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A Novel Tryptophanyl-tRNA Synthetase Gene Confers High-Level Resistance to Indolmycin▽†

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Indolmycin, a potential antibacterial drug, competitively inhibits bacterial tryptophanyl-tRNA synthetases. An effort to identify indolmycin resistance genes led to the discovery of a gene encoding an indolmycin-resistant isoform of tryptophanyl-tRNA synthetase. Overexpression of this gene in an indolmycin-sensitive strain increased the indolmycin MIC 60-fold. Its transcription and distribution in various bacterial genera were assessed. The level of resistance conferred by this gene was compared to that of a known indolmycin resistance gene and to those of genes with resistance-conferring point mutations.

Aminoacyl-tRNA synthetases frequently have been mentioned as targets for a new class of antimicrobial drugs (17, 21, 25, 28, 34). Much of the interest in these enzymes as drug targets is based on the efficacy of mupirocin, an isoleucyl-tRNA synthetase inhibitor that is clinically used as a topical antibacterial agent. Two well-characterized inhibitors of bacterial tryptophanyl-tRNA synthetase are the naturally occurring antibiotics chuangxinmycin and indolmycin (Fig. 1). Indolmycin is produced by *Streptomyces griseus* ATCC 12648 (23, 29), and *Actinoplanes tsinanensis* produces chuangxinmycin (3). Indolmycin is of particular interest because it is active against human pathogens, including methicillin-resistant *Staphylococcus aureus* and *Helicobacter pylori* (16, 19). The indolmycin MICs for methicillin-resistant *Staphylococcus aureus* and *H. pylori* are 0.5 and 0.016 μg/ml, respectively (16, 19).

A concern in the development of any antimicrobial drug is the emergence of resistance (5). The efficacy of mupirocin has been compromised by the distribution of *mupA* and *mupM*, two genes that encode isoleucyl-tRNA synthetase isoforms that are resistant to mupirocin (4, 8, 15, 32). *mupM* is a resistance gene from the mupirocin producer *Pseudomonas fluorescens*, and *mupA* has been observed in *Staphylococcus aureus*, but its origins are unknown (7, 35, 39). Bacteria that have acquired *mupA* or *mupM* retain their chromosomal copy of mupirocin-sensitive isoleucyl-tRNA synthetase. Thus, auxiliary aminoacyl-tRNA synthetase genes are highly correlated with antibiotic resistance. Another example is the auxiliary tryptophanyl-tRNA synthetase gene (*trpRS1*) in *Streptomyces coelicolor* that confers high-level resistance to indolmycin (22, 36). The horizontal transfer of antibiotic-resistant aminoacyl-tRNA synthetase genes could negatively impact the clinical development and use of antibacterials that inhibit aminoacyl-tRNA synthetases (2, 9). Given the pharmacological potential of indolmycin, the identification of native resistance genes is of interest. In efforts to identify additional indolmycin-resistant synthetases, we performed a bioinformatic search and discovered a novel auxiliary tryptophanyl-tRNA synthetase gene (SGR3809) in *Streptomyces griseus* NBRC 13350, which is best known as the producer of streptomycin (26). This gene is distinct in sequence from *trpRS1*, yet it also confers high-level resistance to indolmycin. Homologs of this gene were found in several other bacterial species, including the indolmycin producer *Streptomyces griseus* ATCC 12648. The level of resistance conferred by this gene was compared to those of *trpRS1* and tryptophanyl-tRNA synthetase genes with resistance-conferring point mutations.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this work are provided in Table 1. *Escherichia coli* strains DH5α and ET12567/pUZ8002 were grown in Luria-Bertani medium at 37°C for routine subcloning (30). *Streptomyces* species were grown at 30°C on mannitol soya flour medium (SFM), Difco nutrient agar medium, yeast extract-malt extract medium (YEME), or minimal liquid medium (NMMP) (20). SFM was used for conjugations between *S. coelicolor* and *E. coli* and for generating spore suspensions. For transcriptional analysis, *S. coelicolor* and *Streptomyces avermitilis* strains were grown in NMMP. The indolmycin producer *S. griseus* ATCC 12648 was grown in NMMP without polyethylene glycol (PEG) for transcriptional analysis and the liquid chromatography-mass spectrometry (LC-MS) analysis of culture supernatants. For selecting *E. coli*, ampicillin, apramycin, chloramphenicol, hygromycin, and kanamycin were employed at 100, 50, 25, 80, and 50 μg/ml, respectively. Nalidixic acid was used at 20 μg/ml to counterselect *E. coli* in conjugations with *S. coelicolor*. Apramycin and hygromycin were used at 50 μg/ml for selecting *S. coelicolor*.

Plasmids and primers. Standard cloning procedures were employed in generating the plasmids listed in Table 2 (30). The site-specific integrating vector pMS81 (10) was used for genetic complementation as previously described (36). pIJ10257 was used for overexpression in *S. coelicolor* (12). DNA sequencing was performed by Davis Sequencing (Davis, California). Table 3 shows the primers used in this work, all of which were synthesized by Invitrogen. PCR was performed with *Taq* (Invitrogen) and *Pfu* (Stratagene, Agilent Technologies). All PCRs were performed with 5% (vol/vol) dimethylsulfoxide (DMSO) (20).

Indolmycin and chuangxinmycin. (±)-Indolmycin was chemically synthesized according to the procedure developed by Hasuoka et al. (11). The ^{1}H and ^{13}C nuclear magnetic resonance spectra, as well as mass spectra, of the synthetic material were identical to those reported for authentic indolmycin (11). All amounts of (±)-synthetic indolmycin described herein are corrected to reflect only the active enantiomer. Chuangxinmycin was generously provided as a gift from Richard L. Jarvest of GlaxoSmithKline (3).

Selection of indolmycin-resistant mutants. An *S. coelicolor* B725 (*trpRS1*::*apr*; indolmycin sensitive) spore suspension containing 10^{10} spores was spread over SFM supplemented with 1 μg/ml indolmycin. Several spontaneous low-level indolmycin-resistant mutants arose after incubation at 30°C for 7 days. Fifteen mutants were randomly harvested, and the *trpRS2* locus of each mutant was

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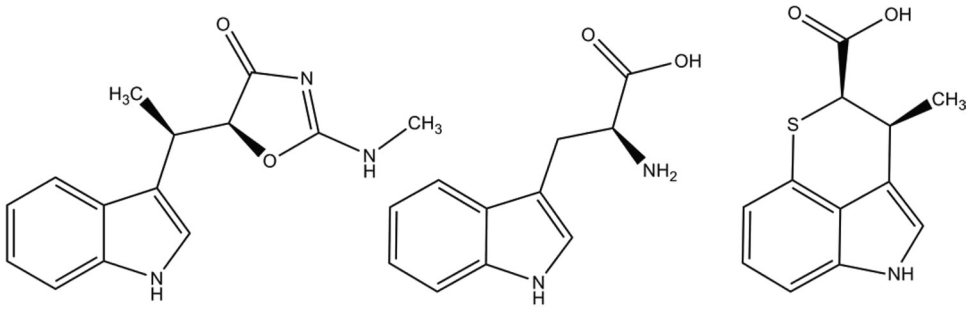

FIG. 1. Indolmycin, tryptophan, and chuangxinmycin. As structural analogs of tryptophan, indolmycin and chuangxinmycin competitively inhibit bacterial tryptophanyl-tRNA synthetases (3, 38). Chuangxinmycin was provided as a gift from GlaxoSmithKline. The indolmycin used in these studies was chemically synthesized (see Materials and Methods).

analyzed by PCR using genomic DNA as the template with primers 15 and 16 (Table 3). Four low-level indolmycin-resistant mutants were chosen for selection for higher-level resistance (strains B730 to B733) (Table 1). A suspension containing 5×10^9 spores of each strain was spread over SFM supplemented with 10 μg/ml indolmycin; spontaneous high-level indolmycin-resistant mutants arose from each strain after incubation at 30°C for 7 days (strains B734 to B737) (Table 1).

Site-directed mutagenesis. Mutations were created in the *S. coelicolor trpRS2* open reading frame (ORF) using the Stratagene QuikChange site-directed mutagenesis kit by following the manufacturer's protocol. Using plasmid pJS330 (Table 2) as the template with primers 23 and 24 (Table 3), the TrpRS2 [G(−13)T/L9F] locus was created. The TrpRS2[G(−13)T/H48Q] locus was created using pJS330 as the template with primers 27 and 28 (Table 3). Plasmid pJS329 (Table 2) was used as the template with primers 25 and 26 (Table 3) to create the TrpRS2(L9F/Q13K) ORF, and the TrpRS2(L9F/H48Q) ORF was created using pJS329 as the template with primers 27 and 28. The TrpRS2(Q13K) ORF was created using plasmid pJS328 (Table 2) as the template with primers 25 and 26.

MIC assays. All MIC assays were performed on Difco nutrient agar medium supplemented with the indicated concentrations of indolmycin. Growth was assessed after incubation at 30°C for 48 h.

RNA isolation and RT-PCR. The transcriptional analyses of the *S. coelicolor trpRS1* and *trpRS2* genes were performed as described previously (36). To analyze the transcription of the tryptophanyl-tRNA synthetase genes in other *Streptomyces* species, two NMMP cultures were inoculated for each organism to test transcription in the presence or absence of indolmycin. Strains were grown for 21 h prior to treatment with 40 μg/ml indolmycin for 3 h. Cells were washed once with 10.3% (wt/vol) aqueous sucrose, resuspended in L (lysis) buffer (20), and incubated for 15 min at 30°C. Total RNA then was isolated using the RNeasy Mini kit (Qiagen) by following the manufacturer's protocol. The concentration of each purified, DNA-free total RNA isolate was measured with a NanoDrop ND-1000 spectrophotometer. An equal quantity of total RNA (1.8 μg) was employed in all reverse transcription-PCRs (RT-PCRs). RT-PCR was performed with the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol for transcripts with high GC content (which requires the use of the Q-solution). Primers used for RT-PCR analyses are listed in Table 3 (primers 1 to 14). The PCR program used for the detection of all transcripts was 50°C for 30 min, 95°C for 15 min, *N* cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, and a final elongation at 72°C for 10 min. For the analysis of SAV3417, 35 cycles was employed. All other genes were analyzed at 25 cycles except SAV4725 and SGR3809, which were analyzed at 20 cycles. No signals were detected in control experiments with *Pfu* polymerase, indicating that the RT-PCR signals correspond to the amplification of transcripts.

Detection of indolmycin in culture media by LC-MS. A published procedure was adapted for the isolation and detection of indolmycin (37). For the LC-MS analysis of secreted indolmycin in culture supernatants, *S. griseus* ATCC 12648 was grown in NMMP without PEG. Culture filtrates then were adjusted to pH 3 with 4N HCl and extracted twice with 20 ml ethyl acetate. The organic phases were combined and extracted with 4 ml of a 10% (wt/vol) Na_2CO_3 solution and then dried over Na_2SO_4. Solvent was removed under reduced pressure to leave residues that were purified twice by solid-phase extraction using strata-x 60-mg, 3-ml columns (part no. 8B-S100-UBJ-TN; Phenomenex) by following the manufacturer's protocol. Purified residues were dissolved in 100 μl methanol for LC-MS analysis. LC-MS analysis was performed with a Thermo LCQ DECA XP MAX ion trap mass spectrometer (C_{18} column; gradient: 5 to 65% mobile phase B for 30 min, where mobile phase B is acetonitrile plus 0.1% formic acid and mobile phase A is water plus 0.1% formic acid; Waters part no. WAT058965).

Sequence accession numbers. All sequences described in this work are available at the NCBI database under the accession numbers listed in Table S1 in the supplemental material. The nucleotide sequences of both tryptophanyl-tRNA synthetase genes from the indolmycin producer *S. griseus* ATCC 12648 were deposited in the GenBank database under accession numbers FJ744752 (SGI3809) and FJ744751 (SGItrpRS2).

RESULTS

An auxiliary tryptophanyl-tRNA synthetase gene from *S. griseus* NBRC 13350 encodes an indolmycin-resistant enzyme. Bioinformatic analysis revealed that *Streptomyces griseus* NBRC 13350 has a unique auxiliary tryptophanyl-tRNA synthetase gene (SGR3809) (Fig. 2A). The tryptophanyl-tRNA synthetase encoded by SGR3809 is only 34% identical in amino acid sequence to TrpRS1, a known indolmycin resistance determinant from *S. coelicolor*. Curiously, auxiliary tryptophanyl-tRNA synthetases homologous to SGR3809 were observed in several bacterial genera (Fig. 2B). We detected a cDNA 93% identical in sequence to SGR3809 by the RT-PCR analysis of RNA from the indolmycin producer, *Streptomyces griseus* ATCC 12648 (see Fig. S1 in the supplemental material). The indolmycin producer is classified distinctly from *S. griseus* NBRC 13350, which does not produce indolmycin.

The observation of an SGR3809 homolog in the indolmycin producer and the robust growth of *S. griseus* NBRC 13350 in media supplemented with inhibitory concentrations of indolmycin suggested that SGR3809 encodes an antibiotic-resistant enzyme. *S. griseus* NBRC 13350, like all other indolmycin-resistant streptomycetes, also grows in the presence of chuangxinmycin (Fig. 3); these are the only bacteria known to be resistant to chuangxinmycin. To determine if SGR3809 is an antibiotic resistance gene, it was heterologously expressed in *Streptomyces coelicolor* B725, the indolmycin-sensitive *trpRS1* null strain (Table 1) (36). In this genetic background, its capacity to confer indolmycin resistance was compared to that of *trpRS1* and its homolog in *S. avermitilis* (SAV4725). The indolmycin MIC for *S. coelicolor* B725 increased from 0.25 to 15

TABLE 1. Strains used in this study

Strain	Genotype and/or description	Reference or source
S. avermitilis ATCC 31267		18, 27
S. coelicolor		
M600	Prototroph, SCP1$^-$ SCP2$^-$	1
B725	M600 *trpRS1*::*apr*; indolmycin sensitive	20
B730	B725 *trpRS2*(L9F), spontaneous mutant selected at 1 μg/ml indolmycin (parent of B737)	36
B731	B725, spontaneous mutant selected at 1 μg/ml indolmycin (unknown mutation[s], parent of B734)	This study
B732	B725, spontaneous mutant selected at 1 μg/ml indolmycin (unknown mutation[s], parent of B735)	This study
B733	B725, spontaneous mutant selected at 1 μg/ml indolmycin (unknown mutation[s], parent of B736)	This study
B734	B725 TrpRS2(H48N), spontaneous mutant selected at 10 μg/ml indolmycin	This study
B735	B725 TrpRS2(H48Q), spontaneous mutant selected at 10 μg/ml indolmycin	This study
B736	B725 TrpRS2[G(−13)T], spontaneous mutant selected at 10 μg/ml indolmycin	This study
B737	B725 TrpRS2[C(−17)A/L9F], spontaneous mutant selected at 10 μg/ml indolmycin	This study
B738	B725 pJS310	This study
B739	B725 pJS311	This study
B740	B725 pJS312	This study
B741	B725 pJS313	This study
B742	B725 pJS314	This study
B743	B725 pJS315	This study
B744	B725 pJS316	This study
B745	B725 pJS317	This study
B746	B725 pJS318	This study
B747	B725 pJS319	This study
B748	B725 pJS320	This study
B749	B725 pJS321	This study
B750	B725 pJS322	This study
B751	B725 pJS323	This study
B752	B725 pJS324	This study
B753	B725 pJS325	This study
S. griseus		
NBRC 13350		26
ATCC 12648		28
S. lividans TK24		
S. venezuelae ATCC 10712		
E. coli		
DH5α	F$^-$ ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^-) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
ET12567	*dam dcm hsdS cat tet*	36

μg/ml upon the expression of the novel tryptophanyl-tRNA synthetase gene SGR3809 (Table 4). A similar MIC elevation was observed upon the expression of *trpRS1* in this background (Table 4). Thus, SGR3809 encodes an antibiotic-resistant tryptophanyl-tRNA synthetase.

Antibiotic-resistant tryptophanyl-tRNA synthetase genes are transcribed differently. The transcription of the *S. coelicolor trpRS1* gene is induced in the presence of indolmycin and chuangxinmycin (22, 36). Transcriptional analyses of the antibiotic-resistant tryptophanyl-tRNA synthetase genes in other streptomycetes were performed to determine if they are regulated in a similar fashion. Using RT-PCR analysis, we found that the transcription of the *trpRS1* ortholog in *S. avermitilis* (SAV4725) also is induced by indolmycin (Fig. 4A). In contrast, SGR3809 in *S. griseus* NBRC 13350 and its ortholog in the indolmycin producer were constitutively transcribed (Fig. 4B). Curiously, the transcript of the SGR3809 ortholog from the indolmycin producer was present when secreted indolmycin could not be detected by LC-MS (Fig. 4B; also see Fig. S5 in the supplemental material). This observation is noteworthy because in antibiotic-producing organisms, resistance genes often are cotranscribed with genes encoding enzymes for antibiotic biosynthesis (13, 33).

Selection of indolmycin-resistant mutants yields point mutations in the tryptophanyl-tRNA synthetase ORF and promoter. We used the indolmycin-sensitive *S. coelicolor trpRS1* null strain B725 to select for spontaneous indolmycin-resistant mutants. We sought to compare the identities of resistance-conferring substitutions to the sequences of the native indolmycin-resistant enzymes. This strain lacks *trpRS1* but has the *trpRS2* gene that encodes an indolmycin-sensitive tryptophanyl-tRNA synthetase. In the initial selection, the indolmycin-sensitive *trpRS1* null mutant (MIC, 0.25 μg/ml) was grown on solid medium supplemented with 1 μg/ml of indolmycin. Spontaneous indolmycin-resistant mutants were observed at a frequency of approximately 10^{-8}. Fifteen in-

TABLE 2. Plasmids used in this study

Plasmid	Description	Vector backbone	Reference or source
pUZ8002	RP4 derivative, OriT, Kan^r		36
pGemT	pUC-derived, *lacZ′*, Amp^r		Promega
pBluescript KS+	pUC *ori* Amp^r		Agilent Technologies (Stratagene)
pMS81	*oriT*, ΦBT1 *attB-int*, Hyg^r	pSET152	10
pIJ10257	*oriT*, ΦBT1 *attB-int*, Hyg^r, *ermEp**	pMS81	12
pJS310	*ermEp**-SCO3334 ORF (*trpRS1*)	pIJ10257	This study
pJS311	*ermEp**-SAV4725 ORF	pIJ10257	This study
pJS312	*ermEp**-SGR3809 ORF	pIJ10257	This study
pJS313	*S. coelicolor* TrpRS2(WT) locus	pMS81	This study
pJS314	*S. coelicolor* TrpRS2[G(−13)T] locus	pMS81	This study
pJS315	*S. coelicolor* TrpRS2[G(−13)T/L9F] locus	pMS81	This study
pJS316	*S. coelicolor* TrpRS2 [G(−13)T/H48Q] locus	pMS81	This study
pJS317	*S. coelicolor* TrpRS2[C(−17)A] locus	pMS81	This study
pJS318	*S. coelicolor* TrpRS2[C(−17)A/L9F] locus	pMS81	This study
pJS319	*S. coelicolor* TrpRS2[C(−17)A/H48Q] locus	pMS81	This study
pJS320	*ermEp*-S. coelicolor* TrpRS2(L9F) ORF	pIJ10257	This study
pJS321	*ermEp*-S. coelicolor* TrpRS2(H48Q) ORF	pIJ10257	This study
pJS322	*ermEp*-S. coelicolor* TrpRS2(L9F/H48Q) ORF	pIJ10257	This study
pJS323	*ermEp*-S. coelicolor* TrpRS2(Q13K) ORF	pIJ10257	This study
pJS324	*ermEp*-S. coelicolor* TrpRS2(L9F/Q13K) ORF	pIJ10257	This study
pJS325	*ermEp*-S. coelicolor* TrpRS2(H48N) ORF	pIJ10257	This study
pJS326	SGI *trpRS2* full-length cDNA from indolmycin producer	pGemT	This study
pJS327	SGI3809cDNA fragment from indolmycin producer	pGemT	This study
pJS328	*S. coelicolor* TrpRS2(WT) ORF	pBluescript KS+	This study
pJS329	*S. coelicolor* TrpRS2(L9F) ORF	pBluescript KS+	This study
pJS330	*S. coelicolor* TrpRS2[G(−13)T] locus	pBluescript KS+	This study

dolmycin-resistant mutants were isolated, and their TrpRS2 loci were cloned and sequenced. Only 1 of the 15 strains had a mutation in the *trpRS2* locus; mutations in the other strains were not identified. This strain (B730; see Table S1 in the supplemental material) had a single point mutation in the *trpRS2* ORF that changed leucine at position 9 to phenylalanine [TrpRS2(L9F)].

A number of high-level resistant strains were selected from the low-level resistant strains (i.e., strain B730 and strains B731 to B733 with unidentified resistance-conferring mutations). The selected high-level resistant strains had point mutations in the *trpRS2* ORF and/or upstream of the *trpRS2* transcription start site. Two strains selected at 10 μg/ml had single point mutations in the *trpRS2* ORF that resulted in substitutions of the histidine at position 48 of the synthetase; one strain (B734) had TrpRS2(H48N) and the other (B735) had TrpRS2 (H48Q).

To enable the direct comparison of the *trpRS2* point mutants and the *trpRS1* and SGR3809 genes, they were cloned into pIJ10257 for expression under the control of the constitutive *ermE** promoter (*ermE*p*) and introduced into the *S. coelicolor trpRS1* null strain (B725). TrpRS2 L9F increased the MIC for the null strain 20-fold (Table 5); by comparison, TrpRS1 increased the MIC 40-fold (Table 4). It is remarkable that TrpRS2(H48N) and TrpRS2(H48Q) increased the MIC for the null strain more than 300-fold (Table 5), far more than the native resistance genes (Table 4).

Two spontaneous high-level indolmycin-resistant strains had point mutations immediately upstream of the *trpRS2* ORF. These mutations were predicted to lie within the *trpRS2* promoter region (36) (Fig. 5). One strain (B736) (Table 1) had a point mutation (G to T) 13 nucleotides upstream of the predicted *trpRS2* start codon. This mutation changed the sequence of the predicted −10 region (**G**AGAAT to **T**AGAAT; the change is in boldface) such that it matched the −10 consensus (T-A-G-purine-purine-T) for streptomycete promoters (31) (Fig. 5). The other strain (B737) (Table 1) had a point mutation (C to A) in the spacer region between the −10 and −35 regions of the *trpRS2* promoter, as well as a mutation in the ORF that substitutes phenylalanine for leucine at position 9 (Fig. 5). To compare the resistance-conferring promoter mutations, *trpRS2* loci with either the mutation in the −10 region or the mutation in the spacer region were cloned and introduced into the *S. coelicolor trpRS1* null strain B725 via the integrative plasmid pMS81. The G-to-T mutation in the −10 region increased the MIC 20-fold, while the C-to-A mutation in the −10 and −35 spacer increased the MIC only 8-fold (Table 5). The results of these selections demonstrate that point mutations in the *trpRS2* promoter can contribute significantly to indolmycin resistance.

Combinations of mutations in the *trpRS2* locus synergistically enhance indolmycin resistance. We used site-directed mutagenesis to determine if specific combinations of mutations in the *trpRS2* locus would act synergistically with respect to indolmycin resistance. Indeed, promoter mutations and ORF mutations enhanced indolmycin resistance synergistically (Table 5). We also examined combinations of resistance-conferring point mutations in the *trpRS2* ORF. The mutation causing the L9F substitution was combined with a mutation that changes glutamine at position 13 to lysine (Q13K). The former mutation increased the MIC for the null strain 20-fold (Table 5). The latter mutation was reported to confer weak resistance to *trpRS2* (22), and in our studies it enhanced the

TABLE 3. Primers used in this study

Entry	Primer name	Application or function	Sequence (reference)
1	TrpRS1 MK54	Detection of the *S. coelicolor trpRS1* (SCO3334) transcript via RT-PCR	5′-CAG GAG GTG GAC ATA TGA CAC GGG TTT TCA G-3′ (22)
2	TrpRS1 MK134	Detection of the *S. coelicolor trpRS1* (SCO3334) transcript via RT-PCR	5′-CAC GTA CCC GGG ATC GGC GGA CAG C-3′ (22)
3	TrpRS2 MK133	Detection of the *S. coelicolor trpRS2* (SCO4839) transcript via RT-PCR	5′-GCG TCT CCG GGT CGT CCA GGT ACT G-3′ (22)
4	TrpRS2 MK135	Detection of the *S. coelicolor trpRS2* (SCO4839) transcript via RT-PCR	5′-CGC GAC CTC GCG GAC CGC TTC AAC CAG-3′ (22)
5	SAV3417 RT FOR	Detection of the *S. avermitilis* SAV3417 transcript via RT-PCR	5′-CCA CCT CGG CAA CTA CCT C-3′
6	SAV3417 RT REV	Detection of the *S. avermitilis* SAV3417 transcript via RT-PCR	5′-TTG CCG GTG TAC TTC TCC TC-3′
7	SAV4725 RT FOR	Detection of the *S. avermitilis* SAV4725 transcript via RT-PCR	5′-ATC TGA CGC TGG GGA ACT AC-3′
8	SAV4725 RT REV	Detection of the *S. avermitilis* SAV4725 transcript via RT-PCR	5′-CCG TAC CGC TGG TTG AAG-3′
9	SGR2702 RT FOR	Detection of the *S. griseus* NBRC 13350 SGR2702 transcript and corresponding transcript from the indolmycin producer *S. griseus* ATCC 12648 via RT-PCR	5′-ACG ACG CCT TCT ACA TGG TC-3′
10	SGR2702 RT REV	Detection of the *S. griseus* NBRC 13350 SGR2702 transcript and corresponding transcript from the indolmycin producer *S. griseus* ATCC 12648 via RT-PCR	5′-GCC TGG TAG AGC AGG ATG TC-3′
11	SGR3809 RT FOR 1	Detection of the *S. griseus* NBRC 13350 SGR3809 transcript via RT-PCR	5′-ACA CCC GCC GTC ACC AC-3′
12	SGR3809 RT REV 1	Detection of the *S. griseus* NBRC 13350 SGR3809 transcript via RT-PCR	5′-GAG AGC ATG ATG TCC GGT TC-3′
13	SGR3809 RT FOR 2	Detection of the *S. griseus* ATCC 12648 SGR3809 homolog transcript via RT-PCR	5′-GGC TGC TCC TGG ACT ATC TG-3′
14	SGR3809 RT REV 2	Detection of the *S. griseus* ATCC 12648 SGR3809 homolog transcript via RT-PCR	5′-GTC GGG GTC GTA GGT GAT G-3′
15	SCO4839 KO DET FOR	Cloning of the *S. coelicolor trpRS2* locus located 93 nucleotides upstream of the translational start site	5′-CCC CGC CCT TGT TAC AC-3′
16	SCO4839 KO DET REV	Cloning of the *S. coelicolor trpRS2* locus located 96 nucleotides downstream of the translational stop site	5′-CAA CCG GAG TGA TGT GCA G-3′
17	SAV4725 FOR	Cloning of the *S. avermitilis* SAV4725 ORF	5′-CAT ATG AAG CGG ATC TTC AGC-3′[a]
18	SAV4725 REV	Cloning of the *S. avermitilis* SAV4725 ORF	5′-CTC GAG TTA CGC CTC CAG CAA CC-3′[b]
19	SCO3334 FOR	Cloning of the *S. coelicolor trpRS1* ORF	5′-CAT ATG ACA CGG GTT TTC AGT G-3′[a]
20	SCO3334 REV	Cloning of the *S. coelicolor trpRS1* ORF	5′-CTC GAG CTA CCG GGC CGC GTT C-3′[b]
21	SGR3809 FOR	Cloning of the *S. griseus* NBRC 13350 SGR3809 ORF	5′-CAT ATG ACC ACA CCC GCC G-3′[a]
22	SGR3809 REV 2.0	Cloning of the *S. griseus* NBRC 13350 SGR3809 ORF	5′-CTC GAG CTA CCG GAA CGC GCC CAT C-3′[b]
23	SCO4839 L9F FOR	Site-directed mutagenesis of the *S. coelicolor trpRS2* locus, creation of L9F mutation	5′-GAA CGT CCC CGT GTG TTC TCC GGT ATC CAG C-3′
24	SCO4839 L9F REV	Site-directed mutagenesis of the *S. coelicolor trpRS2* locus, creation of L9F mutation	5′-GCT GGA TAC CGG AGA ACA CAC GGG GAC–3′
25	SCO4839 Q13K FOR	Site-directed mutagenesis of the *S. coelicolor trpRS2* locus, creation of Q13K mutation	5′-GTG CTC TCC GGT ATC AAG CCG ACC TC-3′
26	SCO4839 Q13K REV	Site-directed mutagenesis of the *S. coelicolor trpRS2* locus, creation of Q13K mutation	5′-GAG CCG GAG GTC GGC TTG ATA CCG GAG-3′
27	SCO4839 H48Q FOR	Site-directed mutagenesis of the *S. coelicolor trpRS2* locus, creation of H48Q mutation	5′-GGT CGT CGA CCT GCA GGC GAT CAC GG-3′
28	SCO4839 H48Q REV	Site-directed mutagenesis of the *S. coelicolor trpRS2* locus, creation of H48Q mutation	5′-CGG GAC CGT GAT CGC CTG CAG GTC G-3′

[a] The engineered NdeI site is underlined.
[b] The engineered XhoI site is underlined.

MIC for the null strain only 12-fold (Table 5). These mutations acted synergistically; the MIC for the null strain expressing the double mutant was 32-fold higher than that for the parent (Table 5). It is interesting that this TrpRS2 (L9F/Q13K) double mutant confers nearly as much indolmycin resistance as TrpRS1 (Tables 4 and 5). The combination of L9F and H48Q did not have an obvious effect on indolmycin resistance below 300 μg/ml (Table 5), but the strain expressing this double mutant grew poorly compared to the growth of the wild-type strain (data not shown).

A

```
                                        F5  K9    HIGH                          H44
Streptomyces coelicolor  (trpRS1)    3 RVFSGVKPTGHLTLGNYLGAMRRWAAVDQHRSDALFCVVDLHALTVD 49
Streptomyces avermitilis (SAV4725)   3 RIFSGVKPTGHLTLGNYLGAVRRWVDVDQHGADALFCVVDLHALTVD 49
Streptomyces griseus     (SGR3809)  51 RVLTGDRPTGPLHLGHYFGSLLNRVRLQNLGVEVVVLIPDYQVITDR 97
                                                                              Q92
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B

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                                        HIGH                          Q92
Streptomyces griseus (SGR3809)    51 RVLTGDRPTGPLHLGHYFGSLLNRVRLQNLG-VEVVVLIPDYQVITDR 97
Aeromonas salmonicida              5 IILTGDRPTGKLHIGHYVGSLRQRVEAQHH--YRQFVMIADLQALTDN 50
Burkholderia oklahomensis         13 IALTGDRPTGALHLGHYIGSLKTRVALQDT--HRQFILVADTQALTDN 58
Chromobacterium violaceum         10 IILTGDRTTGQLHLGHYVGSLRNRVILQER--CAQFLLLADAQALTDN 55
Citrobacter koseri                 6 TILTGDRPTGALHLGHYVGSLRQRVALQHT--HQQFVLIADLQGLTDN 51
Deinococcus radiodurans           24 RVLTGDRPTGALHLGHLAGSLQNRVRLQDE--AELFVLLADVQALTDH 69
Enterobacter cancerogenus          5 TILTGDRPTGPLHLGHFVGSLRQRVALQQT--HNQFVLIADLQGLTDN 50
Enterococcus faecalis              3 TILTGDRPTGKLHLGHYVGSLKKRVEMQADPTNQLFVMIADLQALTDN 50
Klebsiella pneumoniae              6 TILTGDRPTGQLHLGHYVGSLRQRVALQHN--HQQFILIADLQGLTDN 51
Parvibaculum lavamentivorans       5 VILTGDRTTGPLHIGHFAGSLRNRLRFQES--HEQFLLLADTQALTDN 50
Photobacterium profundum          11 KILTGDRATGPLHLGHLVGSLQQRVLLQEQ--YDQTVLVADLQGLTDN 56
Salmonella typhimurium             6 IILTGDRPTGSLHLGHYVGSLRQRVALQQD--HQQYVLIADLQGLTDN 51
Serratia proteamaculans            4 TILTGDRPTGSLHLGHYVGSLRQRVELQQH--HQQTVLVADLQGLTDN 49
Vibrio parahaemolyticus           12 IILTGDRATGPLHLGHYVGSLQQRVALQSE--HDQTILVADMQGLTDN 57

                                        F5  K9                        H44
Streptomyces coelicolor (trpRS1)  3 RVFSGVKPTGHLTLGNYLGAMR-RWAAVDQHRSDALFCVVDLHALTVD 49
Bacillus anthracis                5 IMLTGIKPTGYPHLGNYIGAIKPALQMSKRNEGKALYFIADYHALNAV 52
Desulfitobacterium hafniense      4 RIFSGMRPTGSLHIG-HLSVIQNWAALQSEYQS--YYGIVDQHALTTG 48
```

FIG. 2. (A) Alignment of *trpRS1* with other indolmycin resistance genes. The aligned sequences show the region surrounding the HIGH signature consensus of class I aminoacyl-tRNA synthetases. Positions found to correlate to indolmycin resistance in this study are shaded yellow, and the class I aminoacyl-tRNA synthetase signature sequence HIGH is shaded blue. *trpRS1* is highly similar in sequence to the indolmycin-resistant tryptophanyl-tRNA synthetase gene SAV4725 from *S. avermitilis* but is dissimilar to the indolmycin-resistant tryptophanyl-tRNA synthetase gene SGR3809 from *S. griseus* NBRC 13350 (SGR3809). NCBI accession numbers are provided in the supplementary information (see Table S1 in the supplemental material). (B) Translated sequences of the *trpRS1* and SGR3809 genes aligned with homologs from various bacterial species. NCBI accession numbers are provided in the supplementary information (see Table S1 in the supplemental material).

DISCUSSION

We identified a new, indolmycin-resistant tryptophanyl-tRNA synthetase gene (SGR3809) in *S. griseus* NBRC 13350 whose product has low homology to a known antibiotic-resistant tryptophanyl-tRNA synthetase (*trpRS1* in *S. coelicolor*) and high homology to a gene in the indolmycin producer *S. griseus* ATCC 12648. Selections for indolmycin-resistant bacterial strains led to the discovery of point mutations in the tryptophanyl-tRNA synthetase ORF and promoter that confer indolmycin resistance. In some cases, there were novel point mutations in the *trpRS2* gene that resulted in products with sequences similar to those of the products of the native resistance genes. For instance, the resistance-conferring L9F substitution in *S. coelicolor* TrpRS2 has never been reported, but a phenylalanine is located at the analogous position of the indolmycin-resistant enzyme TrpRS1. Likewise, the H48Q substitution in *S. coelicolor* TrpRS2 that we identified has never

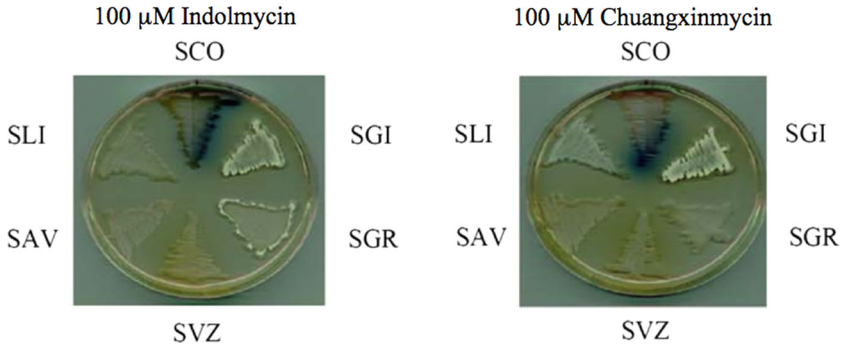

FIG. 3. Various *Streptomyces* species grown in the presence of inhibitory concentrations (100 μM) of indolmycin and chuangxinmycin. SAV, *Streptomyces avermitilis* ATCC 31267; SCO, *Streptomyces coelicolor* M600; SGI, *Streptomyces griseus* ATCC 12648; SGR, *Streptomyces griseus* NBRC 13350; SLI, *Streptomyces lividans* TK24; SVZ, *Streptomyces venezuelae* ATCC 10712.

TABLE 4. Comparison of indolmycin resistance genes from *Streptomyces* species

Entry	ORF[a] expressed in B725 (indolmycin-sensitive *S. coelicolor*)	MIC[b] (μg/ml)
1	*S. coelicolor* B725 (*trpRS1* null strain)	0.25
2	Wild-type *S. coelicolor*	>150
3	*S. coelicolor trpRS1* (SCO3334)	10
4	*S. avermitilis* SAV4725 (*trpRS1* homolog)	5
5	*S. griseus* NBRC 13350 SGR3809	15

[a] All ORFs were introduced into strain B725 under the *ermE** promoter for equalized expression in vivo (see Materials and Methods).
[b] The MIC is the concentration of indolmycin sufficient to inhibit the growth of each strain after 2 days on Difco nutrient agar (see Materials and Methods).

TABLE 5. Comparison of resistance-conferring point mutations in the *S. coelicolor trpRS2* locus

Entry	ORF[a] or locus[b] introduced into B725 (indolmycin-sensitive *S. coelicolor*)	MIC[c] (μg/ml)	Fold increase in MIC[d]
1	*S. coelicolor trpRS2* ORF	0.25	1
2	*S. coelicolor* TrpRS2(L9F) ORF	5	20
3	*S. coelicolor* TrpRS2(H48Q) ORF	>75	>300
4	*S. coelicolor* TrpRS2(H48N) ORF	>75	>300
5	*S. coelicolor* TrpRS2(Q13K) ORF	3	12
6	*S. coelicolor* TrpRS2(L9F/Q13K) ORF	8	32
7	*S. coelicolor* TrpRS2(L9F/H48Q) ORF	>75	>300
8	*S. coelicolor trpRS2* locus	0.25	1
9	*S. coelicolor* TrpRS2[C(−17)A] locus	2	8
10	*S. coelicolor* TrpRS2[C(−17)A/L9F] locus	5	20
11	*S. coelicolor* TrpRS2[C(−17)A/H48Q] locus	>75	>300
12	*S. coelicolor* TrpRS2[G(−13)T] locus	5	20
13	*S. coelicolor* TrpRS2[G(−13)T/L9F] locus	12	48
14	*S. coelicolor* TrpRS2[G(−13)T/H48Q] locus	>75	>300

[a] All ORFs were introduced into strain B725 under the *ermE** promoter for equalized expression in vivo (see Materials and Methods).
[b] Locus refers to the region spanning 93 nucleotides upstream and 96 nucleotides downstream of the *trpRS2* ORF (see Materials and Methods).
[c] MIC is the concentration of indolmycin sufficient to inhibit the growth of each strain after 2 days on Difco nutrient agar (see Materials and Methods).
[d] Fold increase in MIC relative to that of indolmycin-sensitive *S. coelicolor* strain B725 (MIC = 0.25 μg/ml). Note that wild-type *S. coelicolor* (MIC > 150 μg/ml) is more than 600-fold more resistant to indolmycin than strain B725.

been reported, but a glutamine is located at the analogous position of the SGR3809 product. The H48N substitution in the indolmycin-resistant TrpRS2 that we observed is analogous to the H43N substitution observed in indolmycin-resistant tryptophanyl-tRNA synthetases of *Bacillus stearothermophilus* (22) and *Staphylococcus aureus* (16). A consensus of substitutions at this position reflects the fact that the histidine makes a critical hydrogen bond to indolmycin (22). In addition to the ORF mutations, we observed promoter mutations. To our knowledge, this is the first time that promoter mutations have been reported in resistance to aminoacyl-tRNA synthetase inhibitors. Mutations in the *trpRS2* promoter presumably en-

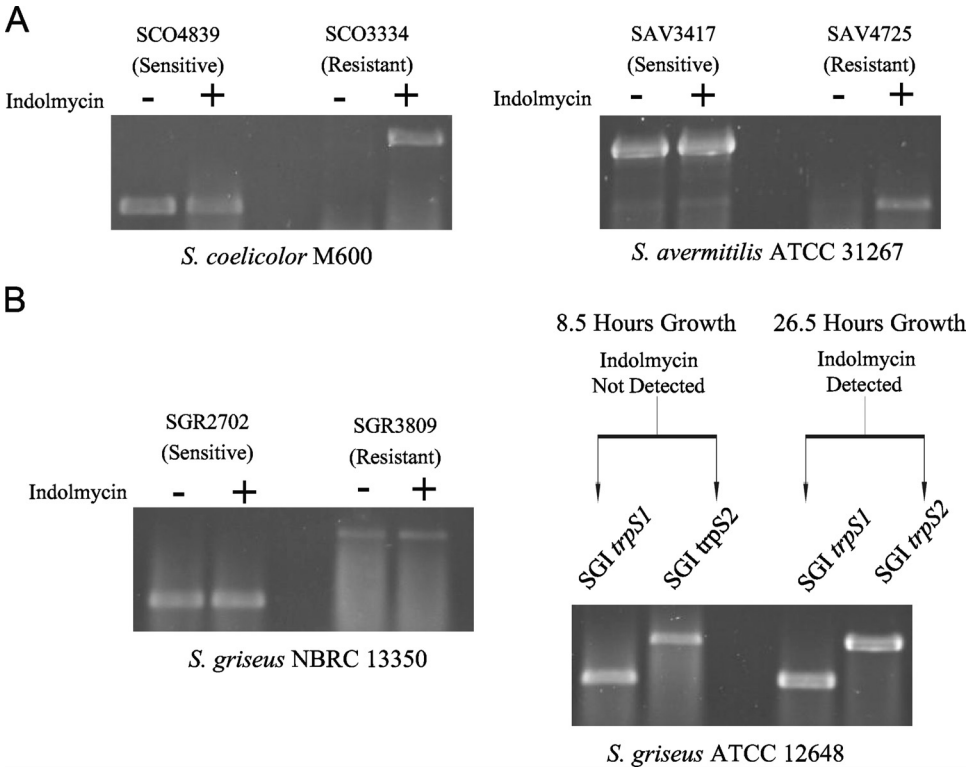

FIG. 4. RT-PCR analyses of the tryptophanyl-tRNA synthetase genes from indolmycin and chuangxinmycin-resistant *Streptomyces* species. (A) RT-PCR analyses of tryptophanyl-tRNA synthetase genes in *S. avermitilis* and *S. coelicolor*. The transcription of the *trpRS1* homolog SAV4725 also is induced in *S. avermitilis* upon exposure to indolmycin. (B) RT-PCR analyses of tryptophanyl-tRNA synthetase genes in the streptomycin producer *S. griseus* NBRC 13350 and in the indolmycin producer *S. griseus* ATCC 12648. The transcription of these genes was not influenced by indolmycin.

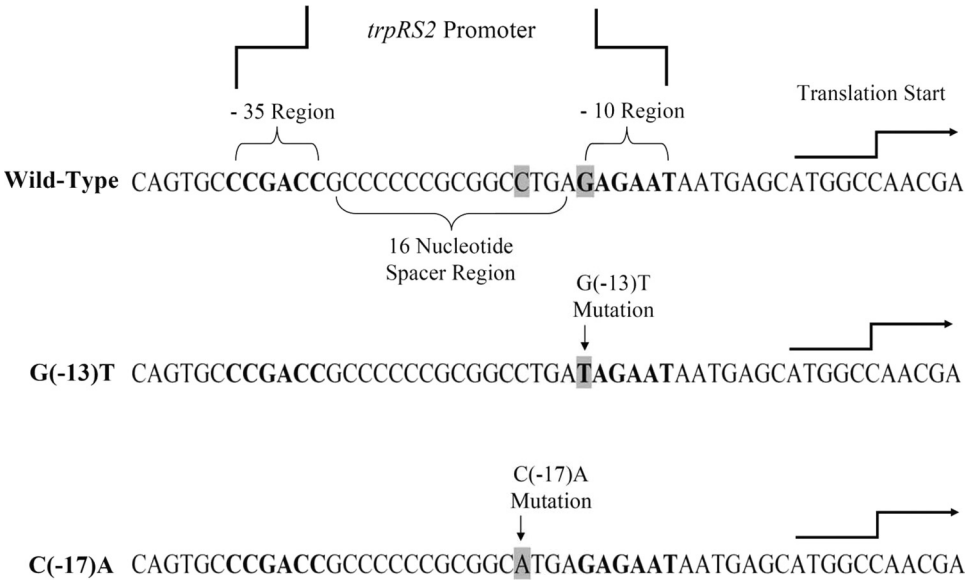

FIG. 5. Mutations in the *trpRS2* promoter that confer resistance to indolmycin. Brackets mark the boundaries of the *trpRS2* promoter. The −10 and −35 regions are in boldface, and the spontaneous mutation sites are shaded gray.

hance the expression of the tryptophanyl-tRNA synthetase gene to levels that confer resistance via titration.

In this work, analyses of streptomycete genome sequences led to the discovery of a new indolmycin resistance determinant. As producers of nearly half of the 10,000 known antibiotics, *Streptomyces* bacteria have been particularly important in drug discovery and are a major source of drug resistance genes (14). It is tempting to speculate that homologs of SGR3809 in other bacterial genera encode antibiotic-resistant tryptophanyl-tRNA synthetases. In any case, it is intriguing that genome mining can be used to predict resistance and/or guide the development of aminoacyl-tRNA synthetase inhibitors and other types of drugs (6, 24). Accordingly, one can predict that resistance to indolmycin and chuangxinmycin is widespread.

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