

## Mutations in Conserved Helix 69 of 23S rRNA of *Thermus thermophilus* That Affect Capreomycin Resistance but Not Posttranscriptional Modifications<sup>∇</sup>

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**Translocation during the elongation phase of protein synthesis involves the relative movement of the 30S and 50S ribosomal subunits. This movement is the target of tubercactinomycin antibiotics. Here, we describe the isolation and characterization of mutants of *Thermus thermophilus* selected for resistance to the tubercactinomycin antibiotic capreomycin. Two base substitutions, A1913U and mU1915G, and a single base deletion, ΔmU1915, were identified in helix 69 of 23S rRNA, a structural element that forms part of an interribosomal subunit bridge with the decoding center of 16S rRNA, the site of previously reported capreomycin resistance base substitutions. Capreomycin resistance in other bacteria has been shown to result from inactivation of the TlyA methyltransferase which 2'-O methylates C1920 of 23S rRNA. Inactivation of the *tlyA* gene in *T. thermophilus* does not affect its sensitivity to capreomycin. Finally, none of the mutations in helix 69 interferes with methylation at C1920 or with pseudouridylation at positions 1911 and 1917. We conclude that the resistance phenotype is a consequence of structural changes introduced by the mutations.**

The translocation step of protein synthesis involves the concerted movement of tRNAs through the ribosome and a relative motion the 30S and 50S subunits (8, 15, 43). A ratcheting motion of the subunits has been observed by cryoelectron microscopy (8, 43), consistent with the hybrid states model (30). Translocation is the target of a number of antibiotics acting by a variety of mechanisms (reviewed in reference 10). For example, fusidic acid stabilizes EF-G on the ribosome in the posttranslocational state (40), while the peptide antibiotic thiostrepton prevents binding of EF-G to the ribosome (2). Capreomycin and viomycin, members of the tubercactinomycin family of cyclic peptide antibiotics, freeze the 70S ribosome in an intermediate state of translocation (7) and slow the transition of tRNAs from the classical to the hybrid state (17). While the mechanism is not understood, genetic and biochemical evidence suggests that these antibiotics contact both 16S and 23S rRNA (16, 29, 45) thereby stabilizing an intermediate state.

The earliest evidence suggesting ribosomal subunit interaction as the target of tubercactinomycin was the finding that viomycin resistance can be conferred by altering either the 30S or the 50S subunit, although the nature of such mutations was not established (45). It was also shown that viomycin stabilizes subunit association (46). The ability of tubercactinomycin to compete with aminoglycosides for binding to the ribosome (28) and the effect these drugs have on translational fidelity (24) implicated the decoding site of the 30S subunit as one of the binding sites. This implication is strengthened by the finding

that aminoglycoside resistance mutations in the decoding site of 16S rRNA confer cross-resistance to viomycin (11, 25, 41), and spontaneous capreomycin resistance mutations have been found in the 16S rRNA gene of *Mycobacterium tuberculosis* (25), *Escherichia coli* (44), and *Thermus thermophilus* (11).

No capreomycin resistance mutations were reported in 23S rRNA until recently, when a spontaneous mutant of *M. tuberculosis* was found to carry a deletion of A1916 of 23S rRNA (16). An identical mutation, isolated in *E. coli* (34), has been confirmed as capreomycin resistant (M. O'Connor, personal communication). Viomycin resistance mutations in the large subunit have been known for some time (45), and the involvement of the large subunit in tubercactinomycin action was implied by chemical footprinting of viomycin on helix 38 of 23S rRNA (29), although this is, very likely, a secondary effect of the drug binding the subunits together. More recently, the lack of methylation of G745 in 23S rRNA was reported to produce a weak viomycin resistance phenotype (12), although later studies indicated that G745 is not involved in viomycin interaction (16). Neither G745 nor the helix 38 site is particularly close to the decoding center of the 30S subunit.

Mutations in the *tlyA* gene of *M. tuberculosis* (26) also confer capreomycin resistance. TlyA is a 2'-O-ribose methyltransferase that is responsible for the modifications at both Cm1920 in helix 69 of 23S rRNA and Cm1409 in helix 44 of 16S rRNA (16). This latter position is in the decoding center, and two base substitutions, C1409G (11) and C1409U (25), have been found to confer resistance to capreomycin. Orthologs of *tlyA* are found in various bacterial and plant genomes, and *tlyA* inactivation may be a more common mechanism of tubercactinomycin resistance than is generally recognized. Interestingly *T. thermophilus* TlyA modifies only Cm1920 in helix 69 of 23S rRNA (13, 27), indicating that the sensitivity of *T. thermophilus* to capreomycin (11) does not involve methylation of C1409.

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TABLE 1. *T. thermophilus* strains and plasmids used in this study<sup>a</sup>

Strain or plasmid	Genotype	Phenotype	Source
HB8	Wild type		ATCC 27634
TLK1	<i>rplK(P22T)</i>	Thiostrepton resistant	3
TLK17	<i>rrLAB(A1095G)</i>	Thiostrepton resistant	3
TM200	<i>ΔrrlB-rrfB-glyT::htk1</i>		This study
TM220	<i>ΔrrlB-rrfB-glyT::htk1 rrlA(A1095G)</i>	Thiostrepton resistant	This study; TM200 transformed with DNA from TLK17
TM240	<i>ΔrrlB-rrfB-glyT::htk1 rrlA(A2059G)</i>	Tylosin resistant	This study; TM200 selected for spontaneous tylosin resistance
TM473	<i>ΔrrlB-rrfB-glyT::htk1 rplK(P22T)</i>	Thiostrepton resistant	This study; TM200 transformed with DNA from TLK1
TM401	<i>ΔrrlB-rrfB-glyT::htk1 rrlA(A1913U)</i>	Capreomycin resistant	This study
TM402	<i>ΔrrlB-rrfB-glyT::htk1 rrlA(mU1915G)</i>	Capreomycin resistant	This study
TM415	<i>ΔrrlB-rrfB-glyT::htk1 rrlA(ΔmU1915)</i>	Capreomycin resistant	This study
TM469	<i>ΔtlyA::htk1</i>		This study
pUC18			47
pUC18ΔB::htk	<i>ΔrrlB-rrfB-glyT::htk1</i>		This study
pUC18ΔtlyA::htk	<i>ΔtlyA::htk1</i>		This study

<sup>a</sup> All strains bearing the *ΔrrlB-rrfB-glyT::htk1* or the *ΔtlyA::htk1* allele are kanamycin resistant.

The conserved helix 69 is highly modified in various bacteria. In *T. thermophilus*, there are four modifications: pseudouridine (Ψ)1911, mU1915, Ψ1917, and Cm1920 (27). Nucleotides 1911, 1915, and 1917 are pseudouridylylated by RluD in *E. coli* (37), but Ψ1915 has not been definitively established in *T. thermophilus*. There is no evidence as to whether modifications of Ψ1911, mU1915, and Ψ1917 affect tuberactinomycin sensitivity.

To further examine the role of helix 69 in tuberactinomycin sensitivity, we developed a genetic enrichment regimen to obtain mutations in the 23S rRNA gene of *T. thermophilus*. Our previous study (11) had failed to produce 23S rRNA mutations, so we selected for capreomycin resistance in a strain containing only one of the two 23S rRNA genes, followed by an enrichment by transformation and crossing out of linked markers. Having a genetic background with a single 23S rRNA gene facilitated the genetic mapping of capreomycin resistance mutations relative to two other antibiotic resistance mutations. Here, we report capreomycin resistance in mutant strains containing either of two single-base substitutions or a deletion in the terminal loop of helix 69 of 23S rRNA. We show that these mutations do not affect the status of methylation of C1920 by TlyA, methylation of U1915, or pseudouridylation of 1911 and 1917. Furthermore, we show that inactivation of *T. thermophilus tlyA* does not confer capreomycin resistance, indicating that methylation of helix 69 in this organism does not contribute to capreomycin sensitivity.

MATERIALS AND METHODS

**Bacterial strains, growth conditions, and genetic manipulation.** All strains and mutants are derived from *T. thermophilus* HB8 (ATCC 27634 [36]) and are described in Table 1. Cultures were grown at 72°C on ATCC medium 1598 (*Thermus* enhanced medium). Antibiotics were used at the following concentrations: kanamycin, 30 μg/ml; thiostrepton, 0.1 μg/ml; tylosin, 100 μg/ml; and capreomycin, 200 μg/ml. Transformation of *T. thermophilus* with either plasmid or chromosomal DNA was performed as described by Koyama et al. (20).

**Inactivation of the *rrlB-rrfB-glyT* operon.** A 420-bp region upstream and a 561-bp region downstream of *rrlB-rrfB-glyT* (locus tags TTHAr05, TTHAr04, and TTHAt16, respectively; positions 3168557 to 3167966 in the *T. thermophilus* HB8 genome sequence; GenBank accession number AP00826) were amplified by PCR from *T. thermophilus* genomic DNA, using oligonucleotide primer sets

TF1/TR1 and TF2/TR2, respectively, and inserted into pUC18 as KpnI-PstI and PstI-HindIII fragments, respectively, to generate the plasmid pUC18ΔB. The *htk* gene (a 1,096-bp PCR product amplified using primers HTK1 and HTK2) (Table 2) was inserted as a PstI fragment into pUC18ΔB to generate the pUC18ΔB::htk plasmid harboring the *ΔrrlB-rrfB-glyT::htk1* allele. This plasmid was used to transform *T. thermophilus* HB8 to kanamycin resistance. The resulting strain harboring the *ΔrrlB-rrfB-glyT::htk1* allele was designated TM200.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')
23S-A	GGCCAAGGCGGAAAGGTGCATGG
23S-B	GGAGACGGAGTGGAACGCCAGC
23S-C	CGCCAAGGAAGTCTGCAAGTTGGCC
23S-D	GTCGCGAGGCCAGCAATGCGAAAAGC
23S-G	TTTTCGCTGCCCTGAGG
23S-H	CCAGCCTAGCCGAAGCTGTTGG
23S-I	GACCCGAAACCGGGCGAG
23S-J	GCTGTCTCGCGAGGGACCCGGTG
23S-K	GTTACCCCGGGGATAACAGGC
23S-L	CGCTCAAGCCGGTAAGACC
23S-M	GGGAGAAATAGCAATGAGTACGGC
23S-N	CCGGTTGAACGTCTCAAGGAACCGC
23S-O	ACGGGACTTGAACCCGCGACC
Bla1	GAAGAGTATGAGTATTCAACATTCC
Bla2	CCAATGCTTAATCAGCTTGACCCACC
HTK1	CCACTGCAGGGTACCCGTTGACGGCGG ATATG
HTK2	GGTCTGCAGCGTAACCAACATGATTAAC
SJ14	GGAATTTTCGCTACCTTAG
TF1	CCAGGTACCTCAAGCACCCCTGCGCCAC
TF2	CCACTGCAGTCCCCTCTCCCCTCC
TR1	GGTCTGCAGCGCAAAGGGGAAGATACC
TR2	CGCAGCCGTAATCAGCTTGACCCGCC
tlyA1	CCAGGTACCTCTCTAGAAGGTCCTT CACC
tlyA2	GGTCTGCAGGGCTCCGAGGAGCTTG TAGG
tlyA3	CCACTGCAGCCAAGGTCCTGGAGCTCCT CAAGC
tlyA4	GGTAAGCTTCCACGCCGAGGCGGTGAG CGTGG
tlyA5	GGCCACGCCGAAGGCCAAGACC
tlyA6	GAGACGACACCATCCGGATCAGC

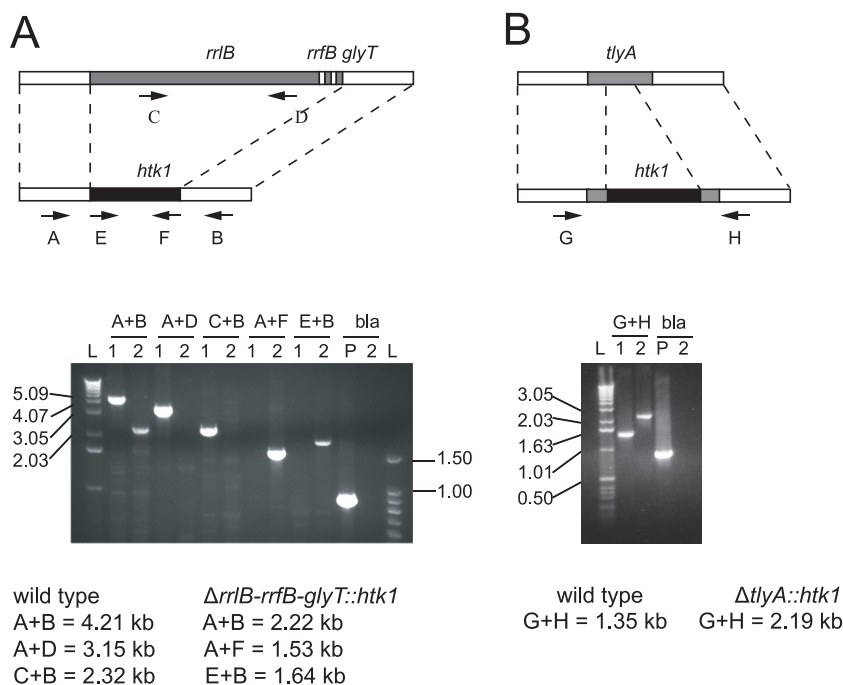


FIG. 1. Deletion of genes for 23S RNA and TlyA. Wild-type HB8 was transformed with plasmid DNA containing the appropriate deletion allele, selecting kanamycin resistance. (A) Deletion of *rrlB-rrfB-glyT*. Lanes: 1, wild-type HB8; 2, TM200; P, PCR using pUC18 plasmid DNA template; bla, PCR using primers specific for the *bla* gene carried by pUC18. Primers A, B, C, D, E, and F are primers 23S-A, 23S-B, 23S-C, 23S-D, HTK1, and HTK2, respectively. (B) Deletion of *tlyA*. Lanes: 1, wild-type HB8; 2, TM469; P, PCR using pUC18 template; bla, PCR for the *bla* gene. Primers G and H are primers tlyA5 and tlyA6, respectively. Lane L and lane values, molecular weight markers (in thousands).

**Inactivation of *tlyA*.** The *tlyA* gene sequence (locus tag TTHA0546) was obtained from the *T. thermophilus* HB8 genome sequence. Plasmid pUC18 $\Delta tlyA::htk$ , used for inactivation of *tlyA*, was constructed by PCR amplifying a 525-bp upstream segment and a 520-bp downstream segment of *tlyA* from genomic DNA, using primer sets tlyA1/tlyA2 and tlyA3/tlyA4, respectively, and inserting these segments into pUC18. The *htk* gene (a 1,096-bp PCR product amplified using primers HTK1 and HTK2) was inserted between the two genomic segments to generate pUC18 $\Delta tlyA::htk$ . This construct was then used to transform *T. thermophilus* HB8 and confer kanamycin resistance. The resulting strain harboring  $\Delta tlyA::htk1$  was designated TM469.

**DNA sequencing.** The entire *rrlA-rrfA-glyT* operon was amplified using primers 23S-G and 23S-O. The PCR product was used as a template for automated DNA sequencing (University of California, Davis, sequencing facility), using sequencing primers 23S-C, 23S-D, 23S-G, 23S-H, 23S-I, 23S-J, 23S-K, 23S-L, 23S-M, 23S-N, and 23S-O.

**Primer extension analysis.** Primer extension to detect 2'-O methylation was performed as described previously (23). DNA primer SJ14, 5' end labeled with [ $\gamma$ - $^{32}$ P]ATP was hybridized to rRNA and extended with avian myeloblastosis virus (AMV) reverse transcriptase. Pausing at 2'-O-ribose-methyl-C1920 is enhanced by decreasing dGTP concentrations (1  $\mu$ M, 0.5  $\mu$ M, and 0.1  $\mu$ M) in the presence of dATP, dCTP, and ddTTP at 40  $\mu$ M. Extension products were separated by electrophoresis in 8 M urea, 13% polyacrylamide gels. Detection of  $\Psi$ 1911 and  $\Psi$ 1917 was carried out as described previously (35). rRNA was incubated for 15 min at 50°C prior to modification by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT). The modified rRNA was precipitated, and the rRNA pellet was dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub> (pH 10.4) and incubated at 37°C for 4 h. After ethanol precipitation, rRNA was dissolved in distilled water and analyzed by primer extension using  $^{32}$ P-labeled SJ14 primer and AMV reverse transcriptase in the presence of 1  $\mu$ M of each deoxynucleoside triphosphate. The extension products were resolved by 8% polyacrylamide gels containing 8 M urea.

**Sucrose gradient analysis.** Cells from mid-log phase cultures were harvested, resuspended in 25 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, and 60 mM KCl (pH 8.0) and lysed by a single passage through a French press. Cell debris was pelleted by centrifugation at 14,000 rpm at 4°C in a Beckman Ti50.2 rotor. Lysates were fractionated over 10 to 40% sucrose gradients in 25 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 60 mM KCl, 3 mM dithiothreitol (pH 8.0) by centrifugation in a SW28

rotor at 18,000 rpm for 18 h at 4°C. Gradient fractions were monitored with an ISCO gradient fractionator.

## RESULTS

**Inactivation of the 23S rRNA-5S rRNA-tRNA<sup>Gly</sup> operon and enrichment for spontaneous capreomycin-resistant mutants.** Previously, we isolated capreomycin resistance mutations that mapped exclusively to 16S rRNA (11), suggesting that capreomycin resistance mutations in the 23S rRNA gene arise at a much lower frequency. We therefore employed a two-step enrichment strategy using a strain containing a single 23S rRNA gene and a transformation and recombination procedure that targeted this gene. The *T. thermophilus* HB8 genome (GenBank accession number AP00826; Masui et al., unpublished) has two unlinked 16S rRNA genes, designated *rrsA* and *rrsB*, and two unlinked 23S rRNA-5S rRNA-tRNA<sup>Gly</sup> operons, designated *rrlA-rrfA-glyT* and *rrlB-rrfB-glyT* (11). The *rrlB-rrfB-glyT* operon was inactivated with a plasmid harboring a heat-stable kanamycin-adenyltransferase gene cassette, *htk*, flanked by the 420 bp of sequence upstream and the 561 bp of sequence downstream of the *rrlB-rrfB-glyT* operon (see Materials and Methods). Since this plasmid lacks a *T. thermophilus* active replication origin, kanamycin resistance results from the integration of the plasmid into the chromosome at a site of homology. Resolution of the cointegrate occurs with high efficiency, and the single integration has never been detected (4). Diagnostic PCR (Fig. 1A) and DNA sequencing analysis confirmed that the entire coding sequence of the *rrlB-rrfB-glyT* operon was deleted and replaced with *htk* (hereafter referred

TABLE 3. Phenotypes of capreomycin-resistant mutants<sup>a</sup>

Strain	Mutation	<i>k</i> value ± SD (min)	Capreomycin MIC (μg/ml)
HB8		43 ± 5	50
TM200		43 ± 3	50
TM401	A1913U	44 ± 4	1,000
TM402	mU1915G	53 ± 4	1,000
TM415	ΔmU1915	90 ± 10	3,000
TM469	Δ <i>tlyA</i> :: <i>htk1</i>	45 ± 4	50

<sup>a</sup> TM200 and all mutants also carry the Δ*rrlB-rrfB-glyT::htk1* allele. Doubling time (*k*, in min) values ± standard deviations (SD) were determined using a Klett-Summerson photometer. Capreomycin MICs were determined by streaking for single colonies on plates containing the indicated concentrations of capreomycin.

to as Δ*rrlB-rrfB-glyT::htk1*). A single transformant, designated TM200, was used for all subsequent experiments. The doubling time of TM200 is not significantly different from that of wild-type *T. thermophilus* HB8 (Table 3), indicating that a single 23S rRNA-5S rRNA-tRNA<sup>Gly</sup> operon can support optimal cell growth under the conditions used in this study.

Spontaneous capreomycin-resistant mutants were obtained by plating 10<sup>9</sup> cells from each of 12 independent cultures of TM200 onto *Thermus* enhanced medium plates containing 200 μg/ml capreomycin. Between 100 and 200 resistant mutants arising on each selection plate were pooled. To enrich for mutations in the single intact 23S rRNA gene, *rrlA*, DNA extracted from each of the pooled selections was used to transform TM220, a version of the TM200 strain carrying the thiostrepton resistance mutation A1095G in *rrlA*, and by selecting capreomycin resistance and screening for the loss of thiostrepton resistance. A similar enrichment was performed by transforming TM240, a version of the TM200 strain carrying the tylosin resistance mutation A2059G in *rrlA*, and by selecting capreomycin resistance and screening for the loss of tylosin resistance. About 5 to 10% of capreomycin-resistant transformants tested were thiostrepton or tylosin sensitive. This fre-

quency reflects both the predominance of mutations in the 16S rRNA gene and the recombination frequency between the capreomycin resistance mutations and either the thiostrepton or tylosin resistance mutations. Six transformants from each enrichment were characterized further.

**Genetic analysis of mutants.** The entire *rrlA-rrfA-glyT* operon of each of the 12 independent capreomycin-resistant mutants was amplified by PCR, and the DNA was sequenced. Three different *rrlA* alleles were identified: A1913U (eight independent isolates), mU1915G (two independent isolates), and ΔmU1915 (two independent isolates). All three alleles are located in helix 69 of 23S rRNA, which is also the site of methylation by TlyA (Fig. 2). One representative of each allele was characterized by DNA sequencing: TM401 [*rrlA*(A1913U)], TM402 [*rrlA*(mU1915G)], and TM415 [*rrlA*(ΔmU1915)]. None had any mutations in the 16S rRNA genes (*rrsA* and *rrsB*). To confirm that the three single mutations are responsible for the capreomycin resistance phenotype, we genetically mapped the three alleles relative to the thiostrepton resistance allele [*rrlA*(A1095G)] and the tylosin resistance allele [*rrlA*(A2059G)]. Strains TM220 [Δ*rrlB-rrfB-glyT::htk rrlA*(A1095G)] and TM240 [Δ*rrlB-rrfB-glyT::htk rrlA*(A2059G)] were transformed with chromosomal DNA of strains TM401, TM402, and TM415, selecting for capreomycin resistance and screening for the loss of either thiostrepton resistance or tylosin resistance, as appropriate. The frequency of transformants screening thiostrepton sensitivity or tylosin sensitivity is a measure of genetic linkage between the capreomycin resistance mutation and the thiostrepton resistance or tylosin resistance mutation. As shown in Table 4, all three capreomycin resistance alleles mapped closer to A2059 than to A1095. These results correlate well with the physical distance among these mutations, supporting the conclusion that the mutations are responsible for the capreomycin resistance phenotype.

**Identification and inactivation of *tlyA*.** The *T. thermophilus* HB8 ortholog of TlyA was identified in a BLASTp search of the *T. thermophilus* HB8 annotated genome sequence, using

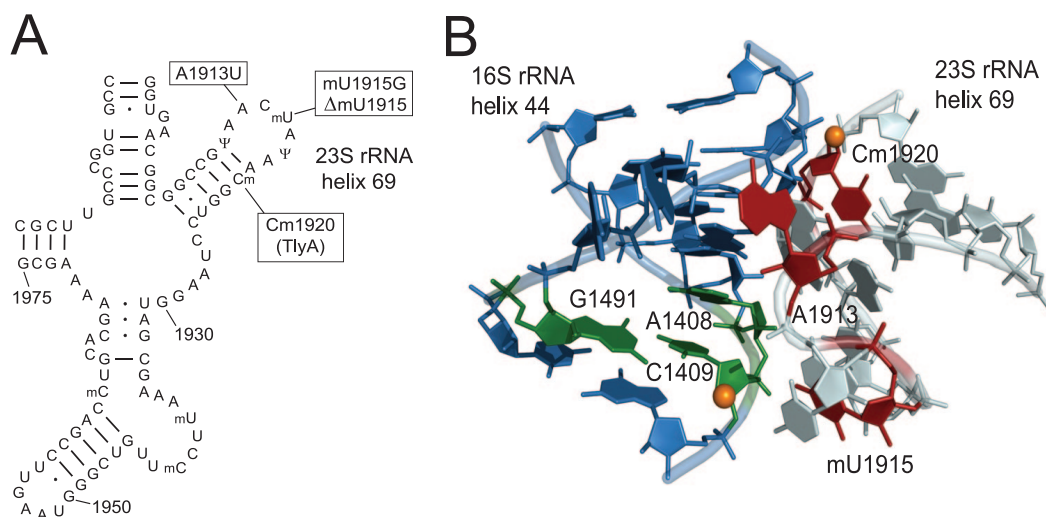


FIG. 2. Capreomycin resistance mutations. (A) Locations of capreomycin resistance mutations in the secondary structure of *T. thermophilus* 23S rRNA (31). (B) Locations of sites of capreomycin resistance mutations in 23S rRNA identified in this study, as well as sites of mutations in 16S rRNA identified previously (11) in the crystal structure of the *T. thermophilus* 70S ribosome (39). Also shown are sites of 2'-O methylation by TlyA (16, 27).

TABLE 4. Recombination mapping of mutant alleles<sup>a</sup>

Donor	Recipient	Selection	Screen	Recombination frequency (n/total)	Distance (bp)
<i>rrlA</i> (A1913U)	<i>rrlA</i> (A1095G)	Capreomycin	Thiostrepton	24/100	818
<i>rrlA</i> (A1913U)	<i>rrlA</i> (A2059G)	Capreomycin	Tylosin	6/100	146
<i>rrlA</i> (A1913U)	<i>rplK</i> (P22T)	Capreomycin	Thiostrepton	100/100	227,562
<i>rrlA</i> (mU1915G)	<i>rrlA</i> (A1095G)	Capreomycin	Thiostrepton	22/100	820
<i>rrlA</i> (mU1915G)	<i>rrlA</i> (A2059G)	Capreomycin	Tylosin	2/100	144
<i>rrlA</i> (mU1915G)	<i>rplK</i> (P22T)	Capreomycin	Thiostrepton	100/100	227,560
<i>rrlA</i> (ΔmU1915)	<i>rrlA</i> (A1095G)	Capreomycin	Thiostrepton	20/100	820
<i>rrlA</i> (ΔmU1915)	<i>rrlA</i> (A2059G)	Capreomycin	Tylosin	2/100	144
<i>rrlA</i> (ΔmU1915)	<i>rplK</i> (P22T)	Capreomycin	Thiostrepton	100/100	227,560
<i>rrlA</i> (A1095G)	<i>rplK</i> (P22T)	Thiostrepton	Tylosin	32/100	964
<i>rrlA</i> (A2059G)	<i>rrlA</i> (A1095G)	Tylosin	Thiostrepton	30/100	964
<i>rrlA</i> (A2059G)	<i>rplK</i> (P22T)	Tylosin	Thiostrepton	100/100	226,598
<i>rplK</i> (P22T)	<i>rrlA</i> (A2059G)	Thiostrepton	Tylosin	100/100	226,598

<sup>a</sup> Recipients were transformed with DNA from the donor, selecting for the donor phenotype, and screened for retention of the recipient phenotype to give the recombination frequency; this number represents the fraction of transformants which have undergone a recombination event between the two alleles. The distance is the physical distance in base pairs based on the genome sequence (GenBank accession number AP00826).

the *M. tuberculosis* TlyA protein as the target sequence. The protein encoded by TTHA0546 is the ortholog (e value,  $9e^{-43}$ ) and has been shown to methylate C1920 of 23S rRNA when expressed in vivo in *E. coli* (S. Johansen and S. Douthwaite, unpublished results). Alignment of the two sequences, using ClustalW (42), shows that the *T. thermophilus* TlyA protein lacks the C-terminal 22 amino acid residues present in the *M. tuberculosis* TlyA protein (data not shown). We deleted *tlyA* by using the same methodology as that described for construction of the  $\Delta rrlB$ -*rrfB*-*glyT*::*htk1* knockout allele. About a third of the central region of *tlyA* was deleted and replaced by *htk* (Fig. 1B). The doubling time of the mutant is 45 min, essentially the same as that of the wild-type strain (43 min).

**Phenotypes of capreomycin-resistant mutants and the Δ*tlyA*::*htk1* deletion mutant.** The A1913U mutation had no effect on doubling time, while mU1915G and ΔmU1915 increased doubling time from 43 to 53 and 90 min, respectively (Table 3). That a deletion mutation has a more pronounced phenotype is perhaps not surprising, as it is expected to have a more extensive effect on loop conformation. The MIC of capreomycin for cells with mutations A1913U or mU1915G increased 20-fold, while ΔmU1915 produced a 60-fold increase (Table 3). All three mutations also conferred resistance to viomycin but not to macrolides, aminoglycosides, or lincosamides, as determined by disc assay (Table 5).

Unexpectedly, deletion of *tlyA* had no effect on sensitivity to capreomycin (Table 3). This result was at variance with a similar deletion in *M. tuberculosis* that increased resistance (26). This difference is not due to an intrinsic property of the enzyme, since expression of *T. thermophilus* TlyA in *E. coli*, which lacks an endogenous *tlyA* ortholog, modifies 23S rRNA and confers increased susceptibility to capreomycin (S. Johansen and S. Douthwaite, unpublished observation).

**Methylation status of C1920 on helix 69 in mutant ribosomes.** The deletion of A1916 of *M. tuberculosis* 23S rRNA confers capreomycin resistance and also prevents methylation of C1920 of 23S rRNA but not C1409 of 16S rRNA (16). We therefore examined the methylation status of Cm1920 in our mutant strains by primer extension. This method is based on the observation that, under conditions of limiting deoxynucleoside triphosphates, reverse transcriptase pauses at 2'-*O*-ribose-methylated residues (23). Ribosomes from each of the three rRNA mutants, including the mutant with deletion of mU1915, showed methylation at Cm1920, while ribosomes from the *tlyA* deletion mutant showed no methylation at C1920 (Fig. 3A). These results indicate that their capreomycin resistance phenotypes are not due in any way to effects of the mutations on modification. They also suggest that recognition of 23S rRNA by *T. thermophilus* TlyA is not significantly influenced by the identity of these residues.

TABLE 5. Susceptibility of *htk* mutants to antibiotics determined by disc assay<sup>a</sup>

Strain	Mutation	Inhibition zone diam (mm)													
		Str	Apr	Par	Neo	Kan	Gen	Hyg	Cap	Vio	Lin	Cli	Tyl	Ery	Amp
HB8		30	28	29	20	25	26	18	15	23	33	37	30	27	53
TM200		32	30	27	17	—	28	25	16	24	31	34	35	30	50
TM401	A1913U	31	26	28	17	—	25	19	—	—	30	36	38	25	47
TM402	mU1915G	28	26	25	16	—	25	18	—	—	32	38	36	23	51
TM415	ΔmU1915	34	32	30	25	9	28	25	—	—	39	42	36	34	56
TM415	Δ <i>tlyA</i> :: <i>htk1</i>	34	28	30	16	—	26	25	16	24	29	32	37	32	52

<sup>a</sup> Discs containing 100 μg of each antibiotic (exceptions, 30 μg of neomycin and 20 μg of ampicillin) were placed on TEM plates spread with 100 μl of overnight culture. Zones of growth inhibition were measured. TM200 and all mutants carry the  $\Delta rrlB$ -*rrfB*-*glyT*::*htk1* allele. Str, streptomycin; Apr, apramycin; Par, paromomycin; Neo, neomycin; Kan, kanamycin; Gen, gentamicin; Hyg, hygromycin B; Cap, capreomycin; Vio, viomycin; Lin, lincomycin; Cli, clindamycin; Tyl, tylosin; Ery, erythromycin; Amp, ampicillin. The — indicates no inhibition.

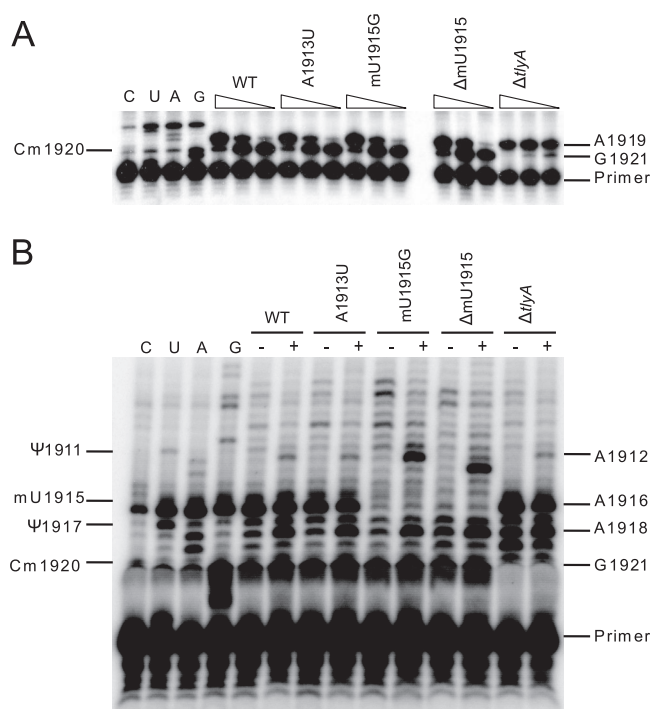


FIG. 3. Effects of mutations in helix 69 of 23S rRNA on posttranscriptional modifications. (A) Primer extension analysis of Cm1920 in capreomycin-resistant mutants A1913U, mU1915G, and  $\Delta$ mU1915 and in the  $\Delta$ tlyA::htk1 deletion mutant ( $\Delta$ tlyA). rRNA from the wild-type (WT) strain was used for dideoxynucleotide sequencing (lanes C, U, A, and G). Decreasing concentrations of dGTP in the extension reaction mixture (1  $\mu$ M, 0.5  $\mu$ M, and 0.1  $\mu$ M) are indicated by wedges. Extension reactions contained dATP, dCTP, and ddTTP at 40  $\mu$ M. ddTTP caused termination at A1919 of all products extending past Cm1920. (B) Primer extension analysis to detect the presence of pseudouridine ( $\Psi$ ) at positions 1911 and 1917 and of methylation at U1915, using CMCT. The presence or absence of CMCT modification is indicated by + and -, respectively.

**Absence of effects of mutations on other modifications of helix 69.** Helix 69 of 23S rRNA is a highly modified secondary structural element conserved in all ribosomes. A recent analysis of *T. thermophilus* 23S rRNA identified four modifications on helix 69:  $\Psi$ 1911, mU1915,  $\Psi$ 1917, and Cm1920 (27). This modification pattern differs from that of helix 69 of *E. coli* 23S rRNA only in that *E. coli* contains *N*<sup>3</sup>-methylpseudouridine m $\Psi$ 1915 and lacks Cm1920. Mutants of *E. coli* deficient in RluD, the enzyme responsible for the formation of  $\Psi$ 1911,  $\Psi$ 1915, and  $\Psi$ 1917, have a strong growth defect (37) and interfere with recognition by termination factor RF2 (6). Loss of multiple modifications of helix 69 of *Saccharomyces cerevisiae* 28S rRNA causes a variety of detrimental effects on ribosome function and biogenesis (21). Thus, it was imperative to establish the modification status of this loop in our study to distinguish direct effects of mutations from indirect effects due to the loss of modification. We examined the *T. thermophilus* helix 69 mutants for the methylation status of mU1915 by primer extension and for  $\Psi$ 1911 and  $\Psi$ 1917 formation, using chemical modification with CMCT, followed by primer extension (see Materials and Methods). In Fig. 3B, we show that none of the helix 69 mutations or the loss of methylation on Cm1920 by

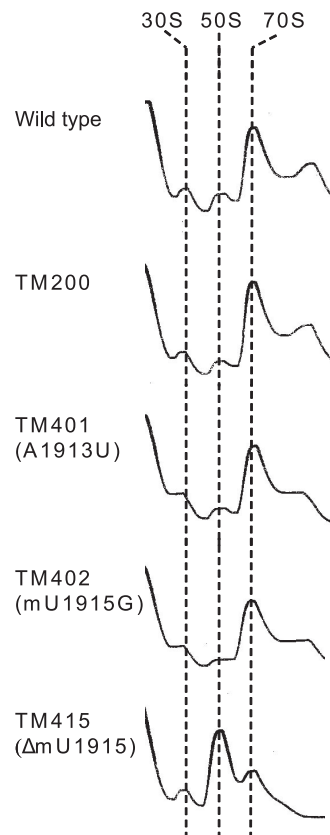


FIG. 4. Subunit association defect of the 23S rRNA ( $\Delta$ mU1915) mutation. Ribosomes and ribosomal subunits were separated on a 10 to 40% sucrose gradient containing 10 mM MgCl<sub>2</sub> as described in Materials and Methods.

$\Delta$ tlyA prevented the formation of  $\Psi$ 1911, mU1915, and  $\Psi$ 1917, except where mU1915 was mutated (mU1915G and  $\Delta$ mU1915). The modifications mU1915 and  $\Psi$ 1917 are apparent in all helix 69 mutants.  $\Psi$ 1911 was clearly detected only where mU1915 was mutated (mU1915G or  $\Delta$ mU1915).  $\Psi$ 1911 was only weakly detected in strains with wild type, A1913U or  $\Delta$ tlyA due to the strong termination of reverse transcription at mU1915.

**The ribosomal subunit association defect caused by the  $\Delta$ mU1915 mutation.** Given that helix 69 is a component of bridge B2a between the ribosomal subunits, we examined the capreomycin resistance mutations for effects on subunit association. The  $\Delta$ A1916 mutation (34) and mutations at neighboring nucleotides in helix 69 of *E. coli* 23S rRNA (22) have been demonstrated to have a significant effect in this regard. Figure 4 shows that sucrose gradient profiles of ribosomes with A1913U or mU1915G are similar to those of wild-type ribosomes. In contrast, profiles of ribosomes with  $\Delta$ mU1915 show an excess of free 50S subunits and a deficiency of 70S ribosomes. Thus, for these helix 69 mutations there is a correlation between the growth defect and the impact on subunit association.

## DISCUSSION

Here, we describe three mutations in helix 69 of *T. thermophilus* 23S rRNA that confer resistance to the antibiotic capreomycin. Helix 69 engages in a hydrogen bonding interac-

tion with 16S rRNA helix 44 at the decoding center, forming the 70S ribosomal bridge B2a (9, 38, 39, 48). Mutations in 16S rRNA that confer capreomycin resistance include A1408G, C1409G, and G1491A, G1491U, and G1491C (11, 25, 44). These mutations are in close proximity to the site of contact with helix 69 and the mutations described in this study (Fig. 2). The subunit association defect associated with deletion of mU1915 is consistent with a role for this region in maintaining the integrity of the 70S ribosome. This phenotype was reported previously in a helix 69 deletion in *E. coli*,  $\Delta$ A1916, originally isolated as a frameshift suppressor (34), and is lethal unless present in only a subset of the cell's ribosome complement or suppressed by mutations in 16S rRNA (33). In contrast, the same mutation in *M. tuberculosis*, which has only one copy of the 23S rRNA gene, is viable and produces capreomycin resistance (16). These observations are consistent with a model for tubercinomycin action wherein the drug stabilizes an intermediate state of the 70S ribosome during translocation (46) and prevents the interribosomal subunit rotation required for translocation (7). Presently, it is not clear whether the mutations confer resistance by interfering with the subunit interaction or by directly altering the sequence to which the drug binds.

Helix 69 of 23S rRNA has been studied extensively, using genetic, biochemical, and biophysical approaches. Mutations in the helix have been identified as suppressors of frameshift mutations (34), a reflection of helix 69's participation in an intersubunit bridge with the decoding center of the 30S subunit and its direct contact with tRNA (38, 39, 48), RF1, and RF3 (5, 18, 19) and competition with IF3 (6). Genetic selections from a randomized library of helix 69 sequences in *E. coli* found that A1912 and  $\Psi$ 1917 are essential residues, while an m $\Psi$ 1915A substitution produced a severe growth phenotype and a defect in translational fidelity (14). As noted above, deletion of A1916 is lethal in *E. coli* unless suppressed (32, 33). Deletion of the entire helix is also lethal, although the mutant ribosomes tested in vitro show no defect in decoding (1).

In *T. thermophilus*, methylation of C1920 in helix 69 by TlyA is unaffected by the A1913U, mU1915G or  $\Delta$ mU1915 mutations. This indicates that the loop bases are unlikely to be important for recognition by TlyA in *T. thermophilus*, despite the fact that deletion of A1916 prevents C1920 methylation in *M. tuberculosis* (16). This raises the questions of what features are likely to be recognized by TlyA and why these features are apparently not the same in different bacteria. *M. tuberculosis* TlyA normally methylates both helix 44 and helix 69, but *T. thermophilus* TlyA methylates only helix 69. Structurally, the *T. thermophilus* TlyA protein differs from the *M. tuberculosis* enzyme by having a C-terminal deletion of 22 amino acids. Expression of *T. thermophilus* TlyA in *E. coli*, which has no endogenous TlyA homolog, leads to modification of helix 69 but not helix 44 of 16S rRNA (S. Johansen and S. Douthwaite, unpublished results).

The observation that mutations in the helix 69 loop do not prevent methylation and pseudouridylation in *T. thermophilus* indicates it is the mutations and not the lack of modifications in the loop that determine resistance. The mutations are in the loop that interacts with 16S rRNA helix 44 and may function to perturb the stability of the bridging interaction, thus disrupting the stabilization of subunit association by capreomycin. The

fact that methylation of C1920 by TlyA does not affect the sensitivity of *T. thermophilus* ribosomes to capreomycin but does so in *E. coli* may reflect differences in the interactions between ribosomal subunits of mesophilic and thermophilic organisms.

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