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Mechanism of Resistance to Amikacin and Kanamycin in *Mycobacterium tuberculosis*

GEORGE J. ALANGADEN,[1]* BARRY N. KREISWIRTH,[2] ARLETTE AOUAD,[1] MINOO KHETARPAL,[1] FELICITAS R. IGNO,[1] SORAYA L. MOGHAZEH,[2] ELIAS K. MANAVATHU,[1] AND STEPHEN A. LERNER[1,3]

Division of Infectious Diseases[1] and Department of Biochemistry and Molecular Biology,[3] Wayne State University School of Medicine, Detroit, Michigan 48201, and Public Health Research Institute TB Center, New York, New York 10016[2]

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An A1400G mutation of the *rrs* gene was identified in *Mycobacterium tuberculosis* (MTB) strain ATCC 35827 and in 13 MTB clinical isolates resistant to amikacin-kanamycin (MICs, >128 μg/ml). High-level cross-resistance may result from such a mutation since MTB has a single copy of the *rrs* gene. Another mechanism(s) may account for high-level amikacin-kanamycin resistance in two mutants and lower levels of resistance in four clinical isolates, all lacking the A1400G mutation.

We examined resistance in *Mycobacterium tuberculosis* (MTB) to the deoxystreptamine aminoglycosides amikacin (AK) and kanamycin (KM) in contrast to its resistance to streptomycin (SM), a streptidine drug. In MTB cross-resistance occurs between AK and KM (2) but not between AK-KM and SM (21).

High-level SM resistance in MTB is associated with alterations of the ribosomal target site resulting from mutations in the *rpsL* gene of the S12 ribosomal protein or in the 530 or 915 region of the *rrs* gene of the 16S rRNA (4, 6–9, 13, 16, 17). In *Escherichia coli*, ribosomal binding of KM is affected by mutation in the 1400 region of the *rrs* gene (14), and mutations in this region produce resistance to various aminoglycosides (5). We had identified an A1400G mutation in the *rrs* gene in a KM-resistant strain of MTB (ATCC 35827) (1). To study further the mechanism(s) of AK-KM resistance, we selected AK-resistant mutants of H37Rv (a standard susceptible strain of MTB) and characterized the 1400 region of the *rrs* gene in these mutants and in clinical isolates of MTB resistant to AK-KM. A subsequent report (19) noted a similar mutation in AK-resistant strains of *Mycobacterium smegmatis*, *M. bovis*, and MTB.

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H37Rv served as the wild-type strain of MTB susceptible to all drugs. ATCC 35827 is an in vitro mutant of H37Rv resistant to KM. Aminoglycoside-resistant mutants of H37Rv were selected on Middlebrook 7H10 agar plates containing SM and AK at 1, 2, 4, and 8 μg/ml. Inoculum titers were determined by plating diluted aliquots of cells onto drug-free agar. Seventeen clinical isolates of MTB resistant to various antituberculosis agents, including AK and KM, were obtained from PHRI TB Center, New York, N.Y. Testing of susceptibility to AK, KM, and SM was performed twice by the proportional method (11). Susceptibilities of clinical isolates to other drugs were determined in various clinical laboratories. Resistance to AK, KM, or SM was defined as an MIC of >2 μg/ml. Resistance to other antituberculosis agents was as described elsewhere (11, 20).

Chromosomal DNA from each MTB strain was genotyped by using a standardized Southern blot hybridization method based on the insertion sequence IS6110 (22). DNA fingerprint patterns were compared by using a scanning densitometer with the BioImage Whole Band Analyzer software (version 3.3), and the strains were catalogued as described elsewhere (12). PCR amplification of the genomic DNA was performed with primers ML51 and ML52 for the *rpsL* gene (306-bp product) (9). Flanking primers RRS30 (GGCTCCCTTTTCCAAAGG GAG) and RRS1539 (GGGGCGTTTTGCTGGTGCTCC) were used to amplify the entire *rrs* gene (1,589-bp product) (10), or primers RRS1096 (GCGCAACCCTTGTCTCATGT TG) and RRS1539 were used to amplify just the 1400 region (464-bp product) of this gene (10). Amplification was carried out for 40 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) using *Taq* polymerase. PCR products were cloned (18) with the pGEM-T vector system (Promega). Plasmid DNA of selected clones was sequenced by using T7 DNA polymerase (Sequenase 2.0; USB). Analysis of nucleotide sequences was performed with PC Gene software (IntelliGenetics). PCR amplification of DNA from each isolate was done in duplicate, and each product was sequenced. Numbering of nucleotides was based on the MTB *rpsL* and *rrs* genes (9, 10).

Mutants of parental strain H37Rv (MICs of AK, KM, and SM = 1 μg/ml) appeared at a frequency of 2×10^{-6} on agar containing 2 μg of AK or SM per ml. At 4 μg/ml of either drug, the frequency was 2×10^{-8}. No mutants ($<10^{-9}$) were obtained at 8 μg of AK or SM per ml.

Strain ATCC 35827 (Table 1) and strains A2B and A4B selected from H37Rv at 2 and 4 μg of AK per ml, respectively, displayed resistance to both AK and KM, but only modest cross-resistance to SM. The 17 clinical isolates (Table 1) included 14 strains differentiated on the basis of IS6110 DNA fingerprinting and displayed either low-level (MIC, 4 to 64 μg/ml) (four isolates) or high-level (MIC, >256 μg/ml) (13 isolates) cross-resistance to AK and KM. All 17 isolates were resistant to SM. Phenotypic differences in drug susceptibility among isolates within strain designations W and W1 may indicate these isolates are possibly different strains.

AK-resistant mutants A2B and A4B had no mutations in either the *rpsL* or *rrs* gene. However, strain ATCC 35827,

* Corresponding author. Mailing address: Division of Infectious Diseases, Wayne State University, School of Medicine, Suite 2140, 4160 John R, Detroit, MI 48201. Phone: (313) 745-9131. Fax: (313) 993-0302. E-mail: galangaden@oncgate.roc.wayne.edu.

TABLE 1. Drug susceptibility profile of amikacin-kanamycin-resistant laboratory strains and clinical isolates of *M. tuberculosis* and their mutations in the *rrs* gene

Laboratory strain and clinical isolate of *M. tuberculosis*[a]	Aminoglycoside susceptibility (MIC in μg/ml)			Drug susceptibility profile[b]								Mutation in the *rrs* gene[c]
	AK	KM	SM	INH	RIF	EMB	PZA	ETH	CIP	CAP	CYC	
H37Rv (wild type)	1	1	1	S	S	S	S	NA	S	NA	NA	None
ATCC35827 (in vitro KM^r mutant)	>128	>128	8	S	S	S	S	NA	S	NA	NA	A1400G
A2B (in vitro AK^r mutant)	128	>128	4	S	S	S	S	NA	S	NA	NA	None
A4B (in vitro AK^r mutant)	64	128	4	S	S	S	S	NA	S	NA	NA	None
TN758 (P)	8	4	R	R	R	S	NA	R	S	R	R	None
TN933 (CB)	8	4	R	R	R	R	NA	NA	NA	NA	NA	None
TN2793 (C)	32	32	R	R	R	R	R	R	S	R	R	None
TN718 (AY)	64	32	R	R	R	R	R	R	S	S	S	None
TN4728 (BN)	>256	>256	R	R	S	S	NA	S	S	R	S	A1400G
TN810 (001/19)	>256	>256	R	R	R	R	R	S	S	S	S	A1400G
TN1521 (BU)	>256	>256	R	R	R	R	NA	S	S	S	S	A1400G
TN2229 (N2)	>256	>256	R	R	R	R	NA	S	S	R	S	A1400G
TN2508 (001/16)	>256	>256	R	R	R	R	NA	S	S	S	S	A1400G
TN4826 (H)	>256	>256	R	R	R	R	NA	S	S	R	S	A1400G
TN3360 (W)	>256	>256	R	R	R	R	NA	S	S	S	S	A1400G
TN3432 (W)	>256	>256	R	R	R	R	NA	R	R	R	S	A1400G
TN3187 (W1)	>256	>256	R	R	R	R	S	R	S	S	S	A1400G
TN3605 (W1)	>256	>256	R	R	R	R	NA	R	R	R	S	A1400G
TN3806 (W1)	>256	>256	R	R	R	R	NA	S	NA	NA	NA	A1400G
TN3218 (W3)	>256	>256	R	R	R	R	NA	S	S	S	S	A1400G
TN4800 (W33)	>256	>256	R	R	R	R	NA	R	S	R	S	A1400G

[a] Clinical isolates are indicated by the prefix TN and have the genotyped strain designation in parentheses.
[b] INH, isoniazid; RIF, rifampin; EMB, ethambutol; PZA, pyrazinamide; ETH, ethionamide; CIP, ciprofloxacin; CAP, capreomycin; CYC, cycloserine; S, susceptible; R, resistant; NA, not available. The drug concentrations tested were as described in the text.
[c] The entire *rrs* gene of H37Rv and the in vitro mutants were sequenced. Only region 1119 to 1536 of the *rrs* gene of the clinical isolates was sequenced. The numbering of base 1400 refers to *M. tuberculosis* (11).

which is resistant to KM, displayed an A1400G mutation in the *rrs* gene (10) and had the wild-type *rpsL* gene. The same A1400G mutation was noted in all 13 clinical isolates with MICs of AK-KM of >256 μg/ml. None of our four clinical isolates with MICs of AK-KM of ≤64 μg/ml displayed any mutation in the 1400 region.

Mutants of strain H37Rv resistant to SM or AK appeared at frequencies of 2×10^{-6} and 2×10^{-8} at concentrations two and four times the parental MICs, respectively. The magnitude of frequencies for both SM (streptidine drug) and AK (deoxystreptamine drug) suggests that resistance to each subclass of aminoglycoside results from a single mutation. We found no mutants of H37Rv in the presence of SM and AK at 8 μg/ml, yet mutants A2B and A4B, selected with 2 and 4 μg of AK per ml, respectively, displayed MICs of AK of 64 to >128 μg/ml. The reason for our failure to obtain mutants by using 8 μg of AK per ml is not clear.

Mutants and clinical isolates displayed cross-resistance to AK and KM. The fourfold rise in MICs of SM among the AK-KM-resistant mutants is comparable to that noted in KM-resistant MTB (21). The SM resistance in many of the clinical isolates is associated with a mutation in the *rpsL* gene that produces a Lys-43-Arg mutation in the S12 ribosomal protein (4). The presence of the A1400G mutation in these isolates might contribute in part to the observed SM resistance.

Strain ATCC 35827, with high-level resistance to only AK-KM, had an A1400G mutation of the *rrs* gene of the 16S rRNA (10) (corresponding to position 1408 in the *E. coli rrs* gene) (5, 15). All 13 clinical isolates (10 different strains) with MICs of AK-KM of >256 μg/ml had this mutation. Ribosomal binding of KM in *E. coli* occurs in the 1400 region (15), and methylation of adenine at this position (1408) in *Streptomyces tenjimariensis* results in resistance to KM and apramycin (3, 5). MTB has only a single copy of the *rrs* gene (10), so such a point mutation results in resistance to AK-KM. A recent report (19) supports these findings. The same A-to-G mutation at position 1408 (*E. coli* numbering) was present in in vitro mutants of *M. smegmatis* and *M. bovis* resistant to AK, gentamicin, and tobramycin (MICs, >500 μg/ml) and in eight AK-KM-resistant clinical isolates of MTB (19). Allelic exchange experiments in an *M. smegmatis* mutant harboring a single rRNA operon demonstrated that the A1408G mutation confers resistance to AK, gentamicin, and tobramycin (19). Therefore, it seems that high-level resistance to both AK and KM in our MTB isolates results from a point mutation in the 1400 position of the *rrs* gene.

Resistance to AK-KM apparently arises also from a mutation(s) in another gene(s), as in mutants A2B and A4B (MICs of AK-KM of 64 to >128 μg/ml). A similar mechanism or another mechanism(s) may account for AK-KM resistance in the four clinical isolates with MICs of AK-KM of ≤64 μg/ml and no mutations in the 1400 region of the *rrs* gene, although we have not ruled out mutation in the other 73% of the nucleotide sequence of the gene that was not examined.

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