Indolmycin Resistance of *Streptomyces coelicolor* A3(2) by Induced Expression of One of Its Two Tryptophanyl-tRNA Synthetases*

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Aminoacyl-tRNA synthetases, a family of enzymes essential for protein synthesis, are promising targets of antimicrobials. Indolmycin, a secondary metabolite of *Streptomyces griseus* and a selective inhibitor of prokaryotic tryptophanyl-tRNA synthetase (TrpRS), was used to explore the mechanism of inhibition and to explain the resistance of a naturally occurring strain. *Streptomyces coelicolor* A3(2), an indolmycin-resistant strain, contains two *trpS* genes encoding distinct TrpRS enzymes. We show that TrpRS1 is indolmycin-resistant in vitro and in vivo, whereas TrpRS2 is sensitive. The lysine (position 9) in the enzyme tryptophan binding site is essential for making TrpRS1 indolmycin-resistant. Replacement of lysine 9 by glutamine, which at this position is conserved in most bacterial TrpRS proteins, abolished the ability of the mutant *trpS* gene to confer indolmycin resistance in vivo. Molecular modeling suggests that lysine 9 sterically hinders indolmycin binding to the enzyme. Tryptophan recognition (assessed by *K*_*m*/*K*~cat~) by TrpRS1 is 4-fold lower than that of TrpRS2. Examination of the mRNA for the two enzymes revealed that only TrpRS2 mRNA is constitutively expressed, whereas mRNA for the indolmycin-resistant TrpRS1 enzyme is induced when the cells are exposed to indolmycin.

The aminoacyl-tRNA synthetases (aaRSs) catalyze the covalent attachment of amino acids to their cognate tRNAs. The remarkable specificity of aaRSs for the amino acid and tRNA substrates ensures the accuracy of protein synthesis (1). While preserving the core architecture of their active sites (1–4), these essential enzymes evolved into finely tuned structures with significant divergence among the three domains of the tree of life (5). For example, while sharing the class I synthetase signature sequences, HIGH and KMSKS, the human and *Escherichia coli* TrpRS proteins have only slight similarity. In contrast to bacterial TrpRS, the human enzyme is autophosphorylated (6) and has a 30–40 residue N-terminal extension (7), which is widely seen in the TrpRSs of higher eukaryotes (5).

Furthermore, a natural fragment of human TrpRS acts as a regulator of angiogenesis (8). These divergences and their indispensable role for cell viability position the aaRSs as promising targets of species-specific inhibitors (9). In fact, some antibiotics inhibit aminoacyl-tRNA formation by binding to the active sites of the aaRSs as amino acid or aminoacyl-adenylate analogs. Best known is mupirocin, an antibiotic produced by *Pseudomonas fluorescens* (10) that selectively inhibits the prokaryotic isoleucyl-tRNA synthetases (11). It is widely used as a topical antimicrobial agent against methicillin-resistant *Staphylococcus aureus*. However, methicillin-resistant *S. aureus* strains exhibiting mupirocin resistance are already known (12, 13). Mupirocin resistance is caused by a plasmid encoding a gene for an altered isoleucyl-tRNA synthetase that discriminates mupirocin in the active site (14).

The antibiotic indolmycin (5(15)-5-[(1R)-1-indol-3-ylthyl]-2-(methylamino)-Δ^2^-oxazolin-4-one) is a second metabolite produced by *Streptomyces griseus* ATCC 12648 (formerly named *Streptomyces albus* BA3972A), which was isolated from a sample of African soil (15, 16). To date, indolmycin has been reported to be produced by at least four strains of *Streptomyces* species. A structural analog of L-tryptophan (Fig. 1), indolmycin competitively inhibits bacterial TrpRS enzymes (17). Although it is effective on various microorganisms, from Gram-positive to Gram-negative bacteria, it shows only weak inhibition of mammalian TrpRS (17). Thus, new compounds are being synthesized based on the structure of indolmycin (18–20), with the hope of introducing a new class of antibacterial agents. A recent paper reported that clinical isolates of *Helicobacter pylori*, which is known as a major causative agent of chronic active gastritis and is considered to be an important etiological factor of peptic ulcers in humans (21), are highly sensitive to indolmycin in vivo (22), suggesting the possible usefulness of indolmycin for *H. pylori* treatment.

Here we report the analysis of two TrpRS enzymes found in the indolmycin-resistant *Streptomyces coelicolor* A3(2) strain. One of the two enzymes is indolmycin-resistant, and its expression is induced when the *S. coelicolor* is exposed to indolmycin; the sensitive TrpRS is constitutively expressed. The possible significance of this finding on indolmycin production by this strain is discussed.
Indolmycin Resistance of S. coelicolor A3(2)

MATERIALS AND METHODS

General—[14C]Tryptophan (2.1 Gbq/mmol) was purchased from Amersham Biosciences. Indolmycin was a kind gift from Pfizer, Inc. Oligonucleotides were purchased from Amersham Biosciences or from the Keck Biotechnology Resource Laboratory at Yale University. S. coelicolor A3(2) genomic DNA was provided by Dr. J. Cullum (University of Kaiserslautern). The Bacillus stearothermophilus trpS gene was described earlier (23).

Bacterial Strains—The E. coli strains used in this study included W3110 (F−, Δ[nirR-nirE1]), DH5α (F−, Φ80damZam15, Δ(lacZ丫argF-U169, deoR, recA1, endA1, hsdS7(rKm−mK+), phaA, supE44, λ, thi-1, gyrA96, relA1), 42C [argB, lac−, B1, relA], trpS42Δ(24), and BL21 (DE3) (F−, ompT, hsdS (rB>B1, mB>B4), λ (λd557, ind1, Sim7, nin5, lacUV5-T7gene1), dcm (DE3)). Many of the Streptomyces strains were from the collection of A. Demain; S. coelicolor A3(2) was from Dr. J. Piret (Northeastern University) and also from Dr. H. Kinashi (Hiroshima University). Streptomyces ambofaciens was from Dr. J. Piret, and S. griseus FD11901 was from Pfizer, Inc. The tryptophan auxotrophic strain S. coelicolor no. 2179 was from Dr. D. Hopwood (John Innes Centre). E. coli strain 42C was described elsewhere (25).

Culture Media—To prepare seed cultures of Streptomyces, YM medium (10 g of malt extract, 4 g of yeast extract, 4 g of glucose per liter, pH 7.3) was used (26). The minimal medium for Streptomyces growth was NMMp [2 g of (NH4)2SO4, 5 g of Difco casamino acids, 0.6 g of MgSO4·7H2O, 50 g of polyethylene glycol 6000, 1 mL of minor elements solution, 15 mM NaH2PO4·K2HPO4 buffer (pH 6.8), 0.5% glucose per liter] (see reference (26) for details). For the growth of E. coli, LB medium (10 g of tryptone peptone, 5 g of yeast extract, 5 g of NaCl per liter, pH 7.0) (27) was used. The minimal medium for the indolmycin susceptibility assay of E. coli transformants was M9 medium (27) supplemented with 0.2% glucose, 1 mg/liter thiamine, 1 mM MgSO4, 1 μM FeCl3, 100 μM CaCl2, and 20 μg/liter of t-arginine.

Indolmycin Susceptibility of Streptomyces—The susceptibility tests of Streptomyces were done by the filter diffusion method. Each Streptomyces strain was assayed with discs containing different concentrations of indolmycin in hopes of finding the minimal level of compound required to inhibit the strain. A 200-μl aliquot of bacteria was placed on each plate (100 × 15 mm). Subsequently, 15 ml of a specific agar medium were added to each plate in a swirling manner to distribute the bacteria homogeneously throughout the agar. After the agar solidified, 200 μl of the antibacterial compound were applied to a filter paper disc (0.25 in diameter), and four such discs were placed on each plate. The plates were then incubated at 4 °C for 24 h to allow the antibiotics to diffuse and placed in a 30 °C incubator for 24–72 h. The zones of inhibition were identified as circular, clear areas around the disc where indolmycin inhibited bacterial growth.

Cloning of Genes Encoding TrpRS and tRNA\(^{Trp}\)—The genes for S. coelicolor A3(2) TrpRS1 (SC03334 in the data base at the Sanger Centre) and TrpRS2 (SC04839) were first amplified by PCR cycles (94 °C for 0.5 min, 55 °C for 1 min, 72 °C for 3 min for 30 cycles). The PCR products were then digested by NdeI and BglII and were gel-purified by 0.7% agarose electrophoresis in Tris-acetate-EDTA buffer (27). These purified fragments were ligated into the NdeI-BglII sites of the pCB11 vector (28) to allow constitutive expression. The pCB11 derivative with TrpRS1 was designated as pCB2-SCW1, and that with TrpRS2 was named pCB2-SCW2. The transformants were obtained by electroporation, and those with the correct length inserts were sequenced. For protein overproduction, the NdeI-BglII fragments of pCB2-SCW1 and pCB2-SCW2 were recloned into the NdeI-BamHI digested plasmids, pET-3a and pET-16b, respectively. The overproducing plasmids were designated as pET-SCW1 and pET-SCW2, respectively. The gene for the tRNA\(^{Trp}\) was constructed by the Khorana method (29). The T7 promoter sequence was placed in front of the first nucleotide of the tRNA\(^{Trp}\); and a BsuNI site was introduced at the CCA-3′ end of the tRNA gene. The ligated DNA was digested by BamHI and was cloned into the pHSG399 plasmid (30). The insert was confirmed by DNA sequencing. This plasmid was designated as pSCR.

Overexpression and Purification of TrpRS1—S. coelicolor TrpRS1 and TrpRS2 were produced in E. coli strain BL21(DE3). TrpRS1 was purified in its native form as described (23) with a few modifications. A W3110 strain, harboring an anti-E. coli TrpRS gene, was used. In only one of the two TrpRS activity peaks (210 m MKCl) of the first UNO-Q chromatography, indicating that those fractions contained TrpRS from the E. coli host strain. Thus, the active fractions around 300 mM KCl were pooled and used for further purification. Fractions that contained electrophoretically pure 37-kDa protein after the final chromatography were analyzed (100 μg protein) for KCl, 5 mM MgCl2, 4 mM dithiothreitol, 50% glycerol (pH 7.0) and kept at −20 °C. The fraction of active TrpRS protein was determined by active site titration (25).

Purification of TrpRS2—Attempts to purify native TrpRS2 showed that it had different chromatographic properties than TrpRS1. Therefore we introduced an affinity tag at the N terminus of the protein by cloning the gene into pET-SCW2, a pET-16b construct with a His\(_6\) tag sequence. Enzyme purification was achieved on a HiTrap column (1 mL, Amersham Biosciences) chelated by Ni\(^{2+}\) and a HiTrap-benzamidine column (1 mL, Amersham Biosciences). A transformant of BL21(DE3) with pET-SCW2 was grown in LB medium containing 100 μg/liter ampicillin at 30 °C to an \(A_{600}\) of 0.6–0.8. Isopropyl-1-thio-β-D-galacto- pyranoside (1 mM) was added and the cultures further incubated for 4 h at 30 °C. After harvesting, an extract was prepared in the Ni\(^{2+}\) column binding buffer (25 mM Tris/HCl (pH 8.0), 300 mM NaCl, 5 mM β-mercaptoethanol) and applied to the HiTrap-chelating column, washed, and eluted with a gradient of 10–500 mM imidazole. Factor Xa (Amersham Biosciences) was then added to the eluted protein in digestion buffer (50 mM Tris/HCl, 1 mM CaCl2, 100 mM NaCl (pH 8.0)) and incubated at 25 °C for 20 h. The digest was applied to the HiTrap-chelating column to remove the cleaved tag and the non-cleaved protein. Finally, the flow-through was applied to the HiTrap-benzamidine column to remove the Factor Xa from the solution. A single 37-kDa band was seen when this fraction was analyzed by SDS-PAGE.

In Vitro tRNA Transcription—The plasmid pSCR was prepared by ultrafiltration with a CAC density gradient. The purified plasmid was digested with BstNI to serve as a DNA template for transcription of the tRNA\(^{Trp}\). Transcription, purification, and refolding of tRNA was performed as described (31).

Assay for Tryptophanyl-tRNA Synthetase Activity—Aminoacylation assays were carried out as described (23), except that 4 μM transcript tRNA\(^{Trp}\) was used instead of 2 μM. All assays were performed at 30 °C, the optimal growth temperature of Streptomyces. The kinetic parameters were determined using concentrations of tryptophan that covered the range of 0.2–5 times the \(K_{\text{m}}\) values. For the \(K_{\text{m}}\) values, the amounts of indolmycin that partially inhibit TrpRSs were first determined, and the apparent \(K_{\text{m}}\) values for tryptophan were measured in the presence of the determined amount of indolmycin. The \(K_{\text{m}}\) values were then calculated by the real and apparent \(K_{\text{m}}\) values.

Site-Directed Mutagenesis—Point mutations of the S. coelicolor and B. stearothermophilus TrpRS genes were introduced by the overlap extension method (32). The sequences were confirmed before the following experiments. The pCBS1 derivative with a mutant S. coelicolor TrpRS1(K9Q) was designated pCBS1-SCW1-K9Q, and that with mutant B. stearothermophilus TrpRS(9Q) was designated pCBS1-BSW-QK9.

Complementation and Susceptibility Test of E. coli Harboring the trpS Plasmid—To assess the in vivo activity of the various trpS genes, pCB2-SCW1, pCBS2-SCW2, pCBS2-SCW1-K9Q, and pCBS-BSW-QK9 were separately transformed into the E. coli trpS mutant strain 42C (24). This strain is tryptophan auxotroph because of its mutant TrpRS with the high \(K_{\text{m}}\) for tryptophan (25). The transformants were selected on LB plates containing 100 μg/ml ampicillin at 30 °C and were grown in LB medium with the same concentration of ampicillin at 30 °C. Aliquots (3 μl) of the overnight cultures were then spotted onto two M9 glucose plates supplemented by L-arginine (25) and incubated at 30 °C. The efficiency of complementation was judged after 40 h. For the susceptibility tests, these plasmids were transformed into the W3110 strain 42C. The plate was incubated at 30 °C, and 3 μl of aliquots were spotted on an M9 glucose plate containing 0.1 mM indolmycin. The plate was incubated at 30 °C. mRNA Detection by RT-PCR—RT-PCR was carried out using the

2 M. Kitabatake and K. Ali, unpublished results.
FIG. 2. Sequence alignments of the N-terminal region of bacterial TrpRS enzymes. Sequences were aligned by ClustalW version 1.81. Q9, H43, and the HIGH motif are shaded.

Access RT-PCR Introductory System (Promega) following the manufacturer’s instructions. The RNAs used in this study were prepared by the SV Total RNA Isolation System (Promega) with slight modifications, following the notes provided by the manufacturer. The primers for TrpRS1 were: MK54 (5’-CGACGATGTGGATGAGCGG-3’) and MK134 (5’-CACGTCACCCCGATGGCCGCAGAC-3’); for TrpRS2 MK133 (5’-CGACCGGTATCGTCCAGACTG-3’) and MK135 (5’-CGACAGCTCCGCCAGCCCTGTTAG-3’).

RESULTS AND DISCUSSION

Distribution of Indolmycin Resistance in Streptomyces strains—We assumed S. griseus to be indolmycin-resistant as it produces this compound. To confirm this assumption, we examined the in vivo indolmycin sensitivity of several Streptomyces strains. Of the ten tested Streptomyces strains, we found that five strains, S. griseus 7-455F3, S. griseus FE11901, Streptomyces cattleya, S. coelicolor, and Streptomyces lavendulae, showed resistance at 500 µg/ml indolmycin (see “Materials and Methods”). At the same concentration, the other five strains, Streptomyces lividans, Streptomyces clavuligerus, Streptomyces glaucus, Streptomyces hygroscopicus, and S. ambofaciens, were indolmycin-sensitive. However, this indolmycin concentration is higher than the minimum inhibitory concentration values of previously reported indolmycin-sensitive bacteria, for example: Pseudomonas aeruginosa (31–250 µg/ml, Ref. 33), Mycobacterium tuberculosis (6–16 µg/ml, Ref. 33), and S. aureus Oxford (0.125 µg/ml, Ref. 19). Therefore, it is possible that Streptomyces strains are generally resistant to indolmycin.

Cloning of the Two trpS Genes and Purification of the Enzymes—To shed light on the mechanism of the resistance of the Streptomyces strains, we chose S. coelicolor A3(2) for further analysis, as its genome was being sequenced (www.sanger.ac.uk/Projects/Scoelicolor/). Because indolmycin is a prokaryotic TrpRS inhibitor, and given that S. coelicolor is indolmycin-resistant, we decided to biochemically examine S. coelicolor TrpRS and the effect of the inhibitor. BLAST searches revealed two trpS orthologs in the S. coelicolor A3(2) genome. The start codons of each open reading frame were predicted by the examination of a multiple sequence alignment of bacterial TrpRSs (Fig. 2) and an analysis of the third letter of each codon. Based on the two open reading frames, the TrpRS enzymes are quite similar with 47% amino acid identity. After PCR amplification and cloning into the PET expression vectors and heterologous expression in E. coli, the proteins were extensively purified. TrpRS2 was well expressed (giving >20 mg of the pure protein/liter of culture), whereas TrpRS1 was not (only 0.1 mg of pure protein/liter of culture); this may be due to the presence in the trpS1 gene encoding TrpRS1 of 16 CGG codons, which are relatively minor in E. coli.

Biochemical Characterization of the Two TrpRSs—The S. coelicolor genome sequence indicated only one tRNA<sup>TTP</sup> gene in the organism. Therefore the aminoacylation properties of both TrpRS enzymes were determined with the in vitro transcript of the S. coelicolor tRNA<sup>TTP</sup>. Both enzymes were equally active (Table I). The <i>K<sub>i</sub></i> values for tryptophan of TrpRS1 and TrpRS2 were 11.7 µM and 3.7 µM, respectively. The former value is comparable with that of the indolmycin-sensitive E. coli TrpRS (12.4 µM) (25), but the <i>k<sub>cat</sub></i> of S. coelicolor TrpRS1 (0.78 s<sup>-1</sup>) is less than one-half that of the E. coli enzyme (2.0 s<sup>-1</sup>) (25). As for TrpRS2, although the rate constant (0.90 s<sup>-1</sup>) was only slightly better than that of TrpRS1, the <i>K<sub>m</sub></i> value for tryptophan (3.7 µM) was three times lower than that of TrpRS1. These results indicate that TrpRS2 catalyzes tryptophanyl-tRNA formation more efficiently than TrpRS1 in vitro.

However, the enzymes showed a profound difference in the presence of indolmycin. Preliminary experiments suggested that indolmycin concentrations of 1 µM (TrpRS1) and 10 nM (TrpRS2) partially inhibit Trp-tRNA formation (at 1 µM tryptophan). We then measured the apparent <i>K<sub>M</sub></i> and <i>k<sub>cat</sub></i> values of the two enzymes in the presence of those amounts of indolmycin. Although the <i>k<sub>cat</sub></i> values were not greatly affected by the addition of indolmycin, the <i>K<sub>M</sub></i> values were elevated several times from the real <i>K<sub>M</sub></i> value of each enzyme. The results indicate that the activities of TrpRS1 and TrpRS2 are both competitively inhibited by indolmycin, but the susceptibilities of the two enzymes are different from each other. The inhibition constant (<i>K<sub>i</sub></i>) values of the two enzymes were then determined from the real <i>K<sub>M</sub></i> and the apparent <i>K<sub>M</sub></i> values. The <i>K<sub>i</sub></i> of TrpRS1 (900 nM) was greater than that of TrpRS2 (60 nM) by about 150-fold, suggesting that TrpRS1 is indolmycin-resistant by reducing the affinity of its tryptophan binding site to indolmycin. The <i>K<sub>i</sub></i> value of TrpRS2 (6 nM) is comparable with those of the indolmycin-sensitive TrpRSs, i.e. E. coli TrpRS (24 nM) (34) and B. stearothermophilus TrpRS (9 nM)<sup>2</sup> showing that the S. coelicolor TrpRS2 is indolmycin-sensitive, like other bacterial TrpRSs. The <i>K<sub>i</sub></i> value of TrpRS1 (900 nM), however, is as high as a mutant B. stearothermophilus TrpRS (800 nM, data not shown) that lacks a hydrogen bond between the active site His-43 and indolmycin.<sup>3</sup> Therefore, we concluded that TrpRS1 is indolmycin-resistant and that TrpRS2 is indolmycin-sensitive.

Multiple Sequence Alignment and Structure Modeling—Because the biochemical analysis revealed that the resistance of TrpRS1 is probably caused by reduced affinity of indolmycin to the tryptophan binding site, we thought it likely that this had

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2. M. Kitabatake, unpublished results.
resulted from changes of one or a few amino acids. To examine this possibility and to identify the potential key substitution(s), we analyzed a multiple sequence alignment of bacterial TrpRS proteins (Fig. 2). Both S. coelicolor enzymes aligned well with the other TrpRSs and shared the characteristic HIGH and KMSKS motifs (1–4) of class I aminoacyl-tRNA synthetases. We looked for specific amino acids in S. coelicolor TrpRS1 that differed from the otherwise conserved positions. The location of these residues was then examined in the crystal structure of the wild type B. stearothermophilus TrpRS (35) so that only those residues in close proximity to the tryptophan binding site were considered. Of several candidates examined, we felt that Lys-9 may be the most important residue. In the crystal structure of B. stearothermophilus TrpRS complexed with indolmycin and ATP, 4 Gln-9 is in the tryptophan binding pocket, and the O\(_{\text{a}}\) atom of the glutamine is only 3.2 Å away from the methylenamino group of indolmycin (Fig. 1).

To examine the effect of Lys-9 in S. coelicolor TrpRS1 on indolmycin binding, we modeled a mutant TrpRS structure replacing Gln-9 with Lys-9 based on the crystal structure of B. stearothermophilus TrpRS-indolmycin complex. When C\(_{\text{a}}\) and C\(_{\text{b}}\) of Lys-9 were superimposed on those of Gln-9 and the long side chain of lysine remained in free form, the C\(_{\text{a}}\) atom of Lys-9 was too close to the imido nitrogen of indolmycin. If the side chain of Lys-9 is stretched, then the minimum distance between Lys-9 and indolmycin would be only 1.8 Å, which is too close for proper interaction. In addition, the \(\epsilon\)-amino group of Lys-9 in the model is only 2.7 Å away from the nitrogen atom of the methylenamino group of indolmycin. This may lead to electrostatic repulsion, because the imino nitrogen is presumably positively charged at neutral pH. These observations support the idea that the unique Lys-9 residue of TrpRS1 would prevent indolmycin association with the tryptophan binding pocket.

**Examination of the S. coelicolor TrpRS Enzymes in Vivo**—To test this idea in vivo, we constructed two mutants of TrpRS. The first one is the S. coelicolor TrpRS1 K9Q mutant, and the second is the B. stearothermophilus TrpRS Q9K mutant. The wild type and mutant S. coelicolor and B. stearothermophilus trpS genes were cloned into the pCBS1 plasmid under the control of the E. coli TrpS promoter, which has been shown to be suitable for constitutive expression of various aminoacyl-tRNA synthetase genes (36). The recombinant plasmids were transformed into the tryptophan auxotrophic E. coli strain 42C to test for complementation of its trpS gene or into E. coli strain W3110 for the indolmycin resistance test.

As shown in Fig. 3A, the auxotrophy of the 42C strain was complemented by all of the TrpRS genes tested. Although the strain with the empty plasmid pBR322 did not grow well, all strains with the TrpRS genes could grow on the same minimal plate. Thus, all the cloned trpS genes gave rise to active enzymes in vivo. It is noteworthy that TrpRS1 functions better than TrpRS2 in E. coli, indicating the lower species specificity of TrpRS1 as compared with that of TrpRS2 (Fig. 3A).

Indolmycin resistance was checked with the W3110 transformants at 30 °C for 40 h. They all grew normally in the absence of indolmycin (Fig. 3C). In contrast, only the strain that expressed the wild type S. coelicolor TrpRS1 grew well in the presence of 0.1 mM indolmycin (Fig. 3B). This strongly suggests that TrpRS1 is sufficient for the indolmycin resistance.

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4 W. Yin and C. Carter, Jr., unpublished results.
of \textit{S. coelicolor}. The \textit{S. coelicolor} TrpRS1 K9Q mutant, which complemented the \textit{E. coli} mutant strain 42C as efficiently as the \textit{S. coelicolor} wild type TrpRS1 at 30 °C (Fig. 3A), did not confer any indolmycin resistance on the W3110 host strain (Fig. 3B). This indicates that Lys-9 in the \textit{S. coelicolor} TrpRS1 gene is essential for indolmycin resistance of the \textit{E. coli} transformant. Thus, the results of the \textit{in vivo} experiment support the suggestion from structure modeling on the role of Lys-9. However, the \textit{B. stearothermophilus} TrpRS Q9K mutant did not show clear resistance \textit{in vivo} only a faint trace of growth was observed on the selection plate (Fig. 3B). This suggests that Lys-9 is necessary for indolmycin resistance in TrpRS1 but not sufficient for general indolmycin resistance \textit{in vivo}.

**Differential Expression of the Two TrpRS Enzymes in \textit{S. coelicolor}**—The kinetic analyses described above revealed the functional complementarity of the two \textit{S. coelicolor} TrpRS enzymes. These observations suggested that TrpRS2 should play the major role in Trp-tRNA synthesis in the absence of indolmycin in the growth medium and that TrpRS1 would assume this role when the presence of indolmycin inhibits TrpRS2. To investigate this postulate, we analyzed the expression of the two TrpRS mRNAs in \textit{S. coelicolor}.

As shown in Fig. 4A, the mRNA of TrpRS1 was observed only when the strain was grown in a minimal medium supplemented with 0.1 mM of indolmycin (lane 7), which is the non-permissible concentration for \textit{E. coli} but not for \textit{S. coelicolor}. The addition of 0.01 mM of indolmycin (lane 5) or tryptophan starvation in the cell (lane 3) did not induce the transcription of the TrpRS1 gene. The emergence of the 901-bp band, corresponding to the TrpRS1 mRNA was dependent on the presence of avian myeloblastosis virus reverse transcriptase (compare lane 7 and lane 8), indicating that this band is not a product of genomic DNA contamination. No TrpRS1 mRNA was detected in a fresh culture of cells grown in rich medium (Fig. 4A). In contrast the mRNA for TrpRS2, which may be the organism's housekeeping TrpRS because of its more efficient biochemical properties, was indeed observed (in the presence of reverse transcriptase) under all conditions examined (Fig. 4B, lanes 1, 3, 5, and 7). These results suggest that TrpRS2 is the major enzyme that is constitutively expressed and catalyzes Trp-tRNA formation in \textit{S. coelicolor}, whereas the indolmycin-resistant TrpRS1 is induced only when the cells are exposed to indolmycin. As a consequence, \textit{S. coelicolor} can grow in a medium with 0.1 mM indolmycin as the induced TrpRS1 would take over Trp-tRNA formation.

**The Induction Mechanism of TrpRS1**—The molecular mechanism of TrpRS1 mRNA induction by indolmycin addition was not investigated in this work. When MFOLD (37), an RNA secondary structure prediction tool, was applied to the 850-bp non-coding sequence in front of the trpS1 gene, several mutually exclusive secondary structures in the region were revealed (data not shown). No conserved sequences for T-Box antiterminator (38), trp-RNA-binding attenuator protein binding site (39), or A-factor-binding protein recognition motif (40) were found in the region, suggesting that this trpS gene may have a different mechanism of transcriptional regulation compared with other known systems for the gene expression regulation.

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