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Characterization of *rpsL* and *rrs* Mutations in Streptomycin-Resistant *Mycobacterium tuberculosis* Isolates from Diverse Geographic Localities

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Two genes (*rpsL* and *rrs*) with mutations associated with streptomycin resistance in *Mycobacterium tuberculosis* were characterized in 78 streptomycin-resistant and 61 streptomycin-susceptible isolates recovered from patients living in the United States, South America, Europe, Africa, and Asia. Fifty-four percent of the 78 resistant organisms had missense mutations in codon 43 of *rpsL* resulting in a K-43→R substitution. Mutations in codon 88 of *rpsL* were also identified in four Asian isolates.

It is estimated that 1.8 billion people worldwide are infected with *Mycobacterium tuberculosis* (18). In addition to an increase in the number of United States tuberculosis cases (4), the proportion of drug-resistant organisms has also increased (3). Bacteria cultured from 13.4% of new tuberculosis cases are resistant to at least one primary antituberculosis drug. In addition, 5.6% of new cases and 7.7% of recurrent tuberculosis cases are due to organisms that are resistant to streptomycin (3).

Streptomycin, an aminocyclitol glycoside, is commonly used in tuberculosis treatment. The drug binds to the 16S rRNA, interferes with translation proofreading, and thereby inhibits protein synthesis (2, 9). Mutations associated with streptomycin resistance have been identified in the genes encoding 16S rRNA (*rrs*) (6) and ribosomal protein S12 (*rpsL*) (7, 8, 10–12, 14, 16). Ribosomal protein S12 stabilizes the highly conserved pseudoknot structure formed by 16S rRNA (17). Amino acid substitutions in RpsL affect the higher-order structure of 16S rRNA (2) and confer streptomycin resistance. Alterations in the 16S rRNA structure disrupt interactions between 16S rRNA and streptomycin, a process that results in resistance (5).

Despite progress in identification of *rpsL* and *rrs* mutations in streptomycin-resistant *M. tuberculosis*, susceptible and resistant isolates from many geographically diverse localities have not yet been studied. Therefore, the goal of the present study was to determine the frequency and geographic distribution of *rpsL* and *rrs* mutations among streptomycin-susceptible and -resistant *M. tuberculosis* isolates.

Strains and streptomycin susceptibility testing. One hundred thirty-nine *M. tuberculosis* strains obtained from patients in different geographic localities were investigated (Table 1). DNA was isolated from bacteria grown in liquid or on solid media as described previously (7) and shipped to the laboratory of J.M.M. for sequencing. Mycobacterial culturing and DNA isolation were conducted in laboratories equipped with biosafety level 3 facilities. Isolates from Japan and Yemen were tested for streptomycin susceptibility by the proportion method using Ogawa egg-based medium (similar to Lowenstein-Jensen medium). Two drug concentrations were tested (20 and 200 μg/ml), and the critical proportion of growth was 1%. Susceptibility to 20 μg of streptomycin per ml, as assessed with this testing strategy, has been shown to correlate with a favorable clinical response, and this is the standard technique used for susceptibility testing in Japan (1). All other isolates were tested for streptomycin susceptibility with either the BACTEC radiorespiratory method with Middlebrook 7H12 medium (6 μg of drug per ml) or the proportion method with Middlebrook 7H10 medium (2 and 10 μg/ml).

Automated DNA sequencing of the *rpsL* and *rrs* genes. The strategies used to amplify and sequence the entire open reading frame of *rpsL* and a 1,042-bp segment of *rrs* have already been described in detail (12). The oligonucleotide primers used to amplify *rpsL* were as follows: forward, 5′-GGCCGACAAACAGAACGT-3′; reverse, 5′-GTTCACCAACTGGGTGAC-3′. Those used for *rrs* were as follows: forward, 5′-GAGAGTTTGATCCTGGCTCAG-3′; reverse, 5′-TGCACACAGGCCACAAGGGA-3′. The 1,042-bp segment of *rrs* encompasses two regions with resistance-associated mutations (15). An automated DNA sequencing instrument (model 373A; Applied Biosystems, Inc., Foster City, Calif.) was used to characterize the regions of *rpsL* and *rrs* studied. Sequences were compared with those in the GenBank database under accession numbers L08011 for *rpsL* and X52917 for *rrs*. The data were assembled and edited with the EDITSEQ, MEGALIGN, and SEQMAN programs (DNASTAR, Madison, Wis.).

***rpsL* mutations in streptomycin-resistant isolates.** Streptomycin resistance in *M. tuberculosis* is associated with substitution of amino acid 43 or 88 of ribosomal protein S12. Amino acid 43 and 88 replacements were identified in strains from widespread geographic localities. For example, four of eight resistant isolates from Japan had *rpsL* changes, including two with codon 43 mutations (AAG→AGG; K-43→R) and two with codon 88 substitutions (AAG→CAG; K-88→Q). In addition, one isolate each from Yemen and Peru had a codon 43 mutation. Consistent with previously published data (10), or-

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TABLE 1. Characteristics of 78 streptomycin-resistant and 61 streptomycin-susceptible *M. tuberculosis* isolates

Source	No. of isolates	No. resistant to streptomycin	No. with mutation[a]		Mutation position(s)	
			rpsL[b]	*rrs*[c]	*rpsL* codon	*rrs* nucleotide
Asia						
Hong Kong	10	1	1	ND[d]	9, 43	
Philippines	3	0	0	0		
Japan	10	8	4	2	43, 88	876, 904
Vietnam	1	1	1	ND	88, 93	
Africa (Rwanda)	14	11	0	0		
Europe (Belgium)	1	0	0	0		
Middle East (Yemen)	6	5	1	1	43	906
North America						
New York	61	43	34	3	43	491, 512, 798
Texas	27	6	0	2		513, 516
South America (Peru)	6	3	1	ND	43	
Total	139	78	42	8		

[a] All isolates with mutations were resistant to streptomycin.
[b] Gene encoding ribosomal protein S12.
[c] Gene encoding 16S rRNA.
[d] ND, not done; *rrs* was not sequenced in these isolates.

ganisms with codon 43 mutations were resistant to streptomycin at >200 μg/ml. Two isolates were unusual because they had two distinct *rpsL* mutations. One isolate from Vietnam had both a codon 88 mutation (AAG→AGG; K-88→R) and a codon 93 substitution (GTG→ATG; V-93→M), and a Hong Kong isolate had a codon 9 substitution (CGC→CAC; R-9→H) and a codon 43 mutation (AAG→AGG; K-43→R). In the aggregate, 42 (54%) of the 78 resistant organisms had missense mutations in codon 43 or 88.

On the basis of studies conducted with *Escherichia coli*, ribosomal protein S12 interacts with the 530 loop of 16S rRNA and maintains the higher-order structure of 16S rRNA. Changes in the amino acid sequence in RpsL alter the RpsL-16S rRNA interaction and result in streptomycin resistance. In addition to confirming the association of amino acid 43 and 88 substitutions with streptomycin resistance in strains from intercontinental sources, we also identified nonsynonymous changes in codons 9 and 93. However, because the organisms with amino acid 9 and 93 mutations also had commonly occurring codon 43 and 88 mutations, respectively, the significance of these paired amino acid changes in streptomycin-resistant *M. tuberculosis* is unknown.

***rrs* mutations in streptomycin-resistant isolates.** Alterations in the 530 loop or 915 region of *rrs* are also associated with streptomycin resistance. Specifically, point mutations located at positions 491, 512, 513, and 516 (7, 13) and 904 and 905 (6, 11, 13) have been found in streptomycin-resistant *M. tuberculosis*. Of the 78 streptomycin-resistant isolates examined, 8 (10%) had *rrs* mutations and these nucleotide changes were found at position 491 (C→T), 512 (C→T), 513 (A→T), 798 (C→T), 877 (G→A), 904 (A→G), or 906 (A→C). The mutations located at positions 798, 877, and 906 in the 915 region of *rrs*, a streptomycin-binding site on 16S rRNA, have not been previously identified in *M. tuberculosis*. Hence, our data confirm and extend the association of 530 loop and 915 region mutations with streptomycin resistance in *M. tuberculosis*. Although this analysis identified several previously undescribed mutations in resistant organisms, their association with streptomycin resistance can only be inferred.

Only one New York isolate among the 78 resistant bacteria had mutations in both *rrs* (798; C→T) and *rpsL* (AAG→AGG; K-43→R). Inasmuch as each mutation alone is associated with resistance, the significance of this double substitution is unclear. However, Meier et al. (13) also identified one strain with a mutation in both *rrs* (A→G at nucleotide 904) and *rpsL* (AAG→CAG; K-88→Q), and the MIC of streptomycin for this strain was unusually high (60 μg/ml).

***rpsL* and *rrs* in streptomycin-susceptible isolates.** None of the 61 streptomycin-susceptible isolates examined had alterations in *rpsL* or *rrs*.

The finding that *rpsL* and *rrs* mutations were present in 54 and 10%, respectively, of resistant organisms is closely consistent with data published by Heym et al. (10) and Morris et al. (14), who found point mutations in *rpsL* (52 and 56.8%, respectively) and *rrs* (8 and 15.6%, respectively) in resistant strains. Currently available strategies for antimicrobial agent susceptibility testing are culture based and frequently require many weeks to identify the resistance pattern. Rapid and accurate antimicrobial susceptibility testing is critical for the treatment of infected patients and for limiting pathogen spread to others. The demonstration that about two-thirds of streptomycin-resistant *M. tuberculosis* isolates can be identified by using DNA sequence data from both *rpsL* and *rrs* and the lack of mutations in susceptible organisms suggest that this approach may have clinical utility.

Our data confirm that 25 to 35% of streptomycin-resistant isolates have the identical wild-type sequence encoding ribosomal protein S12 and the region of the 16S rRNA gene characterized (7, 11, 14). This observation indicates that additional mechanisms mediating streptomycin resistance remain to be discovered. It is possible that cell envelope permeability changes, production of aminoglycoside-modifying enzymes, and alterations in other ribosomal molecules may be responsible for drug resistance in *M. tuberculosis* organisms without

rpsL or *rrs* changes. The availability of well-defined resistant strains lacking *rpsL* or *rrs* substitutions will be valuable in the search for alternate resistance mechanisms.

In conclusion, our data strongly confirm and extend the findings of other investigators (6, 7, 10–14, 16) that point mutations in *rpsL* and *rrs* are associated with streptomycin resistance in a majority of *M. tuberculosis* isolates. More studies are needed to identify additional mechanisms conferring streptomycin resistance in this pathogen.

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