

## Screening and Characterization of Mutations in Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates Obtained in Brazil

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We investigated mutations in the genes *katG*, *inhA* (regulatory and structural regions), and *kasA* and the *oxyR-ahpC* intergenic region of 97 isoniazid (INH)-resistant and 60 INH-susceptible *Mycobacterium tuberculosis* isolates obtained in two states in Brazil: São Paulo and Paraná. PCR-single-strand conformational polymorphism (PCR-SSCP) was evaluated for screening mutations in regions of prevalence, including codons 315 and 463 of *katG*, the regulatory region and codons 16 and 94 of *inhA*, *kasA*, and the *oxyR-ahpC* intergenic region. DNA sequencing of PCR amplicons was performed for all isolates with altered PCR-SSCP profiles. Mutations in *katG* were found in 83 (85.6%) of the 97 INH-resistant isolates, including mutations in codon 315 that occurred in 60 (61.9%) of the INH-resistant isolates and 23 previously unreported *katG* mutations. Mutations in the *inhA* promoter region occurred in 25 (25.8%) of the INH-resistant isolates; 6.2% of the isolates had *inhA* structural gene mutations, and 10.3% had mutations in the *oxyR-ahpC* intergenic region (one, nucleotide –48, previously unreported). Polymorphisms in the *kasA* gene occurred in both INH-resistant and INH-susceptible isolates. The most frequent polymorphism encoded a G<sub>269</sub>A substitution. Although KatG<sub>315</sub> substitutions are predominant, novel mutations also appear to be responsible for INH resistance in the two states in Brazil. Since ca. 90.7% of the INH-resistant isolates had mutations identified by SSCP electrophoresis, this method may be a useful genotypic screen for INH resistance.

Isoniazid (INH), a first-line antituberculosis drug, is bactericidal and has a simple chemical structure consisting of a pyridine ring and a hydrazide group. INH is a prodrug that enters actively growing tubercle bacilli by passive diffusion (2). The bifunctional bacterial enzyme catalase-peroxidase (KatG) converts INH to a range of oxygenated and organic toxic radicals that attack multiple targets in the mycobacterial cell (35, 36, 48). The best-characterized target of these radicals is the cell wall mycolic acid, but DNA, carbohydrates, lipids, and NAD metabolism may be targeted as well (16, 36, 50).

The tuberculosis case rate in Brazil is the 15th highest in the world, with an estimated prevalence of 64 cases per 100,000 population; moreover, ~0.9% of the new cases are multidrug resistant (45). A recent nationwide investigation of primary INH resistance found a national frequency of 3.8% (29); however, the percentages varied greatly between geographic regions of the country. The incidence of tuberculosis cases in Brazil also varies widely among geographic regions, with 18,112 new reported cases in São Paulo State (51.40 cases per 100,000 population) in 1998 (38) and 2,684 new cases in Paraná State (28.99 cases per 100,000 population) in the same year (37).

Molecular studies of the mechanisms of resistance to INH in *Mycobacterium tuberculosis* demonstrated that a significant number of drug-resistant strains have mutations in the *katG* gene, which encodes the KatG enzyme. Initial investigations of *katG* found large deletions in resistant strains (48, 49), but subsequent studies showed this to be rare. Mutations reduce the ability of KatG to activate the prodrug INH, thus leading to resistance (11, 17, 24, 42). In addition, mutations in other genes, including *inhA* and *kasA*, and in the *oxyR-ahpC* intergenic region have been associated with INH resistance but in much lower percentages of strains (26, 32, 33, 50).

An activated INH radical appears to inhibit the InhA enzyme by reacting with the NAD(H) cofactor bound to the InhA active site, which compromises the mycolic acid synthesis (23). Mutation at the InhA enzyme's site of interaction can reduce its affinity for NAD(H) and confer INH and ethionamide resistance to strains (1). The overexpression of InhA because of an upregulation mutation in the promoter region of *inhA* (preceding the *mabA-inhA* operon) can also cause resistance to INH by a titration mechanism (1, 2, 3, 8, 16, 23). Mutations in the *oxyR-ahpC* intergenic region, where the putative promoter of *ahpC* is located, are considered to be a compensatory mechanism for the loss of KatG function in resistant strains (18, 33, 35, 46, 47). These mutations may be used as surrogate markers for the detection of INH resistance in *M. tuberculosis* (33, 39, 41, 50).

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Mdluli et al. (25) reported that the ketoacyl acyl carrier protein synthase (KasA), encoded by the *kasA* gene, which is involved in the biosynthesis of mycolic acids, is a likely target for INH. They found an association between mutations in the *kasA* gene and resistance to INH in *M. tuberculosis*. However, Lee et al. (22) observed mutations in the *kasA* gene in resistant and in susceptible *M. tuberculosis* strains from Singapore. Recently, Larsen et al. (21) demonstrated no correlation between resistance to INH and overexpression of KasA.

A variety of methods have been used to facilitate the rapid detection of mutations in mycobacteria. One widely used method is PCR–single-strand conformational polymorphism (PCR-SSCP) (7, 28, 43). If any two single strands of DNA differ by one or more nucleotides, differences in the secondary structure of these strands may be identified by their electrophoretic mobilities in nondenaturing polyacrylamide gels (9), offering a convenient and cost-efficient method for analyzing mutations in PCR products. The PCR-SSCP method has been demonstrated to be useful for screening mutations associated with anti-tuberculosis drug resistance (7, 10, 15, 30, 46).

We investigated the prevalence of mutations in the genes, *katG*, *kasA*, and *inhA* (regulatory and structural regions) and in the *oxyR-ahpC* intergenic region. We evaluated the usefulness of SSCP electrophoresis for the detection of those mutations among INH-resistant isolates from São Paulo and Paraná, Brazil.

#### MATERIALS AND METHODS

**Mycobacterial isolates and drug susceptibility testing.** We obtained 157 *M. tuberculosis* isolates (97 INH resistant and 60 INH susceptible) from the culture collections of the Instituto Adolfo Lutz of Ribeirão Preto, the Instituto Adolfo Lutz of Sorocaba, and the Instituto Clemente Ferreira, São Paulo State, and the Clinical Bacteriology Laboratory, Department of Clinical Analysis, Paraná State, Brazil. The isolates came from patients in São Paulo and Paraná, Brazil, and were originally cultured from 1997 and 2001. All 60 susceptible and 97 resistant isolates were identified by biochemical tests (19) and typed by the spoligotyping method (26). Susceptibility to isoniazid was determined by using the 1% proportion method in Löwenstein-Jensen medium containing 0.2 µg of INH/ml, which is the critical concentration (4). All isolates were maintained by subculture on Löwenstein-Jensen medium (BBL/Becton-Dickinson Microbiology Systems, Sparks, Md.) at 8°C and in Middlebrook 7H9 with oleic acid-albumin-dextrose-catalase (OADC) Enrichment (BBL/Becton-Dickinson) at –80°C. INH MICs were determined for all resistant and six susceptible isolates by using the microplate Alamar blue assay (6, 12). The isolates were cultured in Middlebrook 7H9 broth containing twofold concentrations of INH ranging from 0.25 to 32 µg/ml. We chose 0.25 µg of INH/ml as the lowest test concentration on the basis of previous research (12) and our experience. The MIC was defined as the lowest concentration of INH that prevented a color change from blue to pink. *M. tuberculosis* strain H<sub>37</sub>Rv (ATCC 27294) was used as a susceptible control for each test.

**DNA extraction.** Chromosomal DNA was extracted from isolates cultured for 30 days at 35°C on Löwenstein-Jensen medium, as described by Gonzales-Merchand et al. (13), with some modifications. Briefly, colonies were suspended in 6 M guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), and bacilli were lysed by freezing in nitrogen, followed by heating at 65°C for 10 min; we then repeated this procedure. The DNA was extracted twice by using 2 volumes of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]) and twice with chloroform-isoamyl alcohol (24:1 [vol/vol]). DNA in the aqueous phase was precipitated with 2 volumes of absolute ethanol, washed with 70% ethanol, dried, dissolved in Tris-EDTA buffer, and stored at –20°C.

**PCR-SSCP analysis.** Regions of the genes, *katG* (codons 315 and 463), *kasA*, and *inhA* (regulatory region and codons 16 and 94), and the *oxyR-ahpC* intergenic region were analyzed by using PCR-SSCP electrophoresis in all 157 isolates. A single set of oligonucleotide primers were selected for each of these regions except for *kasA*, which required six pairs of primers to generate overlapping amplicons encompassing the entire gene (Table 1). The PCR mixes

contained 75 mM Tris-HCl (pH 9.0); 50 mM KCl, 2.0 mM MgCl<sub>2</sub>; 0.2 mM concentrations (each) of dATP, dCTP, dGTP, and dTTP; 100 nM concentrations (each) of the primers; 1 U of DNA polymerase (Biotools/B&M Laboratories, S.A., Uniscience do Brasil, São Paulo, Brazil); and 1 µl of template DNA in a final volume of 50 µl. Thermocycling was performed with a GeneAmp System 2400 thermal cycler (PE Applied Biosystems Corp., Foster City, Calif.) under the following conditions: 94°C for 5 min, 30 cycles of 94°C for 1 min, with annealing temperatures as shown in Table 1 for 1 min and 72°C for 1 min; and a final elongation step at 72°C for 10 min. Then, 1 µl of each PCR was denatured with 25 µl of formamide solution (95% deionized formamide, 20 mM EDTA, 0.005% xylene cyanole FF, 0.005% bromophenol blue), and 5 µl of each sample was examined by polyacrylamide gel SSCP electrophoresis by using the GenePhor System (GeneGel Excel 12.5/24; Amersham Biosciences, Uppsala, Sweden) and conditions specific for each amplicon (Table 2). The gels were stained by using Bio-Rad silver stain (Bio-Rad Laboratories, Calif.), according to the manufacturer's instructions (Fig. 1).

**DNA sequencing.** The oligonucleotide primers used for PCRs and for sequencing PCR products are listed in Table 3. PCR products used as templates for sequencing were prepared by using a Hotstart *Taq* master mix kit (Qiagen, Inc., Valencia, Calif.) according to the manufacturer's instructions, with 1 µl of template genomic DNA in a final volume of 25 µl. Thermocycling was performed by using a GeneAmp System 2400 thermal cycler (PE Applied Biosystems Corp., Foster City, Calif.) under the following conditions: 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, followed by annealing at the temperatures shown in Table 3 for 30 s and at 72°C for 30 s, and a final elongation step at 72°C for 5 min. For reactions that used a 68°C annealing temperature, we set the following conditions: 96°C for 15 min, followed by 35 cycles of 96°C for 30 s and 68°C for 75 s, with a final elongation at 68°C for 5 min. The sequencing of the 2,223-bp *katG* open reading frame (ORF) was accomplished by generating four PCR amplicons and by using the following pairs of primers: KatG-1 and KatG-5, KatG-4 and KatG-9, KatG-8 and KatG-13, and KatG-12 and KatG-14 (Table 3). Except for the last of these primer sets, thermocycling conditions were as follows: 96°C for 15 min, 35 cycles of 96°C for 30 s and 68°C for 75 s, and a final elongation at 68°C for 5 min. Conditions for KatG-12 and KatG-14 were as follows: 96°C for 15 min; 35 cycles of 95°C for 30 s, 60°C for 15 min, and 72°C for 30 s; and a final elongation at 72°C for 5 min. The six primers used only for sequencing *katG* are shown in Table 3. Three regions of *kasA* designated 3, 4, and 5 and located from nucleotides 31066 to 31333, 31290 to 31552, and 31507 to 31729, respectively, were amplified by using the primer pairs KasA-1/KasA-5 (region 3) and KasA-4/KasA-8 (regions 4 and 5) (Table 3). The forward and reverse sequencing primers for each of these three regions were KasA-2 and –5 for region 3, KasA-4 and –7 for region 4, and KasA-6 and –8 for region 5. Sequencing reactions were performed by using an ABI Prism BigDye terminator cycle sequencing kit (PE Applied Biosystems Corp., Foster City, Calif.), according to the manufacturer's instructions in a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems) and were electrophoresed by using an ABI Prism 373XL automatic sequencer (PE Applied Biosystems). The sequence data were assembled and edited by using ABI Prism DNA Sequencing Analysis Software v 3.0 (PE Applied Biosystems), and the results were compared to the published sequences for *inhA*, *kasA*, *katG*, and *oxyR-ahpC* (the GenBank accession numbers are U41388 for *inhA* regulatory and structural genes, Z70692 for *kasA*, X68081 for *katG*, and Z81451 for *oxyR-ahpC*).

#### RESULTS

Among the 157 *M. tuberculosis* isolates examined, 97 were resistant to INH as determined by the proportion method. Thirty-nine different spoligotype patterns were found in the 97 INH-resistant isolates: 25 occurring once and 14 occurring in clusters ranging from 2 to 17 isolates. The three largest clusters were comprised of 12, 13, and 17 isolates each. Among isolates within the 14 spoligotype clusters, 29 (41%) had a set of INH resistance-associated mutations that differed from the other members of their respective clusters. Of the 60 INH-susceptible isolates spoligotyped, 32 patterns were identified; 12 of these also occurred in the resistant isolates.

INH MICs of resistant isolates, as determined by microplate Alamar blue assay, ranged from 1 to >32 µg/ml. Mutations within the *katG* gene were found in 83 (85.6%) of the 97

TABLE 1. Oligonucleotide primers used for PCR-SSCP analyses of *katG*, *inhA* (regulatory and structural regions), *kasA*, and the *oxyR-ahpC* intergenic region

Accession no.	Primer	Primer sequence (5' to 3')	Nucleotide location <sup>a</sup>	PCR annealing temp (°C)	PCR product size (bp)
Z97193	katG-315F	AGA GCT CGT ATG GCA CCG GA	32059	58	145
	katG-315R	CCA GCA GGG CTC TTC GTC AG	31915		
	katG-463F	CTG CTG TGG CAG GAT CCG GT	31636	60	162
	katG-463R	GCG CTT GTC GCT ACC ACG GA	31494		
V66801	inhA-1F	GCT GAG TCA CAC CGA CAA ACG	909	60	187
	inhA-1R	CCA GGA CTG AAC GGG ATA CGA	1075		
V02492	inhA-16F	TGA CAC AAC ACA AGG ACG CA	1779	52	222
	inhA-16R	GTT TTG CAC GTC GAG TTC GA	1981		
AF106077	inhA-94F	GCA AAA CGA GGA GCA CCT GGC	1994	63	182
	inhA-94R	AAT ACG CCG AGA TGT GGA TGC	2156		
V16243	ahpC-F	CTT GCG GCA CTG CTG AAC CAC	7147	60	264
	ahpC-R	ACA GGT CAC CGC CGA TGA GAG	7389		
Z70692	kasA-1F	AAC GTT CAG GCG AGG CTT GA	30631	68	262
	kasA-1R	ATG TCG AGT CGG CCC ATG TG	30873		
	kasA-2F	CGG TCA CCT CAA GGA TCC GG	30844	58	267
	kasA-2R	GCA CCG TTG GGC ATG ATC AT	31092		
	kasA-3F	GGA AGG TGT CCC CGC TGG CC	31066	63	269
	kasA-3R	TCA GGC TCG TCG TTG CGG GT	31314		
	kasA-4F	TTC TCC ATG ATG CGG GCC AT	31290	68	263
	kasA-4R	AGC TCC AGC GAG CGA GTC AT	31533		
	kasA-5F	CCG ATG GTG TTC GTG CCG GT	31507	63	223
	kasA-5R	ACC GAC TCG AGC GCA CCG AC	31710		
	kasA-6F	GTC TGC GTC GGG CCA CTC GA	31682	63	255
	kasA-6R	CTC GCG GCG ACC CGC GAT GTC	31917		

<sup>a</sup> Numbers refer to the nucleotide position within the referenced GenBank sequence of the 5' primer terminus.

INH-resistant isolates; 60 (61.9%) of these occurred in the *katG* codon 315, with base substitutions at nucleotide 944 predominating (57 isolates: 58.8%). The remaining 23 (23.7%) *katG* mutants had changes in other codons, some of which were not previously reported (Table 4). Of the *katG* mutants, 15 isolates (15.5%) also had mutations in the *inhA* regulatory region: 1 (1.1%) in the *inhA* structural gene, 9 (9.3%) in the *oxyR-ahpC* intergenic region, 1 (1.1%) in *ahpC*, and 11 (11.3%) in *kasA*. Fourteen (14.4%) INH-resistant isolates (MIC of 1 to >32 µg/ml) had no *katG* mutation; 12 (12.4%) of these had mutations in one or more of the other genetic regions examined. All 60 KatG<sub>315</sub> mutants were identified by SSCP. Each of

the six different mutations detected in this codon (AGC → ACC, AAC, CGC, ATC, GGC, and AGG) presented characteristic and reproducible SSCP electrophoretic mobility shifts (Fig. 1A). The *katG* mutation in codon 341 (W→S) also presented a characteristic electrophoretic mobility shift (Fig. 1A).

Analysis of the *inhA* regulatory region showed base substitutions at nucleotide positions -15 in 23 (23.7%) and at -17 in 2 (2.1%) resistant isolates. Of these, 5 isolates (5.2%) had mutations only in the *inhA* regulatory region (INH MICs of 2 to >32 µg/ml), 1 had an additional *kasA* mutation (INH MIC of 1 µg/ml), and 19 had additional mutation(s) in the *inhA* structural and *katG* gene and in the *oxyR-ahpC* intergenic region (Table 4). One of two mutations in the *inhA* structural gene (at either codon 21 or 44) was found in six resistant isolates; for all of these strains the INH MICs were ≥8 µg/ml. Each of these six isolates had one or more additional mutation(s) within the other genetic regions examined. The mutations in the *inhA* regulatory (*n* = 2) or structural gene (*n* = 2) showed different SSCP mobility shifts compared to the wild-type controls (Fig. 1B and C).

Nucleotide substitutions in the *oxyR-ahpC* intergenic region (Table 4) were found in 10 (10.3%) resistant isolates (INH MICs of ≥8 µg/ml). Nine of these had additional mutations in *katG* codons other than 315, and one had no additional mutation. Mutations in the *oxyR-ahpC* intergenic region were readily identified by SSCP analysis of a 264-bp PCR product. A silent nucleotide substitution in the *ahpC* structural region (I<sub>10</sub>I) was detected by SSCP in an INH-resistant isolate (INH

TABLE 2. Conditions used for SSCP electrophoresis<sup>a</sup>

Primer pair(s) (forward/reverse)	Volts	Temp (°C)
katG-315F/315R	350	12
katG-463F/463R	400	8
inhA-F/1R	350	12
inhA-16/17R	350	12
inhA-94/94R	400	12
ahpC-F/ahpC R	400	12
kasA-F/3R and -5F/5R	600	13
kasA-2F/2R and -6F/6R	450	8
kasA-1F/1R and -4F/4R	500	13

<sup>a</sup> GenePhor electrophoresis system using GeneGel Excel 12.5/24 gel (Amersham Biosciences). All PCR amplicons were run for 2 h under the indicated conditions.

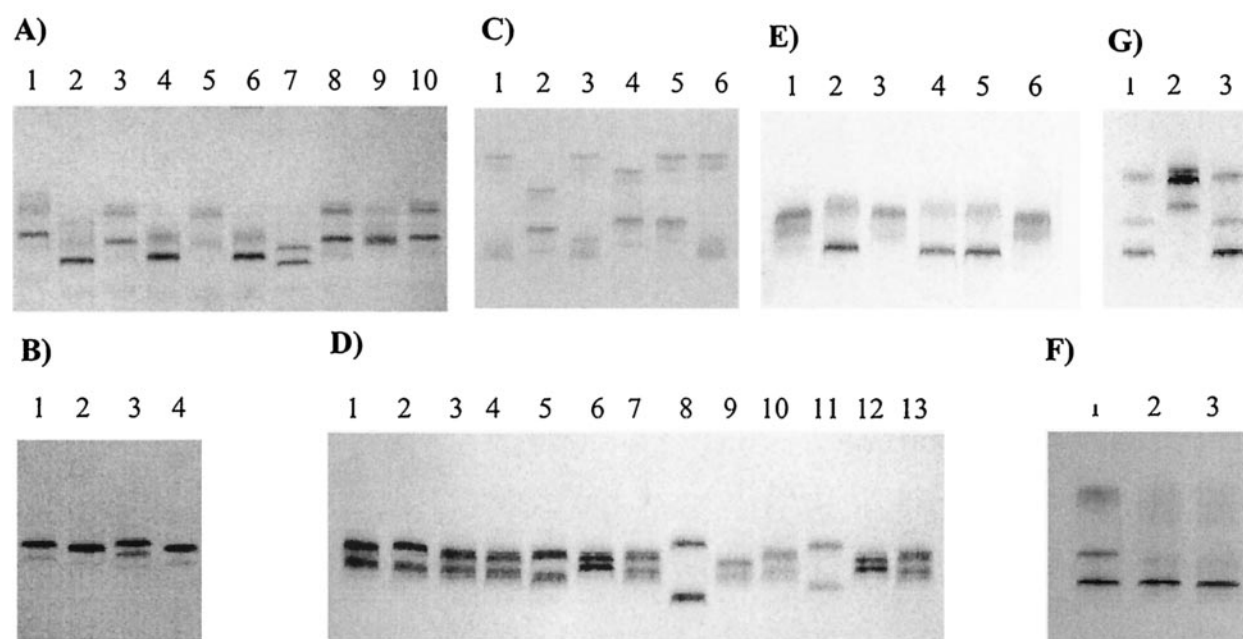


FIG. 1. SSCP analysis of *katG*<sub>315</sub>, *inhA* (promoter and structural ORFs), *oxyR-ahpC* intergenic region, and three regions of *kasA* in INH-resistant *M. tuberculosis*. All PCR products were denatured in the presence of 95% deionized formamide and electrophoresed by using specific conditions shown in Table 2 and by using a GenePhor electrophoresis system (Amersham Biosciences). (A) *katG*<sub>315</sub> (145-bp PCR products). Lanes: 3 and 10, wild-type control (H<sub>37</sub>Rv); 1, W<sub>341</sub>S; 2, S<sub>315</sub>R; 4 and 6, S<sub>315</sub>N; 7, S<sub>315</sub>G; 5 and 8, wild type (susceptible isolates); and 9, S<sub>315</sub>T. (B) *inhA* promoter (187-bp PCR products). Lanes: 1, wild-type control (H<sub>37</sub>Rv); 2 and 4, C<sub>-17</sub>T; 3, C<sub>-15</sub>T. (C) *inhA* structural gene (codon 16) (222-bp PCR products). Lanes: 1 and 6, wild-type control (H<sub>37</sub>Rv); 2, I<sub>21</sub>V; 3, wild type (susceptible isolate); 4, L<sub>44</sub>L; 5, I<sub>21</sub>T. (D) *oxyR-ahpC* intergenic region (264-bp PCR products). Lanes: 1, 7, and 13, wild-type control (H<sub>37</sub>Rv); 2, *oxyR*; 5, C<sub>-15</sub>T; 6, C<sub>-39</sub>T; 8, C<sub>-10</sub>T; 9, G<sub>-48</sub>A; 10, C<sub>-12</sub>T; 11, G<sub>-9</sub>A; 12, C<sub>-39</sub>T; 3, and 4, wild-type (susceptible isolates). (E) *kasA* region 3 (269-bp PCR products). Lanes: 1, wild-type control (H<sub>37</sub>Rv); 2, 4, and 5, H<sub>180</sub>H; 3, G<sub>149</sub>G; and 6, wild type (susceptible isolates). (F) *kasA* region 4 (263-bp PCR products). Lanes: 1, wild-type control (H<sub>37</sub>Rv); 2 and 3, G<sub>269</sub>S. (G) *kasA* region 5 (223-bp PCR products). Lanes: 1, wild-type control (H<sub>37</sub>Rv); 2, G<sub>318</sub>G; and 3, wild type (susceptible isolates).

MIC of 4  $\mu$ g/ml), which also had a KatG<sub>315</sub> substitution, and in four INH-susceptible isolates.

All 157 isolates were examined by *kasA* SSCP electrophoresis. All isolates with shifts in electrophoretic mobility compared to the wild-type control (H<sub>37</sub>Rv) were found to have *kasA* mutations when sequenced. Mutations in the *kasA* gene were observed in 13 (13.4%) INH-resistant isolates (INH MICs of 1 to >32  $\mu$ g/ml) and in 15 (25%) INH-susceptible isolates. Polymorphisms in codons other than 269 were silent and occurred only in susceptible isolates. Additional mutations among the INH-resistant KasA<sub>269</sub> mutants occurred in the *katG* gene in 11 (11.3%) isolates, 8 of which affected codon 315 (MICs of 4 to 16  $\mu$ g/ml) and 1 in the *inhA* regulatory region at position -15 (MIC of 1  $\mu$ g/ml). The same mutation in *kasA* codon 269 (G<sub>805</sub>A) was also found in 10 INH-susceptible isolates. Three additional silent *kasA* mutations were observed in susceptible isolates: GGT to GGC at codon 149 (in an isolate which also had the G<sub>269</sub>S substitution), CAC to CAT at codon 180, and GGC to GGA at codon 318.

Two INH-resistant isolates for which the IHN MICs were >32  $\mu$ g/ml had mutations only in codon 269 of the *kasA* gene or in the *oxyR-ahpC* intergenic region (C<sub>-39</sub>T). Two other isolates had no mutation in the gene regions examined here. In these four isolates, the entire *katG* structural gene was sequenced.

## DISCUSSION

We identified 47 different mutations in three structural genes and two promoter regions (*ahpC* and *inhA*) among 97 INH-resistant *M. tuberculosis* isolates from two states in Brazil (São Paulo and Paraná), which underscores the diversity of mutations associated with resistance to this drug. Mutations in the *katG* gene, occurring in 83 isolates, predominated as expected. A total of 34 different mutations were identified; 25 of these have not been previously reported (Table 4). Six different missense mutations were identified within codon 315 of the *katG* gene. This diversity of mutations in codon 315 is consistent with previous findings (5, 14, 15, 27, 31). We also observed *katG* mutations in codons other than 315 in isolates with high-level resistance (INH MICs of 8 to 32  $\mu$ g/ml) and in regions that have been previously associated with INH resistance. Rouse et al. (34) used site-directed mutagenesis to induce changes in *katG*, including R<sub>104</sub>L, H<sub>108</sub>Q, N<sub>138</sub>S, L<sub>148</sub>R, H<sub>270</sub>Q, T<sub>270</sub>P, S<sub>315</sub>T, W<sub>321</sub>G, and D<sub>381</sub>G. Codons 104 and 108 encode amino acids located near the enzyme's catalytic site, and the residues encoded by codons 270, 275, and 315 participate in the bonding of the enzyme's heme group. Mutations in these regions, therefore, result in loss of KatG enzymatic function (32). Amino acid substitutions in these *katG* regions that conferred high-level resistance in our study included W<sub>91</sub>R, A<sub>109</sub>V, H<sub>97</sub>R, G<sub>273</sub>C, G<sub>279</sub>D, S<sub>302</sub>R, L<sub>293</sub>V, and G<sub>299</sub>S; the

TABLE 3. Oligonucleotide primers used for PCR and the sequencing reaction of *katG*, *inhA* (regulatory and structural), the *oxyR-ahpC* intergenic region, and *kasA*

Accession no.	Primer	Primer sequence (5' to 3')	Nucleotide location <sup>a</sup>	PCR annealing temp (°C)	PCR product size (bp)
U41314	BC 48	TGG CCG CGG CGG TCG ACA TT	725	58	322
	BC 51R	CCA GCA GGG CTC TTC GTC AG	1027		
Z79701	InhA-1	CCT CGC TGC CCA GAA AGG GA	870	60	248
	InhA-2	ATC CCC CGG TTT CCT CCG GT	1098		
	InhA-5	TGG ACG GCA AAC GGA TTC TGG	1813	60	366
	InhA-6	ACG AAT ACG CCG AGA TGT GGA	2158		
Z81451	AhpC-1	GCC TGG GTG TTC GTC ACT GGT	7455	60	359
	AhpC-2	CGC AAC GTC GAC TGG CTC ATA	7117		
Z70692	KasA-1	CGT TCA GGC GAG GCT TGA GGC	93	68	700
	KasA-5	CAG GCT CGT CGT TGC GGG TC	773		
	KasA-4	TGC CCA TCG CGG CGT TCT CCA	736	68	703
	KasA-8	GTC CGA CTC GCC CCC GCA AGC	1418		
	KasA-2	CCA GAC CGG TTC GCC GTT GTT	435		
	KasA-6	CGC CGG CGG ACA TCG ACC AC	1024		
	KasA-7	GCC GTG CCG TGC GCG TTG A	1045		
X68081	KatG-1	GCC CGA TAA CAC CAA CTC CTG	1947	68	680
	KatG-5	CAG ATC CCG CTA CCG CTG TA	2606		
	KatG-2	CGC CGA CTA CGG CCA CTA	2254		
	KatG-3	GCC ACG CCA TCC GGA TAA	2238		
	KatG-4	CCT GGC TCG GCG ATG A	2586	68	648
	KatG-9	CTC GGT GGA TCA GCT TGT ACC	3213		
	KatG-6	CTC GAT GGC ACC GGA ACC	2884	68	667
	KatG-7	CGT CGG GGT GTT CGT CCA TAC	2936		
	KatG-8	GAG GAA TTG GCC GAC GAG TT	3182		
	KatG-13	TCT CAG GGG CAC TGA GCG TAA	3828		
	KatG-10	CAA GTC GGG TGG GAG GTC AA	3482		
	KatG-11	CTC TTC CAG GGT GCG AAT GAC	3527		
	KatG-12	GCC GAG TAC ATG CTG CTC GAC	3794		
	KatG-14	CGG CGG GTT GTG GTT GA	4229		

<sup>a</sup> Numbers refer to the nucleotide position within the referenced GenBank sequence of the 5' primer terminus. Oligonucleotides KasA-2, KasA-6, KasA-7, KatG-2, KatG-3, KatG-6, KatG-7, KatG-10, and KatG-11 were used only for sequencing reactions.

specific effects of these mutations on KatG function warrant further analyses.

The KatG R<sub>463</sub>L polymorphism, which is believed to have no association with INH resistance (44), was observed in two (2.1%) of our resistant isolates from the Instituto Clemente Ferreira, São Paulo, Brazil. These two isolates had a common spoligotype pattern belonging to the "Beijing" group, which has been frequently found, particularly among multidrug-resistant strains in eastern Asia. The low occurrence of the R<sub>463</sub>L polymorphism in *katG* in our study agrees with a previous study in which 85% of the *M. tuberculosis* strains from Mexico, Honduras, Guatemala, Peru, and several other Latin American countries had arginine, rather than leucine, at this codon (J. M. Musser, author's reply to A. S. Lee, L. L. Tang, I. H. K. Lim, L. Tay, and S. Y. Wong, Letter, J. Infect. Dis. **176**:1125–1127, 1997).

We found mutations in the *inhA* regulatory region or structural gene in 25 and 6, respectively, of the INH-resistant isolates. Our results support previous findings regarding the role of the *inhA* gene (regulatory and structural regions) in INH resistance (35) because we found that, overall, 26.8% of the INH-resistant isolates had mutations in *inhA*, and no mutations were found in susceptible isolates.

Biochemical studies of the kinetics of InhA enzyme inacti-

vation by activated INH (3) demonstrated that *inhA* mutation resulting in the amino acid substitution I<sub>21</sub>V conferred resistance to INH in *M. tuberculosis*. We found one isolate with an I<sub>21</sub>V substitution (MIC, 16 µg/ml) and four isolates in which threonine was substituted for the isoleucine residue. The presence of mutations in codons 94 and 95 of the *inhA* gene has also been associated with resistance to INH (3, 10, 50); however, we did not find such mutations in either resistant or susceptible isolates.

Ten INH-resistant isolates had mutations in the *oxyR-ahpC* intergenic region; nine of these isolates had additional mutations in *katG*, and two had *kasA* mutations. This is consistent with the hypothesis that increased expression of the AhpC protein, which was caused by upregulation mutations in the *ahpC* promoter, may compensate for loss of KatG catalase-peroxidase activity (32, 33, 39, 46, 47). We did not observe mutations in the *oxyR-ahpC* intergenic region of the KatG<sub>315</sub> mutants, suggesting that there may be less impairment of KatG enzymatic activity among these mutants and therefore no requirement for compensatory AhpC activity (34, 39). Among the 24 isolates with *katG* mutations in regions other than codon 315, 9 also had mutations in the *oxyR-ahpC* intergenic region, including one novel mutation (G<sub>-48</sub>A). The remaining 15 isolates did not have mutations in the *oxyR-ahpC* region,

TABLE 4. Mutations found in *katG*, *kasA*, *inhA*, and the *ahpC* regulatory region in 97 INH-resistant *M. tuberculosis* isolates

Gene	No. of isolates	Mutation <sup>a</sup>		INH MICs in µg/ml (no. of isolates if >1)	Additional mutation(s)	
		Nucleotide no.	Amino acid			
<i>katG</i>	1	A inserted at position 17	Frameshift*	32		
	1	T→C at position 271	W <sub>91</sub> R*	>32		
	1	A→G at position 290	H <sub>97</sub> R*	8	<i>katG</i>	
	1	C→T at position 326	A <sub>109</sub> V*	16	<i>oxyR-ahpC</i>	
	1	C→T at position 329	A <sub>110</sub> A*	16	<i>katG</i> , <i>inhA</i> P	
	1	C→T at position 431	A <sub>144</sub> V*	1		
	2	A→G at position 598	K <sub>200</sub> E*	8, >32	<i>katG</i> , <i>inhA</i> P, <i>oxyR-ahpC</i>	
	1	C→G at position 695	P <sub>232</sub> R*	32	<i>inhA</i> P	
	1	G→T at position 761	R <sub>254</sub> L*	NG	<i>kasA</i>	
	1	G→T at position 817	G <sub>273</sub> C*	>32	<i>oxyR-ahpC</i>	
	1	G→A at position 836	G <sub>279</sub> D*	>32	<i>inhA</i> P, <i>oxyR-ahpC</i>	
	1	C→G at position 877	L <sub>293</sub> V*	16	<i>inhA</i> P	
	1	G→A at position 895	G <sub>299</sub> S*	>32	<i>oxyR-ahpC</i> , <i>kasA</i>	
	1	A→C at position 904	S <sub>302</sub> R	8	<i>oxyR-ahpC</i>	
	1	A→G at position 943	S <sub>315</sub> G	8	<i>inhA</i> P	
	1	A→C at position 943	S <sub>315</sub> R	>32		
	49	G→C at position 944	S <sub>315</sub> T	4 (23), 8 (15), 16 (5), 32 (3), >32 (3)	<i>inhA</i> P, <i>kasA</i> , <i>katG</i>	
	7	G→A at position 944	S <sub>315</sub> N	4 (3), 16, >32 (3)	<i>inhA</i> P, <i>katG</i>	
	1	G→T at position 944	S <sub>315</sub> I	16		
	1	C→G at position 945	S <sub>315</sub> R	32		
	1	G→C at position 1022	W <sub>341</sub> S*	>32	<i>inhA</i> P	
	1	G→T at position 1255	D <sub>419</sub> Y*	16	<i>inhA</i> P	
	2	A→C at position 1256	D <sub>419</sub> A*	2, 8	<i>InhA</i> P	
	1	C→A at position 1257	D <sub>419</sub> E*	>32	<i>katG</i> , <i>inhA</i> , <i>oxyR-ahpC</i>	
	1	T insertion at position 1311	Frameshift*	>32	<i>oxyR-ahpC</i>	
	1	A insertion at position 1329	Frameshift*	16	<i>katG</i>	
	1	C del at position 1339	Frameshift*	16	<i>katG</i>	
	2	G→T at position 1388	R <sub>463</sub> L	>32 (2)	<i>katG</i>	
	1	G→A at position 1468	G <sub>490</sub> S*	16		
	1	C→T at position 1833	L <sub>611</sub> L	4	<i>inhA</i> P	
	1	Deletion after position 1845	NA*	>32	<i>oxyR-ahpC</i> , <i>kasA</i>	
	1	T→G at position 1985	L <sub>662</sub> R*	32		
	1	C deletion at position 2139	Frameshift*	>32	<i>oxyR-ahpC</i>	
	1	G→A at position 2176	A <sub>726</sub> T*	16	<i>inhA</i> P	
	<i>inhA</i> promoter (P)	23	C→T at position -15	NA	1, 2 (3), 4, 8 (6), 16 (5), 32, >32 (6)	<i>katG</i> , <i>kasA</i> , <i>oxyR-ahpC</i> , <i>inhA</i>
		2	G→T at position -17	NA	16, 32	<i>katG</i>
	<i>inhA</i> structural	1	A→G at position 61	I <sub>21</sub> V	16	<i>katG</i> , <i>kasA</i>
		4	T→C at position 62	I <sub>21</sub> T	8, 16 (2), >32	<i>InhA</i> P
		1	C→T at position 130	L <sub>44</sub> L	>32	<i>katG</i> , <i>inhA</i> P, <i>oxyR-ahpC</i>
	<i>oxyR-ahpC</i>	2	G→A at position -9	NA	16, >32	<i>katG</i>
		1	C→T at position -10	NA	>32	<i>katG</i> , <i>kasA</i>
		1	C→T at position -12	NA	>32	<i>katG</i>
		1	C→T at position -15	NA	>32	<i>katG</i>
		4	C→T at position -39**	NA	8, >32 (3)	<i>katG</i> , <i>kasA</i>
		1	G→A at position -48*	NA	>32	<i>katG</i>
	<i>ahpC</i>	1	T→C at position 30	I <sub>10</sub> I*	4	
	<i>kasA</i>	13	G→A at position 805	G <sub>269</sub> S	1, 4 (3), 8 (3), 16 (2), >32 (3), NG <sup>b</sup>	<i>katG</i> , <i>inhA</i> P, <i>oxyR-ahpC</i> , <i>inhA</i> , <i>kasA</i>

<sup>a</sup> \*, novel mutation; \*\*, one isolate had no additional mutation; NA, not applicable.

<sup>b</sup> NG, no growth.

and 1 of these had a *katG* frameshift mutation (A<sub>17</sub> insertion), which likely would greatly diminish KatG activity and increase the need for AhpC activity. Although a strict correlation between loss of KatG function and AhpC overexpression in clinical *M. tuberculosis* isolates has been reported (39), our findings correlate more closely with those of Sreevatsan et al. (41), who

reported a low frequency of mutations in this region (~5.3% of 169 *M. tuberculosis* complex isolates). One of the four *oxyR-ahpC*<sub>-39</sub> mutants with high-level INH resistance (MIC of >32 µg/ml) was wild type at all of the other loci examined. Mutations in this nucleotide of the *oxyR-ahpC* intergenic region have been previously associated with INH resistance in *M.*

*tuberculosis* (41), but its role in the absence of any structural *katG* involvement in this strain is intriguing and merits further investigation.

The *M. tuberculosis kas* operon is comprised of five genes, all of which are transcribed in the same direction to encode enzymes that participate in the elongation of the main carbonic chain of mycolic acid (20). Mutations in one of these genes, *kasA*, have been postulated to play a role in resistance to INH (25). We found *kasA* mutations in susceptible and resistant isolates. All INH-resistant isolates with a missense mutation in the *kasA* gene also had additional mutation(s) in the other loci examined. We found *kasA* mutations that were not reported in previous studies (22, 25), but the most common amino acid substitution was G<sub>269</sub>S, which occurred in 13.4% of the resistant isolates and 25% of the susceptible isolates. This mutation has been reported to be a gene polymorphism that is unrelated to INH resistance (22, 50), and our results substantiate this conclusion. Our finding of *kasA* gene mutations in both INH-resistant and INH-susceptible isolates combined with the recent demonstration that *InhA*, not *KasA*, is the primary target of INH (21) lead us to speculate that there is no value in including *kasA* analysis in our PCR-SSCP strategy.

Despite the elucidation of multiple mechanisms and genes that are involved in INH resistance, strains exist that have had no mutation identified; some of these have high-level resistance (INH MIC of >50 µg/ml) (32, 33, 50). We found 3 (3.1%) of the 97 INH-resistant isolates with no resistance-associated mutations in the examined regions. Two of these had no mutations in the *katG*, *inhA*, or *kasA* genes or in the *oxyR-ahpC* intergenic region examined, and one had only a *kasA* codon 269 polymorphism. These strains are good candidates for further investigation into possible novel mechanisms of INH resistance.

In comparing the results obtained by PCR-SSCP to screen mutations in codon 315 of *katG* with sequencing, we observed an agreement of 100%. No false-negative or false-positive result was observed when the PCR-SSCP and sequencing results were compared. All 60 INH-susceptible strains had an SSCP pattern identical to that of the wild-type reference strain, and all 60 of the codon 315 mutants had a unique and discernibly different pattern. PCR-SSCP was also highly accurate in detecting mutations within the *inhA* gene. The commonly seen C-to-T transition at nucleotide -15 was identified in all isolates possessing this mutation. Ten resistant strains had mutations within the *oxyR-ahpC* intergenic region; all were identified by SSCP. Mutations in the *katG* gene outside of the PCR amplicon encompassing codon 315 occurred in nine of these mutants. Thus, *oxyR-ahpC* PCR-SSCP proved to be a good surrogate for detecting resistance to INH, as previously suggested (18, 41). Altogether, mutations within the *katG* codon 315, *inhA* promoter, or *oxyR-ahpC* regions were found in 90.7% of these INH-resistant isolates. The ability of PCR-SSCP to identify INH resistance-associated mutations not found in our study is unknown.

Five isolates had missense mutations within the *inhA* structural gene; all had additional mutations in either the *inhA* promoter or *katG* gene. The isolates with both *inhA* promoter and ORF mutations had higher MICs than isolates with only promoter mutations. Although amino acid changes within the *InhA* target of INH confer resistance to this drug, our finding

that their occurrence was relatively infrequent and always in conjunction with mutation within either *katG* or the *inhA* promoter questions the value of including this marker in a routine PCR-SSCP screening stratagem. None of the isolates in the present study correctly identified as INH resistant by SSCP would have been incorrectly called sensitive if we had examined only the *katG* codon 315, *inhA* promoter, and *oxyR-ahpC* PCR amplicons.

A serious theoretical shortcoming of PCR-SSCP analysis, compared to direct sequencing, is its inability to differentiate between biologically relevant mutations (e.g., missense or nonsense) and silent mutations. Strains with silent mutations would be incorrectly identified as resistant by PCR-SSCP. We found one isolate each with a silent mutation in either *inhA* (L<sub>44</sub>L) or *ahpC* (I<sub>10</sub>I). Both of these resistant isolates also had *katG* mutations in codons 279 and 315, respectively, and therefore our PCR-SSCP strategy correctly identified them as INH resistant. Identification of silent mutation by SSCP, leading to the incorrect diagnosis of a phenotypically INH-sensitive strain as resistant, may not present a serious problem in the case of *M. tuberculosis* because of the striking reduction of silent nucleotide substitutions in this species compared to other bacterial human pathogens (40).

Although SSCP conditions must be carefully evaluated, particularly in regard to the selection of primers for each gene region to be examined (7, 28), we found a single set of SSCP conditions, including polyacrylamide gel composition, buffer, and temperature and time of electrophoresis (350 to 400 V, 12°C, 2 h), that was optimal for identifying mutations in each of the three regions associated with INH resistance (the *katG* codon 315, *inhA* regulatory, and *oxyR-ahpC* intergenic regions). The INH susceptibility status of 88 (90.7%) of the isolates in the present study was accurately determined by PCR-SSCP analysis of these three markers. These results further demonstrate the potential utility of PCR-SSCP analysis as a rapid screen for INH resistance. Although previous studies evaluated only a few mutations, we conducted a more comprehensive evaluation of a broader set of mutations within seven gene regions. The reagents and equipment used in our PCR-SSCP procedure are considerably less expensive than those required for automated DNA sequencing, making the SSCP method a viable candidate for laboratories that intend to perform genotypic drug resistance identification methods but do not have access to more expensive automatic sequencing instruments. PCR-SSCP may prove to be an especially useful complement to culture-based susceptibility testing in countries, such as Brazil, that have an high overall incidence of antituberculosis drug resistance. We demonstrated that SSCP electrophoresis offers a convenient and rapid genotypic screen for mutations associated with INH resistance.

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