Resistance to ketolide antibiotics by coordinated expression of rRNA methyltransferases in a bacterial producer of natural ketolides

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Ketolides are promising new antimicrobials effective against a broad range of Gram-positive pathogens, in part because of the low propensity of these drugs to trigger the expression of resistance genes. A natural ketolide pikromycin and a related compound methymycin are produced by Streptomyces venezuelae strain ATCC 15439. The producer avoids the inhibitory effects of its own antibiotics by expressing two paralogous rRNA methylase genes pikR1 and pikR2 with seemingly redundant functions. We show here that the PikR1 and PikR2 enzymes mono- and dimethylate, respectively, the N6 amino group in 23S rRNA nucleotide A2058. PikR1 monomethylation is constitutively expressed; it confers low resistance at low fitness cost and is required for ketolide-induced activation of pikR2 to attain high-level resistance. The regulatory mechanism controlling pikR2 expression has been evolutionarily optimized for preferential activation by ketolide antibiotics. The resistance genes and the induction mechanism remain fully functional when transferred to heterologous bacterial hosts. The anticipated wide use of ketolide antibiotics could promote horizontal transfer of these highly efficient resistance genes to pathogens. Taken together, these findings emphasized the need for surveillance of pikR1/pikR2-based bacterial resistance and the preemptive development of drugs that can remain effective against the ketolide-specific resistance mechanism.

Significance

Studies of antibiotic resistance are usually initiated in earnest only after resistance has become established in clinical pathogens. Here, we forewarn of a resistance mechanism to the novel antibiotics ketolides, which are only coming into broad medical practice. We show that the balanced activities and coordinated expression of two genes, pikR1 and pikR2, provide efficient protection to Streptomyces venezuelae, a bacterial producer of natural ketolides. Expression of the more potent gene, pikR2, is supported by pikR1 and specifically induced by ketolides. The resistance mechanism remains fully functional when pikR1 and pikR2 are transferred to other bacterial species and affords protection against clinical ketolides. These findings emphasize the need for the preemptive development of antibiotics that can overcome this resistance mechanism.

Acknowledgments and disclosures

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two putative resistance genes, pikR1 and pikR2, arranged tail to tail (Fig. 1A). The protein products PikR1 and PikR2 are 42% identical (Fig. S1) and show similarity to Ern-type rRNA methyltransferases (e.g., 30% and 28% identities with ErmE for PikR1 and PikR2, respectively) (14, 15). Given the significant fitness cost associated with erm-based resistance (6), the duplication of PikR enzymatic function in S. venezuelae is puzzling. Other than being of general biological interest, the mechanism by which a natural producer of ketolides attains resistance is of significant medical importance. Not in the least, it can be envisioned that selective pressure imposed by broad medical use of antibiotics could promote the transfer of natural resistance genes to pathogens. Understanding the function and regulation of pikR1/pikR2 and similar genes would facilitate their surveillance and possibly, help curb the dissemination of individual functions. The binding sites of MTM and PKM in the bacterial ribosome have not been biochemically defined previously. However, structural evidence from the Deinococcus radiodurans large ribosomal subunit suggests that MTM binds in the PTC (17) rather than in the NPET, where all conventional macrolides and ketolides bind (2–4). Therefore, the site of action and the nature of the modifications introduced by PikR1 and PikR2 could not be predicted with any certainty. The majority of the investigated Ern-type enzymes confer resistance to macrolides by dimethylating the exocyclic amines of A2058 in the 23S rRNA (E. coli numbering throughout) (5). A2058 dimethylation (but not monomethylation) stalls the progress of reverse transcriptase (RT) on the RNA template and is readily detected by primer extension. A strong band corresponding to RT pausing at A2058 on rRNA extracted from E. coli cells expressing PikR2 indicated that it probably dimethylates this nucleotide (Fig. 2A and Fig. S2A). In contrast, under the standard primer extension conditions, no RT stop was detected at either A2058 or any other site within the PTC and the NPET on rRNA from cells with pPikR (Fig. 2A). However, when we optimized primer extension conditions for detecting N6 monomethylation (Materials and Methods and Fig. 2B), RT pausing at A2058 was observed on rRNA from cells expressing PikR1 (Fig. 2B, lane 2), supporting the hypothesis that PikR1 monomethylates A2058.

The nature of the reactions catalyzed by PikR1 and PikR2 was corroborated by MS. A 50-nt-long 23S rRNA fragment encompassing A2058 was isolated from rRNA extracted from E. coli cells expressing PikR1 or PikR2 and subjected to RNase A digestion and MALDI-TOF analysis. In the PikR1 sample, the peak corresponding to the unmodified RNA fragment GGA_{2055}AAGAC (m/z 2,675) was almost absent, and a new peak at m/z 2,689 appeared (Fig. 2C), indicating that a methyl group had been added. In the corresponding 23S rRNA fragment from the PikR2 sample, the unmethylated RNA peak was also absent and replaced by a peak at m/z 2,703 showing the addition of two methyl groups (Fig. 2D). Combined with the results of primer extension analysis, the MS data showed that PikR1 and PikR2 target the same rRNA nucleotide but generate two different products: PikR1 monomethylates A2058, whereas PikR2 dimethylates this nucleotide.

**pikR1 Is Constitutively Expressed in the Native Host, Whereas pikR2 Is Activated only When Antibiotics Are Produced.** Why does a ketolide producer that is equipped with the A2058 dimethyltransferase gene pikR2 also need the pikR1 gene, which has a product that merely monomethylates the same nucleotide? To address this question, we examined the regulation of expression of pikR1 and
**Fig. 2.** PikR1 and PikR2 RNA methyltransferases target A2058 in the 23S rRNA. (A) Primer extension analysis of m$_6$A modification of rRNA extracted from WT E. coli cells (W; lane 3) or those constitutively expressing pikR1 (R1; lane 2) and pikR2 (R2; lane 1) genes. Sequencing lanes are marked C, U, A, G. Full gels are shown in Fig. S2A. (B) Primer extension analysis of the same samples as in A but carried out under conditions optimized for detection of m$_6$A modification (Materials and Methods). The E. coli ΔrnlU mutant, which lacks the native m$_6$A modification of A2030 (33), was used as a control (Δ; lane 4). (C and D) MALDI-TOF analysis of the RNaseA-generated 23S rRNA fragment encompassing nucleotide A2058. RNA samples were prepared from cells expressing (C) PikR1 or (D) PikR2.

**Fig. 3.** Expression of pikR2 in S. venezuelae is activated during antibiotic production. (A and B) Primer extension analysis of rRNA extracted from WT S. venezuelae ATCC 15439 carried under conditions specific for detection of (A) m$_6$A and (B) m$_6$A + m$_6$A modification. Full gels are shown in Fig. S2B. RNA was extracted from WT cells or mutants unable to produce active antibiotics because of deletion of the pikAI-pikAIV (ΔpikAI) or desI (ΔdesI) genes. Sequencing lanes in A and B are marked C, U, A, G. (C–E) MALDI-TOF analysis of 23S rRNA fragments from the WT or the ΔpikAI and ΔdesI KO mutants of S. venezuelae; the fragments were generated with RNaseA and encompass nucleotide A2058.

pikR2 in *S. venezuelae*. When production of antibiotics was inactivated by deletion of either the pikAI-pikAIV genes (ΔpikAI) or the desI gene (ΔdesI) in the biosynthetic operon (leaving the pikR1 and pikR2 resistance genes intact) (Table S2), A2058 was found to be fully monomethylated (Fig. 3 and Fig. S2B), but there was essentially no dimethylation of this residue (Fig. 3A, D, and E). This result indicated that pikR2 is constitutively expressed, whereas pikR2 remains inactive when no antibiotic is produced. In contrast, in the antibiotic-producing WT *S. venezuelae* ATCC 15439, a significant fraction (~30%) of A2058 was shown by primer extension to be converted to m$_6$A (Fig. 3A and Fig. S3), an observation confirmed by MS (Fig. 3C). Thus, pikR2 is activated only when MTM and/or PKM are present in the cell. Because dimethylation of A2058 is known to reduce cell fitness (6), the inducible nature of pikR2 is consistent with an evolutionary adaptation by the antibiotic-producing host to achieve secure protection against endogenously produced ketolides while maintaining a low fitness cost for this service.

**Expression of pikR2 Is Activated in the Natural Host by Clinically Relevant Ketolides.** Induction of pikR2 by ketolides of its native host suggested that the gene could also be activated by medically relevant ketolides. We tested whether preincubation with the clinical ketolide TEL could induce pikR2 expression in *S. venezuelae*. Derivative strains containing only pikR1 (DHS328) or only pikR2 (DHS330) or lacking both genes (DHS332, designated here as R1, R2, or Δ, respectively) were prepared from the ΔpikR1 strain (DHS2001; designated here as WT*) (18). Without preincubation, the WT* and R1 cells showed an intermediate level of resistance to TEL [minimal inhibitory concentration (MIC) = 8 μg/mL], whereas R2 and Δ were highly sensitive (MIC = 0.25 μg/mL). Preincubation of the WT* and R2 strains with TEL concentrations of one-fourth MICs raised their respective resistance levels 8- and 64-fold, respectively (Table 1). The higher resistance of the WT* and R2 cells on exposure to TEL correlated with increased modification of nucleotide A2058 (Fig. S4). The microbiological and biochemical data thus show that, although constitutive expression of pikR1 provides some protection from the clinical ketolide TEL, resistance is dramatically increased by drug-mediated activation of pikR2.

**Molecular Mechanism of pikR2 Induction.** Macrolide resistance genes are activated by programmed ribosome stalling at the upstream leader ORFs (5, 19). Many of the resistance genes are induced exclusively or preferentially by cladinose-containing macrolides, whereas ketolides are poor inducers (10, 20). Indeed, ketolides owe their high activity against many clinical isolates to their low propensity for activation of inducible resistance genes.

We were interested in elucidating the molecular mechanism of ketolide-dependent induction of the pikR2 gene. Examination of the pikR2 upstream regions revealed the presence of a 16-codon ORF (pikR2L) 157 bp upstream of pikR2 (Fig. 4A). The role of pikR2L in ketolide-mediated inducibility was investigated using a plasmid construct containing the pikR2L ORF, the intergenic region, and the first five codons of pikR2 fused to the lacZα reporter gene (Fig. 4B). An E. coli Ptac promoter drives transcription of the reporter system. Induction of the pikR2-lacZα chimera was tested in E. coli by a disk diffusion assay (21). Natural and semisynthetic clinical ketolide antibiotics but notably, not the cladinose-containing macrolide ERY activated expression of the pikR2L-based reporter system (Fig. 4B). These data showed that ketolide-specific induction of pikR2 occurs at the level of translation and is likely controlled by the upstream leader ORF.

The mechanism of translational induction was investigated in a cell-free transcription-translation system using a primer extension inhibition assay (22, 23) to test for ketolide- and macrolide-induced ribosome stalling on the pikR2L mRNA. Both MTM and PKM induced strong translation arrest at the Leu13 codon of pikR2L.
genes is expected to confer a marked selective advantage and expression. Ketolide-pikR2 gene controlled by its regulatory region or the entire leader ORF. Computational analysis shows that a stable stem-loop configuration of miRNA in the pikR2-pikR2 intergenic region may sequester the pikR2 ribosome binding site (Fig. S5A). Ketolide-induced ribosome stalling at the Leu13 codon of pikR2, which destabilizes the proximal mRNA stem and promote an alternative conformation, in which the initiation region of pikR2 is accessible for translation (Fig. S5B).

### Inducible Expression of pikR2 Alone or Combined with pikR1 Confers a High Level of Ketolide Resistance in a Clinically Relevant Host.

After establishing that the natural resistance mechanisms conferred by pikR1 and pikR2 in the Streptomyces ketolide producer remain operational on transfer to E. coli, we extended the investigation to a heterologous Gram-positive model, because Gram-positive pathogens are the primary clinical targets of macrolides and ketolides. Mycobacterium tuberculosis is the etiological agent of tuberculosis and considered a possible target for ketolide therapy (24). We constructed strains of Mycobacterium smegmatis, a laboratory model of the pathogenic mycobacteria, which carried either the pikR2 gene controlled by its regulatory region or the entire pikR1-pikR2 cluster on a plasmid (Fig. 1A). The presence of pikR2 alone elevated the resistance of M. smegmatis to clinical ketolides by 16- to 32-fold (Table 2). The combination of pikR1 and pikR2 conferred a much higher level of resistance (exceeding 1 mg/mL for TEL). Constitutive monomethylation of A2058 by PicR1 most likely facilitates continued ribosome activity and expression of pikR2 on abrupt exposure of the cells to high concentrations of ketolides. When M. smegmatis cells with pikR2 or pikR1-pikR2 were preincubated with subinhibitory concentrations of TEL, resistance reached even higher levels, exceeding those of the control cells by several hundredfold (Table 2). Thus, the resistance genes originating in the producer of natural ketolides can render heterologous bacteria resistant to high concentrations of clinical ketolide antibiotics.

### Discussion

In this paper, we present evidence that the genes pikR1 and pikR2 have been evolutionarily optimized to confer resistance to ketolide antibiotics. These genes not only render S. venezuelae ATCC 15439 resistant to its endogenously produced ketolides but also confer appreciable resistance to clinical ketolides. The resistance conferred by pikR1 and pikR2 can be transferred to heterologous Gram-negative and -positive hosts. We further show that the fitness cost of resistance is economized by the ketolide-inducible expression of pikR2. Taking these factors into account, acquisition of the pikR1 and pikR2 genes is expected to confer a marked selective advantage on bacteria undergoing repeated exposure to ketolides, which could promote the transfer of these genes to pathogens when ketolides become more widely used in a clinical setting.

Our studies provide insights into the important question of why S. venezuelae, the producer of the natural ketolides MTM and PKM, maintains two resistance genes with seemingly overlapping functions. The constitutively expressed PikR1 monomethylates 23S RNA nucleotide A2058 located at the site of ketolide action, and this modification confers an intermediate level of resistance. Higher levels of resistance are attained with the inducible PikR2, which adds a second methyl group to the same nucleotide. The combined action of two differentially expressed genes ensures active protein synthesis in the ketolide-producing cells across a broad range of the inhibitors concentrations.

The inducibility of pikR2 is significant for the operation of the resistance mechanism. Although dimethylation of A2058, at least in Staphylococcus aureus, is known to significantly reduce cell fitness (6), monomethylation of the same nucleotide seems to have little, if any, effect on S. venezuelae fitness (Fig. S6). Thus, with a minimal toll in fitness cost for the constitutive PikR1 monomethyltransferase, S. venezuelae easily tolerates low concentrations of MTM and/or PKM. However, when S. venezuelae augments its production of these drugs, activation of the more costly PikR2 enzyme is required, resulting in A2058 dimethylation and higher resistance levels. Producers of other natural antibiotics, which carry more than one resistance gene, may use a similar strategy for achieving the cost-effective protection from inhibitors (25).

In contrast to other inducible erm genes, which respond primarily to macrolides bearing a C3-cladinose, the regulation of pikR2 has been evolutionarily optimized for specific activation by ketolides (Fig. 4). Ketolide-specific pikR2 induction is most likely controlled by programmed translation arrest within the pikR2L leader ORF. Cladinose-containing macrolides are prone to promote peptidyl-tRNA drop off occurring within the first 6–10 codons (26). Consistently, the sites of programmed macrolide-dependent translation arrest within the leader ORFs of the resistant genes are located within the ORF’s first 10 codons, so that the drug-bound ribosome is able to reach the arrest site without prematurely dissociating from the mRNA (5, 19, 23, 27). Ribosomes translating pikR2L have to travel slightly farther and polymerize 13 amino acids before reaching the stalling codon. Because ketolides are less prone to promoting peptidyl-tRNA drop off (28), it is easier for ketolide-bound ribosomes than for ERY-bound ribosomes to polymerize the 13-aa-long nascent PikR2L chain. Indeed, the ERY-bound ribosome does not reach the stalling site (Fig. 4 and Fig. S5) and thus, fails to trigger pikR2 expression. Ketolide-induced stalling in the pikR2L ORF occurs within the motif RLR, which represents one of the most problematic sequences for translation by ketolide-bound ribosomes (28, 29). We note that a short ORF is also found in front of the constitutively expressed

### Table 1. MIC (micrograms per milliliter) of TEL or chloramphenicol for S. venezuelae ΔpikA strains containing different pikR resistance genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Preincubation</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT*</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Δ</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>R1</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>R2</td>
<td>0.25</td>
<td>16</td>
</tr>
</tbody>
</table>

1The ΔpikA strain, which contained both pikR1 and pikR2 resistance genes but was unable to produce antibiotics (DHS5201), was designated WT* control. Formal names of the other strains are DHS332 (Δ), DHS3328 (R1), and DHS3330 (R2).

2Cells were grown for 8 h in LB supplemented with one-fourth MIC of TEL (0.0625 μg/mL for Δ and R2 strains or 2 μg/mL for WT* and R1 strains) before MIC testing.
The properties and regulation of the pikR1 and pikR2 genes are principally important for tolerating the broad range of inhibitor concentrations experienced by S. venezuelae. Constitutive monomethylation of A2058 by PikR1 renders ribosomes moderately resistant to ketolides, affording protection to S. venezuelae at the early stages of antibiotic production. Monomethylation of A2058 is a critical step toward acquisition of a high level of resistance, facilitating efficient translation of pikR2, despite the presence of a ketolide inhibitor. Indeed, on abrupt exposure of the S. venezuelae pikR1-null mutant (ΔpikR1) to TEL, the lone pikR2 gene was unable to provide any significant protection (Table 1), but when both pikR genes were present and cells were preincubated with low ketolide concentrations (mimicking the onset of antibiotic production), a high level of resistance was achieved. In the absence of pikR1, the resistance of S. venezuelae to TEL afforded by pikR2 alone, even on preincubation with the antibiotic, was no higher than that provided by the constitutively expressed monomethylase (Table 1). This intermediate level of resistance may be caused by either dimethylation of only a small fraction of ribosomes or mixed mono- and dimethylations, because Erm dimethyltransferases are known to add one methyl group to A2058 in two consecutive reactions (20).

The synergy between the pikR1 and pikR2 genes was observed to an even greater extent in M. smegmatis, which was used to model the expression of the pikR genes in clinical pathogens. Here, the pikR2 gene alone conferred considerable resistance to clinical ketolides. However, it was the simultaneous presence of both pikR1 and pikR2 genes that provided the highest resistance (Table 2). The resistance to TEL afforded by pikR1 and pikR2 was significantly higher than in S. venezuelae. Factors that could contribute to this effect include the increased dosage of the resistance genes introduced into mycobacteria on a plasmid, the slower subunit assembly resulting in a higher proportion of methylated rRNA, or the lower affinity of the antibiotic for modified mycobacterial ribosomes. The inducibility of pikR2, which lowers the fitness cost of the two-gene resistance mechanism, may facilitate not only its acquisition by a new host through horizontal gene transfer but also, its maintenance on discontinuation of the antibiotic treatment. Horizontal transfer of the pikR1/pikR2 gene pair may be further facilitated by their close physical proximity in the S. venezuelae chromosome (Fig. S7). Despite the high GC content of pikR2, our results strongly suggest that their transfer into at least some clinical strains would render the strains highly resistant to ketolide therapy. These findings, thus, provide additional justification for renewed drug discovery efforts to identify novel antibiotics capable of overcoming protection rendered by natural resistance genes.

Materials and Methods

For isolation of the total RNA from S. venezuelae, cells were grown overnight in SGGP media, then diluted 1:100 in SCM media (31), and grown for 5 d at 30 °C with constant shaking. Cells were pelleted from 5-mL cultures, resuspended in 1 mL Tris-EDTA (TE) buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) containing 5 mg/mL lysozyme, incubated for 10 min at room temperature, and then, shaken for 10 min in the Minibead Breaker (Biospec Products) with 50 μL glass beads (particle size ≤106 μm; Sigma-Aldrich). Total RNA was isolated using the RNeasy Maxi Kit (Qiagen).

The RNeasy Plus Mini Kit was used to prepare total RNA from exponentially growing E. coli following the standard manufacturer’s protocol. Primer extension analysis under the standard conditions suitable for detection of adenine N6 dimethylation was carried out as previously described (21). In experiments where black arrows. The ribosomes arrested with the Leu13 codon in their P site are shown by the red arrows. The ribosomes that reached the pikR2 13th codon but failed to arrest translation were captured at the next Arg14 codon (blue arrow) because of the depletion of Ile-tRNA from the translation reaction by the presence of the Ile-tRNA synthetase inhibitor, mupirocin. The stop codon or the ORF is indicated with an asterisk. (C, Lower) Stalling efficiency calculated from the ratios of the intensity of the bands representing ketolide-dependent arrest (codon 13) vs. read through (codon 14). Error bars indicate data spreads in two independent experiments.

Fig. 4. The pikR2 regulatory region controls the inducible expression of the pikR2 resistance gene. (A) The putative leader ORF pikR2L precedes the pikR2 resistance gene. The nucleotide sequence of the ORF and the amino acid sequence of the encoded leader peptide are shown. (B) Antibiotic disk diffusion assay reveals inducibility of pikR2 in E. coli. In the reporter construct in E. coli cells, the pikR2 regulatory region controls expression of the lacZα reporter. The antibiotic disks contained TEL, MTM, PKM, ERY, or chloramphenicol (CHL). The clear areas around the disks contain antibiotic concentrations that inhibited cell growth. Blue disks contained TEL, MTM, PKM, ERY, or chloramphenicol (CHL). (C, Upper) Toe-printing analysis shows ketolide-induced ribosome stalling at the Leu13 codon of the pikR2L ORF. The band of the ribosomes stalled by the control antibiotic thiostrepton (THS) at the initiation codon is indicated by black arrows. The ribosomes arrested with the Leu13 codon in their P site are shown by the red arrows. The ribosomes that reached the pikR2 13th codon but failed to arrest translation were captured at the next Arg14 codon (blue arrow) because of the depletion of Ile-tRNA from the translation reaction by the presence of the Ile-tRNA synthetase inhibitor, mupirocin. The stop codon or the ORF is indicated with an asterisk. (C, Lower) Stalling efficiency calculated from the ratios of the intensity of the bands representing ketolide-dependent arrest (codon 13) vs. read through (codon 14). Error bars indicate data spreads in two independent experiments.
Table 2. MIC (micrograms per milliliter) of clinically relevant ketolides for *M. smegmatis* harboring the pikR2 gene alone or the combination of pikR1 and pikR2 genes

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>TEL Preincubation</th>
<th>SOL Preincubation</th>
<th>CET Preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes*</td>
<td>No</td>
</tr>
<tr>
<td>pMR12 (vector)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pMR2</td>
<td>64</td>
<td>1,024</td>
<td>1,024</td>
</tr>
<tr>
<td>pMR1R2</td>
<td>2,048</td>
<td>2,048</td>
<td>2,048</td>
</tr>
</tbody>
</table>

*Antibiotics used were TEL, solithromycin (SOL), and cethromycin (CET). The *M. smegmatis* strain used in MIC testing was a derivative of the strain mc²155, in which the endogenous macrolide resistance gene ermB38 was inactivated by allelic exchange (34).

*Cells were grown for 72 h in the medium supplemented with one-eighth MIC of TEL (0.25 μg/mL) for cells transformed with the empty vector, 8 μg/mL for cells transformed with pMR2, or 12 μg/mL for cells transformed with pMR1R2) before MIC testing.

Other experimental details are provided in *SI Materials and Methods.*

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detected N6 monomethylation was required, the concentration of dTTP was reduced from 1 to 0.01 mM with extension for 15 min at 37 °C instead of the standard 30 min at 42 °C. Primers L2180, L2563, and L2667 (Table S3) were used to examine domain V of 23S rRNA of *E. coli.* Primer L2405-R was used to check the modification status of A2058 in 23S RNA of *S. venezueae.*

Toe printing was carried out as described previously (23). The status of rRNA modification in *E. coli* and *S. venezueae* was analyzed using the approach described in ref. 32.