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Molecular Characterization of Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates Collected in Australia

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Elucidation of the molecular basis of isoniazid (INH) resistance in *Mycobacterium tuberculosis* has led to the development of different genotypic approaches for the rapid detection of INH resistance in clinical isolates. Mutations in *katG*, in particular the S315T substitution, are responsible for INH resistance in a large proportion of tuberculosis cases. However, the frequency of the *katG* S315T substitution varies with population samples. In this study, 52 epidemiologically unrelated clinical INH-resistant *M. tuberculosis* isolates collected in Australia were screened for mutations at *katG* codon 315 and the *fabG1-inhA* regulatory region. Importantly, 52 INH-sensitive isolates, selected to reflect the geographic and genotypic diversity of the isolates, were also included for comparison. The *katG* S315T substitution and *fabG1-inhA* −15 C-to-T mutation were identified in 34 and 13 of the 52 INH-resistant isolates, respectively, and none of the INH-sensitive isolates. Three novel *katG* mutations, D117A, M257I, and G491C, were identified in three INH-resistant strains with a wild-type *katG* codon 315, *fabG1-inhA* regulatory region, and *inhA* structural gene. When analyzed for possible associations between resistance mechanisms, resistance phenotype, and genotypic groups, it was found that neither the *katG* S315T nor *fabG1-inhA* −15 C-to-T mutation clustered with any one genotypic group, but that the −15 C-to-T substitution was associated with isolates with intermediate INH resistance and isolates coresistant to ethionamide. In total, 90.4% of unrelated INH-resistant isolates could be identified by analysis of just two loci: *katG315* and the *fabG1-inh*A regulatory region.

Isoniazid (INH) is one of the most effective antimycobacterial agents available for the treatment of tuberculosis (TB). However, soon after its introduction in 1952, strains of *Mycobacterium tuberculosis*, the causative agent of TB, resistant to INH emerged (17). INH inhibits the synthesis of mycolic acids, which are integral components of the mycobacterial cell wall, by inhibiting a key enzyme involved in mycolic acid biosynthesis, InhA (3). INH is a prodrug (11, 12) that is converted to its active form in vivo by the *katG*-encoded *M. tuberculosis* catalase-peroxidase KatG (35). Resistance to INH is predominantly associated with mutations in *katG*, particularly at codon 315. The genes *fabG1* and *inhA* both encode mycolic acid biosynthetic enzymes and are cotranscribed (4). Mutations within the *inhA* structural gene and the *fabG1-inhA* regulatory region have also been identified and associated with INH resistance (3, 7, 13). There remain a proportion of isolates resistant to INH that are not accounted for by mutations in either *katG* or *inhA*. The frequency distributions of INH resistance-associated mutations have been shown to vary according to geographic region, population, and genotype (20, 22).

The introduction of molecular epidemiological typing methods such as IS*6110* restriction fragment length polymorphism, spoligotyping, and more recently mycobacterial interspersed repetitive units (MIRUs), has led to the identification and characterization of groups of related *M. tuberculosis* strains described as families, lineages, or clades, including the Beijing, Haarlem, East-African-Indian (EAI), Latino-American and Mediterranean (LAM), X, T, and Central Asian (CAS) families (9). These families are likely to have originally been endemic within specific geographical areas, and while some remain circumscribed to the particular regions, others such as the Beijing family have become widely disseminated (27). An alternative scheme for classifying isolates is based on the likely evolutionary pathway of the *M. tuberculosis* complex. According to this scheme, strains of *M. tuberculosis* fall into three major genetic groups: genetic group 1 is evolutionarily the oldest and the precursor of groups 2 and 3 (28). More recent phylogenetic analysis has led to the proposal of "ancestral" and "modern" strains of *M. tuberculosis* (6). Genetic group 1 comprises both ancestral and modern strains, including isolates representative of the Beijing, CAS, and EAI families, whereas groups 2 and 3 contain only modern strains, including isolates belonging to the Haarlem, LAM, and X families (6).

Evaluation of the frequency distribution of resistance-associated mutations in different clinical settings is a prerequisite for the large-scale implementation of genotypic approaches for the rapid detection of INH resistance. This paper presents the results of a study of 52 INH-resistant (INH^r) and 52 INH-susceptible (INH^s) epidemiologically unrelated *M. tuberculosis* clinical strains of diverse geographic origins and genotypes isolated in Victoria, Australia. The aims of the study were twofold: first, to investigate the prevalence of mutations in the two most commonly reported loci associated with INH resistance, *katG* codon 315 and the *fabG1-inhA* regulatory region; and second, to analyze whether INH resistance and/or mutations in these regions were associated with a particular geographic origin, genotypic group, or drug resistance profile.

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MATERIALS AND METHODS

Mycobacterial isolates. The Victorian Infectious Diseases Reference Laboratory (VIDRL) serves as the reference laboratory for all mycobacterial infections in Victoria, Australia. Six hundred forty-five new laboratory-confirmed cases of TB caused by *M. tuberculosis* were collected during the study period (2001 to 2003). All isolates were tested for susceptibility to first-line drugs INH (0.1 μg/ml), ethambutol (EMB) (5 μg/ml), and rifampin (RIF) (1 μg/ml), using the BACTEC MGIT 960 system. Resistance to pyrazinamide (PZA) was initially tested using Wayne's method (33), with confirmation of resistance in the BACTEC 460 (100 μg/ml). Those strains resistant to one or more first-line drugs were tested for their susceptibility to second-line drugs amikacin, ciprofloxacin, kanamycin, ethionamide (ETH) (5 μg/ml), and rifabutin in the BACTEC 460 system. All strains resistant to RIF were also resistant to rifabutin. As defined by the BACTEC MGIT 960 manual, 42 isolates were resistant to INH at 0.4 μg/ml and 10 isolates were resistant to INH at 0.1 μg/ml but sensitive at 0.4 μg/ml (defined as having intermediate resistance to INH and denoted as INH^I). For the purposes of this study, unless specified, those isolates displaying intermediate resistance have been classified as INH^r. Each matched pair of INH^r and INH^s isolates belonged to the same genotypic group, had similar MIRU-exact tandem repeat (ETR) profiles, and was isolated from patients born in the same country or region. DNA was extracted from *M. tuberculosis* cultures by the method described by Ross et al. (24). Patient country-of-origin data were kindly provided by the Department of Human Services, Victoria, Australia.

MIRU-ETR typing. ETR loci A, B, and C were amplified using the primers and amplification conditions described by Frothingham and Meeker-O'Connell (10). MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 were amplified using the primers and amplification conditions described by Supply et al. (30). PCRs were performed singly rather than using the multiplex format. The number of tandem repeats at each locus was determined by estimating the amplicon sizes after electrophoresis on 2% (wt/vol) agarose Tris-acetate-EDTA gels.

Classification of strains into major genetic groups and ancestral and modern strains. For the purpose of association studies, the isolates were each assigned to either genetic group 1 or groups 2/3 according to the classification scheme described by Sreevatsan et al. (28). (Genetic groups 2 and 3 were not separated due to the small number of isolates.) The presence of *katG463* CTG (Leu) (group 1) or CGG (Arg) (groups 2/3) was determined by PCR-restriction fragment length polymorphism as described by Sreevatsan et al. (28). Genetic group 1 isolates were divided into "ancestral" and "modern" strains based on the number of repeats at MIRU locus 24 (29). Ancestral isolates have more than one repeat at locus 24, while modern strains have one repeat.

Identification of the Beijing and CAS genotype. Strains belonging to the Beijing family were identified based on the scheme proposed by Ferdinand et al. (8), by comparing the MIRU profiles of the study isolates with those of previously published Beijing isolates (18) or by comparison of IS*6110* profiles to those described by Kremer et al. (15; data not shown). The *aphC* −46A polymorphism has been reported to be associated with the Delhi/CAS family of strains (2). The presence of either G or A at *aphC* −46A was determined by nucleotide sequencing of the 200-bp PCR product obtained using primers FSQ AphC-67 (5′-GTC GACTGGCTCATATCGAGA-3′) and R4OxyR-AhpC (5′-GGTTAGCAGTG GCATGACTCT-3′) (M. Hazbon; personal communication). PCR products were sequenced using BigDye terminator 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions, and precipitated reaction products were run on an Applied Biosystems 3730 DNA analyzer.

Cluster analysis. The unweighted-pair group method using average linkages (UPGMA) tree was constructed using the Sequence Type Analysis and Recombinational Tests (START) program (http://outbreak.ceid.ox.ac.uk/software.htm). This algorithm, while not appropriate for the generation of a phylogenetic tree, grouped the isolates according to similarities in their profiles based on the 15 MIRU-ETR loci.

Statistical analyses. Tests for association were performed using the chi-square distribution.

***katG315* MAS-PCR.** Screening for mutations at *kat*G codon 315 was carried out using the multiplex allele-specific PCR (MAS-PCR) assay developed and described by Mokrousov et al. (20). In this assay, the codon 315 region is PCR amplified with two outer primers and an inner reverse primer specific for the *katG315* wild-type allele (AGC). Isolates with a wild-type codon 315 produce two PCR products, while those isolates with a mutation at codon 315 produce only one PCR product. All 52 INH^r and 52 INH^s isolates were screened using this method. Those isolates identified as not having a wild-type *katG* codon 315 (AGC) were then screened using a modified MAS-PCR assay, which, instead of the inner reverse primer specific for the *katG315* wild-type sequence (20), used an inner reverse primer designed to detect the presence of either the *katG315* ACC mutation, primer 523 (5′-ATACGACCTCGATGCCGG-3′), or the ACA mutation, primer 534 (5′-ATACGACCTCGATGCCTG-3′).

Sequencing of the *fabG1-inhA* regulatory region, *inhA* ORF, and *katG* ORF. The *fabG1-inhA* regulatory region was PCR amplified using primers 519 (5′-C CTCGCTGCCCAGAAAGGGA-3′) and 520 (5′-ATCCCCCGGTTTCCTCCG GT-3′). The *inhA* open reading frame (ORF) was amplified using primers 534 (5′-TCCGGTGCGGTCATCCCG-3′) and 535 (5′-AACGGCCGCACCTGCTC G-3′). The *katG* ORF was amplified in three overlapping segments: segment 1 was amplified using primers 527 (5′-ACACTTCGCGATCACATCCG-3′) and 528 (5′-ACCTCGATGCCGCTGGTG-3′), segment 2 with primers 540 (5′-CG GTCACACTTTCGGTAAGA-3′) and 541 (5′-GGCGAAGGACACTTTGAT GT-3′), and segment 3 using primers 542 (5′-GCCAGCCTTAAGAGCCAGA T-3′) and 543 (5′-ACGCGGGGTCTGACAAAT3′). PCR products were sequenced as described previously.

RESULTS

Detection of mutations at *katG* codon 315 and in the *fabG1-inhA* regulatory region. The 52 INH^r and 52 matched INH^s sensitive control strains were screened for mutations at *katG* codon 315 and the *fabG1-inhA* regulatory region by MAS-PCR and DNA sequence analysis, respectively. A complete list of specific mutations identified is provided in Table 1. Thirty-four (65.4%) INH^r isolates had mutations at *katG* codon 315. The wild-type codon, AGC (Ser), was altered to ACC (Thr) in 31 strains and ACA (Thr) in three strains. Mutations in the *fabG1-inhA* regulatory region were identified in 13 (25.0%) of the 52 INH^r isolates (Table 1). All but one of these was the substitution of C for T 15 nucleotides upstream from the *fabG1* start codon. The one exception was a G-to-C substitution at position −17. None of the INH^r isolates had mutations in both *katG315* and the *fabG1-inhA* regulatory region. No mutations at either *katG* codon 315 or in the *fabG1-inhA* regulatory region were identified in any of the INH^s isolates.

Identification of mutations in the *katG* and *inh*A ORFs. Five INH^r isolates had a wild-type sequence at both *katG315* and in the *fabG1-inhA* regulatory region. The entire *katG* and *inhA* ORFs of these isolates were sequenced together with their matched INH^s control strains. All five INH^r and five INH^s isolates had a wild-type *inhA* structural gene. Mutations in *katG* were identified in three of the five INH^r isolates (Table 1). Isolate 2285 had an Asp117Ala substitution, isolate 2562 a Gly491Cys substitution, and isolate 2938 a Met257Ile substitution. These mutations were not present in the three INH^s control strains tested; however, a more extensive survey of INH^r and INH^s isolates would need to be performed for an association between these mutations and INH resistance to be determined. No mutations in *katG*, the *fabG1-inhA* regulatory region, or the *inhA* structural gene were identified in isolate 2919 or 2956. Thus, resistance in these isolates is unlikely to be associated with either of these enzymes.

Drug resistance pattern, geographic origin, and strain differentiation. The drug resistance phenotype, MIRU-ETR profile, genotypic group, geographic origin, and mutation identified for each of the 52 INH^r isolates are presented in Table 2. Twenty-three isolates were INH monoresistant; 16 isolates were resistant to both INH and streptomycin; 5 isolates were multidrug resistant (resistant to INH and rifampin); 4 isolates were resistant to both INH and ETH; 3 isolates were resistant to INH, pyrazinamide, and streptomycin; and 1 isolate was resistant to INH and ethambutol. Ten isolates displayed intermediate resistance to INH.

TABLE 1. Mutations detected in *M. tuberculosis* study isolates

DNA target	No. and type of isolates tested	No. of isolates with mutation	Nucleotide change	Amino acid change
katG codon 315	52 INH^r	31	AGC→ACC	Ser315→Thr
		3	AGC→ACA	Ser315→Thr
	52 INH^s	0		
fabG1-inhA regulatory region	52 INH^r	12	−15C→T	
		1	−17G→T	
	52 INH^s	0		
katG ORF	5 INH^r	1	GAC→GCC	Asp117→Ala
		1	GGC→TGC	Gly491→Cys
		1	ATG→TGC	Met257→Ile
	5 INH^s	0		
inhA ORF	5 INH^r	0		
	5 INH^s	0		

Although the isolates were collected from patients resident in Australia, only 7.7% ($n = 4$) of patients were born in Australia. The majority of overseas-born patients originated from Vietnam ($n = 16$), India ($n = 8$), China ($n = 4$), and the Philippines ($n = 4$), with 11 other countries represented. To determine whether INH resistance was associated with isolates from particular geographic origins, the proportion of strains resistant to INH from each country was compared to the proportion in the rest of the population (data not shown). Patients born in Vietnam were more likely to be infected with INH^r isolates than non-Vietnamese-born patients ($P = 0.001$). This finding cannot be attributed to patient-to-patient transmission, as the isolates were epidemiologically unrelated.

The UPGMA tree (Fig. 1) based on MIRU-ETR patterns (Table 2) illustrates the genetic relationship, genotypic group, drug resistance profile, and mutations identified in the 52 INH^r *M. tuberculosis* isolates. The 16 INH^r isolates identified as ancestral genetic group 1 represented 9.5% (16 of 168) of the total ancestral strains collected during the study period. In comparison, 11.6% (25 of 215) of modern group 1 and only 4.2% (11 of 262) of group 2/3 strains were found to be resistant to INH. Group 2/3 isolates were therefore less likely to be resistant to INH than strains belonging to group 1 ($P = 0.008$). All five multidrug-resistant isolates were modern strains: one isolate belonged to groups 2/3, and four belonged to group 1. Beijing family strains accounted for 30.8% ($n = 16$) of the INH^r isolates (Fig. 1), which was slightly higher than the proportion of Beijing strains in the total collection (22.6%). With the exception of strain 2817, the remaining modern group 1 strains ($n = 8$) were of the Delhi/CAS genotype (Fig. 1).

Association between mutations at *katG* codon 315 and in the *fabG1-inhA* regulatory region, genotypic group, and drug resistance phenotype. The proportions of isolates within each genotypic group with the *katG* S315T substitution were as follows: ancestral, 12/16; group 1, 15/25; and groups 2/3, 7/11. The proportion of Beijing isolates harboring the S315T substitution (68.8%) was slightly higher than that of non-Beijing strains (63.9%). The numbers of isolates with the *fabG1-inhA* −15C→T mutation in each of the genotypic groups were as follows: ancestral, 4/16; group 1, 6/25; and groups 2/3, 2/11. Thus, neither the *katG* S315T substitution nor the *fabG1-inhA* −15C→T mutation clustered with any one genetic group. However, there was an association between the −15C→T substitution and those isolates with intermediate resistance to INH, with the mutation identified in eight of the 10 INH^I isolates. In addition, the −15C→T substitution was strongly associated with resistance to ETH, with four of the five ETH-resistant isolates having the mutation.

DISCUSSION

From 2001 to 2003, 52 (8.1%) of the 645 new laboratory-confirmed *M. tuberculosis* cases in Victoria were resistant to INH. Isolates belonging to genetic groups 2/3 were less likely to be resistant to INH than genetic group 1 strains ($P = 0.008$). This is consistent with a number of studies that reported higher rates of INH or multidrug resistance in Beijing strains (which belong to genetic group 1) than isolates from other families (1, 16, 31, 32).

Mutations at *katG* codon 315 were identified in 65.4% of the isolates studied. The S315T substitution was observed in 68.8% of Beijing strains compared with 63.9% of non-Beijing strains. This difference in frequency of the S3125T substitution between Beijing and non-Beijing strains is smaller than those in previous studies (19, 21). The prevalence of the S315T substitution among this set of diverse isolates highlights the selective advantage conferred by the S315T substitution, which provides the basal catalase-peroxidase activity to protect the cell from oxidative stress while reducing the conversion of the INH prodrug to its active form by KatG (25, 26, 34). The three other mutations identified in *katG*, D117A, M257I, and G491C, represent novel mutations that to our knowledge have not previ-

TABLE 2. Characteristics of INH-resistant *M. tuberculosis* isolates

Strain no.	Resistance pattern	MIRU-ETR[a]	Genetic group[b]	Country of birth	Mutation identified[c]
2285	INH, RIF	424 221325173433	1 M	South Korea	D117A
2316	INH	424 223325173533	1 M	Vietnam	S315T
2357	INH, ETH	464 254226223433	1 A	Philippines	−15C→T
2370	INH, STR	324 224125113322	2/3	Ethiopia	S315T
2376	INH[I]	622 226425133533	1 M	Sudan	−15C→T
2377	INH	464 274326223632	1 A	Philippines	S315T
2381	INH	323 226325153321	2/3	Indonesia	S315T
2382	INH	224 223226153321	2/3	Somalia	S315T
2405	INH[I], ETH, RIF, STR	424 223315173533	1 M	Vietnam	−15C→T
2413	INH, STR	424 222325173543	1 M	Vietnam	S315T
2458	INH[I], STR	424 223325173633	1 M	Vietnam	−15C→T
2475	INH	424 223325163533	1 M	Vietnam	S315T
2482	INH, EMB	614 274326223434	1 A	India	S315T
2492	INH	464 254326223432	1 A	China	S315T
2498	INH	663 254226223522	1 A	India	S315T
2501	INH	424 223325171431	1 M	Vietnam	S315T
2503	INH, STR	952 252326223513	1 A	Australia	S315T
2523	INH	424 223425173542	1 M	China	−15C→T
2558	INH	422 226525163543	1 M	India	−17G→T
2562	INH	324 223326152321	2/3	England	G491C
2591	INH	424 223425173543	1 M	India	S315T
2603	INH[I], ETH	644 274225223534	1 A	Laos	−15C→T
2620	INH[I]	323 225323153323	2/3	Somalia	−15C→T
2628	INH	842 254326223523	1 A	Indonesia	S315T
2640	INH, ETH	464 254326223442	1 A	Philippines	−15C→T
2643	INH, EMB, PZA, RIF	424 223325173533	1 M	Korea	S315T
2683	INH	422 226525153543	1 M	India	S315T
2779	INH	424 223326171531	1 M	Vietnam	S315T
2783	INH, STR	023 223125173533	1 M	China	S315T
2786	INH, STR	424 223325173532	1 M	Australia	S315T
2790	INH[I], STR	323 225325153323	2/3	India	−15C→T
2802	INH, STR	424 242325152322	2/3	Vietnam	S315T
2803	INH, STR	424 223325143533	1 M	Vietnam	S315T
2807	INH	744 263225223532	1 A	Australia	S315T
2813	INH, PZA, STR	744 394226233533	1 A	Vietnam	S315T
2817	INH, STR	635 228225123433	1 M	Vietnam	−15C→T
2847	INH[I]	464 254326223432	1 A	Philippines	−15C→T
2868	INH, STR	744 394225223533	1 A	Vietnam	S315T
2873	INH, PZA, STR	424 223326171533	1 M	Vietnam	S315T
2896	INH	744 364225223534	1 A	Vietnam	S315T
2899	INH, STR	422 224425173533	1 M	India	S315T
2908	INH, PZA, STR	024 242325132322	2/3	Vietnam	S315T
2919	INH[I]	024 242325152322	2/3	Australia	ND
2927	INH[I], STR	424 222325163553	1 M	PNG[d]	−15C→T
2938	INH[I], STR	422 246425163534	1 M	Sudan	M2571
2951	INH	544 284225223533	1 A	Cambodia	S315T
2956	INH, ETH	422 225425143533	1 M	India	ND
2960	INH, PZA, RIF, STR	422 225425133533	1 M	China	S315T
2966	INH, STR	422 227425113434	1 M	Eritrea	S315T
2974	INH, RIF, STR	424 223315153321	2/3	Vietnam	S315T
2999	INH, STR	324 225125113322	2/3	Ethiopia	S315T
3015	INH	952 353326223513	1 A	Zambia	S315T

[a] Number of repeats at variable-number tandem repeat-ETR loci A, B, and C and MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40, respectively.
[b] According to the scheme described by Sreevatsan et al. (28), genetic group 1 includes *katG463* CTG (Leu) and genetic groups 2/3 include *katG463* CGG (Arg). According to the scheme described by Sun el al. (29), A represents ancestral (> 1 repeat at MIRU 24) and M represents modern (1 repeat at MIRU 24).
[c] D117A, *katG117* GAC (Asp) to GCC (Ala); S315T, *katG315* AGC (Ser) to ACC (Thr); −15C→T, cytosine-to-thymine substitution 15 bases upstream of *fabG1* start codon; −17G→T, guanine-to-thymine substitution 17 bases upstream of *fabG1* start codon; G491C, *katG491* GGC (Gly) to TGC (Cys); M257I, *katG257* ATG (Met) to TGC (Ile); ND, not detected.
[d] PNG, Papua New Guinea.

ously been reported in the literature. None of these mutations maps near either the active site or the proposed INH binding site of KatG (5). Therefore, these mutations may cause resistance to INH by causing conformational changes to KatG, rather than by direct inhibition of INH binding.

Thirteen of the 52 INH^r isolates had mutations flanking the putative *fabG1* ribosome binding site. While the absence of mutations in this region in the INH^s control strains indicates that *fabG1-inhA* regulatory region mutations may play a role in INH resistance, the effect(s) of these mutations on FabG1 and/or InhA protein synthesis and in turn the mechanism of resistance conferred by these mutations are yet to be eluci-

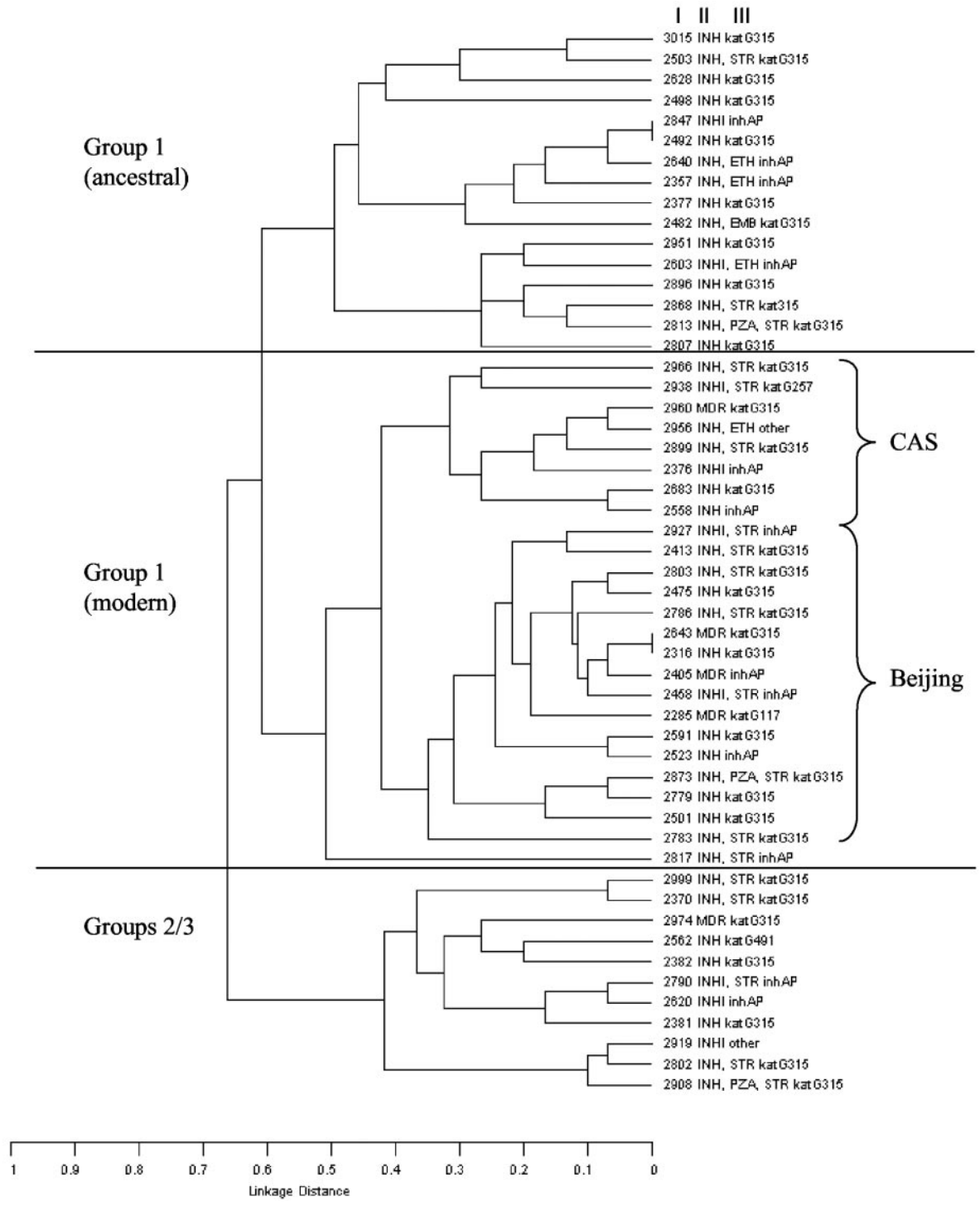

FIG. 1. Genetic relationship, genotypic group, drug resistance profile, and mutations identified in the 52 INH-resistant *M. tuberculosis* isolates. This UPGMA tree was generated using the START program based on the 50 distinct MIRU-ETR patterns (Table 2) that represented the 52 isolates. I, isolate identification number; II, drug resistance phenotype; INHI, INH intermediate resistance; III, inhAP, *fabG1-inhA* regulatory region mutation; katG117, *katG* D117A substitution; katG257, *katG* M257I substitution; katG315, *katG* S315T substitution; katG491, *katG* G491C substitution.

dated. Since experimental studies have shown that overexpression of the *M. tuberculosis inhA* gene confers resistance to INH and ETH in *Mycobacterium smegmatis*, *Mycobacterium bovis*, and *M. tuberculosis* (3, 4), it is possible that the mutations identified in the *fabG1-inhA* regulatory region in clinical isolates could lead to the increased expression of the InhA protein, producing INH resistance via a titration mechanism.

The strong association between mutations in the *fabG1-inhA*

regulatory region and intermediate INH resistance supports previous studies in which mutations within the *inhA* promoter and structural regions were associated with low-level resistance to INH (14, 23, 36). In their 2003 study, Ramaswamy et al. (23) identified one INH^r isolate with a *fabG1-inhA* regulatory region mutation that also had a S315N substitution in *katG* and had a very high MIC, suggesting that in certain isolates with multiple mutations, INH resistance may develop in a stepwise fashion. Therefore, it is possible that those isolates in the study by Ramaswamy et al. with either the −15C→T or −17G→T mutation that were resistant to INH at 4.0 μg/ml have an additional mutation in *katG*, which cumulatively causes the higher level of resistance to INH.

There was also an association between mutations in the *fabG1-inhA* regulatory region and resistance to ETH, with four of the five ETH-resistant isolates having the −15C→T mutation. Other authors have reported that mutations in the *fabG1-inhA* regulatory region occurred in a disproportionately higher percentage of strains resistant to both INH and ETH than in isolates resistant to INH but susceptible to ETH. However in our study, 9 of the 13 isolates with the −15C→T mutation were susceptible to ETH, indicating that this mutation is not predicative of ETH resistance. Furthermore, the observation that a number of ETH-resistant isolates do not have mutations in the *fabG1-inhA* regulatory region indicates that other, ETH-specific mechanisms of resistance are involved in ETH resistance.

Understanding the nature and frequency of mutations associated with drug-resistance in *M. tuberculosis* in different clinical settings is important for the development and large-scale implementation of rapid, genetics-based assays for the detection of drug resistance. In this study, mutations at *katG* codon 315 or the *fabG1-inhA* regulatory region were identified in 90.4% of the INH^r isolates and in none of the 52 INH^s control strains selected to reflect the genotypic and geographic diversity of the isolates. Therefore, mutations in these regions are highly predictive of INH resistance in this population. Nevertheless, the identification of three novel *katG* mutations in three of the five isolates with wild-type *katG* codon 315 and *inhA-fabG1* regulatory regions highlights the importance of bacteriological susceptibility testing methods in achieving comprehensive identification of resistance among clinical isolates.

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