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Molecular Analysis of Cross-Resistance to Capreomycin, Kanamycin, Amikacin, and Viomycin in *Mycobacterium tuberculosis*

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Capreomycin, kanamycin, amikacin, and viomycin are drugs that are used to treat multidrug-resistant tuberculosis. Each inhibits translation, and cross-resistance to them is a concern during therapy. A recent study revealed that mutation of the *tlyA* gene, encoding a putative rRNA methyltransferase, confers capreomycin and viomycin resistance in *Mycobacterium tuberculosis* bacteria. Mutations in the 16S rRNA gene (*rrs*) have been associated with resistance to each of the drugs; however, reports of cross-resistance to the drugs have been variable. We investigated the role of *rrs* mutations in capreomycin resistance and examined the molecular basis of cross-resistance to the four drugs in *M. tuberculosis* laboratory-generated mutants and clinical isolates. Spontaneous mutants were generated to the drugs singularly and in combination by plating on medium containing one or two drugs. The frequencies of recovery of the mutants on single- and dual-drug plates were consistent with single-step mutations. The *rrs* genes of all mutants were sequenced, and the *tlyA* genes were sequenced for mutants selected on capreomycin, viomycin, or both; MICs of all four drugs were determined. Three *rrs* mutations (A1401G, C1402T, and G1484T) were found, and each was associated with a particular cross-resistance pattern. Similar mutations and cross-resistance patterns were found in drug-resistant clinical isolates. Overall, the data implicate *rrs* mutations as a molecular basis for resistance to each of the four drugs. Furthermore, the genotypic and phenotypic differences seen in the development of cross-resistance when *M. tuberculosis* bacteria were exposed to one or two drugs have implications for selection of treatment regimens.

Antituberculosis therapy involves the administration of combinations of drugs, and cross-resistance to the drugs can be a concern, particularly when dealing with multidrug-resistant tuberculosis. The cyclic peptides capreomycin (CAP) and viomycin (VIO) and aminoglycosides kanamycin (KAN) and amikacin (AMK) are important second-line antibiotics used to treat patients with multidrug-resistant tuberculosis. To date, the studies on cross-resistance to these drugs in *Mycobacterium tuberculosis* isolates have been contradictory. There are reports of cross-resistance to CAP and KAN, CAP and VIO, KAN and VIO, and KAN and AMK (5, 8, 16, 17); however, there are also reports of drug-resistant isolates that do not exhibit these cross-resistance relationships (2, 6, 9, 16, 18).

We recently demonstrated that mutation of the *tlyA* gene, encoding a putative rRNA methyltransferase, confers resistance to CAP and to VIO in both *M. tuberculosis* and *Mycobacterium smegmatis* bacteria (7). We also identified CAP-resistant clinical isolates that did not have *tlyA* mutations but did have an A1401G change in their *rrs* genes (7). (In this report, the new numbering of the *rrs* gene in the updated *M. tuberculosis* H37Rv complete genome [3] will be used.) Alangaden et al. also reported *M. tuberculosis* clinical isolates with this *rrs* mutation; however, only half of such mutants were CAP resistant (1). The A1401G (A1400G in the referenced reports) mutation in the *rrs* gene has been associated with high-level KAN resistance in *M. tuberculosis* (14, 15), *M. smegmatis* (15), and *Escherichia coli* (4). The A1401G mutation has also been associated with AMK resistance in *M. tuberculosis* (1) and in other *Mycobacterium* species including *M. smegmatis* (12). Suzuki et al. (14) reported two other mutations in the *rrs* gene associated with resistance in *M. tuberculosis*; resistance to KAN was associated with a C1402T (C1401T in the referenced reports) *rrs* gene mutation, and resistance to KAN, CAP, and VIO was associated with a G1484T (G1483T in the referenced reports) *rrs* gene mutation.

The information from studies addressing resistance and cross-resistance to these drugs at the molecular level is limited (1, 6, 12, 14, 15), and it is still unclear whether mutations in the 16S rRNA gene are associated with CAP resistance and what role the various mutations play in cross-resistance in *M. tuberculosis*. To address these issues, we examined, in detail, the role of the various *rrs* gene mutations in CAP resistance and investigated the molecular genetic basis of cross-resistance to CAP, KAN, VIO, and AMK. Such information could provide insight into how these drugs bind and have implications for the selection of drug treatment regimens.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. tuberculosis* strains H37Rv and CDC1551 and 16 clinical isolates were obtained from the culture collection at the Mycobacteriology Laboratory Branch, Centers for Disease Control and Prevention, and grown in complete Middlebrook 7H9 broth (7H9) supplemented with 10% (vol/vol) albumin-dextrose-catalase (Difco Laboratories, Detroit, MI) and 0.05% (vol/vol) Tween 80 (Sigma, St. Louis, MO) at 37°C or on Middlebrook 7H10 agar (7H10) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (Difco). Kanamycin (Sigma,), capreomycin (Sigma), amikacin (Sigma), and viomycin (U.S. Pharmacopeia, Rockville, MD) were added to the media at concentrations stated in subsequent methods.

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TABLE 1. Mutations found in *M. tuberculosis* mutants selected on a single drug

Selection on 7H10 with drug[a]	No. of mutants with mutation:									
	rrs A1401G ($n = 37$)		*rrs* C1402T ($n = 0$)		*rrs* G1484T ($n = 13$)		*tlyA* ($n = 52$)		Neither *rrs* nor *tlyA* ($n = 19$)	
	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551
KAN5	4	0	0	0	0	1	0	0	6	9
CAP10[b]	0	0	0	0	0	0	10	11	1	0
VIO10	0	0	0	0	1	5	17	14	2	1
AMK4	19	14	0	0	0	6	0	0	0	0

[a] KAN5, 5 μg/ml kanamycin; CAP10, 10 μg/ml capreomycin; VIO 10, 10 μg/ml viomycin; AMK4, 4 μg/ml amikacin. The MICs for wild-type parent strains were ≤5 μg/ml kanamycin, ≤10 μg/ml capreomycin, ≤10 μg/ml viomycin, and ≤4 μg/ml amikacin.
[b] The isolation and characterization of the Cap[r] mutants listed here were described in the work of Maus et al. (7).

DNA isolation, manipulations, sequencing, and PCR. Genomic DNA was purified as previously described (11). Oligonucleotide primers were synthesized at the Biotechnology Core Facility, National Center for Infectious Diseases, Centers for Disease Control and Prevention (sequences available upon request). DNA amplification, sequencing, and evaluation were performed as previously described (7).

Selection of antibiotic-resistant mutants on single-drug plates. Spontaneous KAN-resistant (Kan[r]), AMK-resistant (Amk[r]), and VIO-resistant (Vio[r]) mutants were generated from pan-susceptible *M. tuberculosis* strains H37Rv and CDC1551. Each parent strain was susceptible to 10 μg/ml CAP, 5 μg/ml KAN, and 4 μg/ml AMK, which are the critical concentrations for the drugs (10), and to 10 μg/ml VIO. Portions of a concentrated cell suspension of each strain were spread on plates, in quadruplicate, containing 7H10 agar with 5 μg/ml KAN (7H10-KAN5), 4 μg/ml AMK (7H10-AMK4), 10 μg/ml VIO (7H10-VIO10), 10 μg/ml CAP and 5 μg/ml KAN (7H10-CAP10/KAN5), 10 μg/ml CAP and 4 μg/ml AMK (7H10-CAP10/AMK4), 10 μg/ml CAP and 10 μg/ml VIO (7H10-CAP10/VIO10), 5 μg/ml KAN and 4 μg/ml AMK (7H10-KAN5/AMK4), 5 μg/ml KAN and 10 μg/ml VIO (7H10-KAN5/VIO10), or 10 μg/ml VIO and 4 μg/ml AMK (7H10-VIO10/AMK4). Serial 10-fold dilutions were plated on 7H10 media without drug to determine the number of viable cells in the suspension. Isolated colonies were picked from the drug media and inoculated into 7H9 broth containing the concentration of drug(s) on which they were selected. DNA lysates were made from the cultures and analyzed by PCR and sequencing.

Sequential selection of antibiotic-resistant mutants on single-drug plates. H37Rv *tlyA* mutant C-211 and CDC1551 *tlyA* mutant C-307 were originally selected on 7H10-CAP10 and are resistant to CAP and VIO but susceptible to KAN and AMK (7); each has a Gln 22 Stop mutation in *tlyA* (7). The mutants were plated on 7H10 agar containing 80 μg/ml KAN (7H10-KAN80) and on 7H10 containing 64 μg/ml AMK (7H10-AMK64). H37Rv mutants KC-204 and KC-205 (both selected on 7H10-CAP10/KAN5), which have an *rrs* C1402T mutation and are resistant to KAN (MIC, 10 μg/ml), CAP (MIC, 160 μg/ml), and VIO (MIC, 10 μg/ml) but susceptible to AMK, were plated on 7H10-KAN80 and on 7H10-AMK64. Well-isolated colonies were picked and inoculated into 7H9 broth containing the concentration of drug(s) on which they were selected. DNA lysates were made from the cultures and analyzed by PCR and sequencing.

Determination of MICs. Cultures were grown and processed according to the current guidelines used for susceptibility testing of antituberculosis drugs (10). MICs of the drugs were determined using 7H10 agar containing the following: 10, 20, 40, 80, or 160 μg/ml CAP; 5, 10, 20, 40, or 80 μg/ml KAN; 4, 8, 16, 32, or 64 μg/ml AMK; or 10, 20, 40, or 80 μg/ml VIO. Plates were sealed and incubated at 37°C. After 3 to 6 weeks, the plates were inspected for the presence of visible colonies. The MIC was defined as the lowest concentration of drug resulting in complete inhibition of growth or in growth of <1% of the inoculum.

RESULTS

Selection of antibiotic-resistant mutants on single-drug plates. The frequencies of recovery of mutants selected on media containing one drug were consistent with single-step mutational events and ranged from 1.36×10^{-7} to 3.85×10^{-6}. Most (31 of 40) of the mutants analyzed that were selected on 7H10-VIO10 contained mutations in the *tlyA* gene (Table 1); 13 of 31 had the same *tlyA* mutation. Of the mutants selected on 7H10-KAN5, 25% (5 of 20) had an *rrs* mutation (A1401G or G1484T) and 75% (15 of 20) could not be explained by mutation in the *rrs* gene (Table 1). All 39 of the sequenced mutants selected on 7H10-AMK4 had an *rrs* gene mutation (A1401G or G1484T) (Table 1).

Selection of antibiotic-resistant mutants on dual-drug plates. The frequencies of recovery for the mutants selected on media containing a combination of two drugs were consistent with single-step mutational events and ranged from 2.17×10^{-8} to 4.54×10^{-7}. The majority (65/83) of the mutants selected on media containing two drugs had a mutation in their *rrs* gene (Table 2). Of the 64 mutants with only an *rrs* mutation, 24 had an A1401G mutation, 5 had a C1402T mutation, and 35 had a G1484T mutation (Table 2). Sixteen mutants selected on 7H10-CAP10/KAN5 had a *tlyA* mutation, and one had both an

TABLE 2. Mutations found in *M. tuberculosis* mutants selected on two drugs

Selection on 7H10 with drug[a]	No. of mutants with mutation:											
	rrs A1401G ($n = 24$)		*rrs* C1402T ($n = 5$)		*rrs* G1484T ($n = 35$)		*tlyA* ($n = 16$)		Neither *rrs* nor *tlyA* ($n = 2$)		*rrs* A1401G and *tlyA* ($n = 1$)	
	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551	H37Rv	CDC1551
CAP10/VIO10	0	0	0	0	0	6	0	0	0	2	0	0
CAP10/AMK4	0	1	0	0	0	4	0	0	0	0	0	0
CAP10/KAN5	0	2	3	2	2	3	7	9	0	0	0	1
KAN5/VIO10	0	0	0	0	3	11	0	0	0	0	0	0
KAN5/AMK4	13	8	0	0	1	1	0	0	0	0	0	0
VIO10/AMK4	0	0	0	0	0	4	0	0	0	0	0	0

[a] CAP10, 10 μg/ml capreomycin; VIO10, 10 μg/ml viomycin; AMK4, 4 μg/ml amikacin; KAN5, 5 μg/ml kanamycin. The MICs for wild-type parent strains were ≤10 μg/ml capreomycin, ≤10 μg/ml viomycin, ≤5 μg/ml kanamycin, and ≤4 μg/ml amikacin.

TABLE 3. Mutations found in *M. tuberculosis* mutants following sequential selection[a]

Isolate	Parent	Drug for selection	*rrs* mutation	MIC (μg/ml)			
				CAP	KAN	VIO	AMK
CA64-201	H37Rv *tlyA*	AMK64	A1401G	>160	>80	80	>64
CA64-202	H37Rv *tlyA*	AMK64	A1401G				
CA64-203	H37Rv *tlyA*	AMK64	A1401G				
CA64-204	H37Rv *tlyA*	AMK64	A1401G	>160	>80	80	>64
CA64-301	CDC1551 *tlyA*	AMK64	A1401G	>160	>80	80	>64
CA64-302	CDC1551 *tlyA*	AMK64	A1401G	>160	>80	80	>64
CKN-207	H37Rv *tlyA*	KAN80	A1401G	>160	80	>80	>64
CKN-301	CDC1551 *tlyA*	KAN80	A1401G	>160	>80	>80	>64
CKN-302	CDC1551 *tlyA*	KAN80	A1401G				
KCK-201	H37Rv *rrs* C1402T	KAN80	C1402T	>160	>80	>80	32
KCK-202	H37Rv *rrs* C1402T	KAN80	A1401G and C1402T	>160	>80	>80	>64
KCK-204	H37Rv *rrs* C1402T	KAN80	C1402T				
KCK-205	H37Rv *rrs* C1402T	KAN80	C1402T				
KCK-206	H37Rv *rrs* C1402T	KAN80	C1402T				
KCK-207	H37Rv *rrs* C1402T	KAN80	C1402T	>160	>80	80	8
KCA-301	H37Rv *rrs* C1402T	AMK64	A1401G and C1402T	>160	>80	>80	>64

[a] The medium used for each drug was 7H10. The mutation in the H37Rv and CDC1551 *tlyA* mutants is Gln 22 Stop (7). Mutants are designated based on the antibiotic(s) used to select them. CA64, selected on CAP10 and then AMK64; CKN, selected on CAP10 and then KAN80; KCK, selected on CAP10/KAN5 and then KAN80; KCA, selected on CAP10/KAN5 and then AMK64. MICs of strains resistant to the highest concentration tested are indicated with >. AMK64, 64 μg/ml amikacin; KAN80, 80 μg/ml kanamycin; CAP10, 10 μg/ml capreomycin; KAN5, 5 μg/ml kanamycin. The MICs for wild-type H37Rv and CDC1551 strains were ≤10 μg/ml capreomycin, ≤10 μg/ml viomycin, ≤5 μg/ml kanamycin, and ≤4 μg/ml amikacin.

A1401G *rrs* mutation and a *tlyA* mutation (Table 2). Two mutants selected on 7H10-CAP10/VIO10 had neither a *tlyA* nor an *rrs* mutation (Table 2).

Sequential selection of antibiotic-resistant mutants on single-drug plates. The previously described (7) H37Rv mutant C-211 and CDC1551 mutant C-307 have Gln 22 Stop mutations in the *tlyA* gene and are resistant to CAP and VIO and susceptible to KAN and AMK. When plated on 7H10-AMK64, C-211 and C-307 Amk^r mutants were recovered at a frequency of 2.08×10^{-9} and 1.02×10^{-9}, respectively. Six of six of these mutants had the *tlyA* mutation plus an A1401G *rrs* mutation (Table 3). When plated on 7H10-KAN80, C-211 and C-307 Kan^r mutants were recovered at a frequency of 3.82×10^{-9} and 2.04×10^{-9}, respectively. Three of three of these mutants had the *tlyA* mutation and an A1401G *rrs* mutation (Table 3).

H37Rv strains KC-204 and KC-205, obtained from the selection on 7H10-CAP10/KAN5, have the *rrs* C1402T mutation and are resistant to KAN (MIC, 10 μg/ml), CAP (MIC, 160 μg/ml), and VIO (MIC, 10 μg/ml) but susceptible to AMK. When plated on 7H10-KAN80, KC-204 and KC-205 Kan^r mutants were recovered at a frequency of 1.93×10^{-7} and 1.17×10^{-7}, respectively. One of six of these mutants had the original C1402T mutation and had acquired an A1401G mutation of the *rrs* gene; however, the remaining five mutants had only the original C1402T *rrs* mutation (Table 3). When plated on 7H10-AMK64, KC-204 and KC-205 Amk^r mutants were recovered at a frequency of 5.45×10^{-7} and 1.48×10^{-8}, respectively. Only one of the colonies picked grew well enough to obtain DNA for sequencing. This mutant had both the original C1402T mutation and the A1401G *rrs* gene mutation (Table 3).

MICs. The MIC of each drug was determined for representative mutants with each of the individual *rrs* mutations (A1401G, C1402T, or G1484T), mutants with *tlyA* mutations, mutants with *tlyA* plus *rrs* A1401G mutations, mutants with both *rrs* A1401G and C1402T mutations, and Kan^r mutants which had no *rrs* mutations. The representative mutants were chosen according to the drug(s) upon which each was originally selected. The results are grouped by mutation and displayed in Table 4. Of the *rrs* A1401G mutants 14/15 were susceptible to 10 μg/ml VIO and had CAP MICs of 20 to 80 μg/ml; all 15 had high-level resistance to KAN and AMK (MICs, >80 and >64 μg/ml, respectively). The one *rrs* A1401G mutant, CA-302, which was resistant to VIO (MIC 40 μg/ml) also had the highest CAP MIC (>160 μg/ml) of this group of mutants.

The *rrs* C1402T mutants were susceptible to AMK (MIC, ≤4 μg/ml), had low-level KAN resistance (MICs, 10 to 20 μg/ml), high-level CAP resistance (MICs, ≥160 μg/ml), and VIO MICs of 40 to 80 μg/ml. Most of the *rrs* G1484T mutants had high-level resistance to each of the four drugs; all had CAP MICs of >160 μg/ml, KAN MICs of >80 μg/ml, and VIO MICs of >80 μg/ml, and 74% (17/23) had AMK MICs of ≥64 μg/ml. The *tlyA* mutants selected on 7H10-VIO10 had MICs to CAP and VIO of 40 to 80 and 20 μg/ml, respectively, and were susceptible to KAN and AMK. However, a *tlyA* mutant, KC-305, recovered from 7H10-CAP10/KAN5 was susceptible to AMK but resistant to KAN (MIC, 40 μg/ml) and had high-level CAP and VIO resistance (160 and >80 μg/ml, respectively). Mutant KC-302 and the sequentially selected mutants with *tlyA* plus *rrs* A1401G mutations were highly resistant to all four drugs. The sequentially selected mutants with both an A1401G and a C1402T *rrs* mutation were also highly resistant to all four drugs (Table 3). The C1402T sequentially selected mutants recovered from 7H10-KAN80, in which only the original C1402T mutation was found, were highly resistant to CAP, KAN, and VIO but had low-level resistance to AMK. All four of the Kan^r mutants that did not have *rrs* gene mutations had low-level KAN resistance (MIC, 20 to 40 μg/ml), and two of those had CAP MICs of 20 μg/ml and were susceptible to VIO

TABLE 4. MICs of kanamycin, capreomycin, viomycin, and amikacin for each type of *M. tuberculosis* mutant recovered[a]

Isolate(s)	Mutation	MIC (μg/ml)			
		CAP	KAN	VIO	AMK
A-210, A-211, K-210, KA-201, KA-216	*rrs* A1401G	20	>80	≤10	>64
A-201, A-220, A-319, K-202, KA-301, KA-308, KC-307, KC-310	*rrs* A1401G	40	>80	≤10	>64
A-301	*rrs* A1401G	80	>80	≤10	>64
CA-302	*rrs* A1401G	>160	>80	40	>64
KC-202	*rrs* C1402T	160	20	80	≤4
KC-204, KC-205, KC-306	*rrs* C1402T	160	10	40	≤4
KC-328	*rrs* C1402T	>160	20	40	≤4
A-302, A-316, K-310, KA-203, KA-309, KC-203, KV-301, VA-304	*rrs* G1484T	>160	>80	>80	>64
CA-301, CA-305, CV-301, CV-308, KC-201, KC-301, KC-304, KV-311, V-314	*rrs* G1484T	>160	>80	>80	64
KV-201, V-212, VA-301	*rrs* G1484T	>160	>80	>80	32
KC-330, KV-203, V-301	*rrs* G1484T	>160	>80	>80	16
V-211, V-304, V-305	*tlyA*	80	≤5	20	≤4
V-201, V-306, V-307	*tlyA*	40	≤5	20	≤4
K-203, K-302	*tlyA*	20	20	≤10	≤4
KC-206	*tlyA*	40	10	20	≤4
KC-305	*tlyA*	160	40	>80	≤4
KC-302	*rrs* A1401G and *tlyA*	>160	>80	80	>64
K-203, K-302	None found	20	40	≤10	≤4
K-204	None found	≤10	40	≤10	≤4
K-301	None found	≤10	20	≤10	≤4

[a] Mutants are designated based on the antibiotic(s) used to select them. MICs of strains resistant to the highest concentration tested are indicated with >, and MICs of strains susceptible to the lowest concentration tested are indicated with ≤. The MICs for wild-type parent strains were ≤10 μg/ml capreomycin, ≤10 μg/ml viomycin, ≤5 μg/ml kanamycin, and ≤4 μg/ml amikacin.

and AMK and two were susceptible to CAP, VIO, and AMK (Table 4).

Analysis of drug-resistant clinical isolates. Sixteen clinical isolates resistant to at least one of the four drugs (CAP, KAN, VIO, and AMK) were studied. The gene mutations and MICs for each drug for the clinical isolates were similar to those obtained for the laboratory-generated mutants (Table 5). Nine of the isolates (isolates 23, 24, 25, 26, 33, 38, 39, 41, and 43) had an *rrs* A1401G mutation and were resistant to CAP (MIC, 20 to 80 μg/ml), KAN (MIC, >80 μg/ml), and AMK (MIC, >64 μg/ml) but susceptible to VIO (MIC, ≤10 μg/ml). One isolate (isolate 28) had both A1401G and A514C *rrs* mutations, and another (isolate 40) had three *rrs* mutations, A514C, T1239C, and A1401G; both were resistant to CAP (MIC, 20 μg/ml), KAN (MIC, >80 μg/ml), and AMK (MIC, >64 μg/ml) but susceptible to VIO. One isolate (isolate 29) had both an *rrs* G1484T and C1105G mutation and was highly resistant to all four drugs. Two isolates (isolates 31 and 35) had an *rrs* C517T mutation and were resistant to CAP (MIC, 20 to 80 μg/ml) and KAN (MIC, 20 to 40 μg/ml) but susceptible to VIO and AMK; both of these isolates had wild-type *tlyA* genes. Two isolates had no *rrs* mutations (isolates 27 and 30); however, one (isolate 30) was resistant to CAP (MIC, 20 μg/ml), KAN (MIC, 40 μg/ml), and AMK (MIC, 8 μg/ml) but susceptible to VIO, and the other (isolate 27) was resistant to CAP (MIC, 80 μg/ml), KAN (MIC, 40 μg/ml), and VIO (MIC, 80 μg/ml) but susceptible to AMK; both of these isolates had wild-type *tlyA* genes.

TABLE 5. Mutations and MICs in *M. tuberculosis* drug-resistant clinical isolates

Clinical isolate no.	*rrs* mutation(s)	MIC[a] (μg/ml)			
		CAP	KAN	VIO	AMK
23	A1401G	20	>80	≤10	>64
24	A1401G	20	>80	≤10	>64
25	A1401G	20	>80	≤10	>64
26	A1401G	20	>80	≤10	>64
33	A1401G	40	>80	≤10	>64
38	A1401G	20	>80	≤10	>64
39	A1401G	40	>80	≤10	>64
41	A1401G	80	>80	≤10	>64
43	A1401G	20	>80	≤10	>64
28	A1401G, A514C	20	>80	≤10	>64
40	A1401G, T1239C, A514C	20	>80	≤10	>64
29	G1484T, C1105G	>160	>80	>80	>64
31	C517T	80	10	≤10	≤4
35	C517T	20	40	≤10	≤4
30	None	20	40	≤10	8
27	None	80	40	80	≤4

[a] The MICs for the H37Rv control strain were ≤10 μg/ml capreomycin, ≤10 μg/ml viomycin, ≤5 μg/ml kanamycin, and ≤4 μg/ml amikacin.

DISCUSSION

The results reported here provide new information regarding the previously reported association of 16S rRNA gene mutations with resistance to CAP, KAN, VIO, and AMK (1, 6, 7, 12, 14, 15) and explain some of the conflicts in the literature (1, 2, 5, 6, 8, 9, 13, 16, 17, 18), in part because the data show a clear association between individual A1401G, C1402T, and G1484T *rrs* mutations and specific patterns of resistance to the various drugs. For example, many studies reported variable results with respect to cross-resistance to CAP and VIO in *M. tuberculosis* isolates (5, 9, 13, 16, 17). The molecular data presented here suggest that isolates resistant to CAP and VIO could have *tlyA*, C1402T, or G1484T *rrs* mutations, whereas

isolates resistant to CAP but not VIO could have an A1401G *rrs* mutation. Similarly, A1401G mutations could account for isolates reported to be resistant to KAN and CAP but susceptible to VIO (17). Other studies (2, 6) reported Kan^r, AMK-susceptible isolates, which may correspond to strains with a C1402T *rrs* mutation or to strains that do not have any *rrs* mutations. Our work, as well as published reports (2, 6), identified isolates in which resistance to low levels of KAN (and susceptibility to the three other drugs) is associated with mutations in an as-yet-unidentified gene(s). Finally, previous studies described isolates resistant to CAP, KAN, and VIO (5, 17), which is consistent with mutants carrying either a C1402T or a G1484T mutation in the *rrs* gene.

The molecular data presented here are also consistent with cross-resistance patterns seen in isolates obtained from patients treated with different drug regimens. Tsukamura (16) described isolates recovered from patients treated with KAN as being resistant to KAN and CAP but susceptible to VIO, and the resistance of these strains to CAP varied with the level of KAN resistance. Strains with low-level KAN resistance were generally CAP susceptible (16), which is consistent with the Kan^r mutants that have no *rrs* mutations. The isolates with a high level of KAN resistance were generally CAP resistant, which is consistent with mutants that have either an A1401G or a G1484T *rrs* mutation. Tsukamura (16) also found that, after patients infected with *M. tuberculosis* bacteria highly resistant to KAN were treated with CAP (susceptibility of the initial isolates to CAP was not reported), the recovered bacteria were CAP resistant but VIO susceptible. This is consistent with *rrs* A1401G mutants.

The MICs of the spontaneous mutants suggest that other factors or genes may be involved in resistance to the studied drugs and that these factors may be additive or synergistic when combined with mutations in the 16S rRNA gene. Although the MICs for each drug associated with the individual mutations are generally similar, there are some isolates that appear to be outliers. For instance, most mutants with an *rrs* A1401G mutation have MICs of CAP, KAN, and AMK of 20 to 80 μg/ml, >80 μg/ml, and >64 μg/ml, respectively, and are VIO susceptible (MIC, ≤10). However, isolate CA-302 (A1401G; selected on 7H10-CAP10/AMK4) has a CAP MIC of >160 μg/ml and a VIO MIC of 40 μg/ml. Isolate KC-305 (*tlyA* mutation, no *rrs* mutation; selected on 7H10-CAP10/KAN5) is resistant to CAP (MIC, 160 μg/ml), VIO (MIC, >80 μg/ml), and KAN (MIC, 40 μg/ml) but susceptible to AMK (MIC, ≤4 μg/ml). While the *tlyA* mutation could account for the resistance to CAP and VIO, the MICs of each drug are about fourfold higher than those seen for most *tlyA* mutants (7). Mutants were also recovered which do not have an *rrs* mutation, exhibit low-level resistance to KAN (MIC, 20 to 40 μg/ml), and are susceptible to the other three drugs. It is possible that isolates CA-302 and KC-305 both have an as-yet-unidentified mutation conferring KAN resistance. Interactions between the as-yet-unidentified mutation(s) and the A1401G mutation might account for the increased MICs of CAP and VIO for CA-302. Similarly, interaction of the as-yet-unidentified mutation(s) with a *tlyA* mutation might account for the increased MICs of CAP and VIO for KC-305. The as-yet-unidentified mutations associated with resistance to these drugs could also explain clinical isolates 27 (Cap^r, Kan^r, and Amk^r) and 30 (Cap^r, Kan^r, and Vio^r), which do not have mutations in their *rrs* or *tlyA* genes. Further work is needed to determine the molecular basis and mechanism(s) of the unexplained KAN resistance and the apparently synergistic relationships observed.

Mutants with both a *tlyA* mutation and an *rrs* A1401G mutation display KAN and AMK MICs similar to MICs of mutants with only an A1401G mutation but have higher CAP and VIO MICs than do mutants with only a *tlyA* mutation or the majority of mutants with only the *rrs* A1401G mutation. Further evidence for an interaction of these mutations is provided by the observation that plating *tlyA* mutants on media containing a high concentration of AMK (64 μg/ml) or on media containing a high concentration of KAN (80 μg/ml) generated single-step mutants that had an A1401G *rrs* mutation and displayed approximately fourfold-increased MICs for CAP and VIO.

Interestingly, two *rrs* mutations, C1402T and A1401G, also appear to interact to generate MICs higher than those for mutants containing only one of the two mutations. The MICs for CAP and VIO for the C1402T A1401G double mutants were slightly higher than those for mutants containing either the C1402T or the A1401G mutation alone.

The C1402T *rrs* mutation may also interact with other mutations. Five mutants were recovered after plating on 7H10-KAN80 in which only the original *rrs* C1402T mutation was found. However, MICs for the subset of these mutants displayed resistance to significantly higher levels of KAN and slightly increased resistance to VIO than the C1402T parent strains and were resistant to AMK (MIC, 8 to 32 μg/ml). These data suggest that there is another mutation, possibly the as-yet-unidentified mutation associated with low-level KAN resistance, which may be interacting with the C1402T *rrs* mutation.

Although clinical isolates resistant to the second-line antituberculosis drugs often have been exposed to many drugs and have acquired a number of mutations, we found similar associations of mutations and MICs for each drug in the clinical isolates. Not surprisingly, additional combinations of mutations in the *rrs* gene were also found which may reflect genetic polymorphisms unrelated to drug resistance, mutations associated with other selective pressures (e.g., treatment with streptomycin), or mutations associated with resistance to the drugs being studied. For example, one clinical isolate had a G1484T *rrs* mutation and a C1105G *rrs* mutation and displayed high-level resistance to all four drugs similar to the MICs for the laboratory-generated mutants that contain only the G1484T mutation. Additionally, one clinical isolate had T1239C, A514C, and A1401G mutations, and the MICs of all four drugs were similar to the MICs of those drugs for the laboratory-generated mutants that contain only the A1401G mutation. The lack of difference in the MICs would suggest that the C1105G and T1239C mutations do not play a role in resistance to the drugs studied, although a role cannot be ruled out from these data.

Since the 1940s, streptomycin has been used throughout the world as a first-line antituberculosis drug, and all of the clinical isolates that we tested were resistant to streptomycin. Mutations in the 530 loop region of 16S rRNA (e.g., C517T and A514C) have been associated with resistance to streptomycin (6), and these mutations were also found in four of the clinical

isolates tested. The two isolates with the A514C mutation also contained the A1401G mutation and displayed MICs similar to those of mutants carrying only the A1401G mutation, suggesting that the A514C mutation does not play a significant role in resistance to CAP, VIO, KAN, or AMK. The C517T mutation was the only mutation found in two clinical isolates which were resistant to streptomycin, CAP, and KAN. Previous work (6) revealed an association of the C517T mutation with streptomycin resistance but also described isolates with a C517T mutation that were resistant to KAN but susceptible to streptomycin. The role, if any, of the C517T and A514C mutations in resistance to CAP, KAN, or AMK remains to be elucidated.

The data presented in this report show clear associations between drug resistance and the various mutations, which were recovered at the frequencies expected for single-step mutations. However, the associations do not actually prove a role for the individual mutations in drug resistance. Using gene replacement approaches, our previous work (7) demonstrated the role of *tlyA* mutations in CAP resistance. Using an *M. smegmatis* conjugation system to generate defined mutations, Taniguchi et al. (15) showed that the A1401G (A1400G) mutation confers resistance to KAN and that the G1484T (G1483T) mutation confers resistance to KAN and VIO. Resistance or susceptibility to the other drugs was not reported. Similar genetic studies for the C1402T mutation have not been reported.

In conclusion, the genotypes associated with resistance to the cyclic peptides CAP and VIO and aminoglycosides KAN and AMK are overlapping. Because of this, using a combination of a cyclic peptide (CAP or VIO) and an aminoglycoside (KAN or AMK) is equivalent to using a single drug with respect to the development of drug resistance. The results of this study reveal an important role of 16S rRNA mutations in CAP resistance and provide data that explain the variability in the patterns of cross-resistance to CAP, KAN, VIO, and AMK. This information also provides some insight into where and how these drugs may bind, presumably to the ribosome, which may be useful for future drug design studies. The cross-resistance patterns and MICs varied among the different mutations reported here, with the notable exception of G1484T, which in most instances was associated with high-level resistance to all four drugs. Therefore, these data question the practice of generalizing resistance to a class of drugs, e.g., cyclic peptides or aminoglycosides, based solely on the resistance to a single drug in the class.

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