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Streptomycin Resistance and Lineage-Specific Polymorphisms in *Mycobacterium tuberculosis gidB* Gene▽

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Mutations related to streptomycin resistance in the *rpsL* and *rrs* genes are well known and can explain about 70% of this phenotypic resistance. Recently, the *gidB* gene was found to be associated with low-level streptomycin resistance in *Mycobacterium tuberculosis*. Mutations in *gidB* have been reported with high frequency, and this gene appears to be very polymorphic, with frameshift and point mutations occurring in streptomycin-susceptible and streptomycin-resistant strains. In this study, mutations in *gidB* appeared in 27% of streptomycin-resistant strains that contained no mutations in the *rpsL* or *rrs* genes, and they were associated with low-level streptomycin resistance. However, the association of certain mutations in *gidB* with streptomycin resistance needs to be further investigated, as we also found mutations in *gidB* in streptomycin-susceptible strains. This occurred only when the strain was resistant to rifampin and isoniazid. Two specific mutations appeared very frequently in this and other studies of streptomycin-susceptible and -resistant strains; these mutations were not considered related to streptomycin resistance, but as a polymorphism. We stratified the strains according to the different phylogenetic lineages and showed that the *gidB*^{16} polymorphism (16G allele) was exclusively present in the Latin American-Mediterranean (LAM) genotype, while the *gidB*^{92} polymorphism (92C allele) was associated with the Beijing lineage in another population. In the sample studied, the two characterized single-nucleotide polymorphisms could distinguish LAM and Beijing lineages from the other lineages.

Acquired drug resistance in *Mycobacterium tuberculosis* arises from spontaneous chromosomal mutations. Clinical drug-resistant tuberculosis (TB) occurs when these genetic alterations are selected for during disease treatment. This can occur via erratic drug supply, suboptimal physician prescription, and poor patient adherence (34).

The aminoglycoside antibiotic streptomycin (STR) was the first antibiotic used to control TB. It interacts directly with the small ribosomal subunit 16S rRNA and interferes with translational proofreading, thereby leading to inhibition of protein synthesis (14, 33). Mutations associated with high-level STR resistance in *M. tuberculosis* have been identified in the genes encoding ribosomal protein S12 (*rpsL*) and 16S rRNA (*rrs*) (9). However, mutations in these two genes explain only about 70% of the cases of STR resistance in clinical isolates (22, 26), implying that there must be other loci involved or another mechanism for STR resistance.

Recently, it was shown that mutations in the gene encoding a 7-methylguanosine (m7G) methyltransferase (*gidB*) specific for the 16S rRNA resulted in low-level STR resistance (20). However, in clinical isolates of *M. tuberculosis*, mutations in *gidB* have been observed in strains that are both susceptible and resistant to STR (20, 25).

Genotyping of *M. tuberculosis* strains is useful to answer evolutionary questions and for surveying its transmission dynamics in epidemiological studies (2). In addition, molecular typing improves our understanding of the basic biology of bacterial pathogens, including differences in virulence and transmissibility and the variable effectiveness of vaccines (7). Spoligotyping is a PCR-based genotyping technique that exploits the variability of the direct repeat (DR) region in *M. tuberculosis* (16). Since 1999, genetic diversity databases have been organized for the *M. tuberculosis* complex DR locus as an attempt to analyze population structure and to assess the complexity of global TB transmission underlying the spatial and temporal evolution of the TB genetic landscape (3). The accumulated data demonstrate that a few major lineages of conserved spoligotypes are well distributed throughout the world, whereas others are specific for certain geographic regions (3, 24).

Single-nucleotide polymorphisms (SNPs) are the most robust and appropriate phylogenetically informative markers (7). Therefore, in recent years, some authors have proposed SNPs in phylogenetic studies (2, 8, 12). Some SNPs have been linked to specific *M. tuberculosis* phylogenetic lineages, such as an SNP in the *mgtC* gene (R182H) that can differentiate between Haarlem and non-Haarlem lineages (1), the variation in Rv2629 (N64A), which is found exclusively in lineages

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from Beijing (5, 13), and an SNP in *fadD28* codon 507, which acts as a specific marker for the East Asia lineage (6).

In the present study, we investigated mutations in the *gidB* gene in clinical isolates representative of the genetic diversity of *M. tuberculosis* to explore its possible involvement in STR resistance in strains from southern Brazil. Furthermore, we stratified the strains according to the different phylogenetic lineages and showed that the *gidB*[16] polymorphism (16G allele) was exclusively present in the Latin American-Mediterranean (LAM) genotype and that the *gidB*[92] polymorphism (92C allele) was associated with the Beijing lineage in another population.

MATERIALS AND METHODS

Strains. One hundred six *M. tuberculosis* strains originating from the south of Brazil were isolated between 2005 and 2006 at LACEN-RS (Central Laboratory of Rio Grande do Sul State, Brazil), and 22 *M. tuberculosis* strains of Beijing genotype were acquired from the collection at the Institute of Tropical Medicine (ITM) in Belgium.

MIC determinations. The resazurin microtiter assay (REMA) was used for MIC determinations (21). Briefly, 96-well plates were filled with 7H9-oleic acid-albumin-dextrose-catalase medium. Serial 1:2 dilutions of STR (125 μg/ml to 0.12 μg/ml), rifampin (RIF; 16 μg/ml to 0.03 μg/ml), and isoniazid (INH; 12.8 μg/ml to 0.01 μg/ml) were performed in each column. The breakpoint used to determine STR resistance was an MIC of ≥1 μg/ml; INH and RIF resistance was considered as an MIC of ≥0.25 μg/ml (19).

DNA extraction and sequencing. DNA was isolated from mycobacterial cultures by a lysozyme/proteinase K cetyltrimethylammonium bromide procedure (28). A 306-bp fragment of the *M. tuberculosis rpsL* gene (GenBank accession number L08011) and the 530 region (238 bp) and 912 region (238 bp) of the *rrs* gene (GenBank accession number X52917) were amplified as described by Tracevska et al. (27), and a 675-bp fragment of the gene *gidB* (GenBank accession number AAK48404) was amplified as described by Spies et al. (25). Amplification products were sequenced using the ABI Prism 3100 DNA sequencer (Applied Biosystems) and MegaBACE 1000 DNA analysis system (GE Healthcare Life Sciences). Nucleotide sequences were analyzed using the programs PREGAP and GAP4 of the STADEN software package ver. 10.0. Nucleotide sequences with Phred values of >20 were considered for analysis.

Genotyping. Spoligotyping was performed using a commercial kit (Isogen Biosciences B.V., Netherlands) according to the manufacturer's instructions. Spoligopatterns obtained were entered in a binary format as Excel spreadsheets (Microsoft, Redmond, WA) and compared to information in the SITVIT database (http//www.pasteur-guadaloupe.fr:0881/SITVITDemo/) (3).

LAM-specific PCR. The strains were further characterized by a LAM-specific PCR to classify strains that could not be assigned to an internationally recognized genotype lineage and to confirm the spoligotyping result. In this assay, PCR primers identified the presence of an IS*6110* insertion (position 932204 according to the whole H37Rv genome sequence), which is unique to all members of the LAM lineage (17). PCR amplification products were electrophoretically fractionated in a 3.0% agarose gel at 85 V for 2 h. The presence of a LAM strain was represented by a band of 205 bp, while a non-LAM strain was represented by a band of 141 bp (17).

RESULTS

Mutations in the *rpsL*, *rrs*, and *gidB* genes and STR resistance. To analyze if mutations in the *gidB* gene were related to STR resistance, we compared the mutations present in resistant and susceptible strains with sequencing data from *rpsL* and *rrs*, genes known to be related to STR resistance.

Among the 40 STR-resistant strains, 10 highly STR-resistant strains (MIC ≥ 125 μg/ml) presented only the mutation in codon 43 (AAG→AGG; K43R) of the *rpsL* gene. One strain highly resistant to STR presented a mutation in codon 88 (AAG→AGG; K88Q) of the *rpsL* gene and a mutation in codon 183 of the *gidB* gene (GCG→ACG; A183T), while another strain highly resistant to STR contained a silent mutation in codon 81 of the *rpsL* gene (CTG→TTG; L81L), a frameshift mutation in codon 117 of the *gidB* gene (nucleotide 350 G insertion) and a mutation in position 905 of the *rrs* gene (Table 1).

Three resistant strains presented a single C→T mutation in position 513 of the *rrs* gene, and one strain contained a C→G mutation in position 904. The C→T mutation in position 491 of the *rrs* gene appeared in four low-level resistant strains: three without mutations in the other studied genes and one with a mutation in codon 200 of the *gidB* gene (GCG→GAG; A200E) (Table 1). This mutation in position 491 of the *rrs* gene is also present in two STR-susceptible strains, one of which also contains a mutation in codon 115 (GTG→GGG; V155G) of the *gidB* gene (Table 2).

Ten strains with low-level STR resistance presented different mutations in the *gidB* gene (TGG→TCG [W45S], CCG→CTG [P84L], CAT→TAT [H48Y], GGT→CGT [G30R], TGC→TGA [C52stop], 51 AAC→ACC [N51T], 39 frameshift; TTG→TTT [L79F], CTA→CCA [L49P], 164 GGC→TGC [G164C]), and one with intermediate-level resistance presented the mutation GGG→GAG (G117E) (Table 1). Nine STR-resistant strains presented no mutations in the fragments of the genes studied (STR MICs from 8 to 2 μg/ml).

Of the 66 STR-susceptible strains, 60 did not present any mutation in the DNA fragments of the studied genes. Six strains presented mutations, five of which contained mutations in the *gidB* gene (34 frameshift; CCG→CTG [P84L], CCG→CGG [P93R], TCT→TTT [S100F], GTG→GGG [V115G]). Additionally, all five of the strains containing *gidB* mutations were RIF and INH resistant (Table 2). No susceptible strain had mutations in the *rpsL* gene.

Highly frequent *gidB*[16] polymorphism and LAM lineage. Apart from the several *gidB* mutations that appeared in single strains (Tables 1 and 2), one particular mutation, *gidB*[16] (CTT→CGT; L16R), appeared frequently (n = 63; 59%) in this study population and was identified in 24 resistant and 39 susceptible strains. Therefore, this mutation was not considered a mutation related to STR resistance but as a polymorphism and was not included in Tables 1 and 2.

The presence of this polymorphism was compared according to the distribution of spoligotype lineages, and we observed a very strong association between the 16G allele and the LAM lineage (Table 3). To confirm this, all strains were analyzed using a LAM-specific PCR test.

Of the 54 strains for which the spoligopattern was determined to be LAM, 53 presented the 16G allele and were confirmed by the LAM-specific PCR test. One strain that belongs to the LAM3 and S/convergent sublineage had a 16T allele in the *gidB* gene and was not considered to belong to the LAM lineage by the LAM-specific PCR (Table 3).

All strains belonging to the Haarlem, T, S, and X lineages were considered non-LAM by the LAM-specific PCR test and contained the 16T allele in *gidB* gene (Table 3).

From the 10 strains belonging to the unknown lineage, 6 were classified as LAM by the LAM-specific PCR test, and all 6 also presented the 16G allele. Nine strains presented no shared international type (SIT), and four of them also had the same *gidB*[16] polymorphism (16G allele) and were identified as LAM by the LAM-specific PCR test (Table 3).

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TABLE 1. Mutations in *rpsL*, *rrs*, and *gidB* genes in resistant strains

Phenotypic resistance[a]	Mutation found in:			No. of strains	STR MIC (μg/ml)
	rpsL	*rrs*	*gidB*		
R, H, S					
	AAG→AGG, K43R			8	>125
	AAG→AGG, K88R		GCG→ACG, A183T	1	125
	CTG→TTG, L81L	905 A-G	117 frameshift (insG350)	1	125
		513 C-T		1	8
		513 C-T		1	72
		491 C-T	GCG→GAG, A200E	1	15
		491 C-T		1	8
		491 C-T		1	4
			TGG→TCG, W45S	1	2
			CCG→CTG, P84L	1	2
			CAT→TAT, H48Y	1	4
				6	2–8
H, S					
		513 C-T		1	31
			GGT→CGT, G30R	1	15
			TGC→TGA, C52stop	1	15
		491 C-T		1	8
			AAC→ACC, N52T	1	4
S					
	AAG→AGG, K43R			1	>125
	AAG→AGG, K43R			1	125
		904 C-G		1	125
			GGG→GAG, G117E	1	72
			39 frameshift (delC115)	1	8
			TTG→TTT, L79F	1	2
			CTA→CCA, L49P	1	8
			GGC→TGC, G164C	1	8
				3	2–8
Total				40	

[a] R, rifampin; H, isoniazid; S or STR, streptomycin.

Polymorphism in *gidB*[92] and Beijing family. Interestingly, previous reports identified a different *gidB* mutation that appears frequently within a population. According to Via et al. (29), the majority of the isolates studied (85%) contained an A-C SNP at nucleotide 276 (GAA→GAC; E92D). According to Okamoto et al. (20), the majority of the clinical isolates studied (70%) had this same polymorphism, E92D, and they did not consider this alteration to be related to STR resistance.

This polymorphism was not found in our Brazilian sample, and because there is a different distribution of lineages around the world, we decided to test the possible relationship of this polymorphism with the Beijing genotype. We sequenced the *gidB* gene in 22 Beijing strains to better understand this correlation and found that all of these strains had the 92C allele in the *gidB* gene (GAA→GAC; E92D) as well as the 16T allele. Consequently, they were classified as non-LAM strains and confirmed the relationship of the *gidB*[92] polymorphism with the Beijing genotype.

TABLE 2. Mutations in *rrs* and *gidB* genes in STR-susceptible strains

Phenotypic resistance[a]	Mutation found in:		No. of strains	STR MIC (μg/ml)
	rrs	*gidB*		
R, H				
			32	0.12–1.0
		34 frameshift (delG100)	1	0.5
	491 C-T	GTG→GGG, V115G	1	≤0.12
		CCG→CTG, P84L	1	1
		CCG→CGG, P93R	1	1
		TCT→TTT, S100F	1	0.25
H				
			2	0.5
			26	≤0.12–1.0
	491 C-T		1	1
Total			66	

[a] R, rifampin; H, isoniazid; STR, streptomycin.

DISCUSSION

Genetic resistance to antituberculosis drugs is due to spontaneous chromosomal mutations, because no mobile genetic elements, such as plasmids or transposons, have been characterized in *M. tuberculosis.* Therefore, it is of critical importance to determine the mutations related to STR resistance.

Mutations associated with STR resistance in *rpsL* and *rrs* are well known. For example, the mutation in codon 43 of *rpsL* (found in 10 strains in this study) is the most frequent mutation

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TABLE 3. Spoligotyping of the strains and $gidB^{16}$ polymorphism

Spoligotyping lineage[a]	LAM-specific PCR result	Total no. of isolates	No. of isolates with:	
			16G allele	16T allele
H1	Non-LAM	2		2
H2	Non-LAM	1		1
H3	Non-LAM	10		10
S	Non-LAM	2		2
T1	Non-LAM	4		4
T1 (T4-CE1 ancestor?)	Non-LAM	4		4
T2	Non-LAM	1		1
T3	Non-LAM	2		2
T5_MAD2	Non-LAM	5		5
X1	Non-LAM	1		1
X2	Non-LAM	1		1
LAM1	LAM	9	9	
LAM2	LAM	8	8	
LAM3	LAM	4	4	
LAM3 and S/convergent	Non-LAM	1		1
LAM4	LAM	3	3	
LAM5	LAM	14	14	
LAM6	LAM	3	3	
LAM9	LAM	12	12	
U	6 LAM	10	6	
	4 non-LAM			4
No SIT in SpolDB4	4 LAM	9	4	
	5 non-LAM			5
Total (%)		106 (100)	63 (60)	43 (40)

[a] H, Haarlem; LAM, Latin American-Mediterranean; U, unknown; SIT, shared international type; SpolDB4, Spoligotyping Database 4.

associated with high-level STR resistance (4, 10, 26), while the mutation at position 491 of *rrs* (found in 4 strains in this study) is not related to STR resistance (30, 31). However, mutations in the *gidB* gene of *M. tuberculosis* possibly related to STR resistance have been described in other studies (20, 25, 29).

In this study, we analyzed 106 *M. tuberculosis* clinical isolates from Brazil and found that 18% (19/106) had mutations in *gidB* (without the L16R polymorphism). Only three resistant strains also had mutations in other genes associated with STR resistance. Eleven of 40 STR-resistant strains (27%) had mutations only in *gidB*; 10 had a low level of STR resistance and 1 had intermediate resistance. According to other reports (22, 26), approximately 30% of STR-resistant strains lack mutations in *rpsL* or *rrs*. In our study, *gidB* mutations may explain the 27% of strains that were resistant to STR without mutations in *rpsL* or *rrs*. However, nine strains (22%) considered STR resistant had no mutations in *rpsL*, *rrs* or *gidB*.

We found five STR-susceptible strains with mutations in *gidB* that occurred only in RIF- and INH-resistant strains. No *gidB* mutation was observed in fully susceptible strains. Okamoto et al. (20) came to the same conclusion: *gidB* mutations in STR-susceptible strains occurred in 15/51 strains resistant to INH and RIF but in only 1 (1/24) fully susceptible strain.

Mutations in *gidB* have been reported frequently in other studies. Okamoto et al. (20) reported *gidB* mutations in 33% of STR-resistant strains. Spies et al. (25) found that 73% of isolates presented nucleotide mutations, and 5% with low-level STR resistance had mutations only in *gidB*. In the study by Via et al. (29), 15 different polymorphisms in *gidB* from 21 isolates were described, including drug-susceptible and -resistant strains.

In addition to being frequently mutated, *gidB* seems to be very polymorphic. Apparently, mutations are not present at conserved sites, with the exception of the $gidB^{16}$ and $gidB^{92}$ polymorphisms, which are not associated with STR resistance. Taking into account all published studies (20, 25, 29) and this study, a total of 414 *M. tuberculosis* strains have been sequenced for the *gidB* gene, comprising strains from Japan (132), South Korea (97), and Brazil (185).

The frameshift mutations at codons 14, 36, and 64 each appeared in one susceptible strain. The frameshift mutations at codons 34, 39, 117, and 118 appeared in several strains each, both in STR-resistant and -susceptible strains. The frameshift mutation at codon 40 appeared only in one resistant strain which contained no additional mutations in the other STR resistance-associated genes.

Point mutations (substitutions) are more variable than the frameshift mutations and did not occur at conserved sites. Fifty-five different point mutations have been found in the *gidB* gene. Forty-three of them occurred only once: 21 in susceptible strains (L35R, W45R, V65A, V66M, L74S, V74A, V77A, R83L, P84R, P84C, P93R, P93Q, S100F, V115G, R116P, R118C, D132V, R137G, V139M, A183E, and V188M), 10 in resistant strains with mutations in *rpsL* and/or *rrs* (E40stop, R47Q, I55S, E57stop, R96R, E103stop, L128S, K147T, A183T, and V188G), and 12 in resistant strains with no mutations in the *rpsL* and/or *rrs* genes (G30R, W45C/V110V/W148R, W45S, H48Y, L49P, N51T, C52stop, D67H, P75S/V110V/A141A, P75A, G117E, and G164C).

While investigating the evolution of drug resistance in the KwaZulu-Natal family of *M. tuberculosis*, Ioerger et al. (15) reported a distinct *gidB* mutation, a 130-bp deletion (spanning amino acids 50 to 93 encompassing the SAM-binding site) that causes a frameshift in the C-terminal remainder, which they presumed to completely abrogate the function of the protein. This deletion was found in both drug-resistant (multidrug resistant and extremely drug resistant) and in one STR-susceptible strain.

It will be necessary to sequence more clinical isolates of *M. tuberculosis* to better understand the role of *gidB* gene mutations in STR resistance, thus allowing a more accurate identification of mutations relevant for STR resistance. Nevertheless, *gidB* gene mutations appearing in STR-resistant strains with no additional mutations in other genes seem to be associated with low-level resistance to STR. Low-level antibiotic resistance is frequently required as an initial step for the emergence of high-level resistance. If the bacterial population is not killed effectively by a given antibiotic, the cells will remain under stress, and this may increase the mutation rate (18).

Okamoto et al. (20) found that 70% (93/132) of clinical isolates had the amino acid substitution E92D in *gidB*. We previously found the L16R substitution in 49% (39/79) of clinical isolates (25). Via et al. (29) found 85% (83/97) of isolates contained a point mutation in E92D of *gidB*.

Taking all this into account, we can report that polymorphism $gidB^{92}$ (GAA→GAC; E92D) was found more frequently among strains from Asia and polymorphism $gidB^{16}$ (CTT→CGT; L16R) was very frequent among strains from Brazil. According to Brudey et al. (3), Beijing and Beijing-like

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strains represent about 50% of the strains from Far East Asia (such as Japan and South Korea), and the LAM lineage corresponds only to 5% of the strains. In South America, about 50% of the strains belong to the LAM lineage, and the Beijing genotype is rarely observed. In Brazil, only 0.8% of the strains have been reported as Beijing genotype (23).

We have successfully characterized two SNPs that can distinguish the LAM and Beijing lineages. All strains belonging to the LAM lineage by LAM-specific PCR had the SNPs in codon 16 (G allele) and codon 92 (A allele) of *gidB*, indicating their belonging to the LAM and non-Beijing lineages. Similarly, all Beijing strains characterized by spoligotyping had the SNPs in codon 16 (T allele) and codon 92 (C allele) in *gidB*; thus, they were classified as non-LAM Beijing strains. However, since we have analyzed only samples from Brazil and Asian countries, it will be important to extend these studies to samples from other geographic regions.

One strain gave discordant results; it was classified as LAM3 and S/convergent sublineage by spoligotyping but as non-LAM by the LAM-specific PCR and by the $gidB^{16}$ polymorphism. Spoligotypes evolve through successive loss of spacer DNA sequences (16), and since lineages are defined by spacer patterns, the independent loss of similar spacers sets can lead to the convergent evolution of spoligotypes (32). This is problematic, because not all strains may truly represent their designed spoligotype lineage (11). This may explain the discrepant result found in this study, indicating that a convergence of spoligotypes may have occurred. The same has been reported from a study by Gibson et al. (11), where strains classified as LAM by spoligotyping were considered non-LAM by LAM-specific PCR and SNP analysis.

When some spoligotyping patterns could not be assigned to an internationally recognized genotype lineage, we were able to differentiate these strains as LAM or non-LAM by the $gidB^{16}$ polymorphism and LAM-specific PCR, indicating that spoligotyping alone is not always the best method to differentiate LAM strains.

In conclusion, we have described two SNPs in the *gidB* gene that allowed us to distinguish LAM and Beijing lineages from the other lineages for *M. tuberculosis*. The previously reported association of certain mutations in *gidB* with resistance to STR needs to be further investigated.

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