

## Detection of Multidrug Resistance in *Mycobacterium tuberculosis*<sup>▽</sup>

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We developed a DNA sequencing-based method to detect mutations in the genome of drug-resistant *Mycobacterium tuberculosis*. Drug resistance in *M. tuberculosis* is caused by mutations in restricted regions of the genome. Eight genome regions associated with drug resistance, including *rpoB* for rifampin (RIF), *katG* and the *mabA* (*fabG1*)-*inhA* promoter for isoniazid (INH), *embB* for ethambutol (EMB), *pncA* for pyrazinamide (PZA), *rpsL* and *rrs* for streptomycin (STR), and *gyrA* for levofloxacin, were amplified simultaneously by PCR, and the DNA sequences were determined. It took 6.5 h to complete all procedures. Among the 138 clinical isolates tested, 55 were resistant to at least one drug. Thirty-four of 38 INH-resistant isolates (89.5%), 28 of 28 RIF-resistant isolates (100%), 15 of 18 EMB-resistant isolates (83.3%), 18 of 30 STR-resistant isolates (60%), and 17 of 17 PZA-resistant isolates (100%) had mutations related to specific drug resistance. Eighteen of these mutations had not been reported previously. These novel mutations include one in *rpoB*, eight in *katG*, one in the *mabA*-*inhA* regulatory region, two in *embB*, five in *pncA*, and one in *rrs*. *Escherichia coli* isolates expressing individually five of the eight *katG* mutations showed loss of catalase and INH oxidation activities, and isolates carrying any of the five *pncA* mutations showed no pyrazinamidase activity, indicating that these mutations are associated with INH and PZA resistance, respectively. Our sequencing-based method was also useful for testing sputa from tuberculosis patients and for screening of mutations in *Mycobacterium bovis*. In conclusion, our new method is useful for rapid detection of multiple-drug-resistant *M. tuberculosis* and for identifying novel mutations in drug-resistant *M. tuberculosis*.

The emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis*, especially multidrug-resistant (MDR) strains, are serious threats to the control of tuberculosis and comprise an increasing public health problem (40). Patients infected with MDR strains, which are defined as strains resistant to both rifampin (RIF) and isoniazid (INH), are difficult to cure and are more likely to remain sources of infection for a longer period of time than are patients with drug-susceptible strains (40).

It is essential that rapid drug susceptibility tests be developed to prevent the spread of MDR *M. tuberculosis*. The time necessary for culture of specimens was reduced by the radiometric BACTEC 460TB system (BD Biosciences, Sparks, MD), the nonradiometric ESP II system (Trek Diagnostics, Westlake, OH), and other rapid broth methods, such as BACTEC MGIT 960 SIRE (BD Biosciences) (20). These drug susceptibility tests, however, still require 1 to 2 weeks for final determination and reporting to the clinician (23). Additional reductions in the detection period are needed.

Drug resistance in *M. tuberculosis* is caused by mutations in relatively restricted regions of the genome (17, 39). Mutations associated with drug resistance occur in *rpoB* for RIF, *katG* and the promoter region of the *mabA* (*fabG1*)-*inhA* operon for INH, *embB* for ethambutol (EMB), *pncA* for pyrazinamide (PZA), *rpsL* and *rrs* for streptomycin (STR), and *gyrA* for fluoroquinolones (FQs) such as ofloxacin (OFX) and levofloxacin (LVX) (17, 39). For example, 96% to 100% of RIF-resistant *M. tuberculosis* isolates have at least 1 mutation in *rpoB*, which encodes the RNA polymerase  $\beta$ -subunit (17, 31, 39). Of INH-resistant isolates, 42% to 58% have at least 1 mutation in *katG*, which encodes catalase-peroxidase, and 21% to 34% carry at least 1 mutation in the promoter of *mabA*, a synonym for *fabG1* (10), which encodes a 3-ketoacyl reductase (3, 17, 38, 39). Of EMB-resistant isolates, 47% to 65% have at least one mutation in *embB*, which encodes arabinosyltransferase (17, 32, 39). Seventy-two to 97% of PZA-resistant isolates have at least one mutation in *pncA*, which encodes pyrazinamidase (17, 26, 39). Of STR-resistant isolates, 52% to 59% and 8% to 21% have mutations in *rpsL*, which encodes ribosomal protein S12, and *rrs*, which encodes 16S rRNA, respectively (17, 19, 39). Of FQ-resistant isolates, 75% to 94% have mutations in *gyrA*, which encodes the A subunit of DNA gyrase (17, 30, 39).

Various molecular methods have been used to identify the

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TABLE 1. Bacterial strains used in the present study

Strain	Characteristic(s) or susceptibility pattern	No. of isolates	Strain	Characteristic(s) or susceptibility pattern	No. of isolates												
<i>M. tuberculosis</i> IMCJ	Clinical strains isolated in Japan	105	<i>M. kansasii</i> JCM 6379 (ATCC 124878)	ND	1												
	Rif <sup>r</sup> Inh <sup>r</sup> Emb <sup>r</sup> Pza <sup>r</sup> Str <sup>r</sup> Ofx <sup>r</sup>	3		<i>M. marinum</i> GTC 616 (ATCC 927)	ND	1											
	Rif <sup>r</sup> Inh <sup>r</sup> Emb <sup>r</sup> Pza <sup>r</sup> Str <sup>r</sup>	3			<i>M. nonchromogenicum</i> JCM 6364 (ATCC 19530)	ND	1										
	Rif <sup>r</sup> Inh <sup>r</sup> Pza <sup>r</sup> Str <sup>r</sup>	3				<i>M. phlei</i> RIMD 1326001 (ATCC19249)	ND	1									
	Rif <sup>r</sup> Inh <sup>r</sup> Emb <sup>r</sup> Str <sup>r</sup>	4					<i>M. scrofulaceum</i> JCM 6381 (ATCC 19981)	ND	1								
	Rif <sup>r</sup> Inh <sup>r</sup> Emb <sup>r</sup> Pza <sup>r</sup>	2						<i>M. simiae</i> GTC 620 (ATCC 25275)	ND	1							
	Inh <sup>r</sup> Emb <sup>r</sup> Str <sup>r</sup>	2							<i>M. smegmatis</i> ATCC 19420	ND	1						
	Rif <sup>r</sup> Inh <sup>r</sup>	2								<i>M. szulgai</i> JCM 6383 (ATCC 35799)	ND	1					
	Inh <sup>r</sup> Str <sup>r</sup>	4									<i>M. terrae</i> GTC 623 (ATCC 15755)	ND	1				
	Pza <sup>r</sup> Str <sup>r</sup>	1										<i>Escherichia coli</i> ATCC 8739	ND	1			
	Rif <sup>r</sup> Str <sup>r</sup>	2											<i>Haemophilis influenzae</i> IID <sup>f</sup> 984 (ATCC 9334)	ND	1		
	Rif <sup>r</sup>	2												<i>Klebsiella pneumoniae</i> IID5209 (ATCC 15380)	ND	1	
	Inh <sup>r</sup>	5													<i>Legionella pneumophila</i> GTC 745	ND	1
	Pza <sup>r</sup>	2														<i>Mycoplasma pneumoniae</i> IID 817	ND
Str <sup>r</sup>	6	<i>Pseudomonas aeruginosa</i> ATCC 27853	ND														1
Susceptible to all drugs tested	64		<i>Rhodococcus equi</i> ATCC 33710	ND													1
<i>M. tuberculosis</i> P	Clinical strains isolated in Poland			33	<i>Staphylococcus aureus</i> N315												ND
	Rif <sup>r</sup> Inh <sup>r</sup> Emb <sup>r</sup> Pza <sup>r</sup>			1		<i>Streptococcus pneumoniae</i> GTC 261											ND
	Rif <sup>r</sup> Inh <sup>r</sup> Emb <sup>r</sup>			2			<i>M. intracellulare</i> JCM 6384 (ATCC 13950)										ND
	Rif <sup>r</sup> Inh <sup>r</sup> Pza <sup>r</sup>			1				<i>M. tuberculosis</i> H37Rv (ATCC <sup>a</sup> 27294)									Susceptible to all drugs tested
	Rif <sup>r</sup> Inh <sup>r</sup>			2					<i>M. tuberculosis</i> H37Ra (ATCC 25177)								Susceptible to all drugs tested
	Inh <sup>r</sup> Str <sup>r</sup>			1						<i>M. avium</i> ATCC 25291							ND <sup>g</sup>
	Rif <sup>r</sup>			1							<i>M. bovis</i> BCG <sup>b</sup> (Japanese strain 172)						Pza <sup>r</sup>
	Inh <sup>r</sup>			3								<i>M. chelonae</i> JCM <sup>c</sup> 6390 (ATCC 14472)					ND
	Emb <sup>r</sup>			1									<i>M. fortuitum</i> RIMD <sup>d</sup> 1317004 (ATCC 6841)				ND
	Pza <sup>r</sup>			1										<i>M. gastri</i> GTC <sup>e</sup> 610 (ATCC 15754)			ND
Str <sup>r</sup>	1			<i>M. intracellulare</i> JCM 6384 (ATCC 13950)	ND										1		
Susceptible to all drugs tested	19				<i>M. tuberculosis</i> H37Rv (ATCC <sup>a</sup> 27294)	Susceptible to all drugs tested									1		

<sup>a</sup> American Type Culture Collection, Rockville, MD.

<sup>b</sup> Japan BCG Laboratory, Tokyo, Japan.

<sup>c</sup> Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan.

<sup>d</sup> Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

<sup>e</sup> Gifu Type Culture Collection, Department of Microbiology-Bioinformatics, Regeneration and Advanced Medical Science, Gifu University, Graduate School of Medicine, Bacterial Genetic Resources, Gifu, Japan.

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<sup>g</sup> ND, not determined.

mutations in *rpoB*, *katG*, *rpsL*, *rrs*, *embB*, *pncA*, *gyrA*, and other genes (7, 17). Among these methods, DNA sequencing is the most direct and reliable for detection of both known and novel mutations. The conventional methods, nevertheless, are not applicable for analysis of strains that may have multiple muta-

tions in genes related to drug resistance because different PCR conditions are required for amplification of each target region. We describe here a new PCR-based method for simultaneous detection of mutations in eight genes responsible for resistance to six antitubercular drugs.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** One hundred five and 33 clinical isolates of *M. tuberculosis* were obtained from patients with pulmonary tuberculosis in Japan and Poland, respectively. All of the bacterial strains used in this study, except for those used in cloning experiments, are listed in Table 1. *Escherichia coli* UM262 (*recA katG::Tn10 pro leu rpsL hsdM hsdR endl lacY*) (13) was provided by Barbara L. Triggs-Raine (University of Manitoba, Manitoba, Canada) and was used as a host for the expression of *katG* derived from *M. tuberculosis* clinical isolates and H37Rv, an *M. tuberculosis* reference strain. *E. coli* TOP10 (Invitrogen, Carlsbad, CA) and BL21-AI (Invitrogen) were used as hosts for cloning and protein overexpression studies, respectively. pCRT7/NT (Invitrogen) was used as a cloning and protein expression vector.

**Drug susceptibility test.** All clinical isolates of *M. tuberculosis*, *M. tuberculosis* strains H37Rv and H37Ra, and *Mycobacterium bovis* BCG Japanese strain 172 were tested for drug susceptibility. Strains were analyzed by three different methods. Two methods were agar proportion methods: the Middlebrook 7H10 agar medium method recommended by the United States Public Health Service (20) and the egg-based Ogawa medium method recommended by the Japanese Society for Tuberculosis (Vit Spectrum-SR; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan). The third method was a rapid broth method (BD BACTEC MGIT 960 SIRE, BD Biosciences, Sparks, MD) (20). The proportion method with 7H10 agar medium was used to assess susceptibilities to RIF, INH, EMB, STR, and OFX. Ogawa medium was used to test susceptibilities to RIF, INH, EMB, SM, and LVX. The broth method was applied to assess susceptibilities to RIF, INH, EMB, STR, and PZA. All isolates were tested by all three methods.

**Assay for PZase activity.** Pyrazinamidase (PZase) activity was determined as described previously (34). *M. tuberculosis* strain H37Rv, which is susceptible to PZA and positive for PZase, was used as a positive control for the assay. *M. bovis* strain BCG, which is resistant to PZA and negative for PZase, was used as a negative control. Each test tube was read and classified by three independent observers. There were no discrepancies between the classifications for any of the isolates tested.

**DNA extraction.** Genomic DNAs from bacteria were extracted as described previously (21).

**Clinical samples.** Six samples of mycobacterial staining-positive sputa from six patients with relapsed active tuberculosis and four samples of staining-negative sputa from four patients who had been treated previously with antitubercular drugs, were treated with *N*-acetyl-L-cysteine-NaOH solution according to the procedure of the BBL MycoPrep mycobacterial system digestion/decontamination kit (BD Diagnostic Systems, Franklin Lakes, NJ). Each sample was resuspended in 1.5 ml phosphate buffer. One milliliter of the suspension was transferred to a 1.5-ml tube for PCR. The remaining suspension was inoculated into Ogawa medium and MGIT 960 broth and cultured for mycobacterial examination. The 1 ml of suspension for PCR was centrifuged for 15 min at 13,000 × *g*, and the supernatant was removed with a pipette. Tris-EDTA (TE) buffer (500 μl) was added to resuspend the sediment, and the solution was again centrifuged for 15 min at 13,000 × *g*. The sediment was resuspended in 100 μl of a 10% solution of Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) in distilled water. The sample was resuspended by vortexing and incubated at 45°C for 45 min followed by incubation at 100°C for 10 min. The sample was vortexed again, allowed to cool, and centrifuged at 12,000 × *g* for 5 min to clarify the supernatant, which was transferred to another 1.5-ml tube and used for PCR.

**DNA sequencing of drug resistance-related genes.** Eight pairs of PCR primers were designed to amplify simultaneously regions of eight genes associated with resistance to six antituberculosis drugs. Sixteen primers were designed to determine the DNA sequences of the amplicons. The sequences of oligonucleotide primers for PCR, PR1 to PR16, and for DNA sequencing, PR17 to PR32, and the regions analyzed are listed in Table 2.

A two-temperature PCR consisting of 30 cycles of 95°C for 1 s for denaturation and 68°C for 30 s for annealing and elongation was performed with a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA) for DNA amplification, according to the instructions in the manufacturer's manual. Each PCR primer pair in TE (1.0 μl of 10 μM) listed in Table 2 was added to an individual reaction tube (0.2 ml Thermo-Tube; Advanced Biotechnologies, Epsom, Surrey, United Kingdom). Hence, eight reaction tubes containing the different primer pairs were prepared. Forty-nine microliters of a solution containing 1.0 μl DNA template, 1.25 U *Z*-*Taq* polymerase (Takara Bio, Ohtsu, Shiga, Japan), 4 μl of 2.5 mM each deoxynucleotide triphosphate, and 5 μl of 10× *Z* *Taq* PCR buffer (Takara Bio) was added to each reaction tube. The *Z*-*Taq* polymerase offers unmatched

PCR productivity, with a processing speed five times faster than those of other commercially available *Taq* polymerases, which allowed us to reduce the annealing and elongation times.

PCR products were purified with MicroSpin S-300 HR columns (Amersham Biosciences, Uppsala, Sweden) or a DyeEx 2.0 spin kit (QIAGEN K.K., Tokyo, Japan) and used as templates for DNA sequencing. PCR products were sequenced with the appropriate gene-specific primers (Table 2). Sequencing was performed with an ABI PRISM BigDye terminator cycle sequencing ready reaction kit for a 96-well format (Applied Biosystems). Five microliters of pre-mixed reagents from the kit (Terminator Ready Reaction mix; Applied Biosystems) and 13.5 μl of 1× reaction buffer were added to each tube containing 100 ng of templates and 5 pmol sequencing primer and mixed with a pipette. Amplification conditions were 25 cycles of 96°C for 10 s for denaturation, 50°C for 5 s for annealing, and 60°C for 4 min for elongation. It took 2.5 h to complete the entire reaction. Centri-Sep spin columns (Applied Biosystems) were used to remove unincorporated reagents and primers. Purified products were dried in a vacuum centrifuge, resuspended in Hi-Di formamide (Applied Biosystems), heated for 2 min at 95°C for denaturation, immediately cooled on ice, and loaded into a 96-well plate (MicroAmp 96-well reaction plate; Applied Biosystems). The purified samples were then analyzed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems). DNA sequences were collected and edited with Data Collection version 1.01 and Sequencing Analysis version 3.7 software (Applied Biosystems) and compared with those of *M. tuberculosis* H37Rv (GenBank accession no. NC\_000962) with Genetyx-WIN (version 5; Software Development Co., Tokyo, Japan). The codon numbers of *rpoB* were designated on the basis of alignment of the *E. coli rpoB* sequence with a portion of the *M. tuberculosis* H37Rv sequence and are not the positions of the actual *M. tuberculosis rpoB* codons (14, 31).

**Cloning of *katG*.** The coding regions of *katG* from six INH-resistant clinical isolates of *M. tuberculosis*, two INH-susceptible isolates, and the H37Rv strain were cloned. *katG* was amplified by PCR with 2.5 U of Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) and primers PR3 and PR4 (Table 2). The PCR products were ligated into the pCRT7/NT vector downstream of the region encoding a His<sub>6</sub> tag.

**KatG enzyme assays.** For expression of *M. tuberculosis* KatG, *katG*-deficient *E. coli* UM262 (13) cells were transformed with pCRT7/NT carrying cloned *katG* genes derived from eight clinical isolates and the H37Rv strain. KatG-mediated catalase activity was assayed spectrophotometrically by monitoring the decrease in H<sub>2</sub>O<sub>2</sub> concentration at A<sub>240</sub> (ε<sub>240</sub> = 0.0436 mM<sup>-1</sup> cm<sup>-1</sup>) as described previously (18).

**Assay for free-radical formation from INH oxidation.** Rates of KatG-mediated free-radical formation from INH oxidation in the presence of H<sub>2</sub>O<sub>2</sub> were monitored spectrophotometrically by following the reduction of nitroblue tetrazolium (NBT) as described previously (35).

**Purification of KatG.** *M. tuberculosis* KatG from strain H37Rv was overexpressed in *E. coli* BL21-AI cells and purified with chelating Sepharose (Ni Sepharose 6 Fast Flow; Amersham Biosciences) loaded with Ni<sup>2+</sup> in a column. The purity of the KatG protein was more than 95% by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Enzyme-linked immunosorbent assay and Western blotting.** Purified His-tagged KatG was used as an antigen to raise polyclonal antibodies in a male Japanese white rabbit. Antiserum against KatG was used for Western blotting and enzyme-linked immunosorbent assay.

**Data analysis.** The correlations between mutation data from the DNA sequence-based assays and data from conventional culture methods with drugs or PZase activities were assessed by the index of test efficiency. The efficiency of a test was defined as the percentage of times that the test gave the correct answer compared to the total number of tests.

## RESULTS

**Drug susceptibility patterns.** The susceptibility patterns of the 138 clinical isolates for six drugs, RIF, INH, EMB, PZA, STR, and OFX, are shown in Table 1. Among the 138 clinical isolates, 55 were resistant to at least one drug and 23 were MDR strains displaying resistance to both INH and RIF. Twenty of the 23 MDR strains were resistant to at least one other drug in addition to INH and RIF. Eighty-three clinical isolates and 2 laboratory strains, H37Rv and H37Ra, were susceptible to all of the drugs tested. The BCG strain

TABLE 2. Primers used to detect MDR tuberculosis

Target gene <sup>a</sup>	Primer set (direction)	Nucleotide sequence	Positions <sup>b</sup>	Product size (bp)
<b>PCR primers</b>				
<i>rpoB</i>	PR1 (forward)	5'-CCGCGATCAAGGAGTTCTTC-3'	1256–1275	315
	PR2 (reverse)	5'-ACACGATCTCGTCGCTAAC-3'	1570–1551	
<i>katG</i>	PR3 (forward)	5'-GTGCCCAGCAACACCCACCCATTACAGAAAC-3'	1–32	2,223
	PR4 (reverse)	5'-TCAGCGCACGTCGAACCTGTGCGAG-3'	2223–2200	
<i>mabA</i> promoter	PR5 (forward)	5'-ACATACCTGCTGCGCAATTC-3'	–217 to –198	1,362
	PR6 (reverse)	5'-GCATACGAATACGCCGAGAT-3'	1145–1126	
<i>embB</i>	PR7 (forward)	5'-CCGACCACGCTGAAACTGCTGGCGAT-3'	640–665	2,748
	PR8 (reverse)	5'-GCCTGGTGCATACCGAGCAGCATAG-3'	3387–3303	
<i>pncA</i>	PR9 (forward)	5'-GGCGTCATGGACCCATATC-3'	–80 to –61	670
	PR10 (reverse)	5'-CAACAGTTCATCCCGGTTTC-3'	590–572	
<i>rpsL</i>	PR11 (forward)	5'-CCAACCATCCAGCAGCTGGT-3'	4–23	572
	PR12 (reverse)	5'-GTCGAGAGCCCGCTTGAGGG-3'	575–556	
<i>rns</i> (16S RNA)	PR13 (forward)	5'-AAACCTCTTTACCATCGAC-3'	428–447	1,329
	PR14 (reverse)	5'-GTATCCATTGATGCTCGCAA-3'	1756–1737	
<i>gyrA</i>	PR15 (forward)	5'-GATGACAGACACGACGTTGC-3'	–1–19	398
	PR16 (reverse)	5'-GGGCTTCGGTGTACCTCAT-3'	397–379	
<b>Sequencing primers</b>				
<i>rpoB</i>	PR17	5'-TACGGCGTTTCGATGAAC-3' (complementary strand)	1529–1512	
<i>katG</i>	PR18	5'-ACGTAGATCAGCCCCATCTG-3' (complementary strand)	689–670	
	PR19	5'-GAGCCCGATGAGGTCTATTG-3'	574–593	
	PR20	5'-CCGATCTATGAGCGGATCAC-3'	1162–1181	
	PR21	5'-GAACAAACCGACGTGGAATC-3'	1729–1748	
<i>mabA</i> promoter	PR22	5'-ACATACCTGCTGCGCAATTC-3'	–217 to –198	
<i>embB</i>	PR23	5'-ACGCTGAAACTGCTGGCGAT-3'	646–665	
	PR24	5'-GTCATCCTGACCGTGGTGT-3'	1462–1481	
	PR25	5'-GGTGGGCAGGATGAGGTAGT-3' (complementary strand)	1596–1577	
	PR26	5'-CACAATCTTTTCGCCCTGT-3'	2007–2026	
	PR27	5'-GCGTGGTATCTCCTGCCTAAG-3'	2581–2601	
<i>pncA</i>	PR28	5'-GGCGTCATGGACCCATATC-3'	–80 to –61	
<i>rpsL</i>	PR29	5'-CCAACCATCCAGCAGCTGGT-3'	4–23	
<i>rns</i> (16S RNA)	PR30	5'-CAGGTAAGGTTCTTCGCGTTG-3' (complementary strand)	979–959	
	PR31	5'-GTTCCGATCGGGGTCTGCAA-3'	1291–1310	
<i>gyrA</i>	PR32	5'-GATGACAGACACGACGTTGC-3'	–1–19	

<sup>a</sup> The complete sequences of target genes in *M. tuberculosis* H37Rv are in the GenBank database under accession no. NC\_000962.

<sup>b</sup> Numbering based on nucleotide position relative to the initiation codon of each gene.

was sensitive to all drugs tested except PZA. When the results of the proportion method were compared with those of the Vit Spectrum-SR and BD BACTEC MGIT 960 SIRE methods, there was full agreement for all drugs. However, there were some differences in the degrees of susceptibility to INH between the methods. One hundred isolates were susceptible to 0.2 µg/ml of INH when they were assessed with the solid media, whereas only 6 of these isolates were resistant to 0.1 µg/ml of INH when they were tested by the broth method.

**Two-temperature PCR.** We optimized a two-temperature PCR strategy to amplify regions of eight drug resistance-related genes in *M. tuberculosis*. The target regions varied in length from 315 to 2,748 bp (Table 2). Genomic DNA (approximately 100 ng) from *M. tuberculosis* strain H37Rv was amplified with eight primer pairs simultaneously. The entire procedure, including PCR, took less than 60 min. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide (Fig. 1A). Genomic DNAs from strain H37Ra and 138 clinical isolates of *M. tuberculosis*



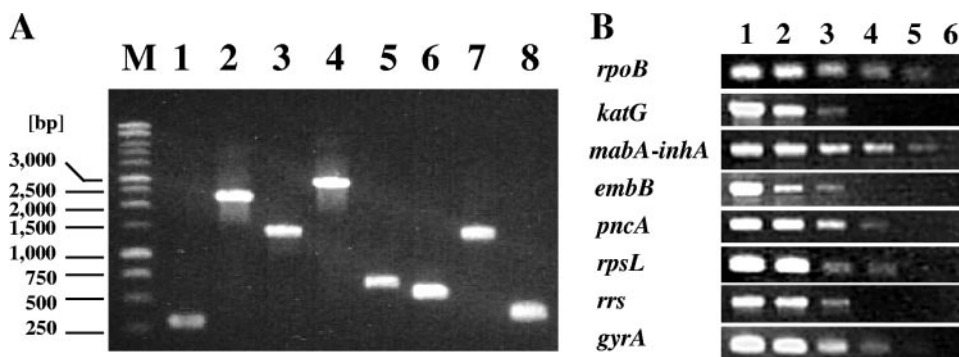


FIG. 1. (A) Amplification products from two-temperature PCR of *M. tuberculosis* H37Rv. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. Lane M, 1-kbp ladder as a molecular size marker; lane 1, *rpoB*; lane 2, *katG*; lane 3, *mabA-inhA* locus; lane 4, *embB*; lane 5, *pncA*; lane 6, *rpsL*; lane 7, *rrs*; and lane 8, *gyrA*. (B) Determination of the sensitivity of two-temperature PCR with serially diluted *M. tuberculosis* H37Rv DNA as a template. Experiments were repeated twice with similar results. Lane 1, 100 ng of template DNA; lane 2, 10 ng; lane 3, 1 ng; lane 4, 100 pg; lane 5, 10 pg; lane 6, 1 pg.

were then amplified by PCR. Each PCR yielded a single band of the expected length (data not shown). These results indicate that the PCR is reliable for use in clinical isolates of *M. tuberculosis*.

**Sensitivity and specificity of two-temperature PCR.** To determine the sensitivity of the two-temperature PCR for the target DNA, 100, 10, and 1 ng and 100, 10, and 1 pg of genomic DNA from *M. tuberculosis* H37Rv were amplified with the PCR assay. As shown in Fig. 1B, the limits of detection for *mabA-inhA* and *rpoB*, for *gyrA*, *pncA*, and *rpsL*, and for *embB*, *katG*, and *rrs* were 10 pg, 100 pg, and 1 ng of DNA, respectively.

To determine the species specificity of the PCR, genomic DNA (100 ng) was isolated from various species of bacteria, including *Mycobacterium* spp. and additional pathogenic bacterial species listed in Table 1. DNA was amplified with the PCR primer pairs shown in Table 2. The PCR patterns for *M. bovis* BCG were identical to those of *M. tuberculosis* (Table 3). Some mycobacterial species were positive for the PCR with primer pairs for *rpsL*, *rrs*, and *gyrA*; however, all mycobacterial species except *M. tuberculosis* and *M. bovis* were negative for all eight gene targets.

**Sequencing of *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. tuberculosis*.** PCR products were purified and then sequenced with the 16 sequencing primers listed in Table 2. Sequencing yielded 8.8 kb of sequence for each *M. tuberculosis* strain. Sequences were obtained for regions of the *rpoB*, *katG*, *mabA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* genes and the promoters of *mabA-inhA* and *pncA*. The mutations identified in the 138 clinical isolates of *M. tuberculosis* are shown in Table 4.

(i) ***rpoB*.** We sequenced a 240-bp fragment containing the "81-bp core region" of *rpoB*. One hundred nine isolates had no mutations in *rpoB*. The remaining 25 each had a single point mutation, and 4 isolates each had two point mutations. All of the detected mutations resulted in amino acid substitutions. Of these mutations, two, S450L (TCG→TTG at nucleotide [nt] positions 1348 to 1350) and S509R (AGC→AGG at nt positions 1525 to 1527) were novel (Table 4).

(ii) ***katG*.** Thirty-seven isolates had no mutations in *katG*, whereas 81 isolates each had a single point mutation, 11 isolates had two point mutations each, 1 isolate had three point mutations, and 2 isolates had a 3-bp insertion each. One mutation was a silent mutation (CTG→TTG at nt positions 1957 to 1959 [L653L]), and all others caused amino acid substitutions. We identified 10 novel point mutations and the novel L390 insertion.

(iii) ***mabA-inhA* locus.** We found no mutations in the *mabA* gene and the regulatory region of *mabA-inhA* in 126 isolates. Ten isolates had a C-to-T transition -15 bp upstream of the *mabA* initiation codon, 1 isolate had a T-to-A transition 8 bp upstream of the initiation codon, and 1 isolate had a T-to-A transition 5 bp upstream of the initiation codon. The T-to-A transition -5 bp upstream of the initiation codon was novel.

(iv) ***embB*.** We found no mutations in *embB* in 107 isolates. Twenty-six isolates each had a single point mutation, 3 each had two point mutations, and 2 each had three point mutations. Several isolates had silent mutations (D345D, D534D, L355L, and P1075P). Five of these point mutations were novel.

(v) ***pncA*.** One hundred nineteen isolates had no mutations in *pncA* or the *pncA* regulatory region. Nineteen isolates each had a single point mutation. Five of these mutations were novel.

(vi) ***gyrA*.** All isolates tested contained the E21Q mutation of *gyrA*. Eighteen isolates each had one point mutation, 117 isolates each had two point mutations, 2 isolates each had three point mutations, and 1 isolate carried four mutations.

(vii) ***rpsL*.** All isolates carried the K121K mutation of *rpsL*. One hundred twenty-five isolates each had a single point mutation, and the remaining 13 isolates each had two point mutations.

(viii) ***rrs*.** One hundred thirty-three isolates had no mutations in *rrs*. Two isolates had a C-to-T transition at nt position 516. One isolate had an A-to-G transition at nt position 1400. One isolate had two point mutations, an A-to-G transition at nt position 1400 and an A-to-G transition at nt position 1539. One isolate had an insertion of a cytosine at position 1061 of *rrs*.

TABLE 3. Species specificity of two-temperature PCR

Bacterium	Origin	Results of PCR with various primer pairs <sup>g</sup>							
		<i>rpoB</i>	<i>katG</i>	<i>mabA-inhA</i>	<i>embB</i>	<i>pncA</i>	<i>rpsL</i>	<i>rrs</i>	<i>gyrA</i>
<i>M. tuberculosis</i> IMCJ	105 clinical isolates from Japan	+	+	+	+	+	+	+	+
<i>M. tuberculosis</i> P	33 clinical isolates from Poland	+	+	+	+	+	+	+	+
<i>M. tuberculosis</i> H37Rv	ATCC <sup>a</sup> 27294	+	+	+	+	+	+	+	+
<i>M. tuberculosis</i> H37Ra	ATCC 25177	+	+	+	+	+	+	+	+
<i>M. bovis</i> BCG Japanese strain 172	Japan BCG Laboratory <sup>b</sup>	+	+	+	+	+	+	+	+
<i>M. avium</i>	ATCC 25291	-	-	-	-	-	+	+	-
<i>M. gastri</i>	GTC <sup>c</sup> 610 (ATCC 15754)	-	-	-	-	-	+	+	+
<i>M. intracellulare</i>	JCM <sup>d</sup> 6384 (ATCC 13950)	-	-	-	-	-	+	+	-
<i>M. kansasii</i>	JCM 6379 (ATCC 124878)	-	-	-	-	-	+	+	-
<i>M. marinum</i>	GTC 616 (ATCC 927)	-	-	-	-	-	+	+	+
<i>M. simiae</i>	GTC 620 (ATCC 25275)	-	-	-	-	-	-	-	+
<i>M. scrofulaceum</i>	JCM 6381 (ATCC 19981)	-	-	-	-	-	-	+	-
<i>M. szulgai</i>	JCM 6383 (ATCC 35799)	-	-	-	-	-	+	+	+
<i>M. nonchromogenicum</i>	JCM 6364 (ATCC 19530)	-	-	-	-	-	+	-	-
<i>M. terrae</i>	GTC 623 (ATCC 15755)	-	-	-	-	-	-	-	-
<i>M. chelonae</i>	JCM 6390 (ATCC 14472)	-	-	-	-	-	+	-	-
<i>M. fortuitum</i>	RIMD <sup>e</sup> 1317004 (ATCC 6841)	-	-	-	-	-	+	-	-
<i>M. phlei</i>	RIMD 1326001 (ATCC 19249)	-	-	-	-	-	-	-	-
<i>M. smegmatis</i>	ATCC 19420	-	-	-	-	-	+	-	-
<i>Escherichia coli</i>	ATCC 8739	-	-	-	-	-	-	-	-
<i>Haemophilus influenzae</i>	IID <sup>f</sup> 984 (ATCC 9334)	-	-	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	IID5209 (ATCC 15380)	-	-	-	-	-	-	-	-
<i>Legionella pneumophila</i>	GTC 745	-	-	-	-	-	-	-	-
<i>Mycoplasma pneumoniae</i>	IID 817	-	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	ATCC 27853	-	-	-	-	-	-	-	-
<i>Rhodococcus equi</i>	ATCC 33710	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i>	N315	-	-	-	-	-	-	-	-
<i>Streptococcus pneumoniae</i>	GTC 261	-	-	-	-	-	-	-	-

<sup>a</sup> American Type Culture Collection, Rockville, MD.

<sup>b</sup> Japan BCG Laboratory, Tokyo, Japan.

<sup>c</sup> Gifu Type Culture Collection, Department of Microbiology-Bioinformatics, Regeneration and Advanced Medical Science, Gifu University, Graduate School of Medicine, Bacterial Genetic Resources, Gifu, Japan.

<sup>d</sup> Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan.

<sup>e</sup> Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

<sup>f</sup> Institute of Medical Science, University of Tokyo, Tokyo, Japan.

<sup>g</sup> Amplification results were determined by agarose gel electrophoresis. Symbols: +, presence of amplification products; -, absence of amplification products.

**Correlation between drug susceptibility and mutation(s) in *M. tuberculosis*.** (i) **RIF resistance and *rpoB*.** Mutations in the 81-bp core region of *rpoB* are responsible for resistance in at least 96% of RIF-resistant *M. tuberculosis* isolates (17, 31, 39). In the present study, we identified two novel mutations, S450L (TCG→TTG at nt positions 1348 to 1350) and S509R (AGC→AGG at nt positions 1525 to 1527). S450L was located upstream of the 81-bp core region, and the isolate with S450L was susceptible to RIF, indicating this mutation is not associated with RIF resistance. Isolates with both S509R and H526R (CAC→CGC at nt positions 1576 to 1578) mutations were RIF resistant. However, it is unclear whether S509R is associated with RIF resistance because H526R is known to be associated with RIF resistance (39).

(ii) **INH resistance and *katG* and *mabA-inhA*.** INH resistance is related to mutation(s) in *katG*, *inhA*, and/or the promoter region of *mabA-inhA* (17, 22, 38, 39). In the present study, we found 11 novel mutations and a CTA insertion at nt position 1170 in *katG*. Among these mutations, Q295P and G297V conferred INH resistance. Two INH-resistant isolates carried the L141F and R463L mutations. R463L is known not to be associated with INH resistance (33, 39), and L141F may confer INH resistance. The A65T, A245V, and V725A mutations did not influence INH susceptibility. Isolates carrying the

T324P, L48Q, or M257T mutation and -15C→T upstream of *mabA* were resistant to INH. However, it is unclear whether T324P, L48Q, or M257T is related to INH resistance because the -15C→T upstream of *mabA* is known to confer INH resistance (10, 22, 39).

(iii) **EMB resistance and *embB*.** EMB resistance is related to mutations in *embB* (17, 32, 39). In the present study, we found five novel mutations in *embB*. Among these, D354A conferred EMB resistance. V492L, A680T, and A1007V were not associated with EMB resistance. An isolate with both N296Y and M306I was resistant to EMB. However, it is unclear whether N296Y is related to EMB resistance because the M306I mutation is known to confer EMB resistance (39).

(iv) **PZA resistance and *pncA*.** It is known that PZA resistance is related to mutations in *pncA* (17, 26, 39). In the present study, we identified five novel mutations in *pncA*. Among these, A3E, D53N, P54L, C72W, and M175V conferred PZA resistance. It will be necessary to determine whether the PZA activities of various mutants are correlated with PZA susceptibility. We then evaluated the PZase activities of *M. tuberculosis* clinical isolates, strains H37Rv and H37Ra, and *M. bovis* strain BCG. The BCG strain was included as a negative control as described in Materials and Methods. One hundred twenty-one clinical isolates and H37Rv

TABLE 4. Nucleotide and amino acid changes found in 138 clinical isolates of *M. tuberculosis*<sup>a</sup>

Gene	No. of isolates (n = 138)	Isolate origin	Changes		% Resistant (no. of isolates displaying resistance)	Other mutations
			Nucleotide	Amino acid (silent mutation)		
<i>rpoB</i> <sup>b</sup>	83	Japan	None	None	0	
	26	Poland	None	None	0	
	1	Japan	TCG→TTG	S450L*	0	
	1	Poland	CAA→CTA	Q513L	100	
	1	Japan	GAC→GTC	D516V	100	
	1	Japan	TCG→TTG	S522L	100	
	1	Japan	CAC→TAC	H526Y	100	
	2	Japan	CAC→CGC	H526R	100	
	1	Poland	CAC→ACC	H526T	100	
	7	Japan	TCG→TTG	S531L	100	
	5	Japan	TCG→TGG	S531W	100	
	5	Poland	TCG→TTG	S531L	100	
	2	Japan	AGC→AGG and CAC→CGC	S509R* and H526R	100	
	2	Japan	ATG→ATT and GAC→TAC	M515I and D516Y	100	
	<i>katG</i>	14	Japan	None	None	0
23		Poland	None	None	4.4 (1)	
1		Japan	CTG→TTG	(L653L)	0	
1		Japan	GCC→ACC	A65T*	0	
1		Japan	GCG→CTG	A245V*	0	
1		Poland	CAG→CCG	Q295P*	100	
1		Poland	GGC→GTC	G297V*	100	
4		Japan	AGC→ACC	S315T	100	
2		Japan	AGC→AAC	S315N	100	
3		Poland	AGC→ACC	S315T	100	
1		Japan	GTC→GCC	V725A*	0	
63		Japan	CGG→CTG	R463L	3.2 (2)	
2		Japan	CGG→CTG	R463L	100	-15C→T upstream of <i>mabA</i>
1		Poland	ACC→CCC	T324P*	100	-15C→T upstream of <i>mabA</i>
7		Japan	AGC→ACC and CGG→CTG	S315T and R463L	100	
2		Japan	TTG→TTC and CGG→CTG	L141F* and R463L	100	
1		Japan	ATG→ACG and CGG→CTG	M257T* and R463L	100	-5T→A* upstream of <i>mabA</i>
1		Japan	CTG→CAG and CGG→CTG	L48Q* and R463L	100	-15C→T upstream of <i>mabA</i>
1		Japan	ATG→ACG, CGG→CTG, and GTC→GCC	M257T*, R463L and V708P*	100	-15C→T upstream of <i>mabA</i>
2		Japan	CTA insertion at position 1170	L390 insertion*	100	
<i>mabA-inhA</i> operon <sup>c</sup>	97	Japan	None	None	0	
	29	Poland	None	None	3.4 (1)	
	2	Japan	-15C→T upstream of <i>mabA</i>	None	100	R463L in <i>katG</i>
	1	Poland	-15C→T upstream of <i>mabA</i>	None	100	T324P* in <i>katG</i>
	1	Japan	-15C→T upstream of <i>mabA</i>	None	100	L48Q* and R463L in <i>katG</i>
	1	Japan	-15C→T upstream of <i>mabA</i>	None	100	M257T*, R463L and V708P* in <i>katG</i>
	1	Japan	-15C→T upstream of <i>mabA</i>	None	100	
	4	Poland	-15C→T upstream of <i>mabA</i>	None	100	
	1	Japan	-8T→A upstream of <i>mabA</i>	None	100	
1	Japan	-5T→A* upstream of <i>mabA</i>	None	100	M257T* and R463L in <i>katG</i>	
<i>embB</i>	77	Japan	None	None	0	
	30	Poland	None	None	3.3 (1)	
	2	Japan	GAC→GAT	(D345D)	100 (2)	
	1	Japan	GAC→GAT	(D534D)	0	
	5	Japan	ATG→GTG	M306V	100	
	1	Japan	ATG→ATT	M306I	100	
	1	Japan	ATG→ATC	M306I	100	
	2	Poland	ATG→ATA	M306I	100	
	2	Japan	GAC→GCC	D354A*	100	

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TABLE 4—Continued

Gene	No. of isolates (n = 138)	Isolate origin	Changes		% Resistant (no. of isolates displaying resistance)	Other mutations
			Nucleotide	Amino acid (silent mutation)		
	4	Japan	GAG→GCG	E378A	0	
	1	Japan	GTG→TTG	V492L*	0	
	1	Poland	CAG→CGG	Q497R	100	
	2	Japan	GCC→ACC	A680T*	0	
	3	Japan	GCC→GTC	A1007V*	0	
	1	Japan	GAC→AAC	D1024N	0	
	2	Japan	CTG→CTA and GAG→GCG	(L355L) and E378A	0	
	1	Japan	AAT→TAT and ATG→ATA	N296Y* and M306I	100	
	2	Japan	ATG→CTG, GAG→GCG, and CCC→CCA	M306L, E378A, and (P1075P)	100	
<i>pncA</i>	89	Japan	None	None	0	
	30	Poland	None	None	0	
	2	Japan	TCC→TCT	(S65S)	0	
	1	Japan	GCG→GAG	A3E*	100	
	1	Poland	CAG→CCG	Q10P	100	
	1	Japan	GAC→GCC	D12A	100	
	2	Japan	CAC→CAA	H51Q	100	
	1	Poland	CAC→CAG	H51Q	100	
	3	Japan	CCG→CTG	P54L*	100	
	1	Japan	TGC→TGG	C72W*	100	
	2	Japan	GGT→AGT	G132S	100	
	2	Japan	ATT→ACT	I133T	100	
	1	Poland	CGC→AGC	R148S	100	
	1	Japan	ATG→GTG	M175V*	100	
	1	Japan	GAC→AAC	D53N*	100	
<i>gyrA</i>	7	Japan	GAG→CAG	E21Q*	0	
	11	Poland	GAG→CAG	E21Q*	0	
	95	Japan	GAG→CAG and AGC→ACC	E21Q* and S95T	0	
	22	Poland	GAG→CAG and AGC→ACC	E21Q* and S95T	0	
	1	Japan	GAG→CAG, GAC→GGC, and AGC→ACC	E21Q*, D94G, and S95T	100	
	1	Japan	GAG→CAG, GCG→GTG, and AGC→ACC	E21Q*, A90V, and S95T	100	
	1	Japan	GAG→CAG, GCG→GTG, GAC→GCC, and AGC→ACC	E21Q*, A90V, D94A, and S95T	100	
<i>rpsL</i>	87	Japan	AAA→AAG	(K121K)	11.5 (10)	
	33	Poland	AAA→AAG	(K121K)	6.1 (2)	
	2	Japan	AAA→AAG	(K121K)	100	516C→T in <i>rps</i>
	1	Japan	AAA→AAG	(K121K)	100	1,061C insertion* in <i>rps</i>
	1	Japan	AAA→AAG	(K121K)	100	1,400A→G in <i>rps</i>
	1	Japan	AAA→AAG	(K121K)	100	1,400A→G and 1539A→G in <i>rps</i>
	13	Japan	AAG→AGG and AAA→AAG	K43R and (K121K)	100	
<i>rps</i>	33	Poland	None		6.1 (2)	(K121K) in <i>rpsL</i>
	87	Japan	None		11.5 (10)	(K121K) in <i>rpsL</i>
	13	Japan	None		100	K43R and (K121K) in <i>rpsL</i>
	2	Japan	516C→T		100	(K121K) in <i>rpsL</i>
	1	Japan	1,061C insertion*		100	(K121K) in <i>rpsL</i>
	1	Japan	1,400A→G		100	(K121K) in <i>rpsL</i>
	1	Japan	1,400A→G and 1539A→G		100	(K121K) in <i>rpsL</i>

\*, mutation not previously reported.

<sup>b</sup> The codon numbering system of RpoB initially described by Telenti et al. (31) was used. The codon numbers of RpoB are designated on the basis of alignment of translated *E. coli rpoB* sequence with a portion of translated *M. tuberculosis* sequence and are not the positions of the actual *M. tuberculosis rpoB* codons.

<sup>c</sup> Nucleotide numbering based on nucleotide position relative to *mabA* start codon.

and H37Ra were positive for PZase activity (data not shown). The remaining 17 *M. tuberculosis* clinical isolates and *M. bovis* BCG were negative for PZase activity. All PZase-positive bacilli were sensitive to PZA, and all PZase-negative bacilli were

resistant to PZA. These data were consistent with previously published results (15, 39).

(v) **STR resistance and *rpsL* and *rps*.** STR resistance is related to mutations in *rpsL* and *rps* (17, 19, 39). In the present



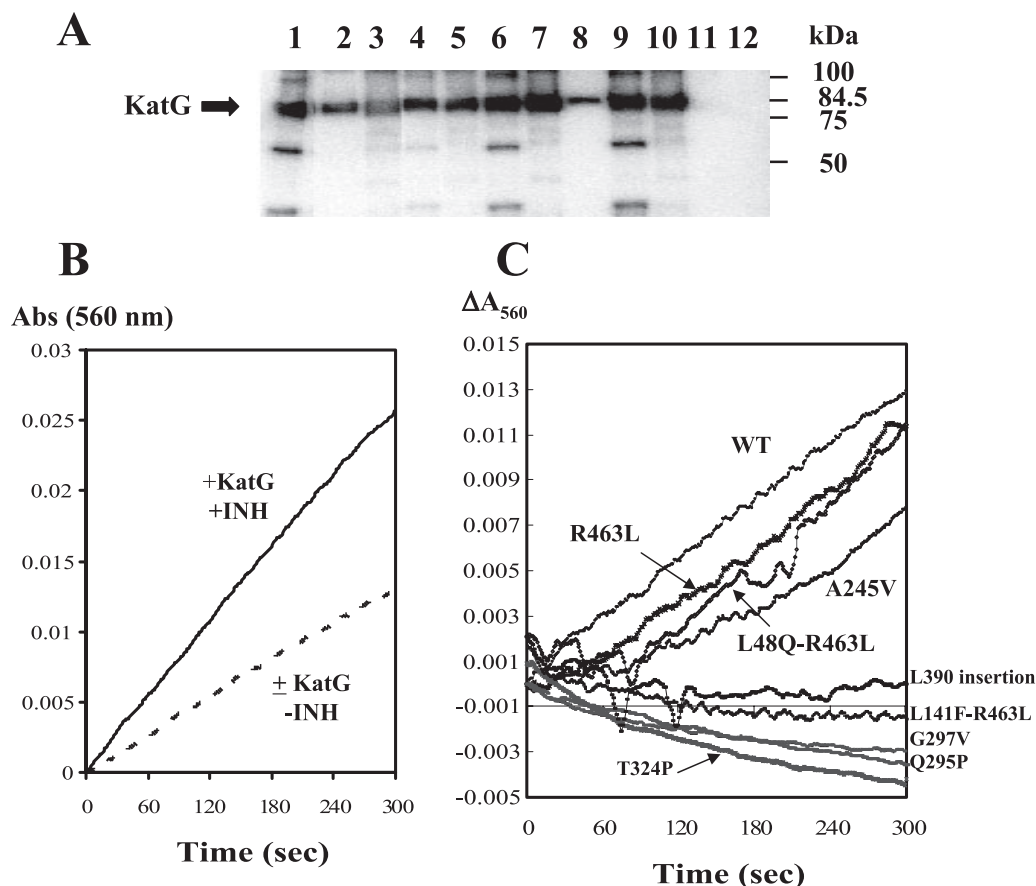


FIG. 2. (A) Western blot analysis of whole-cell protein extracts from KatG-deficient *E. coli* strain UM262 complemented with a control plasmid, pCRT7/NT, or recombinant plasmids expressing various KatG mutants. Lane 1, wild-type KatG; lane 2, KatG<sup>Q295P</sup>; lane 3, KatG<sup>T324P</sup>; lane 4, KatG<sup>L48Q-R463L</sup>; lane 5, KatG<sup>R463L</sup>; lane 6, KatG<sup>G297V</sup>; lane 7, KatG<sup>A245V</sup>; lane 8, KatG<sup>L390 insertion</sup>; lane 9, KatG<sup>L141F-R463L</sup>; lane 10, purified KatG protein; lane 11, control plasmid pCRT7/NT; and lane 12, *E. coli* strain UM262. The positions of molecular mass markers are shown. (B) Time course of NBT reduction with and without the addition of 2.4 mM INH with wild-type KatG. Abs, absorbance. (C) Time course of net INH-dependent NBT reduction by wild-type KatG (WT) and eight KatG mutants. For each KatG sample tested, NBT reduction in the absence of INH was subtracted from that in the presence of INH to obtain the net INH-dependent NBT reduction over time. The concentration of KatG was determined by enzyme-linked immunosorbent assay.

study, all isolates, regardless of STR resistance status, had a silent mutation (K121K) in *rpsL*, and, therefore, the K121K mutation is not associated with STR resistance.

We found a novel insertional mutation in *rrs*. The insertion is the likely cause of STR resistance, because the isolate with the mutation was resistant to STR.

(vi) **FQ resistance and *gyrA***. Mutations in the FQ resistance-determining region (QRDR) in *gyrA* are responsible for resistance in at least 96% of FQ-resistant *M. tuberculosis* isolates (17, 30, 39). In the present study, we found one novel mutation, E21Q, in *gyrA*, and all isolates tested, except the H37Rv strain, contained this mutation. However, some isolates were susceptible to FQs and others were resistant. Therefore, it is not clear that this mutation is associated with resistance to FQs. E21Q is located upstream of the QRDR.

**Catalase and INH oxidation activities of recombinant KatG mutants.** KatG, catalase-peroxidase, converts INH to its biologically active form (38). Some mutations in *katG* reduce or eliminate the enzymatic activity that is associated with INH resistance (9, 39). To measure catalase and INH oxidation activities, we expressed wild-type KatG and the A245V,

Q295P, G297V, T324P, L48Q-R463L, L141F-R463L, R463L, and L390 insertion mutants of KatG in *katG*-deficient *E. coli* (Fig. 2A and Table 5). The catalase activities of these mutants were determined at various H<sub>2</sub>O<sub>2</sub> concentrations. The  $k_{cat}$ ,  $K_m$ , and  $k_{cat}/K_m$  ratio values are shown in Table 5. Catalase activity was not detected for the KatG<sup>Q295P</sup>, KatG<sup>G297V</sup>, KatG<sup>T324P</sup>, KatG<sup>L141F-R463L</sup>, or KatG<sup>L390 insertion</sup> mutants. The  $k_{cat}$  of the KatG<sup>L48Q-R463L</sup> mutant was 26% lower than that of wild-type KatG. In contrast, the KatG<sup>A245V</sup> and KatG<sup>R463L</sup> mutants showed activities similar to that of wild-type KatG.

The INH oxidation activities of the mutants were determined in the presence of H<sub>2</sub>O<sub>2</sub> by monitoring the free radical generation in the NBT reduction reaction. When wild-type KatG was tested in this assay, there was a significant background activity of NBT reduction in the absence of INH, whereas the NBT reduction was increased significantly in the presence of INH (Fig. 2B). We subtracted the background activity to obtain the net INH oxidation/NBT reduction. The net values are shown in Fig. 2C. The KatG<sup>Q295P</sup>, KatG<sup>G297V</sup>, KatG<sup>T324P</sup>, KatG<sup>L141F-R463L</sup>, and KatG<sup>L390 insertion</sup> mutants did not show enhanced activity, whereas wild-type KatG and

TABLE 5. Catalase activity of wild-type and mutant KatG, KatG-mediated INH-converting activity, and INH susceptibility

Recombinant KatG <sup>a</sup>	Catalase activity			KatG-mediated INH-converting activity <sup>b</sup>	INH susceptibility <sup>c</sup>
	$k_{cat}$ (S <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}/K_m$ ratio (mutant/wild type)		
Wild type	2,403 ± 440	60.1 ± 9.5	1.00	+	S
A245V*	2,666 ± 530	62.8 ± 10.8	1.06	+	S
Q295P*	ND <sup>d</sup>	ND	NA <sup>e</sup>	-	R
G297V*	188 ± 36	68.7 ± 7.0	0.07	-	R
T324P*	ND	ND	NA	-	R
L48Q*-R463L	1,776 ± 310	64.8 ± 12.5	0.69	+	R
L141F*-R463L	ND	ND	NA	-	R
R463L	2543 ± 450	64.0 ± 11.0	1.01	+	S
L390 (CTA) insertion*	ND	ND	NA	-	R

<sup>a</sup> \*, novel mutation.

<sup>b</sup> Time courses of net KatG-mediated INH conversion are shown in Fig. 2C.

<sup>c</sup> INH susceptibilities shown are those of *M. tuberculosis* H37Rv strains having the *katG* gene of the wild type and those of clinical strains having the *katG* gene with the respective mutation(s). S, susceptible; R, resistant.

<sup>d</sup> ND, not detected.

<sup>e</sup> NA, not applicable.

the KatG<sup>L48Q-R463L</sup>, KatG<sup>A245V</sup>, and KatG<sup>R463L</sup> mutants of KatG showed significant enhancement of activity in the presence of INH.

Collectively, these results for the enzymatic activities of KatG mutants indicate that the Q295P, G297V, T324P, L141F, and L390 insertion mutants cause loss of enzymatic activity, whereas the A245V and R463L mutants have no effect on the enzymatic activity. The L48Q mutation has little effect on enzymatic activity; however, there was no isolate that carried only the L48Q mutation in KatG in the present study. These mutations and enzymatic activities, except for those of the L48Q-R463L mutant, correlated well with INH susceptibility (Table 5). The L48Q-R463L mutant also carried the -15C→T transition upstream of *mabA* (Table 4). Therefore, the INH resistance of this mutant is likely due to the -15C→T mutation, which is known to be related to INH resistance (10, 22, 39).

**Sequencing of *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. bovis* BCG.** PCR products amplified from *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. bovis* BCG were sequenced with the same sequencing primers as those for *M. tuberculosis* (Table 2). When the nucleotide sequences of BCG were compared with those of *M. bovis* AF2122/97 (GenBank accession no. NC\_002945) (8), the sequences were identical. When the sequences of *M. bovis* BCG were compared with those of *M. tuberculosis* H37Rv, the sequences of *rpoB*, the promoter region of the *mabA-inhA* operon, and *rrs* were identical. The R463L (CGG→CTG at nt positions 1387 to 1389) and silent P29P (CCC→CCA at nt positions 85 to 87) mutations were found in *katG* of BCG. E378A (GAG→GCG at nt positions 1159 to 1161) in *embB*, H57D (CAC→GAC at nt positions 169 to 171) in *pncA*, K121K (AAA→AAG at nt positions 361 to 363 [silent]) in *rpsL*, and S95T (AGC→ACC at nt positions 283 to 285) in *gyrA* were identified in *M. bovis* BCG.

**Correlation between drug susceptibility and mutations in BCG.** We next compared the sequences of BCG and *M. tuberculosis* H37Rv and found four mutations, R463L in *katG*, E378A in *embB*, H57D in *pncA*, and S95T in *gyrA*, that caused amino acid substitutions in *M. bovis* BCG. R463L in *katG* is known not to be associated with INH resistance in *M. tuber-*

*culosis* (33) and may not be associated with INH resistance in *M. bovis*. E378A in *embB* is not associated with EMB resistance in *M. tuberculosis* (32, 39). H57D in *pncA* was reported previously and is characteristic of PZA resistance in *M. bovis* (39). S95T in *gyrA* is not associated with FQ resistance in *M. tuberculosis* (39). Therefore, in *M. bovis*, mutations except H57D in *pncA* may be polymorphisms not associated with drug resistance.

**Detection and sequencing of drug resistance-related genes of *M. tuberculosis* in sputa from tubercular patients.** We tested a total of 10 sputa from 10 tuberculosis-diagnosed patients. These patients had been received treatment with antitubercular drugs. Of these samples, six were positive for acid-fast bacilli (AFB; >101/field in two samples, 26 to 50/field in two samples, 1/field in one sample, and 1/several fields in one sample) under microscopic observation, and four were negative. Five of the six samples that were positive for AFB were positive for all eight genes tested by PCR. One sample which was positive for AFB (one/several fields) was positive for five genes (*rpoB*, *pncA*, *rpsL*, *rrs*, and *gyrA*) by PCR. The four AFB-negative samples yielded no PCR products and were negative by culture, suggesting that tuberculosis was not active in these patients who had received treatment.

PCR products (a total of 45) were subjected to DNA sequencing. No mutations were identified in 38 of the PCR products. The remaining seven PCR products contained nine mutations. The seven PCR products were obtained from one sputum sample. The sputum sample was cultured, and after several weeks, an isolate of *M. tuberculosis* was obtained and analyzed. We conducted PCR analysis of this isolate and detected the same nine mutations. This isolate was resistant to RIF, INH, EMB, STR, FQs, and PZA. These results indicate that our DNA sequencing-based method can be used to detect MDR strains of *M. tuberculosis* in sputa obtained from clinical tuberculosis patients.

## DISCUSSION

Our novel DNA sequencing-based method described here is useful for detection and diagnosis of drug-resistant strains of *M. tuberculosis*, especially MDR strains. Our method has

several advantages. First, it allows simultaneous detection of mutations in eight genes associated with resistance to six antituberculosis drugs. Second, the entire assay from DNA extraction to the DNA sequencing can be completed within 1 working day. Third, this method is sensitive enough to detect 1 ng of genomic DNA (i.e.,  $3 \times 10^5$  *M. tuberculosis* cells). We found that this method worked well even in positive sputum containing few bacilli, as determined by acid-fast staining. Fourth, our DNA sequencing-based method allows detection of both novel and known mutations. In the present study, we identified 25 novel mutations by PCR-based analysis.

This strategy does have a few disadvantages. The DNA sequencer and sequencing are costly, and the procedure is somewhat complicated. However, this issue may be addressed if DNA sequencing costs are reduced by new sequencing methods and equipment. Also, this strategy may not be able to detect very low numbers of bacilli in sputa. We did not have the opportunity to test AFB smear-negative but culture-positive samples. Among the smear- and culture-positive samples we tested, one sample with small numbers of bacilli (one/several fields) was negative for three of the eight genes tested, indicating that the sensitivity of this method is limited. However, the sensitivity and accuracy of our method are comparable to those of traditional drug susceptibility tests, and this is sufficient for use in the clinical setting.

The method described here was excellent for diagnosis of RIF-resistant *M. tuberculosis* isolates with 100% specificity, sensitivity, and test efficiency. RIF interferes with the synthesis of mRNA by binding to the  $\beta$ -subunit of RNA polymerase (RpoB) in bacterial cells (39). The RIF-binding site is a pocket in the upper wall of the main channel for double-stranded DNA entry just upstream of the polymerase catalytic center. The various RIF-resistant mutations are clustered around this pocket (39). Mutations in *rpoB* have been found in 95% to 100% of clinical RIF-resistant isolates of *M. tuberculosis* (39). Most of the mutations found in the 28 RIF-resistant isolates tested here were located between nucleotides 1276 and 1356 (codons 507 to 533) of *rpoB*, which is the 81-bp core region of this gene (Table 4) (31, 39). Two other mutations, V146F and E562A, were located outside of the 81-bp core region. Isolates with V146F were reported to show low-level resistance to RIF (MIC,  $\leq 4$   $\mu\text{g/ml}$ ) (39). It is not known whether the E562A mutation is involved in resistance because the isolate with this mutation also had another mutation in the 81-bp core region (39). The V146F mutation could not be detected by the DNA sequencing method described here. Although we were able to detect the E562A mutation by our sequencing method, we did not find this mutation in any of 138 isolates tested in the present study.

Our sequencing method is applicable for diagnosis of INH-resistant isolates with 89.5% sensitivity, 100% specificity, and 97.1% test efficiency (Table 6). The sensitivity of the two-temperature PCR for *katG* was lower than that of the PCR for *rpoB* or *mabA-inhA* (Fig. 1B), and, therefore, we will need to increase the sensitivity to detect *katG* mutations to assess INH resistance. The mode of INH action is one of the most complicated among all antibiotics. INH is a prodrug that requires activation of the bacterial catalase-peroxidase enzyme (KatG) (38) to generate a range of reactive radicals, which then affect multiple systems, including cell wall mycolic acid synthesis and

TABLE 6. The diagnostic performance of the DNA sequencing-based method in comparison with drug susceptibility testing<sup>a</sup>

Drug susceptibility test result <sup>b</sup>	No. of isolates (n = 138)		% Sensitivity <sup>c</sup>	% Specificity <sup>d</sup>	% Test efficiency <sup>e</sup>
	Mutation positive	Mutation negative			
RIF					
Resistant	28	0	100	100	100
Susceptible	0	110			
INH					
Resistant	34	4	89.5	100	97.1
Susceptible	0	100			
EMB					
Resistant	15	3	83.3	100	97.8
Susceptible	0	120			
PZA					
Resistant	17	0	100	100	100
Susceptible	0	121			
STR					
Resistant	18	12	60.0	100	91.3
Susceptible	0	108			
OFX					
Resistant	3	0	100	100	100
Susceptible	0	135			

<sup>a</sup> The diagnostic performance of the DNA sequencing-based method in comparison with drug susceptibility testing was determined after resolution of polymorphisms.

<sup>b</sup> Drug susceptibility for antituberculosis agents except for PZA was determined by the agar proportion method according to NCCLS (now CLSI) guidelines, and that for PZA was determined by PZase activity.

<sup>c</sup> Sensitivity: no. of drug-resistant isolates with mutations/(no. of drug-resistant isolates with mutations + no. of drug-resistant isolates without mutations).

<sup>d</sup> Specificity: no. of drug-susceptible isolates without mutations/(no. of drug-susceptible isolates with mutations + no. of drug-susceptible isolates without mutations).

<sup>e</sup> Test efficiency: (no. of drug-resistant isolates with mutations + no. of drug-susceptible isolates without mutations)/no. of all isolates tested.

lipid peroxidation and NAD metabolism, and cause DNA damage (39). Deficient efflux of INH radicals and defective antioxidative defenses may underlie the susceptibility of *M. tuberculosis* to INH (39). Mutations in *katG* are among the most frequently detected mutations in INH-resistant clinical isolates. Mutations in *inhA* and its promoter region, which is located upstream of the *mabA-inhA* operon, are also common (16, 17, 39). Our sequencing method should identify a majority of INH-resistant isolates. We are able to detect mutations in *katG* and the region upstream of *mabA* in 90% (34/38) of INH-resistant isolates. Ten different mutations (L48Q, L141F, M257T, Q295P, G297V, S315T, S315N, T324P, R463L, and V708P) were detected in *katG* of INH-resistant isolates, and 3 mutations (A65T, A245V, and V725A) were identified in INH-susceptible isolates. The L48Q, A65T, L141F, A245V, M257T, Q295P, G297V, T324P, V708P, V725A, and L390 insertion mutations are novel (Table 4).

To date, several mutations in *katG* in MDR isolates have been reported (9, 16, 17, 38, 39). Rouse et al. (24) reported previously that codons 104 and 108 encode amino acids located near the catalytic site of KatG and that the residues encoded by codons 270, 275, and 315 participate in binding the heme group

of KatG (24). Mutations in these regions, therefore, are thought to cause loss of KatG enzymatic function (24). Yu et al. (36) reported that residue W321 of KatG was important for substrate binding and that residue Y229 was critical to protect the catalase activity of KatG (37). We compared the catalase and INH oxidation activities of eight KatG mutants identified in this study with those of the wild type. Although we were able to express all of the KatG mutants (Fig. 2A), we were unable to detect the catalase and INH oxidation activities of four KatG mutants, KatG<sup>O295P</sup>, KatG<sup>T324P</sup>, KatG<sup>L141F-R463L</sup>, and KatG<sup>L390 insertion</sup> (Table 5 and Fig. 2C). The specific effects of these mutations on KatG function need to be analyzed further. The lack of activity or lower activity of these mutants, however, correlated quite well with the INH-resistant phenotype of their respective *M. tuberculosis* isolates. The enzymatic activity of KatG<sup>L48Q-R463L</sup> was not correlated with INH susceptibility (Table 5), and this mutation had little effect on the measured activities (Table 5 and Fig. 2C). The isolate carrying the L48Q-R463L mutations also had the -15C→T transition upstream of *mabA* (Table 4), which is known to be associated with INH resistance (10, 22, 39). The KatG<sup>A245V</sup> and KatG<sup>R463L</sup> mutants showed activities similar to those of wild-type KatG, and these results are consistent with the INH-susceptibility phenotypes of their respective isolates. Therefore, we concluded that the L48Q, A245V, and R463L mutations are merely polymorphisms that do not influence INH resistance.

We found mutations in the region upstream of *mabA* or in the regulatory region of the *mabA-inhA* operon in 12 of 38 INH-resistant isolates. Five of these isolates had no other mutations within *katG* (Table 4). Our present results support those of Morris et al. (16), who examined the *inhA* locus for sequence polymorphisms by single-strand conformation polymorphism analysis and DNA sequencing of 42 INH-resistant isolates. They found no alterations in the coding portion of *inhA*, but five isolates had mutations in the regulatory region of the *mabA-inhA* operon (16).

Mutations in *kasA*, which encodes  $\beta$ -ketoacyl ACP synthase (11), and *ndh*, which encodes NADH dehydrogenase (12), have been found in a small proportion of clinical isolates, and we plan to modify our sequencing method to analyze *ndh* and *kasA*.

Our method was sufficient for diagnosis of EMB-resistant isolates, although a limited portion (80%) of *embB* was sequenced. EMB inhibits polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (39). Three homologues of arabinosyltransferases, EmbC, EmbA, and EmbB, have been proposed to be the targets of EMB (32, 39). Mutations in *embB* are found in 47% to 69% of EMB-resistant isolates of *M. tuberculosis* (28, 32). Most EMB-resistant isolates with *embB* mutations exhibited high-level resistance (2, 27). The 35% of EMB-resistant isolates that do not have *embB* mutations showed decreased resistance to EMB (2). We were able to detect a majority (15/18 isolates) of EMB-resistant isolates with our sequencing-based analysis. In addition, we identified two novel mutations, D354A and N296Y, in EMB-resistant isolates in the present study.

*pncA* is known to be associated with PZA resistance (17, 39). In the present study, we sequenced the complete open reading frame of *pncA* and its promoter region. PZA enters the organism through passive diffusion and is converted to pyrazinoic

acid by cytoplasmic PZase. Despite recent progress, the targets of pyrazinoic acid are still not known (39). All PZA-resistant *M. tuberculosis* isolates tested in the present study contained at least one mutation within *pncA* and showed no PZase activity (Table 4). Our results are consistent with those of previous studies that showed 72% to 95% of PZA-resistant clinical isolates of *M. tuberculosis* carried *pncA* mutations (25). All of the *pncA* mutations identified in the present study of PZA-resistant isolates caused amino acid substitutions. Among these mutations, 5A3E, D53N, P54L, C72W, and M175V were novel. The *pncA* mutations were highly diverse and scattered across the gene.

STR, an aminoglycoside, inhibits initiation of mRNA translation. The site of action is the small 30S subunit of the ribosome, especially ribosomal protein S12 and the 16S rRNA (17). *M. tuberculosis* becomes resistant when targets of STR in the ribosomes are mutated. The principal site of mutation is the *rpsL* gene, which encodes ribosomal protein S12 (6, 19, 27). The most frequently observed mutation in *rpsL* was K43R. In the present study, 13 of 30 STR-resistant isolates tested had the K43R mutation. Mutation of the *rrs* gene is also associated with STR resistance in *M. tuberculosis*. *M. tuberculosis* has only a single copy of the *rrs* gene, which encodes the 16S rRNA. Thus, the loops of 16S rRNA that interact with the S12 protein constitute an easily selected mutation site. Such *rrs* mutations are clustered in the highly conserved 530 loop and in the adjacent 915 region (6). In addition, a 1400A→G mutation of *rrs* was identified in both amikacin- and kanamycin-resistant clinical isolates of *M. tuberculosis* (1, 29). These isolates were resistant to STR, indicating that this mutation may contribute to STR resistance (1, 29). In the present study, one STR-resistant isolate had two mutations, 1400A→G and 1539A→G. Because the STR resistance of the isolate can be explained by the 1400A→G mutation, it is unclear whether the 1539A→G mutation is associated with STR resistance.

FQs are active in vitro against *M. tuberculosis* isolates (5) and are increasingly being used in combination with other agents to treat tuberculosis. The principal mechanism of resistance to FQs identified in other bacterial species is alteration of the target proteins DNA gyrase and topoisomerase IV. DNA gyrase is composed of two A and two B subunits, which are encoded by *gyrA* and *gyrB*, respectively (39). Mutations in *gyrA* are associated with high-level resistance of *M. tuberculosis* to FQs (39). *gyrB* mutations associated with resistance have only been identified in laboratory mutants of *M. tuberculosis* (39). Mutations associated with FQ resistance occur within a relatively restricted region of *gyrA*. We identified three mutations, A90V, D94GA, and D94G, in FQ-resistant isolates. We also identified a polymorphism, S95T, that is not associated with FQ resistance. The G88C, D89G, S91P, and D94A, -N, -H, or -Y mutations in *gyrA* have also been found in FQ-resistant isolates (4, 30). These mutations are presumed to be located in the FQ-binding region (4, 30).

Some researchers have described mutations that caused amino acid substitutions but not drug resistance (30, 33, 39). In the present study, we identified several novel mutations that cause amino acid substitutions but do not confer drug resistance. Except for these mutations and silent mutations, the drug resistance profiles of the isolates tested correlated quite



well with the various mutations that we identified (Table 6). The sensitivities of the DNA sequencing-based method (i.e., the ability to detect true drug resistance) were 100%, 89.5%, 83.3%, 100%, 60%, and 100% for the RIF-, INH-, EMB-, PZA-, STR-, and OFX-resistant strains, respectively. The specificities (i.e., the ability to detect true drug susceptibility) were 100% for all drugs tested. The test efficiencies (i.e., the ability to give the correct answer in all samples tested) were 100%, 97.1%, 97.8%, 100%, 91.3%, and 100% for the RIF-, INH-, EMB-, PZA-, STR-, and OFX-resistant strains, respectively. These results indicate that our DNA sequencing-based method is effective for detection of MDR strains. However, when novel mutations in drug resistance-related genes are detected by our method, it is essential to also perform drug susceptibility testing, because novel mutations are not necessarily associated with drug resistance. Of the 25 novel mutations we detected, we cloned 7 novel mutations in KatG. Significant information could be gained if all novel mutations were cloned. For practical purposes, it would be helpful to know the phenotypic manifestations of specific mutations.

In conclusion, we have shown the usefulness of our DNA sequencing strategy for drug susceptibility screening of various targets. Most MDR *M. tuberculosis* strains, which are defined as those strains resistant to both RIF and INH, are resistant to other antitubercular drugs. Our new sequencing-based method can rapidly and efficiently assess MDR of *M. tuberculosis*. The method can also be used to detect MDR *M. tuberculosis* in sputa from patients. Further studies will focus on the clinical application of this method for diagnosis of drug-resistant *M. tuberculosis*.

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