Mutation of *tlyA* Confers Capreomycin Resistance in *Mycobacterium tuberculosis*

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Capreomycin, an important drug for the treatment of multidrug-resistant tuberculosis, is a macrocyclic peptide antibiotic produced by *Saccharothrix mutabolis* subspecies *capreolus*. The basis of resistance to this drug was investigated by isolating and characterizing capreomycin-resistant strains of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Colonies resistant to capreomycin were recovered from a library of transposon-mutagenized *M. smegmatis*. The transposon insertion site of one mutant was mapped to an open reading frame in the unfinished *M. smegmatis* genome corresponding to the *tlyA* gene (Rv1694) in the *M. tuberculosis* H37Rv genome. In *M. smegmatis* spontaneous capreomycin-resistant mutants, the *tlyA* gene was disrupted by one of three different naturally occurring insertion elements. Genomic DNAs from pools of transposon mutants of *M. tuberculosis* H37Rv were screened by PCR by using primers to the *tlyA* gene and the transposon to detect mutants with an insertion in the *tlyA* gene. One capreomycin-resistant mutant was recovered that contained the transposon inserted at base 644 of the *tlyA* gene. Complementation with the wild-type *tlyA* gene restored susceptibility to capreomycin in the *M. smegmatis* and *M. tuberculosis* *tlyA* transposon mutants. Mutations were found in the *tlyA* genes of 28 spontaneous capreomycin-resistant mutants generated from three different *M. tuberculosis* strains and in the *tlyA* genes of capreomycin-resistant clinical isolates. In in vitro transcription-translation assays, ribosomes from *tlyA* mutant but not *tlyA*+ strains resist capreomycin inhibition of transcription-translation. Therefore, TlyA appears to affect the ribosome, and mutation of *tlyA* confers capreomycin resistance.

Tuberculosis is the only disease that the World Health Organization has declared a global public heath emergency. Worldwide in 2000, there were about 9 million new cases and 2 million deaths due to tuberculosis (12, 13). The emergence of multidrug-resistant (MDR) tuberculosis has further complicated the treatment and control of the disease (14). Treatment of MDR tuberculosis requires the use of second-line drugs, such as capreomycin, kanamycin, or amikacin (4). In contrast to several antituberculosis drugs, relatively little is known about the mechanism of action or molecular basis of resistance for capreomycin, an important second-line antibiotic (28, 30).

Capreomycin is a macrocyclic peptide antibiotic produced by *Saccharothrix mutabolis* subspecies *capreolus* (31, 45) and appears to interfere with translation in mycobacteria (34). Capreomycin can inhibit phenylalanine synthesis in an in vitro translation assay using mycobacterial ribosomes (34). Similar inhibition was seen whether or not ribosomes were preincubated with mRNA, suggesting that capreomycin does not interfere with mRNA binding to the ribosome (34).

Capreomycin is structurally similar to viomycin (26), and many studies show complete cross-resistance between viomycin and capreomycin in *Mycobacterium tuberculosis* (2, 20, 21, 35, 36). Viomycin has been shown to bind to both the 30S and 50S ribosome subunits (42) and to affect the dissociation of the

70S ribosome of *Mycobacterium smegmatis* (41). Furthermore, it inhibits ribosomal translocation by arresting peptidyl-tRNA in the ribosomal acceptor site (23). Additionally, changes in the 30S and 50S ribosomal subunits alter binding and confer viomycin resistance in *M. smegmatis* (8, 22, 33, 40, 41, 43).

Previous reports have linked mutations in the 16S rRNA gene to capreomycin resistance (32, 33); however, these reports are variable and do not completely account for capreomycin resistance in *M. tuberculosis*. Transposon mutagenesis is a useful tool to search for nonessential genes encoding products that alter a drug target, are involved in drug transport, or convert a prodrug to its active form. To investigate capreomycin resistance in mycobacteria, libraries of transposon mutants from *M. smegmatis* and *M. tuberculosis* were utilized. Characterization of transposon and spontaneous *M. smegmatis* and *M. tuberculosis* capreomycin-resistant mutants as well as capreomycin-resistant clinical isolates revealed that mutation of the *tlyA* gene confers capreomycin resistance in mycobacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. smegmatis* strain LR222 and *M. tuberculosis* strains H37Rv, CDC1551, and Beijing F2 and 18 clinical isolates were obtained from the culture collection of the Tuberculosis/Mycobacteriology Branch, Centers for Disease Control and Prevention (CDC). *M. tuberculosis* cultures were grown in complete Middlebrook 7H9 broth (7H9) (Remel, Lenexa, Kans.) at 37°C or on Middlebrook 7H10 agar (7H10). *M. smegmatis* was grown in 7H9 broth or on Trypticase soy agar (TSA). In TSA-capreomycin (TSA-Cap; Sigma, St. Louis, Mo.), 7H9-capreomycin (7H9-Cap), 7H10-capreomycin (7H10-Cap), or 7H9-viomycin (7H9-Vio; U.S. Pharmacopeia, Rockville, Md.) medium, each drug was used at a concentration of 10 μg/ml unless stated otherwise. Hygromycin (Invitrogen, Carlsbad, Calif.) was used in the media at a final

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concentration of 50 μg/ml for mycobacteria when needed. Acetamide (Sigma) was incorporated into media at a final concentration of 0.2% as appropriate. The *Escherichia coli* EC100 (Epicentre, Madison, Wis.) strain was grown in Luria broth or Luria agar, and hygromycin at a concentration of 200 μg/ml was used when appropriate.

DNA isolation, manipulations, sequencing, PCR, and Southern blotting. Genomic and plasmid DNAs were purified as previously described (27). All enzymatic reactions were performed as recommended by the manufacturer (Invitrogen, Life Technologies). Oligonucleotide primers were synthesized at the Biotechnology Core Facility, National Center for Infectious Diseases, CDC (sequences available upon request). Amplification reactions (100 μl) were performed as recommended by the manufacturer (PE Applied Biosystems, Foster City, Calif.) in a GeneAmp PCR system 2400 thermocycler (PE Applied Biosystems) by using a three-step cycle of denaturation for 30 s at 96°C, annealing for 30 s at 60 to 68°C (optimized for each primer pair), and extension for 2 min at 72°C. Amplification products were analyzed by electrophoresis through 0.8 to 2.0% agarose–Tris-borate-EDTA gels and visualized by ethidium bromide staining (29). Prior to DNA sequencing, amplicons were purified and concentrated by using a DNA Clean and Concentrator kit (Zymo Research, Orange, Calif.). DNA Sequencing was carried out by using a CEQ Dye Terminator Cycle Sequence Quick Start kit (Beckman Coulter, Fullerton, Calif.) according to the manufacturer's directions. Samples were analyzed on the CEQ8000 sequencer (Beckman). Sequence data were evaluated by using Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Tom Hall, Department of Microbiology, North Carolina State University). Southern blot analysis was carried out by using an ECL nucleic acid labeling and detection system (Amersham, Arlington Heights, Ill.) as previously described (27).

Transposon-mutant libraries. The *M. smegmatis* LR222 library was constructed by using an EZ::TN Kan Transposome Kit (Epicentre Technologies, Madison, Wis.). Wild-type *M. smegmatis* electrocompetent cells were electroporated with 1 μl of the transposome as described previously (23); then 1 ml of 7H9 broth was added. After an 18-h incubation at 37°C, aliquots of the cell mixture were spread on 10 TSA plates containing 10 μg of kanamycin (TSA-Kan) per ml. After 72 h at 37°C, 40 colonies were picked for evaluation, and the remaining growth from each plate was resuspended in 7H9 broth with 10 μg of kanamycin (7H9-Kan) per ml and incubated for 18 h at 37°C. The 10 pools were stored at −70°C. The *M. tuberculosis* H37Rv library was generated in a similar manner except that a total of seven samples of electrocompetent cells were electroporated and outgrowth in 7H9 broth was for 24 to 72 h. The cell mixture from each electroporation was plated on 10 7H10 plates containing 10 μg of kanamycin (7H10-Kan) per ml and held at 37°C for 21 days. Eighteen colonies from each set were picked for evaluation, and those remaining were resuspended in 7H9-Kan broth and incubated 30 days at 37°C. DNA was extracted from a 1.5-ml sample of the culture from each pool. Pools were stored at −70°C. DNA was extracted from the individual colonies selected for evaluation and analyzed by PCR by using primers specific to the Tn*903* kanamycin resistance gene (Kan^r) in the EZ::TN transposon.

Generation of capreomycin-resistant spontaneous mutants. Spontaneous capreomycin mutants were generated from *M. smegmatis* LR222 and pan-susceptible *M. tuberculosis* strains H37Rv, CDC1551, and Beijing strain F2. All parent strains were susceptible to 10 μg of capreomycin per ml. Portions of a concentrated cell suspension of each strain were spread on each of four plates containing 10 μg of capreomycin (TSA-Cap for *M. smegmatis* or 7H10-Cap for *M. tuberculosis*) per ml. Serial 10-fold dilutions of the concentrated cell suspension were plated on media without drug to determine the number of viable cells in the suspension. Once colonies grew on the drug media, isolated colonies were picked and inoculated into 7H9-Cap broth. DNA lysates were made from the cultures and analyzed by PCR and sequencing.

Cloning of mutant genomic DNA into pBluescript. Genomic DNAs from two capreomycin-resistant *M. smegmatis* transposon mutants were digested with EcoRI and ligated into an EcoRI restriction site of pBluescript KS(+) (Stratagene, La Jolla, Calif.). The libraries were electroporated into *E. coli*, and colonies were selected for kanamycin resistance. Plasmid DNAs from three kanamycin-resistant transformants were sequenced by using outward facing primers specific to the Tn*903* Kan^r gene in the EZ::TN transposon.

Construction of *tlyA* expressing plasmids. The open reading frames (ORFs) of the *M. smegmatis* and *M. tuberculosis* wild-type *tlyA* genes were amplified by using primers with BglII restriction sites. The amplicons were purified, digested with BglII, and ligated into the BamHI site of pMF1 (provided by Mark Fisher) which contains the *E. coli* origin of replication from pUC18, the mycobacteriophage L5 integrase gene and *attP* site, the *Streptomyces hygroscopicus* hygromycin resistance selectable marker, and the *M. smegmatis* acetamidase promoter (M. Fisher, personal communication). This cloning strategy places the *tlyA* ORF under the

control of the acetamidase promoter. The resulting plasmids were designated pMF1-tlyA/sm and pMF1-tlyA/tb for the *M. smegmatis tlyA* ORF and *M. tuberculosis tlyA* ORF, respectively.

Susceptibility testing. Susceptibilities to antituberculosis drugs were determined according to NCCLS guidelines and definitions (24) by using 7H10 agar containing the following: isoniazid (at concentrations of 0.2, 1, and 5 μg/ml; Sigma), rifampin (1 μg/ml; Sigma), ethambutol (5 μg/ml; Sigma), streptomycin (2 and 10 μg/ml; Sigma), rifabutin (2 μg/ml; Pharmacia and Upjohn, Kalamazoo, Mich.), ciprofloxacin (2 μg/ml; Bayer, West Haven, Conn.), kanamycin (5 μg/ml; Sigma), ethionamide (10 μg/ml; Sigma), capreomycin (10 μg/ml; Sigma), *p*-aminosalicylic acid (2 μg/ml; Sigma), ofloxacin (2 μg/ml; Sigma), or amikacin (4 μg/ml; Sigma). MICs were determined in 7H10 agar by using the following drugs at the indicated concentrations: capreomycin at 10, 20, 40, 80, and 160 μg/ml; viomycin at 10, 20, 40, and 80 μg/ml; kanamycin at 5, 10, 20, 40, and 80 μg/ml; and amikacin at 4, 8, 16, 32, and 64 μg/ml. The MIC was defined as the lowest concentration of drug resulting in complete inhibition of growth or in growth of <1% of the inoculum. MIC controls for spontaneous capreomycin-resistant mutants and clinical isolates consisted of wild-type strains *M. tuberculosis* H37Rv, CDC1551, and Beijing F2. All control strains were susceptible to 10 μg of capreomycin per ml, which is the critical concentration for the drug as stated by NCCLS (24).

Growth analysis. Wild-type *M. smegmatis* bacteria and the *tlyA* EZ::TN-kan mutant were used separately to inoculate, in triplicate, 7H9 and 7H9-Cap media to a starting optical density at 600 nm (OD$_{600}$) of 0.2 as measured by an Ultrospec 1100 pro visible spectrophotometer (Biochrom Ltd., Cambridge, England). The cultures were incubated at 37°C for up to 52 h on a tissue culture roller drum. OD$_{600}$ readings were taken every 4 h. The mean OD$_{600}$ ± standard deviation (SD) for each time point was calculated.

In vitro transcription-translation. Coupled in vitro transcription-translation assays were performed by using S30 extracts obtained from the *M. smegmatis* wild-type and EZ::TN-kan *tlyA* mutant. Bacteria were harvested by centrifugation at 6,000 × g for 15 min from 1 liter of 7H9 cultures grown to mid-log phase. The pellets were washed twice with buffer A (10 mM Tris acetate [pH 8.2], 14 mM Mg acetate, 60 mM K acetate, 1 mM dithiothreitol). Pellets were stored at −70°C, subjected to five cycles of freezing and thawing, and then resuspended in 1.82 ml of buffer A per 1 g of pellet (wet weight). The suspensions were passed through a Parr bomb at 2,000 lb/in^2 for 45 min on ice to break cells (19). The resulting lysates were treated with 2 μl of 1 M dithiothreitol per 1 ml of lysate and centrifuged twice at 30,000 × g for 30 min to obtain the S30 extracts.

Components of an *E. coli* S30 Extract System for Linear Templates kit (Promega, Madison, Wis.) were used for the coupled transcription-translation assay. The plasmid pTS73 which contains the luciferase gene expressed from the Tn*5* promoter was the DNA template in the assay. The reaction mixture contained 0.08 μg of pTS73/μl reaction mixture, 5 μl of complete amino acid mixture, 20 μl of S30 premixture without amino acids, and 20 μl of *M. smegmatis* S30 extract. *E. coli* S30 extract was used as a reagent control. The samples were incubated at 37°C for 2.5 h, and then the reactions were stopped by incubation at 4°C for 20 min. Luciferase activity was measured by using a Luciferase Assay System (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.).

RESULTS

Generation of EZ::TN libraries in *M. smegmatis* and *M. tuberculosis*. Electroporation of 1 μl of the EZ::TN Kan transposome into *M. smegmatis* yielded a total of 5 × 10^3 kanamycin-resistant colonies that were combined into 10 pools. Each of the 40 kanamycin-resistant colonies studied contained the Tn*903* Kan^r gene of the EZ::TN transposon in a differently sized fragment (data not shown), suggesting random insertion within the genome. The seven electroporation reactions in *M. tuberculosis* yielded a total of 6.9 × 10^3 kanamycin-resistant colonies. A total of 126 colonies were selected for evaluation, and the remaining colonies were combined into 64 pools. Southern blot analysis of 126 mutants revealed that 114 (90%) had an insertion of the EZ::TN transposon into the chromosome and that 12 appeared to be spontaneous Kan^r mutants (data not shown). The distribution of the size of the fragments detected with the Tn*903* Kan^r gene probe (data not shown) suggested that the insertion sites were random.

TABLE 1. Complementation of *tlyA* mutants

Strain	Plasmid	Growth on 7H10 agar[a]			
		Hygromycin (50 µg/ml)		Capreomycin (10 µg/ml)	
		Uninduced	Induced	Uninduced	Induced
M. smegmatis tlyA	pMF1	+	+	+	+
M. smegmatis tlyA	pMF1-tlyA/sm	+	+	+	−
M. tuberculosis tlyA	pMF1	+	+	+	+
M. tuberculosis tlyA	pMF1-tlyA/tb	+	+	+	−

[a] Growth was scored after 7 and 28 days for the *M. smegmatis* and *M. tuberculosis* strains, respectively, on 7H10 media containing the indicated antibiotic and no acetamide (uninduced) or 0.2% acetamide (induced) to induce expression of the plasmid-encoded *tlyA* gene. +, visible colonies, −, no growth.

Isolation of capreomycin-resistant *M. smegmatis* transposon mutants. Portions of the library of *M. smegmatis* EZ::TN transposon mutants were spread on TSA plates containing 10 µg of capreomycin per ml, and resistant colonies were recovered at a frequency of about 1 in 5.5×10^4 bacteria plated. Initially, three capreomycin-resistant mutants were selected, and all three mutants displayed the same 8-kb fragment when EcoRI-digested DNA was hybridized with a probe specific for the Tn*903* Kan^r gene in the EZ::TN transposon. Genomic DNAs from two of the mutants were cloned into pBluescript and transformed into *E. coli*, and colonies were selected for kanamycin resistance. Plasmid DNAs from three kanamycin-resistant transformants were sequenced, and the insertion site of the transposon for each was mapped to an ORF in the unfinished *M. smegmatis* genome sequence, which corresponds to the *tlyA* gene (Rv1694) of the *M. tuberculosis* H37Rv sequence (10). One of the EZ::TN-kan capreomycin-resistant mutants was designated P2U and used in further studies.

Isolation of a capreomycin-resistant *M. tuberculosis* transposon mutant. DNAs were extracted from each of the 64 pools of *M. tuberculosis* H37Rv EZ::TN transposon mutants and amplified by using a primer to the *tlyA* gene and a primer to the *aph* (Kan^r) gene of the EZ::TN transposon. Two of the 64 pools produced PCR products of about 300 bp when a *tlyA* reverse primer and Tn*903* forward primer were used and approximately 700 bp when a *tlyA* forward primer and Tn*903* reverse primer were used. Capreomycin-resistant colonies were obtained from one of the two pools by plating on 7H10 plates containing 10 µg of capreomycin per ml. Amplification and sequencing of DNAs from the capreomycin-resistant colonies confirmed that the EZ::TN transposon was inserted in the *tlyA* gene and had inserted at base 644 (amino acid Ala). One of these mutants was designated 315-A and utilized for further study. The *tlyA* transposon mutant 315-A had a MIC for capreomycin of 20 µg/ml.

Complementation of *tlyA* transposon mutants. Integrating plasmids were constructed that expressed either the wild-type *M. smegmatis* or *M. tuberculosis tlyA* ORF by using the inducible *M. smegmatis* acetamidase promoter (pMF1-tlyA/sm or pMF1-tlyA/tb, respectively). This promoter is induced approximately eightfold in the presence of 0.2% acetamide (25). The plasmid containing the appropriate *tlyA* ORF as well as the parent plasmid without the *tlyA* ORF (pMF1) were electroporated into either the EZ::TN-kan *tlyA* mutant *M. smegmatis* P2U or *M. tuberculosis* 315-A and plated on media containing

hygromycin (Hyg agar). Hygromycin-resistant transformants were recovered, grown in 7H9-Hyg broth, and plated on Hyg agar, Hyg-acetamide agar, capreomycin (Cap agar), and Cap-acetamide agar. All strains grew equally well on Hyg agar with and without acetamide (Table 1). The *M. smegmatis* P2U and *M. tuberculosis* 315-A transposon mutants containing pMF1 grew on Cap agar and Cap-acetamide agar (Table 1). *M. smegmatis* P2U with pMF1-tlyA/sm and *M. tuberculosis* 315-A with pMF1-tlyA/tb grew on Cap agar (uninduced) but not on Cap-acetamide agar (induced) (Table 1), indicating that expression of the integrated wild-type *tlyA* gene restored capreomycin susceptibility.

Generation of spontaneous capreomycin-resistant mutants. Spontaneous capreomycin-resistant *M. smegmatis* mutants were recovered at a frequency of 1.2×10^{-5}. Amplification and sequencing of the *tlyA* gene of nine spontaneous *M. smegmatis* mutants revealed that a naturally occurring *M. smegmatis* transposon IS*6120* (17), IS*1549* (27), or IS*1096* (9) had inserted into the *tlyA* gene in each of the nine mutants (Fig. 1). The insertion elements were found in both the forward and reverse direction in relation to the *tlyA* ORF. Spontaneous capreomycin-resistant mutants of *M. tuberculosis* were recovered at a frequency of 4.9×10^{-7} and 7.8×10^{-7} for H37Rv and CDC1551, respectively. The frequency of mutants in the Beijing strain F2 could not be determined accurately due to the slow growth of the mutants. Of the mutants studied, 10 of 11 H37Rv mutants, 11 of 11 CDC1551 mutants, and 7 of 8 Beijing strain F2 mutants contained changes within the *tlyA* gene (Table 2). Among these 28 *tlyA* mutations, 10 introduced a stop codon, 9 generated a frameshift, and 9 generated a missense mutation (Table 2). For the two isolates that contained wild-type *tlyA* genes, no mutations were found in the 500 bp upstream of the *tlyA* ORF. Furthermore, sequence analysis of all 30 *M. tuberculosis* mutants found that none had mutations in the region of the 16S rRNA gene (*rrs*) associated with resistance to aminoglycosides (32, 33). Ten *M. tuberculosis tlyA* spontaneous mutants representing missense, nonsense, and frameshift mutations (Table 2) were evaluated with a panel of 12 antituberculosis drugs including kanamycin and found to be susceptible to all 12 drugs and to have capreomycin MICs of 40 µg/ml (Table 2).

Mutations in capreomycin-resistant clinical isolates. Eighteen capreomycin-resistant clinical isolates were plated on me-
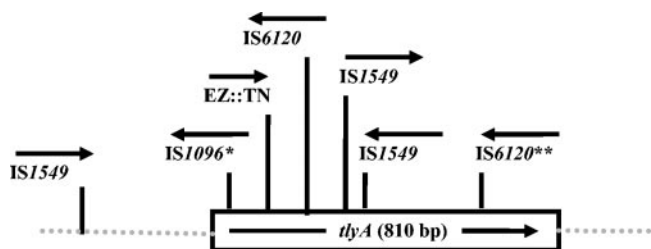

FIG. 1. Insertion sites in spontaneous capreomycin-resistant *M. smegmatis* LR222 mutants. The arrows indicate the orientation of the IS*1096*, IS*1549*, or IS*6120* insertion element in or near the *tlyA* ORF. *, two mutants with the same insertion element in the same insertion site; **, three separate mutants with the same insertion element in the same insertion site. The location and orientation of the EZ::TN transposon insertion are also noted.

TABLE 2. *tlyA* mutations in spontaneous
capreomycin-resistant *M. tuberculosis* mutants

Strain[a]	Mutation site (base no.)	Nucleotide change	Amino acid change	MIC (μg/ml)[b] CAP	MIC (μg/ml)[b] VIO
H37Rv[c]					
C-201	758	ΔC	Frameshift	40	20
C-202	7	C→T	Arg 3 Stop	40	20
C-203	397	C insertion	Frameshift	40	20
C-206	555	T→G	Phe 185→Leu		
C-210	353	T→C	Leu 118 Pro	40	40
C-211	64	C→T	Gln 22 Stop		
C-212	52	C→T	Arg 18 Stop		
C-213	383	T→A	Val 128 Glu		
C-214	26	ΔC	Frameshift		
C-215	200	C→A	Ala 67 Glu		
C-216	None	N/A	N/A		
CDC1551[c]					
C-302	550	C→T	Gln 184 Stop		
C-307	64	C→T	Gln 22 Stop		
C-308	586	ΔG	Frameshift	40	20
C-309	550	C→A	Gln 184→Lys		
C-310	653	ΔT	Frameshift		
C-311	550	C→T	Gln 184 Stop		
C-312	64	C→T	Gln 22 Stop	40	≤10
C-313	310	ΔG	Frameshift		
C-314	205	A→G	Lys69→Glu		
C-315	548	C→T	Pro183→Leu	40	20
C-316	400	ΔA	Frameshift		
Beijing F[c]					
C-501	272	C→A	Ala91→Glu	40	≤10
C-502	712	G→A	Glu238→Lys		
C-503	673–674	ΔGAT	Frameshift		
C-504	550	C→T	Gln 184 Stop	40	≤10
C-505	477	ΔG	Frameshift	40	≤10
C-507	550	C→T	Gln 184 Stop		
C-509	550	C→T	Gln 184 Stop		
C-510	None	N/A	N/A		

[a] Mutants were selected on 7H10 containing 10μg of capreomycin/ml.
[b] MICs were determined for a missense, nonsense, and frameshift mutant from each of the three different parent strains.
[c] All parent strains were susceptible to 10 μg of capreomycin/ml and 10 μg of viomycin/ml. CAP, capreomycin; VIO, viomycin; N/A, not applicable.

dia containing 10 μg of capreomycin per ml, individual colonies were recovered, and their *tlyA* and 16S rRNA (*rrs*) genes were sequenced (Table 3). Four of the isolates, (1, 10, 21, and 22) had mutations in the *tlyA* gene but not the *rrs* gene (Table 3). Isolates 10 and 21 had a capreomycin MIC of 80 and 40 μg/ml, respectively, and were kanamycin susceptible. Isolates 1 and 22 had a capreomycin MIC of 80 μg/ml and a kanamycin MIC of 20 μg/ml. Isolate 11 contained a mutation in the *tlyA* gene and an A1401G mutation in the 16S rRNA gene and had higher MICs for both capreomycin (>160 μg/ml) and kanamycin (>80 μg/ml). Each of the remaining 13 isolates contained an A1401G mutation in the *rrs* gene and had various levels of capreomycin resistance and high-level kanamycin resistance (MIC of >80 μg/ml) (Table 3); none of the 13 had mutations in the 500 bp upstream of the *tlyA* ORF. The *rrs* A1401G mutation in these isolates corresponds to the A1400G mutation in *M. tuberculosis* and the A1408G mutation in *E. coli* which have been previously been observed in kanamycin-resistant strains (10, 27, 31, 32). Throughout this report this mutation will be referred to as A1401G in accordance with the revised numbering in the updated *M. tuberculosis* H37Rv com-

plete genome, accession number NC_000962, National Center for Biotechnology Information (7).

Cross-resistance to viomycin. *M. smegmatis* P2U and *M. tuberculosis* 315-A *tlyA* transposon mutants were resistant to viomycin at a concentration of 10 μg/ml. Susceptibility to viomycin at a concentration of 10 μg/ml was restored by complementation with pMF1-tlyA/sm or pMF1-tlyA/tb, respectively (the *M. smegmatis* and *M. tuberculosis* wild-type parent strains are susceptible to viomycin at a concentration of 10 μg/ml). Among the 10 spontaneous capreomycin-resistant mutants examined, resistance to viomycin was found in 6 of 7 of the *tlyA* spontaneous mutants obtained from H37Rv and CDC1551 (Table 2). However, the three spontaneous mutants tested that were obtained from the Beijing strain F2 were not resistant to viomycin (Table 2). Viomycin resistance was seen in three (1, 10, and 22) of the four clinical isolates that had only a *tlyA* mutation (Table 3).

Growth rate of *tlyA* mutant. The generation times of *M. smegmatis* wild-type and *tlyA* mutant P2U were approximately 3.5 h in 7H9 broth with no drug (Fig. 2). When capreomycin was included in the media, the growth rate of the *tlyA* mutant P2U bacteria was significantly slower, with a generation time of approximately 6 h (Fig. 2).

In vitro transcription-translation assays. In vitro transcription-translation assays were done by using S30 extracts from *M. smegmatis* wild-type and *tlyA* mutant P2U, and transcription-translation activity was measured without capreomycin and in the presence of capreomycin at five concentrations ranging from 4.6 to 65.3 ng/ml (Fig. 3). In the presence of 4.6 ng of capreomycin per ml, transcriptional-translational activity of the wild-type S30 extracts was only 13.6% of the activity seen without capreomycin, whereas the P2U S30 had 91.1% of the activity seen with no drug. A capreomycin concentration of 65.3 ng/ml was required to reduce the activity of the S30 from the *tlyA* mutant P2U to 5% of the activity seen with no drug.
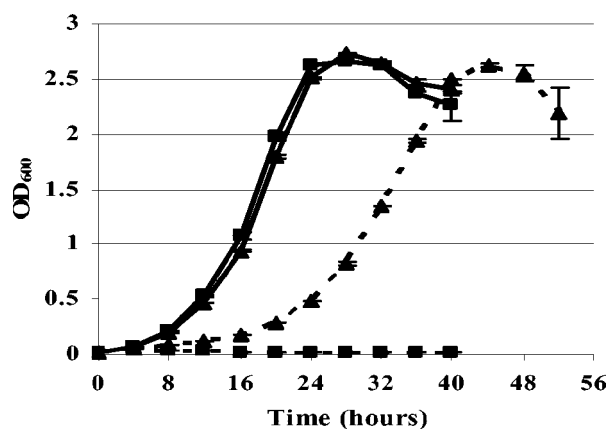

FIG. 2. Comparison of growth in the presence and absence of capreomycin. The growth of *tlyA*+ capreomycin-susceptible *M. smegmatis* bacteria (■) was compared to that of *tlyA* mutant capreomycin-resistant *M. smegmatis* bacteria (▲). Each was grown in 7H9 alone (solid line) or 7H9-Cap (10 μg/ml) (dashed line). The experiment under each growth condition was performed in triplicate. The OD_{600} was determined every 4 h, up to 52 h. Results are presented as the mean OD_{600} ± SD. Similar results were observed in a second independent experiment.

TABLE 3. Mutations and drug susceptibility in capreomycin-resistant *M. tuberculosis* clinical isolates

Clinical isolate no.	Mutation		Drug susceptibility[a]									
			MIC (μg/ml)[b]				INH	RIF	EMB	STR	CIP	ETH
	tlyA	*rrs*	CAP	KAN	VIO	AMK						
1	C insert in 218L	None	80	20	20	≤4	R	R	R	R	S	S
10	N236K	None	80	≤5	20	≤4	R	S	S	R[c]	S	S
21	L150P	None	40	≤5	≤10	≤4	R	R	S	R	S	S
22	ΔA codon 23	None	80	20	40	≤4	S	R	R	R	S	S
11	R14W	A1401G	>160	>80	>80	>64	R	R	R	R	S	R
2	None	A1401G	80	>80	≤10	>64	R	R	R	R	S	S
3	None	A1401G	80	>80	≤10	>64	R	R	R	R	R	S
4	None	A1401G	20	>80	≤10	>64	R	R	R	R	S	S
5	None	A1401G	20	>80	≤10	>64	R	S	S	R	S	S
7	None	A1401G	40	>80	≤10	>64	R	R	R	R	S	S
9	None	A1401G	>160	>80	20	>64	R	R	R	R	S	R
12	None	A1401G	>160	>80	≤10	>64	R	R	R	R	S	S
13	None	A1401G	>160	>80	40	>64	R	R	R	R	S	S
14	None	A1401G	>160	>80	40	>64	R	S	R	R	S	R
15	None	A1401G	>160	>80	20	>64	R	R	R	R	R	R
16	None	A1401G	20	>80	≤10	>64	R	R	R	R	S	S
17	None	A1401G	40	>80	20	>64	R	R	S	R	S	R
18	None	A1401G	20	>80	20	>64	R	R	R	R	S	R

[a] R, resistant; S, susceptible; CAP, capreomycin; KAN, kanamycin; VIO, viomycin; AMK, amikacin; INH, isoniazid (5 μg/ml); RIF, rifampin; EMB, ethambutol; STR, streptomycin (10μg/ml); CIP, ciprofloxacin; ETH, ethionamide.
[b] Control strains were susceptible to 10 μg of CAP/ml, 5 μg KAN/ml, 10 μg of VI O/ml, and 4 μg of AMK/ml.
[c] Resistant to 2 μg of streptomycin/ml.

DISCUSSION

Although transposon mutagenesis cannot be used to identify essential genes, which are often the targets of drugs, transposon mutagenesis can be useful to identify genes whose products may alter the drug target to allow drug binding, may activate the drug, or may be involved in drug transport. In the work presented here, transposon mutagenesis was used to identify a gene, *tlyA*, that is not essential for survival but plays a role in capreomycin resistance in mycobacteria. Complementation of a capreomycin-resistant *tlyA* transposon mutant with *tlyA* expressed from an inducible promoter restored capreomycin susceptibility and confirmed the role of the *tlyA* gene product in capreomycin resistance.

The frequency of recovering spontaneous *tlyA* mutations in the *M. smegmatis* strain was approximately 100-fold higher than that in the *M. tuberculosis* strains. The *tlyA* mutations in the *M. smegmatis* mutants were due to the insertion of naturally occurring transposons into the *tlyA* ORF. However, the *tlyA* mutations found in the *M. tuberculosis* mutants were point mutations. A high level of transposon mobility has been reported in *M. smegmatis* but is relatively rare in *M. tuberculosis* (27), which could account for the higher frequency of mutation in *M. smegmatis* compared to *M. tuberculosis*.

Capreomycin is structurally similar to viomycin, which has been shown to bind to both the 30S and 50S ribosome subunits (42), to affect the dissociation of the 70S ribosome of *M. smegmatis* (44), and to inhibit ribosomal translocation by arresting peptidyl-tRNA in the ribosomal acceptor site (23). Furthermore, changes in either the 16S rRNA or 50S ribosomal subunit alters binding and confers resistance to viomycin in *M. smegmatis* (33, 44). Because capreomycin also interferes with ribosomal function (34) and resistance to it involves the ribosome (33, 42, 44), ribosomal proteins or RNA may be the target of capreomycin, and TlyA may act to alter this target.

To further define the role of TlyA in capreomycin resistance, in vitro transcription-translation assays using S30 extracts containing ribosomes from *M. smegmatis* wild type and the *tlyA* mutant were used. The resistance of the mutant extract to inhibition by capreomycin in these assays suggests that resistance is not due to the loss of a transporter for the uptake of capreomycin. Additionally, the dose response suggests that resistance is not due to a loss of function required to activate capreomycin. Rather, the results suggest that the loss of TlyA
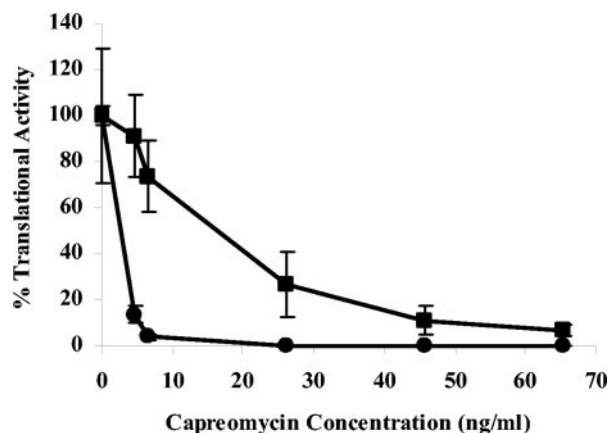

FIG. 3. Inhibition of translation by capreomycin. Capreomycin was added to S30 extracts from *tlyA*+ capreomycin-susceptible *M. smegmatis* (●) and from *tlyA* mutant capreomycin-resistant *M. smegmatis* (■) at final concentrations of 4.57 to 65.3 ng/ml. The production of luciferase activity in a coupled transcription-translation assay was measured in triplicate samples. Results are presented as the mean percent translational activity ± SD of the activity in the presence of drug to the activity in the absence of drug. Similar results were observed in a second independent experiment.

function has reduced the ability of capreomycin to interact with its target.

The translated product of the *tlyA* ORF was originally annotated as a hemolysin because extracts of *E. coli* that express the *M. tuberculosis tlyA* gene have hemolytic activity (39). More recent in silico data show that the TlyA ORF contains motifs that align well with domains predicted to be involved in rRNA methyltransferase reactions and RNA binding (3, 6), and the ORF displays homology with FtsJ/RrmJ (23S rRNA methylase) of *E. coli* (6). Furthermore, in silico research by Feder et al. (15) categorized TlyA as an rRNA methyltransferase, and the National Center for Biotechnology Information lists TlyA as a member of COG1189 (clusters of the orthologous groups of proteins), which is a group of predicted rRNA methylases (http://www.ncbi.nlm.nih.gov/genomes/altik.cgi?db = G&gi = 135). Although the possible rRNA methyltransferase activity of TlyA has not been assessed experimentally, it is interesting that 9 of the 12 missense mutations seen in either the *M. tuberculosis* spontaneous capreomycin-resistant mutants or capreomycin-resistant clinical isolates map at or near the four highly conserved residues of the putative FtsJ/RrmJ catalytic tetrad (K-D-K-E) or in the region implicated in S-adenosylmethionine binding (5, 15, 18).

Given the homology of TlyA to rRNA methyltransferases (15), the reported ability of capreomycin to affect ribosomes (34), and the data presented here indicating that *tlyA* related capreomycin resistance involves the ribosome, it is possible that TlyA-mediated methylation of rRNA may be required for capreomycin susceptibility. This suggests that ribosomes lacking the methylation would be capreomycin resistant. This is an unusual mechanism, given that resistance to most drugs that interact with ribosomes has been associated with changes in rRNA sequences, acquisition of methylated bases, alterations in ribosomal proteins, or acquisition of activities that inactivate the drug (28, 30, 38). Although unusual, it is not unprecedented as resistance to kasugamycin, an aminoglycoside antibiotic, is due to the loss of a methylase, RsmA (also known as KsgA), which methylates bases 1518 and 1519 of the *E. coli* ribosomal 16S rRNA (16). Mutation of 16S rRNA at bases 794, 926, and 1519 can also confer kasugamycin resistance (37).

Because capreomycin is typically used clinically after failure of therapy with first-line drugs (4), capreomycin-resistant clinical isolates are usually resistant to several antituberculosis drugs including kanamycin. Cross-resistance between kanamycin and capreomycin was previously observed in a variable fraction of kanamycin-resistant *M. tuberculosis* isolates and strains (21, 36) though the molecular mechanism of this resistance was not known. More recent reports show that most isolates with high-level kanamycin resistance have mutations in the 16S rRNA gene, with more than 90% of the mutations being A1401G (designated A1400G in the publications) (11, 28, 32, 33). However, isolates with low-level kanamycin resistance usually do not have changes in the *rrs* gene, and the molecular basis of resistance is not known (28). Each of the 14 clinical isolates studied that contained the *rrs* A1401G mutation was also resistant to capreomycin; however, the capreomycin MICs varied from 20 to >160 μg/ml. Based on these data, it is tempting to speculate that the *rrs* A1401G mutation can confer resistance to both drugs, but it is difficult to explain how a single mutation can yield such a large variation in ca-

preomycin MICs. Also, in a previously published report (1), the presence of the A1401G (A1400G in reference 1) mutation correlated with resistance to 10 μg of capreomycin per ml in only 6 of 12 clinical isolates with this mutation. A caveat with this study is that the susceptibility to capreomycin was determined in one of several submitting laboratories. The fact that all of the clinical isolates in both studies were MDR also limits the ability to draw a conclusion about the A1401G (A1400G) mutation and capreomycin resistance. If the 1401 *rrs* mutation is truly involved in capreomycin resistance and if TlyA is an rRNA methyltransferase, then a reasonable hypothesis is that capreomycin resistance in mycobacteria is due to the loss of the ability of capreomycin to bind to and inhibit ribosomes because of the lack of methylation of rRNA (*tlyA* mutation) or changes in the 16S rRNA (*rrs* mutation).

Overall, the results reported here demonstrate that disruption of the *tlyA* ORF can confer capreomycin resistance. This was the predominant means of resistance observed in laboratory-generated spontaneous capreomycin-resistant mutants and was also observed in four capreomycin-resistant clinical isolates. However, *tlyA* or *rrs* mutations were not found in two of the spontaneous mutants, and only the *rrs* A1401G mutation was found in 13 of the 18 capreomycin-resistant MDR clinical isolates examined. Further work is needed to elucidate the role of mutations in *rrs* in capreomycin resistance and to discover the basis of resistance in capreomycin-resistant strains that do not have mutations in *tlyA* or *rrs*.

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