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Polymorphisms Associated with Resistance and Cross-Resistance to Aminoglycosides and Capreomycin in *Mycobacterium tuberculosis* Isolates from South Korean Patients with Drug-Resistant Tuberculosis[▽]

Laura E. Via,[1] Sang-Nae Cho,[5] Soohee Hwang,[2] Hyeeun Bang,[4] Seung Kyu Park,[2] Hyung Seok Kang,[2] Doosoo Jeon,[2,3] Seon Yeong Min,[3] Taegwon Oh,[3] Yeun Kim,[4] Young Mi Kim,[5] Vignesh Rajan,[1] Sharon Y. Wong,[1] Isdore Chola Shamputa,[1] Matthew Carroll,[1] Lisa Goldfeder,[1] Song A. Lee,[3] Steven M. Holland,[6] Seokyong Eum,[3] Hyeyoung Lee,[4] and Clifton E. Barry III[1]*

Tuberculosis Research Section, Laboratory of Clinical Infectious Disease, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland[1]; National Masan Tuberculosis Hospital, Masan, Republic of Korea[2]; International Tuberculosis Research Center, Masan, Republic of Korea[3]; Department of Biomedical Laboratory Science, Yonsei University, Wonju, Republic of Korea[4]; Department of Microbiology, Yonsei University College of Medicine, Seoul, Republic of Korea[5]; and Laboratory of Clinical Infectious Disease, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland[6]

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The aminoglycosides streptomycin, amikacin, and kanamycin and the cyclic polypeptide capreomycin are all widely used in second-line therapy for patients who develop multidrug-resistant tuberculosis. We have characterized a set of 106 clinical isolates of *Mycobacterium tuberculosis* using phenotypic drug susceptibility testing (DST) to determine the extent of resistance to each agent and cross-resistance between agents. These results were compared with polymorphisms in the DNA sequences of ribosome-associated genes previously implicated in resistance and with the clinical outcomes of subjects from whom these isolates were obtained. Thirty-six (34%) of these isolates displayed resistance to one or more of these agents, and the majority of these (20 of 36) showed cross-resistance to one or more agents. Most (33 of 36) of the resistant isolates showed polymorphisms in the 16S ribosome components RpsL and *rrs*. Three resistant strains (3 of 36) were identified that had no known polymorphisms in ribosomal constituents. For kanamycin and streptomycin, molecular DST significantly outperformed phenotypic DST using the absolute concentration method for predicting 4-month sputum conversion (likelihood ratios of 4.0 and 2.0, respectively) and was equivalent to phenotypic DST using the National Committee for Clinical Laboratory Standards (NCCLS)-approved agar proportion method for estimating MIC (likelihood ratio, 4.0). These results offer insight into mechanisms of resistance and cross-resistance among these agents and suggest that the development of rapid molecular tests to distinguish polymorphisms would significantly enhance clinical utility of this important class of second-line antituberculosis drugs.

Drug-resistant tuberculosis (TB) is an emerging issue in global TB control, with multidrug-resistant (MDR) and extensively drug-resistant (XDR) disease threatening to overwhelm existing initiatives (8). The difference between MDR TB (resistant to at least isoniazid [INH] and rifampin [RIF]) and XDR TB (MDR plus resistant to any fluoroquinolone and at least one of the injectable second-line drugs amikacin [AK], kanamycin [KM], and capreomycin [CM]) is often life or death for patients suffering from drug-resistant TB (7, 14, 15). Nonetheless, the diagnosis of MDR and XDR TB remains a lengthy, technically demanding laboratory procedure relying on culturing bacteria isolated from patient sputum on solid or liquid medium in the presence of individual drugs. To make matters worse, such highly resistant strains occasionally show growth differences in *in vitro* culture that may be related to fitness costs imposed by the mutations conferring resistance (9). These growth differences, and the complexity of *in vitro* assessment of drug resistance, result in laboratory determinations of XDR of questionable reliability (16). There is a growing movement toward molecular drug susceptibility testing using a variety of detection platforms that show robust performance for the first-line agents that define MDR TB (10).

Molecular drug susceptibility tests are limited in accuracy only by knowledge of the single nucleotide polymorphisms (SNPs) associated with conferring drug resistance. Since these are relatively well defined for the two front-line agents, INH and RIF, such tests perform well for these two agents, and one has even recently received regulatory approval for use in the diagnosis of MDR TB (3, 12, 18). The performance of molecular DST for the diagnosis of XDR TB is far inferior to that for the diagnosis of MDR TB principally due to the lack of detailed knowledge of the SNPs associated with resistance (11). The SNPs associated with fluoroquinolone resistance are somewhat established, and the sensitivities of such tests have been reported to range from 70 to 90% (2, 21, 28).

* Corresponding author. Mailing address: CEB, Bldg. 33, Rm. 2W20D, 33 North Drive, Bethesda, MD 20892. Phone: (301) 435-7509. Fax: (301) 480-5712. E-mail: cbarry@mail.nih.gov.

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TABLE 1. Sequences of primers used

Gene (annealing temp)	Forward primer	Reverse primer
rrs-1F, 1R (60°C)	5′ TCCTTAAAAGCCGGTCTCAG	5′ ACAGACAAGAACCCCTCACG
rrs-420F, 990R (65°C)	5′ TTCACCATCGACGAAGGTCC	5′ CTAGACGCGTCCTGTGCATGT
tlyA-1F, 1R (60°C)	5′ CATCGCACGTCGTCTTTC	5′ AATACTTTTTCTACGCGCCG
rpsL-1F, 1R (60°C)	5′ GGCCGACAAACAGAACGT	5′ GTTCACCAACTGGGTGAC
gidB-1F, 1R (58°C)	5′ CGTAATGTCTCCGATCGAGC	5′ CTTTGATGGCGAGCATTCG

Far less is known about the mechanisms by which TB develops resistance to aminoglycosides, including KM, AK, and streptomycin (SM). Despite the knowledge that the molecular target of all three is the bacterial ribosome, and despite the availability of the three-dimensional structure of the ribosome in complex with several aminoglycosides, we still lack a basic understanding of which mutations confer which patterns of resistance and cross-resistance in isolates from patients (6). In addition, CM, a structurally unrelated cyclic polypeptide, also binds the bacterial ribosome and there is relatively little information relating cross-resistance between the aminoglycosides and this class of molecule. In one study of 17 drug-resistant clinical isolates, 13 were found to have a SNP in the *rrs* gene (A1401G) and all 13 were found to be fully cross-resistant to KM, AK, and SM, while there were no polymorphisms to which to attribute the resistance of the remaining 4 isolates (1). In another study of 16 drug-resistant clinical isolates, 9 were identified as having exclusively the same *rrs*(*A1401G*) SNP while 5 other strains had multiple *rrs* polymorphisms and the remaining 2 had none. Levels of cross-resistance depended on the exact SNPs in each strain, with at least one strain appearing cross-resistant to CM as well as KM and AK (19). In a study of 18 CM-resistant mutants selected *in vitro* by transposition, an rRNA methyltransferase encoded by *tlyA* was implicated in CM resistance, with 4 of these isolates retaining full susceptibility to KM and AK (20). More recently, polymorphisms in *gidB*, another rRNA methyltransferase, were found to be closely associated with low-level SM resistance (20). These authors report that of 132 randomly selected clinical isolates, 57 were SM resistant and most contained polymorphisms in the *gidB* gene. Interestingly, 22 of the 57 SM-resistant isolates were also resistant to KM but did not contain the *rrs*(*A1401G*) SNP, thereby suggesting that *gidB* polymorphisms may mediate some level of cross-resistance between SM and KM.

In this study we examine the cross-resistance, molecular genetics, and outcomes of therapy for 106 subjects, most of whom were undergoing retreatment with second-line antitubercular agents. These subjects are part of an ongoing, longitudinal study of the factors that contribute to the development, diagnosis, and cure of XDR TB (13).

MATERIALS AND METHODS

***Mycobacterium tuberculosis* strains and clinical isolates.** The *M. tuberculosis* strain H37Rv was used as a standard in all experiments. Clinical isolates included in this analysis were collected from subjects sequentially enrolled in cohort B of a prospective observational cohort study (ClinicalTrials.gov identifier: NCT00341601) conducted in collaboration with the National Masan Tuberculosis Hospital (NMTH), a tertiary TB care center in South Korea. The characteristics of the subjects enrolled in this study and who yielded these isolates have been previously described by Jeon et al. (13). Cohort B enrolled consenting subjects with more than 30 days of previous treatment prior to their current diagnosis of TB. While all subjects enrolled in the trial were sputum smear positive for acid-fast bacilli (AFB+), some had begun treatment prior to enrollment; thus, not all subjects yielded cultivable *M. tuberculosis*. The 106 isolates described here were isolated from the first postenrollment sputum samples provided by 145 subjects enrolled in cohort B. Among 39 subjects whose samples not evaluated in this study, 1 subject withdrew prior to producing a sputum sample, and specimens from another 24 subjects were not successfully cultured initially. In addition, 3 isolates were identified as nontuberculous mycobacteria and 11 did not grow consistently on 7H10 agar supplemented with oleic acid albumin complex upon attempted resuscitation from frozen stocks.

The cohort study that yielded these isolates was reviewed and approved by the Institutional Review Boards of the NMTH and by the National Institute of Allergy and Infectious Disease, National Institutes of Health. All subjects gave written informed consent. Data collection, quality, and protocol compliance were monitored by an independent clinical research organization.

Drug susceptibility testing. The clinical laboratory of NMTH (lab A) performs drug susceptibility testing (DST) using the absolute concentration method on Löwenstein-Jensen (LJ) slants containing the relevant anti-TB drugs (4, 5), and these data are collected as part of this observational study. Growth of more than 1% of the control in LJ medium indicated resistance. Isolates were tested for resistance to INH (0.2 μg/ml), RIF (40 μg/ml), ethambutol (EMB) (2.0 μg/ml), SM (10 μg/ml), KM (40 μg/ml), ofloxacin (OFX) (2.0 μg/ml), ethionamide (ETH) (40 μg/ml), cycloserine (CS) (30 μg/ml), and *p*-aminosalicylic acid (PAS) (1 μg/ml). Resistance to pyrazinamide (PZA) was determined by the pyrazinamidase assay (29).

The MICs of KM (5 to 160 μg/ml), AK (4 to 64 μg/ml), CM (10 to 160 μg/ml), and SM (4 to 64 μg/ml) were determined for each isolate using the current standard for susceptibility testing using Middlebrook 7H10 agar in labs B (International Tuberculosis Research Center) and C (Yonsei University College of Medicine) using the agar proportion method as described by the National Committee for Clinical Laboratory Standards (NCCLS). The MIC was defined as the lowest concentration of drug resulting in growth of <1% of the inoculum.

When conflicting results were observed between lab A and lab B (25% of the strains for SM and 7% of the strains for KM), repeated MIC measurements for each isolate were performed in lab C using the NCCLS method and all four drugs. If the two NCCLS determinations were in agreement, then that was the MIC reported for that strain. If the NCCLS method performed in lab C disagreed with that from lab B but agreed with the result of the absolute concentration method test done in lab A, then the MIC was repeated again in lab C and the MIC reported accordingly. In addition, lab C randomly repeated an additional 25 strains with concordant results (between lab A and lab B) all of which agreed with the original determination.

Genotyping and DNA sequencing. DNA samples were prepared as described previously (17). Direct sequencing of the *rpsL*, *rrs*(420-980), *rrs*(1293-1537), *tlyA*, and *gidB* genes was performed on PCR products obtained from amplification of chromosomal DNA with the primers shown in Table 1. Both DNA strands of the resulting PCR products were sequenced. The BLAST2 sequence database available from the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD) was used for DNA sequence comparison.

Statistical methods. The Fisher exact test was used to compare the proportions of drug-resistant and drug-sensitive isolates with the SNPs of interest, and the odds ratios (OR), 95% confidence intervals (CI), and *P* values were calculated using Prism 5 (GraphPad Prism Software). The Fisher exact test with calculations for sensitivity, selectivity, and likelihood analysis was used to compare predictive ability of the DST methods with subject sputum culture conversion.

RESULTS

Phenotypic drug susceptibility testing for the aminoglycosides and CM. We have analyzed 106 isolates from consecu-

TABLE 2. Concordance of drug susceptibility testing between and within labs

Lab	% concordance[c]					
	Lab A[a]		Lab B[b]		Lab C[b]	
	SM	KM[c]	SM	KM	SM	KM
Lab A						
Lab B	75	93				
Lab C	63	91	83	92	85	100

[a] Drug susceptibility testing determined via the absolute concentration method on LJ slants (4).
[b] Drug susceptibility testing determined via the agar proportion method per NCCLS standards.
[c] Percentages of isolates whose DST results were concordant between the two labs. SM, streptomycin; KM, kanamycin.

tively enrolled South Korean subjects with a history of at least 30 days of prior TB chemotherapy. These subjects were selected from our ongoing longitudinal study of MDR and XDR TB from a tertiary referral hospital that specializes in the treatment of patients with highly drug-resistant strains (13). From this sample, 73 (68%) isolates were resistant to both INH and RIF (MDR); among these, 18 isolates were pre-XDR (being resistant to either KM or a fluoroquinolone but not both) and 14 (13%) fit the formal definition of XDR TB (23). All but one of the isolates were found to be members of the Beijing clade by spoligotyping, and many had atypical Beijing restriction fragment length polymorphism patterns (data not shown). To determine susceptibility to SM, KM, AK, and CM, we first obtained susceptibility data for SM and KM using the absolute concentration method on LJ slants (lab A) from the medical chart (5). This testing method, however, does not provide quantitative information on the MIC of each of these agents. Therefore, we performed MIC testing in a second laboratory (lab B) using the standard agar proportion method as described by the NCCLS (30). Because we observed discordance between these two sets of results for 25% of the strains tested with respect to SM resistance and with 7% of the strains tested with respect to KM resistance, we repeated the MICs for the discordant strains along with random concordant strains in a third laboratory (lab C) in order to achieve consensus. We consistently observed poorer concordance between laboratories with SM than with KM, a finding that held true even when comparing results from the same lab (lab C) repeating the susceptibility testing another time (Table 2). Concordance between lab B and lab C for results from AK and CM was above 90% for both drugs. Consensus MICs were obtained as described in Materials and Methods. Our results showed, as expected, that about 20% of the isolates were resistant to various levels of each of the four agents tested (Table 3).

Polymorphisms in genes encoding components of the mycobacterial 16S ribosome. From this set of 106 isolates we sequenced the entire *rpsL*-encoded ribosomal S12 protein as well as the central domain (nucleotides 420 to 980) and the 3′ minor domain (nucleotides 1293 to 1537) of the 16S rRNA encoded by the *rrs* gene (Table 3). Among these isolates, 12 contained an A→G transition at nucleotide (nt) 128 of *rpsL* resulting in the mutation of lysine 43 to arginine in the RpsL protein. Two other strains with *rpsL* SNPs were observed; one had a T→C transition at nt 51, and the other had an A→G transition at nt 363, both of which were silent with respect to altering amino acid composition.

The 3′ minor domain of the rRNA encoded by *rrs* was found to have polymorphisms in 28 isolates from this strain collection. As expected, alteration of nt 1401 in helix 44 of the mature rRNA was by far the most common, with an A→G transition occurring in all 16 of the isolates in which any alteration was found in this region. A second SNP in this domain was also found at high frequency. In 16 isolates an A→C transversion was observed at nt 1338, which occupies a position at the base of helix 43 in the rRNA structure. In addition, a single strain had SNPs in nearby nt 1332 (helix 29) and 1445 (helix 44).

Seven isolates were found to have SNPs in the rRNA central domain that includes both the 530 loop region of helix 18 and a region in helix 26. Five isolates had SNPs in nt 514; four had A→C transversions, while one showed an A→T transversion. In the same loop at the tip of helix 18, two other isolates showed 517 C→T transitions. No SNPs were detected in the 900 region of helix 27; however, a single strain showed a C→T transition at nt 832.

The correlation between ribosomal gene polymorphisms and SM resistance. To understand the relationship between the observed polymorphisms and the DST results, we compared the distribution of SNPs between SM-resistant and SM-sensitive strains at each allele (Fig. 1A). More than 20% of the 106 isolates were resistant to 8 μg/ml or more SM. Among 80 SM-susceptible isolates, SNPs at nt 128 in the *rpsL* gene were never observed, while among 15 isolates showing high-level SM resistance (≥64 μg/ml), 12 showed an A→G transition resulting in the replacement of the wild-type lysine by arginine at position 43 in the protein. Thus, *rpsL*(*A128G*) appears to be associated with high-level SM resistance as expected (OR = 138.8, 95% CI = 7.774 to 2.478, $P < 0.0001$). The remaining two observed SNPs in *rpsL* (nt 363 A→G and nt 51 T→C) were also found in isolates that were fully sensitive to SM.

In the *rrs* gene the A→G transition at nt 1401 was observed in 8 out of 80 fully SM-susceptible isolates, as well as 4 out of 11 moderately resistant strains and 4 out of 15 highly resistant strains. A similar pattern of distribution characterizes polymorphisms at nt 1338, which are also found in both SM-susceptible and SM-resistant isolates, suggesting no correlation with resistance. Seven isolates having SNPs in helix 18 were identified at nt 514 and 517. These SNPs were found in 4 out of 11 and 2 out of 15 strains that were moderately or highly SM resistant, respectively. Interestingly, one of these SNPs occurred in an isolate that was SM susceptible by the agar proportion method. However, this isolate was scored as SM resistant by the absolute concentration method and this result was repeated several times with comparable outcomes. Thus, all seven isolates containing changes in the pseudoknot region of helix 18 were SM resistant to some extent. Three additional *rrs* singleton SNPs were observed, one at nt 832 in helix 26 in a SM-resistant strain that was also carrying a SNP at *rrs* 514 and two others at nt 1332 and 1445, both of which occurred in the same moderately SM-resistant isolate (32 μg/ml). Thus, from this data, two regions of *rrs*, the pseudoknot region of helix 18 (OR = 23.7, 95% CI = 2.7 to 208, $P = 0.008$) and the 3′ domain, including both helix 44 and helix 26 (OR = 4, 95% CI = 1.3 to 12.1, $P = 0.022$), are likely to be associated with SM resistance.

TABLE 3. Polymorphisms in the *rrs*, *rpsL*, and *gidB* genes of TB clinical isolates

Phenotypic resistance pattern	Polymorphism in gene			No. of isolates	Drug MIC (μg/ml) (no. of strains)			
	rrs	*rpsL*	*gidB*[a]		KM	AK	CM	SM
KM/AK/CM/SM	514 A→C, 1338 A→C, 1401 A→G			1	>80	>64	20	32
	1332 G→A, 1445 A→G			1	20	32	160	32
	1401 A→G	128 A→G (K→R)		4	>80	>64	20 (2); 160 (2)	>64
	1401 A→G, 1338 A→C		ΔG102	2	>80	>64	40	8
	1401 A→G		254 A→G (D→G)	1	>80	>64	80	8
KM/AK/CM	1338 A→C, 1401 A→G			2	160	>64	40	≤4
	1401 A→G		ΔG102; 230 T→C (V→A); 104 T→G (L→R); 286 C→T (R→C)	4	>80	>64	20 (2); 40 (2)	≤4
	1401 A→G			2	>80	>64	20 (1); 80 (1)	≤4
KM/CM/SM	514 A→C			1	10	≤4	40	>64
KM	NM[b]	NM	341 T→C (I→T); ΔC115	2	10 (1); >40 (1)	≤4	≤10	≤4
SM		128 A→G (K→R)		7	≤5	≤4	≤10	>64
	514 A→T			1	≤5	≤4	≤10	64
	514 A→C, 832 C→T			1	≤5	≤4	≤10	8
	517 C→T			1	≤5	≤4	≤10	>64
	517 C→T, 1338 A→C			1	≤5	≤4	≤10	16
	1338 A→C	128 A→G (K→R)		1	≤5	≤4	≤10	>64
	1338 A→C		341 T→C (I→T)	1	≤5	≤4	≤10	8
	1338 A→C		440 A→C (K→T)	1	≤5	≤4	≤10	8
			169 G→T (stop)	1	≤5	≤4	≤10	32
	NM	NM	NM	1	≤5	≤4	≤10	>64
Sensitive	1338 A→C			6	≤5	≤4	≤10	≤4
	514 A→C			1	≤5	≤4	≤10	≤4
	1338 A→C		ΔG193; 278 C→A (P→Q)	2	≤5	≤4	≤10	≤4
		51 T→C; 363 A→G[c]		2	≤5	≤4	≤10	≤4
			ΔG102; ΔC115; 133 T→C (W→R); 251 C→G (P→R); 254 A→G (D→G); 413 C→T (A→V); 413 C→A (A→E)	8	≤5	≤4	≤10	≤4
	NM	NM	NM	51	≤5	≤4	≤10	≤4

[a] All of the *gidB* polymorphisms occur as single polymorphisms in individual strains but are grouped for brevity.
[b] NM, no mutation.
[c] Both polymorphisms are silent and occur in different strains.

The correlation between ribosomal gene polymorphisms and KM/AK resistance. KM and AK resistance appeared to be almost identical across the entire set of strains in which polymorphisms were found; therefore, the data were analyzed for correlation with SNPs simultaneously for KM and AK (Fig. 2). Eight strains that were fully susceptible to KM/AK had SNPs in the *rpsL* gene at nt 128, while only 4 out of the 16 that were highly resistant to KM/AK showed such SNPs and all 4 of these strains contained both the *rrs*(*A1401G*) allele and the *rpsL*(*A128G*) allele and were also highly resistant to SM (Table 3). The two other SNPs in *rpsL* observed occurred in KM- or AK-susceptible strains. Therefore, we found little evidence of an association between any *rpsL* gene SNP and resistance to KM/AK (OR = 2.4, 95% CI = 0.7 to 9.1, $P = 0.22$).

Sixteen isolates were identified that had SNPs in *rrs* in the region of nt 1401, and all were highly resistant to KM/AK; in contrast, this SNP in helix 44 was never found in a KM/AK-susceptible strain. Therefore, as expected, there was a strong correlation between KM/AK resistance and the presence of the *rrs*(*A1401G*) allele (OR = 619, 95% CI = 31.8 to 12,075, $P < 0.0001$) (Fig. 1B). Similar to what was observed for SM, the 16 SNPs observed at nt 1338 occurred in both susceptible and KM/AK-resistant isolates, without an apparent strong association with drug-resistant strains (OR = 2.2, 95% CI = 0.69 to 7.5, $P = 0.178$). Further, the 1338 A→C transversion was observed in eight fully drug-susceptible isolates, suggesting that this SNP at the base of helix 43 may not be associated with resistance to any of these agents. Five out of seven isolates showing SNPs in the pseudoknot region of helix 18 [*rrs*(*A514C*), *rrs*(*A514T*), and *rrs*(*C517T*)] were fully KM/AK susceptible, and the remaining two isolates were also SM resistant. Therefore, polymorphisms in this region do not appear to be associated with KM/AK resistance (OR = 0.70, 95% CI = 0.08 to 6.1, $P = 1$). Likewise the isolate containing the SNP in helix 26 (nt 832) was also KM/AK sensitive. The strain carrying the double SNPs *rrs*(*A1445G*, *G1332A*) was

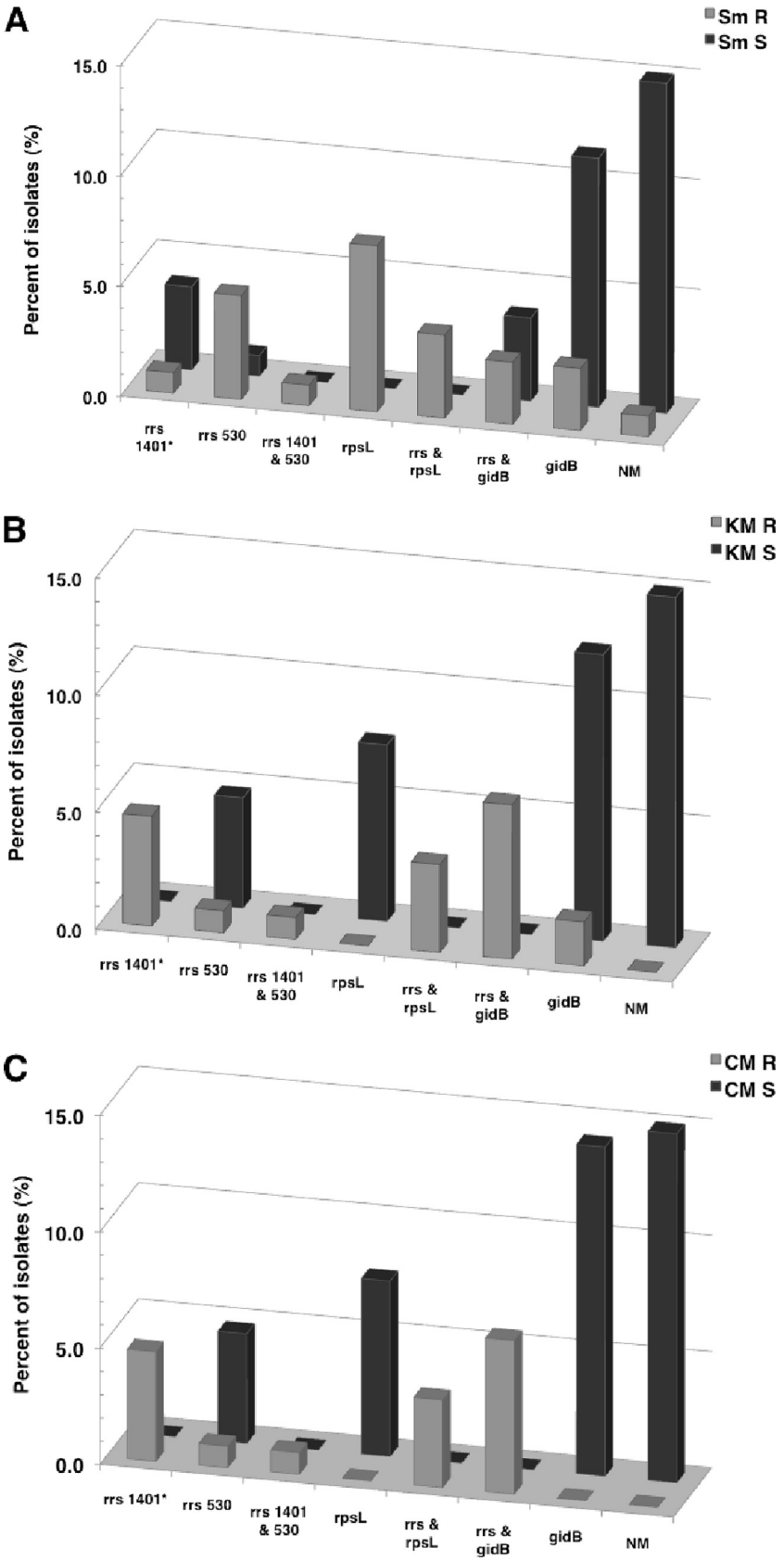

FIG. 1. Frequency of observed polymorphisms observed in the *M. tuberculosis* isolates as a function of resistance to SM (A), KM (B), or CM (C). The bars show the percentage of isolates with polymorphisms in the four indicated alleles or allele combinations that are fully susceptible

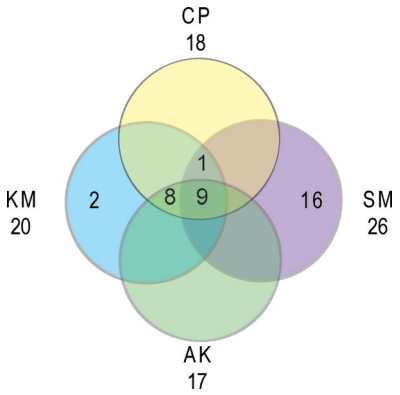

FIG. 2. Cross-resistance of isolates to injectable antibiotics. Phenotypic drug resistance of isolates diagrammed to depict overlapping resistance pattern with any polymorphism profile. Each circle represents the set of isolates resistant to that drug. Isolates resistant to both SM and KM are depicted within the overlap of those two circles.

found to be low-level KM/AK resistant (20 μg/ml KM and 32 μg/ml AK).

The correlation between ribosomal gene polymorphisms and CM resistance. Two out of 5 high-level CM-resistant isolates and 2 out of 13 moderately CM-resistant isolates showed the *rpsL*(*A128G*) allele; however, 8 out of 89 fully CM-susceptible strains also showed this allele and all four of the CM-resistant isolates with *rpsL* polymorphisms were also highly resistant to SM (Table 3). This suggests that the *rpsL*(*A128G*) SNP is not associated with CM resistance. Likewise, the two other observed *rpsL* alterations, at nt 51 and nt 363, were both found in CM-sensitive strains and were therefore not associated with CM resistance.

Polymorphisms in helix 44 at nt 1401 were found in 4 out of 5 high-level CM resistant isolates as well as in 12 out of 13 moderately CM-resistant strains (Table 3 and Fig. 1C). All 16 strains carrying this SNP were resistant to CM, strongly supporting that this SNP is associated with CM resistance (OR = 1,168, 95% CI = 53 to 25,500, $P < 0.0001$). SNPs at nt 1338 were present in 11 CM-sensitive strains and 5 out of 13 moderately resistant strains but absent from all 5 of the highly CM-resistant isolates, suggesting that this SNP was not associated with CM resistance. Likewise, 5 out of 7 of the SNPs in the pseudoknot region of helix 18 were in CM-sensitive strains and the two isolates moderately resistant to CM with SNPs in this region were both SM resistant. The *rrs*(*C832T*) allele was fully CM susceptible. Interestingly, however, a single strain with the double lesion in *rrs* at both nt 1445 and 1332 was highly CM resistant. All the isolates found to be resistant to CM in this strain collection were found to have at least one SNP in the *rrs* gene.

Polymorphisms in *tlyA* and association with resistance to CM. The *tlyA* gene was also sequenced in the entire set of isolates. Several polymorphisms were detected, including a ubiquitous codon 11 CTA-to-CTG transition that was found in all isolates compared with the *M. tuberculosis* reference strain H37Rv. Only three additional unique polymorphisms were noted in this strain collection: *tlyAΔ144-155*, *tly*(*C152T*) (codon 51 GCG→GTG [Ala-Val]), and *tly*(*A344T*) (codon 115 TAC→TTC [Tyr-Phe]). Notably, all three of these strains remained fully CM susceptible.

Polymorphisms in *gidB* and association with low-level resistance to SM. We determined the complete sequence of the *gidB* gene for 97 isolates for which DNA amplification was successful, including all those isolates resistant to the drugs examined in this study, and detected a total of 18 differences in comparison to the sequence of H37Rv. All of the isolates showed a SNP at nt 299 with a T replacing the 299C allele in the reported sequence of H37Rv. In addition, the majority of isolates ($n = 83$) contained an A→C SNP at nt 276 (codon 92 E→D). The 16 remaining polymorphisms were present at a frequency of 0.227 (22 of 97). A deletion of G at nt 102, which resulted in a frameshift at codon 34, was observed in four samples, three of which were resistant to at least three drugs, but only two of them showed low-level SM resistance, with an MIC of 8 μg/ml. These two samples also had two SNPs in the *rrs* gene (1401 A→G and 1338 A→C). The third drug-resistant sample that was SM susceptible had only the *rrs* (1401 A→G) SNP in addition to the G102 deletion. The remaining fourth sample had only the G102 deletion and was fully susceptible to all drugs. Another isolate with a SM MIC of 32 μg/ml had no polymorphisms detected in the *rpsL* or *rrs* genes but had a 169 G→T SNP in *gidB*, which resulted in a stop signal at codon 57. Two additional isolates had either a nt 341 T→C or a 440 A→C SNP causing amino acid changes in GidB in combination with the *rrs*(*A1338C*) SNP and were resistant to 8 μg/ml SM. These two SNPs in *gidB* were not seen among the susceptible isolates. Two isolates had C115 deletions and 1 isolate had a G193 deletion, causing frameshifts at codons 39 and 65, respectively, yet all isolates were susceptible to 4 μg/ml SM. Interestingly, two KM-resistant isolates that had no *rrs* polymorphism were found to have *gidB* polymorphisms (Table 3) so it is possible that GidB activity can also influence KM resistance. The remaining SNPs observed in the isolates substituted one amino acid for another in GidB in susceptible strains. While we have observed a number of polymorphisms in GidB in SM-resistant strains, some of these polymorphisms are also found in susceptible strains so it is difficult to assess the role of GidB in resistance to SM and KM with the data available. It will be necessary to construct these polymorphisms in susceptible strains before we can draw firm conclusions.

(S) or resistant (R). The column representing susceptible isolates without a detected polymorphism describes 56 to 60% of isolates but has been truncated at 15%. Category *rrs* 1401* includes those isolates with a polymorphism in this location as well as the single isolate with a double polymorphism at *rrs-1332* and *-1445*. (A) Isolates with the indicated codon 43 polymorphism in RpsL are significantly more likely to have high-level drug resistance ($P < 0.0001$) than isolates with other polymorphisms. (B and C) The resistance to KM and CM is most prevalent in isolates with the A1401G transition or with both an *rpsL* gene polymorphism and an *rrs* polymorphism in the 1401 region ($P < 0.0001$).

TABLE 4. Predictive value of phenotypic and molecular drug susceptibility testing (DST) for 4-month sputum culture conversion[a]

DST method and result	No. with treatment outcome		Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)	Likelihood ratio
	Success	Failure						
MIC								
Sensitive	52	11						
Resistant	11	25	69.5	82.5	72.6	69.4	82.5	4.0
Critical concn								
Sensitive	38	6						
Resistant	26	29	82.9	59.4	37.2	52.7	86.4	2.0
Genotyping								
Sensitive	53	13						
Resistant	10	23	63.8	84.1	71.6	69.7	80.3	4.0

[a] PPV, positive predictive value; NPV, negative predictive value; likelihood ratio = sensitivity/(1−specificity).

The predictive value of resistance-associated alleles in determining sputum culture conversion. Ultimately the importance of a given clinical test is in the utility of such a test in predicting the outcome of the clinical decision taken as a result of a positive or negative finding of the test. In the case of drug resistance, a test can be evaluated based upon whether the result correlates with the outcome of the patients being treated. In this case (a population of mostly MDR and some XDR patients), we considered a successful outcome to be sputum culture conversion by 4 months or less and a negative outcome to be failure to achieve sputum culture conversion. Since nearly all of these patients were being treated with aminoglycosides, we explored the ability of the phenotypic DST using two different methods (the absolute concentration and the NCCLS methods) to predict the outcome in these patients. There were 100 patients in this study with complete data on sputum culture conversion (≥2 consecutive negative cultures and no recurrence of a positive culture within the next 4 months). When one considers the outcome of treatment, a finding of resistance for either KM or SM using the absolute concentration method correlates with treatment failure only 53% of the time, whereas a finding of resistance using the more accurate NCCLS method correlates with treatment failure 69% of the time (Table 4). Likewise, the predictive value of the test for a positive outcome can be estimated. In the same way, a finding of either SM or KM resistance or susceptibility from the molecular test (using the reported *rrs* polymorphisms in helix 44 and the 530 pseudoknot and rpsL 128 alleles) can be compared with outcome and the three tests can therefore be directly compared in terms of their accuracy and predictive value. In terms of overall accuracy, results of the molecular test and the NCCLS method are indistinguishable at about 76% and clearly superior to those of the absolute concentration method (67%). The three methods have similar negative predictive values (i.e., a finding of sensitive correlates well with treatment success) and differ most strongly in their positive predictive values (i.e., a finding of resistant correlates with failure). This allows one to calculate a likelihood ratio for each of the tests that estimates the fold increase in the probability that the test can accurately predict the clinical result. In this calculation, the molecular susceptibility test significantly outperforms the commonly used absolute concentration method and is equivalent to the NCCLS MIC method.

DISCUSSION

Establishing causality for SNPs observed in drug-resistant clinical isolates of MDR TB is nontrivial; the often incomplete treatment history of subjects and the inaccuracy of phenotypic DST for second-line agents lead to the potential for misinterpretation of observed SNPs as functional when they in fact represent natural sequence variation. In many cases, it can also be difficult to establish whether the observed polymorphism represents the mutation that gave rise to resistance or a compensatory mutation selected afterward to improve fitness of the resistant isolate (9). Selection of single-step resistant mutants *in vitro* can facilitate this process but in many cases gives rise to mutations that are not representative of the situation *in vivo* during infection. Comparing the results of the two different selective environments offers some hope to rationalize the observed resistance. For example, in this study we found that the majority of KM-resistant isolates from this subject cohort that were resistant to KM were also resistant to AK and CM and the majority of these isolates showed the 1401 A→G transition (Fig. 2). In fact 16 out of 16 isolates with this polymorphism were resistant to KM and were fully cross-resistant to AK and CM. This is in accordance with previous reports that have selected for *in vitro* mutants, either simultaneously or sequentially, to combinations of these drugs and have observed similar causality of the 1401 allele (19). In addition, this SNP is biologically plausible as this polymorphism occurs very near to the known binding site for KM (Fig. 3). For this allele, therefore, we can be fairly confident in predicting that the SNP at nt 1401 conferred resistance to all three drugs. Unfortunately, even in this relatively small strain collection, more than 15% (3 of 20 strains) do not have this SNP yet are highly resistant to one of these agents.

Likewise, our finding that the most common polymorphism associated with SM resistance was the A128G substitution in *rpsL* is consistent with many previous reports from both *in vitro* and clinical samples (26, 27). The location of this polymorphism on the bacterial ribosome is likewise very close to the binding site for SM, making this also biochemically reasonable

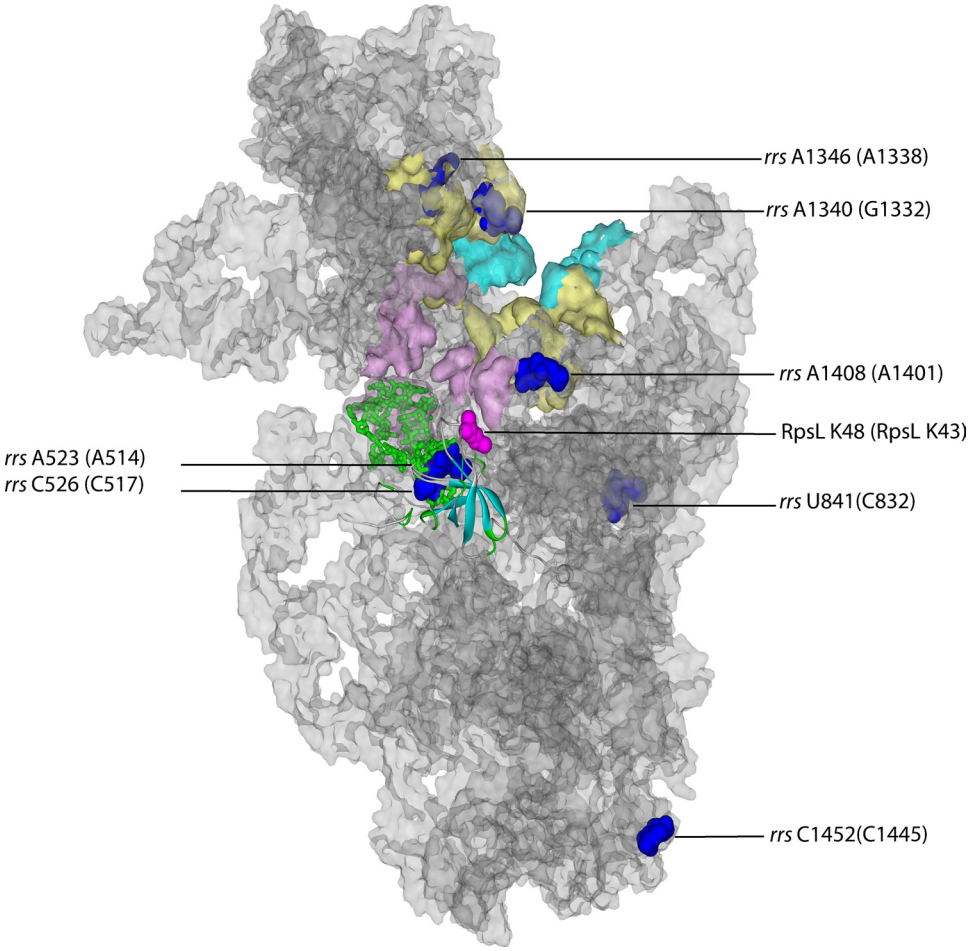

FIG. 3. The location of polymorphisms identified in this study on components of the bacterial ribosome. The structures of the 16S rRNA and RpsL from *Thermus thermophilus* (Protein Data Base no. 2hgr) were used to construct this schematic (produced using Discovery Studio 2.0, Accelrys). The ribosomal A, P, and E sites that form the active center of the complex are shaded pink, yellow, and blue, respectively. The RpsL protein is shown in ribbon form. The 530 loop region is shown as green balls and sticks. Numbering shown is according to the *T. thermophilus* convention followed by the corresponding position in the *M. tuberculosis* sequence. Polymorphisms observed in this study of *rrs* are colored blue and those of *rpsL* in pink.

(Fig. 3). Many other studies have also focused on the role of *rrs* alterations in the 530 loop region similar or identical to the ones described here that appear to be associated with SM resistance (25). It is noteworthy that the 530 loop region is very close in space to the lysine at position 43 of RpsL in the crystal structure and substitutions here are therefore likely to exert a direct effect on drug binding. Confirmation of the causality of these polymorphisms in the 530 loop region will require moving these specific changes into isogenic strains.

What was striking in this sample set was the extent of polymorphism in the *gidB* gene. GidB was identified as an *S*-adenosyl-*L*-methionine (SAM)-dependent, rRNA methyltransferase associated with low-level SM resistance (22, 24). The GidB protein mediates methylation of G527, another residue in the 530 pseudoknot that may affect the SM binding site directly (22). We saw 15 different polymorphisms in *gidB* among 21 isolates, including drug-sensitive as well as drug-resistant strains. Most of these polymorphisms mapped to conserved residues of the protein, including the SAM-binding site. Interestingly, the G102 deletion occurred in four different strains and was the only polymorphism that was also observed by others in both SM-resistant and SM-susceptible clinical strains (22, 24). Further investigation is needed to probe the association between the G102 deletion, the resultant frameshift at codon 34, and SM susceptibility. It seems rather unlikely that polymorphisms in highly conserved regions of this protein that methylates rRNA very close to the binding site for SM are unrelated to drug selective pressure; however, our analysis using standard DST methodology does not provide any direct confirmation of this prediction. In contrast to the observation by Okamoto et al. (22) that some isolates carrying a *gidB* polymorphism were both SM and KM resistant, despite lacking the *rrs*(*A1401G*) SNP, we did not observe this same genetic profile in the present set of isolates, although we did identify two isolates with *gidB* polymorphisms that were KM resistant and SM susceptible in the absence of the *rrs*(*A1401G*) SNP. Thus, at present, we have no evidence supporting the possibility that *gidB* polymorphisms may mediate some level of cross-resistance between SM and KM.

This study did identify a potential new double polymorphism

in the 3′ minor domain that may be associated with low-level KM/AK/CM cross-resistance, *rrs*(*A1445G*, *G1332A*). Confirmation of this will require allelic exchange in congenic strains. Notably, A1332 is located in the middle of the P site (Fig. 3), suggesting a possible effect on overall catalytic rates of protein synthesis that may confer relative drug insensitivity. Perhaps more importantly, we have identified two strains with resistance to the 2-deoxystreptamines for which there are no apparent polymorphisms in genes other than *gibB*. If this can be established, the basis for the molecular resistance of these two strains will help to improve the sensitivity of a molecular approach to diagnosing XDR disease. There are also a considerable number of strains for which SM resistance cannot be explained. Of the 26 moderately to highly resistant isolates, 8 do not contain polymorphisms in either the 530 loop of *rrs* or the RpsL protein.

CM is the agent for which the least is known in terms of resistance mechanisms in clinical isolates. Transposon mutagenesis of *tlyA* (Rv1694) in *M. tuberculosis* resulted in CM-resistant colonies that when complemented with wild-type *tlyA* recovered susceptibility to the drug (20). The *tlyA* gene encodes a 2′-*O*-methyltransferase that creates methylations in helix 44 of the 16S and helix 69 of the 23S rRNA. When the ribosomal subunits form a complex, these methylations are brought together, creating the binding site of CM, which, once bound, inhibits ribosome translocation. Loss of function of *tlyA* or mutation of its targets confers resistance to CM. Due to scarce use of CM in this population, SNPs in the *tlyA* gene associated with CM resistance were not observed. There remains the possibility that patients resistant to KM but lacking a SNP in *rrs* in the region of nt 1401 will still be sensitive to CM even though in this cohort it appears that this is rare. It also appears as though the strong selection presented by KM/AK use generates very high levels of CM cross-resistance, limiting the utility of this drug in this patient population.

As elegantly shown *in vitro*, both the order and the concentration of the drug selective pressure make a difference on the mutations observed (19). In the context of the isolates examined in this study, CM is relatively rarely used due to availability of this agent in South Korea and its expense; therefore, KM/AK and SM have exerted the predominant selective pressure. These results therefore not only expand our knowledge of the extent of cross-resistance between various ribosomal inhibitors that have been used clinically but also suggest that some additional thought might be appropriate for the order of drug addition in selecting patient regimens. CM, for example, might be better added prior to treatment with KM or AK, since resistance to CM (if generated via *tlyA*) may be less likely to give cross-resistance to the 2-deoxystreptamines, whereas application of KM and AK seems to result predominantly in mutations that confer cross-resistance to CM.

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