

Population Genetics Study of Isoniazid Resistance Mutations and Evolution of Multidrug-Resistant *Mycobacterium tuberculosis*†

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The molecular basis for isoniazid resistance in *Mycobacterium tuberculosis* is complex. Putative isoniazid resistance mutations have been identified in *katG*, *ahpC*, *inhA*, *kasA*, and *ndh*. However, small sample sizes and related potential biases in sample selection have precluded the development of statistically valid and significant population genetic analyses of clinical isoniazid resistance. We present the first large-scale analysis of 240 alleles previously associated with isoniazid resistance in a diverse set of 608 isoniazid-susceptible and 403 isoniazid-resistant clinical *M. tuberculosis* isolates. We detected 12 mutant alleles in isoniazid-susceptible isolates, suggesting that these alleles are not involved in isoniazid resistance. However, mutations in *katG*, *ahpC*, and *inhA* were strongly associated with isoniazid resistance, while *kasA* mutations were associated with isoniazid susceptibility. Remarkably, the distribution of isoniazid resistance-associated mutations was different in isoniazid-mono-resistant isolates from that in multidrug-resistant isolates, with significantly fewer isoniazid resistance mutations in the isoniazid-mono-resistant group. Mutations in *katG315* were significantly more common in the multidrug-resistant isolates. Conversely, mutations in the *inhA* promoter were significantly more common in isoniazid-mono-resistant isolates. We tested for interactions among mutations and resistance to different drugs. Mutations in *katG*, *ahpC*, and *inhA* were associated with rifampin resistance, but only *katG315* mutations were associated with ethambutol resistance. There was also a significant inverse association between *katG315* mutations and mutations in *ahpC* or *inhA* and between mutations in *kasA* and mutations in *ahpC*. Our results suggest that isoniazid resistance and the evolution of multidrug-resistant strains are complex dynamic processes that may be influenced by interactions between genes and drug-resistant phenotypes.

Isoniazid (INH) is one of the most effective and specific agents for the treatment of infections with *Mycobacterium tuberculosis*. INH is the cornerstone of treatment for drug-susceptible tuberculosis, and it is also widely used to treat latent

M. tuberculosis infections. Recent increases in INH-resistant (INH^r) and multidrug-resistant (MDR) tuberculosis are jeopardizing the continued utility of this drug (13, 61). Furthermore, the development of INH resistance is a common first step in the evolution to MDR (11). Thus, there has been considerable interest in identifying the molecular basis of INH resistance in clinical *M. tuberculosis* isolates.

INH is a prodrug that requires activation by the catalase-peroxidase enzyme encoded by the *katG* gene (65). Activated INH appears to disrupt the synthesis of essential mycolic acids by inhibiting the NADH-dependent enoyl-ACP reductase enzyme encoded by *inhA* (45). INH resistance is likely to arise

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TABLE 1. Clinical *M. tuberculosis* isolates included in the study

Geographic origin	No. (%) of isolates								
	Total	Pan-susceptible	INH ^r	Mono-INH ^r	MDR	Resistant to indicated no. of drugs			
						1	2	3	4
Australia	56	26 (46)	29 (52)	20 (36)	4 (7)	21 (38)	6 (11)	3 (5)	0
Colombia	302	159 (53)	133 (44)	3 (1)	128 (46)	10 (3)	32 (11)	68 (23)	33 (11)
India	45	10 (21)	22 (49)	2 (4)	16 (36)	12 (27)	12 (27)	8 (17)	3 (7)
Mexico	197	73 (37)	121 (61)	43 (22)	65 (33)	46 (23)	54 (26)	15 (8)	9 (5)
New York	146	119 (82)	22 (15)	3 (2)	18 (12)	7 (5)	8 (5)	2 (1)	10 (7)
Spain	109	97 (89)	11 (10)	1 (1)	9 (8)	2 (2)	6 (5)	4 (4)	0
Texas	156	91 (58)	65 (41)	30 (19)	32 (21)	30 (19)	10 (7)	8 (5)	17 (11)
Total	1011	575 (57)	403 (40)	102 (10)	272 (27)	128 (13)	128 (13)	108 (11)	72 (7)

through multiple molecular mechanisms, only a subset of which have been fully characterized. Between 40 and 95% of INH^r clinical *M. tuberculosis* isolates have mutations in *katG*, 75 to 90% of which are located in codon 315, with 10 to 25% of mutations located in other *katG* loci (12, 35, 37, 42–44, 57, 64). Mutations in *katG*315 may be favored because mutations at this location appear to decrease INH activation without abolishing catalase-peroxidase activity, a potential virulence factor (22, 41, 49, 59). *M. tuberculosis* may compensate for *katG* mutations by overexpressing the *ahpC* gene (52, 54). INH resistance can also develop through alterations or overexpression of the INH drug target *InhA*, and 0 to 5% of INH^r *M. tuberculosis* isolates have mutations in the *inhA* open reading frame (ORF), while 8 to 20% have mutations in the *inhA* promoter (38, 42, 43, 64). Mutations in *ndh*, a gene encoding an NADH dehydrogenase, were recently found to confer resistance to INH and ethionamide in *M. bovis* (58). The *ndh* mutants had altered NADH/NAD ratios, which appeared to protect them from INH-mediated toxicity. Mutations in at least 16 other genes have been reported to be associated with INH resistance in clinical isolates (42–44). However, the roles of these genes in INH resistance (if any) remain unclear. Furthermore, approximately 10% to 25% of INH-resistant strains do not contain mutations in any known gene targets for INH resistance (42–44, 64). These results demonstrate the need for further investigations in this field.

The sequencing studies that form the basis of our understanding of INH resistance-associated mutations in clinical *M. tuberculosis* isolates have had a number of important limitations. Most studies have examined relatively small numbers of isolates or have failed to include sufficient numbers of drug-susceptible controls (10, 12, 25, 27–29, 31, 38–40, 43, 44, 47, 55, 64). Even studies that were somewhat larger did not usually include enough INH^r and INH-susceptible (INH^s) isolates to demonstrate statistically significant associations between specific mutations and INH resistance (10, 12, 25, 27–30, 33, 38, 43, 44, 47), and none of these have been large enough to perform more detailed analyses. Furthermore, much of what is known about INH resistance-associated mutations was discovered by testing collections of predominantly MDR isolates (14, 31, 47). However, as demonstrated in a large study of ethambutol (EMB) resistance (18), MDR isolates may not have the same distribution of mutations as mono-resistant *M. tuberculosis* isolates (4, 19, 25).

Here we present an in-depth study of INH resistance-asso-

ciated mutations in a large and geographically diverse set of INH^s and INH^r clinical isolates of *M. tuberculosis*. Our goals were to determine if any point mutation previously classified as an INH resistance-associated mutation was detectable in INH^s isolates and to test for statistically valid associations between mutations and drug resistance. Interactions between different mutations were also studied as a mean to better understand the biology of INH resistance. Finally, we aimed to test the hypotheses that certain mutations were concentrated in MDR isolates compared to mono-resistant isolates and that MDR isolates had an excess accumulation of multiple INH resistance-associated mutations. Our results have important implications in understanding how INH resistance arises and continues to evolve in MDR isolates.

MATERIALS AND METHODS

***M. tuberculosis* clinical isolates.** A total of 1,011 clinical *M. tuberculosis* isolates were obtained from reference laboratories in Australia, Colombia, India, Mexico, New York City, Spain, and Texas (Table 1). The 56 samples from Australia consisted of all isolates resistant to at least INH collected in the state of Victoria at the Victorian Mycobacterium Reference Laboratory in Melbourne during 2001 and 2002 and 26 randomly selected pan-susceptible control isolates from the same period (27). The samples from Colombia consisted of 133 isolates resistant to at least INH, 159 pan-susceptible isolates, and 7 rifampin (RIF)-mono-resistant isolates that were selected from the strain bank of the Mycobacterial Group, Instituto Nacional de Salud, composed of clinical *M. tuberculosis* isolates collected from all regions of Colombia between 1992 and 2000. The samples from India consisted of all 35 drug-resistant isolates of any type identified in the Vallabhbhai Patel Chest Institute in New Delhi between January 2001 and January 2002. These included 10 streptomycin (STR)-mono-resistant, 22 INH^r (2 of which were mono-INH^r), and 10 randomly selected pan-susceptible control isolates from the same period. The samples from Mexico consisted of 121 INH^r (42 of which were mono-INH^r) and 76 INH^s clinical isolates randomly selected from genotyped isolates collected between 1991 and 1996 in Mexico City, Huachuquingo, Puebla, Orizaba, and Veracruz, 3 of which were mono-resistant to STR and 73 of which were pan-susceptible controls (7, 16, 23), obtained from the Laboratory of Clinical Microbiology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City. The samples from Spain consisted of all 109 isolates collected at the General Penitentiary Hospital, Microbiology Laboratory, from a longitudinal study of tuberculosis performed between January 1993 and June 1994 (8). These samples comprised 11 INH^r isolates (1 of which was mono-INH^r), 1 STR-mono-resistant isolate, and 97 pan-susceptible controls. The 65 samples from Texas consisted of all the INH^r isolates (30 mono-INH^r) collected by the Texas Department of Health and the University of Texas Health Center at Tyler between May 1992 and August 1994 and 91 INH^s isolates collected in a population-based study of Tarrant County between May 1992 and December 1996 (60, 62) which were submitted to the mycobacteriology research laboratory of the Central Arkansas Veterans Healthcare System for genotype analysis. Samples from New York City consisted of all INH^r isolates (22 of which were mono-resistant) for which DNA was available

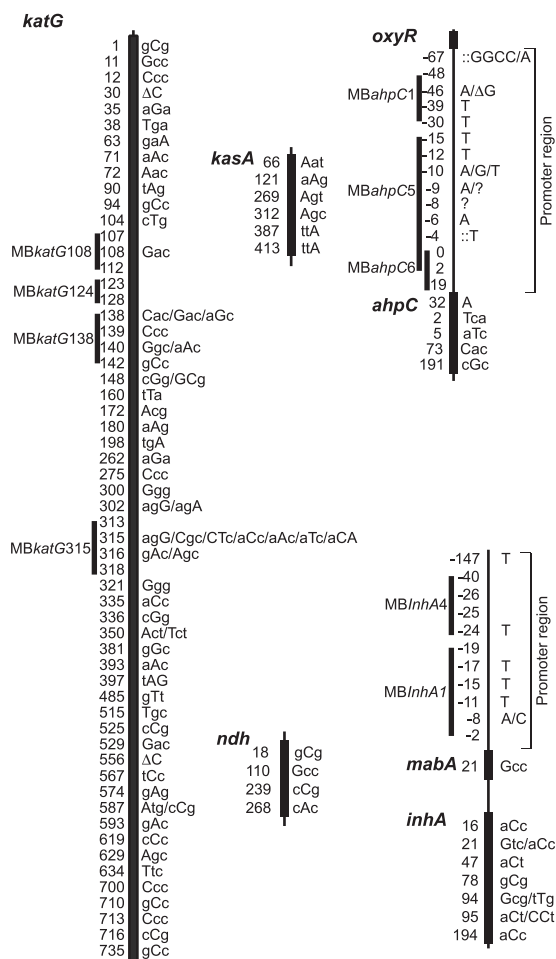


FIG. 1. Alleles screened in this study. The ORF of each gene is shown as a thick line. Intergenic regions are indicated by thin lines. The position of each allele examined in this study is indicated to the left of the lines. Alleles tested inside an ORF are indicated by their codon positions; alleles tested within an intergenic region are indicated by their nucleotide positions relative to the start of the following ORF, except for *ahpC*, which is shown relative to the transcription start. The specific mutations examined in the HP assays are indicated in capital letters to the right of each gene. Several mutations are indicated when more than one mutant allele was tested at the same codon or nucleotide position. Deletions are indicated with the symbol “Δ,” and insertions are indicated with colons. Question marks (?) indicate the presence of a mutation detected and confirmed by a molecular beacon assay, but the exact nucleotide change was not identified because the sequencing results are inconclusive at that position. The total sequence examined by each molecular beacon is indicated by a line labeled with the name of that molecular beacon, starting with an MB prefix. Sequence accession numbers for *katG*, *ahpC*, *kasA*, *ndh*, and *inhA* are Z97193, Z79701, Z70692, BX842577, and Z79701, respectively. Genes and upstream regions are not drawn to scale. The sequences of the primers, molecular beacons, and sequencing primers used to detect or confirm these alleles are shown in Tables S1 to S3 in the supplemental material.

among isolates collected at the Montefiore Medical Center, Bronx, N.Y., between 1989 and 1996 and 124 INH^s randomly selected controls (1).

Drug susceptibility testing and strain typing. All isolates included in this study were subjected to susceptibility testing and DNA fingerprinting as described below. Each center or laboratory performed susceptibility testing with at least INH, RIF, STR, and EMB by the agar proportions method (24) (Colombia, India, New York, and Spain), the BACTEC MGIT 960 method (20) (Australia), or the radiometric BACTEC 460 method (30) (Mexico and Texas). The break-

point concentrations used for INH were 0.2 μg/ml for the agar proportions method and 0.1 μg/ml for the BACTEC MGIT 960 system and BACTEC 460 methods. DNA fingerprinting (either IS6110 or variable-number tandem repeat fingerprinting and spoligotyping [1, 6, 15, 21]) demonstrated that this study population included 343 unique and 265 clustered INH^s isolates and 196 unique and 207 clustered INH^r isolates.

Detection of INH resistance-associated mutations in clinical isolates of *M. tuberculosis*. We tested each isolate for virtually all single nucleotide polymorphism (SNP) mutations in the *M. tuberculosis* *katG*, *kasA*, *mabA*, *inhA*, *oxyR*, *ahpC*, and *ndh* genes (or their upstream promoter regions) that have been found in association with INH resistance in previously published studies (Fig. 1) (3, 10, 12, 14, 25, 28, 29, 31, 34, 35, 37, 38, 40, 42–44, 46, 48, 51, 52, 54, 56, 58, 59, 65). A total of 204 INH resistance-associated alleles were detected by using 106 high-throughput hairpin-shaped-primer (HP) assays (see Table S1 in the supplemental material) as described previously (17). Regions with high concentrations of potential SNPs (e.g., the promoter regions of *inhA* and *ahpC*) and positions 103 to 113, 123 to 128, and 137 to 143 of *katG* (which account for 36 alleles) were screened using nine molecular beacon assays that were designed to detect only wild-type DNA sequences in those regions (see Table S2 in the supplemental material). Resistance-associated SNPs in negative molecular beacon assays (which indicated that the sequence diverged from the wild-type sequence) were confirmed by DNA sequencing. Additional screening for mutations in *katG315* was confirmed by using a previously described molecular beacon assay (40). Finally, the *katG* gene was tested for the presence of large deletions that could also be associated with INH resistance by scanning for regions where adjacent HP assays suggested the absence of DNA target sequences. Each deletion in *katG* was confirmed by Southern blot analysis.

DNA sequencing and other confirmatory testing. During the course of the study, we performed 220 different sequencing reactions (using BigDye Terminator kits from ABI) to confirm the alleles assigned by the HP assays and to identify the specific mutations indicated by the molecular beacon assay results. Sequencing primer sequences and conditions are indicated in Table S3 in the supplemental material. We also retested the susceptibility patterns to INH, RIF, STR, and EMB of 103 isolates, including all INH^s isolates with mutations in *katG*, *inhA*, *ahpC*, or *ndh*. Totals of seven, one, four, and three discrepancies in the repeat susceptibility results were detected for INH, RIF, EMB, and STR susceptibility, respectively. The drug resistance of each isolate was reconfirmed after performing a third drug susceptibility test in all cases. These final drug susceptibility results were used in all analyses.

Statistical analysis. Basic descriptive statistics and graphics were employed. Chi-square tests were used to test for individual associations and differences between proportions. Logistic regression was used to build models, with the area under the receiver operating curve used as a general measure of goodness of fit. Chi-square tests for trend were also employed. SAS, version 8.02, was used for all statistical analyses.

RESULTS

Distribution of mutations in INH^s and INH^r isolates. In this study, we tested 609 INH^s and 402 INH^r *M. tuberculosis* isolates for 240 alleles in five genes that were previously reported to be associated with INH resistance. The most common mutations were found at *katG315*, as reported previously (3, 40, 42, 44, 56, 57). Mutations in *katG315* were identified in 184/403 (46%) INH^r isolates (Table 2). The *katG* S315T mutation was detected in one INH^s isolate in this study; however, this isolate was not viable for repeat testing. We suspect that this isolate was truly INH^r, and we reclassified it as INH^r for subsequent analyses. Five additional INH^r isolates (1%) were found by HP PCR scanning and Southern blot analysis to contain deletions of various sizes in the *katG* gene (data not shown). These five isolates were all catalase negative (32), as would be predicted by the gene deletions. No *katG* deletions were detected in any of the INH^s isolates. Mutations at other *katG* locations were uncommon, comprising only 17/238 (7%) *katG* mutants (4% of the total INH^r isolates). *katG* G316S and L587P mutations were found in INH^s isolates, while all other *katG* mutations were found only in INH^r isolates.

TABLE 2. Mutations detected among INH^r and INH^s isolates

Mutation ^a	No. of isolates			Mutation ^a	No. of isolates		
	INH ^r	INH ^s	Total		INH ^r	INH ^s	Total
<i>katG</i> mutations							
123-128-? ^b	2		2	-46 g→A	14	11	25
R128P (cgg→cCg)	1		1	-46 ΔG		1	1
N138H (aac→Cac)	1		1	-39 c→T	4		4
N138D (aac→Gac)	2		2	-30 c→T	1		1
N138S (aac→aGc)	1		1	-15 c→T	1		1
A172T (gcg→Acg)	2		2	-12 c→T	2		2
S315R (agc→agG)	1		1	-10 c→A	2		2
S315T (agc→aCc)	184		184	-10 c→G	2		2
S315N (agc→aAc)	9		9	-10 c→T	7		7
S315I (agc→aTc)	6		6	-9 g→A	2	1	3
S315T (agc→aCA)	21		21	-9 g→? ^b	2		2
G316D (ggc→gAc)	1		1	-8 a→? ^b	1		1
G316S (ggc→Agc)		2	2	-6 g→A	4		4
W321G (tgg→Ggg)	1		1	-4::T	3		3
L336P (ctg→cGg)	1		1	ORF mutations			
R515C (cgc→Tgc)	1		1	T5I (acc→aTc)	1		1
L587P (ctg→cCg)		1	1	D73H (gac→Cac)	7	6	13
D735A (gac→gCc)	1		1	Total	54	19	73
Total	235	3	238	<i>inhA</i> mutations			
<i>kasA</i> mutations				Promoter mutations ^d			
D66N (gat→Aat)	1	1	2	-17 g→T	3	1	4
G269S (ggt→Agt)	36	87	123	-15 c→T	31		31
G312S (ggc→Agc)	7	12	19	-8 t→A	3		3
Total	44	100	144	-8 t→C	1		1
<i>ndh</i> mutations				ORF mutations			
V18A (gtg→gCg)	12	16	28	I21V (atc→Gtc)	1		1
R268H (cgc→cAc)	2		2	I21T (atc→aCc)	2		2
Total	14	16	30	I47T (att→aCt)		1	1
<i>oxyR-ahpC</i> mutations				S94A (tcg→Gcg)	5		5
Promoter mutations ^c				I194T (atc→aCc)	2	1	3
-67 g→A	1		1	Total	48	3	51
				All mutations	395	141	536

^a The mutant codon is indicated, followed by the nucleotide change, shown in capital letters. When the mutation occurs in an intergenic region, the nucleotide change is indicated relative to the start site of the following ORF.

^b Sequences that were confirmed to be mutants by a molecular beacon assay but whose exact mutation is not known because sequencing results were inconclusive or DNA was unavailable for sequencing.

^c Positions in the *ahpC* promoter are situated relative to the transcriptional start residue.

^d Positions in the *inhA* promoter are situated relative to the start of the *mabA* ORF (*mabA* is the first gene in the *mabA-inhA* operon).

A total of 144 mutations were detected in *kasA*, at positions 66, 269, and 312, in 100/608 (16%) INH^s isolates and 44/403 (11%) INH^r isolates (Table 2). These results definitively show that none of the common mutant *kasA* alleles are uniquely present in INH^r *M. tuberculosis*. Thus, it appears unlikely that these alterations in *kasA* result in INH resistance, as has been reported previously (2, 40, 44).

We detected 51 mutations in *inhA*, in 3/608 (0.5%) INH^s isolates and 48/403 (12%) INH^r isolates. All promoter mutations (except the -17 G-to-T mutation) were confined to INH^r isolates, with the C-to-T mutation at position -15 being the most abundant (Table 2). Two ORF mutations, I47T and I194T, were detected in INH^s isolates; however, all other ORF mutations were only detected in INH^r isolates. Importantly, *inhA* S94A mutants, which appear to alter binding of the INH-NADH adduct to InhA (45), were confined to INH^r isolates. These results are consistent with the known structural interactions between INH and InhA and add further support to the role of *inhA* mutations in clinical INH resistance.

There were a total of 73 mutations detected in *ahpC*, in 19/608 (3%) INH^s isolates and 54/403 (13%) INH^r isolates.

The most common mutation was found at position -46 in both INH^s and INH^r isolates (Table 2), confirming that this mutation is not associated with INH resistance (3, 27). The D73H mutation and the G-to-A mutation at position -9 were also detected in both susceptible and resistant isolates. The T5I mutation was the only ORF mutation detected and was present in only one INH^r isolate. These results help to confirm a role for *ahpC* promoter mutations in INH resistance but suggest that mutations in the *ahpC* ORF make little or no contribution to INH resistance.

We detected a total of 30 *ndh* mutations, in 16/608 (3%) INH^s isolates and 14/403 (3%) INH^r isolates. The most common mutation, V18A, was detected in both INH^s and INH^r isolates. The R268H mutation was only detected in two INH^r isolates. These results suggest that these *ndh* mutations play a small role, at most, in clinical INH resistance.

In order to limit any potential bias introduced by sampling or geographic origin of the isolates tested, we examined the prevalence of the mutations detected by gene in each country. The proportions were comparable for all countries with substantial (>30) sample sizes (data not shown).

Associations between mutations and drug resistance. The large number of isolates analyzed in our study permitted us to test the statistical strength of reported associations between certain genes and INH resistance for the first time. We observed strong individual associations between any mutations in the *katG*, *inhA*, and *ahpC* genes and resistance to INH ($P = 0.0001$). Conversely, any mutations in the *kasA* gene were associated with susceptibility to INH ($P = 0.012$), while any mutations in *ndh* were not associated with INH resistance ($P = 0.412$) or susceptibility.

Logistic regression modeling was conducted using the five genes. The results of this analysis confirmed that any mutation in *ndh* was not associated with INH resistance or susceptibility ($P = 0.68$), while mutations in the other four genes were associated either with INH resistance (for *katG*, odds ratio [OR] = 214.2, standard deviation [SD] = 92.5, and $P = 0.0001$; for *inhA*, OR = 55.0, SD = 33.8, and $P = 0.0001$; and for *ahpC*, OR = 8.0, SD = 2.5, and $P = 0.001$) or with INH susceptibility (for *kasA*, OR = 0.5 [for resistance], SD = 0.2, and $P = 0.036$), with an area under the curve of 0.876. In order to address the potential clustering of samples within a country, the final logistic regression model was rerun, allowing for correlation within the country. The model did not alter significantly, supporting the representative nature of the overall sample. These results strongly implicate *katG*, *ahpC*, and *inhA* mutations in the mechanism of INH resistance in clinical isolates. The association between *kasA* mutations and INH susceptibility has no obvious biological explanation and will need to be explored in further studies.

We next examined the association between mutations in INH resistance-associated genes and resistance to other anti-tuberculosis drugs. The development of MDR *M. tuberculosis* has long been attributed to the independent acquisition of resistance to individual drugs. However, INH resistance in itself (i.e., by any mechanism) appears to be associated with other types of drug resistance (9, 36). We desired to test the hypothesis that certain INH resistance-associated mutations might be more strongly associated with resistance to other drugs than other mutations. This type of unequal association might be observed if particular INH resistance-associated mutations also aided in the development of MDR. The fact that we had already observed this phenomenon in a previous study of *embB306* mutations and drug-resistant *M. tuberculosis* (18) supported this line of analysis.

RIF resistance and STR resistance were associated with mutations in *katG*, *inhA*, and *ahpC*, both individually and in logistic regression models (P values were as follows: RIF, 0.0001, 0.0001, and 0.002, respectively; STR, 0.0001, 0.0001, and 0.005, respectively). RIF resistance was not associated with mutations in *kasA* ($P = 0.69$) or *ndh* ($P = 0.07$), and STR resistance was not associated with mutations in *kasA* ($P = 0.68$). Thus, each of the genes found to be associated with INH resistance was also associated with RIF resistance. This observation suggests that no individual INH resistance-associated mutation predisposes *M. tuberculosis* to the development of RIF resistance.

In contrast, resistance to EMB was associated with mutations in *katG* (OR = 5.10 [1.08]; $P = 0.0001$) and *ahpC* (OR = 2.16 [0.70]; $P = 0.024$) but not in *inhA* ($P = 0.16$), *kasA* ($P = 0.127$), or *ndh* ($P = 0.422$). This suggests that INH^r *katG*

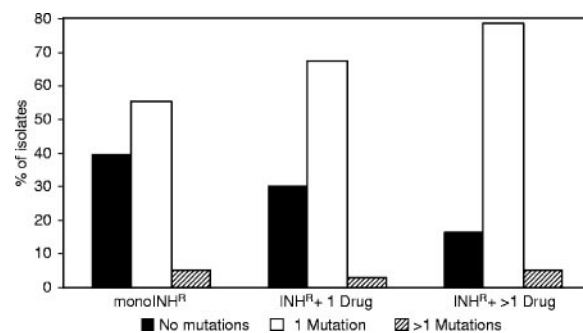


FIG. 2. Distribution of INH resistance-associated mutations in drug-resistant clinical *M. tuberculosis* isolates. The proportion of *M. tuberculosis* isolates with detectable INH resistance-associated mutations and the number of mutations detected in each isolate are shown according to the drug susceptibility pattern of the *M. tuberculosis* isolate.

mutants may be particularly prone to developing EMB resistance compared to other INH^r isolates. We postulate that *inhA* mutations are not well tolerated by some EMB-resistant isolates.

Differences between mono-INH^r and MDR isolates. The genetic mechanisms responsible for INH resistance are commonly thought to be identical in mono-INH^r and INH^r MDR *M. tuberculosis*. However, it has been suggested that some drug resistance-associated mutations occur at higher frequencies in MDR *M. tuberculosis* than in mono-INH^r clinical isolates (4, 19, 40). The size of our current investigation made it possible to rigorously test for differences between mono-INH^r and MDR isolates for the first time.

We examined the proportion of INH^r-associated mutations that could be detected in clinical isolates as a function of the total number of drugs to which each isolate was resistant. In this analysis, we only examined isolates that were resistant to INH or INH plus another drug. We also excluded from the analysis all mutations observed in INH^s isolates. The results (Fig. 2) show that INH resistance-associated mutations were less common in mono-INH^r isolates (61/101 [60%]) than in isolates that were resistant to INH plus one other drug (84/120 [70%]; $P = 0.09$) or isolates that were resistant to INH plus two or more drugs (148/177 [84%]; $P = 0.001$). Conversely, the proportion of isolates without any detectable mutation decreased as isolates became resistant to INH plus an increasing number of drugs. We repeated the analysis within each country, and the distributions observed were similar, limiting the possibility that bias may have been introduced by sampling or geographic origin (data not shown).

It has been suggested that Beijing clade isolates (which fall into principal genetic group 1 [PGG-1] [53]) with *katG315* mutations are overrepresented among MDR *M. tuberculosis* isolates (4, 19). We determined the PGGs for 74% (295/398) of the mono-INH^r and MDR isolates in the study (Table 3) to test for this potential confounder. The results were used to study the PGG distributions among all isolates and among the *katG315* mutants (Table 3). We found that PGG-1 isolates were not overrepresented in the MDR isolates or in the sample limited to isolates with *katG315* mutations. In fact, only 23% of the MDR isolates were in PGG-1, compared to 46% of the

TABLE 3. Distribution of clinical isolates by PGG

Study approach	Isolates included	Resistance pattern	No. of isolates in PGG/total no. of isolates (%) ^a		
			PGG-1	PGG-2	PGG-3
Counting all isolates	All	Mono-INH ^R	31/68 (46)	31/68 (46)	6/68 (8)
	All	INH ^R + resistance to ≥1 drug	52/227 (23)	142/227 (62)	33/227 (15)
	<i>katG</i> 315 mutants	Mono-INH ^R	17/32 (53)	14/32 (44)	1/32 (3)
	<i>katG</i> 315 mutants	INH ^R + resistance to ≥1 drug	38/143 (27)	90/143 (63)	15/143 (10)
Counting one isolate per cluster ^b	All	Mono-INH ^R	30/65 (46)	29/65 (45)	6/65 (9)
	All	INH ^R + resistance to ≥1 drug	41/192 (21)	122/192 (64)	29/192 (15)
	<i>katG</i> 315 mutants	Mono-INH ^R	17/31 (55)	13/31 (42)	1/31 (3)
	<i>katG</i> 315 mutants	INH ^R resistance to ≥1 drug	27/110 (25)	71/110 (64)	12/110 (11)

^a Results based on 68/101 (67%) and 227/297 (76%) mono-INH^R isolates and MDR isolates, respectively.

^b Results using all unique isolates plus one isolate from each cluster. In cases where a cluster contained isolates with different drug susceptibility profiles and/or mutations, each variation was counted as a new cluster.

mono-INH^R isolates. This difference remained when we analyzed our data by including only nonclustered isolates and one isolate from each cluster (to control for bias due to clustering) (Table 3).

The increase in detectable mutations in MDR isolates was almost entirely due to an increase in the proportion of *katG*315 mutations in MDR isolates (Fig. 3A). This observation did not change when the results were reanalyzed with a sample that included all unique isolates and only one isolate from each cluster (Fig. 3B). In contrast, we found that the proportion of isolates with *inhA* promoter mutations decreased as resistance increased ($P = 0.57$ for the difference between mono-INH^R and resistance to INH plus one other drug; $P = 0.011$ for the difference between mono-INH^R and resistance to INH plus two or more drugs); however, this trend was lost when the results were reanalyzed with a sample that included all unique isolates and only one isolate from each cluster, and a larger sample will be needed to make a conclusion about the significance of this finding. In contrast, no differences were observed when mono-INH^R and MDR isolates were compared regarding the proportions of isolates with mutations in *katG* other than those in codon 315 and of *ahpC* and *inhA* ORF mutants. A similar analysis was performed to determine if MDR isolates were more likely to have multiple INH resistance-associated mutations than mono-INH^R isolates (Fig. 2). No association was seen between mutation number and increasing drug resistance.

These results support the hypothesis that *katG*315 mutations

maintain or increase the fitness of *M. tuberculosis* isolates as they evolve from INH monoresistance to MDR compared to other categories of INH resistance-associated mutations. They do not support the hypothesis that *M. tuberculosis* accumulates multiple mutations as it evolves to MDR. These results also suggest that *inhA* promoter mutations may attenuate *M. tuberculosis* isolates, decreasing the likelihood that these isolates result in clusters of MDR disease.

Interactions between mutations. Totals of 1/608 (0%) INH^S isolates and 80/403 (20%) INH^R isolates contained more than one mutation in *katG*, *inhA*, *ahpC*, *kasA*, and *ndh*. This suggested that mutations in these genes might interact in a way that increases the drug resistance or general fitness of the isolate. In fact, an interaction between *ahpC* promoter mutations and mutations in *katG* that occur outside of the *katG*315 codon has been postulated previously (52, 54, 63). This hypothesis was based on the observation that *ahpC* mutations are not sufficient in themselves to confer INH resistance, yet these mutations could be found in isolates containing *katG* mutations at loci other than *katG*315. We reasoned that our study would be sufficiently powered to identify statistically significant interactions between certain mutations. Our results (Table 4) demonstrated a significant association between *ahpC* promoter mutants and mutations in *katG* that were not at position 315, in contrast to the case for mutations at *katG*315. A total of 9/221 (4%) *katG*315 mutants had mutations in the *ahpC* promoter, while 5/15 (33%) non-*katG*315 mutants had similar

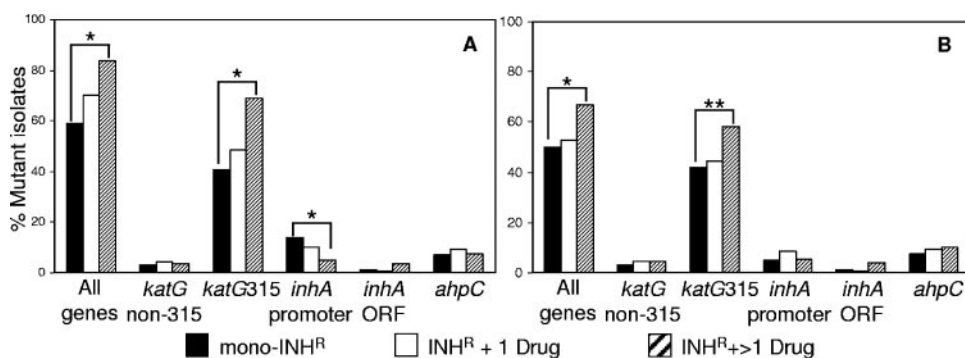


FIG. 3. Distributions of mutations in specific genes. The proportions of isolates with INH resistance-associated mutations in the *katG*, *inhA*, and *ahpC* genes are shown according to the drug susceptibility patterns of the *M. tuberculosis* isolates. (A) Results using all isolates in the study; (B) results using all unique isolates plus one from each cluster. In cases where a cluster contained isolates with different drug susceptibility profiles and/or mutations, each variation was counted as a new cluster. *, $P < 0.01$; **, $P = 0.034$.

TABLE 4. Frequency and distribution of multiple mutations in individual *M. tuberculosis* isolates

Gene	Location of mutation	No. (%) of isolates with mutations in indicated gene location													
		<i>katG</i>			<i>inhA</i>			<i>ahpC</i>				<i>kasA</i>			<i>ndh</i>
		All	Position 315	Positions other than 315	All	Promoter	ORF	All	Promoter	ORF	D73H	All	Position 269	Position 312	
<i>katG</i>	All	238 (100)	221 (100)	17 (100)	5 (11)	2 (5)	3 (25)	20 (27)	14 (24)	6 (43)	5 (38)	34 (24)	29 (24)	5 (26)	9 (30)
	Position 315	221 (93)	221 (100)	0	5 (11)	2 (5)	3 (25)	15 (21)	9 (15)	6 (43)	5 (38)	34 (24)	29 (24)	5 (26)	9 (30)
	Positions other than 315	17 (7)	0	17 (100)	0	0	0	5 (7)	5 (8)	0	0	0	0	0	0
<i>inhA</i>	All	5 (2)	5 (2)	0	46 (100)	39 (100)	12 (100)	6 (8)	6 (10)	0	0	5 (3)	3 (2)	2 (11)	1 (3)
	Promoter	2 (1)	2 (1)	0	39 (85)	39 (100)	5 (42)	6 (8)	6 (10)	0	0	3 (2)	1 (1)	2 (11)	0
	ORF	3 (1)	3 (1)	0	12 (26)	5 (13)	12 (100)	1 (2)	1 (2)	0	0	3 (2)	2 (2)	1 (5)	1 (3)
<i>ahpC</i>	All	20 (8)	15 (7)	5 (29)	6 (13)	6 (15)	1 (8)	73 (100)	59 (100)	14 (100)	13 (100)	0	0	0	2 (7)
	Promoter	14 (6)	9 (4)	5 (29)	6 (13)	6 (15)	0	59 (81)	59 (100)	0	0	0	0	0	1 (3)
	ORF	6 (3)	6 (3)	0	0	0	1 (8)	14 (19)	0	14 (100)	13 (100)	0	0	0	1 (3)
	D73H	5 (2)	5 (2)	0	0	0	0	13 (18)	0	13 (93)	13 (100)	0	0	0	0
<i>kasA</i>	All	34 (14)	34 (15)	0	5 (11)	3 (8)	3 (25)	0	0	0	0	144 (100)	123 (100)	19 (100)	0
	Position 269	29 (12)	29 (13)	0	3 (7)	1 (3)	2 (17)	0	0	0	0	123 (85)	123 (100)	0	0
	Position 312	5 (2)	5 (2)	0	2 (4)	2 (5)	1 (8)	0	0	0	0	19 (13)	0	19 (100)	0
	All	9 (4)	9 (4)	0	1 (2)	0	1 (8)	2 (3)	1 (2)	1 (7)	0	0	0	0	30 (100)

mutations in the *ahpC* promoter ($P = 0.03$). After removing mutants with mutations at *ahpC* position 46 (which are not associated with INH resistance), only 1/221 (0.5%) *ahpC* promoter mutants were found to have a mutation at *katG315*, while 4/16 (25%) had a non-*katG315* mutation ($P < 0.0001$).

We also observed a strong negative association between mutations in *katG315* and mutations in the promoter region of *inhA* ($P < 0.0088$). This result suggests that this type of double mutant is selected against during strain evolution or clustering. Interestingly, we noted a very strong negative association between mutations in the promoter region of *ahpC* and mutations in *kasA* ($P < 0.0001$), mainly due to *kasA269* mutations ($P < 0.0006$). In fact, these mutations were never found in the same INH^s or INH^r isolate, despite the high overall prevalence of these mutations in our sample. The significance of this observation is unclear. A weaker but still statistically significant positive association between mutations in *ndh* and mutations in *kasA269* was also observed ($P < 0.041$).

DISCUSSION

This study provides new insights into the population genetics and evolution of drug-resistant *M. tuberculosis* while also confirming a number of previously held hypotheses. There are several reasons why this investigation represents a significant advance.

First, we tested a large number of drug-susceptible isolates for INH resistance-associated mutations, unlike many previous studies that mostly focused on INH^r isolates. This approach enabled us to discover that the *katG* G316S, *kasA* D66N, *ahpC* -9 G-to-A, *inhA* -17 G-to-T, *inhA* I47T, and *inhA* I194T mutations, previously seen only in INH^r *M. tuberculosis* isolates, could also be found in INH^s isolates. It also confirmed that *kasA269*, *kasA312*, *aphC* -46, *ahpC* D73H, and *ndh* V18A mutations could be detected in INH^s isolates. KasA is a protein involved in mycolic acid biosynthesis (50), and its role in resistance to INH is still controversial (26). These results provide strong evidence in clinical isolates that none of the above mutations are causally involved in INH resistance.

Second, we tested a much larger number of INH^r *M. tuberculosis* isolates than any previous study. The large number of observations available for analysis enabled us to use statistical techniques to search for associations between mutations and drug resistance and to test for interactions between mutations. This type of analysis is likely to have been underpowered with the smaller data sets previously available. Our results show a strong statistical association between mutations in the *katG*, *ahpC*, and *inhA* genes and INH resistance. This confirms previously held assumptions about the roles of these genes (26, 49, 54). We also discovered a surprisingly strong inverse association between *kasA* mutations and INH resistance. Our results suggest that these mutations did not evolve because they conferred INH resistance. Perhaps this newly discovered association with INH^s isolates will provide a clue to the roles of these mutations in *M. tuberculosis* evolution.

We found that *katG*, *ahpC*, and *inhA* mutations were associated with RIF resistance but that only *katG* mutations were associated with EMB resistance. These results do not directly implicate INH resistance mutations as a cause of resistance to either RIF or EMB. Rather, we believe that these results

suggest that the type of mutation causing resistance to one drug can influence the probability of developing resistance to other drugs. Our results suggest that *katG* mutations provide a more favorable environment for the development of EMB resistance than do mutations in other genes. These results are consistent with our observation that *inhA* promoter mutations were inversely associated with *katG* mutations and that *inhA* promoter mutations occurred less frequently in highly MDR isolates than in mono-INH^r isolates. We suggest below that this observation is due to a fitness advantage conferred by *katG315* mutations and by a possible attenuating effect of *inhA* promoter mutations.

Third, we studied both mono-INH^r and MDR isolates for INH resistance-associated mutations in sufficiently large numbers that it was possible to perform statistical comparisons between these groups. Prior studies, which focused principally on MDR isolates, appear to have overestimated the importance of *katG* mutations (and especially *katG315* mutations) in causing INH monoresistance. Almost 56% of the mono-INH^r isolates in our study did not have *katG* mutations, and 34% of the mono-INH^r isolates did not have any previously identified mutation that was likely to have caused INH resistance. This suggests that many of the genetic causes of primary INH resistance remain to be discovered.

Our comparison between mono-INH^r and MDR *M. tuberculosis* isolates also showed that *katG315* mutations occurred with increasing frequency as clinical *M. tuberculosis* isolates became progressively more drug resistant. Thus, our results confirm the primary hypothesis of this investigation and corroborate previous reports (4, 19, 40). We speculate that two different mechanisms could account for this observation. One hypothesis suggests that *katG* mutations may have an important role as secondary mutations that develop after a (often unknown) primary mutation has caused an isolate to become INH^r. Under this hypothesis, mutations in *katG* may be delayed until after an isolate has already become MDR. An alternate hypothesis suggests that *katG315* mutations confer a survival advantage on INH^r isolates. Under this hypothesis, *katG315* mutants would be more likely to survive in a treated patient, to be transmitted, and to evolve into MDR strains because they have increased fitness. This second hypothesis is supported by our observation that *inhA* promoter mutations were observed with diminished frequency in highly MDR isolates when all clustered samples were included. Laboratory studies have suggested that expression of the *inhA* gene may be tightly regulated, as *inhA* cannot be overexpressed on a multicopy plasmid using its native promoter (5). If *inhA* overexpression occurs at a cost to the bacterium, then this mutation may be selected against compared to mutations in *katG315*. These results point to a continuing evolutionary process in MDR isolates and suggest that future MDR isolates may be even more fit and difficult to control than those currently encountered. These results also suggest that future INH analogues might select for MDR *M. tuberculosis* less frequently if they were able to circumvent *katG315* resistance and prevent the selection of this mutation.

In addition to the insights that our analysis brings to the evolution of INH resistance, our results may also contribute to the design of genetic assays to detect INH resistance. We would have been able to detect INH resistance in 295 of 397

(74%) of the INH^r isolates in our study by simply testing for mutations at *katG315*, *inhA494*, the promoter regions of *inhA* (spanning the positions between -19 and -2), and the *ahpC* promoter (except position -46). Only three INH^r isolates would have been misclassified as INH^r using this approach. Thus, these regions could be used as molecular markers for INH resistance, and they could be screened easily with only five assays. Our results also suggest that most of the positions evaluated in the ORFs of *katG*, *kasA*, *inhA*, *ahpC*, and *ndh* have little value in predicting resistance to INH.

This study has a number of potential limitations. We only analyzed the *M. tuberculosis* isolates for mutations that had been associated with INH resistance in previous studies. Additional mutations might have been discovered if we had completely sequenced each of the relevant genes. However, the SNPs targeted by this study included almost all SNPs identified in a large body of literature spanning ~15 years. Furthermore, the frequency of *katG* mutations outside of codon 315 was sufficiently low to suggest that additional sequencing would have yielded only a small number of additional SNPs. It is very unlikely that the results would have changed any of the conclusions brought forth in this study. We were only able to detect mutations in 78% (309/397) of the INH^r isolates in this study. It has been suggested that a number of other genes could be involved in INH resistance (44), and these additional genes were not examined here. However, few of these potential INH resistance genes contained mutations in *M. tuberculosis* isolates that did not also have mutations in *katG*, *ahpC*, *inhA*, *kasA*, or *ndh*, and most mutations were quite rare. Thus, it is unlikely that the inclusion of these genes would have changed the results of our study.

Future population genetic studies based on even larger sample sizes will have the power to examine mutations and other genetic variations among clinical isolates in fine detail, ultimately providing information that will aid in the development of improved treatments for tuberculosis.

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