or RNAs were purified from 1% low-melting-point agarose gels.

**Assay of SCO5745 activity.** Fifteen-microliter assay mixtures contained 1X New England BioLabs restriction buffer 3 (100 mM NaCl, 50 mM Tris-\( \text{HCl} \), 10 mM MgCl\(_2\), 1 mM dithiothreitol [DTT], pH 7.9), typically with 1.2 \( \mu \)g SCO5745 (ca. 1 \( \mu \)M) and 1 pmol [\( \gamma ^{-32}\)]P-labeled (67 nM) substrate. This enzyme-to-substrate ratio was the same as that used by Mathy et al. \((10)\) in their studies of the \( B. \) subtilis RNAse J1/J2 heterotrimer. Reaction mixtures were incubated at 37°C and terminated by the addition of 10 \( \mu \)l of sequencing stop solution, containing formamidine and gel tracking dyes (U.S. Biochemical Corp.). Products were separated on 5% or 12% denaturing polyacrylamide gels, which were subjected to autoradiography. Reaction products were also separated by thin-layer chromatography (TLC) as described by Portnoy et al. \((23)\), using 0.9 M guanidine hydrochloride as a solvent.

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**Assay for CDA.** The calcium-dependent antibiotic (CDA) assay essentially followed that described by Yang et al. (24). Inocula of $10^{9}$ spores of M145, JSE2301, JSE2301/pJSE2255, and JSE2301/pIJ8600 were plated on Oxoid nutrient agar (10 ml) and incubated at 30°C for 3 days. The plates were then frozen at $-80^\circ$C overnight and slowly thawed to room temperature. Mycelium and medium were centrifuged (13,000 g, 10 min), and the resulting supernatant was collected for assay of CDA production. CDA-sensitive B. subtilis BG267 was used as an indicator strain. For each strain, a 75-µl aliquot of an overnight culture of B. subtilis was added to 25 ml melted soft nutrient agar supplemented with 16 mM Ca(NO$_3$)$_2$. As the negative control, a similar set of plates was prepared without calcium. Agar plugs were removed from the plates, creating wells into which 40 µl of the collected supernatant was added. Calcium-dependent antibiotic bioactivity was observed as a zone of inhibition after overnight incubation at 37°C.

**RESULTS**

The S. coelicolor genome encodes a protein with high similarity to B. subtilis RNases J1 and J2. A BLAST (25) search of the S. coelicolor genome, using RNases J1 and J2 as query sequences, revealed SCO5745 (SC9A10.09), which encodes a 561-amino-acid protein whose sequence is 39% identical and 60% similar to the B. subtilis RNase J1 protein and 38% identical and 60% similar to B. subtilis RNase J2. A PILEUP multiple-sequence alignment (26) is shown in Fig. 1. Analysis of the amino acid sequence showed conservation of the three major domains of RNase J necessary for RNase activity: the ß-lactamase core (motifs 1 to 4), the ß-CASP (elements A to C), and the C-terminal domain. These findings strongly suggest that SCO5745 is a member of the RNase J family.

To characterize the biochemical activity of SCO5745, the gene was cloned into pET19B and overexpressed in E. coli. The protein, with a decahistidine tag, was purified by immobilized metal ion chromatography. A homogeneous protein with an M$_r$ of ca. 62,000 was obtained as judged by LDS-PAGE and Coomassie blue staining. Given the importance of the 5'-end phosphorylation state of substrates for RNase J activity, the enzyme preparation was

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**FIG 1** PILEUP analysis (26) of SCO5745 and B. subtilis RNases J1 and J2. ß-Lactamase domain motifs are labeled M1 to M4. ß-CASP domain elements are labeled A, B, and C. Residues that are identical in the three proteins are highlighted in black, while similar residues are highlighted in gray.
assayed for pyrophosphatase contamination. Briefly, [α-32P]ATP was incubated with SCO5745 for 90 min. Reaction mixtures contained increasing amounts of cold ATP to facilitate enzyme activity, and the products were compared to ATP and CIP-treated ATP via their separation by thin-layer chromatography. SCO5745 digests produced no AMP, and thus there appears to be no contaminating pyrophosphatase in our preparations (see Fig. S1 in the supplemental material). In an additional attempt to detect phosphatase or pyrophosphatase contamination, we incubated SCO5745 with a chromogenic nonspecific substrate for phosphatases, p-nitrophenylphosphate (pNPP). The SCO5745 preparation was inactive against this substrate. Calf intestine alkaline phosphatase served as the positive control for the reaction and cleaved pNPP efficiently (data not shown). Thus, our enzyme preparation does not appear to contain contaminating phosphatase or pyrophosphatase activities. One possibility that we have not been able to exclude definitively is contamination of the SCO5745 preparation with RNA pyrophosphohydrolase (RppH) from the E. coli strain used for overexpression. However, we note that we obtained results identical to those presented below (see Fig. 4 and 5) with an enzyme which had been subjected to additional purification steps (ion-exchange chromatography and gel filtration) for crystallization studies (data not shown). Taken together, our analyses suggest that SCO5745 itself, rather than contaminating enzymes, is responsible for the activities we observed in the studies reported here.

**The exonuclease activity of SCO5745 has 5′-to-3′ directionality.** To address the question of exonuclease directionality, we used the thrS RNA that is derived from the leader region of the threonyl-tRNA synthetase gene of *B. subtilis* (15, 16). A schematic structure of the thrS leader is shown in Fig. 2. Site 1 and site 2 are the mapped 5′ ends resulting from RNase J1 processing. Site 1 was originally thought to be produced by internal cleavage (4). However, processing is sensitive to the 5′ phosphorylation state and is produced via exonucleolytic activity (27). Site 2 is cleaved endonucleolytically by RNase J1 (4), by the *M. smegmatis* RNase J (5), and by *E. coli* RNase E (28). Curiously, the RNase J1/J2 heterotrimer does not cleave at site 2 but rather at a site immediately downstream of the terminator (10).

If SCO5745 functions as a 5′- to-3′ exonuclease, our expectations were that label would be released and accumulate more rapidly from a 5′-end-labeled substrate than from a 3′-end-labeled substrate. To examine the directionality of exonuclease digestion, therefore, we utilized 5′-end-labeled, monophosphorylated thrS and thrS labeled at the 3′ end with [32P]pCp. As indicated in Materials and Methods, the two radioactive substrates were adjusted to the same specific activity using nonradioactive thrS. Digestion products were resolved by thin-layer chromatography and observed by autoradiography. Figure 3A shows that by 60 min, there was significant accumulation of both 5′- and 3′-end labels. Figure 3B shows the quantification of that accumulation by densitometry of the product spots. As expected, the rate of product accumulation was greater for the 5′-end-labeled substrate than for the 3′-end-labeled substrate. We conclude from this analysis that, like other RNases J, the exonucleolytic activity of SCO5745 proceeds 5′ to 3′.

**SCO5745 has both 5′-to-3′ exonucleolytic and endonucleolytic activities.** The endonucleolytic activity originally ascribed to RNases J1 and J2 (4) is now recognized as dependent upon high *in vitro* concentrations of enzyme (29, 30). Thus, we began the biochemical characterization of SCO5745 with an enzyme concentration curve. Since the exonuclease activity measured for other RNases J is strongly dependent upon the phosphorylation state of the 5′ end (4, 9, 11, 12), we used 5′-end-labeled monophosphorylated substrate. The results (Fig. 4) show that SCO5745 had robust exonuclease activity, as reflected by release of the monophosphate label at lower protein levels and decreased release of monophosphate at higher protein concentrations. We return to the latter observation below. In contrast, the endonucleolytic activity of SCO5745, reflected by the formation of 5′-end-labeled bands with mobilities between those of the full-length substrate and the monophosphate bands, was increased at higher concentrations of protein. Based upon these results, we chose to use an enzyme-to-substrate ratio of ca. 1.2 µg (19 pmol) SCO5745 per pmol of thrS in further experiments. This enzyme-to-substrate (E:S) ratio of 19:1 is close to that used by Mathy et al. (10).

Activity of SCO5745 against 5′-monophosphorylated and 5′-triphosphorylated substrates. As mentioned above, the exo- and

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**FIG 2** Schematic diagram of the thrS leader region. J1 site 1 and J1 site 2 indicate the *B. subtilis* internal cleavage sites as originally identified, with structure and numbering adapted from a report by Even et al. (4). Site 1 cleavage is now known to be produced by exonucleolytic activity.
endonuclease activities of other characterized RNases J have been shown to depend on the phosphorylation state of the RNA substrate. It was therefore of considerable interest to determine whether 5′-end phosphorylation affected the activity of SCO5745. Figure 5 presents the results of assays that compared the activities of SCO5745 on mono- and triphosphorylated 5′-end-labeled thrS RNA. To obtain maximum resolution of the products of these assays, reaction mixtures were fractionated on a 12% sequencing gel, with two nucleoside phosphate standards, GMP and GTP. Monophosphorylated substrate was digested to GMP by SCO5745 in a time-dependent manner (Fig. 5, lanes 1 to 4). This substrate also produced internal cleavage products consisting of a few nucleotides proximal to the 5′ end, as well as a major product created by cleavage presumably near site 2 of Fig. 2 (Fig. 5, arrows). This larger product was also produced from the triphosphorylated substrate (Fig. 5). Endonucleolytic activity proximal to the 5′ end produced a ladder of small 5′ triphosphorylated products, 2 to 10 nucleotides in length. To our surprise, GTP, the expected product of exonuclease activity acting upon the triphosphorylated substrate, did not accumulate when that RNA was utilized in the reactions (Fig. 5, lanes 5 to 8). Thus, under our reaction conditions, SCO5745 is a bifunctional RNase. The predominant activity with a 5′ monophosphorylated substrate is exonucleolytic, while the predominant activity with a 5′ triphosphorylated substrate is endonucleolytic.

SCO5745 is not essential, and disruption of the gene affects antibiotic production. Model organisms that contain different sets of RNases have already revealed important alternative mechanisms of RNA metabolism (1, 31, 32). In E. coli, RNase J is absent and RNase E is the key enzyme for initiating mRNA degradation (31, 32). RNase E is absent in B. subtilis, but the organism contains a different endoribonuclease, RNase Y (33). As indicated above, RNase J was first identified in B. subtilis and for some years it was thought that both RNases J1 and Y were essential in that organism (33, 34). Recent studies have shown, however, that neither is essential in B. subtilis (35). In the actinomycete M. smegmatis, RNase E is essential while RNase J is not (5). S. coelicolor is known to contain RNase E, which is not essential (36), and the results reported here indicate that the organism also contains RNase J. It was of considerable interest, therefore, to determine whether SCO5745 was essential in S coelicolor.

We replaced sco5745 with a viomycin resistance cassette utilizing PCR-targeted mutagenesis as described by Gust et al. (21) to create the disruptant strain JSE2301. Positive disruptants were verified by PCR using primers 5745OutF1 and 5745OutR1 (Table 1) located ca. 100 bp upstream and downstream, respectively, from the sco5745 open reading frame. Successful disruption of the gene predicted the amplification of a 1,670-bp product, while the parental allele should produce a 1,856-bp product. As shown in Fig. 6A, we observed products of the expected sizes using chromosomal DNAs from the parental and disrupted strains as the tem-
antibiotics that we examined JSE2301 for antibiotic production. Of the three antibiotics produced by cleavage at site 2 by RNase J1.

FIG 5 Characterization of SCO5745 exo- and endonucleolytic activities. A time course reaction was run with 1.2 µg of enzyme and 1 pmol thrS substrate with either a 5’ monophosphorylated (lanes 1 to 4) or 5’ triphosphorylated end (lanes 5 to 8). Reaction products were resolved on a 12% denaturing polyacrylamide gel. Nucleotide standard lanes are labeled GMP and GTP. The arrows indicate the internal cleavage product that is similar in size to that produced by cleavage at site 2 by RNase J1.

FIG 6A shows that it was nonessential and allowed characterization of a phenotype for the RNase J knockout.

Since S. coelicolor is a model organism for the study of antibiotic production and the regulation of secondary metabolism (14, 37), we examined JSE2301 for antibiotic production. Of the three antibiotics that S. coelicolor M145 normally produces under the growth conditions we employed, two are growth phase regulated and pigmented and thus can be easily visualized in the growth medium (38). The red antibiotic undecylprodigiosin normally appears at about 24 to 36 h after inoculation into R2YE medium, while the blue antibiotic actinorhodin appears later. Our comparison of antibiotic production by the parental M145 strain and by JSE2301 was done on R2YE agar. By 3 days postinoculation, M145 had made much undecylprodigiosin but actinorhodin production was delayed beyond day 5, and the darker color seen at 5 days in Fig. 6B is caused by overproduction of undecylprodigiosin. We also assessed antibiotic production in the complemented strain JSE2301/pJSE2255 and the vector control strain JSE2301/pIJ8600. Complementation with sco5745 restored the production of actinorhodin at day 3. We also note the failure of the JSE2301/pIJ8600 control to produce normal levels of antibiotic even by day 5. This strain was grown on three antibiotics, viomycin, apramycin, and thiostrepton, and unlike JSE2301/pJSE2255, it lacks a wild-type RNase J gene and so might not be expected to grow as well.

We also assessed the ability of JSE2301 to make calcium-dependent antibiotic, in comparison with that of control strains. CDA levels were measured after 3 days of growth and showed that production of this antibiotic was also dramatically reduced in JSE2301 (Fig. 6C). A similar rationale as that indicated above may apply to the CDA assay, in which the JSE2301/pIJ8600 control also did not make as much CDA as either the wild type or JSE2301/pJSE2255. Thus, RNase J joins RNase III (39–41) as an RNase that dramatically affects the production of antibiotics in S. coelicolor.

We did not observe any obvious effect of the SCO5745 mutation on the rate of growth of JSE2301 in solid or liquid media, nor did we observe any obvious deficiencies in the ability of the mutant to sporulate.

DISCUSSION

Two model pathways for RNA degradation have emerged based primarily upon the different combinations of enzymes present in E. coli and B. subtilis. As a first step in E. coli, 5’ triphosphorylated ends of primary transcripts are cleaved to the monophosphorylated state by the RNA pyrophosphohydrodase RppH (42). This step creates the preferred substrate for RNase E, an endonuclease that degrades RNA into short segments, thus creating new 5’ monophosphorylated ends. Since E. coli possesses no 5’-to-3’ exonucleases, further degradation proceeds via 3’-to-5’ exonucleases such as PNPase and RNase II (1, 43). In B. subtilis, degradation begins in a similar manner with cleavage by RppH (44). The endonuclease RNase Y also prefers substrates with 5’ monophosphorylated ends, and it too becomes active downstream of RppH (45). Exonucleolytic degradation occurs 5’ to 3’ via RNase J1/J2 that, like RNase E, prefers 5’ monophosphorylated substrates, and exonuclease degradation also occurs 3’ to 5’ via PNPase. In contrast, S. coelicolor, although possessing an RNase E homolog, does not contain RNase Y, and no evidence for the presence of RppH has yet been adduced.

We have shown here that SCO5745 functions as both a 5’-to-3’ exonuclease and an endonuclease. Furthermore, we show that endonucleolytic activity can efficiently degrade the 5’ triphosphorylated thrS transcript without the release of GTP. Taverniti et al. (5) have proposed a model in which RNase J acts as a sliding endonuclease. Since the 5’ triphosphate moiety cannot fit into the phosphate binding pocket of the RNase J active site (27), Taverniti et al. suggest that the RNA slides past the catalytic center and is then endonucleolytically cleaved at downstream nucleotides close to the 5’ end (5). Our results are consistent with this model, as shown by the formation of small, ladderlike, 2- to 10-nucleotide bands resolved on denaturing polyacrylamide gels (Fig. 5). Taverniti et al. (5) further observed the release of GTP by what they claim is endonucleolytic cleavage, as presumably the 5’ triphosphate suppressed exonucleolytic activity. They also report a clear bias for two endonuclease cleavage sites: the first at site 2 (Fig. 2) and the...
FIG 6 (A) PCR analysis of the sco5745 region in S. coelicolor M145 and the disruptant strain JSE2301. Lane 1, size standards; lane 2, PCR product from S. coelicolor M145; lane 3, PCR product from strain JSE2301. (B) Comparison of antibiotic production in S. coelicolor M145 and sco5745 disruptant strains. Equivalent amounts of spores were streaked onto R2YE medium and incubated at 30°C to permit comparison of undecylprodigiosin (red) and actinorhodin (blue) antibiotic production. Plates streaked with strains bearing derivatives of pIJ8600 contained apramycin (50 μg ml⁻¹) and thiostrepton (50 μg ml⁻¹) to induce expression from the tipA promoter. All plates contained nalidixic acid (25 μg ml⁻¹) and nystatin (245 U ml⁻¹). Plates bearing JSE2301 and its derivatives also contained viomycin (30 μg ml⁻¹). (C) CDA assay. Zones of inhibition of B. subtilis BG267 appeared after overnight incubation at 37°C of the indicator strain, BG267, in the presence of 16 mM Ca(NO₃)₂. The center wells contained 40 μl of supernatant from S. coelicolor strains grown for 3 days on Oxoid nutrient agar. No inhibition was seen when calcium nitrate was omitted from the medium (data not shown).
other encompassing the first four nucleotides proximal to the 5’ end of thrS. We saw a very similar internal cleavage pattern (Fig. 5); however, there was no release of GTP. de la Sierra-Galley et al. (27) also reported internal cleavage at site 2 but no release of GTP.

Was our failure to observe the release of GTP due to the relatively high protein concentrations used in our study, thus favoring endonuclease activity and inhibiting exonuclease activity? Our E:S ratio was 19, while Taverniti et al. (5) used an E:S ratio of 0.1. Daou-Chabo and Condon (36), using an E:S ratio similar to ours, described endonuclease activity as nonspacially cleaving all single-stranded regions. Thus, one might expect endonuclease cleavage to release GTP even at high protein concentrations. We observed no GTP release (Fig. 5). This raises at least two questions. Was the release of GTP reported by others through exonuclease or endonuclease activity? Is the mechanism by which exonuclease activity is inhibited by the 5’ triphosphate moiety the same mechanism that causes stimulation of endonucleolytic activity by high E:S ratios? The answer to these questions will require further experimentation. Studies to determine the crystal structure of SCO5745 are in progress and should shed light on the answers to these mechanistic questions.

Ultimately, the important question is what does SCO5745 do in vivo? It has been shown in several systems that RNases J are active in rRNA maturation (5, 7). Chloroplas RNase J functions as an RNA surveillance mechanism for antisense RNA (9). In B. subtilis, an RNase J null mutation combined with reduced expression of RNase J1 globally affected the expression of over 600 genes (46). We have shown here that S. coelicolor contains only a single copy of RNase J, which is not essential, and that knocking out sco5745 delays the onset of actinorhodin production while undecylprodigiosin is overproduced and CDA production is greatly reduced. Thus, at the very least, it is clear that SCO5745 functions in the control of antibiotic production in S. coelicolor.

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