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Molecular Mechanisms of Isoniazid Resistance in *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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Genetic and biochemical studies have suggested a link between reduced catalase activity and resistance to isoniazid in *Mycobacterium tuberculosis*. In this study, we examined the molecular mechanisms of resistance to isoniazid with six in vitro mutants of the *M. tuberculosis* complex (*Mycobacterium bovis* and *M. tuberculosis*). Five of six mutants resistant to isoniazid were negative by catalase assays. Immunoblot analyses using a polyclonal antibody against the *katG* gene product (catalase-peroxidase) demonstrated that the enzyme is not produced in four of these isoniazid-resistant strains. A complete deletion of the *katG* gene was detected in only one of these isoniazid-resistant *M. tuberculosis* complex strains by Southern blot analyses. In two other resistant strains, partial deletions of the *katG* gene were identified. A point mutation which resulted in the insertion of a termination codon in the *katG* coding sequence caused a catalase-negative phenotype in a fourth strain. Of the two resistant strains which produce the enzyme, one was shown to be negative by a catalase assay. Single-stranded conformational polymorphism and DNA sequence analyses identified a mutation in the *katG* gene of this strain which may contribute to reduced enzymatic activity and subsequent isoniazid resistance. These data demonstrate that genetic alterations to the *katG* gene other than complete deletions are prevalent and may contribute significantly to the number of cases of isoniazid-resistant tuberculosis.

Primarily because of the increase in the number of AIDS cases and worsening urban social and economic conditions, tuberculosis has resurged in the United States in the past decade (2, 3). In particular, recent outbreaks of multidrug-resistant tuberculosis, with mortality rates of 72 to 89% and median intervals from diagnosis to death of 4 to 16 weeks, have elevated public health concerns about the reemergence and control of tuberculosis (5, 14). The increasing prevalence of multidrug-resistant tuberculosis has dramatically demonstrated that the development of a more efficacious vaccine as well as improved rapid diagnostic protocols and therapeutic regimens is required to meet the challenge of tuberculosis in the 1990s.

An improved understanding of the targets of drug interactions and the mechanisms of resistance should facilitate the design of more efficacious drugs and better procedures to detect drug-resistant organisms. Isoniazid is currently among the most effective and inexpensive antituberculosis medications and is an essential component of multiple-drug chemotherapeutic regimens for the treatment of tuberculosis. Recent molecular and genetic studies have identified at least two possible mechanisms of isoniazid resistance. Banerjee et al. (1) have shown that mutations within the mycobacterial *inhA* gene can confer resistance to isoniazid and ethionamide. Cell-free assays indicate that the *inhA* gene product may be involved in mycolic acid biosynthesis. Zhang et al. (27, 28) have shown that deletions of the *katG* gene, which encodes the mycobacterial catalase-peroxidase enzyme, can confer resistance to isoniazid in *Mycobacterium tuberculosis*. Moreover, transfer and expression of the wild-type *M. tuberculosis katG* gene into isoniazid-resistant and *katG* mutant strains of *M. tuberculosis* and *Mycobacterium smegmatis* can increase sensitivity to isoniazid. These genetic data are consistent with earlier reports by Middlebrook et al. (9, 10), which demonstrated that a significant proportion of isoniazid-resistant strains of *M. tuberculosis* have reduced catalase activity.

In this study, we examined the molecular mechanisms of isoniazid resistance in six in vitro mutants of *M. tuberculosis* complex strains. Using DNA hybridization techniques, single-stranded conformational polymorphism (SSCP), and DNA sequence analyses, we assayed for *katG* deletions or mutations in these six resistant strains. The presence or absence of a functional *katG* gene product was evaluated by immunoblot and catalase assays. Although our results confirm that a complete deletion in the *katG* gene may be associated with resistance to isoniazid, this does not appear to be the most common perturbation of the gene. Data presented in this study demonstrate that more subtle mutations in the *katG* gene can result in the absence of a functional gene product.

MATERIALS AND METHODS

Bacterial strains. All of the *Mycobacterium bovis* and *M. tuberculosis* strains used in this study were obtained from the American Type Culture Collection (Rockville, Md.). A list of the strains used, strain histories, and drug resistance patterns are shown in Table 1. The drug-resistant variants were originally selected in a single-step process at the Trudeau Institute, Saranac Lake, New York. Briefly, individual cultures were plated onto a single agar plate containing a gradient of isoniazid. Initial selection was based upon a relative level of resistance. Drug susceptibility testing of these strains was then performed to better define the level of resistance for each isolate. These results were recently confirmed by the MIC method at the Korean Institute of Tuberculosis in Seoul, South Korea. Mycobacterial culture techniques, preparation of mycobacterial sonicates, and DNA isolation procedures have been described previously (13, 17).

Immunoblot analyses. A polyclonal antibody enriched for anticatalase activity was produced by affinity chromatography. Briefly, the recombinant *Mycobacterium intracellulare* catalase-peroxidase enzyme was overexpressed in *Escherichia coli*, purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroeluted as described previously (11). The purified protein was coupled to an activated Sepharose 4B column with reagents and protocols supplied by the manufacturer (Pharmacia, Piscataway, N.J.). Hyperimmune anti-*M. intracellulare* burro serum was then absorbed to the column, which was then washed in 10 mM Tris (pH 8.0)–0.5 M NaCl. Nonspecific antibodies were eluted

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TABLE 1. Bacterial strains

Strain	Drug resistance(s)[a]	Strain history	INH MIC (μg/ml)
ATCC 25177	None	H37Ra (avirulent)	0.02
ATCC 27294	None	H37Rv (virulent)	0.02
ATCC 35822	INH	in vitro mutant of H37Rv	50
ATCC 35823	INH, SM	in vitro mutant of H37Rv	50
ATCC 35825	INH, SM, PAS	in vitro mutant of H37Rv	5
ATCC 35835	INH	in vitro mutant of H37Ra	50
ATCC 35720	None	Virulent *M. bovis* Ravenal	0.02
ATCC 35727	INH	in vitro mutant of 35720	50
ATCC 35735	None	*M. bovis* BCG Montreal	0.02
ATCC 35747	INH	in vitro mutant of 35735	50

[a] INH, isoniazid; SM, streptomycin; PAS, *para*-amino-salicylic acid.

with 10 mM Tris (pH 8.0)–2.0 M NaCl. Next, polyclonal antibodies reactive against the catalase-peroxidase protein were eluted from the column with 4 M $MgCl_2$. The protocols for immunoblot detection have been outlined previously (17). Catalase-reactive antibodies were detected by using an anti-horse alkaline phosphatase conjugate (Sigma Chemicals, St. Louis, Mo.) and alkaline phosphatase substrates (Gibco/BRL, Gaithersburg, Md.).

Catalase assays. In vivo whole-cell catalase assays of the mycobacterial strains were performed using a protocol described by Vestal (19). Briefly, individual colonies were removed from Lowenstein-Jensen slants after 7 to 10 days of growth and inoculated into 0.5 ml of phosphate solution (0.041 M Na_2HPO_4, 0.026 M KH_2PO_4 [pH 7.0]). A solution containing equal amounts of 30% hydrogen peroxide and 10% Tween 80 was prepared, and 0.5 ml of the peroxide–Tween 80 solution was added to the 0.5 ml of mycobacterial suspension. The evolution of O_2 gas caused frothing indicative of catalase activity, whereas the absence of O_2 gas bubbles demonstrated the loss of enzymatic activity.

DNA hybridizations. Approximately 3 μg of DNA from each mycobacterial strain was restricted with 30 to 50 units of *Eco*RI for 3 to 4 hours at 37°C to ensure complete digestion. Samples were precipitated with a twofold volume of ethanol and 10 μg of mussel glycogen at −20°C for 16 to 18 h. Restricted samples were electrophoresed on a 0.8% agarose gel and then blotted to Illuminator nylon membranes (Stratagene, La Jolla, Calif.) by the Southern technique. The protocols for fluoresceinated probe production, membrane hybridization, and the detection of bound probe are included in the Illuminator nonradioactive detection system (Stratagene). Briefly, the *rpsL* gene from *M. tuberculosis* (12) and an 850-bp fragment representing approximately one third of the *katG* gene from *M. intracellulare* (11) were fluoresceinated by using fluor-12-dUTP in a random primer-based system. Fluoresceinated DNA probes were purified from unincorporated nucleotides with a Sephadex G-50 spun column. Nylon membranes (75 cm^2) were prehybridized in 5 ml of a solution containing 50% formamide, 1 M NaCl, 1% SDS, 500 μg of salmon sperm DNA per ml, and 2% high-molecular-weight dextran sulfate for 2 to 4 h at 42°C. Approximately 1 ml of the hybridization fluid was retained, while the remainder was discarded. The probe was added to 100 μl of salmon sperm DNA (10 mg/ml) and boiled for 5 min. After addition of the probe to the hybridization fluid, the membrane was incubated at 42°C for 16 to 18 h. Nonspecifically bound probe DNA was removed by washing the filters one time for 15 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature followed by two 15-min washes in the same solution at 60°C. The membrane was then blocked and developed according to the manufacturer's protocol. To detect bound probe, the membrane was exposed to X-ray film for times ranging from 1 to 20 min.

PCR amplification and cloning of *katG* gene fragments. A group of eleven different overlapping primer sets (Lofstrand Laboratories, Gaithersburg, Md.) that spans the entire length of the *M. tuberculosis katG* gene was generated (8) (Table 2). These primers were used along with reagents and protocols supplied by the manufacturer (Perkin-Elmer) to amplify gene products from the *katG* gene of *M. bovis* and *M. tuberculosis* genomic DNA. Thermocycler reaction conditions were 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C for 40 cycles. PCR products generated from these primer sets were analyzed on 1.5% agarose gels. Fragments selected for further analysis were purified with the Wizard PCR Preps DNA purification system (Promega, Madison, Wis.). The PCR products were then cloned with the TA cloning system (Invitrogen, San Diego, Calif.).

Construction of the partial library of *M. bovis* ATCC 35747. Southern blot and PCR analyses suggested that *M. bovis* ATCC 35747 has a deletion within the *katG* gene. The *katG* probe was shown to react with a 2.5-kb *Eco*RI fragment in this strain. In order to isolate this reactive fragment, 10 μg of DNA was digested with *Eco*RI and run on a 1.2% low-melting-point agarose gel (Gibco/BRL). An area corresponding to 2 to 3 kb was excised from the gel and then melted in a 65°C water bath. DNA was extracted from the agarose with the Wizard PCR Preps DNA purification system (Promega). Recovered fractions were pooled and precipitated with 10 μg of mussel glycogen and twice the volume of ethanol at −20°C for 16 to 18 hours. The fractionated DNA was pelleted by centrifugation for 5 min at 14,000 × *g*, washed in 70% ethanol, and dried under a vacuum. The pellets were suspended in a minimum volume of 10 mM Tris (pH 8.0)–1 mM EDTA. Approximately 200 ng of *Eco*RI-restricted and dephosphorylated pUC19 vector DNA (Gibco/BRL) was ligated to 100 ng of the *Eco*RI-fractionated chromosomal DNA for 5 h at 12°C by using 1 U of T4 DNA ligase (Gibco/BRL). The ligation mixture was then transformed into competent *E. coli* DH5α and plated onto β-galactosidase indicator plates containing 100 μg of ampicillin per ml. The plasmid DNAs from recombinant clones were isolated with the Wizard miniprep system (Promega). Recombinant plasmids were restricted with *Eco*RI and evaluated by Southern blot hybridization protocols with the *katG* gene probe. Plasmid DNA from the clone that reacted with the *katG* probe was sequenced by using the double-stranded DNA sequencing protocol of the *Bst*I system (Bio-Rad, Richmond, Calif.) and α-^{35}S-dATP (NEN/Dupont, Wilmington, Del.). The *katG* deletion was identified by comparison of the sequence with the parental *katG* gene, using the GAP program of the Genetics Computer group software package (4).

PCR-SSCP analyses. SSCP is a sensitive technique for identifying mutations (15). The SSCP technique can be briefly described as follows: radioactively labeled PCR products are amplified from the appropriate gene segment and then denatured into single-stranded products with formamide and heat. The denatured products are loaded onto an MDE gel, and any aberrant electrophoretic mobility relative to the parental strain is an indication of differences in nucleotide sequence. Specifically, DNA isolated from *M. bovis* and *M. tuberculosis* was amplified for SSCP analysis in a 50-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 200 μM dATP, 100 μM dCTP, 200 μM dGTP, 200 μM dTTP, 0.5 μl (0.5 μCi) of [α-^{32}P]dCTP (NEN/Dupont), 1 μM each primer, and 2.5 U of native *Taq* polymerase (18). The reaction mixtures were subjected to 40 cycles of amplification (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C). Subsequently, 25 μl of the PCR product was diluted with 75 μl of dilution buffer (10 mM EDTA, 0.1% SDS). A 3-μl aliquot of the diluted product was then mixed with 3 μl of nucleotide sequencing stop buffer, heated at 95°C for 10 min, and then placed on ice. The products were loaded onto a nondenaturing 0.5× MDE gel (AT Biochem, Malvern, Pa.) with a Model S2 DNA sequencing apparatus (BRL). The gel was electrophoresed at 3 to 4 W for 16 to 18 h. The gels were dried and exposed to X-ray film for 1 to 3 days. PCR products showing aberrant SSCP mobilities were cloned by the TA cloning system and sequenced as described above. To verify the existence of specific mutations, products from three independent PCR amplifications were cloned and sequenced.

TABLE 2. *katG* overlapping primer sets

Set no.	Forward (5′-3′)	Reverse (5′-3′)
1	CTTCGCGATCACATCCGTG	GCGTCAGGGCGTCAAGTCGA
2	GCCGCGGAGGTCGCGACCA	AAGCGGCGCGAACCGCTGC
3	GGCATGCAGCGGTTCGCGC	GTCTCCCACTGGTCGACGC
4	CGGGTTCGGCTTCGGGCGT	ACGTCTCGCGAATGTGCAC
5	CGCGGCCGGTCGACATTCG	CTGGTGATCGCGTCCTTAC
6	GCTCGTATGGCACGGGAAC	GAACGGGTCCGGGATGGTG
7	GTTCGGCGGGCCAGGGCGC	CGCGCACGAGGTCGTGCTG
8	TGGCAGGATCCGGTCCCTGCG	CTGCAGGCGGATGCGACCACC
9	CGCCAACGGTGGTCGCATC	CGGAGCCGCCTTTGCTGCT
10	TGGCTGTGCGCCACTAGAG	GCAGGTTCGCCTTGTCGAC
11	GCCGAGTACATCGCTGCTC	GCGCACGTCGAACCTGTCG

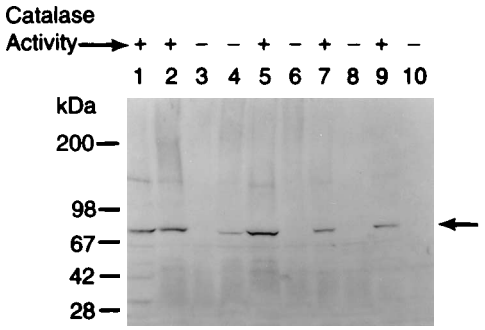

FIG. 1. Immunoblot of isoniazid-sensitive and -resistant *M. tuberculosis* complex strains incubated with a polyclonal antibody enriched for catalase-peroxidase activity. Ten micrograms of mycobacterial sonic extract was applied to each well of a 4 to 20% polyacrylamide gradient gel. Molecular size protein standards (in kilodaltons) are indicated on the left. The 80-kDa catalase-peroxidase is indicated by an arrow. Lanes 1 to 6: *M. tuberculosis* H37Ra, H37Rv, ATCC 35822, ATCC 35823, ATCC 35825, and ATCC 35835, respectively. Lanes 7 to 10: *M. bovis* ATCC 35720, ATCC 35727, ATCC 35735, and ATCC 35747, respectively. The catalase activity of each strain is noted at the top of each lane.

RESULTS

Immunoblot analyses and catalase activity assays. To assay for the presence of the *katG* gene product, mycobacterial sonicates from the ten *M. tuberculosis* complex strains were separated by SDS-PAGE and immunoblotted with an absorbed polyclonal antibody reactive against the 80-kDa catalase-peroxidase enzyme. Among the strains evaluated were four isoniazid-sensitive parental strains (*M. tuberculosis* H37Ra or ATCC H37Rv, *M. bovis* Ravenal, and *M. bovis* BCG Montreal) and six isoniazid-resistant mutants (*M. tuberculosis* ATCC 35822, ATCC 35823, ATCC 35825, and ATCC 35835 and *M. bovis* ATCC 35727 and ATCC 35747) (Table 1). Five of these strains—ATCC 35822, ATCC 35823, ATCC 35835, ATCC 35727, and ATCC 35747—are resistant to at least 50 μg of isoniazid per ml, whereas strain ATCC 35825 is resistant to isoniazid at the 5-μg/ml level. The anticatalase polyclonal antibody detected a reactive band at approximately 80 kDa in six of these sonicates, including the four parental strains. The 80-kDa band was not seen in sonicates of the *M. tuberculosis* isoniazid-resistant strains, ATCC 35822 and ATCC 35835, and the resistant *M. bovis* strains, ATCC 35727 and ATCC 35747 (Fig. 1). These data suggest that a full-length catalase-peroxidase enzyme is produced in all four parental strains as well as in two isoniazid-resistant strains but is not being synthesized in the isoniazid-resistant strains ATCC 35822, ATCC 35835, ATCC 35727, and ATCC 35747.

As shown in Fig. 1, whole-cell in vivo catalase assays were performed with each of the ten *M. tuberculosis* complex strains. All of the parental strains and the isoniazid-resistant strain ATCC 35825 had catalase activity, whereas all four of the strains that did not have an 80-kDa reactive band in the immunoblot assay did not have catalase activity (Fig. 1). In addition, strain ATCC 35823, which synthesizes an apparent full-length enzyme, did not have catalase activity.

Hybridization of isoniazid-resistant *M. tuberculosis* complex strains to the *katG* and *rpsL* gene probes. To evaluate whether these isoniazid-resistant strains had complete *katG* gene deletions, *Eco*RI-digested chromosomal DNAs from the resistant and parental strains were probed with a fluorescein-labeled amino-terminal *M. intracellulare katG* gene probe. As seen in Fig. 2A, the *katG* gene probe hybridized to an approximately 11-kb *Eco*RI fragment in 8 of the 10 strains analyzed. The *katG* gene probe did not react with chromosomal DNA from *M. tuberculosis* ATCC 35822, suggestive of a complete deletion of the *katG* gene (Fig. 2A, lane 3). This result was confirmed by the observed lack of reactivity of DNA from strain ATCC 35822 to the full-length *M. intracellulare katG* gene probe (data not shown). Southern blot analyses with the amino-terminal *katG* gene probe also revealed that isoniazid-resistant *M. bovis* ATCC 35747 has an aberrant *katG* gene (Fig. 2A, lane 10). A 2.5-kb *Eco*RI restriction fragment from this strain hybridizes to the *katG* gene probe, whereas the drug-sensitive parental strain (ATCC 35735) exhibits a reactive band at approximately 11 kb. As a control, an analogous Southern blot was probed with the *M. tuberculosis rpsL* gene, which encodes the ribosomal S12 protein. All of the strains, including ATCC 35822 and ATCC 35747, reacted with a 5.5-kb *Eco*RI fragment when probed with the *rpsL* gene (Fig. 2B).

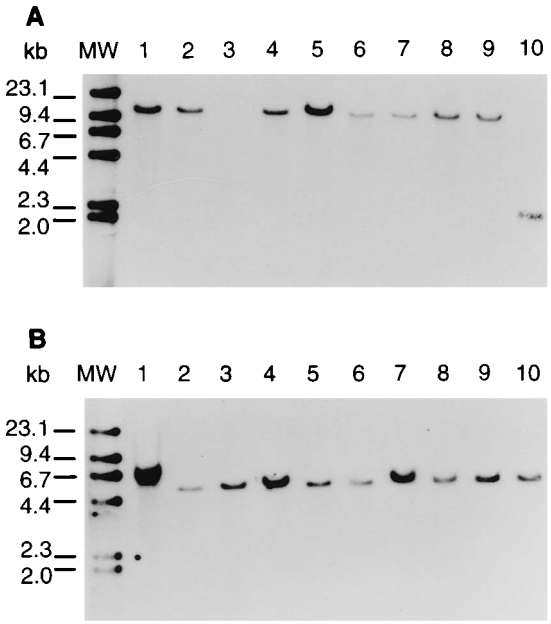

FIG. 2. (A) Southern blot of 3 μg of genomic DNA digested with *Eco*RI and electrophoresed through a 0.8% agarose gel. This blot was hybridized to a fluorescein-labeled *M. intracellulare katG* gene probe. Fluoresceinated λ/*Hin*dIII molecular mass markers (in kilobases) are indicated on the left. Lanes 1 to 6: *M. tuberculosis* H37Ra, H37Rv, ATCC 35822, ATCC 35823, ATCC 35825, and ATCC 35835, respectively. Lanes 7 to 10: *M. bovis* ATCC 35720, ATCC 35727, ATCC 35735, and ATCC 35747, respectively. (B) Southern blot identical to the blot described above for panel A that has been hybridized to a fluorescein-labeled *rpsL* gene probe from *M. tuberculosis.*

PCR amplification, cloning, and sequencing of *katG* gene products from *M. bovis* ATCC 35747. PCR analyses performed with the 11 overlapping sets of catalase primers which encompass the entire *katG* gene demonstrated that the primer sets 1 and 2 generated PCR products (Fig. 3, lanes 2 and 4) with *M. bovis* ATCC 35747 DNA as a template. However, downstream primer sets 3 to 6 did not amplify appropriate PCR products (Fig. 3, lanes 6, 8, 10, and 12) with the same template. These PCR results are consistent with the hybridization data, indicating a *katG* gene deletion in this isoniazid-resistant strain of *M. bovis*. The deletion was further characterized, by cloning and sequencing the fragment containing the truncated *katG* gene from *M. bovis* ATCC 35747. As described in Materials and Methods, a pUC19 partial genomic library was constructed from 2- to 3-kb *Eco*RI fragments of strain ATCC 35747 chromosomal DNA and transformants were screened for reactivity with the *katG* probe. Southern blot data indicated that one of

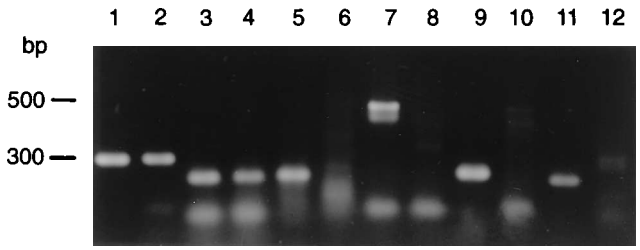

FIG. 3. PCR products from the *katG* genes of *M. bovis* ATCC 35735 (isoniazid-sensitive parental strain) and ATCC 35747 (isoniazid-resistant derivative). Genomic DNA from strain ATCC 35735 was the template for the PCR product in the odd-numbered lanes of the 1.5% agarose gel, and genomic DNA from strain ATCC 35747 was the template in the even-numbered lanes. Overlapping PCR primer sets that span the entire *katG* gene from *M. tuberculosis* were used to approximate the location of the gene deletion in the isoniazid-resistant mutant. The results from the first six primer sets are shown. PCR products were amplified from both the drug-sensitive parent and drug-resistant mutant with the primer sets 1 and 2 (lanes 1 to 4). PCR products were also generated in the parental strain with primer sets 3, 4, 5, and 6 (lanes 5, 7, 9, and 11, respectively), whereas no product was detected with DNA isolated from the resistant strain (lanes 6, 8, 10, and 12).

FIG. 4. SSCP analysis of three strains of *M. bovis* with primer set 1. The genomic DNA used is from strains ATCC 35720 (lane 1), ATCC 35727 (lane 2), and ATCC 35735 (lane 3). Each DNA template was subjected to standard PCR protocols with the exception of the inclusion of a radioactive nucleotide ([α-^{32}P]dCTP). The PCR products were treated as described in Materials and Methods and then electrophoresed onto a nondenaturing gel with a standard DNA sequencing apparatus. Exposure to X-ray film and subsequent development revealed distinctly different electrophoretic mobilities for the isoniazid-sensitive parent (lane 1) and isoniazid-resistant mutant (lane 2).

the recombinant clones had a 2.5-kb *Eco*RI fragment containing the *katG* gene. Nucleotide sequence analysis of this clone indicated that only the first 507 nucleotides encoding the amino portion of the enzyme were present in this strain.

Characterization of the *katG* genes of isoniazid-resistant strains ATCC 35823, ATCC 35825, ATCC 35835, and ATCC 35727. Southern blot analyses did not indicate a significant deletion of the *katG* gene in the four other isoniazid-resistant strains examined (*M. tuberculosis* ATCC 35823, ATCC 35825, and ATCC 35835 and *M. bovis* ATCC 35727). To identify small deletions or point mutations in these *katG* genes, chromosomal DNA from these strains as well as their parental strains was analyzed by SSCP. Briefly, *katG* gene segments were amplified by PCR in the presence of a radioactive nucleotide and then denatured with formamide and heat. The single-stranded molecules were loaded onto the appropriate matrix and resolved by electrophoresis. On SSCP gels, altered electrophoretic mobilities relative to those of the parental strain often result from point mutations, insertions, or deletions. Figure 4 shows that the SSCP profile for *M. bovis* ATCC 35727 (lane 2) is markedly altered relative to the SSCP profile for the isoniazid-sensitive strain (ATCC 35720) from which it was derived (lane 1) and to that of the isoniazid-sensitive *M. bovis* BCG ATCC 35735 (lane 3). Since only two bands are usually detected by SSCP analyses, the presence of five bands in each lane of Fig. 4 was unexpected. Although the exact nature of the five SSCP bands has not been defined, it has been shown that more than one single-stranded metastable conformation is sometimes allowed in SSCP conditions (15). It is therefore likely that the three additional bands observed in these lanes are single-stranded conformers of the same sequence. Because the primer set 1 SSCP profiles for strain ATCC 35727 and its parental strain ATCC 35720 differed significantly, these PCR products were cloned and sequenced as described previously. A DNA sequence comparison revealed a 76-bp deletion in this region of the *katG* gene of the drug-resistant mutant ATCC 35727 (Fig. 5).

Similar analyses comparing the multiple-drug-resistant *M. tuberculosis* strains, ATCC 35823 and ATCC 35825 with their drug-sensitive parental strain (H37Rv) were performed. A summary of the nonsilent mutations detected by SSCP analyses is shown in Table 3. In strain ATCC 35823, different SSCP mobilities were observed in primer sets 5, 6, and 7. DNA sequence analyses revealed silent mutations in primer sets 6 and 7 that did not change the amino acid sequence of the catalase-peroxidase enzyme produced by strain ATCC 35823. However, a transversion mutation observed in primer set 5 (codon 274) results in a nonconservative amino acid change from a threonine to a proline. Recent structural analyses of the *M. tuberculosis katG* gene product have predicted that the threonine at position 274 is involved in H_2O_2 binding and substrate catalysis (8). Therefore, the threonine-to-proline change may be responsible for the absence of catalase activity in this strain. In addition, we analyzed the *katG* gene of the

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                .         .         .         .         .
  1 atgctgtgcccgagcaacacccacccattacagaaaccaccaccggagcc 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 atgctgtgcccgagcaacacccacccattacagaaaccaccaccggagcc 50
                .         .         .         .         .
 51 gctagcaacggctgtcccgtcgtgggtcatatgaaatacccagtcgaggg 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 gctagcaacggctgtcccgtcgtgggtcatatgaaatacccagtcgaggg 100
                .         .         .         .         .
101 cggcggaaaccaggactggtggcccaaccggctcaatctgaaggtactgc 150
    ||||||||||
101 cggcggaaac..................................... 110
                .         .         .         .         .
151 accaaaacccggccgtcgctgacccgatgggtgcggcgttcgactatgcc 200
                                    ||||||||||||||
111 .................................cgttcgactatgcc 124
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FIG. 5. DNA sequence analysis of the PCR products of *M. bovis* ATCC 35720 and ATCC 35727 generated by primer set 1 shown in Table 2. Primer set 1 amplifies a *katG* fragment from −56 to +232 bp, relative to the initiation codon. A representative portion of this fragment is shown. The nucleotide sequence of strain ATCC 35720 is represented in each top row, and the sequence of strain ATCC 35727 is given in each bottom row. Initiation begins at the GTG located at nucleotides 6 to 8. The isoniazid-resistant strain ATCC 35727 has a deletion of 76 bp of the *katG* gene at nucleotides 111 through 186 (nucleotides 106 through 181 of the coding sequence).

TABLE 3. Mutations, detected in primer sets by SSCP, resulting in amino acid changes

Strain[a]	Result for primer set no.[b]:										
	1	2	3	4	5	6	7	8	9	10	11
H37Ra	−	−	−	−	−	−	−	−	−	−	−
H37Rv	−	−	−	−	−	−	−	−	−	−	−
ATCC 35822[c]											
ATCC 35823	−	−	−	−	+	−	−	−	−	−	−
ATCC 35825	−	−	−	−	−	−	−	+	−	−	−
ATCC 35835	−	−	−	++	−	+	−	−	−	−	−
ATCC 35720	−	−	−	−	−	−	−	+	−	−	−
ATCC 35727[d]											
ATCC 35735	−	−	−	−	−	−	−	+	−	−	−
ATCC 35747[d]											

[a] ATCC 35835 is an H37Ra mutant; ATCC 35822, ATCC 35823, and ATCC 35825 are H37Rv mutants; ATCC 35727 is a 35720 mutant; and ATCC 35747 is a 35735 mutant.

[b] +, amino acid change; ++, termination codon; −, silent mutation or no mutation.

[c] Complete deletion of *katG* gene.

[d] Partial deletion of *katG* gene.

catalase-positive strain ATCC 35825. The *katG* gene fragments amplified from strain ATCC 35825 chromosomal DNA with primer sets 6 and 8 exhibited different SSCP patterns. However, only a mutation in primer set 8 (codon 461) resulted in an amino acid change (arginine to leucine). Although the amino acid change is nonconservative, it should be noted that a leucine is normally present at this position in catalase-positive and isoniazid-sensitive strains of *M. bovis* described in this paper as well as *M. intracellulare* catalase-positive strains (11). To evaluate the potential contribution of *inhA* gene mutations to isoniazid resistance in strains ATCC 35823 and ATCC 35825, we analyzed the *inhA* structural genes of these strains by SSCP. These studies suggested that the *inhA* genes of these strains were not mutated. Additionally, the entire *inhA* coding region of strain ATCC 35825 was nucleotide sequenced and no mutations were observed (data not shown).

The isoniazid-resistant strain ATCC 35835 is a derivative of the avirulent strain H37Ra. SSCP analyses indicated that nucleic acid differences were present in the PCR products of primer set 4. A transition mutation detected in primer set 4 substitutes a termination codon (TAG) for the tryptophan residue (TGG) at codon 198. The presence of a termination codon in the 5′ terminus of the coding region is consistent with the immunoblot data, which indicated that the full-length enzyme is not produced, and also with the absence of catalase activity observed in this strain. In addition, SSCP analyses of the *katG* genes from strains ATCC 35835 and H37Ra also included a comparison with the H37Rv *katG* gene. This SSCP study revealed no differences between the H37Ra and H37Rv *katG* genes.

DISCUSSION

For more than four decades, isoniazid has been an essential component of international tuberculosis control programs. Despite its importance in tuberculosis chemotherapy, the mode of action and the cellular target(s) of isoniazid have not been unequivocally elucidated. Recent genetic studies that have examined the mechanisms of resistance to isoniazid have begun to unravel the mystery of its specific antituberculosis activity. Banerjee et al. (1) have recently shown that low-level resistance to isoniazid in some mycobacterial strains is associated with mutations in the *inhA* gene which encodes a protein involved in mycolic acid biosynthesis. This result is consistent with findings of previous studies which showed that isoniazid interferes with the synthesis of mycolic acids (16, 23). In contrast, high-level resistance to isoniazid appears to be associated with alterations of the *katG* gene. Earlier studies in the 1950s by Middlebrook and colleagues (9, 10) had established an inverse correlation between levels of isoniazid resistance and catalase-peroxidase activity in drug-resistant strains of *M. tuberculosis*. Zhang et al. (27, 28) recently defined the molecular basis for the association between catalase activity and isoniazid resistance by demonstrating that some catalase-negative *M. tuberculosis* strains had *katG* deletions and that expression of the wild-type *M. tuberculosis katG* gene product could increase isoniazid sensitivity in catalase-negative and isoniazid-resistant mycobacterial strains.

Our results extend the relationship between catalase-peroxidase activity and resistance to isoniazid. Five *M. tuberculosis* complex strains, which had been selected for isoniazid resistance in vitro and are highly resistant to isoniazid, have significant alterations in the *katG* gene. Southern blot hybridization, SSCP, and DNA sequence analyses have demonstrated that three of these mutants have deletions in the *katG* gene. *M. tuberculosis* ATCC 35822 has a complete deletion of the *katG* gene, whereas both resistant strains of *M. bovis* examined have partial deletions of the *katG* gene. *M. bovis* ATCC 35727 has a 76-bp deletion in the amino terminus of the coding sequence, and approximately 1800 bp of the coding sequence in the *katG* gene of strain ATCC 35747 was shown to be deleted. By SSCP analyses, point mutations were detected in the *katG* genes of the three remaining isoniazid-resistant strains. A transition mutation at codon 198 resulted in the conversion of tryptophan (TTG) into a termination codon (TAG) in *M. tuberculosis* ATCC 35835. This result is consistent with the observations that this strain does not produce an 80-kDa protein which reacts with an anticatalase polyclonal antibody or produce measurable catalase activity. The catalase-negative *M. tuberculosis* ATCC 35823 has a transversion mutation at codon 274 which substitutes a proline (CCC) for a threonine (ACC) in a conserved region of the protein. Recent comparisons of the *M. tuberculosis katG* primary sequence with the yeast cytochrome *c* peroxidase sequence (8, 21) have identified a number of structurally and catalytically important residues in the *katG* gene. Thr-274, Arg-104, Trp-107, His-108, Asn-138, His-269, His-275, and Arg-308 are residues that are predicted to be involved in H_2O_2 binding and substrate catalysis (8). Our demonstration that single mutations at Thr-274 in strain ATCC 35823 as well as mutations at His-108 and Asn-138 in two isoniazid-resistant *M. tuberculosis* clinical isolates (11a) are associated with loss of catalase-peroxidase activity and reduced sensitivity to isoniazid supports these predictions.

In contrast, the association between *katG* alterations and isoniazid resistance in strain ATCC 35825, which is 10-fold less resistant to isoniazid than the other strains examined, has not been clearly defined. The single nonsilent mutation detected in the multidrug-resistant strain ATCC 35825 was a transversion mutation at codon 461 that converts an arginine (CGG) into a leucine (CGT). However, the relevance of this substitution to isoniazid resistance needs further study. Strain ATCC 35825 produces a full-length catalase-peroxidase enzyme and has normal levels of catalase activity. Furthermore, isoniazid-resistant and -sensitive strains of *M. bovis* as well as *M. intracellulare* strains normally have a leucine at this position. Therefore, reduced sensitivity to isoniazid in this strain may not be associated with this *katG* alteration and may be the result of an alternative resistance mechanism. DNA sequence analyses did not detect mutations in the coding sequence of the *inhA* gene

in this strain. This result does not preclude the possibility of mutations in the promoter sequence of the *inhA* gene that affect expression of the gene product. Alternatively, *M. tuberculosis* ATCC 35825 may be resistant to isoniazid via a mechanism that has not been defined to date.

Although these molecular analyses of isoniazid-resistant in vitro mutants and clinical isolates have clearly established that mutations in the *katG* gene contribute to the problem of isoniazid resistance in *M. tuberculosis*, the molecular mechanisms by which the catalase-peroxidase enzyme interacts with isoniazid and enhances drug toxicity have not been defined. At least two modes of action have been proposed. First, the enzyme may facilitate transport of isoniazid into the bacterium. Studies with *E. coli*, *M. bovis* BCG, and *M. smegmatis* have demonstrated that catalase-peroxidase encoded by *katG* is probably surface associated and thus may be involved in transport processes (7, 22, 26). Reduced uptake of isoniazid has been demonstrated in many isoniazid-resistant, catalase-negative *M. tuberculosis* strains (25). Moreover, the expression of the *katG* gene product (hydroperoxidase I [HPI]) in *E. coli* allows the rapid recovery of transport processes which had been inhibited by oxidative stress (6). Further studies will be needed to ascertain whether the mycobacterial *katG* gene product participates directly in the transport of isoniazid or is involved in the maintenance of the membrane structure and function necessary for transport activity. Secondly, the *katG* gene product may chemically modify isoniazid and convert isoniazid into a more active form. For instance, Youatt and coworkers have suggested that pigmented metabolites, which are presumed to result from the interactions of the *katG* gene product with isoniazid, NAD, and peroxide, may contribute to the bactericidal activity of isoniazid (24, 26). Reactive oxygen metabolites that have been generated by the metabolism of isoniazid by catalase-peroxidase may also be deleterious to the cell (23).

Another important issue related to the association between the *katG* gene product and isoniazid sensitivity is the nature of hypersensitivity of the *M. tuberculosis* complex to isoniazid. *M. tuberculosis* complex strains are often sensitive to 0.02 μg of isoniazid per ml. Most other species of mycobacteria are much less sensitive to isoniazid, and bacteria from other genera are relatively insensitive to the drug. A probable drug target being essentially restricted to mycobacteria, such as the mycolic acid biosynthesis pathway, could explain the specificity of isoniazid for mycobacteria. However, the reason for the hypersensitivity of the *M. tuberculosis* complex relative to the rest of the genus is still unknown. While most species of mycobacteria produce two catalases, an HPI-like heat-labile T catalase and a heat-stable M catalase, organisms in the *M. tuberculosis* complex produce only the T catalase (20). The presence of the single T catalase may partially explain the increased sensitivity of the *M. tuberculosis* complex to isoniazid. Moreover, the structure and reactivity of the *M. tuberculosis katG* gene could be unique. A comparison of the *M. tuberculosis* catalase-peroxidase amino acid sequence with the homologous sequence from the naturally isoniazid-resistant *M. intracellulare* shows that these protein homologs are only 60% identical (8). These differences in protein structure may contribute to the observed differences in isoniazid sensitivity. To examine these issues, we are characterizing mutations in the *katG* genes of isoniazid-resistant *M. tuberculosis* clinical isolates by SSCP analyses. By comparing the *katG* genes of drug-resistant and drug-sensitive strains of *M. tuberculosis*, *M. intracellulare*, and other bacteria, amino acid residues which may play a crucial role in the hypersensitivity of *M. tuberculosis* complex organisms to isoniazid are being identified. Given these results, the *M. tuberculosis* and *M. intracellulare katG* genes will be subjected to site-directed mutagenesis and then cloned and expressed in the catalase-negative *M. tuberculosis* complex strains described in this paper. An evaluation of the catalase activity and isoniazid sensitivity of the subsequent transformants should assist in better defining interactions between the *M. tuberculosis katG* gene product and isoniazid. Clarification of the catalase-peroxidase–isoniazid association should provide a more rational basis for the development of improved antituberculosis drugs.

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