Ribosomes provide a target for several antibiotic families, among which is the macrolide-ketolide group. High-resolution crystal structures showed that macrolides and their derivatives bind to a specific pocket of the nascent protein exit tunnel (2, 3, 7, 16, 20, 21, 25, 29), the universal feature of the large ribosomal subunit through which nascent proteins emerge. The same pocket is exploited by all members of the macrolide family, and effective inhibitory action is achieved when the drug consumes a significant portion of the tunnel cross-section (1, 31, 32). Typically, resistance to macrolides is acquired through either efflux or target-based alteration (methylation or mutation of nucleotides involved in drug binding [for reviews, see references 11 and 28]).

Ketolides are an advanced generation of the macrolide antibiotics, which, in part, provide activity against macrolide-resistant pathogens. They are semisynthetic derivatives of erythromycin, the first macrolide in use. Like erythromycin, ketolides are composed of a 14-membered macrolactone ring (Fig. 1). However, their macrolactone ring lacks a cladinose sugar and possesses a keto group at position 3, a cyclic carbamate, and an extended arm. Ketolides and macrolides share their more elaborated chemistry, in addition to their binding to common, albeit not identical inhibitory mechanisms. Owing to their more elaborated chemistry, in addition to their binding to the same pocket is exploited by all members of the macrolide family, and effective inhibitory action is achieved when the drug consumes a significant portion of the tunnel cross-section (1, 31, 32). Typically, resistance to macrolides is acquired through either efflux or target-based alteration (methylation or mutation of nucleotides involved in drug binding [for reviews, see references 11 and 28]).

Ketolides are an advanced generation of the macrolide antibiotics, which, in part, provide activity against macrolide-resistant pathogens. They are semisynthetic derivatives of erythromycin, the first macrolide in use. Like erythromycin, ketolides are composed of a 14-membered macrolactone ring (Fig. 1). However, their macrolactone ring lacks a cladinose sugar and possesses a keto group at position 3, a cyclic carbamate, and an extended arm. Ketolides and macrolides share a similar, albeit not identical inhibitory mechanism. Owing to their more elaborated chemistry, in addition to their binding to the same pocket is exploited by all members of the macrolide family, and effective inhibitory action is achieved when the drug consumes a significant portion of the tunnel cross-section (1, 31, 32). Typically, resistance to macrolides is acquired through either efflux or target-based alteration (methylation or mutation of nucleotides involved in drug binding [for reviews, see references 11 and 28]).

Ketolides are an advanced generation of the macrolide antibiotics, which, in part, provide activity against macrolide-resistant pathogens. They are semisynthetic derivatives of erythromycin, the first macrolide in use. Like erythromycin, ketolides are composed of a 14-membered macrolactone ring (Fig. 1). However, their macrolactone ring lacks a cladinose sugar and possesses a keto group at position 3, a cyclic carbamate, and an extended arm. Ketolides and macrolides share a similar, albeit not identical inhibitory mechanism. Owing to their more elaborated chemistry, in addition to their binding to the same pocket is exploited by all members of the macrolide family, and effective inhibitory action is achieved when the drug consumes a significant portion of the tunnel cross-section (1, 31, 32). Typically, resistance to macrolides is acquired through either efflux or target-based alteration (methylation or mutation of nucleotides involved in drug binding [for reviews, see references 11 and 28]).

Ketolides are an advanced generation of the macrolide antibiotics, which, in part, provide activity against macrolide-resistant pathogens. They are semisynthetic derivatives of erythromycin, the first macrolide in use. Like erythromycin, ketolides are composed of a 14-membered macrolactone ring (Fig. 1). However, their macrolactone ring lacks a cladinose sugar and possesses a keto group at position 3, a cyclic carbamate, and an extended arm. Ketolides and macrolides share a similar, albeit not identical inhibitory mechanism. Owing to their more elaborated chemistry, in addition to their binding to the same pocket is exploited by all members of the macrolide family, and effective inhibitory action is achieved when the drug consumes a significant portion of the tunnel cross-section (1, 31, 32). Typically, resistance to macrolides is acquired through either efflux or target-based alteration (methylation or mutation of nucleotides involved in drug binding [for reviews, see references 11 and 28]).
FIG. 1. Chemical structures of the ketolides and 16-membered macrolides used in the present study.
and rplV (coding for ribosomal protein L22) are given in Table 2. Nucleic acid sequencing was done with fluorescence-labeled nucleotides and the Taq cycle sequencing system of Applied Biosystems. rplD was wild type in all strains, and mutational alterations were limited to rplV and 23S rRNA as specified in the text.

**Genetic characterization of the mutants.** Based on the available crystal structures of D50S complexed with ketolide antibiotics (2, 20), we assumed that the resistance mutations should localize to 23S rRNA domains II and V. Gene amplification of corresponding regions of the 23S rRNA gene and subsequent sequence determination revealed that the telithromycin resistance phenotype was associated with a single point mutation in domain V in *M. smegmatis* rmb, i.e., either 2058C (six of nine isolates) or 2058G (three of nine isolates). Thirteen of the fifteen telithromycin-resistant isolates obtained from *M. smegmatis* rmb A2058G mutant demonstrated the double mutation 2058G/2059G. In two of fifteen of the analyzed mutants, no additional sequence alteration within 23S rRNA domains II or V other than the parental 2058A—G alteration was found, but we observed a deletion in rplV that resulted in the loss of 15 amino acids from protein L22 (Ile85-Arg99) (Fig. 2). Inspection of the nucleotides around the deleted DNA region revealed the presence of flanking direct repeats (Fig. 2), suggesting that either homologous recombination or slipped stranded mispairing (23) resulted in the loss of residues between Ile85 and Arg99.

**Physiological investigations.** We next determined MICs to telithromycin and several 16-membered-ring macrolides (Table 3). As previously shown (16), A2058C confers high-level resistance to telithromycin (relative resistance [RR] > 4,096) and results in a significant resistance to spiramycin, josamycin, and carbomycin but has only a small effect on the interactions of tylosin and desmycosin with the ribosome. The A2058G mutation marginally alters the susceptibility of the ribosome to 16-membered macrolides but confers significant resistance to telithromycin (RR = 512). The double mutation 2058G/2059G obtained with a frequency of 4 × 10⁻⁷. *M. smegmatis* mc2155 SMR5 rnb A2058G mutant was used to select for mutants resistant to more than 512 µg of telithromycin/ml; mutants were obtained with a frequency of 3 × 10⁻⁷.

**RESULTS AND DISCUSSION**

**Isolation of mutants.** *M. smegmatis* mc2155 SMR5 rmb (18) was used to select for mutants resistant to more than 64 µg of telithromycin/ml (for the chemical structure of telithromycin was used to select for mutants resistant to more than 64 µg/ml (for the chemical structure of telithromycin was used to select for mutants resistant to more than 64 µg/ml. The drugs were obtained from Pfizer (carbomycin), Sigma (josamycin, spiramycin, tylosin), Eli Lilly (desmycosin), and Aventis Pharma (telithromycin). The A2058G alteration was found, suggesting that either homologous recombination or slipped stranded mispairing (23) resulted in the loss of residues between Ile85 and Arg99.

**Physiological investigations.** We next determined MICs to telithromycin and several 16-membered-ring macrolides (Table 3). As previously shown (16), A2058C confers high-level resistance to telithromycin (relative resistance [RR] > 4,096) and results in a significant resistance to spiramycin, josamycin, and carbomycin but has only a small effect on the interactions of tylosin and desmycosin with the ribosome. The A2058G mutation marginally alters the susceptibility of the ribosome to 16-membered macrolides but confers significant resistance to telithromycin (RR = 512). The double mutation 2058G/2059G obtained with a frequency of 4 × 10⁻⁷. *M. smegmatis* mc2155 SMR5 rmb A2058G mutant was used to select for mutants resistant to more than 512 µg of telithromycin/ml; mutants were obtained with a frequency of 3 × 10⁻⁷.

**Results and Discussion.**

### TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc2155 SMR5 rmb*</td>
<td>*..</td>
</tr>
<tr>
<td>mc2155 SMR5 rmb†</td>
<td>...</td>
</tr>
<tr>
<td>mc2155 SMR5 rmb‡</td>
<td>...</td>
</tr>
<tr>
<td>mc2155 SMR5 rmb‡</td>
<td>...</td>
</tr>
<tr>
<td>mc2155 SMR5 rmb‡</td>
<td>...</td>
</tr>
<tr>
<td>mc2155 SMR5 rmb‡</td>
<td>...</td>
</tr>
</tbody>
</table>

* †, Parental strain; †, recombinant mutants of *M. smegmatis* mc2155 SMR5 rmb; ‡, spontaneous mutants of *M. smegmatis* mc2155 SMR5 rmb 2058G mutant.

### TABLE 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence (5’ to 3’)</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S rRNA (5s)*</td>
<td>GGC GTC TGG GGG GAA CGC GG</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#625</td>
<td>GGC GTC TGG GGG GAA CGC GG</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#86</td>
<td>GGA GGT AGA GCT ACT GGA TGG</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#696</td>
<td>CCA TCC AGT AGC TCT ACC TCC</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#85</td>
<td>TAC GGC TAC CTT CCT GCG TC</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#601</td>
<td>GTC GCC AAA TTC TCT GTC GGG TA</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#603</td>
<td>GGT GGG TAG TTT AAG TGG GG</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>#604</td>
<td>CGC GCG GCG GAT AGA GAC CG</td>
<td>23S rRNA (5s)</td>
</tr>
<tr>
<td>Downstream of 55 rRNA*</td>
<td>TCT CAC GGG TTA TGG GGG CGG CCT CG</td>
<td>Downstream of rmA</td>
</tr>
<tr>
<td>#630A</td>
<td>TCT CAC GGG TTA TGG GGG CGG CCT CG</td>
<td>Downstream of rmA</td>
</tr>
<tr>
<td>L22 (rplV)*</td>
<td>CGC ACG TTT AAG GGT CAC AT</td>
<td>Upstream of rplV</td>
</tr>
<tr>
<td>#738</td>
<td>TAC TGG TGT TGG CAC TAC CA</td>
<td>Upstream of rplV</td>
</tr>
<tr>
<td>L4 (rplD)*</td>
<td>CGG CAA GAC GGA CGG TTC TG</td>
<td>rplD</td>
</tr>
<tr>
<td>#775</td>
<td>CGG CAA GAC GGA CGG TTC TG</td>
<td>rplD</td>
</tr>
<tr>
<td>#739</td>
<td>TGT TCA AGG GCA CCC GCA TG</td>
<td>Upstream of rplD</td>
</tr>
<tr>
<td>#740</td>
<td>CCG TAC GAT TCC GAG AT</td>
<td>Downstream of rplD</td>
</tr>
</tbody>
</table>

* †, E. coli numbering (with exception of downstream region of rmA operon A which is numbered according to contig:3563_m_smegmatis, positions 5023050 to 5025075 from www.tigr.org); †, numbered according to contig:3563_m_smegmatis, positions 1537075 to 1541332 from www.tigr.org. |
results in high-level resistance to all ketolides and 16-mem-
bered macrolides. For \textit{M. smegmatis} A2058G/rplV \Delta Ile85-
Arg99, a fourfold increase in resistance to telithromycin, tylo-
sin, and desmycosin is found compared to \textit{M. smegmatis} A2058G. In contrast, the deletion in \textit{rplV} did not affect the
MICs for josamycin, carbomycin, and spiramycin. We also
found that the generation time of the 23S rRNA A2058G/
rplV/H9004 Ile85-Arg99 double mutant is similar to that of strain 
\textit{M. smegmatis} A2058G (data not shown), indicating that the partial 
rplV deletion does not grossly affect protein biosynthesis.

\textbf{Structural basis for telithromycin resistance.} Molecular
modeling, based on the crystal structure of telithromycin com-
plexed with the ribosomal large subunit of \textit{D. radiodurans},
provides a feasible structural basis for the telithromycin resis-
tance mechanisms conferred by the alterations isolated. Thus,
both mutations of nucleotide A2058 observed in the \textit{M. smeg-
matis} \textit{rrnB} mutant, namely, A2058G or A2058C, disturb the
interactions of telithromycin with the nucleotide at position
2058, either by steric hindrance (A3G mutation) or by placing
the antibiotic molecule distant from position 2058, thus ham-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{(Top) Multiple sequence alignment of protein L22 of
\textit{Escherichia coli} (Ec; GenBank accession no. X02613), \textit{Thermus thermophilus} (Tt; GenBank accession no. X84708), \textit{Deinococcus radiodurans} (Dr; GenBank accession no. AE001892), and \textit{Mycobacterium smegmatis} (Ms; GenBank accession no. Y13227). The sequences were aligned by using the CLUSTAL W algorithm (17). Amino acids identical to the consensus sequence provided by the CCD (accession no. pfam00237.11) are highlighted with gray boxes (14). Residues forming the conserved \( \beta \)-hairpin (positions 79 to 99 according to \textit{E. coli} numbering) are shown within the black box with the tip of the hairpin highlighted in black. The region deleted in the telithromycin-resistant \textit{M. smegmatis} strains is indicated by a brace. (Bottom panel) (A) Ribbon presentation of the overall fold of L22 from \textit{M. smegmatis} (in black) based on its structure within the large subunit of \textit{D. radiodurans} (in gray) (9). (B) The secondary structure elements are indicated above the amino acid sequence of the L22 protein of \textit{M. smegmatis} (GenBank accession no. Y13227). The amino acids deleted in the telithromycin-resistant mc2155 SMR5 \textit{rrnB} 2058G/rplV \Delta Ile85-Arg99 mutant are highlighted by a gray box. (C) DNA sequence of \textit{M. smegmatis} around the location of the deletion (gray box). The deleted nucleotides are flanked by direct repeats (DR, open boxes).
\end{figure}
When *M. smegmatis* /H9262 shows significant resistance to telithromycin (MIC of 128 µg/ml) and RR in the proximity of domain II is ascribed, partially, to the different conformations of domain II nucleotides involved in telithromycin binding in eubacteria and archaea (Fig. 4). Thus, in the D50S complex nucleotide 790 forms stacking interactions with the aryl-alkyl arm of telithromycin, thus stabilizing its conformation. A similar conformation of the drug’s aryl-alkyl arm would not benefit from this stabilizing interaction in the mH50S complex, since the mH50S equivalent to nucleotide 790 is flipped away from the position that could facilitate interactions with the telithromycin aryl-alkyl arm (Fig. 4). An additional reason for the inconsistency between the large volume of biochemical and crystallographic studies may be linked to the high salinity essential for *H. marismortui* optimal growth and integrity (22), which may mask several potential ribosomal entities that could have interacted with the drug.

### Structural basis for resistance to 16-membered lactone-ring macrolides
All 16-membered lactone ring macrolides share a larger lactone ring compared to the 14-membered lactone ring, pering optimal interactions (A → C mutation). Both mutations are therefore likely to decrease the binding affinity of telithromycin (Table 3). Nucleotide 2058 was found to play a key role in the binding of 14-membered macrolides and ketolides (19). Consistently, crystal structures of complexes of macrolides or ketolides, with eubacterial ribosomes possessing an adenosine at position 2058, show that A2058 is implicated in hydrogen bonding with the drug (2, 3, 16, 20, 21).

As shown in Table 3, the A2058G mutation alone provokes significant resistance to telithromycin (MIC of 128 µg/ml). When *M. smegmatis* A2058G was subjected to selection with 512 µg/ml of telithromycin/mill, resistance was accompanied by additional mutations. One of the additional mutations was found to be 2059A → G (16): consistent with its contribution to macrolides or ketolides binding observed in complexes of the eubacterial large ribosomal subunit (2, 3, 20, 21), the additional A2059G should result in a steric hindrance and binding destabilization. These results, as well as those described below, suggest the existence of multiple steps alongside several types of mutations that can yield telithromycin resistance.

The other mechanism for additional resistance involves the deletion of 15 residues in the protein L22 (Ile85-Arg99), most of which line the D50S tunnel (9), in a location that is rather close to the ketolides long arm (Fig. 3) (2, 21). The A → G mutation of 2058, the key nucleotide for macrolide/ketolide binding, which leads to additional space consumption, should cause a shift in telithromycin position, thus decreasing telithromycin affinity to the 2058 region of the macrolide binding pocket and may, at the same time, facilitate the formation of new drug contacts. The most likely direction of this shift is toward the other side of the tunnel, proximal to the L22 hairpin tip. Consequently, telithromycin may interact with L22, and these interactions may become rather critical for its binding to eubacterial A2058G mutated ribosomes.

We analyzed the resistance mechanism mediated by the deletion in ribosomal protein L22, by modeling its *M. smegmatis* structure using the structure of L22 in *D. radiodurans* as a template. Protein L22 is composed of a globular domain, a long N-terminal extension, and a long β-hairpin, which is a constituent of the ribosome exit tunnel. The Ile85-Arg99 stretch belongs to the tip of L22 β-hairpin (Fig. 2 and 3) and is involved in intensive interactions with the 23S rRNA (for a list of nucleotides located within 4 Å of the region Ile85-Arg99, see Table 4). The modeled L22 protein from *M. smegmatis* shows that a few direct interactions between telithromycin and the L22 β-hairpin are possible and that the L22 residue closest to telithromycin is Gln90, whose side chain is located 4 Å from the drug (Fig. 3). The L22 region Ile85-Arg99 is positively charged; as such, it may contribute to stabilize the local RNA architecture via coulomb effects. As shown in Table 4, in the crystal structure of the telithromycin/D50S complex, the region Ala89-Gly91 interacts with domain II nucleotides U747, G748, A750, and A751 (2, 29). This region was also found to play a role in telithromycin binding by biochemistry, site-directed mutagenesis, and studies of clinical isolates with acquired drug resistance (4, 5, 8, 15, 26, 27, 30). Besides broadening the tunnel cross-section, a deletion of Ile85-Arg99 in L22 may cause a structural rearrangement of these nucleotides, with a resulting decrease in telithromycin binding affinity.

The only structural study that challenges the interaction of telithromycin with the 23S RNA domain II, in the proximity of L22 hairpin tip, is based on the crystal structure of telithromycin in complex with the G2058A mutant of the large ribosomal subunit from the archaeon *Haloarcula marismortui*, called here mH50S (25). Since interactions with domain II nucleotides have neither been observed in the crystal structure of this complex nor been identified by footprinting experiments (A. Mankin, unpublished observations), this structure cannot provide a structural explanation as to the L22 deletion. It is conceivable, however, that the lack of telithromycin contacts with domain II is ascribed, partially, to the different conformations of domain II nucleotides involved in telithromycin binding in eubacteria and archaea (Fig. 4). Thus, in the D50S complex nucleotide 790 forms stacking interactions with the aryl-alkyl arm of telithromycin, thus stabilizing its conformation. A similar conformation of the drug’s aryl-alkyl arm would not benefit from this stabilizing interaction in the mH50S complex, since the mH50S equivalent to nucleotide 790 is flipped away from the position that could facilitate interactions with the telithromycin aryl-alkyl arm (Fig. 4). An additional reason for the inconsistency between the large volume of biochemical and crystallographic studies may be linked to the high salinity essential for *H. marismortui* optimal growth and integrity (22), which may mask several potential ribosomal entities that could have interacted with the drug.

#### Table 3. Drug susceptibility of *M. smegmatis* mutants

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>WT MIC (µg/ml)</th>
<th>2058C&lt;sup&gt;b&lt;/sup&gt;</th>
<th>2058G&lt;sup&gt;b&lt;/sup&gt;</th>
<th>2059G&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔL22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telithromycin</td>
<td>0.25</td>
<td>&gt;1,024</td>
<td>&gt;4,096</td>
<td>128</td>
<td>512</td>
</tr>
<tr>
<td>Tylosin</td>
<td>16</td>
<td>64</td>
<td>4</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Desmycosin</td>
<td>16</td>
<td>256</td>
<td>16</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>32</td>
<td>&gt;1,024</td>
<td>&gt;32</td>
<td>256</td>
<td>8</td>
</tr>
<tr>
<td>Josamycin</td>
<td>4</td>
<td>1,024</td>
<td>256</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Carbomycin</td>
<td>2</td>
<td>512</td>
<td>256</td>
<td>64</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild type. The RR was calculated by dividing MIC for the mutant by the MIC for the wild type.

<sup>b</sup> These data have been reported previously (16) and were experimentally confirmed in the present study. They have been included for comparison.
The additional two atoms seem to allow for higher conformational flexibility and also provide more potential interactions with the ribosome. Both properties should increase binding to altered ribosomes, since the higher flexibility and the increased number of interactions with the binding pocket can stabilize the drug binding and thus compensate for the loss of contacts with a mutated or methylated nucleotide at position 2058.

Among the 16-membered macrolides, tylosin and desmycosin carry a mycinose sugar at position 14 of their macrolactone rings, whereas spiramycin, carbomycin, and josamycin lack this sugar (Fig. 1). Structurally, it was shown that tylosin binds to D50S (29) and to the large ribosomal subunit of H. marismortui, H50S (7), in a similar fashion, with its mycinose sugar involved in interactions both with L22 and with domain II nucleotides. Hence, both binding modes are consistent with the resistance conferred by the Ile85-Arg99 L22 deletion (Table 3).

Our results show that the 16-membered ring macrolides that lack the mycinose sugar (i.e., spiramycin, josamycin, and car-
bomycin) are more susceptible to the A2058C mutation compared to tylosin and desmycosin, indicating that the mycinose sugar can serve as an additional interacting moiety. These findings are in accord with the structures of their complexes with H50S (7). The finding that the Ile85-Arg99 L22 deletion did not confer resistance to spiramycin, josamycin, and carboxymycin, is also in line with this interpretation.

**Conclusions.** Based on the findings reported here, supported by a large volume of biochemical and genetic evidence, as well as by crystallographic observations, we conclude that domain II and protein L22 can stabilize the binding of telithromycin and 16-membered lactone rings containing a mycinose sugar. Consistently, these interactions may become imperative as a result of the A2058G mutation and thus can play a role in resistance to these antibiotics. Our results suggest that the L22 deletion causes alterations in the tunnel cross-section and eliminates possible direct drug-ribosome interactions, thus portraying the way that the L22 deletion exerts its effects in the presence of a mutated 2058A.

**ACKNOWLEDGMENTS**

Funds were provided by the Swiss National Science Foundation (E.C.B.), L.R.5 Regione Campania (R.B.), the University of Zurich (E.C.B.), the U.S. National Institutes of Health (GM34360 [A.Y.]), and the Kimmelman Center for Macromolecular Assembly (A.Y.). A.Y. holds the Helen and Martin S. Kimmel Professorial Chair.

**REFERENCES**


