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Mutations in *katG* Gene Sequences in Isoniazid-Resistant Clinical Isolates of *Mycobacterium tuberculosis* Are Rare

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In this study, a battery of oligonucleotides was directed toward the *katG* gene and PCR–single-stranded conformation polymorphism (SSCP) analysis was used to search for *katG* gene deviations in clinical isolates of *Mycobacterium tuberculosis* from different geographical regions. Since a complete deletion of the *katG* gene was not found, it is suggested that deletion is not a major mechanism of isoniazid (isonicotinic acid hydrazide; INH) resistance in these isolates. However, 7 of 39 isolates (4 of 25 from South Africa and 3 of 14 from other geographical regions) showed mobility shifts by SSCP analysis, suggesting aberrations in the *katG* gene. Direct sequence analysis confirmed that the mobility shifts were due to Thr-275→Ala (Thr275Ala), Arg409Ala, Arg463Leu, and Asp695Ala mutations and a 12-bp deletion in the 5′ region of the *katG* gene. Mutations at codons 275, 463, and 695 created altered restriction sites for *Hha*I, *Msp*I, and *Hae*III, respectively, and sequence results, supported by restriction fragment length polymorphism analysis, suggested that the PCR-SSCP procedure is a good indicator of mutations in PCR-amplified fragments. Identical mutations at codons 463 and 275 were found in isolates from different geographical regions. This may suggest a common evolutionary event, but one of the control isolates (susceptible to INH [3%; $n = 30$]) also had a mutation at codon 463. The results suggest that variations in the *katG* coding gene sequences of INH-resistant isolates of *M. tuberculosis* are infrequent and that defects in other regions of the *M. tuberculosis* genome are of equal or greater importance in contributing to the acquisition of resistance to INH.

It is estimated that at least one third of the world's population is infected with *Mycobacterium tuberculosis* (4, 6, 7), and the current resurgence of tuberculosis complicates control of this disease. Occurrences of institutional outbreaks of tuberculosis caused by drug-resistant strains have become a further concern. In New York, N.Y., approximately 46% of tuberculosis isolates are reportedly resistant to one or more antibiotics (3, 18), with at least one strain resistant to as many as 11 drugs. Mortality rates in these outbreaks of multidrug-resistant tuberculosis can be as high as 80%, with most fatalities occurring among patients infected with human immunodeficiency virus (3, 17). There is an urgent need for a better understanding of the mechanisms of resistance to antituberculous drugs and for rapid means of detecting drug resistance in clinical isolates.

Isoniazid (isonicotinic acid hydrazide; INH) has been used for treating tuberculosis since 1952, but the mechanism of its action and the development of resistance to this drug by *M. tuberculosis* are not fully understood. It has recently been demonstrated (by cloning studies) that the catalase-peroxidase enzyme of *M. smegmatis*, encoded by the *katG* gene, mediates susceptibility to INH (19). Furthermore, the *katG* gene was deleted from some (two of eight) highly drug-resistant clinical isolates of *M. tuberculosis* (19). These findings led to the hypothesis that *katG* deletion is associated with INH resistance. However, this mechanism is not universal, since it does not explain the resistance pattern of isolates from which the gene is not deleted.

The aim of this study was to screen clinical isolates of *M. tuberculosis* from different geographical regions with known resistance patterns for aberrations in the *katG* gene. Different regions of the gene were amplified by PCR, and the products were examined by single-stranded conformation polymorphism (SSCP) analysis. The molecular data obtained were compared with the data for reference strain $H_{37}Rv$, the MICs of INH, and the catalase activities of appropriate isolates.

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MATERIALS AND METHODS

Clinical isolates of *M. tuberculosis*. Heat-inactivated (80°C for 1 h) scrape cultures in sterile saline and DNAs from randomly selected clinical isolates of *M. tuberculosis* from South Africa (25 isolates), Switzerland (3 isolates), and the United States (11 isolates) with known resistance patterns were used in this study. The MICs of INH for the South African isolates and the reference strain $H_{37}Rv$ were determined by the agar dilution method (14). Serial twofold dilutions of INH, ranging from 0.156 to 40 μg/ml and including a final concentration of 50 μg/ml, were incorporated into Middlebrook 7H10 agar medium. Inoculates were prepared from 10-day-old cultures in Dubos broth, which were diluted to a concentration of approximately 2×10^6 CFU/ml. Ten microliters of each culture was then inoculated (in duplicate) onto INH-containing and drug-free control plates, sealed in plastic bags, and incubated at 37°C for 21 days. The lowest concentration of INH that inhibited more than 99% of the bacterial population was considered to be the MIC. A critical INH concentration of 0.156 μg/ml was used as the breakpoint to differentiate between susceptible and resistant isolates. The catalase activities of test organisms were measured by the semiquantitative method (13). The MICs and catalase activities for isolates from other geographical regions were done by the same basic procedures (13, 14), and the results were supplied by K. D. Eisenach. In addition to $H_{37}Rv$, 30 clinical isolates from South Africa susceptible to INH were included as controls for mutational analysis by PCR-SSCP.

PCR procedures. A battery of oligonucleotide primers was designed (Table 1) from the known sequence of the *katG* gene (GenBank accession no. X68081) to

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TABLE 1. Overlapping primer sets used to amplify regions of the *katG* gene by PCR

Primer set	Sequence[a]	Nucleotides[b]	Fragment size (bp)
RTB51	GTGCCCGAGCAACACCCACC	1–221	221
RTB31	TCAAGTCGACTGGTCGCGAC		
RTB512	GTCGCGACCAGTCGACTTGA	202–338	137
RTB312	TAGGTGCCGGCAGCGTGCCA		
RTB511	TGGCACGCTGCCGGCACCTA	319–558	242
RTB311	CGAAGCCGAACCCGAACGTC		
RTB510	GACGTTCGGGTTCGGCTTCG	541–741	201
RTB310	AATGTCGACCGCCGCGGCCA		
RTB59	TGGCCGCGGCGGTCGACATT	722–942	221
RTB39	GCTGGTGATCGCGTCCTTAC		
RTB58	GTAAGGACGCGATCACCAGC	923–1140	218
RTB38	GGTCAGTGGCCAGCATCGTC		
RTB57	CCGACGATGCTGGCCACTGA	1120–1342	223
RTB37	AGGTCGTGCTGACCGCAGGG		
RTB56	CCCTGCGGTCAGCACGACCT	1323–1526	204
RTB36	TCGGGGTCGTTGACCTCCCA		
RTB52	TGGGAGGTCAACGACCCGAC	1507–1718	212
RTB32	TGTTCCTGCGACGCATCCGT		
RTB53	ACGGATGCGTCGCAGGAACA	1699–1915	217
RTB33	CCAGCGCTAAGCGCTTGTAG		
RTB54	CTACAAGCGCTTACCGCTGG	1896–2115	220
RTB34	GACCTCGACAAGCGCCCGCA		
RTB55	TGCGGGCGCTTGTCGAGGTC	2096–2310	215
RTB35	CAAATCGCGCCGGGCAAACA		

[a] Primer sequences are given from 5′→3′, and the complete *katG* sequence is in the GenBank database under accession no. X68081.

[b] Ranges are the first and last nucleotides amplified in each fragment. Nucleotides are numbered consecutively from the start codon GTG (G = position 1).

amplify approximately 200-bp fragments at a time. PCRs were done in 100-μl reaction volumes in which the final concentrations were as follows: $MgCl_2$, 2.5 mM; dATP, dCTP, dGTP, and dUTP, 200 μM each; 5′ primer, 0.2 μM; 3′ primer, 0.2 μM; bovine serum albumin, 0.2 μg/μl; and *Taq* polymerase (Promega), 1.5 U. Five microliters of DNA (approximately 1 μg of DNA) or scrape cultures in sterile saline were used as the template for amplification. Reaction mixtures were initially heated to 93°C for 3 min to lyse cells, with a subsequent heating cycle of 93°C for 1 min, 64°C for 1 min, and 72°C for 2 min, which was repeated 30 times in a thermocycler (MedTech Multigene, Cape Town, South Africa) for all amplifications. To check that DNA was a suitable template for PCR amplification, a primer set (MT51/MT31, referred to as the diagnostic primer set) was used to amplify a 123-bp fragment of the insertion sequence IS*6110* (8) in the PCR procedure. Amplified products were detected by ethidium bromide staining after electrophoresis on 12% polyacrylamide minigels (Mini-Protean II; Bio-Rad).

SSCP analysis. Equal volumes of stop solution (95% formamide, 20 mM EDTA, and 0.005% [each] bromophenol blue and xylene-cyanol) and PCR amplification products (10 μl each) were mixed and heat denatured at 95°C for 2 min. Denatured products were electrophoresed on nondenaturing 6% polyacrylamide slab gels containing 5% glycerol. Electrophoresis was performed at 50 W (constant power) in 0.6× TBE buffer (10× TBE = 900 mM [each] Tris and boric acid and 25 mM EDTA) for 4 to 5 h with cooling from a benchtop fan (15). FMC Gelbond film was used to facilitate the manipulation of gels. For control purposes, the PCR products of the INH-sensitive strain $H_{37}Rv$ and two fully susceptible clinical isolates were always included.

Gels were subsequently fixed for 6 min in a buffer containing 0.5% acetic acid and 10% ethanol and then placed in a 0.1% silver nitrate buffer for 10 min, after which staining was performed on a horizontal orbital shaker for 20 min or until bands were visible. The staining buffer consisted of 0.1 M $NaBH_4$, 1.5 M NaOH, and 0.004% formaldehyde. Fixing was performed in a 0.75% Na_2CO_3 buffer for 10 min.

Sequencing analysis. PCR-amplified products that showed mobility shifts (and products from other samples as specified in text) detected by PCR-SSCP were sequenced directly by the dideoxy chain termination method according to the specifications of the manufacturer (Sequenase PCR sequencing kit; U.S. Biochemicals). The samples used for sequence analysis were amplified in duplicate and sequenced with both forward and reverse primers.

RESULTS

Characteristics of resistant clinical isolates. Thirty-nine clinical isolates resistant to INH from different geographical regions were included in this study (Table 2). The majority of these isolates were resistant to more than one antibiotic. The MICs of INH varied from low-level (0.2 μg/ml) to high-level resistance (>50 μg/ml) (Table 2). Semiquantitative catalase assays were performed on all isolates, when possible, and most isolates showed low catalase activities, compared with that of reference strain $H_{37}Rv$ (Table 2). South African strains 93/3, 93/16, and 93/19 required INH MICs of ≥40 μg/ml with almost undetectable catalase activities, and only one strain (92/3) required an INH MIC of >40 μg/ml and had a catalase activity of >60 mm. Catalase assays showed catalase activities of 60 mm for *M. gordonae* and *M. avium-M. intracellulare*, two mycobacterial species with usually high catalase activities (13) (results not shown).

***katG* gene deletions.** Primer set RTB51/RTB31 was used to amplify a 221-bp fragment in the 5′ region of the *katG* gene by PCR. An additional primer set, MT51/MT31, widely used for the detection of *M. tuberculosis* (8) in clinical samples was used to prove that all samples were amplifiable. Figure 1 shows sample results. All isolates were amplifiable with both sets of primers. In addition, amplification products were obtained with all of the other primer sets (Table 1) directed toward the *katG* gene for all samples. From these results, it was concluded that no complete *katG* gene deletion was identified in the isolates screened.

Point mutations in the *katG* gene. A battery of oligonucleotide primers (Table 1) directed against the *katG* gene was used to amplify different regions in this gene by PCR. Additional confirmation of the amplification of *katG* gene sequences was obtained by specific restriction enzyme analysis of the amplified fragments yielding expected fragments according to the known sequences, as well as Southern hybridization with a *katG* probe (19) (results not shown). This was followed by a search for sequence alterations in the amplified fragments by SSCP analysis. Sample results for primer sets RTB51/RTB31, RTB56/RTB36, and RTB59/RTB39 are shown in Fig. 2. DNA fragments with altered migrations, relative to that of the control ($H_{37}Rv$), are visible in samples 93/17 (Fig. 2A) and TB160 (Fig. 2B). Mobility shifts in the single-stranded bands from sample 113716 are also visible (Fig. 2C). However, it must be noted that the amplified product in this sample was shorter than those in the other samples, possibly because of a small deletion. The same strategy was used with all of the other primer sets, and 7 of 39 samples (4 of 25 from South Africa and 3 of 14 from other geographical areas) showed either mobility shifts (indicating point mutations) or deletions of a few base pairs in the *katG* gene (Table 2). Only primer sets RTB56/RTB36 and RTB59/RTB39 detected aberrations in the *katG* gene in more than one isolate (isolates 93/17, 113560, and 93/21 and TB160, respectively) (Table 2).

Sequence analysis (Fig. 3A to E) showed that the band mobility shifts detected by SSCP analysis were due to the following mutations: CGG to CTG (isolates 93/17 and 113035) at nucleotides 1381 to 1383, GAC to GCC (isolate 93/19) at

TABLE 2. Characteristics and molecular data of clinical isolates

Isolate origin[a]	Isolate	Resistance pattern[b]	INH MIC (μg/ml)[c]	Catalase activity (mm)	Mutation and/or deletion
A	92/3	INH, RMP, ETH	>40.0	>60	—[d]
	92/4	INH, RMP, SM	10.0	35	—
	92/5	INH, RMP, SM, ETH, EMB	10.0	0	—
	92/6	INH, RMP, SM	10.0	1	—
	92/7	INH, RMP, SM	10.0	35	—
	93/1	INH, RMP, SM, EMB, PZA	10.0	8	—
	93/2	INH, SM	10.0	10	—
	93/3	INH, SM	>50.0	0	—
	93/4	INH	20.0	3	—
	93/5	INH, SM	10.0	6	—
	93/6	INH, SM	10.0	1	—
	93/7	INH, SM	10.0	1	—
	93/8	INH, RMP, SM, EMB, PZA	2.5	9	—
	93/9	INH, SM	10.0	10	—
	93/10	INH	5.0	13	—
	93/11	INH	5.0	10	—
	93/12	INH, SM	5.0	8	—
	93/13	INH, SM, PZA	5.0	10	—
	93/14	INH	2.5	12	—
	93/16	INH, RMP, SM, ETH, EMB, PZA	40.0	1	—
	93/17	INH, RMP, SM, PZA	2.5	1	Codon 463
	93/19	INH, RMP, SM	40.0	0	Codon 695
	93/20	INH, RMP, SM, EMB	2.5	0	Codon 409
	93/21	INH, RMP, SM, ETH, EMB	10.0	0	Codon 275
	93/22	INH	5.0	4	—
B	3090	INH	0.2	0	—
	5220	INH	>2.5	25	—
	1046	INH, RMP	5.0	20	—
	3952	INH	>5.0	0	—
	13999	INH	>2.5	0	—
	7945	INH	>2.5	0	—
C	11306	INH, RMP, EMB	5.0	3	—
	113989	INH, RMP, EMB	ND[e]	ND	—
	113035	INH, RMP, EMB	1.0	6	—
	113716	INH, RMP, EMB	1.0	7	12-bp deletion, codons 52–57
	113560	INH, EMB	>5.0	25	Codon 463
D	TB173	INH, RMP	<5.0	3	—
	TB160	INH, RMP	>2.5	3	Codon 275
	TB149	INH, RMP	<5.0	6	—
Reference strain	$H_{37}Rv$	Fully sensitive	<0.15	45	—

[a] A, South Africa; B, Health Dept. (Arkansas); C, Health Dept. (Texas); D, Switzerland.
[b] RMP, rifampin; ETH, ethambutol; SM, streptomycin; EMB, erythromycin B; PZA, pyrazinamide.
[c] On day 21.
[d] —, no gene deviations.
[e] ND, no data available.

nucleotides 2068 to 2070, CGC to GCC (isolate 93/20) at nucleotides 1222 to 1224, ACC to GCC (isolates 93/21 and TB160) at nucleotides 820 to 822, and a 12-bp (CCGTCGCTGA CC) deletion (isolate 113716) between nucleotides 157 and 168.

The *katG* sequence was recently updated in the GenBank database (accession no. X68081). When the sequence data were aligned to the updated version, it was found that the observed changes resulted in Thr-275→Ala (Thr275Ala), Arg409Ala, Arg463Leu, and Asp695Ala replacements. After careful inspection of sequences, it was concluded that the mobility shift observed by SSCP for isolate 93/20 was due to a CGC-to-GCC transition, compared with the original published sequence. The 12-bp deletion caused the replacement of the protein-coding sequence Ala-Val-Ala-Asp-Pro by a new codon for alanine.

These mutations were confirmed not only by both the forward and reverse primers from other independent PCR products but also by restriction enzyme analysis when possible. The mutations at codons 463, 695, and 275 created new restriction sites for *Msp*I, *Hae*III, and *Hha*I, respectively. Figure 3F shows a sample result in which the G-to-T transition in sample 93/17 caused the loss of an *Msp*I restriction site. No additional mutations were detected when the other INH-resistant isolates were screened with *Msp*I for this particular amplified region.

Isolate 93/3 required an exceptionally high INH MIC (>50 μg/ml) and had no catalase activity. Since no *katG* gene deletion was found and no SSCP mobility shift was observed for this isolate, the different regions of aberrations in the *katG* gene (RTB51/RTB31, RTB59/RTB39, RTB57/RTB37, RTB56/RTB36, and RTB54/RTB34) of other isolates in this study

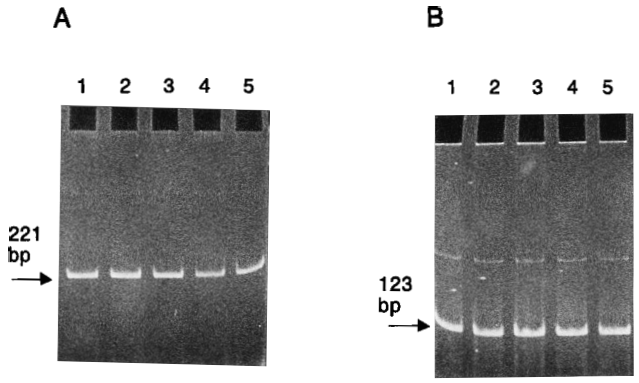

FIG. 1. Detection of *katG* gene deletions by PCR amplification. DNAs from isolates 92/3 (lanes 1), 5220 (lanes 2), 1046 (lanes 3), 3952 (lanes 4), and 13999 (lanes 5) were amplified by PCR with primer sets RTB51/RTB31 (A) and MT51/MT31 (B).

were sequenced in isolate 93/3 and no gene deviations were observed. Although it is possible to miss mutations during SSCP analysis (16), the last two findings suggest that this is not the case in this study.

Thirty clinical isolates susceptible to INH were subjected to PCR-SSCP analysis as controls for those regions in which differences in the *katG* gene were detected. One susceptible isolate gave a band mobility shift with primer set RTB56/RTB36. It was later confirmed by sequence and restriction enzyme analyses (*Msp*I) that this isolate has the same (G-to-T transition [codon 463]) mutation found in INH-resistant isolates 93/17 and 113560. No other band mobility shift was identified for any other region in any sample.

DISCUSSION

Limited experimental evidence has led to the hypothesis that the *katG* gene is associated with INH resistance in *M. tuberculosis* (19). However, since no deletions of the entire *katG* gene were seen in the strains investigated in this study, a deletion of the whole gene must be a rare event. This is in agreement with reports (1, 9, 17) from other geographical areas where whole-gene deletion has not been reported as a frequent event. It therefore suggests that other mechanisms play an important role in INH resistance amongst clinical isolates of *M. tuberculosis.*

It has been speculated that point mutations are another mechanism that could be involved in the acquisition of INH resistance (17). This hypothesis was substantiated when 7 of the 39 isolates (18%) analyzed in this study showed band mobilization shifts by PCR-SSCP analysis. Direct sequencing confirmed these data. Scattered mutations were detected but were predominantly biased toward central regions of the *katG* gene. Although no deletion of the complete *katG* gene was found in any isolate in this study, sequence data for isolate 113716 indicated that a small deletion at the 5′ end of the gene may play a role in INH resistance in *M. tuberculosis.*

Apart from the initial finding that complete *katG* deletion may be involved in resistance to INH (19), limited previous information about other aberrations in *katG* is available. In one study (17), the analysis of *katG* was limited to a 282-bp segment of the 5′ end of the gene. No DNA sequencing was done to identify point mutations, insertions, or deletions. Other studies have shown mutations in a 5′ 237-bp fragment of *katG* (1) and mainly missense mutations in 23 of 36 INH-resistant strains (10, 11). Our study represents a comprehensive analysis of the *katG* gene in multiple clinical isolates of *M. tuberculosis* with a wide range of INH resistance. The functional importance of these mutations is still unknown. Attempts to predict important residues in *katG* of *M. tuberculosis* from the crystal structure of the related enzyme cytochrome-*c* peroxidase from yeasts have been made. A number of structurally and catalytically important residues have been predicted; in this respect, Thr-275, which was replaced in two isolates in this study, is regarded to be involved in active-site modulation and H_2O_2 binding (12).

Approximately equal proportions of INH-resistant isolates from South Africa (16%) and from other geographical regions (21%) showed mutations in the *katG* gene. Identical Arg-463 (isolates 93/17 and 113560) and Thr-275 (isolates 93/21 and TB160) mutations were seen in two separate cases when isolates from South Africa and other regions were analyzed. It has been reported that 44% of *M. tuberculosis* strains whose INH MICs were ≥1.0 μg/ml showed the same Arg-463 mutation (5). This is suggestive of a mutational hot spot. However, one

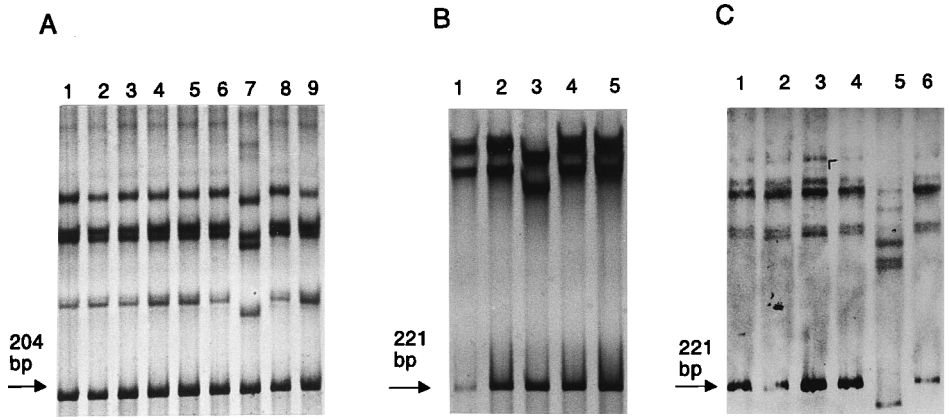

FIG. 2. Search for point mutations in the *katG* gene by PCR-SSCP analysis. Isolates in panel A (lanes 1 to 9: 93/10, 93/11, 93/12, 93/13, 93/14, 93/16, 93/17, 93/19, and $H_{37}Rv$, respectively) and panel B (lanes 1 to 5: 92/5, 93/14, TB160, TB149, and $H_{37}Rv$, respectively) were amplified by PCR with primer sets RTB56/RTB36 and RTB59/RTB39, respectively. Isolates in panel C (lanes 1 to 6: $H_{37}Rv$, 11306, 13989, 113035, 113716, and 113560, respectively) were amplified by PCR with primer set RTB51/RTB31. Band mobility shifts are visible in lane 7 in panel A, lane 3 in panel B, and lane 5 in panel C, compared with reference strain $H_{37}Rv$. Sequence analysis showed that the amplified product in lane 5 in panel C was 12 bp smaller than those of the other isolates and the reference strain. All double-stranded PCR products were compared with molecular weight marker ϕX174 *Hae*III (not shown).

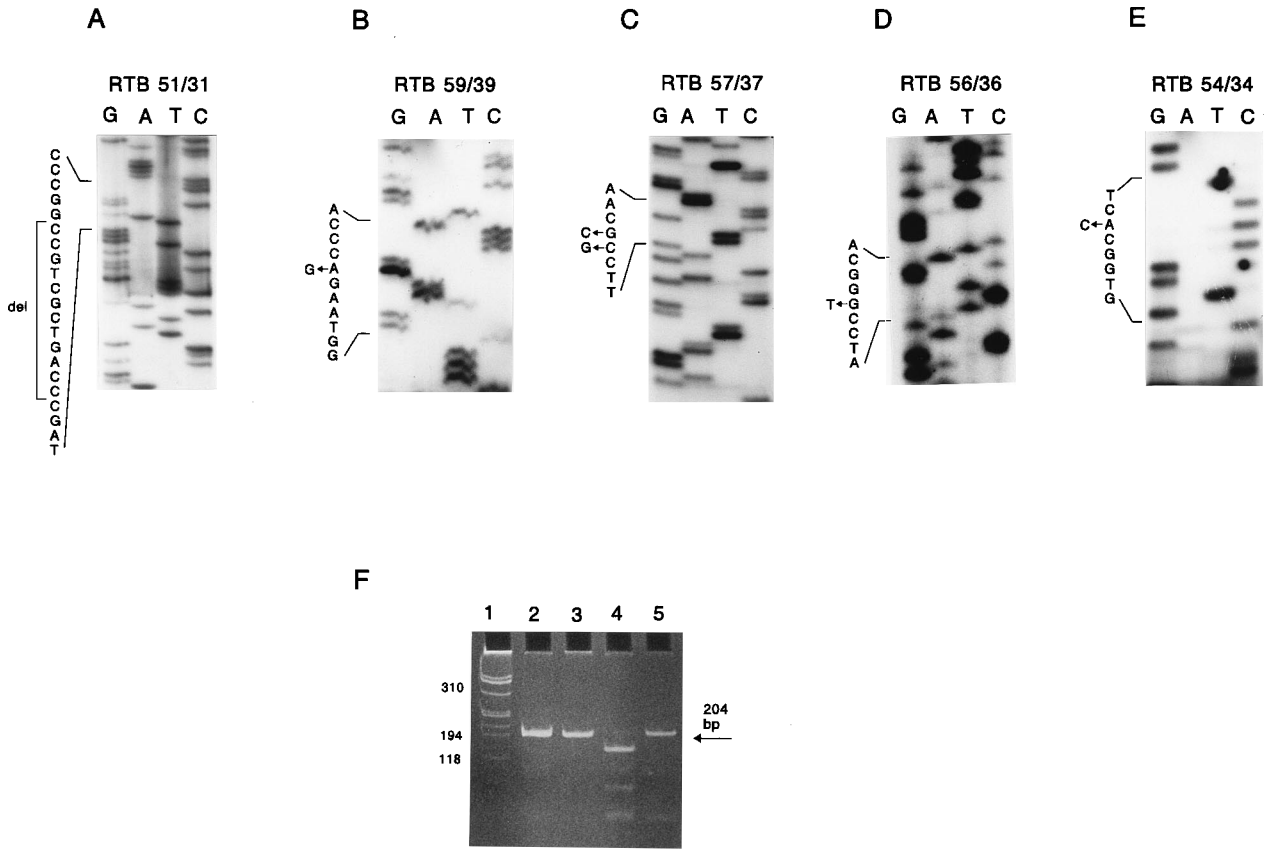

FIG. 3. Confirmation of mutations and small deletions by direct DNA sequencing and restriction fragment length polymorphism analysis. (A) Isolate 113716 was amplified with primer set RTB51/RTB31, and an aliquot of the amplified product was directly sequenced with primer RTB31. A 12-bp deletion (del) between nucleotides 157 and 168 was detected. (B) Isolate 93/21 was amplified with primer set RTB59/RTB39 and sequenced with primer RTB59. A point mutation, ACC to GCC, between nucleotides 820 and 822 was detected. The same mutation was found in isolate TB160. (C) Isolate 93/20 was amplified with primer set RTB57/RTB37 and sequenced with primer RTB57. A point mutation, CGC to GCC, between nucleotides 1222 and 1224 was detected. (D) Isolate 93/17 was amplified with primer set RTB56/RTB36 and sequenced with primer RTB56. A point mutation, CGG to CTG, between nucleotides 1381 and 1383 was detected. The same mutation was found in isolate 113560 and a control isolate (susceptible to INH). (E) Isolate 93/19 was amplified with primer set RTB54/RTB34 and sequenced with primer RTB34. A point mutation, GAC to GCC, between nucleotides 2068 and 2070 was detected. (F) $H_{37}Rv$ and isolate 93/17 were amplified with primer set RTB56/RTB36, and 15 μl of the amplified product was subsequently digested with *Msp*I and analyzed by gel electrophoresis. Lanes 1 to 5: molecular size marker ϕX174 *Hae*III, digested 93/17, undigested 93/17, digested $H_{37}Rv$, and undigested $H_{37}Rv$, respectively. The mutation in isolate 93/17 resulted in the loss of the restriction site for *Msp*I; similar results were found with isolate 113035 and a control isolate (susceptible to INH). The arrow indicates the size of the amplified product.

control isolate (3%; $n = 30$), susceptible to INH, also had the Arg-463 mutation, which was also reported elsewhere (5) for a strain whose INH MIC was ≤1.0 μg/ml. More isolates have to be screened to investigate the role of this mutation in INH-susceptible isolates. Concurrent with our study, a consensus sequence of *katG* has been published (5); it would be interesting to correlate mutational frequencies with larger numbers of samples from different geographical areas with INH resistance.

A comparison was drawn between the molecular data obtained in this study and culture data. The INH MIC and semiquantitative catalase activity were relatively good indicators of resistance, except for isolate 92/3, which showed a catalase activity of >60 mm. For all of the isolates that showed *katG* gene aberrations, catalase activities varied from 0 to 25 mm. However, the INH MICs for these isolates ranged from low-level (1 μg/ml) to high-level resistance (40 μg/ml). This phenomenon may suggest a resistance mechanism that does not involve *katG*. It has been proposed that in addition to catalase involvement, other mechanisms contribute to the evolution of drug resistance in *M. tuberculosis*. One such alternative mechanism of resistance to INH may involve the *inhA* gene, recently reported to be involved in resistance (2). It is possible that in the samples in which no *katG* gene defect was detected, *inhA* mutations are involved. Alternatively, the possibility of other resistance mechanisms, as yet undescribed, cannot be ruled out.

The PCR-SSCP technique is now widely used as a screening procedure for mutations; however, since many factors can influence the sensitivity of this procedure to detect mutations (16), it cannot be regarded as infallible. Nevertheless, it may be an easier screening method for mutation detection than direct DNA sequencing. As the effects of mutations on the conformation of single-stranded DNA are more pronounced when the DNA fragments are small, in this study pairs of PCR primers were designed to generate fragments of approximately 200 bp. The sequence results of this study suggest that the PCR-SSCP procedure is a good indicator of mutations in PCR-amplified regions. This was supported by restriction fragment length polymorphism analysis (Fig. 3F) with *Msp*I, which showed not only that this particular Arg-463 mutation can easily be detected with this system but also that mutations were not missed during the initial PCR-SSCP screening procedure for this locus.

In conclusion, the results of this study show that alterations

in the *katG* gene which lead to INH resistance in clinical isolates of *M. tuberculosis* (other than whole-gene deletion) may occur, although they were infrequent events in the strains tested. Some of these alterations also occur in INH-susceptible isolates of *M. tuberculosis*. The functional importance of these mutations has not yet been elucidated. The results also suggest that other mechanisms, such as defects in elements controlling the expression of catalase, may be involved in the development of resistance to INH in *M. tuberculosis.*

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