Tetracycline is a broad-spectrum bacteriostatic antibiotic. Its usefulness in the early days of antibiotic therapy has subsequently been jeopardized by widespread drug resistance (27). A common mechanism of resistance to tetracycline is referred to as ribosomal protection (for a review, see reference 33). A common mechanism of resistance to tetracycline is referred to as ribosomal protection (for a review, see reference 33). We will confine ourselves to discussing the protein levels (27), and all of these appear to function in a common mechanism of resistance to tetracycline is referred to as ribosomal protection (for a review, see reference 33).

The effects of mutations in host genes on tetracycline resistance mediated by the Tet(O) and Tet(M) ribosomal protection proteins, which originated in Campylobacter spp. and Streptococcus spp., respectively, were investigated by using mutants of Salmonella typhimurium and Escherichia coli. The miaA, miaB, and miaAB double mutants of S. typhimurium specify enzymes for tRNA modification at the adenine at position 37, adjacent to the anticodon in tRNA. In S. typhimurium, this involves biosynthesis of N^6-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms^2io6A). The miaA mutation reduced the level of tetracycline resistance mediated by both Tet(O) and Tet(M), but the latter showed a greater effect, which was ascribed to the isopentenyl (i^6) group or to a combination of the methylthioadenosine (ms^2) and i^6 groups but not to the ms^2 group alone (specified by miaB). In addition, mutations in E. coli rpsL genes, generating both streptomycin-resistant and streptomycin-dependent strains, were also shown to reduce the level of tetracycline resistance mediated by Tet(O) and Tet(M). The single-site amino acid substitutions present in the rpsL mutations were pleiotropic in their effects on tetracycline MICs. These mutations affect translational accuracy and kinetics and suggest that Tet(O) and Tet(M) binding to the ribosome may be reduced or slowed in the E. coli rpsL mutants in which the S12 protein is altered. Data from both the miaA and rpsL mutant studies indicate a possible link between stability of the aminoacyl-tRNA in the ribosomal acceptor site and tetracycline resistance mediated by the ribosomal protection proteins.

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tet

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rpsL

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position 37 (8, 24). The availability of isogenic strains of S. typhimurium LT2 and its miaA and miaB derivatives (17) allowed us to test the effect of the hydroxysopentenyl (io6) tRNA modification described above and to test the effect of the modification in combination with methylthioadenosine (ms2) as the ms2io6 modification on Tet(O) and Tet(M) Tc
dependent on the strain.

Protein gels and immunoblot blots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the plasmids containing tet(O) or tet(M) was performed by the method of Laemmlil (22). The total amount of protein loaded onto each gel was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.), and 7.5 μg of protein was loaded in each lane. Electrophoresis was performed with a Protean II apparatus (Bio-Rad) on a 10% polyacrylamide gel at a constant voltage of 150 V. The gels were stained with Coomassie blue stain. Prestained molecular weight standards (Gibco-BRL or Rainbow Markers; Amersham, Buckinghamshire, United Kingdom) were used throughout.

After SDS-PAGE, the protein was electroblotted onto a NitroPlus nitrocellulose membrane (Micron Separations Inc., Westboro, Mass.). The membrane was then rinsed in wash buffer (phosphate-buffered saline plus 5% skim milk powder and 0.05% Tween 20). The membrane was blocked for 1 h with wash buffer containing 10% skim milk powder and was then incubated for 1 h with primary antibody against Tet(O) (34) diluted 1:500 in wash buffer, followed by three 5-min washes. Goat anti-rabbit conjugate (Sigma Chemicals), which was diluted 1:1,000 in wash buffer, was added, and the membrane was then reincubated for 1 h. Detection was performed with an ECL Western Blotting kit (Amersham) by the method of Bjo¨rkl (17). The presence of plasmids in strains was confirmed by agarose gel electrophoresis.

Antibiotic susceptibility testing. MICs were determined in the following manner. Cultures were grown to a concentration of 106 cells in liquid medium with an appropriate antibiotic to select for plasmid-containing cells. A volume of 10 μl from a 5-ml culture was diluted in 5 ml of 1× phosphate-buffered saline (Oxoid), and 10 μl of this dilution was spotted onto plates containing increasing twofold concentrations of tetracycline ranging from 1 to 128 μg/ml. The plates were incubated at 37°C overnight. For strains that were streptomycin dependent (Strr), streptomycin was added at a concentration of from 100 to 500 μg/ml, depending on the strain.

RESULTS

MICS specified by tet(M) and tet(O) genes in wild-type S. typhimurium. Burdett (11) has shown that mutations in E. coli miaA reduce the level of tetracycline resistance of strains con-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Genotype</th>
<th>Reference</th>
<th>Source</th>
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<td>S. typhimurium LT2</td>
<td>Prototroph</td>
<td>17</td>
<td>G. R. Bjork</td>
</tr>
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<td>miaA</td>
<td>17</td>
<td>G. R. Bjork</td>
</tr>
<tr>
<td>GT2734</td>
<td>S. typhimurium LT2</td>
<td>miaB</td>
<td>17</td>
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</tr>
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<td>GT3635</td>
<td>S. typhimurium LT2</td>
<td>miaB2508::Tn10dCm</td>
<td>6a</td>
<td>G. R. Bjork</td>
</tr>
<tr>
<td>Tx2568</td>
<td>E. coli K-12</td>
<td>Prototroph</td>
<td>13</td>
<td>M. Winkler</td>
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<tr>
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<td>miaA</td>
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<tr>
<td>M83</td>
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<td>strA lac-proAB thi D80 lacZ D15M</td>
<td>42</td>
<td>J. Messing</td>
</tr>
<tr>
<td>M101</td>
<td>E. coli K-12</td>
<td>traD36 proA&quot; proB&quot; lac&quot; lacZ D15M supE thi D( lac-proAB)</td>
<td>42</td>
<td>J. Messing</td>
</tr>
<tr>
<td>M101-S</td>
<td>E. coli K-12</td>
<td>Str derivative of M101</td>
<td>This study</td>
<td>M. Bekkerling</td>
</tr>
</tbody>
</table>

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TABLE 2. Effect of miaA and miaB mutations in S. typhimurium on MICs of tetracycline resistance mediated by Tet(O) and Tet(M)

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
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<td>Wild type (msio&lt;sup&gt;+&lt;/sup&gt; A37)</td>
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<td>16</td>
<td>64</td>
<td>64</td>
<td>ND</td>
<td>256</td>
</tr>
<tr>
<td>GT523</td>
<td>miaA (A37)</td>
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<td>4</td>
<td>16</td>
<td>32</td>
<td>ND</td>
<td>256</td>
</tr>
<tr>
<td>GT2734</td>
<td>miaB (t&lt;sup&gt;+&lt;/sup&gt; A37)</td>
<td>1</td>
<td>16</td>
<td>64</td>
<td>16</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>GT3635</td>
<td>miaAmiaB (A37)</td>
<td>0.5</td>
<td>4</td>
<td>8</td>
<td>32</td>
<td>8</td>
<td>256</td>
</tr>
</tbody>
</table>

* For the biosynthetic pathway in which miaA and miaB genes are involved, see Fig. 1.

<sup>a</sup> Plasmid pRT11 is a pVHS1::Tn10 derivative which specifies tetracycline efflux (19).

<sup>b</sup> ND, the MIC was not determined because it proved to be impossible to transform pUOA2E2 into these two strains.

Effect of host mutations (miaA and miaB) which under-modify A37 in tRNA. We used S. typhimurium mutants in these experiments because of the availability of isogenic mutations in miaA and miaB and the double mutant (miaAB) (Table 1). The miaA mutation in S. typhimurium reduced the tetracycline MIC for both tet(M)- and tet(O)-containing strains (Table 2). The levels of reduction in the MICs for tet(M)- and tet(O)-containing strains were compared and were found to differ, with the MIC for the tet(M)-containing strains, specified by pVB11, being reduced from 16 to 4 μg/ml and those specified by pJ3 being reduced from 64 to 16 μg/ml, but the reduction specified by pUOA2E1 (wild-type tet(O)) was smaller (64 to 32 μg/ml). The difficulties encountered in preparing constructs with pUOA2E2 [upstream deletion of tet(O)] made comparisons more difficult, but a twofold decrease in the MIC (from 16 to 8 μg/ml) for the miaB and miaAB mutants was again found (Table 2). The miaB mutation had no effect on the tetracycline MIC for either tet(M)- or tet(O)-containing strains. The MICs for the strain with the double mutation (miaAB) were approximately identical to those observed for strain with the miaA mutation alone.

E. coli miaA mutants with Km<sup>+</sup> transposon insertions into miaA, mutL, and miaB (14) were also used to determine MICs. With pVB11, the MIC for the E. coli miaA strain was 4 μg/ml, a definite reduction in the MIC (from 16 μg/ml), whereas for the miaB and mutL (located upstream of mia4) strains, there was no reduction in the MIC. In the case of pJ3, the MIC was also reduced to 16 μg/ml (from 64 μg/ml). However, for pUOA2E1, the MIC remained at 64 μg/ml for all E. coli miaA, miaB, and mutL derivatives tested. Therefore, the miaA mutation appears to reduce the MIC for tet(M) strains to a greater extent than it does for tet(O) strains. For S. typhimurium, the MIC specified by tet(O) is reduced only by one-half, whereas for E. coli no reduction in the MIC was observed.

Effect of mutations in the rpsL gene encoding ribosomal protein S12 on MICs of tetracycline. During MIC testing of strains harboring plasmids carrying the tet(M) and tet(O) genes, we observed that the MICs of tetracycline were different for E. coli JM83 and JM101 carrying the same plasmid. For example, for JM83(pVB11) the MIC was 16 μg/ml, but for JM101(pVB11) the MIC was 32 μg/ml. Similar results with other plasmids were observed, with the MIC for the JM83 host always being lower than the MIC for JM101, even though no difference in MICs were found for plasmid-free strains. The major difference between JM83 and JM101 was a strA mutation in the former strain. To test the effect of resistance to streptomycin on the tetracycline MIC, JM101(pVB11) was made resistant to streptomycin by selection on 100 μg of streptomycin per ml. The MIC for streptomycin-resistant (Str<sup>R</sup>) strain JM101(pVB11) on retesting was reduced from 32 to 8 μg/ml. We therefore began to examine the effect of Str<sup>R</sup> and Str<sup>Δ</sup> mutations on the MICs specified by the tet(M) and tet(O) genes.

The strongest evidence that Str<sup>R</sup> and Str<sup>Δ</sup> mutations reduce the MICs specified by tet(M) and tet(O) is shown in Table 3. We used strains with known mutations in ribosomal protein S12; these mutations have been sequenced by Timms and colleagues (35, 36). Almost all rpsL mutations reduce the MICs specified by both tet(M) and tet(O) genes. In general, the level of reduction for each particular mutation for tet(M) and tet(O) is similar, e.g., for strain CM1245 (Str<sup>R</sup>) the MIC is reduced by half for both determinants, whereas for strain CM1236 (Str<sup>Δ</sup>) the MIC is reduced to one-fourth. Nevertheless, the reduction in the MIC for strains with mutations in rpsL is pleiotropic, with the reduction in the MIC for some strains with tet(M) being greater than that for strains with tet(O), as with CM1243 (Str<sup>R</sup>) and CM1238 (Str<sup>Δ</sup>). No reduction in the MIC for strain CM1235(pVB11) was found. In contrast to the effect on tetracycline resistance mediated by Tet(M) and Tet(O), which act by protecting the ribosome, the Tet(B) determinant, which specifies tetracycline efflux, was not affected by the rpsL mutation. The MICs specified by Tet(B) were 256 μg/ml for both parental strains and strains with an rpsL background (Table 3).

We used the CM strains in preference to other rpsL mutants because the rpsL genes have been sequenced and the exact amino acid substitutions in the mutants are known (35, 36). However, other Str<sup>R</sup> mutants in which the exact mutations involved were not known were also tested. Some Str<sup>R</sup> mutations in strains with pVB11, pJ3, or pUOA2E1, or pUOA2E2 had no effect on tetracycline MICs. In contrast, for two Str<sup>Δ</sup> strains known as strM (29), the MICs specified by Tet(M) and Tet(O) plasmids were reduced; e.g., the MIC for CGSC6983(pVB11)
was 2 μg/ml, that for CGSC6983(pJ13) was 4 μg/ml, and that for CGSC6983(pUOA2E1) was 2 to 4 μg/ml (data not shown in Table 3). In contrast to the rpsL mutants, no parental strains were available for the StrM strains.

Effect of addition of streptomycin to growth medium for MIC testing. Since streptomycin must be added to StrR strains for growth to occur, it occurred to us that this might be affecting the MICs of tetracycline. Therefore, streptomycin at 100 μg/ml was added to the tetracycline plates used to determine the MICs (Table 3). The presence or absence of streptomycin had no effect on the tetracycline MICs for the StrR strains listed in Table 3. It was not possible to test StrR strains without streptomycin, since they would not be able to grow. We infer from the results of this experiment that streptomycin itself does not reduce the tetracycline MIC.

Examination of Tet(M) and Tet(O) protein production. Immunoblotting studies were performed with antibody to Tet(O) [which also reacts with Tet(M)] by using the strains listed in Tables 2 and 3. There was no significant difference in the amount of Tet(M) or Tet(O) protein produced by any of the strains tested (data not shown). Consequently, differences in the MICs for particular strains could not be explained by the differences in the levels of ribosomal protection proteins.

DISCUSSION

A major goal of this study was to examine the effect of undermodification of A37 within tRNA on TcR mediated by Tet(O). This was precipitated by the report that modification of tRNA at A37, which was lacking in an E. coli miaA mutant, was necessary for Tet(M)-mediated TetR (11). We found that Tet(O)-mediated TetR was slightly reduced in an S. typhimurium miaA mutant, but the effect was less than that observed with Tet(M)-mediated TetR. Also, our data on the effect of miaA on TetO are not as compelling as those presented in an earlier study (11). The availability of miaA, miaB, and miaAB isogenic mutations enabled us to test different modifications within ms2i6A37 on both Tet(O) and Tet(M)-mediated resistance. The miaA mutants should produce primarily unmodified A37, and miaB mutants accumulate mostly isopentenyl (i6) A37, with only small amounts of io6 A37 accumulating in S. typhimurium (17). The effect on both TetR determinants can be ascribed to the io6 group alone or to the combination of the ms2 and i8 groups but not to the ms2 group alone (Fig. 1). Identical results were found by Esberg and Björk (17) in studying the decoding efficiency of tRNA. Their results indicate that although ms2 and i8 groups contribute to the decoding efficiency of tRNA, the major impact originates from the i8 group or the combination of the i8 and the ms2 groups. The ms2i8 A37 has been shown to stabilize the anticodon-codon interaction by improving the stacking of the hypermodified nucleoside (39).

The effects of the miaA mutation on E. coli are pleiotropic. Mutants grow more slowly, the translation elongation rate is decreased, and the regulation of many operons is affected (16). The ms2i6 modification also influences translational accuracy; fidelity is increased in an miaA mutant, possibly because the absence of the modification which usually stabilizes the codon-anticodon interaction affects the binding of near cognate tRNA more than it affects the binding of cognate tRNA (5).

The effect of undermodification of A37 on Tet(O)- and Tet(M)-mediated TcR is consistent with the interaction of these proteins near the ribosomal A site, the site of tetracycline action. Tetracycline binds near the ribosomal A site and blocks entry of the aminoacyl-tRNA·EF-Tu·GTP ternary complex. Tet(O) and Tet(M) cause tetracycline to be displaced from the ribosome (12, 37). A decrease in aminoacyl-tRNA stability caused by the loss of the ms2i6 modification may decrease the ability of aminoacyl-tRNA to successfully bind to the A site before the rebinding of tetracycline. The difference in the level of resistance noted between the Tet(O) and Tet(M) derivatives of the miaA strains may reflect differences in the kinetic rates of these ribosomal protection proteins which alter the amount of time that the aminoacyl-tRNA has to bind before the rebinding of tetracycline.

In this study, we also discovered that the levels of resistance mediated by Tet(O) and Tet(M) were reduced in E. coli strains with particular mutations in the rpsL gene encoding ribosomal protein S12. For both StrR and StrS strains with either Tet(O) or Tet(M) some reduction in tetracycline MICs was observed. Although the reductions were quite variable, the MICs specified by Tet(O) (pVB11) and Tet(O) (pUOA2E1) are comparable, in that mutations which reduce tetracycline resistance for strains with Tet(M) reduce it by about the same level as that observed for strains with Tet(O). The effect of rpsL mutants on tetracycline resistance could not be explained by the hypersensitivity of these mutants to tetracycline or to poor growth since the presence of a Tet(B) determinant, which acts via an efflux mechanism, resulted in the maintenance of an MIC of 256 μg/ml for both parental and rpsL mutant derivatives.

Streptomycin induces misreading in the translation of the genetic code (21). Mutations in the rpsL gene, which generate StrR or StrS ribosomes, lead to an error-restrictive (or hyperaccurate) phenotype, i.e., the accuracy of translation is increased, as is the case with the miaA mutation. This phenomenon was once thought to relate to increased proofreading in

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### Table 3. Defined mutations in E. coli rpsL gene reduce MICs specified by tet(M) and tet(O) genes

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genotype</th>
<th>Amino acid change</th>
<th>Tetracycline MIC (μg/ml) for strains with following plasmid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No [Tet(M)]</td>
</tr>
<tr>
<td>WP2</td>
<td>Wild type</td>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>CM1234</td>
<td>rpsL (StrR)</td>
<td>Lys 42→Gln</td>
<td>0.5</td>
</tr>
<tr>
<td>CM1283</td>
<td>rpsL (StrR)</td>
<td>Pro 90→Arg</td>
<td>1</td>
</tr>
<tr>
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<td>rpsL (StrR)</td>
<td>Gly 91→Asp</td>
<td>1</td>
</tr>
<tr>
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<td>rpsL (StrR)</td>
<td>Lys 42→Thr</td>
<td>1</td>
</tr>
<tr>
<td>CM1236</td>
<td>rpsL (StrR)</td>
<td>Lys 42→Ile</td>
<td>ND</td>
</tr>
<tr>
<td>CM1237</td>
<td>rpsL (StrR)</td>
<td>Lys 42→Arg</td>
<td>1</td>
</tr>
<tr>
<td>CM1238</td>
<td>rpsL (StrR)</td>
<td>Lys 42→Asn</td>
<td>1</td>
</tr>
</tbody>
</table>

a The codon at which the amino acid substitution occurred in the S12 ribosomal protein is indicated. Data are from references 35 and 36.
b pVB11 is a pV85::TetO derivative (19).
c ND, the MIC was not determined because it proved to be impossible to transform pVB11 into this strain.
rpsL mutants (28), but this is no longer thought to be the complete explanation (20). It is now established that error-restrictive S12 mutations reduce the kinetic efficiency of the interaction between the ternary complex (aa-tRNA · EF-Tu · GTP) and the ribosome (3, 7, 38). The restrictive S12 mutations are thought to reduce the saturation level of the ribosome (21). The effects of mutations in Str' and Str' mutants on translational accuracy and kinetics are presumably related to ternary complex binding at the A site, perhaps indirectly, because the S12 protein affects the 16S RNA which in turn affects ternary complex interactions at the A site (2). Mutant ribosomes with decreased translational accuracy (ribosomal ambiguity mutants) have a higher nonspecific affinity for tRNA at the A site, while the mutants with hyperaccurate mutations, such as the ribosomes of rpsL mutants, have a lower general affinity for tRNA, which makes the codon-anticodon interaction of increased importance (20). In addition, some mutants with mutations in S12 were shown to be abnormal with respect to their capacity to stimulate the GTPase activity of EF-Tu (7). S12 has also been cross-linked to EF-G, and EF-Tu is believed with mutations in S12 were shown to be abnormal with respect to the elongation factor binding site on the ribosome. Data point to an interaction of the ribosomal protection proteins with the elongation factor binding site on the ribosome. Data for both the miaA mutants and the rpsL mutants indicate a possible link between stability of the aminoacyl-tRNA in the A site and tetracycline resistance. The overall accuracy of translation is important for ribosomal protection protein-mediated tetracycline resistance.

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REFERENCES