

# Regulation of an Auxiliary, Antibiotic-Resistant Tryptophanyl-tRNA Synthetase Gene via Ribosome-Mediated Transcriptional Attenuation<sup>∇</sup>

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***cis*-Acting RNA elements in the leaders of bacterial mRNA often regulate gene transcription, especially in the context of amino acid metabolism. We determined that the transcription of the auxiliary, antibiotic-resistant tryptophanyl-tRNA synthetase gene (*trpRS1*) in *Streptomyces coelicolor* is regulated by a ribosome-mediated attenuator in the 5' leader of its mRNA region. This regulatory element controls gene transcription in response to the physiological effects of indolmycin and chuangxinmycin, two antibiotics that inhibit bacterial tryptophanyl-tRNA synthetases. By mining streptomycete genome sequences, we found several orthologs of *trpRS1* that share this regulatory element; we predict that they are regulated in a similar fashion. The validity of this prediction was established through the analysis of a *trpRS1* ortholog (SAV4725) in *Streptomyces avermitilis*. We conclude that the *trpRS1* locus is a widely distributed and self-regulating antibiotic resistance cassette. This study provides insights into how auxiliary aminoacyl-tRNA synthetase genes are regulated in bacteria.**

*Streptomyces* is a large genus of Gram-positive, soil-dwelling bacteria. Interest in these organisms is based largely on the fact that they produce half of the 10,000 known antibiotics and two-thirds of the antibiotics used in clinical medicine (2). Since antibiotic resistance determinants accompany genes for antibiotic biosynthesis (7), it is a widely held notion that these bacteria represent a reservoir of antibiotic resistance genes (8, 28, 38). The mining of streptomycete genomes has led to the discovery of novel antibiotic resistance genes (40). The high frequency of antibiotic resistance observed in many *Streptomyces* species indicates that much lateral transfer of resistance genes has taken place (3, 8, 38, 42). In many cases, a *Streptomyces* species will harbor multiple genes conferring resistance to antibiotics that it does not produce (8). As many resistance genes are needed conditionally, the expression of antibiotic resistance genes often is induced upon exposure to antibiotics (9).

Bioinformatic analysis of the genome sequence of *Streptomyces coelicolor* (the model organism of the genus) revealed several putative genes that confer resistance to antibiotics that the organism does not produce, including erythromycin, vancomycin, chloramphenicol, the pristinamycins, and the lincosamides (2). Many of the bioinformatic predictions have been validated by antibiotic susceptibility assays and by genetic and biochemical analyses (11, 17, 20, 24, 40). The transcription of some resistance genes is tightly regulated. For example, in the presence of vancomycin and the pristinamycins, *S. coelicolor* activates the transcription of the corresponding resistance genes (11, 17, 20).

The focus of this study is an auxiliary tryptophanyl-tRNA synthetase gene (*trpRS1*) in *S. coelicolor* that confers resistance to indolmycin and chuangxinmycin (25, 39), antibiotics that competitively inhibit bacterial tryptophanyl-tRNA synthetases (34). The primary tryptophanyl-tRNA synthetase encoded by the *trpRS2* gene is sensitive to these antibiotics (25). Indolmycin is an antibiotic produced by *Streptomyces griseus* ATCC 12648 (35). Although indolmycin is not used clinically, its selective inhibition of bacterial tryptophanyl-tRNA synthetases and potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Helicobacter pylori* recently has renewed interest in its pharmacological potential (19). Chuangxinmycin, produced by *Actinoplanes tsinanensis*, also has demonstrable activity against multi-drug-resistant pathogens (6). We previously reported that these antibiotics did not affect the transcription of *trpRS2*, whereas the open reading frame (ORF) of the *trpRS1* transcript could be detected only in cultures that were treated with indolmycin or chuangxinmycin (39). These observations indicated that the gene was subject to regulation at the level of transcription (39). Further, our finding that *trpRS1* is transcribed with a 157-nucleotide leader raised the possibility that the gene is transcriptionally regulated via the formation of distinct secondary structures in the leader that either cause or repress the premature termination of transcription. The regulation of gene transcription by *cis*-acting RNA regulatory structures such as T-box riboswitches and ribosome-mediated transcriptional attenuators is a recurring theme in bacteriology, especially in the context of amino acid metabolism (15, 26, 29). Computational analyses often enable the facile classification of RNA sequences into one of these two categories of RNA regulatory elements (13, 29, 37, 41). On this basis, we analyzed the *trpRS1* leader sequence in search of any canonical RNA regulatory elements. Sequence elements resembling those of ribosome-

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TABLE 1. Bacterial strains used in this study

Strain	Genotype/description	Reference/ source
<i>S. avermitilis</i> ATCC 31267		21, 32
<i>S. coelicolor</i>		
M600	Prototroph; SCP1 <sup>-</sup> SCP2	24
B725	M600 <i>trpRS1::apr</i>	39
B734	B725 TrpRS2(H48N)	40
B735	B725 TrpRS2(H48Q)	40
B771	B734 pJS340	This study
B772	B735 pJS340	This study
B774	M600 pJS340	This study
B775	M600 pJS341	This study
B776	M600 pJS342	This study
B779	M600 pJS345	This study
B780	M600 pJS346	This study
B782	M600 pJS348	This study
B784	M600 pJS350	This study
B785	M600 pJS351	This study
B786	M600 pJS352	This study
B788	M600 pJS354	This study
B790	M600 pJS356	This study
B791	M600 pJS357	This study
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) <i>U169 recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>phoA supE44 thi-1 gyrA96</i> <i>relA1</i> $\lambda$ <sup>-</sup>	Invitrogen
ET12567	<i>dam dcm hsdS cat tet</i>	39

mediated transcriptional attenuators were identified in the *trpRS1* leader.

In this study, we used molecular genetic approaches to determine if the apparent attenuator regulates *trpRS1* transcription. Herein, we report the mechanism by which *trpRS1* transcription is induced by antibiotics that inhibit TrpRS2.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** All of the strains that were utilized in this study are listed in Table 1. *S. coelicolor* strains were grown at 30°C on

mannitol soya flour medium (SFM) or Difco nutrient agar medium (DNA) (24). For RNA isolation, *S. avermitilis* ATCC 31267 was grown in minimal liquid medium (NMMP) as previously described (40). *Escherichia coli* strains DH5 $\alpha$  and ET12567/pUZ8002 were grown on Luria-Bertani medium at 37°C (36). The MICs for *Streptomyces* strains were assessed on DNA solid medium after a 48-h incubation period. For selecting *Escherichia coli*, ampicillin, apramycin, chloramphenicol, hygromycin, and kanamycin were employed at 100, 50, 25, 80, and 50  $\mu$ g/ml, respectively. Nalidixic acid was used at 20  $\mu$ g/ml to counterselect *E. coli* in conjugations with *S. coelicolor*. Apramycin and hygromycin were used at 50  $\mu$ g/ml for selecting *S. coelicolor*. Indolmycin was chemically synthesized by the authors as described by Kamiyama et al. (14) and used at 10  $\mu$ g/ml.

**Plasmids and primers.** All plasmids used in this study are listed in Table 2. The plasmids used in this study were constructed using standard cloning procedures (36). DNA sequencing was performed by Davis Sequencing (Davis, CA). The site-specific integrating vector pMS81 (12) was used to introduce the various reporter constructs (various *trpRS1* leader fragments fused to the *neo* open reading frame) into *S. coelicolor*. The primers used in this work, all of which were synthesized by Invitrogen, are listed in Table 3. PCR was performed with either *Taq* (Invitrogen) or *Pfu* (Stratagene, Agilent Technologies) under standard conditions for G-C-rich DNA templates (24).

**Creation of reporter constructs.** Using wild-type strain *S. coelicolor* M600 genomic DNA as the template, a 380-bp region spanning the *trpRS1* leader and promoter was amplified by PCR using primers 1 and 2 (Table 3). The PCR product, with an engineered NdeI site (introduced from primer 2) at its 3' end to enable fusion to the *neo* (kanamycin resistance) gene, was ligated into pBlue-script KS+ to give pJS358 (Table 2). The *trpRS1* leader-*neo* fusion then was subcloned into the integrative vector pMS81, yielding pJS340. pJS340 was introduced into *E. coli* strain ET12567/pUZ8002 and then into *S. coelicolor* strains B734 and B735 (yielding strains B771 and B772 [Table 1]), and M600 (yielding strain B774; Table 1) by way of conjugation (23).

A 280-bp segment spanning the *trpRS1* promoter and the proposed 1:2 hairpin of the *trpRS1* leader was PCR amplified using pJS358 as the template with primers 1 and 4. The native *trpRS1* ribosome-binding site was engineered into primer 4 to enable fusion to the truncated leader (Table 3, boldface segment of primer 4 sequence). The PCR product derived from primers 1 and 4 was fused to the *neo* gene as described above. The truncated *trpRS1* leader-*neo* fusion was subcloned into pMS81 to yield pJS341, which was introduced into wild-type strain *S. coelicolor* M600 by way of conjugation to yield strain B775 (Table 1).

A 322-bp segment spanning the *trpRS1* promoter and leader, with the exception of the four terminal residues of the A+U-rich tract, was PCR amplified using pJS358 as the template with primers 1 and 3. The native *trpRS1* ribosome-binding site was engineered into primer 3 to enable fusion to the truncated leader (boldface segment of primer 3 sequence in Table 3). The PCR product was ligated to the *neo* gene, subcloned into pMS81 to give pJS342, and introduced into wild-type strain *S. coelicolor* M600 by way of conjugation to yield strain B776 (Table 1).

TABLE 2. Plasmids used in this study<sup>a</sup>

Plasmid	Description	Vector backbone	Reference or source
pBluescript KS+	pUC <i>ori</i> Amp <sup>r</sup>		Agilent Technologies (Stratagene)
pGemT	pUC-derived, <i>lacZ'</i> , Amp <sup>r</sup>		Promega
pMS81	<i>oriT</i> $\phi$ <i>attB-int</i> Hyg <sup>r</sup>		12
pJS340	<i>trpRS1</i> wild-type leader- <i>neo</i>	pMS81	This study
pJS341	<i>trpRS1</i> leader lacking 3:4 stem-loop- <i>neo</i>	pMS81	This study
pJS342	<i>trpRS1</i> leader lacking part of A+U-rich tract- <i>neo</i>	pMS81	This study
pJS345	<i>trpRS1</i> leader G(-126)A- <i>neo</i>	pMS81	This study
pJS346	<i>trpRS1</i> leader C(-127)A- <i>neo</i>	pMS81	This study
pJS348	<i>trpRS1</i> leader G(-129)C- <i>neo</i>	pMS81	This study
pJS350	<i>trpRS1</i> leader C(-131)A- <i>neo</i>	pMS81	This study
pJS351	<i>trpRS1</i> leader T(-132)C- <i>neo</i>	pMS81	This study
pJS354	SAV4725 wild-type leader- <i>neo</i>	pMS81	This study
pJS356	SAV4725 leader G(-128)C- <i>neo</i>	pMS81	This study
pJS357	SAV4725 leader G(-125)C- <i>neo</i>	pMS81	This study
pJS358	<i>trpRS1</i> wild-type leader	pBluescript KS+	This study
pJS359	<i>trpRS1</i> wild-type leader- <i>neo</i>	pBluescript KS+	This study
pJS360	SAV4725 wild-type leader	pBluescript KS+	This study

<sup>a</sup> Numbers in parentheses refer to positions relative to the predicted translation start site. Antibiotic resistance markers: Amp<sup>r</sup>, ampicillin resistance; Hyg<sup>r</sup>, hygromycin resistance; *neo*, kanamycin resistance.

TABLE 3. Primers used in this study<sup>a</sup>

Entry	Primer name	Application/function	Sequence
1	SCO3334 (-380) For	Cloning of the <i>trpRS1</i> leader	5'- <u>GATATCTTCTTTCGAACGTGGATTCTCCTC</u> -3' <sup>b</sup>
2	SCO3334 (-1) Rev	Cloning of the <i>trpRS1</i> leader	5'- <u>CATATGCTCTCCACCTCCTGGTCAG</u> -3' <sup>c</sup>
3	SCO3334 (-59)A Rev	Cloning of the <i>trpRS1</i> leader lacking part of the A+U-rich tract	5'- <u>CATATGCTCTCCACCTCCTTACGAGAACGGCCG</u> CC-3' <sup>c,e</sup>
4	SCO3334 (-100)A Rev	Cloning of the <i>trpRS1</i> leader lacking 3:4 hairpin	5'- <u>CATATGCTCTCCACCTCCTTGTGTACGGCCGCCG</u> TC-3' <sup>c,e</sup>
5	TrpRS1 G(-126)A F	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of G(-126)A mutation	5'-GTACCCAGCTCTGGCAGGCCGCCTG-3'
6	TrpRS1 G(-126)A R	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of G(-126)A mutation	5'-CGCCGTCAGGCGGCCTGCCAGAGCTG-3'
7	TrpRS1 C(-127)A F	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of C(-127)A mutation.	5'-GTGTACCCAGCTCTGGAGGGCCGCCTG-3'
8	TrpRS1 C(-127)A R	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of C(-127)A mutation.	5'-GCCGTCAGGCGGCCCTCCAGAGCTG-3'
9	TrpRS1 G(-129)C F	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of G(-129)C mutation	5'-CCAGCTCTCGCGGGCCG-3'
10	TrpRS1 G(-129)C R	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of G(-129)C mutation.	5'-CGGCCCGCAGAGACTG-3'
11	TrpRS1 C(-131)A F	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of C(-131)A mutation	5'-GTACGTGTACCCAGCTATGGCGGGCCGCCTG-3'
12	TrpRS1 C(-131)A R	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of C(-131)A mutation	5'-GTCAGGCGGCCCGCCATAGCTGGGTAC-3'
13	TrpRS1 T(-132)C F	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of T(-132)C mutation	5'-GTACGTGTACCCAGCCCTGGCGGGCCGCCTG-3'
14	TrpRS1 T(-132)C R	Site-directed mutagenesis of the <i>trpRS1</i> leader, creation of T(-132)C mutation	5'-CAGGCGGCCCGCCAGGGCTGGGTAC-3'
15	SAV4725 UF	Cloning of the SAV4725 leader	5'- <u>ACTAGTCGGAATCCTTGCTCCC</u> -3' <sup>d</sup>
16	SAV4725 UR	Cloning of the SAV4725 leader	5'- <u>CATATGCTCTCCACCTCCTGGTCG</u> -3' <sup>c</sup>
17	SAV4725 G(-125)C F1	Site-directed mutagenesis of the SAV4725 leader, creation of G(-125)C mutation	5'-GTACCCAGCAGTGGTCGGCCGCCTGAC-3'
18	SAV4725 G(-125)C R1	Site-directed mutagenesis of the SAV4725 leader, creation of G(-125)C mutation	5'-CGCCGTCAGGCGGCCGACCACTGCTG-3'
19	SAV4725 G(-128)C F1	Site-directed mutagenesis of the SAV4725 leader, creation of G(-128)C mutation	5'-CGTGTACCCAGCAGTCGTGGGCCGCC-3'
20	SAV4725 G(-128)C R1	Site-directed mutagenesis of the SAV4725 leader, creation of G(-128)C mutation	5'-CGTCAGGCGGCCACGACTGCTGGGTAC-3'
21	SAV4725 RC A	SAV4725 transcript, 5' RACE	5'-CTGAAGATCCGCTTCATCTCTC-3'
22	SAV4725 RC C	SAV4725 transcript, 5' RACE	5'-CCGTGCTGATCGACGTC-3'
23	SAV4725 RC D	SAV4725 transcript, 5' RACE	5'-GTCGACGACGCAGAACAG-3'

<sup>a</sup> Numbers in parentheses refer to positions relative to the predicted translation start site.

<sup>b</sup> The engineered EcoRV site is underlined.

<sup>c</sup> The engineered NdeI site is underlined.

<sup>d</sup> The engineered SpeI site is underlined.

<sup>e</sup> The predicted *trpRS1* ribosome-binding site is in boldface.

Using wild-type *S. avermitilis* ATCC 31267 genomic DNA as the template, a 400-bp region spanning the SAV4725 leader and promoter was amplified by PCR using primers 15 and 16 (Table 3). The PCR product was cloned into pBluescript KS+ (yielding pJS360; Table 2). It then was subcloned into pJS341 for fusion to the *neo* gene, yielding pJS354 (Table 2). pJS354 was introduced into wild-type *S. coelicolor* M600 by way of conjugation, yielding strain B788 (Table 1).

**Site-directed mutagenesis.** Specific point mutations in the *S. coelicolor* *trpRS1* leader were generated using the Stratagene QuikChange site-directed mutagenesis procedure. The primer pairs used to generate all point mutations in the *trpRS1* leader are provided in Table 3 (primers 5 to 14). Plasmid pJS358 was used as the template in these reactions. All mutant *trpRS1* leaders were fused to the *neo* gene and subcloned into pMS81, yielding plasmids pJS345 to pJS351 (Table 2). Plasmids pJS345 to pJS351 were independently introduced into wild-type *S. coelicolor* strain M600 via conjugation, yielding strains B779 to B785 (Table 1).

The primer pairs used for the site-directed mutagenesis of the SAV4725 leader are provided in Table 3 (primers 17 to 20). Plasmid pJS360 was used as the template in these reactions. The mutant SAV4725 leaders were fused to the *neo* gene and subcloned into pMS81, yielding plasmids pJS356 and pJS357 (Table 2). Plasmids pJS356 and pJS357 were introduced into wild-type *S. coelicolor* strain M600 by conjugation, yielding strains B790 and B791 (Table 1).

**Mapping of the SAV4725 transcription start site.** The SAV4725 transcription start site was mapped using the 5' rapid amplification of cDNA ends (5'-RACE) system, version 2.0 (Invitrogen), as previously described (39). Primers used in this analysis (21–23) are described in Table 3. The final PCR product was ligated into vector pGemT (Table 2) and identified by sequencing. The location of the transcription start site was corroborated through independent trials.

**Nucleotide sequence accession numbers.** The nucleotide sequences of all *trpRS1* orthologs are available at the NCBI database under the following accession numbers: *Streptomyces albus* (ABYC01000233), *Streptomyces avermitilis* (NC\_003155), *Streptomyces coelicolor* (NC\_003888), *Streptomyces ghanaensis* (ABYA01000261), *Streptomyces griseoflavus* (ACFA01000404), *Streptomyces hygroscopicus* (ACEX01000367), *Streptomyces lividans* (ACEY01000177), *Streptomyces pristinaespiralis* (ABJI01000428), *Streptomyces svicensis* (ABJJ01000233), and *Streptomyces viridochromogenes* (ACEZ01000149).

## RESULTS

**Bioinformatic analysis of the *trpRS1* leader revealed features that are reminiscent of a ribosome-mediated transcriptional attenuator.** Analysis of the *trpRS1* leader revealed fea-



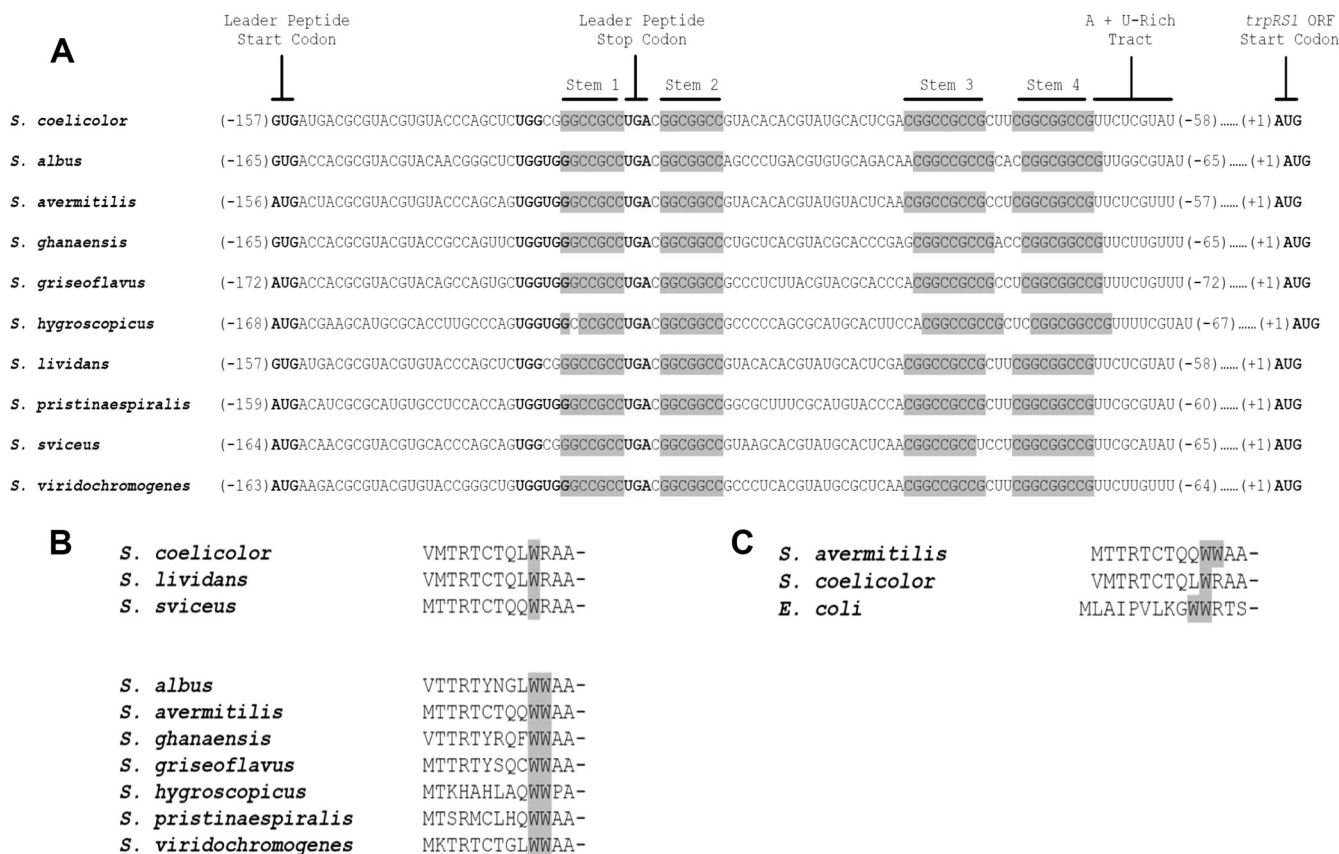


FIG. 2. Sequence alignments of the regions upstream of *trpRS1* and its orthologs in various *Streptomyces* species and of the cognate leader peptides. (A) The regions upstream of the *trpRS1* orthologs are highly homologous to the attenuator elements of the *trpRS1* leader. The sequences of the dyads of symmetry are shaded gray. The start codon (GUG), tryptophan codon (UGG), and stop codon (UGA) of the leader peptide ORFs are in boldface. (B) Alignment of the predicted peptide encoded by the small ORF in the *trpRS1* leader with those of its orthologs. The peptides are grouped based on their tryptophan content. The tryptophan residues are shaded gray. (C) Comparison of the leader peptide sequences of *S. avermitilis* (SAV4725), *S. coelicolor* (*trpRS1*), and *E. coli* (*trpL*).

acids in length and had a tryptophan residue at position 10 (Fig. 2B). Interestingly, some leader peptides had tandem tryptophan residues at positions 10 and 11 (Fig. 2B). On this basis, we organized the leader peptides into two groups (Fig. 2B). The latter group is typical of the canonical attenuators of *trp* operons in bacteria, in that the leader peptide has tandem tryptophan residues (29, 30). We predict that *trpRS1* and these orthologs are regulated by ribosome-mediated transcriptional attenuation.

**Targeted deletions and missense point mutations within the *S. coelicolor trpRS1* leader deregulate transcription.** The sequence and predicted RNA secondary structures of the *trpRS1* leader motivated a series of experiments to examine their significance in the regulation of *trpRS1*. The key to these experiments was the construction of a reporter system in which the promoter and the leader of the *trpRS1* gene were fused to the *neo* gene ORF, which encodes an aminoglycoside phosphotransferase that is a kanamycin resistance determinant. An integrative plasmid harboring the reporter system was introduced into wild-type strain *S. coelicolor* M600, which is kanamycin sensitive. As expected, the resulting strain (*S. coelicolor* B774) exhibited kanamycin resistance only when grown on media supplemented with indolmycin (Fig. 1).

Initially, we tested the hypothesis that the 3:4 hairpin is a *rho*-independent transcriptional terminator. We deleted the sequence comprising the 3:4 hairpin from the aforementioned reporter construct and introduced the resulting plasmid into wild-type *S. coelicolor* M600. The resulting strain (*S. coelicolor* B775) did not require indolmycin for growth on media supplemented with kanamycin (Fig. 3A). The observed deregulation of the *neo* reporter gene was consistent with the proposed function of the 3:4 hairpin as a *rho*-independent transcriptional terminator. This proposed role for the 3:4 hairpin also was based on the fact that it was accompanied by an A+U-rich sequence. These sequences often flank *rho*-independent terminators and are required for the efficient termination of transcription. The truncation of the A+U-rich tract of *rho*-independent terminators has been shown to compromise transcriptional termination (4). To evaluate the significance of the *trpRS1* leader's A+U-rich tract, we prepared a reporter construct in which the four terminal nucleotides of the tract were replaced with the native *trpRS1* ribosome-binding site. *S. coelicolor* strain B776, harboring this truncated reporter construct, exhibited kanamycin resistance in the absence of indolmycin. The deregulated expression of the *neo* gene from this reporter construct indicated that the

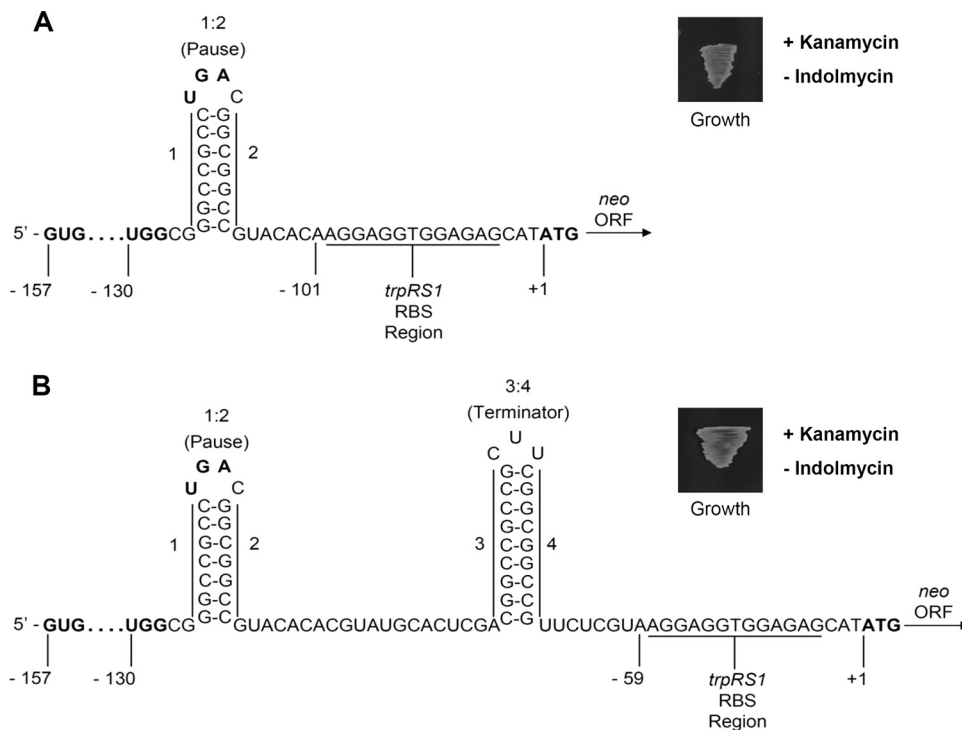


FIG. 3. Schematic representation of the engineered *trpRS1* leaders defective with respect to transcription termination. (A) Schematic representation of the *trpRS1* reporter construct in which the 3:4 hairpin is replaced by the native *trpRS1* ribosome-binding site. The strain harboring this construct grows in an indolmycin-independent fashion on media supplemented with kanamycin. (B) Schematic representation of the *trpRS1* reporter construct in which the terminal four nucleotides of the A+U-rich tract are replaced by the native *trpRS1* ribosome-binding site. The strain harboring this construct grows in an indolmycin-independent fashion on media supplemented with kanamycin. The start codon (GUG), tryptophan codon (UGG), and stop codon (UGA) of the leader peptide ORF are in boldface. The start codon (ATG) of the *neo* open reading frame at position +1 also is in boldface.

A+U-rich tract is required for efficient transcriptional termination. Taken together, these observations support our proposal that the 3:4 hairpin functions as a *rho*-independent terminator.

The presence of a small ORF in the *trpRS1* leader is central to our prediction that the *trpRS1* gene is subject to ribosome-mediated transcriptional attenuation. Our model for antitermination is based on the antibiotic-induced stalling of the ribosome at the tryptophan codon of the leader peptide ORF. On this basis, we hypothesized that missense mutations affecting this codon would deregulate the transcription of *trpRS1*. The wild-type reporter system was used to test this hypothesis. Using site-directed mutagenesis, we introduced a missense mutation (UGG to UCG) that changed the tryptophan codon to a serine codon. This construct was introduced into wild-type *S. coelicolor* M600, yielding strain *S. coelicolor* B782. This strain failed to grow on media supplemented with both kanamycin and indolmycin or with kanamycin alone; apparently, the transcription of the reporter gene no longer was induced by indolmycin. In contrast, missense mutations affecting other codons of the small open reading frame did not perturb the indolmycin dependence of reporter gene transcription (as evident in the phenotype of *S. coelicolor* strains B779 to B780 and B784 to B785; data not shown). Based on these observations, it is clear that antibiotic-induced antitermination is dependent on the tryptophan codon within the small ORF of the *trpRS1* leader.

**Tryptophanyl-tRNA synthetase activity is inversely correlated with antitermination of *trpRS1* transcription.** *S. coelicolor* transcribes the *trpRS1* ORF only in the presence of indolmycin and chuangxinmycin. This phenomenon is not a response to the antibiotics but to their inhibition of TrpRS2 activity (39). We proposed that the reduced availability of charged tryptophanyl-tRNA resulting from TrpRS2 inhibition is directly sensed via the *trpRS1* leader. Accordingly, we suspected that *S. coelicolor* strains with a compromised ability to charge tryptophanyl-tRNA would transcribe the *trpRS1* ORF in the absence of indolmycin. To test this hypothesis, we introduced the wild-type *neo* reporter construct into *S. coelicolor* *trpRS1* null strains with point mutations in the *trpRS2* ORF, encoding TrpRS2(H48N) or TrpRS2(H48Q) (40). In homologous tryptophanyl-tRNA synthetases, this histidine residue directly participates in catalysis, and the replacement of this residue with asparagine is known to compromise enzymatic activity (19, 25). The resulting strains, *S. coelicolor* B771 and *S. coelicolor* B772, exhibited indolmycin-independent growth on media supplemented with kanamycin. Presumably, strains with genetic mutations that affect tryptophanyl-tRNA charging have insufficient charged tryptophanyl-tRNA for the efficient synthesis of the *trpRS1* leader peptide; the ensuing ribosomal stalling leads to antitermination. Similar observations have been made in *E. coli*, where it has been reported that strains harboring mutations in tryptophanyl-tRNA synthetase exhibit

a high degree of antitermination at the *trp* operon via ribosome-mediated transcriptional attenuation (6, 43, 44).

**Analysis of a *trpRS1* ortholog in *S. avermitilis*.** We predict that the *trpRS1* orthologs identified in streptomycete genome sequences also are regulated by ribosome-mediated transcriptional attenuation. Some of these orthologs have two tryptophan codons in their apparent leader peptide ORF rather than one, as in the *trpRS1* leader peptide ORF. To test predictions about the regulation of *trpRS1* orthologs and to investigate the significance of tryptophan content in the leader peptide, we analyzed the *trpRS1* ortholog (SAV4725) in *S. avermitilis* (21, 31).

A series of experiments was performed to characterize the transcription of SAV4725. Using reverse transcription-PCR (RT-PCR), we found that the full-length transcript was detected only in *S. avermitilis* cultures that had been treated with indolmycin (40). 5'-RACE analysis revealed that the SAV4725 transcript has a leader that is 156 nucleotides in length and highly homologous to the *trpRS1* leader (90% identity). The sequences comprising the dyads of symmetry are identical in the *trpRS1* and SAV4725 leaders (Fig. 2A). Furthermore, a positionally conserved small ORF was observed in the SAV4725 leader. As was the case for *trpRS1*, the start codon of the apparent SAV4725 leader peptide was coincident with the transcription start site. There were few differences between the *trpRS1* and SAV4725 leader peptide sequences; most notably, the SAV4725 peptide had two tryptophan residues (Fig. 2C). Both leader peptides resemble the one encoded in the leader of the *E. coli trp* operon (Fig. 2C), which has been extensively studied (29, 30).

Site-directed mutagenesis experiments, analogous to those described in our analysis of the *trpRS1* leader, were performed to characterize the SAV4725 leader. The experiments were based on the construction of a reporter construct in which the SAV4725 promoter region and leader were fused to the *neo* gene ORF. This construct was introduced into wild-type *S. coelicolor* M600. As expected, the resulting strain (*S. coelicolor* B788) exhibited indolmycin-dependent growth on media supplemented with kanamycin. Independent missense mutations were introduced at each of the tryptophan codons of the leader peptide ORF; the codons were changed to serine codons. *S. coelicolor* strains harboring reporter constructs with mutations in either of the tryptophan codons of the leader peptide ORF (i.e., *S. coelicolor* B790 and *S. coelicolor* B791) exhibited indolmycin-dependent growth on media supplemented with kanamycin. These observations indicate that *trp* codons at either position 10 or 11 within the leader peptide ORF enable transcriptional attenuation; the stalling of the ribosome at either of these tryptophan codons will lead to antitermination.

It is noteworthy that the strains harboring reporter constructs with single missense mutations in either of the *trp* codons of the SAV4725 leader peptide ORF (*S. coelicolor* 790 and 791) required 2.5 times more indolmycin (10 versus 25  $\mu$ g/ml) for growth on media supplemented with kanamycin than did the strain harboring the wild-type reporter construct (*S. coelicolor* B788). These findings are consistent with observations of a direct correlation between the number of consecutive regulatory codons in the leader peptide and the sensitivity of ribosome-mediated transcriptional attenuators to changes in levels of charged tRNA (44). Thus, we predict

that *trpRS1* orthologs with two consecutive tryptophan residues in the leader peptide attenuator are more sensitive to antibiotic-induced perturbations in levels of charged tryptophanyl-tRNA than those with a single tryptophan residue.

## DISCUSSION

Auxiliary aminoacyl-tRNA synthetase genes in bacteria often are associated with resistance to antibiotics that inhibit aminoacyl-tRNA synthetases (25, 39, 40, 45). Given that the auxiliary enzyme isoforms are needed only in the presence of antibiotics, one might anticipate that the corresponding genes are regulated in a fundamentally different way than those encoding the primary isoforms. *Streptomyces coelicolor* provided an interesting opportunity to test this premise, since it has two tryptophanyl-tRNA synthetase genes (2). Apparently, the transcription of *trpRS1* (the auxiliary, antibiotic-resistant tryptophanyl-tRNA synthetase) is an adaptive response to indolmycin and chuangxinmycin exposure. The transcription of other tryptophan metabolism genes in *S. coelicolor* is regulated in a growth phase- and growth rate-dependent fashion (16, 18).

The regulated expression of genes encoding aminoacyl-tRNA synthetases and other enzymes involved in amino acid metabolism is a well-known phenomenon in many bacterial genera (29, 30). Often, the messenger RNAs have regulatory structures in their 5' leaders that are affected by regulatory proteins, uncharged tRNA, cofactors, or rates of leader peptide synthesis (1, 29, 30). We determined that the *trpRS1* leader controls transcription via ribosome-mediated attenuation. *trpRS1* orthologs were identified in a number of *Streptomyces* species, and their proposed leader sequences were found to be highly homologous to that of the *trpRS1* leader. We demonstrated that the *trpRS1* ortholog in *S. avermitilis* (SAV4725) is regulated in the same fashion as *trpRS1*. Thus, it appears that the *trpRS1* locus is a widely distributed and self-regulating antibiotic resistance cassette.

The closest analogy to the mode of regulation for *trpRS1* and SAV4725 is that of the *trp* operon in *E. coli* (29, 30). Its transcription is controlled by a 162-nucleotide leader region designated *trpL* (43, 44). In *trpL*, attenuation is governed by the formation of competing terminator and antiterminator RNA secondary structures that either arrest or permit, respectively, the transcription of genes for tryptophan biosynthesis (29, 30, 43). When charged tryptophanyl-tRNA levels are low, the stalling of the ribosome at either of two tandem tryptophan codons in the *trpL* leader peptide ORF precludes the formation of a *rho*-independent terminator and allows the transcription of the tryptophan biosynthesis genes. The results of all of our experiments were consistent with the *trpL* model of transcriptional attenuation (30, 44).

It is interesting that the SAV4725 attenuator, but not the *trpRS1* attenuator, was identified in a bioinformatic search for RNA regulatory elements in actinobacterial genomes (37). These attenuators differ only in the number of *trp* codons in the leader peptide ORF. Our findings suggest that future bioinformatic analyses should account for leader peptide attenuators with a single regulatory codon. Further, we predict that the tryptophan content of the *trpRS1* leader peptide is physiologically meaningful. Generally, leader peptide attenuators play a critical role in cellular responses to amino acid starvation. The

number of regulatory codons in the leader peptide ORF determines the sensitivity of the attenuator to amino acid availability (22). Attenuators with higher numbers of regulatory codons are more sensitive to changes in the availability of charged tRNA than are those with fewer numbers of regulatory codons (44). Since the *trpRS1* leader has a single *trp* codon, we suspect that the *trpRS1* gene is not transcribed under typical starvation conditions. Indeed, the *trpRS1* ORF was not detected in response to tryptophan starvation (25). It appears that the *trpRS1* gene is transcribed only under the adverse physiological conditions brought about by antibiotics that inhibit tryptophanyl-tRNA synthetases. Only under these conditions is the level of charged tryptophanyl-tRNA low enough to cause the ribosome to stall at the single *trp* codon in the *trpRS1* leader. The stringent requirements for *trpRS1* transcription likely minimize the fitness cost of maintaining the gene on the *S. coelicolor* chromosome.

The expression of antibiotic resistance genes is often an adaptive response to the physiological consequence of antibiotic action (9). The molecular logic of *trpRS1* regulation is an ingenious strategy for controlling the expression of an antibiotic-resistant tryptophanyl-tRNA synthetase gene. One can envision that adaptive responses to antibiotics that inhibit aminoacyl-tRNA synthetases are based on the detection of either substrate accumulation (i.e., amino acids or uncharged tRNA) or product diminution (i.e., charged tRNA). The peptide attenuator mechanism for *trpRS1* regulation enables a response to the diminution of charged tRNA levels triggered by indolmycin and chuangxinmycin. In contrast, the presence of a T-box motif upstream of an auxiliary tryptophanyl-tRNA synthetase (homologous to TrpRS1) in *Bacillus anthracis* suggests that its transcription is a response to the uncharged tRNA that would accumulate in the presence of these antibiotics (13, 40). In any case, this study provides insights into how auxiliary aminoacyl-tRNA synthetase genes are regulated in bacteria.

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