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ethA, *inhA*, and *katG* Loci of Ethionamide-Resistant Clinical *Mycobacterium tuberculosis* Isolates

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Ethionamide (ETH) is a structural analog of the antituberculosis drug isoniazid (INH). Both of these drugs target InhA, an enzyme involved in mycolic acid biosynthesis. INH requires catalase-peroxidase (KatG) activation, and mutations in *katG* are a major INH resistance mechanism. Recently an enzyme (EthA) capable of activating ETH has been identified. We sequenced the entire *ethA* structural gene of 41 ETH-resistant *Mycobacterium tuberculosis* isolates. We also sequenced two regions of *inhA* and all or part of *katG*. The MICs of ETH and INH were determined in order to associate the mutations identified with a resistance phenotype. Fifteen isolates were found to possess *ethA* mutations, for all of which the ETH MICs were ≥50 μg/ml. The *ethA* mutations were all different, previously unreported, and distributed throughout the gene. In eight of the isolates, a missense mutation in the *inhA* structural gene occurred. The ETH MICs for seven of the InhA mutants were ≥100 μg/ml, and these isolates were also resistant to ≥8 μg of INH per ml. Only a single point mutation in the *inhA* promoter was identified in 14 isolates. A *katG* mutation occurred in 15 isolates, for which the INH MICs for all but 1 were ≥32 μg/ml. As expected, we found no association between *katG* mutation and the level of ETH resistance. Mutations within the *ethA* and *inhA* structural genes were associated with relatively high levels of ETH resistance. Approximately 76% of isolates resistant to ≥50 μg of ETH per ml had such mutations.

Multidrug-resistant tuberculosis (MDR-TB) is an emerging public health crisis in many regions of the world, particularly in developing nations (29). Tubercle bacilli isolated from patients with MDR-TB are resistant to at least isoniazid (INH) and rifampin (8). These two drugs represent half the standard four-drug tuberculosis treatment regimen along with pyrazinamide (PZA) and either ethambutol or streptomycin (2). These five compounds are referred to as the "first-line" antituberculosis drugs. Infection with MDR-TB complicates tuberculosis treatment by necessitating the selection of substitute drugs, collectively referred to as "second-line" drugs, to replace the ineffective first-line drugs (8). Ethionamide (ETH), one of the most frequently used and efficacious second-line drugs (8), is a structural analog of INH (5). Both compounds are known to inhibit mycolic acid biosynthesis (28). The existence of partially cross-resistant phenotypes has long been known (6, 14, 18). Low-level INH-resistant strains frequently display low-level ETH resistance, while high-level INH-resistant strains typically remain ETH susceptible (6). The structural similarity and existence of cross-resistant phenotypes suggested that these two drugs share a common molecular target (1).

The molecular genetics of INH resistance in *Myobacterium tuberculosis* has been extensively investigated. INH is classified as a prodrug, meaning that it must undergo in vivo transformation to an active form. *katG*-encoded catalase-peroxidase (KatG) performs this function in *M. tuberculosis* (30), and mutations in *katG*, particularly at codon 315, confer INH, but not ETH, resistance (5). The primary target of activated INH is an NADH-dependent enoyl-acyl carrier protein reductase, designated InhA (16). Mutations within the *inhA* structural gene (1, 3, 15, 23) or within the *inhA* promoter (15, 17, 21, 22, 24) have been identified and are associated with both INH and ETH resistance (16, 17). Missense mutations within the *inhA* structural gene cause INH resistance by reducing the NADH binding affinity of InhA and thus protecting the enzyme from INH inactivation (25). The *inhA* promoter mutations upregulate target expression, producing INH and ETH resistance via a drug titration mechanism (1, 16). The structural similarity and shared molecular target of INH and ETH led to the hypothesis that ETH must, like INH, undergo cellular activation (4).

Recently two groups have reported the discovery of an enzyme capable of activating ETH (4, 10). Both groups initially identified a protein that when overexpressed produced ETH resistance. This protein showed homology with members of the TetR family of transcriptional regulators (4, 10). The open reading frame (ORF) encoding this protein is designated Rv3855 in the *M. tuberculosis* genome database. An adjacent, transvergently transcribed ORF (Rv3854c), separated from the other by a 76-bp intergenic region, encodes a protein with homology to known monooxygenases (4, 10). Overexpression of Rv3854c in *Mycobacterium smegmatis* resulted in substantially increased ETH sensitivity relative to wild-type *M. smegmatis* (4, 10). Mycolic acid synthesis was also dramatically inhibited in the Rv3854c expression construct (4). Attempts to overexpress Rv3854c in *M. tuberculosis* were unsuccessful (10).

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TABLE 1. Oligonucleotide primers used in this study

Name	Sequence (5′→3′)[a]	Application		Accession no.
		PCR	Sequencing	
ethA-1	ATC ATC GTC GTC TGA CTA TGG	Yes	Yes	z83864
ethA-2	CGA CAG ACA AAC TCC GAC ACC	No	Yes	z83864
ethA-3	CCT CCT GGA CGC TGA AG	No	Yes	z83864
ethA-4	CCT CGA CCT TCC CGT GA	Yes	Yes	z83864
ethA-5	ACT ACA ACC CCT GGG ACC	Yes	Yes	z83864
ethA-6	CTT GGC GCC CGA GTC C	No	Yes	z83864
ethA-7	CGT CGT GAT CGG CAG TGG C	No	Yes	z83864
ethA-8	GGT GGA ACC GGA TAT GCC TG	Yes	Yes	z83864
ethA-9	CCT CGA GTA CGT CAA GAG CAC	Yes	Yes	z83864
ethA-10	CGT TGA CGG CCT CGA CAT TAC	Yes	Yes	z83864
katG-1	TGG CCG CGG CGG TCG ACA TT	Yes	Yes	x68081
katG-2	CCA GCA GGG CTC TTC GTC AG	Yes	Yes	x68081
inhA-1	CCT CGC TGC CCA GAA AGG GA	Yes	Yes	u41388
inhA-2	ATC CCC CGG TTT CCT CCG GT	Yes	Yes	u41388
inhA-3	AGG TCG CCG GGG TGG TCA GC	Yes	Yes	u41388
inhA-4	AGC GCC TTG GCC ATC GAA GCA	Yes	Yes	u41388

[a] Underlined sequences are in the reverse complement orientation relative to the GenBank sequence.

These results led both groups to conclude that Rv3854c activates ETH and that this enzyme is under the regulatory control of Rv3855. The DeBarber group designated *Rv3855* and *Rv3854c* as *etaR* and *etaA*, respectively, while the Baulard group used the designations *ethR* and *ethA*, respectively.

The identification and characterization of the *ethAR* loci represent a significant advance in understanding the biochemistry of ETH and the mechanistic relationship of this drug to its structural analog, INH. ETH must undergo activation via an EthA-mediated process in a manner analogous to the KatG activation of INH. The putative final metabolites for both drugs are very similar, and they share the same cellular target, namely InhA. Genetic alterations leading to reduced EthA activity would be expected to result in increased ETH resistance, just as *katG* mutations confer INH resistance. The *ethA* genes of 11 ETH-resistant isolates were sequenced, and coding sequence mutations were found in all of them (10).

While the ability of EthA to activate ETH has been convincingly demonstrated, very limited data exist on the occurrence of *ethA* mutations in ETH-resistant *M. tuberculosis* clinical isolates. This investigation was undertaken to provide additional data regarding the relative prevalence of *ethA* and *inhA* mutations in such isolates. We sequenced either all or part of the *ethA*, *inhA*, and *katG* genes of 41 ETH-resistant clinical isolates of *M. tuberculosis*. To evaluate the relative phenotypic impact of mutations within these genes, we determined the MICs of ETH and INH for all isolates.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA isolation. The 41 isolates of *M. tuberculosis* examined in this study were obtained from patients in the United States, Russia, and Brazil. The U.S. isolates ($n = 29$) had been submitted to the Centers for Disease Control and Prevention for routine drug susceptibility testing. The Russian isolates ($n = 6$) had been collected as part of a drug resistance surveillance study and originated from the oblasts of Ivanovo and Vladimir. The Brazilian specimens ($n = 6$) were obtained from a collection of isolates gathered for a study of the molecular genetics of INH resistance in Brazil. All isolates were resistant to 10 μg of ETH per ml when tested by the method of proportions (7) with Middlebrook 7H10 agar. Isolates were stored frozen at −70°C until selected for this study.

Genomic DNA was prepared by a minibead cell disruption protocol. One milliliter of a 2-week-old 7H9 broth culture was added to a 2.0-ml screw-cap microcentrifuge tube containing Lysing Matrix B (Qbiogene, Inc., Carlsbad, Calif.). The tubes were then incubated for 20 min at 95°C to kill the cells. Next, 200 μl of chloroform and 300 μl of Tris-EDTA (TE) buffer were added to each tube. This mixture was vigorously agitated for 1 min with a Mickle cell disrupter (Brinkman Instruments, Inc., Westbury, N.Y.) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase, which contained genomic DNA, was collected and stored at 4°C.

DNA amplification and sequencing. The entire *ethA* ORF was PCR amplified. Because of the large size of this ORF (1,470 bp), three reactions were performed for each sample, producing three overlapping PCR amplicons: ETH1, ETH2, and ETH3. The primers used and the sizes of the amplicons generated are as follows: primers ethA-1 and ethA-5 produced a 667-bp product designated ETH1, primers ethA-4 and ethA-9 produced a 692-bp product (ETH2), and primers ethA-8 and ethA-10 produced a 342-bp product (ETH3). All reaction mixtures contained 12.5 μl of HotStartTaq master mix (Qiagen Inc., Santa Clarita, Calif.), 1.0 μl of template DNA, and each primer at a final concentration of 0.3 μM. Each reaction was adjusted to a final volume of 25 μl with Type 1 water. The amplification profile for ETH1 and ETH2 consisted of an initial 15-min denaturation and enzyme activation at 95°C followed by 35 cycles of 95°C denaturation for 30 s and 65°C annealing and elongation for 1.25 min and a final 5-min elongation. The profile for ETH3 was identical, except that the annealing and elongation temperature was 68°C. All amplifications were performed in a Gene-Amp PCR system 2400 thermal cycler (Perkin-Elmer, Inc., Foster City, Calif.).

A 322-bp fragment of *katG* encompassing codon 315 was PCR amplified with primers katG-1 and katG-2. A 248-bp fragment containing the *inhA* promoter was amplified with primers inhA-1 and inhA-2. Nucleotide residues 13 to 379 of the 810-nucleotide (nt) *inhA* ORF were amplified with primers inhA-3 and inhA-4. The PCR mixtures and thermal cycler used to amplify these three loci were the same as those described for *ethA* amplification. All three loci were amplified with the same thermal cycling profile of 15 min of denaturation and enzyme activation at 95°C followed by 35 cycles of 95°C denaturation for 30 s, 60°C annealing for 30 s, 72°C elongation for 30 s, and a final 5-min elongation. The entire *katG* structural gene was sequenced in a select subset ($n = 12$) of the isolates.

Automated DNA sequencing was performed by dichlororhodamine BigDye terminator chemistry (Perkin-Elmer, Inc.). The protocol supplied by the manufacturer was modified by halving the volume of master mix used and adjusting the ionic strength of the reaction mixture with 5× sequencing buffer (Perkin-Elmer, Inc.). The fluorescent elongation products were electrophoresed on a model 373XL DNA sequencer (Perkin-Elmer, Inc.). Amplicons ETH1 and ETH2 of *ethA* were each sequenced with four internal primers and the *katG*, *inhA* promoter, *inhA* ORF, and ETH3 amplicons were sequenced with the primers used for amplification. A complete list of all PCR and sequencing primers, with their nucleotide sequences, is found in Table 1. All *ethA* primers were designed with Oligo version 6.0 primer analysis software (Molecular Biology Insights, Inc.,

Cascade, Colo.). Sequence analyses were performed with Sequence Navigator version 1.0.1 software (Perkin-Elmer, Inc.), and all sequencing runs included the pan-susceptible strain *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) as a wild-type control. Each sequence was compared with that of both the control strain and the appropriate published sequence.

Strain differentiation. All *M. tuberculosis* isolates were genotyped by the spoligotyping method (19). Each spoligotype pattern was assigned an octal code as described by Dale et al. (9).

ETH and INH susceptibility testing. ETH and INH susceptibility testing was performed with the microplate alamar blue assay (MABA) (12). A 32-mg/ml stock solution of ETH (Sigma Chemical Co., St. Louis, Mo.) was prepared in dimethyl sulfoxide (DMSO; J. T. Baker, Inc., Phillipsburg, N.J.), and a 10-mg/ml stock solution of INH (Sigma Chemical Co.) was prepared in sterile water. The stock solutions were aliquoted and stored at −70°C. The ETH stock was diluted with Middlebrook 7H9 broth to a concentration of 400 μg/ml, and the solution was subsequently twofold serially diluted, resulting in solutions ranging in concentration from 400 to 50 μg/ml. Four additional twofold serial dilutions ranging in concentration from 40 to 5.0 μg/ml were formulated by the same approach.

The perimeter wells of 96-well, clear microtiter plates (Costar 3596; Corning, Inc., Corning, N.Y.) were filled with 200 μl of sterile water to prevent the plates from drying out during incubation. The wells in rows B through F of each column received 100 μl of test medium, with the drug concentration highest in column 2 and diminishing in order through column 9. Columns 10 and 11 received 100 and 200 μl of drug-free medium, respectively. To control for any possible inhibitory effect of the DMSO on cell growth, the wells in row G received 100 μl of DMSO-containing media. The concentration of DMSO in each column was equivalent to that of the ETH-containing wells in that column. The INH assay plate was prepared in a similar manner, with concentrations ranging from 64 to 0.5 μg/ml.

The *M. tuberculosis* strains were cultured for 2 weeks, after which the turbidity was visually adjusted with 7H9 broth to that equivalent to a McFarland no. 1 standard. The inocula were prepared by diluting the standardized cultures 1:25 with 7H9 broth. Each test well received 100 μl of inoculum. The wells in column 11 were not inoculated and served as a sterility control. The plates were sealed with transparent tape. The pan-susceptible *M. tuberculosis* strains $H_{37}Rv$ and "circle 8" (a clinical isolate routinely used as a control in our laboratory) were used as controls.

The plates were incubated at 35°C under ambient conditions. The detection reagent was prepared by diluting a 10× alamar blue (Trek Diagnostic Systems, Inc., Westlake, Ohio) solution 1:1 with freshly prepared 10% Tween 80 (Sigma Chemical Co.). After 9 days of incubation, 25 μl of alamar blue solution was added to the drug-free control wells (columns 10 and 11). The plates were incubated for another 24 h, after which the control wells were examined. A color change from blue to pink in the inoculated wells and no color change in the uninoculated wells validated the controls. Once the controls were validated, 25 μl of alamar blue solution was dispensed into the remaining test wells. The plates were examined, and results were recorded after another 24 h of incubation. The MIC was defined as the lowest drug concentration that prevented a color change. All strains were tested in duplicate on separate occasions.

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RESULTS

DNA sequencing of *ethA*, *inhA*, and *katG* loci. A complete list of specific mutations identified as well as the MICs of ETH and INH for each isolate is provided in Table 2. The entire *ethA* ORFs of the 41 *M. tuberculosis* isolates were sequenced. An *ethA* mutation was identified in 15 (37%) of those isolates; all 15 mutations were unique and had not been previously reported. The mutations were distributed from nt 110 through 1387 of the 1,470-nt ORF. The types of mutations included eight missense mutations, 1 nonsense mutation, four 1-nt deletions, one 2-nt deletion, and one 1-nt insertion. Nine of the *ethA* mutants, including all six of the frameshift mutants, had no *inhA* mutations, while the other six also had *inhA* regulatory mutations. The ETH MICs for the *ethA* mutants ranged from 50 to ≥200 μg/ml.

Two separate regions of the *inhA* gene, one encompassing codons 13 to 119 of the ORF (270 total codons) and another containing the putative promoter, were examined in all isolates. Mutations in the *inhA* ORF were identified in 9 (22%) of the 41 isolates. Among the nine *inhA* ORF mutants, four different point mutations were identified: three were missense (Ile 21→Thr or Val and Ser 94→Ala) and one was silent (Leu 44→Leu). Twenty-seven (66%) of the 41 isolates displayed a mutation in the *inhA* regulatory region. All but one of these involved the substitution of cytosine for thymine at the position 15 nt upstream (−15) of the *mabA* initiation codon. The one exception was a thymine-to-cytosine transition at position −17. Six of the eight *inhA* ORF missense mutants also displayed *inhA* regulatory mutations. Only one isolate (isolate 15) possessed mutations in all of the regions examined; however, the *inhA* ORF mutation was silent. Three isolates were wild type at both the *ethA* and *inhA* loci, two of which were susceptible to the lowest concentration of ETH tested, while the MIC for the third isolate was 50 μg/ml.

The complete *katG* ORF of 12 isolates and a fragment encompassing codons 249 through 342 (741 total codons) of the remaining isolates were sequenced. Mutations in *katG* were found in 15 (37%) of the 41 isolates. Eleven of those mutants had a guanine-to-cytosine transversion at nt 944, resulting in the substitution of threonine for serine at amino acid residue 315. The four other *katG* mutations identified were Asn 138→Thr, Glu 195→Lys, Gly 279→Asp, and Trp 341→Ser. The INH MICs for all but one of the *katG* mutants were ≥32 μg/ml: the INH MIC for the Glu 195→Lys mutant (isolate 35) was 1 μg/ml. Tables 3 and 4 list the number of isolates with mutations in each locus, or combination of loci, stratified according to MIC.

Strain differentiation. Twenty-eight different spoligotype patterns were identified among the 41 study isolates. Twenty-three of these patterns were represented by a single isolate, and the remaining 18 isolates were distributed among five clusters. The largest of these five clusters included eight isolates, one cluster contained four isolates, and three clusters consisted of two isolates each. Among the clustered isolates, none had mutation profiles identical to those of the other members of its cluster. All five of the single-base frameshift mutants were members of the eight-isolate cluster. The spoligotype of each of the isolates is shown in Table 2.

ETH and INH MICs. We determined the MICs of ETH and INH for all 41 isolates in order to compare the types of mutations identified with drug resistance levels. The INH MICs showed perfect concordance between replicates in 39 (95%) of the isolates. The MICs of the two discrepant isolates differed by only 1 dilution. The end points for INH were sharp and unambiguous, as indicated by the absence of a color change from blue to pink in the MIC well. The end points for ETH were somewhat less obvious, since color changes were gradual as the end point was approached. This "trailing" effect typically occurred over 1 to 2 drug dilutions, but did not occur for isolates that were either fully susceptible or resistant to ≥200 μg of ETH per ml. To confirm our 24-h readings, we allowed the plates to incubate for an additional 24 h, after which time, the color change from blue to pink became more pronounced. The ETH MICs for 27 (66%) of the isolates were identical between tests: 10 (24%) differed by 1 dilution, and 4 (10%)

TABLE 2. Characteristics of 41 clinical *M. tuberculosis* isolates

Isolate no. (origin)[a]	Spoligotype	MIC (μg/ml)		Genetic alteration						
				ethA		*katG*[b]		*inhA* ORF		*inhA* regulator (nucleotide no.)
		ETH	INH	Nucleotide no.	Amino acid	Nucleotide no.	Amino acid	Nucleotide no.	Amino acid	
1 (US)	677777477413771	>200	>32	None	NA[c]	**None**	**NA**	T → C62	I21 → T	C (−15) T
2 (US)	000000000003771	>200	8	None	NA	**None**	**NA**	T → G280	S94 → A	None
3 (US)	477777777413771	>200	4	None	NA	None	NA	None	NA	C(−15) T
4 (US)	000000000000771	>200	8	C → T736	Q246 AMB	None	NA	None	NA	C(−15) T
5 (US)	777737776413731	>200	2	None	NA	None	NA	None	NA	C(−15) T
6 (US)	667777477413771	>200	8	None	NA	**None**	**NA**	A → G61	I21 → V	C (−15) T
7 (US)	677777477413771	>200	8	None	NA	**None**	**NA**	A → G61	I21 → V	C (−15) T
8 (R)	000000000003771	>200	>32	T deleted at 703	Frameshift	G → C944	S315 → T	None	NA	None
9 (R)	000000000003771	>200	32	A deleted at 110	Frameshift	G → C944	S315 → T	None	NA	None
10 (R)	000000000003771	>200	>32	G deleted at 768	Frameshift	G → C944	S315 → T	None	NA	None
11 (US)	647777477413771	>200	4	None	NA	None	NA	None	NA	C (−15) T
12 (BZ)	777777777760601	>200	32	A → G1174	T392 → A	G → C944	S315 → T	None	NA	None
13 (BZ)	777741017760771	>200	>32	G → A1154	G385 → D	G → C944	S315 → T	None	NA	C (−15) T
14 (BZ)	677737607760771	>200	>32	None	NA	**None**	**NA**	T → C62	I21 → T	C (−15) T
15 (BZ)	777777777760731	>200	>32	A → C167	D55 → A	G → A836	G279 → D	C → T130	L44 → L	C (−15) T
16 (US)	777777777760740	>200	0.5	GC deleted at 1322–1323	Frameshift	None	NA	None	NA	None
17 (US)	777777776413771	>200	>32	None	NA	**None**	**NA**	None	NA	C (−15) T
18 (R)	777777607760771	200	>32	T → G1013	I338 → S	G → C944	S315 → T	None	NA	C (−15) T
19 (US)	000000000003071	200	32	G → A127	G43 → S	G → C944	S315 → T	None	NA	None
20 (US)	777777774020771	100	8	None	NA	**None**	**NA**	T → G280	S94 → A	C (−15) T
21 (US)	777777777720771	100	8	None	NA	**None**	**NA**	T → C62	I21 → T	G (−17) T
22 (US)	000000000003771	100	32	A inserted at 338	Frameshift	G → C944	S315 → T	None	NA	None
23 (US)	677777477413771	100	2	None	NA	None	NA	None	NA	C (−15) T
24 (US)	700777747413771	100	2	None	NA	None	NA	None	NA	C (−15) T
25 (US)	000000000003771	50	32	C deleted at 1290	Frameshift	G → C944	S315 → T	None	NA	None
26 (US)	777777777760771	50	2	G → A668	E223 → K	None	NA	None	NA	C (−15) T
27 (US)	474377777413771	50	2	G → A1238	G413 → D	**None**	**NA**	None	NA	None
28 (US)	477777777413771	50	2	None	NA	**None**	**NA**	None	NA	None
29 (BZ)	777777607760771	50	>32	C → A1387	R463 → S	G → C1022	W341 → S	None	NA	C (−15) T
30 (R)	000000000003771	25	2	None	NA	None	NA	None	NA	C (−15) T
31 (US)	777777777760771	20	2	None	NA	None	NA	None	NA	C (−15) T
32 (US)	677777477413751	20	2	None	NA	None	NA	None	NA	C (−15) T
33 (US)	776377760000731	20	4	None	NA	None	NA	None	NA	C (−15) T
34 (US)	700036777760711	20	2	None	NA	None	NA	None	NA	C (−15) T
35 (US)	677777477413771	10	1	None	NA	**G → A583**	**E195 → K**	T → G280	S94 → A	None
36 (US)	777777777740711	10	2	None	NA	None	NA	None	NA	C (−15) T
37 (US)	776000003660771	10	2	None	NA	None	NA	None	NA	C (−15) T
38 (BZ)	677777607760771	10	>32	None	NA	G → C944	S315 → T	None	NA	C (−15) T
39 (US)	000000007760771	<2.5	2	None	NA	None	NA	None	NA	C (−15) T
40 (US)	777777774020771	<2.5	>32	None	NA	**A → C413**	**N138 → T**	None	NA	None
41 (R)	000000000003771	<2.5	>32	None	NA	G → C944	S315 → T	None	NA	None
$H_{37}Rv$	777777477760771	<2.5	0.5	None	NA	None	NA	None	NA	None
circle 8	777777774020771	<2.5	<0.25	None	NA	None	NA	None	NA	None

[a] Country where the isolate was collected. US, United States; R, Russia; BZ, Brazil.
[b] Results in boldface indicate the entire *katG* ORF was sequenced.
[c] NA, not applicable.

differed by 2 dilutions. In all cases, we defined the MIC as the lowest concentration that prevented any color change compared with the negative control.

The ETH MICs for 15 isolates with *ethA* mutations, either alone ($n = 9$) or in combination with *inhA* promoter mutations, were ≥50 μg/ml. No isolate for which the ETH MIC was ≤25 μg/ml possessed *ethA* mutations. Six isolates displayed both *inhA* ORF and promoter mutations, and the ETH MIC for all of them was ≥100 μg/ml. Two strains with Ser 94→Ala substitutions in InhA had a wild-type promoter; one of these (isolate 2) was wild type at all other loci examined, while the other (isolate 35) also had a Glu 195→Lys substitution in *katG*. Isolate 35 was much less resistant to both ETH and INH than was isolate 2.

In 14 of the isolates, only *inhA* promoter mutations were identified. The ETH MICs for these isolates varied greatly, with six being resistant to ≥100 μg/ml, seven being in the range of 10 to 25 μg/ml, and one being susceptible to the lowest concentration tested. The INH resistance level of these isolates was much more consistent, with INH MICs for 13 being either 2 or 4 μg/ml. The sole exception (isolate 17) was resistant to >32 μg of INH per ml. This strain was also resistant to 200 μg of ETH per ml.

The INH MICs for 17 (41%) of the study isolates were ≥32 μg/ml. Fourteen of these possessed *katG* mutations, either alone ($n = 9$) or in combination with *inhA* promoter mutations ($n = 5$). Two strains (isolates 1 and 14) resistant to 32 μg of INH per ml had both an Ile 21→Thr substitution in InhA and

TABLE 3. ETH MICs for and mutations in the *ethA* and *inhA* loci of 41 clinical *M. tuberculosis* isolates

ETH MIC (μg/ml)	No. of isolates	No. of isolates with mutations in[a]:					No. of isolates with no mutation
		ethA only	*inhA*			*ethA* and *inhA* promoter	
			ORF only	Promoter only	ORF and promoter		
>200	17	5	1	4	4	3	0
200	2	1	0	0	0	1	0
100	5	1	0	2	2	0	0
50	5	2	0	0	0	2	1
25	1	0	0	1	0	0	0
20	4	0	0	4	0	0	0
10	4	0	1	3[c]	0	0	0
5	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0
<2.5	3	0	0	1	0	0	2[e]
Total	41	9[b]	2	15	6	6[d]	3

[a] Exclusive of mutations in *katG* and silent mutations.
[b] Includes seven isolates with *katG* mutations.
[c] Includes one isolate with a *katG* mutation.
[d] Includes four isolates with *katG* mutations.
[e] Both isolates have *katG* mutations.

an *inhA* promoter mutation. The INH MICs for four strains with both *inhA* ORF and promoter mutations, one strain with only an ORF mutation (isolate 2), and one strain with only a promoter mutation (isolate 4) were 8 μg/ml. At least one example of each of the three InhA substitutions identified in this study is represented in this group. In the case of the Ile 21→Thr substitution, this change occurred in conjunction with a point mutation within the *inhA* promoter, resulting in the replacement of guanine with thymine at position −17. This contrasts with two strains resistant to 32 μg of INH per ml that had the same *inhA* ORF mutation but in combination with the −15 promoter mutation. The INH MIC for one isolate (isolate 35) with a Ser 94→Ala substitution in InhA and a Glu 195→Lys substitution in KatG was 1 μg/ml. No INH resistance-associated mutations were identified in four INH-resistant isolates.

TABLE 4. INH MICs for and mutations in the *katG* and *inhA* loci of 41 clinical *M. tuberculosis* isolates

INH MIC (μg/ml)	No. of isolates	No. of isolates with mutations in[a]:					No. of isolates with no mutation
		katG only	*inhA*			*katG* and *inhA* promoter	
			ORF only	Promoter only	ORF and promoter		
>32	12	4	0	1	2	5[e]	0
32	5	5	0	0	0	0	0
16	0	0	0	0	0	0	0
8	6	0	1	1[d]	4	0	0
4	3	0	0	3	0	0	0
2	13	0	0	11[d]	0	0	2[f]
1	1	1[b]	0	0	0	0	0
0.5	1	0	0	0	0	0	1[f]
0.25	0	0	0	0	0	0	0
<0.25	0	0	0	0	0	0	0
Total	41	10[c]	1	16	6	5	3

[a] Exclusive of mutations in *ethA* and silent mutations.
[b] Isolate also has an *inhA* ORF mutation.
[c] Includes seven isolates with *ethA* mutations.
[d] Includes one isolate with an *ethA* mutation.
[e] Includes four isolates with *ethA* mutations.
[f] Includes one isolate with an *ethA* mutation.

DISCUSSION

Our finding of 15 different mutations among 15 *ethA* mutants combined with the 9 previously described mutations suggests that a high degree of genetic diversity occurs within the *ethA* genes of ETH-resistant *M. tuberculosis* isolates. The 15 *ethA* mutants we describe represent 10 spoligotype patterns, including clusters of two and five isolates. All members of the five-isolate cluster had single-nucleotide frameshift mutations. These results indicate that *ethA* mutations appear to be widely disbursed across the structural gene, with no single nucleotide or codon predominating. This distribution contrasts with the situation seen in *katG*, where the majority of the mutations occur at codon 315, with Ser 315→Thr being the most prevalent. The predominance of the codon 315 mutations has been explained by the need for the cell to maintain a minimum basal level of catalase-peroxidase activity to protect against organic peroxides. Alterations that reduce KatG activity below this critical level would be lethal, and changes that lead to little or no reduction in enzyme activity would result in little or no decrease in INH susceptibility.

The fact that no such well-adapted *ethA* mutation has emerged in the ETH-resistant bacilli investigated suggests the existence of one or more enzymes with functional redundancy to EthA. In fact, the genome of *M. tuberculosis* possibly encodes more than 30 monooxygenases (4). The proliferation of such enzymes in *M. tuberculosis* may have evolved as a protective mechanism against various xenobiotic substances (10). The exact role of EthA is not known, but the gene is highly conserved throughout the genus, suggesting it serves an important function (4). Diminution, or loss, of EthA activity would thus be expected to have a deleterious effect on the cell. Given the proliferation of EthA homologs, it seems likely that one or more of these enzymes may be capable of compensating for a loss of EthA activity.

Clearly further study is needed to substantiate the association between *ethA* mutations and ETH resistance and to establish the extent of genetic diversity in this gene. Should the initial finding that a wide array of mutations occur in ETH-resistant strains be verified by future investigation, such a phenomenon would resemble that seen in the *pncA* gene of *M. tuberculosis* strains resistant to PZA (20). This gene encodes pyrazinamidase, the enzyme responsible for the conversion of the PZA into its metabolically active derivative pyrazinoic acid (26). ETH, INH, and PZA are all nicotinamide analogs, and all three drugs rely on fortuitous enzymatic conversion to their respective active metabolites.

The predominance of a single, well-adapted mutation in the *katG* gene of high-level INH-resistant strains reflects this enzyme's critical function of detoxifying reactive oxygen species. Under those rare circumstances in which KatG expression is completely lost, this loss occurs in conjunction with a mutation in the *ahpC* promoter that up-regulates expression of AhpC, an enzyme also involved in antioxidant defense (27). In contrast, PZA-resistant strains display a wide diversity, both in number and spatial distribution, of *pncA* mutations, and no particular mutation predominates. The *ethA* genes of high-

level ETH-resistant strains appear to possess a similar degree of genetic diversity, and no evidence of selective pressure favoring a particular mutation has emerged.

While all of the *ethA* mutants identified were resistant to ≥50 μg of ETH per ml, together they accounted for only 15 (52%) of the 29 isolates displaying that phenotype. An *inhA* missense mutation was found in half of the remaining 14 isolates. Two isolates (isolates 1 and 7) with a shared spoligotype pattern had mutations in adjacent nucleotides of *inhA* codon 21 that resulted in different amino acid substitutions. These two isolates have very different levels of INH resistance. The higher INH MIC for isolate 1 may result from the replacement of an aliphatic isoleucine residue with a weakly polar hydroxyl-containing threonine residue. Such a replacement can produce a greater disruption of InhA structure than is produced when an aliphatic valine residue is substituted, as in isolate 7. X-ray crystallography of InhA has shown that Ile 21 is located in the NADH binding site (11). The fact that no similar disparity was seen in ETH MICs may result from subtle differences in drug-target interactions between ETH, INH, and the InhA-NADH complex. Alternatively, differences in ETH resistance between the two mutants may have gone undetected because they occur at concentrations >200 μg/ml. An Ile 21→Thr substitution occurred in an unrelated strain from Brazil (isolate 14) that was also resistant to >200 μg of ETH per ml and >32 μg of INH per ml, suggesting that the phenotype associated with that particular mutation is consistent across strains. The phenotype associated with the Ile 21→Val substitution also recurred in a second strain (isolate 6); however, this strain differed from the matched pair by one spacer.

InhA structural gene mutations were far more prevalent in this study than in previous investigations. This inconsistency presumably reflects the different criteria used for selecting the study specimens. We selected our isolates on the basis of ETH resistance, whereas in previous investigations, isolates were selected on the basis of INH resistance (21, 22, 24).

We identified a Ser 94→Ala mutation in the *inhA* structural gene of three strains (isolates 2, 20, and 35). This mutation was first described in the seminal paper identifying InhA as the target of ETH and INH (1). Curiously, we are not aware of any prior report describing the originally identified Ser 94→Ala alteration in clinical isolates. In one strain (isolate 2), this was the only mutation identified, while in the others, it occurred in combination with either a *katG* (isolate 35) or an *inhA* promoter mutation (isolate 20). The resistance phenotypes of these strains differed dramatically. The inconsistency of these results is difficult to reconcile but suggests the involvement of other, strain-specific factors.

An *inhA* promoter mutation was identified in 15 isolates with wild-type *ethA* and *inhA* structural genes. The ETH MICs for eight of these isolates were in the range of 10 to 25 μg/ml, a moderate increase in ETH resistance that is consistent with a drug titration mechanism. Four of the 15 promoter mutants were resistant to >200 μg of ETH per ml, and the MIC for 2 mutants each was100 μg/ml. It is highly improbable that the promoter mutation alone can account for the high-level ETH resistance seen in those isolates. This assertion is supported by the fact that the INH MICs for five of these strains were ≤4 μg/ml. Were the promoter mutation alone responsible for the high-level ETH resistance seen in these strains, we would expect a concomitant and proportional increase in INH resistance.

A more plausible explanation for the high-level ETH resistance of those strains is that other, ETH-specific mechanisms of resistance are involved. Expression of EthA is under the negative regulatory control of the protein repressor EthR. An increase in EthR expression would then down-regulate *ethA*, ultimately leading to less drug activation and increased resistance to ETH. Hyperexpression of EthR has been experimentally proven to cause ETH resistance (4, 10) and could therefore explain the highly ETH-resistant phenotype of the six strains with only an *inhA* promoter mutation. How EthR production is controlled and to what stimuli it responds are unknown. The potentially important involvement of *ethR* in clinical ETH resistance shows the need for additional studies to determine which factors mediate EthR production.

The MABA method proved itself a very useful research tool for correlating specific mutations in ETH and INH drug resistance markers with relative resistance phenotypes. The INH MICs obtained were highly reproducible between tests. Establishing a precise end point for ETH was somewhat technically challenging because of "trailing" effect, but there was good reproducibility between replicates. The different end point characteristics of ETH and INH presumably reflect the in vitro bactericidal potency of the two drugs: INH is considered to be bactericidal at or near its MIC, while ETH is bactericidal at concentrations 2 to 4 times its MIC (13). The MICs we report here are specific to the MABA method, and we caution the reader against extrapolating these results to other drug susceptibility testing methods.

In summary, our finding of *ethA* mutations in 52% of clinical isolates for which ETH MICs were ≥50 μg/ml provides substantial new evidence confirming the role of this gene in ETH resistance. As expected, mutations in *ethA* had no detectable association with INH resistance. The level of INH resistance in the study isolates was explainable by and consistent with mutations in *katG* and *inhA*. Twenty-four percent of the high-level ETH-resistant strains had mutations in the *inhA* structural gene. With the exception of Ser 94→Ala, these mutations always occurred in combination with *inhA* promoter mutations. Only an *inhA* promoter mutation was identified in approximately a third of the isolates. The majority of those isolates displayed intermediate levels of ETH and INH resistance. Six of the promoter mutants were resistant to ≥100 μg of ETH per ml, a high level of resistance that we believe is not exclusively attributable to the promoter mutations but rather results from another mechanism. Because the regulatory protein EthR mediates *ethA* expression, it seems reasonable that activator and target mutations alone cannot account for all observed high-level ETH resistance. While mechanisms of ETH resistance exclusive of the *ethAR* loci cannot be discounted, it seems probable that mutation in *ethA* is not the only ETH resistance-associated mechanism involving these loci. While the identification of the *ethAR* loci has contributed greatly to the understanding of ETH resistance, additional investigation is clearly needed.

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