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Single Nucleotide Polymorphisms in Genes Associated with Isoniazid Resistance in *Mycobacterium tuberculosis*

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Isoniazid (INH) is a central component of drug regimens used worldwide to treat tuberculosis. Previous studies have identified resistance-associated mutations in *katG*, *inhA*, *kasA*, *ndh*, and the *oxyR-ahpC* intergenic region. DNA microarray-based experiments have shown that INH induces several genes in *Mycobacterium tuberculosis* that encode proteins physiologically relevant to the drug's mode of action. To gain further insight into the molecular genetic basis of INH resistance, 20 genes implicated in INH resistance were sequenced for INH resistance-associated mutations. Thirty-eight INH-monoresistant clinical isolates and 86 INH-susceptible isolates of *M. tuberculosis* were obtained from the Texas Department of Health and the Houston Tuberculosis Initiative. Epidemiologic independence was established for all isolates by IS*6110* restriction fragment length polymorphism analysis. Susceptible isolates were matched with resistant isolates by molecular genetic group and IS*6110* profiles. Spoligotyping was done with isolates with five or fewer IS*6110* copies. A major genetic group was established on the basis of the polymorphisms in *katG* codon 463 and *gyrA* codon 95. MICs were determined by the E-test. Semiquantitative catalase assays were performed with isolates with mutations in the *katG* gene. When the 20 genes were sequenced, it was found that 17 (44.7%) INH-resistant isolates had a single-locus, resistance-associated mutation in the *katG*, *mabA*, or *Rv1772* gene. Seventeen (44.7%) INH-resistant isolates had resistance-associated mutations in two or more genes, and 76% of all INH-resistant isolates had a mutation in the *katG* gene. Mutations were also identified in the *fadE24*, *Rv1592c*, *Rv1772*, *Rv0340*, and *iniBAC* genes, recently shown by DNA-based microarray experiments to be upregulated in response to INH. In general, the MICs were higher for isolates with mutations in *katG* and the isolates had reduced catalase activities. The results show that a variety of single nucleotide polymorphisms in multiple genes are found exclusively in INH-resistant clinical isolates. These genes either are involved in mycolic acid biosynthesis or are overexpressed as a response to the buildup or cellular toxicity of INH.

Isoniazid (INH) is one of the primary chemotherapeutic and prophylactic drugs used against *Mycobacterium tuberculosis*, the causative agent of tuberculosis, which remains the leading single cause of death due to an infectious agent throughout the world. Recent studies indicate that the median rate of primary resistance to INH is 7.3% (range, 1.5 to 32%) and that the rates of acquired resistance range from 5.3 to 70% globally (9, 45). The overall rate of resistance to INH is 8.4% in the United States and has remained relatively stable in the last decade (23). Global reports of clusters of tuberculosis cases caused by drug-resistant strains together with the emergence and dissemination of multidrug-resistant tuberculosis have underscored the need for research into the mechanisms of drug resistance and the design of more effective antituberculous agents. Despite the use of INH for several decades, the molecular basis for its bactericidal action and the mechanisms by which INH resistance evolves in *M. tuberculosis* are only beginning to be understood.

INH has a simple chemical structure consisting of a hydrazide group attached to a pyridine ring, but its mode of action is very complex (8). It is proposed that INH enters *M. tuberculosis* as a prodrug by passive diffusion and is activated by catalase-peroxidase, encoded by *katG*, to generate free radicals, which then attack multiple targets in the cells (6). Recent studies have shown that an NADH-dependent enoyl acyl carrier protein (ACP) reductase, encoded by *inhA*, and a β-ketoacyl ACP synthase, encoded by *kasA*, are two potential intracellular enzymatic targets for activated INH; and both of these enzymes are involved in the biosynthesis of mycolic acids (4, 19, 20). Resistance-associated amino acid substitutions have been identified in the *katG*, *inhA*, and *kasA* genes of INH-resistant clinical isolates of *M. tuberculosis* (7, 20, 24, 26, 29). In addition, mutations in the *oxyR-ahpC* intergenic region have been identified in INH-resistant isolates (36). Additional genetic and biochemical studies have shown that certain promoter mutations of alkylhydroperoxide reductase, encoded by *ahpC*, in INH-resistant isolates result in overexpression of *ahpC* as a compensatory mechanism for the loss of catalase activity due to *katG* mutations (15, 32). Recently, missense mutations were identified in *ndh*, a gene encoding NADH dehydrogenase, which is an essential respiratory chain enzyme that regulates the NADH/NAD^+ ratio in cells (18, 22). The molecular mechanism by which mutations in *ndh* confer INH resistance in *M. tuberculosis* is poorly understood. In addition, low-level INH resistance in mycobacteria has been shown to be

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associated with enhanced expression of arylamine *N*-acetyltransferase (NAT), which is believed to inactivate the prodrug INH by acetylating the molecule. However, no INH resistance-associated mutations have been described in the NAT gene (25, 37).

Publication of the complete genome sequence of *M. tuberculosis* has made possible the use of a DNA microarray to monitor changes in gene expression in response to INH (10, 43). Research has been shown that INH induces several genes, including *ahpC*; genes that encode type II fatty acid synthase enzymes involved in mycolic acid biosynthesis; *fbpC*, which encodes trehalose dimycolyl transferase (antigen 85-C), which is involved in mycolate maturation; *fadE23* and *fadE24*, which are presumably involved in fatty acid β-oxidation; and several other genes with unknown functions (43). There have been no studies of the resistance-associated polymorphisms in the genes induced by INH. In an effort designed to better understand the molecular genetic basis of INH resistance, all genes implicated in conferring INH resistance as well as genes induced by INH were sequenced in their entirety to identify resistance-associated mutations in clinical isolates of *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction. Thirty-eight epidemiologically unrelated INH-monoresistant and 86 INH-susceptible isolates of *M. tuberculosis* cultured from patients with pulmonary and extrapulmonary tuberculosis were studied. The resistant isolates were obtained from 35 residents of Texas and 3 residents of Mexico. The Texas residents lived in 11 different counties. Foreign birth was verified for 11 Texas residents and included El Salvador, Honduras, India, the Philippines, and Zaire ($n = 1$ each), Vietnam ($n = 2$), and Mexico ($n = 4$). INH-susceptible isolates were also obtained from residents of Texas and Mexico. The INH-resistant and INH-susceptible isolates were chosen to represent all three major genetic groups of *M. tuberculosis*. Furthermore, on the basis of IS*6110* profiling and genetic group designation, the genotypes of the susceptible control isolates were judged to be closely similar to those of the INH-resistant organisms. Genomic DNA was isolated from bacteria grown on Lowenstein-Jensen (LJ) medium. All bacterial growth and DNA extraction procedures were conducted in a biosafety level 3 laboratory.

Semiquantitative catalase assay. INH-resistant isolates with mutations in *katG* encoding the catalase-peroxidase enzyme were tested for catalase activity, with strain H37Rv used as a positive control. A suspension of the isolates was prepared from freshly growing LJ agar slants in Middlebrook 7H9 broth to a density equivalent to that of a McFarland 1.0 standard. The suspensions were allowed to stand for 30 min so that large particles would settle to the bottoms of the tubes. One hundred microliters of the supernatant was inoculated onto an LJ deep (an agar tube with a horizontal surface) and incubated at 35°C for 2 weeks. One milliliter of a 1:2 mixture of Tween and hydrogen peroxide was then added, and the mixture was incubated at room temperature for 5 min. The height of the bubbles (in millimeters) was then measured.

MIC determination by E-test. All isolates were initially classified as INH monoresistant or susceptible in routine diagnostic laboratories by the BACTEC radiometric method (0.1 μg/ml). The MICs for 32 INH-resistant organisms and H37Rv as a positive control were determined by the E-test (41). The E-test compares favorably with the proportion method in quantifying INH susceptibility results for *M. tuberculosis* (13, 14). Briefly, a suspension of *M. tuberculosis* was prepared from a freshly growing LJ agar slant in Middlebrook 7H9 broth to a density equivalent to that of a McFarland 3.0 standard. Four to five sterile glass beads were added, and the suspension was vigorously vortexed. The suspension was then allowed to stand for 30 min so that large particles would settle to the bottom of the tube. One 90-mm Middlebrook agar plate was inoculated by swabbing the suspension in three directions, taking care to evenly cover the plate. The plates were incubated at 35°C in 5% CO_2 for 24 h, at which time an INH E-test strip (AB Biodisk, Solna Sweden) was placed on each plate. The plates were sealed with CO_2-permeable tape or shrink seals and were reincubated under the same conditions until growth was visible (5 to 10 days). The MIC was read and was the point where the growth of the organism intersected the E-test strip.

IS*6110* profiling. All isolates were assessed for epidemiologic independence by IS*6110* restriction fragment length polymorphism (RFLP) profiling by an internationally standardized method (39). The hybridizing DNA fragments were visualized by enhanced chemiluminescence, and the band patterns were analyzed with the BioImage (Ann Arbor, Mich.) Whole Band Analysis program (version 3.2). The number of hybridizing bands ranged from 1 to 21. Six isolates had five or fewer IS*6110* bands (low-copy-number isolates). Except for two isolates that shared the same print pattern, designated Z (13 IS*6110* copies), all other isolates had unique fingerprints, a result indicating epidemiologic independence.

Spoligotyping and major genetic group assignment. Low-copy-number isolates were subjected to a secondary typing method by use of spoligotyping. This method was performed with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the instructions of the manufacturer. The spoligotype patterns were compared with those in the database maintained at the Houston Tuberculosis Initiative (34). All six low-copy-number isolates had unique spoligotype patterns.

All isolates were assigned to one of three major genetic groups on the basis of the polymorphisms present at *gyrA* codon 95 and *katG* codon 463 (35, 37). The distributions of the INH-resistant isolates on the basis of major genetic groups were as follows: group 1, $n = 11$; group 2, $n = 18$; and group 3, $n = 7$. The group designation was not given for two isolates because of *katG* deletions. The distributions of the INH-susceptible isolates on the basis of major genetic groups were as follows: group 1, $n = 28$; group 2, $n = 35$; and group 3, $n = 23$.

PCR amplification and sequencing strategy. The 20 genes analyzed for nucleotide sequence variation are listed in Table 1. The oligonucleotide primers and PCR conditions used to amplify *katG*, *inhA*, *ahpC*, the *oxyR-ahpC* intergenic region, and the *gyrA* codon 95 region have been described previously (24, 36). The primers used to amplify all other genes are listed in Table 2. DNA was amplified with a GeneAmp system 9700 thermocycler (Applied Biosystems, Inc., Foster City, Calif.). Unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators (Amicon Inc., Beverly, Mass.). DNA sequencing reactions were performed with a BigDye terminator cycle sequencing kit (Applied Biosystems, Inc.). The sequencing reaction products were purified by using Centrisep columns (Princeton Separations, Adelphia, N.J.). The sequence data generated with an ABI 377 automated instrument (Applied Biosystems, Inc.) were assembled and edited electronically with the ALIGN and EDITSEQ programs (DNASTAR, Madison, Wis.), and the sequences were compared with the corresponding sequences in the published sequence of strain H37Rv (10). All nonsynonymous mutations identified in the INH-resistant isolates were confirmed by resequencing.

RESULTS

Overall sequence analysis. Of the 2.6 megabases sequenced for the 124 *M. tuberculosis* isolates, 28 polymorphic synonymous nucleotide sites were identified (Table 1). Synonymous substitutions do not result in amino acid replacements and, hence, are unlikely to participate in drug resistance. These changes will not be discussed further when the sequencing results for the 20 genes studied are described. Of the 47 polymorphic nonsynonymous sites identified, 24 were present exclusively in INH-resistant isolates, 14 were found in both INH-resistant and INH-susceptible isolates, and 9 were found only in INH-susceptible isolates. The observation of small numbers of polymorphic sites in the structural genes is in agreement with previous observations (35). Seventeen (44.7%) of the 38 INH-resistant isolates had a resistance-associated mutation in only 1 of the 20 genes sequenced (Table 3). Seventeen isolates (44.7%) had resistance-associated mutations in more than one gene (Table 4). Six of the 20 genes (*fabD*, *accD6*, *efpA*, *fbpC*, *ndh*, and *nhoA*) sequenced did not have any resistance-associated mutations.

Polymorphisms in the *katG* region. Twenty-nine (76.3%) of the 38 INH-resistant isolates had a resistance-associated mutation in *katG*. Fifteen of those 29 isolates had a resistance-

TABLE 1. Genes analyzed for nucleotide sequence diversity in INH-resistant *M. tuberculosis*

Gene	Product or function	Gene size (bp)	Positions of nucleotides sequenced[a]	Codons with nonsynonymous substitutions[b]	Codons with synonymous substitutions
furA	Ferric uptake regulator	453	−70 to 570	5[r], 115[s]	0
katG	Catalase-peroxidase	2,223	1 to 2311	(90, 91, 110, 138, 315, 336, 397, 434, 438, 529)[r] 463[rs]	322, 411, 436
mabA	3-Ketoacyl reductase	744	−199 to 781	21[r]	34, 203, 241
inhA	Enoyl ACP reductase	810	−75 to 898	(21, 194)[r]	3
ahpC	Alkylhydroperoxide reductase	588	−355 to 608	44[s]	6, 7
Rv0340	Unknown	540	−231 to 619	(101, 143, 149)[s], 163[r]	30, 170, 173
iniB	INH-inducible gene	1,440	−192 to 1440	192[s]	0
iniA	INH-inducible gene	1,923	−36 to 1923	(3, 537)[r], 481[rs]	178
iniC	INH-inducible gene	1,482	1 to 1566	83[r]	60
srmR homolog	Regulatory gene	1,245	−127 to 1509	3[r], 323[rs]	108, 112, 296
fabD	Malonyl-CoA ACP transacylase	909	−109 to 962	(199, 275)[rs]	138
kasA	β-Ketoacyl ACP synthase	1,251	−29 to 1264	(77, 269)[r], 312[rs]	6, 60
accD6	Acetyl-CoA carboxylase	1,422	−15 to 1460	229[rs]	143, 200
fbpC	Trehalose dimycolyl transferase	1,023	−250 to 1084	158[rs]	103, 205, 322
fadE24	Fatty acyl-CoA dehydrogenase	1,404	−168 to 728	0	0
efpA	Efflux protein	1,590	−92 to 718	73[rs]	0
Rv1592c	Unknown	1,338	−232 to 1357	(42, 430)[r], (321, 322)[rs] (60, 355)[s]	56, 321
Rv1772	Unknown	309	−333 to 388	4[r], 49[s]	0
ndh	NADH dehydrogenase	1,392	−186 to 1464	18[rs]	0
nhoA	Arylamine *N*-acetyltransferase	999	−539 to 1030	(67, 207)[rs]	304

[a] The primers used for the *katG* region amplify *furA* and *katG* plus 70 bp upstream from the start codon of *furA* and 88 bp downstream from the end of *katG.* The primers used for the *inhA* region amplify 199 bp upstream from the start codon of *mabA,* the *mabA* and *inhA* genes, and 88 bp downstream from the end of the *inhA* gene. The primers used for the *ahpC* region amplify 355 bp upstream from the start codon of *ahpC,* which includes the *oxyR-ahpC* intergenic regulatory region, the entire *ahpC* gene, and 20 bp downstream from the end of the gene. The primers used for the *ini* locus amplify 231 bp upstream of the start codon of *Rv0340,* the entire *Rv0340* gene, the *Rv0340-iniB* intergenic region, *iniB,* the *iniB-iniA* intergenic region, *iniA, iniC,* and 84 bp downstream from the end of *iniC.* The primers used for the *srmR* homolog amplify 127 bp upstream from the start codon of the *srmR* homolog gene, the entire *srmR* homolog gene, and 264 bp downstream from the end of the gene. The primers used for the *fabD* region amplify 109 bp upstream from the start codon of *fabD,* the entire *fabD* gene, and 53 bp downstream from the end of the gene. The primers used for the *kasA* region amplify 29 bp upstream from the start codon of *kasA,* the entire *kasA* gene, and 13 bp downstream from the end of the gene. The primers used for the *accD6* region amplify 15 bp upstream from the start codon of *accD6,* the entire *accD6* gene, and 38 bp downstream from the end of the gene. The primers used for the *fbpC* region amplify 250 bp upstream from the start codon of *fbpC,* the entire *fbpC* gene, and 61 bp downstream from the end of the gene. The primers used for the *fadE* region amplify 168 bp upstream from the start codon of *fadE24* and 728 bp of the *fadE24* gene. The primers used for the *efpA* region amplify 92 bp upstream from the start codon of *efpA* and 718 bp of the coding region of the gene. The primers used for the *Rv1592c* region amplify 232 bp upstream from the start codon of *Rv1592c,* the entire *Rv1592c* gene, and 19 bp from the end of the gene. The primers used for the *Rv1772* region amplify 333 bp upstream from the start codon of *Rv1772,* the entire *Rv1772* gene, and 79 bp from the end of the gene. The primers used for the *ndh* region amplify 186 bp upstream from the start codon of *ndh,* the entire *ndh* gene, and 72 bp from the end of the gene. The primers used for the *nhoA* region amplify 539 bp upstream from the start codon of *nhoA,* the entire *nhoA* gene, and 31 bp from the end of the gene.

[b] r, nonsynonymous sites found exclusively in INH-resistant isolates; rs, nonsynonymous sites found in both INH-resistant and INH-sensitive isolates; s, nonsynonymous sites found in INH-sensitive isolates only.

associated mutation in *katG* only. Two isolates had complete *katG* gene deletions, based on the reproducible absence of PCR products; two isolates had different termination mutations in codons 90 (TGG→TAG) and 434 (CAG→TAG); one isolate had an insertion of a guanine nucleotide at the third base in codon 124, which resulted in a frameshift mutation; and eight codons had substitution mutations in *katG*. One isolate had two substitution mutations in codons 397 (TGG→TAC, Trp→Tyr) and 529 (AAC→GAC, Asn→Asp) of *katG* and carried no other mutations in the other 19 genes sequenced. The most common substitution mutation occurred in codon 315 (Ser→Thr [AGC→ACC], $n = 13$; Ser→Ile [AGC→ATC], $n = 1$); and (Ser→Asn [AGC→AAC], $n = 1$). None of the polymorphisms identified in *katG* were observed in the susceptible organisms, suggesting that these mutations probably contribute to INH resistance.

It has recently been shown that *katG* is cotranscribed with *furA* from a common regulatory region and that *furA* is a negative regulator of *katG* (27, 46). So, we sequenced *furA* and its promoter region and found two polymorphisms. One of the polymorphisms was located at position 30 upstream of the start site of *furA*, resulting in a T→C change. This mutation was also identified in susceptible organisms and, thus, may not contribute to INH resistance. The second mutation was located in codon 5 (TCC→CCC, Ser→Pro) of a single INH-resistant isolate. This isolate also had other mutations in four different genes (Table 4).

Polymorphisms in the *inhA* region. The *inhA* region consists of two genes, *mabA* and *inhA*, both of which encode enzymes involved in mycolic acid biosynthesis (4, 5). One isolate had two mutations in *mabA*: a C→T change at position 15 upstream of the start site of *mabA* and Thr→Ala substitution at codon 21. This isolate did not have mutations in any of the other genes. Eight isolates had nucleotide changes upstream of the start site of *mabA* at positions 15 ($n = 6$) and positions 8 and 147 ($n = 1$ each) (Table 4). Two isolates with a C→T change at position 15 upstream of the start site of *mabA* also had nonsynonymous substitutions in *inhA*, resulting in Ile194Thr (ATC→ACC) and Ile21Val (ATC→GTC) replacements. One of these isolates also contained a Pro42Leu (CCT→CTT) substitution in *Rv1592c*, and the other isolate did not have any other changes.

Polymorphisms in the *oxyR-ahpC* intergenic region. Two isolates had nucleotide substitutions in the *oxyR-ahpC* inter-

TABLE 2. PCR primers and conditions used to amplify the designated *M. tuberculosis* gene regions[a]

Gene region	Forward primer	Reverse primer	Size (bp)	Length of D(s)	A (temp [°C], time [s])	Length of E(s)
furA	5′-GCGATCGGGTCCTAGCAG-3′	5′-TTCATATGACCCACGACGG-3′	641	40	61.0,30	40
Rv0340	5′-ATGCGTCGTATGCTTGG-3′	5′-CCAAACACCTATCGGGATC-3′	850	40	58.5,30	40
iniB	5′-ATAAGTTCCGGACCGGCG-3′	5′-CGACAGATGAGGCATAGCAG-3′	1,053	40	56.0,30	45
iniB-iniA	5′-TTGAACGGCGCTGCTATG-3′	5′-GTGCTGATGTCATCGACGG-3′	1,070	40	62.0,30	45
iniA	5′-CAACCGCAGCGGTTGACAT-3′	5′-CCGCATGCCGATAATCATT-3′	1,079	40	61.0,30	45
iniA-iniC	5′-GGAATCGAAACCGCTGCG-3′	5′-CCAGCCCACCGATCTGTTTGA-3′	1,090	40	64.0,30	45
iniC	5′-TCCTGTTGCGCACCCTGAAC-3′	5′-AACATGTTCCACCCGGTGGC-3′	1,040	40	65.0,30	45
srmR homolog	5′-GCCAGTACCGGATCGACG-3′ 5′-CCAGGATGTCCGCGACACC-3′	5′-GTGTCGCGGACATCCTGG-3′ 5′-TGTCCGGGTGCGAGCAAC-3′	819 835	30 30	63.5,30 67.5,30	40 40
fabD	5′-AAAAACATAGCTTACAGGCCCG-3′	5′-GTTGTGTACAAATCGAACTGACG-3′	1,071	40	59.5,30	45
kasA	5′-GTTCAGGCGAGGCTTGAG-3′	5′-GCGATGTCGTGCTTCAGTAA-3′	1,293	45	60.5,30	50
accD6	5′-GACAGGAGACCTGCGATGAC-3′ 5′-CGGTTGGTCGGATTGTTC-3′	5′-GGCAGAACAATCCGACCA-3′ 5′-CCTCGCGCGTGCGATTCTG-3′	655 842	30 35	61.5,30 61.0,30	30 35
fadE24	5′-GCTATCAGATGCCTGGCG-3′	5′-GTGGGATCGAATAGTGGCTG-3′	890	35	61.0,30	35
fbpC	5′-GCATGGGTCTCTCCTCTG-3′ 5′-GCCAATCCAGTTTCTACACCG-3′	5′-GCTGATACCAGTCGGTGTAG-3′ 5′-ATGTTCCACACATCGGC-3′	635 732	30 35	56.0,30 57.5,30	30 35
efpA	5′-ACCTCCCGCGATCATCG-3′	5′-CGTCGAGCTTCATCCGTTC-3′	810	35	64.5,30	35
Rv1592c	5′-AACTCGGCGTACCCAACC-3′ 5′-GAGCGTGTCGGGTTGTCC-3′	5′-ACACGCTCGGAATTCAAGG-3′ 5′-CGAGGTTGTGTGCCAGGTC-3′	804 834	35 35	61.5,30 64.0,30	35 35
Rv1772	5′-CGGGTGTTTCTCAACGAC-3′	5′-GGACTGGACTCGCTGATTG-3′	633	30	57.5,30	30
ndh	5′-ATCACCACCGCCGCTGAAGC-3′ 5′-CATTCCCGGGTACCCGAAC-3′	5′-GTTCGGGTACCCGGGAATG-3′ 5′-GTCGACCGTTTTGGCGTTGG-3′	1,134 535	45 30	65.0,30 65.0,30	50 30
nhoA	5′-CTAGTGGCGCGAGCAGAC-3′ 5′-GCTATCTCGTCGACGTCG-3′	5′-CCGACGTCGACGAGATAGC-3′ 5′-GCATTCTACGTCTACGCCG-3′	927 662	40 30	61.5,30 59.0,30	40 30

[a] Abbreviations: D, denaturation at 94°C; A, primer annealing conditions; and E, extension at 72°C. All PCR were run for 25 cycles, were preceded by a denaturation step at 95°C for 15 min, and included a final extension step at 72°C for 7 min.

genic region, which has previously been shown to be involved in INH resistance (29). The sites were at positions 9 (G→A) and 48 (G→A) upstream of the transcriptional start site of *ahpC*. The MICs were high for both isolates, and the isolates had reduced levels of catalase activity, suggesting that these mutations were probably selected as a consequence of the *katG* mutations. One isolate had a complete deletion of *katG*, and the other isolate had an insertion of a guanine nucleotide in codon 124 of *katG*, resulting in a frameshift mutation (Table 4). Three isolates had a substitution mutation at position 100 (G→A) upstream of the transcriptional start site of *ahpC*, but this change was also identified in susceptible organisms.

Polymorphisms in the *kasA* region. The *kasA* genomic region consists of five genes (*fabD*, *acpM*, *kasA*, *kasB*, and *accD6*) in an operon preceded by a regulatory gene (*srmR* homolog) encoding enzymes involved in the fatty acid biosynthetic (type II fatty acid synthase [FAS-II] system) pathway (10, 12, 20, 33). *fabD* and *acpM* encode a malonyl coenzyme A (CoA) ACP transacylase and an ACP, respectively. Both *kasA* and *kasB* encode a β-ketoacyl ACP synthase involved in the production of long-chain mycolic acids. The fifth gene in the cluster, *accD6*, is an acetyl-CoA carboxylase (β-subunit) that is involved in the production of malonyl-CoA. It has previously been shown (43) that these genes are overexpressed in *M. tuberculosis* in the presence of activated INH. The open reading frame immediately upstream of the FAS-II gene cluster encodes a homolog of the regulatory protein, SrmR, that controls the production of polyketide in *Streptomyces ambofaciens* (12). Except for *kasB* and *acpM*, all genes in the *kasA* region were sequenced.

Sequencing analysis showed that two isolates had a substitution mutation in codon 3 (GAC→GGC, Asp→Gly) and codon 323 (ATG→ACG, Met→Thr) of the *srmR* homolog. The nonsynonymous change at codon 323 was also found in susceptible organisms, and the isolate with the codon 3 substitution also contained a mutation in codon 91 (TGG→CGG,

TABLE 3. INH resistance-associated mutations in *M. tuberculosis*

Gene or gene region	No. of isolates with mutations at:		% of isolates with mutation at single locus	MIC range[b] (μg/ml)
	Single locus[a]	Multiple loci		
furA	0	1	0	
katG	15	14	39.5	0.1–>256
mabA	1	8	2.6	2
inhA	0	2	0	
ahpC	0	2	0	
Rv0340	0	1	0	
iniB	0	1	0	
iniA	0	3	0	
iniC	0	1	0	
srmR homolog	0	1	0	
fabD	0	0	0	
kasA	0	3	0	
accD6	0	0	0	
fbpC	0	0	0	
fadE24	0	1	0	
efpA	0	0	0	
Rv1592c	0	2	0	
Rv1772	1	0	2.6	0.125
ndh	0	0	0	
nhoA	0	0	0	
Total	17		44.7	

[a] The 15 *katG* mutations were Trp90STOP (TGG→TAG), Asn138Ser (AAC→AGC), Ser315Ile (AGC→ATC), Trp397Tyr (TGG→TAC) and Asn529Asp (AAC→GAC) (same isolate), Trp438Arg (TGG→CGG), and deletion of *katG* (n = 1 each) and Ser315Thr (AGC→ACC; n = 9). One isolate had two mutations in *mabA*. They were at position −15 (C→T) and Thr21A1a (ACC→GCC). The mutation in *Rv1772* was Thr4A1a (ACA→GCA; n = 1). Seventeen isolates had INH resistance-associated mutations in two or more genes.

[b] The MIC range shown is for the isolates with single-locus mutations. The MICs for isolates with multiple mutations are shown in Table 4.

Trp→Arg) of *katG* (Table 4). Three isolates had substitution mutations in *kasA* at codon 269 (GGT→AGT [Gly→Ser], n = 2) and codon 77 (ATG→ATA [Met→Ile], n = 1) that were not found in the susceptible organisms. However, these mutations were associated with mutations in *katG* and other changes in different genes (Table 4). In addition, one isolate had a Gly312Ser (GGC→AGC) replacement that was also observed in susceptible organisms. No resistance-associated mutations were identified in *fabD* or *accD6*. However, four isolates had Ser275Asn (AGC→AAC, n = 2) and Ala199Thr (GCG→ACG, n = 2) replacements in *fabD* that were also observed in susceptible organisms. Seven group 1 isolates had a GAC→GGC (Asp→Gly) replacement in codon 229 of *accD6* which was also present in group 1 susceptible organisms, indicating that this polymorphism is a surrogate marker for group 1 *M. tuberculosis* isolates and does not participate in INH resistance.

Polymorphisms in the *iniBAC* region. The *ini* chromosomal region has four genes designated *Rv0340*, *iniB*, *iniA*, and *iniC*, of which the last three genes are organized as an operon (2, 3, 10). The *Rv0340* gene is located upstream of the *iniBAC* operon and is transcribed in the same orientation (10). The *ini* genes were originally identified on the basis of induction by INH and ethambutol treatment in vitro (2). On the basis of sequence annotation, the *iniB* gene encodes a protein with weak homology to alanine- and glycine-rich cell wall structural

TABLE 4. INH-resistant *M. tuberculosis* isolates with mutations in two or more genes

Isolate	MIC (μg/ml)	Mutation[a]											
		furA	*katG*	*mabA*	*inhA*	*oxyR-ahpC*	*srmR* homolog	*kasA*	*Rv0340*	*iniB*	*iniA*	*iniC*	*Rv1592c*	*fadE24*
UT739	4		S315T									W83G		
UT1885	12		S315T										V430A	
UT2090	>256		124; G ins			9 UPS; G→A								
UT2204	2		W91R				D3G							
UT3174	>256		DEL			48 UPS; G→A								
UT3230	ND		S315T											64 UPS; 2 bp ins
UT3306	>256		Q434STOP											
UT3315	ND		L336P					G269S	V163I					
UT3437	ND		S315T											
UT3740	ND			147 UPS; C→T										
HN1984	>256		S315N	15 UPS; C→T										
HN1993	0.19		A110V	15 UPS; C→T										
HN2029	0.19			15 UPS; C→T	I194T									
NHN670	1.5		A110V	15 UPS; C→T										
NHN673	0.25		A110V	15 UPS; C→T										
NHN674	<0.016	S5P	A110V	8 UPS; T→A										
NHN681	ND			15 UPS; C→T	I21V			M77I		12-bp DEL at position 222			P42L	

[a] The single-letter amino acid designations used are as follows: A, alanine; D, aspartic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; and W, tryptophan. The single-letter nucleotide designations used are as follows: A, adenine; C, cytosine; G, guanine, and T, thymidine. The other abbreviations are as follows: ins, insertion of bases; UPS, nucleotide position (base pairs) upstream of the start codon; DEL, deletion of the bases; STOP, termination mutation; ND, not determined.

proteins, *iniA* encodes a protein with a phosphopantetheine attachment site motif characteristic of ACPs, and *iniC* encodes a protein that is 34% identical to IniA (2, 3). We have previously demonstrated ethambutol resistance-associated mutations in all four genes and also elected to sequence these genes in this study (30).

Sequence analysis showed that all four genes harbored mutations that were not found in the INH-susceptible organisms. One isolate with a termination mutation in codon 434 of *katG* also had a Val→Ile (GTT→ATT) replacement mutation in codon 163 of the *Rv0340* gene (Table 4). One isolate had a 12-bp deletion beginning with the second base of codon 222 of *iniB*, resulting in a frameshift mutation. This isolate had changes in other genes as well (Table 4). Three isolates had INH resistance-associated mutations in *iniA.* One isolate had an Arg537His (CGC→CAC) replacement, another isolate had a Pro3Ala (CCC→GCC) substitution, and a third isolate had a 5-bp deletion beginning with the third base of codon 94, resulting in a frameshift mutation. All three isolates with resistance-associated changes in *iniA* had mutations in other genes as well (Table 4). Three INH-resistant isolates had a His481Gln (CAT→CAG) substitution that was also found in susceptible organisms (Table 1). One isolate had a resistance-associated mutation in codon 83 (TGG→GGG, Trp→Gly) of *iniC* that was not represented in INH-susceptible organisms. This isolate also had a Ser315Thr replacement in the *katG* gene.

Polymorphisms in other genes induced by INH. *fbpC* is another gene induced by INH. It encodes an exported antigen (antigen 85-C) that has trehalose dimycolyl transferase activity and mediates a terminal step of mycolate maturation by esterifying mycolates to specific carbohydrate moieties in the cell wall of *M. tuberculosis* (43). Sequencing analysis showed that one isolate had a Gly158Ser (GGC→AGC) replacement mutation that was also found in susceptible organisms. Two isolates had a C→T nucleotide substitution at position 63 upstream of the start site of *fbpC* that was also found in INH-susceptible isolates.

Rv1592c and *Rv1772* are two genes with unknown functions and are also transcriptionally induced by INH (10, 43). Sequencing analysis showed that two isolates had Pro42Leu (CCT→CTT) and Val430Ala (GTG→GCG) amino acid substitution mutations in *Rv1592c* that were absent in susceptible isolates. Both isolates had mutations in other genes as well. A group 1 INH-susceptible isolate had a Glu→Asp (GAA→GAC) substitution in codon 60, and a group 3 INH-susceptible isolate had a Asp→Glu (GAC→GAA) replacement in codon 355 of *Rv1592c.* Interestingly, all INH-resistant and -susceptible group 1 isolates also had a synonymous change in codon 321 (GAA→GAG) and a nonsynonymous substitution in codon 322 (ATT→GTT, Ile→Val). A subset of group 2 INH-resistant and -susceptible isolates also had the same changes. These two polymorphisms were not observed in any group 3 organisms. In addition, six INH-resistant group 2 organisms and six INH-susceptible group 2 organisms had a G→A nucleotide substitution at position 29 upstream of the start site of the *Rv1592c* gene. One isolate had a Thr4Ala (ACA→GCA) amino acid replacement mutation in *Rv1772* that was not present in INH-susceptible isolates. This mutation was not accompanied by changes in the other 19 genes sequenced. The MIC for this isolate was low (0.125 μg/ml).

faDE24 is another gene induced by INH which encodes a fatty acyl-CoA dehydrogenase that is presumed to degrade fatty acids into acetyl-CoA subunits by β-oxidation (43). Sequencing analysis showed that one isolate had a 2-bp insertion at position 64 upstream of the translational start site of the gene. This mutation was not found in susceptible isolates but was accompanied by a Ser315Thr mutation in *katG*. In addition, two isolates had an A→C nucleotide substitution 23 bp upstream of the start codon, but this change was also found in INH-susceptible organisms.

An additional gene, *efpA*, encodes an efflux protein whose role in INH metabolism is not known (43). No resistance-associated mutation was identified in this gene. However, one isolate had an Ile→Thr (ATC→ACC) amino acid replacement mutation in codon 73 which was also discovered in INH-susceptible organisms.

Polymorphisms in *ndh* and *nhoA* genes. *ndh* encodes an NADH dehydrogenase which controls the oxidative state of mycobacterial cells by regulating the NADH/NAD^+ ratio (22). Mutations in *ndh* that cause an increase in this ratio result in coresistance to both INH and ethionamide in *Mycobacterium smegmatis* (22). Recently, mutations in *ndh* were reported in INH-resistant *M. tuberculosis* isolates (18). Our sequencing analysis shows no resistance-associated mutations in *ndh*, but two isolates had a Val→Ala (GTG→GCG) substitution mutation in codon 18 which was also identified in INH-susceptible organisms.

A recent study showed that *nhoA* encodes an NAT which acetylates INH in vitro and in *Mycobacterium bovis* BCG (25, 37). It was also shown that this gene was polymorphic at codon 207 and that all isolates with the polymorphism had the same IS*6110* RFLP profile (37). Our sequence data showed a Gly→Glu (GGG→GAG) amino acid replacement mutation in codon 207 of *nhoA* in two isolates, but the mutation was also detected in INH-susceptible organisms. In addition, one isolate had a Gly→Arg (GGC→GGT) substitution at codon 67 which was also found in an INH-susceptible isolate. All the isolates with the two polymorphisms belonged to major genetic group 2.

Relationship between INH MICs, *katG* mutations, and catalase activity. It has been known for a long time that the loss of catalase activity is associated with a high level of INH resistance (21). Our study corroborates this premise by showing that the seven INH-resistant isolates for which the MICs are the highest (>256 μg/ml) also had very low levels of or no catalase activity (Table 5). Two of those isolates lacked *katG* completely, two other isolates had truncated KatG proteins, one isolate had a frameshift mutation at codon 123, and the other two isolates had substitution mutations. The MICs for INH-resistant isolates with single-locus nonsynonymous mutations in *katG* were in the range of 0.19 to 256 μg/ml. Interestingly, for isolates with the Ser315Thr mutation in *katG* (n = 13), the MICs were in the range of 0.38 to 12 μg/ml, and the catalase activities of those isolates ranged from 10 to 26 mm. The MICs for isolates with amino acid replacements other than threonine at codon 315 of *katG* were higher, and the isolates had much lower catalase activities (Table 5). The MICs for two other isolates with single-locus, nonsynonymous, non-

TABLE 5. MICs, catalase activity, and mutations in *katG* for INH-resistant *M. tuberculosis* isolates[a]

Isolate	*katG* mutation[b]	Catalase (activity height of bubbles [mm])[c]	MIC (μg/ml)
UT450	S315I	13	32
UT473	W397Y, N529D	15	>256
UT561	S315T	26	1
UT739	S315T	10	4
UT1885	S315T	25	12
UT1998	S315T	15	0.75
UT2007	S315T	15	6
UT2090	124; G Ins	5	>256
UT2204	L91R	5	2
UT2340	S315T	20	0.38
UT2419	S315T	20	4
UT2883	S315T	10	8
UT2931	S315T	25	0.5
UT3171	W90STOP	No bubbles	>256
UT3174	Del	No bubbles	>256
UT3181	Del	No bubbles	>256
UT3194	S315T	14	19
UT3306	Q434STOP	5	>256
HN1984	S315N	5	>256
HN1993	A110V	10	0.19
NHN670	A110V	9	1.5
NHN673	A110V	10	0.25
NHN674	A110V	10	<0.016
H37Rv	WT	25	<0.016

[a] H37Rv was used as a positive control.
[b] The single-letter amino acid designations used are as follows: A, alanine; D, aspartic acid; I, isoleucine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine. The single-letter nucleotide designation used is as follows: G, guanine. The other abbreviations are as follows: Ins, insertion of bases; Del, deletion of the gene; STOP, termination mutation; WT, wild type.
[c] No bubbles indicates a lack of catalase activity. Only isolates with data on catalase activity are shown.

katG mutations were low (range, 0.125 to 2 μg/ml). There was also no simple correlation between the MICs for isolates with single or multiple resistance-associated mutations.

DISCUSSION

INH has been used as a primary drug to treat tuberculosis since the 1950s (8, 21). Shortly after the introduction of this drug for the treatment of tuberculosis, INH-resistant strains that had lost catalase activity were isolated from patients (21). The observed clinical relationship between the loss of catalase activity and INH resistance was finally explained in 1992 by the isolation and characterization of *katG*, which encodes the bifunctional catalase and peroxidase activities in *M. tuberculosis* (47). Biochemical studies have shown that the catalase-peroxidase activity of *katG* is also required for the activation of INH, which passively enters *M. tuberculosis* cells as a prodrug (6, 7, 11). The identity of the activated INH is unknown, but it has been suggested that it may be a free-radical intermediate (42). The stable end products of KatG oxidation of INH result in the production of isonicotinic acid, isonicotinamide, pyridine-4-carboxyaldehyde, and 4-pyridylmethanol, none of which have antimycobacterial activities (42). On the basis of the hyperfine splitting patterns of electron paramagnetic resonance spin trapping experiments, KatG-mediated oxidation of INH has been shown to produce acyl, acyl peroxo, and pyridyl radicals of INH (42). These free radicals can potentially disrupt various cellular processes, resulting in pleiotropic effects. The leading hypothesis suggests that KatG-activated INH primarily targets at least two components of the FAS-II system of *M. tuberculosis*. An acyl pyridine free-radical derivative of INH has been shown to bind to NAD^+, and this adduct inhibits InhA, an enoyl reductase involved in mycolic acid biosynthesis (11, 19). In addition, a β-ketoacyl ACP synthase encoded by *kasA* has been shown to covalently bind to activated INH (20). Several studies have documented a multitude of mutations in *katG*, including insertions, partial and complete deletions, and termination and substitution mutations, in INH-resistant isolates (1, 24, 29, 31). Also, resistance-associated mutations have been found in the *inhA* and *kasA* genes of INH-resistant clinical isolates (7, 20, 24, 29). Knowledge of the spectrum of mutations in the *katG*, *inhA*, and *kasA* genes that may participate in INH resistance is not complete. Furthermore, several additional chromosomal loci have recently been identified that encode proteins that are induced in *M. tuberculosis* during INH treatment (43). The identification of these genes raises the possibility that mutations in them may confer INH resistance or participate in the survival mechanisms of *M. tuberculosis* due to INH treatment. Moreover, sequence variations in these induced genes have not been studied in either INH-resistant or -susceptible organisms. Information gained from sequencing analysis will be useful in the design of rapid molecular genetic methods that can be used to identify INH-resistant isolates as well as for the recognition of single nucleotide polymorphisms that can be useful in epidemiologic surveillance. To address these questions, we sequenced all 20 genes implicated in INH resistance in a sample of 38 INH-monoresistant isolates independently recovered from patients with tuberculosis in Texas and Mexico.

The results obtained from this study show that the genetic mechanisms of INH resistance in *M. tuberculosis* are highly complex and involve several genes. Twenty-nine of the 38 INH-resistant isolates had complete deletions, a frameshift mutation that caused by an insertion, and termination and point mutations in *katG*. Isolates with insertions and deletions have lost their catalase activities, and the MICs for these isolates are also the highest, confirming previous observations about the loss of catalase activity and a high level of INH resistance (21, 29). This finding also suggests that *katG* is not an essential gene and that *M. tuberculosis* cells have other means to combat oxidative stress. The most common mutation was the Ser315Thr mutation in *katG*, which is in agreement with the bulk of the epidemiologic data (29, 40). Biochemical evidence obtained from studies with purified wild-type and Ser315Thr KatG proteins suggest that the Ser315Thr mutation results in a competent catalase-peroxidase that has a reduced ability to metabolize INH (29). The high frequency with which variants with this mutation are recovered from patients all over the world suggests that *M. tuberculosis* cells achieve a good balance between the need to maintain catalase-peroxidase activity and the need to reduce conversion of the prodrug INH to its activated form without a significant loss of bacterial fitness (27, 28).

The catalase-peroxidase gene (*katG*) is under the positive control of *oxyR*, a global transcriptional regulator of oxidative stress in several bacteria. The *oxyR* sequence in *M. tuberculosis* contains multiple substitution mutations and partial deletions

that render the gene inactive (29, 36). This gene is divergently transcribed with *ahpC*, which encodes the catalytic subunit of alkylhydroperoxide reductase. Biochemical evidence suggests that increased AhpC activity could compensate for the loss of KatG activity in the detoxification of organic peroxides (32, 44). In light of the prevailing view regarding the role of *ahpC* in INH resistance, two isolates that had lost their catalase activities also had compensatory mutations in *ahpC* (29). In *M. tuberculosis* the *katG* gene is adjacent to *furA*, a homolog of the ferric uptake regulator (10). Recently, it was shown that FurA is a negative regulator of *katG*, and both genes are cotranscribed from a common regulatory region (27, 46). In addition, FurA appears to be a dominant regulator of oxidative stress and other genes involved in intracellular survival in mycobacterial species and lacks a functional *oxyR* (27). If *furA* is a negative regulator of *katG*, promoter mutations might lead to decreased levels of expression of KatG, which should then result in INH resistance. Our results show that a promoter mutation at position 30 upstream of the start codon of *furA* was identified in both INH-resistant and -susceptible organisms, indicating that this nucleotide is probably not important for transcriptional activity. Also, another strain with a structural mutation in codon 5 of FurA had mutations in other genes. The MIC for this strain was equal to that for the control organism, suggesting that the *furA* and other mutations in this isolate may contribute to a low-level INH resistance phenotype.

Previous studies have shown that mutations in the upstream region of the *inhA* locus result in increased levels of InhA expression, thereby elevating the drug target levels and producing INH resistance via a titration mechanism (29). Also, the mutations identified in the structural part of *inhA* results in INH resistance due to a reduced binding affinity of the INH-NAD^+ adduct for enoyl reductase (4, 7, 29). Our results show that the MICs for all isolates harboring mutations in the promoter region of the *inhA* locus as well as point mutations in the structural part of *inhA* are low. Four of these isolates also contained an Ala110Val (GCC→GTC) substitution in *katG*, implying that this mutation contributes to low-level INH resistance. Another isolate with the *inhA* promoter mutation contained a Ser315Asn (AGC→AAC) change in *katG* and two different mutations in *iniA*. The MIC for this isolate was very high. This finding suggests that in certain isolates with multiple mutations, INH resistance develops in a stepwise fashion. This study also identified for the first time a structural mutation in *mabA* in an INH-resistant clinical isolate (5, 29). INH has not been shown to bind to MabA and, hence, is not a direct target for activated INH (5, 11). This isolate, for which the MIC was low, also had the promoter mutation, making it difficult to explain the contribution of the structural mutation to INH resistance.

Differential protein analysis of INH-treated and control cells has shown that activated INH covalently binds to AcpM and β-ketoacyl synthase (19). *kasA* and other genes in the FAS-II system are also upregulated by INH treatment (43). Our results show that mutations in *kasA* were accompanied by changes in other genes. The MICs for isolates with *kasA* mutations were low. Piatek et al. (26) have previously shown that Gly269Ser (GGT→AGT) point mutations can also be found in INH-susceptible isolates. We did not observe this change in any of the 86 INH-susceptible isolates in this study. The Met77Ile (ATG→ATA) substitution in *kasA* has also not been observed before. In addition, Lee et al. (17) have observed point mutations in codons 121 and 387 ($n = 1$ each) of *kasA* from two different INH-resistant isolates. On the basis of molecular modeling of the KasA protein, it has been suggested that the known mutations in *kasA* might affect the KasA-INH-AcpM complex, and this issue remains to be investigated. Kremer et al. (16) have described two clinical isolates with the Gly269Ser (GGT→AGT) substitution that are still sensitive to INH, suggesting that this mutation is not clinically significant. It then appears that resistance-associated *kasA* changes occur at a low frequency in INH-resistant clinical isolates and likely reflect different geographical prevalences of specific genotypes (18). Therefore, the routine use of sequencing of *kasA* to identify INH-resistant clinical isolates is not useful. The *srmR* homolog is a gene found upstream of the FAS-II gene cluster and controls the production of polyketide in *S. ambofaciens* (12). Polyketide biosynthesis is mechanistically similar to fatty acid biosynthesis by the FAS-II system (12). Our sequencing analysis showed that an INH-resistant isolate for which the INH MIC was at a medium level contained a mutation in the *srmR* homolog, but it should be noted that this isolate also had a *katG* mutation (Table 4).

The *iniBAC* operon encodes genes that are induced by a broad range of antibiotics including INH and ethambutol (3). The functions of the proteins encoded by the genes of this operon are unknown, and these genes lack close homologs in nonmycobacterial species. It has been shown that the *iniBAC* promoter is specifically induced by a broad range of inhibitors of cell wall biosynthesis in *M. tuberculosis* (3). On the basis of the induction pattern, it is postulated that these genes may participate in the regulation of cell wall growth or have a protective role in cell death (2, 3). Our results show that six isolates contained mutations in the *ini* genes exclusively represented in INH-resistant isolates. However, all these isolates also had changes in other genes. We have previously identified mutations in the *ini* genes in ethambutol-resistant *M. tuberculosis* isolates (30). The mutations identified in *ini* genes in this study are completely different from the ones selected due to ethambutol treatment. Ethambutol inhibits arabinogalactan biosynthesis and brings about cell death, whereas INH treatment leads to the accumulation of mycolic acids and eventual cell death. Thus, it remains plausible that mutations in the *ini* genes are selected as a consequence of cell wall injury due to INH or ethambutol therapy.

The roles of *Rv1592c* and *Rv1772* in the physiology of *M. tuberculosis* are not known (10, 43). Further research is warranted to understand the significance of mutations identified in these genes. The identification of a synonymous substitution and a nonsynonymous substitution (codons 321 and 322, respectively) in *Rv1592c* in all group 1 isolates and a subset of group 2 isolates indicates that these polymorphisms are ancient and conserved in the evolution of the *M. tuberculosis* complex and suggests that this gene may have a role in the typing or classification of group 1 and 2 organisms (35).

Our sequencing analyses showed no resistance-associated mutations in the following genes: *fabD*, *accD6*, *efpA*, *fbpC*, *ndh*, and *nhoA*. However, this does not rule out a role for these genes in causing INH resistance. Additional studies that in-

clude INH-resistant *M. tuberculosis* isolates from global populations are needed to see if mutations in these genes play a role in INH resistance. It should be noted that resistance-associated mutations in *ndh* have been reported in *M. tuberculosis* isolates from Singapore (18). Four INH-resistant isolates in this study did not have resistance-associated mutations in any of the 20 genes sequenced. The MIC for one of those isolates was high (48 μg/ml). This finding suggests that genetic mechanisms other than the ones described in this study are likely involved in the causation of INH resistance. The sequencing data from this limited data set also showed that certain polymorphisms are exclusively present in INH-resistant isolates, while other polymorphisms are found in both INH-resistant and -susceptible isolates (Table 1). With the exception of substitutions in codon 315 of *katG* and the *mabA* promoter substitution at position 15 upstream of the start codon, all other polymorphisms associated with INH resistance occurred at low frequencies. In the absence of knowledge regarding the functions of many of the genes, the low frequency of mutations observed in the isolates makes it difficult to reach firm conclusions about their role in the causation of INH resistance. To solve the problem of associating low-frequency polymorphisms with drug resistance by sequencing alone, screening of a large number of susceptible isolates is required, but this is fiscally constraining. Further biochemical and genetic studies are required to establish a role for the mutations identified in INH resistance. The sequence data provided by analysis of 20 genes from this study is an initial step toward gaining insight into the complex mechanism of action of INH in *M. tuberculosis.*

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