Identification of Mutations Related to Streptomycin Resistance in Clinical Isolates of *Mycobacterium tuberculosis* and Possible Involvement of Efflux Mechanism^{∇†}

Fernanda S. Spies,^{1,2} Pedro E. Almeida da Silva,³ Marta O. Ribeiro,²
Maria Lucia Rossetti,^{2,4} and Arnaldo Zaha^{1,5*}

Programa de Pós-graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil¹;
Fundação Estadual de Produção e Pesquisa em Saúde do Estado do Rio Grande do Sul, Porto Alegre, Brazil²; Programa de
Pós-Graduação em Ciências da Saúde, Universidade Federal do Rio Grande, Campus da Saúde, Rio Grande, Brazil³;
Programa de Pós-graduação em Ciências em Genética e Toxicologia Aplicada-Universidade Luterana do Brasil,
Canoas, Brazil⁴; and Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul,
Porto Alegre, Brazil⁵

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The MIC for streptomycin in the presence of efflux pump (EP) inhibitors and the sequencing of *rpsL*, *rrs*, and *gidB* genes provided evidence for the possible participation of EP in low-level streptomycin (STR) resistance of some isolates without mutations. Mutation in the *gidB* gene and an EP could act synergistically to confer low STR resistance.

Streptomycin (STR), the first antibiotic used to control tuberculosis, acts by binding to the 30S ribosomal subunit, inhibiting polypeptide synthesis (5, 24). Mutations in genes *rpsL* and *rrs*, encoding the ribosomal protein S12 and the 16S rRNA, respectively, are responsible for most of the high-level STR resistance in *Mycobacterium tuberculosis* (7, 9, 24). However, a low level of resistance is found in approximately one-third of the clinical isolates resistant to STR without mutations in these genes (7, 11, 17). Recently, a new STR resistance locus (*gidB*) that encodes a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA was found to confer a low level of STR resistance (12).

Also possibly involved with a low level of STR resistance is the efflux system. This system can grant to bacteria a mechanism which favors their survival in a hostile environment (e.g., the presence of antibiotics), and those bacteria that overexpress efflux pumps (EPs) can be selected (23). It has been shown that the *M. tuberculosis* genome encodes multiple putative EPs (2, 6), and reports have suggested that EPs may also be involved in transporting fluoroquinolones, aminoglycosides, tetracycline, and possibly isoniazid and ethambutol (4, 14, 18, 22).

In this study we have evaluated the possible role of the efflux mechanism as a molecular basis of STR resistance in clinical isolates of *M. tuberculosis*, using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and verapamil as efflux pump inhibitors (EPI) (1, 10).

A total of 79 clinical isolates from the state of Rio Grande do Sul, Brazil, were studied. Resistance to drugs other than

STR was found in 62 isolates (see Table S1 in the supplemental material). The resazurin microtiter assay (13) was used for MIC determination. Microplates were divided into three equal parts: one with 7H9-oleic acid-albumin-dextrose-catalase medium, one with medium and verapamil (100 μ M), and one with medium and CCCP (11 μ M). Serial 1:2 dilutions of STR (250 μ g/ml to 0.25 μ g/ml) were performed in each column. The breakpoint to determine STR resistance was a MIC of $\geq$ 8 μ g/ml.

DNA was isolated from mycobacterial cultures by the lysozyme/proteinase K cetyltrimethylammonium bromide procedure (21). A 306-bp fragment of the *M. tuberculosis* *rpsL* gene (GenBank accession number L08011) and the 530 loop (238 bp) and 912 region (238 bp) of the *rrs* gene (GenBank accession number X52917) were amplified as described by Tracevska et al. (20). A 675-bp fragment of the gene *gidB* (GenBank accession number AAK48404) was amplified using primers *gidB*1 (5'-GTCCCTCCACTCGCCATC3') and *gidB*2 (5'-GCGGAGTGCCTAATGTCTC3'). PCR amplification was performed by the following steps: initial denaturation at 92°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1.5 min; and a final extension at 72°C for 5 min. PCR was performed using 30 pmol of each primer, with 2.5 U of *Taq* polymerase (CBIOT, UFRGS, Brazil), 200 μ M of each deoxynucleoside triphosphate, and 1.5 mM magnesium chloride. Sequencing was performed in the ABI Prism 3100 DNA sequencer (Applied Biosystems) and MegaBACE 1000 DNA analysis system (GE Healthcare Life Sciences). Nucleotide sequences were analyzed using the STADEN package. Nucleotide sequences with Phred values >20 were considered for analysis.

According to the MIC, 47 *M. tuberculosis* isolates were classified as STR resistant and 32 isolates as susceptible. To test the effect of the EPI on the STR MIC, verapamil and CCCP were separately added to the medium. In the presence of verapamil, 36 isolates (48%) had decreased MICs. In the pres-

* Corresponding author. Mailing address: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Rua Bento Gonçalves 9500, Prédio 43421, CEP 91501-970 Porto Alegre, RS, Brazil. Phone: 55 51 33086054. Fax: 55 51 33087309. E-mail: zaha@cbiot.ufrgs.br.

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ence of CCCP, 10 isolates (13%) had decreased MICs. For eight isolates (10%) the MIC was decreased by both inhibitors. The remaining 41 isolates (52%) exhibited no difference of MIC in the presence of the inhibitors (see Table S1 in the supplemental material).

The nucleotide sequences of the *rpsL*, *rrs*, and *gidB* genes were determined for all *M. tuberculosis* isolates included in the present work (see Table S1 in the supplemental material). We identified mutations in codons 43 (AAG→AGG; K43→R) and 88 (AAG→CAG; K88→Q) of the *rpsL* gene sequence as the most common mutations related to STR resistance due to *rpsL* (3, 8, 19). The other mutation found in the *rpsL* gene was a silent mutation in codon 81 (CTG→TTG; L81→L). Nine isolates presented multiple mutations in the *rpsL* gene sequence (90% silent mutations) (3) and did not have high-level STR resistance (see Table S1 in the supplemental material). In these nine isolates the few mutations that cause amino acid changes were not among the mutations described as related to STR resistance. It is possible that isolates with multiple mutations may have, primarily, alterations in genes involved in DNA repair (*mut* genes) (15). With the exception of isolates with multiples mutations, all isolates with mutations in *rpsL* were highly resistant to STR (MIC ≥ 250 µg/ml).

The following mutations were observed in the *rrs* genes of STR-resistant isolates: G to C at position 426 (426 G→C), 491 C→T (16), 513 A→C, 513 A→T, 516 C→T (20), and 905 A→G. The last one has not been described previously. The mutation 461 C→T was found in the *rrs* gene of only one STR-susceptible isolate and probably is not involved in STR resistance (3).

For the *gidB* gene, 58 (73%) isolates presented nucleotide mutations (see Table S1 in the supplemental material), which is in agreement with the data presented by Okamoto et al. (12). In the majority of the clinical isolates studied (49%, 39/79) an amino acid substitution due to mutation of *gidB* (CCT→CGT; L16→R) was observed. This substitution was also observed in STR-susceptible isolates without mutations in genes *rpsL* and *rrs*. Consequently, we do not consider this alteration related to STR resistance. The G102 deletion mutation and the mutation involving the deletion of GAGCCGAGGAG353, as well as the groups of mutations consisting of TGG→TGC (W45→C), GTG→GTT (V110→V), and TGG→CGG (W148→R) and of CCG→TCG (P75→S), GTG→GTT (V110→V), and GCG→GCA (A141→A), each occurred in one isolate (5%, 4/79) with a low level of STR resistance without alterations in the other studied genes (see Table S1 in the supplemental material).

Among the 38 *M. tuberculosis* isolates that presented decreased MICs in the presence of EPI, 27 (71%) had mutations in the *rpsL*, *rrs*, and/or *gidB* gene sequences, 11 (28%) had no mutations, 22 were resistant to STR, and 16 were susceptible (Table 1). Of the 41 *M. tuberculosis* isolates that had no decreased MIC in the presence of EPI, 34 (82%) carried mutations in the gene sequences analyzed, 7 (17%) presented no mutations, 25 were resistant to STR, and 16 were susceptible (Table 1).

To compare the types of treatment (medium, medium plus verapamil, and medium plus CCCP), the nonparametric Kruskal-Wallis statistical analysis was performed. The test was applied in three distinct groups: all of the isolates studied, only

TABLE 1. Overall profile of mutations within the *rpsL*, *rrs*, and *gidB* genes from *M. tuberculosis* clinical isolates and of STR MICs in the presence of EPIs

STR susceptibility ^a	Decreased MIC with EPI	Total no. of isolates	No. of isolates with:			
			Mutations in:			No mutations
			Only <i>gidB</i> ^b	Only <i>rpsL</i> and/or <i>rrs</i>	<i>gidB</i> + <i>rpsL</i> + <i>rrs</i>	
R	Yes	22	4	11	2	5
S	Yes	16	8	2	0	6
R	No	25	0	18	6	1
S	No	16	6	4	0	6
Total		79	18	35	8	18

^a R, resistant; S, susceptible. Susceptibility is based on MICs.

^b The L16R mutation in *gidB* of 32 isolates was not considered for the analysis.

the mutated isolates (for *gidB*, only mutated resistant isolates were included), and only the isolates without mutations ($P < 0.001$). To identify the differences between the groups, the treatments were tested two by two and a chi-square test was performed. The P values for all comparisons were statistically significant. Among the 79 *M. tuberculosis* isolates analyzed in this study, 38 (48%) had decreased MICs when in the presence of EPI and this decrease occurred in both resistant and susceptible isolates, with or without mutations ($P < 0.001$). An important finding was the detection of five isolates (indicated in Table S1 in the supplemental material) with a low level of resistance that did not present any mutations in the *rpsL*, *rrs*, and *gidB* gene sequences but showed decreased MICs only in the presence of EPI. Another interesting result was the observation of four isolates that had low-level STR resistance (indicated in Table S1 in the supplemental material) with mutations only in the *gidB* gene sequence, reinforcing the relation of mutation in this gene to a low level of STR resistance (12). However, the mutations found are not identical to those previously described. The MICs for all four isolates were also diminished in the presence of inhibitors, indicating that both the *gidB* mutation and an EP could act synergistically to confer low STR resistance. Additional experiments are needed to elucidate this aspect.

The results presented in this work suggest that EPs could be involved in STR resistance in *M. tuberculosis*. However, further experimental work is required to determine the types of pumps involved and regulation of the expression of the genes involved.

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