

Characterization of the *katG* and *inhA* Genes of Isoniazid-Resistant Clinical Isolates of *Mycobacterium tuberculosis*

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Received 12 June 1995/Returned for modification 24 July 1995/Accepted 5 September 1995

Resistance to isoniazid in *Mycobacterium tuberculosis* has been associated with mutations in genes encoding the mycobacterial catalase-peroxidase (*katG*) and the InhA protein (*inhA*). Among the 26 isoniazid-resistant clinical isolates evaluated in this study, mutations in putative *inhA* regulatory sequences were identified in 2 catalase-positive isolates, *katG* gene alterations were detected in 20 strains, and 4 isolates had wild-type *katG* and *inhA* genes. Mutations in the *katG* gene were detected in all 11 catalase-negative isolates: one frameshift insertion, two partial gene deletions, and nine different missense mutations were identified. An arginine-to-leucine substitution at position 463 was detected in nine catalase-positive isolates. However, site-directed mutagenesis experiments demonstrated that the presence of a leucine at codon 463 did not alter the activity of the *M. tuberculosis* catalase-peroxidase and did not affect the capacity of this enzyme to restore isoniazid susceptibility to isoniazid-resistant, KatG-defective *Mycobacterium smegmatis* BH1 cells. These studies further support the association between *katG* and *inhA* gene mutations and isoniazid resistance in *M. tuberculosis*, while also suggesting that other undefined mechanisms of isoniazid resistance exist.

Despite major advances in prevention and treatment of the ancient scourge tuberculosis in the past century, this disease remains a global tragedy with enormous public health and economic implications. Tuberculosis accounts for about 7% of all deaths in developing countries and 26% of avoidable adult deaths (2). The World Health Organization estimates that 8 million new cases and 3 million deaths worldwide result each year from tuberculosis and has projected that 30 million people will die from this disease in the next decade (36). Confounding these bleak statistics has been the emergence of the often fatal multiple-drug-resistant tuberculosis. Since multiple-drug-resistant tuberculosis bacilli are unsusceptible to most current antituberculosis medications, disease management of individual patients is difficult, and consequently, their infectiousness is prolonged. Therefore, multiple-drug-resistant tuberculosis has limited the effectiveness of tuberculosis control programs by complicating both treatment and prevention protocols (5, 7, 8).

The continuing global threat of tuberculosis has emphasized the urgent need to design more effective diagnostic procedures and to develop improved antituberculosis therapies. The molecular definition of antituberculosis drug targets and resistance mechanisms should facilitate the rational development of improved diagnostics and antimycobacterial medications. Currently, the most effective and specific agent for the treatment of drug-susceptible *Mycobacterium tuberculosis* is isoniazid (INH). Although INH has been a central component of tuberculosis treatment protocols for several decades and drug-resistant strains have been isolated since the 1950s, genetic markers associated with the resistant phenotype have only recently been elucidated. Zhang et al. have shown that the *katG* gene, which encodes the catalase-peroxidase enzyme, is absent from some INH-resistant *M. tuberculosis* strains and that transformation of the wild-type *katG* gene into *katG*-deleted myco-

bacterial strains restores INH susceptibility (37, 38). Moreover, recent studies have demonstrated that a missense mutation in the *inhA* gene, which encodes an enoyl acyl reductase involved in mycolic acid biosynthesis, is associated with reduced susceptibility to INH in mycobacteria (1, 6). Overexpression of the wild-type InhA protein also conferred increased INH resistance to transformed *Mycobacterium smegmatis* and *Mycobacterium bovis*.

In this study, we have extended the observations from other studies of the genetic markers of INH resistance to 26 clinical isolates of *M. tuberculosis* from the United States and South Korea. We have used single-stranded conformational polymorphism (SSCP), PCR, and nucleotide sequence analyses to detect mutations in the *katG* and *inhA* genes of these clinical isolates (21). These findings have been correlated with results of catalase activity assays and antibiotic susceptibility testing. In addition, we have used site-directed mutagenesis techniques to evaluate the effect of the most frequently observed mutation in INH-resistant clinical isolates on both peroxidase activity and INH resistance.

MATERIALS AND METHODS

Characterization of *M. tuberculosis* clinical isolates. The bacterial isolates were obtained from the following locations: 6 from the Walter Reed Medical Center, Washington, D.C.; 1 from the National Institutes of Health Medical Center, Bethesda, Md.; and 19 from the Korean National Institute of Tuberculosis, Seoul, South Korea. The drug susceptibility profiles were determined by the absolute concentration method (32). Resistance was defined as survival of the tubercle bacilli at the following drug concentrations: INH, 0.2 µg/ml; streptomycin, 4 µg/ml; rifampin, 32 µg/ml; ethionamide, 20 µg/ml; pyrazinamide, 100 µg/ml; kanamycin, 50 µg/ml; ethambutol, 3 µg/ml. Whole-cell qualitative catalase assays were performed on each of the isolates. The catalase activities were recorded as negative (no O₂ release) or positive (visible O₂ bubbling).

Preparation of genomic mycobacterial DNA. The preparation of genomic DNA from mycobacterial strains has been described elsewhere (26). Cell lysates from the Korean isolates which were utilized in PCR studies were prepared by extraction with chloroform (19).

PCR-SSCP analyses. The protocols and reaction components utilized for the PCR-SSCP analyses have been described previously (19, 24). In this study, the PCR-SSCP reaction mixtures were subjected to 40 cycles of PCR amplification (1 min at 95°C, 1 min at 60°C, 1 min at 72°C). The PCR-SSCP products were electrophoresed at 3 to 4 W for 16 to 18 h into a 0.5× MDE gel by using a 0.6×

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TBE buffer (60 mM Tris, 54 mM boric acid, 0.6 mM EDTA [pH 8.4]). PCR products showing aberrant SSCP patterns were purified by using the Wizard purification procedure (Promega, Madison, Wis.) and then cloned by using the TA cloning system (Invitrogen, San Diego, Calif.). The cloned products were analyzed by using the *Bst*I double-stranded DNA sequencing protocols (Bio-Rad, Hercules, Calif.). At least two independent PCR clones were sequenced for each genetic analysis. To verify the presence of a mutational event, the nucleotide sequence of a PCR clone derived from a clinical isolate was compared with the DNA sequence of an *M. tuberculosis* strain H37Rv clone generated with the same PCR primer set. The specific primers utilized in this study have been listed previously (19). The *katG* primers for both PCR and SSCP analyses were designed from the published *M. tuberculosis katG* gene sequence (12). The *inhA* primers were generated on the basis of the published *M. tuberculosis inhA* gene sequence (1) and the *M. bovis* (BCG) *inhA* gene sequence generously provided by Des Collins of AgResearch, Wallaceville Animal Research Center, Upper Hutt, New Zealand.

Site-directed mutagenesis of the *M. tuberculosis katG* gene. PCR primers were designed to amplify a 2.9-kb *EcoRV-KpnI* DNA fragment which contained the *M. tuberculosis katG* gene and to allow cloning into the *XbaI* and *KpnI* sites of the mycobacterial vector pMD31 (4, 12, 38). The sequence of the 5' primer utilized in the PCRs (which has a *XbaI* restriction site tail) was 5'-ACTCTAGATGAT TGTTTCGATATCCGAC-3', and the 3' primer sequence (with a *KpnI* restriction site tail) was 5'-ATGGTACCGCGACCTTCGT-3'. *M. tuberculosis* H37Rv genomic DNA (100 ng) was used as the PCR template. The PCR mixtures were subjected to 35 cycles of amplification (1 min at 94°C, 1 min at 55°C, 2.5 min at 72°C). The expected 2.9-kb product was not detected after PCR amplification under standard reaction conditions. However, when glycerol was added to the reaction mixture to a final concentration of 20%, the full-length 2.9-kb PCR product was amplified. Glycerol has previously been shown to be a cosolvent which can enhance PCR amplification (28).

The amplified *katG* products were initially cloned, using the TA cloning system (Invitrogen). The 2.9-kb insert fragment from these clones was digested with *XbaI* and *KpnI* and then ligated into the mycobacterial plasmid vector pMD31, using standard protocols. The resulting ligation mixture was then transformed into competent *Escherichia coli* DH5 α cells. Recombinant pMD31:*katG* clones were identified by restriction analyses of purified plasmid DNA. Because of the potential infidelity of PCR amplifications, the *katG* inserts of several clones were analyzed by SSCP as described above. The clone ZM1 was chosen for further study because this clone had only a single silent mutation within its *katG* insert.

The Clontech (Palo Alto, Calif.) transformer site-directed mutagenesis kit was used to mutate *katG* codon 463 in clone ZM1. Briefly, this protocol involved simultaneously annealing to one strand of denatured plasmid DNA two primers: a primer which introduced the desired codon 463 mutation and a primer which eliminated the unique *Hind*III site in the pMD31 vector. The restriction site mutation allowed the selection of mutated from nonmutated plasmids because the mutated DNA lacked the *Hind*III restriction site and therefore was resistant to digestion. After standard DNA elongation, ligation, and primary digestion with *Hind*III, the plasmid pool was transformed into the *E. coli* strain, BMH 71-18 (*mutS*), which is defective in mismatched repair. The transformants were pooled, plasmid DNA was prepared, and the isolated DNA was subjected to a second round of *Hind*III digestion. This pool of plasmid DNA that was enriched for the *Hind*III restriction site modification was retransformed into BMH 71-18. The presence of codon 463 mutations in the resulting transformants was demonstrated by using the double-stranded sequencing protocols described above.

Preparation and characterization of *katG* codon 463 mutants. The pMD31:*katG* clone (ZM1) and three codon 463 mutants were electroporated into the highly INH-resistant, catalase-negative *M. smegmatis* BH1. Characterization of the *M. smegmatis* BH1 cells and protocols for the preparation of competent mycobacteria have been described previously (10, 14). Initially, plasmid DNA (2.5 μ g) was mixed with 50 μ l of competent cells, and the mixture was placed on ice for 1 min. The Gene Pulser apparatus (Bio-Rad) was then set to 1,250 V, 25 mF, and 1,000 Ω . The mixture was transferred to a 0.2-cm-diameter cuvette and pulsed for 10 ms. The cells were subsequently suspended in 1 ml of Middlebrook 7H9 broth containing oleic acid-albumin-glucose complex and 0.05% Tween 80 and incubated at 37°C for 3 h. *M. smegmatis* BH1 transformants were selected on Middlebrook 7H9 agar containing 50 μ g of kanamycin per ml.

Solid-phase testing and broth MIC analyses were used to evaluate the drug susceptibility of the *M. smegmatis* BH1 transformants. The solid-phase drug testing consisted of streaking recombinant *M. smegmatis* BH1 cells on Middlebrook 7H9 plates containing 50 μ g of kanamycin per ml and either 0, 5, 10, 50, or 100 μ g of INH per ml. The capacity to restore INH susceptibility was determined by examination of the plates for the presence or absence of cell growth after 24 h of incubation at 37°C. The broth MIC measurements were done by using established protocols. Recombinant *M. smegmatis* BH1 cells (10^3) were inoculated into 5 ml of Middlebrook 7H9 broth containing oleic acid-albumin-glucose complex, 0.05% Tween 80, 50 μ g of kanamycin per ml, and 0, 1.6, 3.1, 6.2, 12, 25, 50, 100, or 200 μ g of INH per ml. Cell growth was evaluated by measuring the A_{600} after 48 h of incubation at 37°C.

The peroxidase activities of the *katG M. smegmatis* BH1 recombinants were determined by using a whole-cell assay. Briefly, 2×10^8 log-phase cells were centrifuged and resuspended in 1 ml of a 33 mM phosphate-buffered solution (pH 6) containing 4.1 mM 2,4-dichlorophenol, 0.67 mM 4-aminoantipyrine, and

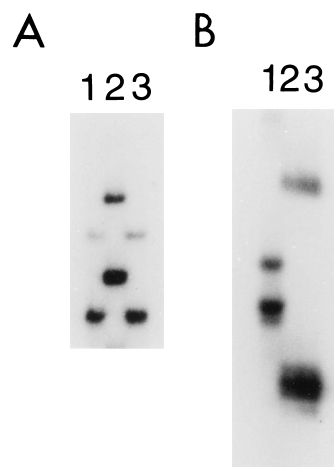


FIG. 1. SSCP analyses of *M. tuberculosis katG* gene fragments. The SSCP profiles of primer set 1 (A) and primer set 2 (B) PCR products amplified from the genomic DNA of strain 28 (lanes 1), strain 32 (lanes 2), and *M. tuberculosis* H37Rv (lanes 3) are shown. The PCR products in lanes 2 and 3 from primer set 2 have identical electrophoretic mobilities and SSCP profiles.

2.9 mM H₂O₂. After a 10-min incubation at 37°C, the cells were pelleted by centrifugation, and the peroxidase activity was determined by measuring the A_{500} (10). To evaluate the relative levels of *katG* expression in the transformants, *M. smegmatis* BH1 sonicates were analyzed by immunoblot assays, using an absorbed polyclonal antibody enriched for reactivity to mycobacterial catalase-peroxidases, as described previously (24).

RESULTS

SSCP analyses. Gene fragments containing mutations in the *katG* and *inhA* genes were initially identified by using SSCP analyses. For these analyses, each isolate was evaluated with the 11 different *katG* and 4 different *inhA* primer sets (19). PCR products with aberrant SSCP profiles were identified by comparison with the control *M. tuberculosis* strain, H37Rv, SSCP results. Figure 1 shows an example of an aberrant SSCP pattern at two different locations within the *katG* gene. The *katG* primer set 1 PCR product shown in lane 2 of Fig. 1A and the primer set 2 PCR product shown in lane 1 of Fig. 1B clearly have SSCP profiles different from that of the control H37Rv (lanes 3, Fig. 1). Subsequent nucleotide sequence analyses of clones derived from these PCR products confirmed that these DNA fragments had *katG* mutations.

Catalase activities and drug resistance levels for clinical isolates. The results of catalase activity assays, drug susceptibility testing, and genetic analyses for the *M. tuberculosis* clinical isolates are summarized in Table 1. The first 11 strains listed are catalase negative, and the remaining 15 isolates have catalase activity. It is apparent from Table 1 that the catalase-negative isolates generally have high drug resistance levels for INH. For six of these strains, INH MICs are at least 25 μ g/ml. In contrast, only 1 of 15 catalase-positive strains (strain 30) is highly resistant to INH.

Characterization of catalase-negative isolates. Several *katG* gene mutations were detected in the catalase-negative clinical isolates and are summarized in Fig. 2A. Alterations in the *katG* gene were detected in all 11 catalase-negative, INH-resistant strains examined. Frameshift mutations were identified in three highly resistant strains. Strain 4 had a single base insertion in codon 521 of the *katG* coding sequence. Small deletions which perturbed the *katG* reading frame were detected in two other strains—strain 31 has an 8-bp deletion from codon 217 to

TABLE 1. *katG* and *inhA* mutations associated with INH resistance in clinical isolates of *M. tuberculosis*

Strain	Drug resistance ^a	MIC (μg/ml)	Gene	Alteration (codon) ^b
Catalase negative				
4	I	125	<i>katG</i>	Insertion
20	I, R, P	50	<i>katG</i>	A→P (717)
28	I	50	<i>katG</i>	H→Q (108)
31	I	50	<i>katG</i>	Deletion
32	I	50	<i>katG</i>	D→E (63)
10	I	25	<i>katG</i>	Deletion
74	I	2	<i>katG</i>	I→T (335)
73	I	2	<i>katG</i>	S→T (315); R→L (463)
5	I	1	<i>katG</i>	A→S (350)
9	I	1	<i>katG</i>	G→S (629)
2	I	1	<i>katG</i>	T→R (262)
Catalase positive				
30	I	50		
68	I	2	<i>katG</i>	R→L (463)
69	I	2		
6	I	1	<i>katG</i>	R→L (463)
7	I	1	<i>katG</i>	R→L (463)
8	I	1	<i>inhA</i>	g→t
14	I, S	1		
15	I, S	1	<i>inhA</i>	t→g
16	I, S	1	<i>katG</i>	R→L (463)
17	I, S	1	<i>katG</i>	R→L (463)
18	I, R, S, E	1	<i>katG</i>	R→L (463)
19	I, R, S, K	1		
24	I, R, S, E, K	1	<i>katG</i>	R→L (463)
25	I, R, E, K	1	<i>katG</i>	R→L (463)
26	I, R, E, K	1	<i>katG</i>	R→L (463)

^a I, INH; R, rifampin; S, streptomycin; E, ethambutol; K, kanamycin; P, *para*-aminosalicylic acid.

^b The numbers in parentheses represent the mutated *katG* codons.

codon 219, and strain 10 has a 22-bp deletion from codon 289 to codon 296.

Missense mutations in the *katG* gene were identified in the other catalase-negative clinical isolates. The following amino acid substitutions were detected: Thr-262 to Arg (strain 2), Ala-350 to Ser (strain 5), Gly-629 to Ser (strain 9), Ala-717 to Pro (strain 20), His-108 to Gln (strain 28), Asp-63 to Glu (strain 32), Ser-315 to Thr and Arg-463 to Leu (strain 73), and Ile-335 to Thr (strain 74). It is of interest that the Asp-63, His-108, Thr-262, Ser-315, Ala-350, and Gly-629 substitutions occurred in amino acid residues that are conserved among the *Mycobacterium intracellulare*, *E. coli*, and *Salmonella typhimurium* KatG proteins (17, 20, 30).

Primary sequence comparisons suggest that the mycobacterial KatG protein and the yeast cytochrome *c* peroxidase are members of the same plant peroxide superfamily (33). The mycobacterial KatG protein consists of two related domains which have strong similarity to the yeast cytochrome *c* peroxidase. The putative amino-terminal active-site regions (*M. tuberculosis* amino acids 94 to 154 and 257 to 276) are 45% identical, and several residues which may be involved in substrate binding or catalysis (Arg-104, His-108, His-270, and His-276) are conserved. Therefore, the structural implications of missense mutations in mycobacterial KatG proteins can be predicted from the known crystal structure of the yeast cytochrome *c* peroxidase enzyme (9, 33, 34). On the basis of this predicted structure, the His-108, Thr-262, Ser-315, Ile-335, and Ala-350 residues are located in helical regions of the *M. tuber-*

culosis KatG protein. Consequently, mutations at these sites should alter protein structure and affect the activity of the KatG enzyme.

Characterization of catalase-positive isolates. Perturbations in the *katG* genes of nine catalase-positive isolates were also detected. All of these isolates with *katG* mutations had a G-to-T transversion which converts Arg-463 to Leu. Since this mutation was detected in a number of catalase-positive isolates, the Arg-to-Leu substitution at codon 463 clearly does not significantly affect the catalase activity of the KatG protein.

Alterations in the *inhA* operon were identified in two of the catalase-positive and in none of the catalase-negative clinical isolates. Extensive SSCP analyses with three overlapping *inhA* gene primer sets did not detect any mutations in the *inhA* coding sequence of these isolates. However, further analyses demonstrated that two strains (strains 8 and 15) had mutations in putative *inhA* regulatory sequences (Table 1 and Fig. 2B). The *inhA* operon consists of two open reading frames: *orf1* encodes a 29-kDa protein, and *orf2* encodes the 32-kDa InhA protein (1). In both of these strains, alterations were identified proximal to the translation start codon of *orf1*. A T-to-G transversion was detected 8 bp upstream of the *orf1* initiation codon in strain 15 and adjacent to a predicted ribosome binding site for the *inhA* operon. A G-to-T transversion was found 24 bp upstream from the translation initiation site of *orf1* in strain 8. Similar transversions in putative *inhA* regulatory sequences have been recently reported in other INH-resistant clinical isolates of *M. tuberculosis* (16). Although the relevance of these genetic alterations has yet to be demonstrated, it is possible that mutations in *inhA* regulatory sequences may cause overexpression of the InhA protein. Increased synthesis of InhA can effectively elevate the levels of the drug target for INH, resulting in a more resistant phenotype (1).

It is interesting to note that neither *katG* nor *inhA* genetic alterations were detected in several clinical isolates, including the highly resistant strain 30. Our failure to identify mutations of known genetic markers in these strains suggests that addi-

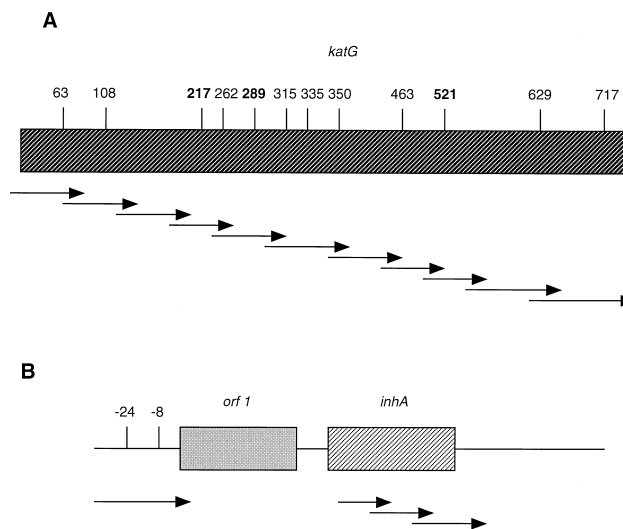


FIG. 2. Summary of mutations detected within the *katG* gene (A) and *inhA* operon (B) and the location of primer sets (arrows) utilized in the SSCP analyses. Sites of missense mutations in the *katG* gene are indicated by codon number in lightface type, and sites where insertions or deletions were detected are shown in boldface type. The *inhA* operon mutations are given as nucleotide base pairs relative to the initiation codon (+1) of the *orf1* gene.

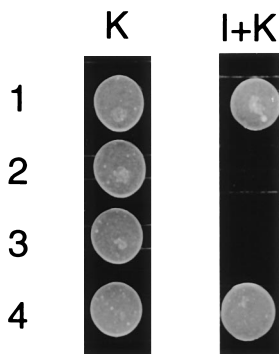


FIG. 3. *M. smegmatis* BH1 cells expressing the *M. tuberculosis* *katG* gene or the *M. tuberculosis* codon 463 mutant *katG* gene have increased susceptibility to INH. *M. smegmatis* BH1 cells were transformed with the following constructs: (1) pMD31 (vector control); (2) pMD31, *M. tuberculosis* *katG* (Arg-463); (3) pMD31, *M. tuberculosis* *katG* codon 463 mutant (Leu-463); or (4) pMD31, irrelevant mycobacterial gene fragment. The transformants were streaked on plates containing 50 μ g of kanamycin per ml (K) or 100 μ g of INH per ml and kanamycin (I+K). The absence or presence of growth was recorded after 24 h of incubation at 37°C.

tional INH resistance mechanisms exist or that certain regulatory defects that affect *katG* or *inhA* expression remain undefined.

Site-directed mutagenesis of *katG* codon 463. The most frequent *katG* gene alteration identified in INH-resistant *M. tuberculosis* clinical isolates is a G-to-T change at codon 463, which results in the substitution of Leu for Arg (3, 9, 19). To evaluate the relative importance of this perturbation, codon 463 of the *M. tuberculosis* *katG* H37Rv gene was changed from an Arg to a Leu by site-directed mutagenesis. Initially, the capacity of this codon 463 mutant to restore INH susceptibility to a catalase-negative, INH-resistant *M. smegmatis* strain, BH1, was assessed by using solid-phase assays. *M. smegmatis* BH1 cells were transformed with the mycobacterial plasmid vector pMD31 containing the *M. tuberculosis* *katG* gene (Arg-463) and with pMD31 containing the *katG* codon 463 mutant (Leu-463). *M. smegmatis* BH1 cells transformed with the vector pMD31 and with pMD31 containing an irrelevant mycobacterial gene fragment served as controls. As seen in Fig. 3, all the transformants grow on Middlebrook 7H9 agar containing kanamycin. However, when these recombinants are streaked onto solid medium containing kanamycin and 100 μ g of INH per ml, only the control *M. smegmatis* BH1 transformants proliferate (Fig. 3, rows 1 and 4). The absence of growth of the *M. smegmatis* BH1 transformants in rows 2 and 3 suggests that expression of either the wild-type or the codon 463 mutant *M. tuberculosis* *KatG* restores INH susceptibility to *M. smegmatis* BH1 cells.

The *M. smegmatis* BH1 transformants were also evaluated for INH susceptibility by using standard broth MIC assays. Consistent with the solid-phase results, the MICs for the control strains exceeded 200 μ g/ml of INH, while the MICs for *M. smegmatis* BH1 transformants expressing the *M. tuberculosis* *KatG* or the codon 463 mutant protein were 10 μ g/ml. Therefore, expression of either the wild-type or the mutant *katG* gene enhanced INH susceptibility in *M. smegmatis* BH1 cells by at least 20-fold.

To assess the relative enzymatic activities of the wild-type and mutant *KatG* proteins, the levels of *katG* expression and the peroxidase activities of the *M. smegmatis* BH1 transformants were determined. Immunoblot assays using a polyclonal antibody enriched for mycobacterial catalase reactivity dem-

onstrated that the levels of expression of wild-type and mutant *KatG* proteins in several *M. smegmatis* BH1 transformants were essentially equivalent. Moreover, no difference in the peroxidase activities of strains expressing wild-type or mutant proteins was detected. However, the peroxidase activities measured in *M. smegmatis* BH1 cells expressing either the wild-type or mutant *KatG* proteins were fivefold higher than for the vector control. These results suggest that the codon 463 Arg-to-Leu substitution does not significantly alter the level of expression of *katG* or the peroxidase activity of the *M. tuberculosis* catalase-peroxidase.

DISCUSSION

Although INH has been a key component of tuberculosis chemotherapeutic regimens for several decades, the mechanism of action of this highly specific and reactive drug remains obscure. Recent reports have suggested that INH or a derivative of INH may act by blocking the synthesis of mycolic acids in drug-susceptible mycobacteria and that this inhibition involves the *inhA* gene product, an enoyl acyl reductase in the mycolic acid biosynthetic pathway (1, 6). Furthermore, the toxicity of the drug seems to be potentiated by its interactions with the mycobacterial catalase-peroxidase (27, 31). In fact, Johnsson et al. have recently shown that oxidation of INH by *KatG* in the presence of *InhA* leads to the inactivation of *InhA* (15). Our studies of INH resistance mechanisms in *M. tuberculosis* clinical isolates support the identification of *inhA* and *katG* as drug targets. Among 26 INH-resistant clinical isolates that we have examined in this study, 20 have *katG* perturbations and 2 have *inhA* regulatory sequence alterations.

It is of interest that neither *katG* nor *inhA* gene mutations were detected in four of the isolates evaluated in these experiments. Heym and coworkers have also reported that a number of clinical isolates had no mutations in these genetic markers of INH resistance (9, 11). Since several of the previously described resistant strains that have wild-type *katG* and *inhA* genes are catalase negative, the expression of *katG* in some strains may be impaired by an undefined regulatory defect. Consistent with this possibility, Rosner and colleagues have shown that *E. coli* *oxyR* mutants (the *oxyR* regulon controls *katG* expression in *E. coli*) have altered susceptibility to INH (22, 23). Alternatively, unknown regulatory defects that affect *InhA* expression may be present in some INH-resistant strains. This class of alteration will be difficult to detect until a reliable assay for measuring *InhA* activity in clinical isolates is developed. It is also possible that other mechanisms of INH resistance exist. This hypothesis is likely, since we have shown that several INH-resistant strains with nonmutated *katG* or *inhA* genes have wild-type catalase activities. To identify new mechanisms of INH resistance, INH-susceptible mycobacterial strains are being transformed with gene libraries prepared from DNA of highly INH-resistant, catalase-positive strains (including strain 30). Genetic analysis of INH-resistant transformants may permit characterization of genes responsible for the resistant phenotype.

Initial observations by Zhang et al. linked INH resistance with complete deletions of the *katG* gene (38). However, several recent studies have found a low frequency of complete *katG* deletions in INH-resistant clinical isolates. For example, we have failed to detect a complete *katG* deletion in any of these 26 resistant isolates. Recently, Wilson and colleagues have demonstrated that integration of a functional *katG* gene into an avirulent *M. bovis* strain restored full virulence (35). This experiment suggested that *KatG* is a virulence factor in *M. bovis* and that the presence of this enzyme in mycobacteria may

enhance persistence in the macrophage and prolong survival of the organism. Therefore, the failure to identify *katG*-deleted isolates in our studies may result from a proliferative disadvantage of these strains within the hostile host environment. It is possible that the absence of *katG* selects against the continued survival of the *katG*-deleted strains.

In this study, small insertions, partial deletions, and nine different missense mutations have been identified in the *katG* genes of INH-resistant *M. tuberculosis* clinical isolates. The consistent correlation between the presence of these reading frame alterations, decreased catalase activity, and elevations of the MIC of INH strengthens the association between *katG* perturbations and INH resistance in isolates with insertions or deletions of the *katG* gene. In contrast, the relevance of specific *katG* missense mutations to drug resistance has generally not been definitively established. It is clear that six of nine substitutions detected, Asp-63, His-108, Thr-262, Ala-349, Ser-315, and Gly-625, occur in residues that are likely to be functionally important because they are conserved among several bacterial hydroperoxidases. Furthermore, comparisons with the known structure of the yeast cytochrome *c* peroxidase suggest that at least five of the mutations identified, His-108, Thr-262, Ser-315, Ile-335, and Ala-350, may have significant structural effects on the *M. tuberculosis* KatG protein (9, 33, 34). Although these predictions strongly suggest that particular mutations are linked to drug resistance, the absence of a consistent genetic background among clinical isolates prevents an absolute delineation of the association between specific changes in the *katG* gene and resistance to INH in these strains. To circumvent this problem, resistant mutants of a reference strain can be isolated and the effect of relevant mutations can be characterized. Using this strategy, we have recently shown in an INH-resistant mutant of H37Rv that substitution of a Pro for a Thr in KatG residue 275 eliminates catalase activity and decreases susceptibility to INH (24). Alternatively, as shown in the codon 463 studies, the effects of mutational events on KatG function can be evaluated by using site-directed mutagenesis. Studies using specific mutagenesis protocols to more definitively identify critical KatG residues are currently in progress.

The most frequent and intriguing amino acid substitution identified in clinical isolates of *M. tuberculosis* is the codon 463 Arg-to-Leu replacement. Some evidence suggests that this substitution may be relevant to drug resistance. Our group and others have detected this change in 20 to 45% of INH-resistant clinical isolates (3, 9, 19). The Arg residue is found only in position 463 of the wild-type *M. tuberculosis* protein, while the more INH-resistant *M. intracellulare* and *Bacillus stearothermophilus* strains have a Leu at an equivalent residue (18, 20). Moreover, the only *katG* gene alteration detected in one resistant mutant of H37Rv resulted in the same codon 463 Arg-to-Leu change (24). In contrast, the site-directed mutagenesis experiments presented in this study demonstrated that the Arg-to-Leu change had no significant effect on the peroxidase activity or the drug susceptibility of recombinant *M. smegmatis* BH1 cells. Furthermore, we have detected this substitution in 5 of 24 INH-susceptible *M. tuberculosis* clinical isolates (25). Whether this codon 463 substitution is a frequent polymorphic event or is relevant to drug resistance needs further clarification. Repeating the site-directed mutagenesis studies in a slowly growing *katG*-deficient mycobacterial strain may be required to more clearly define the effect of this mutation.

In summary, we have detected multiple *katG* and *inhA* gene mutations in clinical isolates that may be associated with INH resistance. We initially speculated that rapid diagnostic tests for identifying INH-resistant bacilli could be developed on the basis of the detection of these alterations in drug target genes.

The consistency of *rpoB* mutations in rifampin-resistant isolates suggests that rapid molecular tests for identifying reduced susceptibility to rifampin will be feasible (29). However, in our studies and other recent reports, at least 20 *katG* mutations (encompassing the entire *katG* gene) and 6 *inhA* alterations have been identified (3, 9, 16, 19). Because of the number of perturbations identified and the variable locations of the *katG* alterations, it is unlikely that useful amplification-based diagnostic methods will be available in the near future for rapidly detecting INH-resistant clinical isolates. Given this circumstance, the continued development of alternative diagnostic approaches for identifying drug-resistant mycobacteria, such as mycobacteriophage reporter assays, is clearly warranted (13).

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