study, may yield important insight into INH resistance in *M. tuberculosis*.

Experimental procedures

Bacterial strains and plasmids

One hundred and eight INH^r *M. tuberculosis* clinical isolates were obtained from single patients with INH^r tuberculosis hospitalized at the National Center for Global Health and Medicine (formerly International Medical Center of Japan) and National Hospital Organization Tokyo National Hospital from 2003 to 2008. Fifty-one INH^s isolates were chosen using a random number list from INH^s isolates obtained from single patients hospitalized in the two hospitals from 2003 to 2008. The *M. tuberculosis* and *Escherichia coli* strains and plasmids used in this study are listed in Table S1. *M. tuberculosis* H37Rv, NCGM2828, NCGM2836 and NCGM2898 were used as controls for drug susceptibility testing, Western blotting analysis and INH oxidase assays. NCGM2836 was also used for β-galactosidase and complementation assays. *E. coli* DH5α and TOP10 (Invitrogen), and BL21(DE3) (Stratagene) were used for cloning and for protein overexpression studies respectively.

Growth conditions

Unless otherwise specified, *M. tuberculosis* clinical isolates and strains were pre-cultured in 125 ml Erlenmeyer flasks (Corning) containing 20 ml of MycoBroth [modified Middlebrook 7H9 broth (BD), Kyokuto] (500 mg of ammonium sulphate, 500 mg of L-glutamic acid, 100 mg of sodium citrate, 1 mg of pyridoxine, 0.5 mg of biotin, 2.5 g of disodium phosphate, 1 g of monopotassium phosphate, 50 mg of magnesium sulphate, 500 mg of calcium chloride, 1 mg of zinc sulphate, 850 mg of NaCl, 5 g of BSA, 2 g of dextrose, 3 mg of catalase and 500 mg of Tween 80 per litre, pH 6.6 ± 0.2) for 14 days at 37°C. The bacterial pre-cultures were inoculated into fresh MycoBroth or 7H10 agar (BD) supplemented with 10% OADC enrichment (BD) and 0.5% glycerol (Nacalai Tesque) and further cultured at 37°C. *E. coli* was grown in Luria–Bertani (LB) medium (BD). When required, kanamycin (KM) (Sigma) was added at 20 μg ml^{-1} to cultures of *M. tuberculosis* or 50 μg ml^{-1} to cultures of *E. coli*.

Drug susceptibility testing

All clinical isolates and *M. tuberculosis* strains were tested for drug susceptibility using the agar proportion method with egg-based Ogawa medium (Vit Spectrum-SR, Kyokuto; or Wellpack, Japan BCG Laboratory) according to the manufacturer's instructions, which were based on a slightly modified WHO protocol and have been recommended by the Japanese Society for Tuberculosis (Fujiki, 2001; WHO, 2003). The medium contained INH (0.2 μg ml^{-1} and 1.0 μg ml^{-1}), rifampin (RIF) (40 μg ml^{-1}), ethambutol (EB) (2.5 μg ml^{-1}), KM (20 μg ml^{-1}), *p*-aminosalicylic acid (PAS) (0.5 μg ml^{-1}), streptomycin (SM) (10 μg ml^{-1}), ethionamide (TH) (20 μg ml^{-1}), enviomycin (EVM) (20 μg ml^{-1}), cycloserine (CS) (30 μg ml^{-1}) and levofloxacin (LVFX) (1.0 μg ml^{-1}). The results are shown in Table 1 and Fig. S1. Clinical isolates harbouring mutations in *furA* or the *furA–katG* intergenic region, including H37Rv, NCGM2828, NCGM2836, NCGM2898 and NCGM 2836-derived strains, were also tested for INH susceptibility using an agar proportion method with 7H10 agar plates according to the protocols of the Clinical and Laboratory Standards Institute (NCCLS, 2003) and a broth dilution method (BrothMIC MTB-I, Kyokuto). The results are shown in Tables 1 and 2.

Isolation of genomic DNA

Genomic DNA from bacteria was extracted as described previously (Otsuka *et al.*, 2004).

DNA sequencing of INH resistance-related genes

The DNA sequences of the oligonucleotide primers used in the present study are listed in Table S2. The *furA–katG*, *fabG1–inhA*, *ndh*, *ahpC* genes and their upstream regions, and *kasA–kasB*, were amplified by two-temperature PCR with the same conditions, i.e. reaction mixtures contained 0.5 U of *Z-Taq* polymerase (Takara), 5 μl of 10× *Z-Taq* buffer (Takara), 4 μl of 2.5 mM dNTP mixture (Takara), 0.5 μl of each primer at 25 mM (Invitrogen), 20 ng of genomic DNA and sterile distilled water to 50 μl. Thermal cycling was performed on a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) with 30 cycles of 98°C for 1 s and 68°C for 30 s. PCR products were purified with Microcon YM-30 centrifugal filter devices (Millipore) and used as templates for direct DNA sequencing. DNA sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). DNA sequences were compared with *M. tuberculosis* H37Rv using Genetyx-Mac (Genetyx Corporation).

Restriction fragment length polymorphism (RFLP)

IS*6110*-probed RFLP was performed as described previously (Otsuka *et al.*, 2004). The fingerprinting patterns were analysed with Fingerprinting II software (Bio-Rad). Each dendrogram was calculated with the unweighted pair group method with average linkage according to the manufacturer's instructions. Patterns with more than 70% similarity were postulated to form a cluster.

Construction of plasmids

The *furA* gene and its variant *furA*^{c41t} were amplified by PCR with the BamHI-furA-F/furA-EcoRI-R primer set (Table S2) from *M. tuberculosis* H37Rv and a clinical isolate harbouring *furA*^{c41t} mutation. The PCR products were digested with BamHI and EcoRI, and ligated into the pGEX-2T vector (GE Healthcare) digested with the same restriction enzymes. Recombinant plasmids were used to transform *E. coli* DH5α and selected on LB plates containing 100 μg ml^{-1} ampicillin (AMP). We utilized pJEM13 (Timm *et al.*, 1994) carrying the fused P_{furA}-*furA–katG′–′lacZ* gene for β-galactosidase assays in *M. tuberculosis*. This plasmid contained a DNA fragment

from 138 bp upstream of the initiation codon of *furA* to the 99th base of *katG*. DNA fragments with or without a mutation in the *furA–katG* intergenic region were prepared by PCR using primer sets ApaI-P_{furA}-F/katG100-KpnI-R (Table S2). These fragments were digested with ApaI and KpnI and cloned into the corresponding site of pJEM13. Plasmids were used to transform *E. coli* TOP10 and selected on LB plates containing KM. For complementation assays of *M. tuberculosis* Δ(*furA–katG*), pMV306-derived plasmids were constructed. The entire *furA–katG* and 500 bp upstream region with or without a mutation was obtained by PCR with primer sets XbaI-(-500)furA-F/katG+40-HindIII-R (Table S2). These PCR products were digested with XbaI and HindIII and cloned into the corresponding site of pMV306. Plasmids were used to transform *E. coli* TOP10 and selected on LB plates containing KM. The DNA sequences of all clones were confirmed by sequencing.

Transformation of M. tuberculosis

INH^r *M. tuberculosis* NCGM2836 was grown in 20 ml of MycoBroth for 14 days as described above. Two-millilitre aliquots of 2 M glycine were added and the cultures were incubated for 24 h at 37°C. Bacteria were harvested by centrifugation at 3000 r.p.m. for 15 min at room temperature (RT), washed twice with 20 ml of 10% glycerol at RT and resuspended in 400 μl of 10% glycerol. Bacteria were electroporated with 1–4 mg of plasmids using a Bio-Rad Gene Pulser with settings of 2.5 kV, 25 mF and 1000 W. After electroporation, the bacteria were added to 4 ml of MycoBroth, incubated for 24 h at 37°C, harvested by centrifugation at 3000 r.p.m. for 15 min at RT and resuspended in 300 μl of MycoBroth. Transformants were selected by plating out on 7H10 agar plates with KM. To check the correct chromosomal structures of the complementary isogenic strains, KM-resistant colonies were examined by PCR with GC buffer, i.e. reaction mixtures contained 2.5 U of *LA Taq* polymerase (Takara), 25 μl of 2× GC buffer I (Takara), 8 μl of 2.5 mM dNTP mixture (Takara), 0.5 μl of each primer (glyV-20-F/lipP+20-R) at 25 mM (Invitrogen), 20 ng of genomic DNA and sterile distilled water to 50 μl. Thermal cycling was performed on a GeneAmp PCR system 9700 thermocycler with 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 10 min.

β-Galactosidase assay

The pJEM13-derived gene fusions were used to transform NCGM2836 and β-galactosidase activity was measured as described (Alland *et al.*, 2000). The transformed *M. tuberculosis* clones were streaked onto plates containing X-gal to monitor *lacZ* expression.

Preparation of total protein extracts

Bacteria were grown in 20 ml of MycoBroth for 14 days as described above, harvested by centrifugation at 3000 r.p.m. for 15 min at RT, washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in 500 μl of the same buffer. Bacteria were then lysed by shaking in a FastPrep FP100A homogenizer (Savant) (speed: 6.5 m s^{-1}; time: 20 s, twice) with 70 mg of Lysing Matrix B (Qbiogene). The supernatant obtained after centrifugation at 12 000 r.p.m. for 1 min was added to Spin-X centrifuge tube filters with cellulose acetate membranes of pore size 0.22 μm (Costar) and centrifuged at 12 000 r.p.m. for 5 min at RT. Total protein extracts were quantified using Protein Assay CBB Solution (Nacalai Tesque).

Western blotting analysis

Proteins separated by SDS-5%–20%-gradient-PAGE were transferred onto Immun-Blot PVDF membranes (Bio-Rad). The membranes were incubated simultaneously with anti-KatG polyclonal antibody (diluted 1:10 000) (Sekiguchi *et al.*, 2007) and anti-HSP70 monoclonal antibody (diluted 1:500) (Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG (diluted 1:10 000) (Amersham Biosciences) and goat anti-mouse IgG (diluted 1:1000) (Santa Cruz Biotechnology). Proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantity One (Bio-Rad) was used to quantify the KatG and HSP70 protein levels.

INH oxidase assay

Total protein extracts were prepared and quantified as above. The INH oxidase activity of KatG was assayed as described previously (Wei *et al.*, 2003; Sekiguchi *et al.*, 2007; Ando *et al.*, 2010). To 1 ml of 50 mM phosphate buffer (pH 7.0) were added 300 mg of total protein extracts, 0.04 mM nitroblue tetrazolium (NBT), 9 mM INH, 0.5 mg of glucose oxidase and 0.4 mM glucose. KatG activity was measured spectrophotometrically by monitoring the reduction of NBT at A_{560}. The absorbance was read 200 s after initiation of the reaction. All assays were performed at 25°C. NBT reduction in the absence of INH was subtracted from that in the presence of INH.

Purification of recombinant FurA and FurA^{A14V}

To purify FurA and FurA^{A14V}, *E. coli* BL21(DE3) carrying the plasmids pGEX-*furA* and pGEX-*furA*^{c41t} were grown in LB medium containing 200 μg ml^{-1} AMP at 37°C. Induction and purification of the GST-fused FurA and FurA^{A14V} were performed according to the manufacturer's instructions (GE Healthcare). Thrombin (GE Healthcare) was used to cleave the GST-Tag at the N-termini of the FurA proteins. The final concentration of protein was determined using a bicinchoninic acid protein assay kit (Pierce).

Electrophoretic mobility shift assay (EMSA)

Binding between purified *M. tuberculosis* FurA or FurA^{A14V} and P_{furA} was assessed by EMSA as described (Sala *et al.*, 2003). DNA fragments for EMSA were amplified by PCR using specific primers, −129furA and furA33-R for the upstream region of *furA*, and −5furA-F and furA166-R as a negative control. Both PCR products were 171 bp in length.

Binding reaction mixtures in 20 μl of binding buffer [20 mM Tris-HCl (pH 8.0), 1 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 50 μg of BSA per ml and 200 μM $NiSO_4$] containing 180 ng DNA fragment were incubated with purified FurA protein for 20 min at RT. Reaction mixtures were analysed by electrophoresis on 7.5% polyacrylamide gels in 40 mM Tris-acetate buffer at RT. DNA was visualized by ethidium bromide staining. For exposure of proteins to H_2O_2 (Santoku), 16 μM FurA protein was mixed with various concentrations of H_2O_2 in reaction mixtures without DNA fragments and incubated for 10 min at RT. DNA fragments were then added and incubation was continued for a further 10 min.

Structure-based sequence alignment

Amino acid sequences of *M. tuberculosis* FurA were aligned using CLUSTALW (Thompson *et al.*, 1994) and edited using ESPript (Gouet *et al.*, 1999). The secondary structure of FurA was determined by ESPript based on the crystal structure of *P. aeruginosa* Fur (PDB ID: 1MZB), a template predicted by automated modelling with the SWISS MODEL server.

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Evaluation of a line probe assay for the rapid detection of *gyrA* mutations associated with fluoroquinolone resistance in multidrug-resistant *Mycobacterium tuberculosis*

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The aim of this study was to establish the importance of detecting fluoroquinolone (FQ) resistance in multidrug resistant (MDR) *Mycobacterium tuberculosis*, and to show the usefulness of a hybridization-based line probe assay (LiPA) for detecting *gyrA* mutations. Thirty-three MDR *M. tuberculosis* isolates were collected from a total of sixty MDR isolates identified in Japan over 6 months during a national surveillance study in 2002. Seventeen MDR isolates were collected by the National Center for Global Health and Medicine in Japan over 6 years from 2003 to 2008. These 50 isolates were examined for FQ susceptibility, and analysed by LiPA and *gyrA* sequencing. Among them, 22 (44 %) showed FQ resistance. All FQ-resistant isolates had at least one mutation in *gyrA*. The results of the LiPA were fully consistent with the DNA sequencing results. Given that on the basis of our results almost half of the MDR *M. tuberculosis* isolates in Japan might have resistance to FQ, it is important to monitor FQ resistance in patients with MDR tuberculosis (TB), as well as with drug-susceptible TB, prior to commencing treatment. For the detection of FQ resistance, LiPA is useful and can rapidly and efficiently assess FQ resistance.

INTRODUCTION

The emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* are a serious threat to the control of tuberculosis (TB), and constitute an increasing public-health problem (Caminero, 2010; Johnston *et al.*, 2009; Sotgiu *et al.*, 2009; Van Deun *et al.*, 2010a). Multidrug resistant (MDR) strains are defined as strains resistant to both rifampicin and isoniazid, and extensively drug-resistant strains are defined as MDR plus resistance to any fluoroquinolone (FQ) and to at least one of the second-line injectable drugs. Patients infected with these strains are difficult to cure and are more likely to remain infected for a longer period of time than patients with drug-susceptible strains (Caminero, 2010; Johnston *et al.*, 2009; Sotgiu *et al.*, 2009). With the emergence of MDR and extensively drug-resistant TB, the need for rapid drug susceptibility testing is now apparent (Van Deun *et al.*, 2010a).

FQ is a class of antimicrobial drug with strong activity against *M. tuberculosis* and is one of the most important second-line antituberculosis drugs used in the treatment of MDR TB (Yew *et al.*, 2010). Newer FQs, including gatifloxacin (GFLX) and moxifloxacin (MFLX), have been developed. A prospective study (Van Deun *et al.*, 2010b) indicated that a treatment regimen including GFLX was highly effective for MDR TB. A phase II trial study (Conde *et al.*, 2009) showed that a regime including MFLX improved culture conversion in the initial treatment of TB.

The main target of FQ in *M. tuberculosis* is the DNA gyrase encoded by *gyrA* and *gyrB*, which is essential for DNA supercoiling (Guillemin *et al.*, 1998). The genetic mechanism of FQ resistance is mainly due to alterations in the DNA gyrase, especially mutations in a short sequence called the quinolone-resistance-determining region (QRDR) of the *gyrA* (Guillemin *et al.*, 1998).

Abbreviations: CPFX, ciprofloxacin; FQ, fluoroquinolone; GFLX, gatifloxacin; JATA, Japan Anti-Tuberculosis Association; LiPA, line probe assay; LVFX, levofloxacin; MDR, multidrug resistant; MFLX, moxifloxacin; NCGM, National Center for Global Health and Medicine; QRDR, quinolone-resistance-determining region; SPFX, sparfloxacin; TB, tuberculosis; TRC, Tuberculosis Research Committee.

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In this study, 50 MDR *M. tuberculosis* isolates collected in Japan were examined for FQ susceptibility and analysed by *gyrA* sequencing. Furthermore, we developed and evaluated a hybridization-based line probe assay (LiPA) for the rapid detection of *gyrA* mutations, which can easily be used in clinical applications. The whole procedure takes only 9 h, and the estimated cost per sample is £22 (US $35).

We found that nearly half of our Japanese MDR *M. tuberculosis* isolates had resistance to FQ. Therefore, it is important to detect FQ resistance in patients with MDR TB, as well as with drug-susceptible TB, prior to commencing treatment.

METHODS

Bacterial strains. Among the 50 MDR *M. tuberculosis* isolates, 33 were obtained from patients with TB in 9 hospitals in Japan located in various regions – 1 in Hokkaido, 1 in Tohoku, 4 in Kanto, 2 in Kinki and 1 in Kyushu – during a national surveillance study [conducted from June to November 2002 by the Tuberculosis Research Committee (Ryoken), Japan] (TRC, 2007). The remaining 17 were obtained from patients diagnosed as MDR TB in the National Center for Global Health and Medicine (NCGM) from 2003 to 2008. During the national surveillance study, 60 MDR *M. tuberculosis* isolates were obtained (TRC, 2007). Of them, 33 were recovered from bacterial stocks and used in this study. *M. tuberculosis* strain H37Rv (ATCC 27294) and a *Mycobacterium bovis* strain (BCG Japanese strain 172), which are susceptible to FQ, were used in this study. To determine the species specificity of the LiPA, the following 18 bacterial strains were also used: *Mycobacterium avium* (ATCC 25291), *Mycobacterium chelonae* (ATCC 19237), *Mycobacterium fortuitum* (RIMD 1317004, ATCC 15754), *Mycobacterium intracellulare* (JCM 6384, ATCC 13950), *Mycobacterium kansasii* (JCM 6379, ATCC 124878), *Mycobacterium nonchromogenicum* (JCM 6364, ATCC 124878), *Mycobacterium scrofulaceum* (JCM 6381, ATCC 19981), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium terrae* (GTC 623, ATCC 15755), *Escherichia coli* (ATCC 8739), *Haemophilus influenzae* (IID 984, ATCC 9334), *Klebsiella pneumoniae* (IID 5209, ATCC 15755), *Legionella pneumophila* (GTC 745), *Mycoplasma pneumoniae* (IID 817), *Pseudomonas aeruginosa* (ATCC 27853), *Rhodococcus equi* (ATCC 33710), *Staphylococcus aureus* strain N315 and *Streptococcus pneumoniae* (GTC 261).

FQ susceptibility testing. Drug susceptibility testing was performed at two institutions [33 isolates at the Japan Anti-Tuberculosis Association (JATA) and 17 at the NCGM]. Susceptibility to levofloxacin (LVFX), sparfloxacin (SPFX) and ciprofloxacin (CPFX) was determined at JATA, and susceptibility to LVFX was determined at the NCGM. A broth dilution method (BrothMIC MTB-I; Kyokuto) was used at JATA, and an agar proportion method with egg-based Ogawa medium (Vit Spectrum-SR; Kyokuto) was used at the NCGM. The proportion method is based on a slight modification of the World Health Organization protocol (Aziz *et al.*, 2003) and is recommended by the Japanese Society for Tuberculosis (Aziz *et al.*, 2003; Fujiki, 2001). The results are shown in Table 1.

Isolation of genomic DNA. Genomic DNA was extracted from bacteria as described previously (Sekiguchi *et al.*, 2007b).

PCR and DNA sequencing. The QRDR of *gyrA* was amplified and sequenced by a previously described method (Sekiguchi *et al.*, 2007a). DNA sequences were compared with H37Rv using GENETYX-MAC, version 14.0.2 (Genetyx).

LiPA. The LiPA was performed as described previously (Ando *et al.*, 2010; Sekiguchi *et al.*, 2007b). Seven oligonucleotide probes were designed for the LiPA to detect *gyrA* mutations (Table 2). Two oligonucleotide probes, S1 and S2, were designed to cover the entire QRDR of *gyrA* of H37Rv. S2 also contained another probe to compensate for a neutral mutation of G to C at nucleotide position 284 (S95T) (Zhang & Telenti, 2000). The remaining five oligonucleotide probes, R1–R5, were designed to detect five mutations that were reported previously in FQ-resistant isolates obtained in Japan (Sekiguchi *et al.*, 2007a). These probes were immobilized on strips of nitrocellulose membrane. The QRDR of *gyrA* was amplified by nested PCR. Immobilized probes were hybridized with the biotinylated PCR products, and then incubated with streptavidin labelled with alkaline phosphatase. The results were visualized using colour development after incubation with 5-bromo-4-chloro-3'-indolyl-phosphate *p*-toluidine and nitro blue tetrazolium. The presence or absence of bands on all strips was determined visually. Among the 20 bacterial strains described above, 2 strains of *M. tuberculosis* and *M. bovis* were positive for PCR, but the other 18 strains were negative. The PCR products from the two strains hybridized with the S probes, but not the R probes (data not shown). These data indicated that the LiPA is specific for *M. tuberculosis* and *M. bovis*. The sensitivity of the LiPA was 24.2 fg *M. tuberculosis* DNA, equivalent to five copies of *gyrA* (data not shown).

RESULTS AND DISCUSSION

Fifty MDR *M. tuberculosis* isolates were collected. Of them, 33 were collected from a total of 60 MDR isolates identified in Japan over 6 months during the national surveillance study in 2002, and 17 were collected in the NCGM in Japan over 6 years from 2003 to 2008. These isolates and H37Rv were examined for FQ susceptibility, and 22 (44 %) of the isolates were resistant to FQ (Table 1). Among the 33 MDR isolates analysed at JATA, 14 (42 %) were resistant to all three FQs used, i.e. LVFX, SPFX and CPFX. None of the isolates were resistant to only one or two of these FQs (Table 1). The MICs of the three FQs for FQ-susceptible isolates ranged from 0.06 to 0.5 mg l^{-1} (median 0.125 mg l^{-1}) for LVFX, from 0.03 to 0.25 mg l^{-1} (median 0.125 mg l^{-1}) for SPFX, and from 0.06 to 0.5 mg l^{-1} (median 0.25 mg l^{-1}) for CPFX. The MICs for FQ-resistant isolates ranged from 2 to 16 mg l^{-1} (median 4 mg l^{-1}) for LVFX, from 1 to 8 mg l^{-1} (median 4 mg l^{-1}) for SPFX, and from 2 to 16 mg l^{-1} (median 8 mg l^{-1}) for CPFX. Among the 17 MDR isolates analysed at the NCGM, 8 (47 %) were resistant to LVFX (Table 1). There were no differences in rates of FQ resistance in MDR isolates between the two institutions ($P=0.4$).

We examined whether the MDR isolates had mutations in the QRDR of *gyrA* by LiPA and DNA sequencing analysis. Hybridization signals visualized as violet bands on the strips were strong and readily discernible with low background (data not shown). As shown in Table 1, regarding the FQ-resistance profile, the LiPA yielded results that were 100 % in agreement with those obtained by culture-based susceptibility testing. Of the 50 isolates, 28 were positive for both S1 and S2, and the remaining 22 were negative for one of the S probes (ΔS1 or ΔS2 in Table 1). Of the 22, 4 were positive for R1 indicating that isolates

Table 1. FQ susceptibility, LiPA profiles and *gyrA* mutations among 50 MDR *M. tuberculosis* isolates

Source of isolate	Strain	FQ MIC (mg l^{-1})			LiPA profile*	Mutation	
		LVFX†	SPFX	CPFX		Nucleotide change	Amino acid change
Control strain	H37Rv	0.25	0.125	0.25	WT (S1, S2)	–	–
JATA	2A-3-14	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-3-47	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-3-84	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-3-142	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-4-30	0.06	0.03	0.06	WT (S1, S2)	–	–
	2A-4-138	0.25	0.125	0.25	WT (S1, S2)	–	–
	2B-7-38	0.5	0.25	0.5	WT (S1, S2)	–	–
	2C-1-3	0.25	0.125	0.25	WT (S1, S2)	–	–
	2E-1-3	0.25	0.125	0.25	WT (S1, S2)	–	–
	2E-1-93	0.25	0.125	0.25	WT (S1, S2)	–	–
	2G-2-5	0.25	0.125	0.25	WT (S1, S2)	–	–
	2G-3-24	0.125	0.06	0.125	WT (S1, S2)	–	–
	2I-11-4	0.25	0.125	0.25	WT (S1, S2)	–	–
	2O-2-16	0.125	0.06	0.125	WT (S1, S2)	–	–
	2P-1-120	0.25	0.125	0.25	WT (S1, S2)	–	–
	2P-5-113	0.25	0.25	0.25	WT (S1, S2)	–	–
	2P-5-253	0.5	0.25	0.5	WT (S1, S2)	–	–
	2R-1-48	0.25	0.125	0.25	WT (S1, S2)	–	–
	2V-5-5	0.125	0.06	0.25	WT (S1, S2)	–	–
	2A-3-11	4	2	4	ΔS1, R1(A90V)	C269T	A90V
	2B-7-33	8	4	8	ΔS2, R4(D94G)	A281G	D94G
	2O-4-41	4	4	8	ΔS2, R5(D94A)	A281C	D94A
	2O-5-21	2	1	4	ΔS2, R5(D94A)	A281C	D94A
	2P-1-57	2	1	2	ΔS2, R5(D94A)	A281C	D94A
	2P-1-114	4	2	8	ΔS2, R4(D94G)	A281G	D94G
	2P-1-118	2	2	4	ΔS2, R5(D94A)	A281C	D94A
	2P-5-58	8	4	8	ΔS1, R1(A90V)	C269T	A90V
	2P-5-65	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-108	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-167	2	1	4	ΔS2, R5(D94A)	A281C	D94A
	2P-5-230	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-233	4	4	8	ΔS2, R5(D94A)	A281C	D94A
	2P-5-254	2	1	4	ΔS2, R5(D94A)	A281C	D94A
NCGM	NCGM2819	S	ND	ND	WT (S1, S2)	–	–
	NCGM2825	S	ND	ND	WT (S1, S2)	–	–
	NCGM2847	S	ND	ND	WT (S1, S2)	–	–
	NCGM2861	S	ND	ND	WT (S1, S2)	–	–
	NCGM2862	S	ND	ND	WT (S1, S2)	–	–
	NCGM2864	S	ND	ND	WT (S1, S2)	–	–
	NCGM2929	S	ND	ND	WT (S1, S2)	–	–
	NCGM2931	S	ND	ND	WT (S1, S2)	–	–
	NCGM2933	S	ND	ND	WT (S1, S2)	–	–
	NCGM2803	R	ND	ND	ΔS2	G280T	D94Y
	NCGM2822	R	ND	ND	ΔS2, R5(D94A)	A281C	D94A
	NCGM2834	R	ND	ND	ΔS1, R1(A90V)	C269T	A90V
	NCGM2835	R	ND	ND	ΔS2, R5(D94A)	A281C	D94A
	NCGM2863	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2888	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2930	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2932	R	ND	ND	ΔS1, R1(A90V)	C269T	A90V

ND, Not determined; R, resistant to LVFX; S, sensitive to LVFX; WT, wild-type.
*Δ indicates a negative signal for the probe.
†S and R results were determined by the agar proportion method.

Table 2. Locations of oligonucleotide probes designed to cover *M. tuberculosis gyrA*

Probe	Amino acid region detected by each probe	Nucleotide sequence*
S1	88−92	GATCGACGCGTCGCC
S2	92–97	CACCAGGSTGTCGTAGAT
R1	A90V mutation	GATCGAC**A**CGTCGCC
R2	D94G mutation	CACCAGGCTG**C**CGTAGAT
R3	D94A mutation	CACCAGGCTG**G**CGTAGAT
R4	D94G-S95T mutations	CACCAGG**G**TG**C**CGTAGAT
R5	D94A-S95T mutations	CACCAGG**G**TG**G**CGTAGAT

*S represents C or G; bold letters indicate mutations.

had an A90V mutation, 8 were positive for R4 (D94G mutation) and 9 were positive for R5 (D94A mutation). However, one isolate, NCGM2803, with ΔS2 was negative for R1–R5 indicating that the LiPA could not identify a mutation associated with FQ resistance. As shown in Table 1, the DNA sequencing data were fully consistent with results obtained by culture-based susceptibility testing. The FQ-resistant NCGM2803 isolate had a mutation of g280t (D94Y). In addition, it was reported that FQ-resistant isolates had mutations in *gyrB* but not in *gyrA* (Aubry *et al.*, 2006). Therefore, the present LiPA will be improved to detect the mutations.

The number of the MDR isolates used in this study is enough to allow estimation of the rate of FQ resistance in Japan. We tested 33 of 60 MDR isolates collected in the national surveillance study. JATA reported that there were 11 933 cases positive for sputum smear in Japan in 2002 (www.jata-org.jp). Whereas, 3122 isolates were collected for 6 months in 2002 during the national surveillance study (TRC, 2007). It is estimated that the number of isolates collected in the study covered more than 50 % of cases positive for sputum smear. Of the 3122 isolates, 60 isolates were reported to be MDR (TRC, 2007).

Occasional monitoring of the drug susceptibility of patients with MDR TB before and during chemotherapy is essential as MDR isolates can easily acquire resistance to additional antituberculosis drugs. We reported previously that more than 50 % of MDR isolates have already acquired PZA resistance (Ando *et al.*, 2010). In the present study, we found that nearly half of our Japanese MDR *M. tuberculosis* isolates were resistant to FQ. However, little information is available regarding resistance to other second-line drugs, and it will be necessary in future to monitor susceptibility to these drugs.

We strongly suggest that FQ susceptibility needs to be monitored as soon as patients are diagnosed with TB, because FQs are widely used for the treatment of bacterial infections. A population of patients with TB who received FQ treatment have been reported by Wang *et al.* (2006). Some other TB patients were initially treated as having community-acquired pneumonia and were administered FQs (Yoon *et al.*, 2005). We could not obtain information about the TB patients whose isolates were tested in the present study, especially about their previous treatments with antituberculosis drugs, including FQs. Nevertheless, it is very likely that these patients received FQs. In Japan, FQs were used for patients with TB, especially MDR TB, before 2008 but LVFX, MFLX, GFLX, SPFX and CPFX were not officially recommended as antituberculosis drugs by the Japanese Society for Tuberculosis until 2008.

It is also necessary to develop a rapid and inexpensive diagnostic method to determine the drug susceptibility of *M. tuberculosis*. The whole procedure of the LiPA described here takes only 9 h, and the estimated cost per sample is £22 (US $35). A DNA sequencing-based method (Sekiguchi *et al.*, 2007a) is also rapid, but is more expensive than the LiPA. Therefore, the LiPA is suitable for this purpose. Clinical trials for *in vitro* diagnosis including non-MDR TB are in progress in Japan.

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Transparency Declaration

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Pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates in Japan

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Abstract

Thirty-six multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates collected in Japan were examined for pyrazinamide susceptibility and pyrazinamidase activity, and analysed by *pncA* sequencing and a hybridization-based line probe assay (LiPA), which was used to detect *pncA* mutations for the rapid identification of pyrazinamide-resistant isolates. Pyrazinamide resistance was found in 19 (53%) of them. All pyrazinamide-resistant isolates had no pyrazinamidase activity and at least one mutation in *pncA*. Among the *pncA* mutations, 11 had not been previously reported. The results of the LiPA were fully consistent with the DNA sequencing results. A majority of MDR *M. tuberculosis* isolates in Japan were resistant to pyrazinamide.

Keywords: Line probe assay, multidrug resistance, *Mycobacterium tuberculosis*, *pncA*, pyrazinamide

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The emergence and spread of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*, which are defined as strains resistant to both rifampin and isoniazid, constitute a serious threat to the control of tuberculosis (TB) [1].

Pyrazinamide is a first-line anti-TB drug that exhibits strong activity against semidormant bacilli sequestered within macrophages, and is used in short-course treatment in combination with rifampin, isoniazid, and ethambutol [2,3]. Pyrazinamide is also one of the most important drugs used in the treatment of MDR TB. It is a prodrug that requires conversion into its active form, pyrazinoic acid, by the bacterial enzyme pyrazinamidase (PZase), which is encoded by *pncA* [4–6]. Mutations in *pncA* lead to the loss of PZase activity and constitute the mechanism of pyrazinamide resistance in *M. tuberculosis* [5,7,8].

It is essential that rapid drug susceptibility testing (DST) be developed to prevent the spread of MDR *M. tuberculosis*. DST of *M. tuberculosis* produces reliable results for most anti-TB drugs [9]. However, conventional DST for pyrazinamide is hampered by poor bacterial growth, because pyrazinamide requires acidic conditions to be active; therefore, DST must be performed under such conditions [5]. Liquid culture-based methods have recently been developed to resolve this problem [10].

Previously, we described a DNA sequencing-based method for detecting mutations in the genome of drug-resistant strains, including pyrazinamide-resistant *M. tuberculosis* [11]. However, the use of this method in ordinary-scale clinical laboratories can present a problem because of its high cost. Therefore, we developed a hybridization-based line probe assay (LiPA) for the rapid detection of *pncA* mutations in pyrazinamide-resistant *M. tuberculosis* that can easily be used for clinical applications [12].

Thirty-six MDR *M. tuberculosis* isolates were collected during the national surveillance study, from June to November 2002, by the Tuberculosis Research Committee (Ryoken), Japan [13]. These isolates were obtained from patients with TB in nine hospitals in Japan located in various regions: one in Hokkaido, one in Tohoku, four in Kanto, two in Kinki and one in Kyushu. *M. tuberculosis* strain H37Rv (ATCC 27294), which is susceptible to pyrazinamide and positive for PZase, was used as a positive control for the assay. *Mycobacterium bovis* strain BCG (Japanese strain 172), which is resistant to pyrazinamide and negative for PZase, was used as a negative control.

Pyrazinamide susceptibility testing was performed with a broth method (BD BACTEC MGIT 960; BD Biosciences, Sparks, MD, USA) [9]. PZase activity was determined using Wayne's method [14], with some modifications [12]. Rifampin and isoniazid susceptibility testing were performed with an egg-based Ogawa medium method. These assays were performed in one institution (Japan Anti-Tuberculosis Association).

Nested PCR was performed to amplify a 670-bp fragment that includes the entire *pncA* gene, as described previously [12]. For DNA sequencing, only the second PCR was performed. DNA sequences of *pncA* and its promoter region (nucleotides −80–572 relative to the initiation codon) were determined as described previously [11] and compared with those of H37Rv using GENETYX-MAC (Genetyx Corporation, Tokyo, Japan).

Forty-seven oligonucleotide probes designed to cover the entire *pncA* gene of H37Rv were immobilized on two strips and used for the LiPA (Table S1). The LiPA was conducted as described previously [15]. Biotinylated PCR products from test samples were hybridized to the immobilized probes, and the strips were washed. The presence or absence of bands on all strips was judged visually.

Of 36 clinical isolates of MDR *M. tuberculosis* tested with the LiPA, 17 were wild type, and the other 19 showed at least one mutation (Table 1 and Fig. 1). As shown by the data in Fig. 1, the 17 wild-type isolates (lanes 1–17) and H37Rv (lane 37) hybridized to all probes. The other 19 isolates did not hybridize to at least one probe (lanes 18–36). Regarding the pyrazinamide resistance profile, the LiPA yielded results that were 100% in agreement with those obtained by culture-based susceptibility testing (Table 1). All PZase-positive bacilli tested were sensitive to pyrazinamide, and all PZase-negative bacilli were resistant to pyrazinamide (Table 1). These data are consistent with those of previously published reports [11,12]. All of the 19 pyrazinamide-resistant isolates were correctly identified as being pyrazinamide-resistant by the LiPA, and all of the 17 pyrazinamide-susceptible isolates were identified as being pyrazinamide-susceptible.

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TABLE 1. **Identification of *pncA* mutations by line probe assay (LiPA) among 36 multidrug-resistant *Mycobacterium tuberculosis* isolates**

Strain	LiPA profile[a]	Pyrazinamide susceptibility	PZase activity	Mutation: Nucleotide change	Mutation: Amino acid change
2A-3-16	Wild type	S	+	–	–
2A-3-83	Wild type	S	+	–	–
2A-3-84	Wild type	S	+	–	–
2A-4-30	Wild type	S	+	–	–
2B-7-38	Wild type	S	+	–	–
2C-1-46	Wild type	S	+	–	–
2G-2-5	Wild type	S	+	–	–
2I-11-4	Wild type	S	+	–	–
2P-5-113	Wild type	S	+	–	–
2P-5-280	Wild type	S	+	–	–
2P-5-420	Wild type	S	+	–	–
2R-1-48	Wild type	S	+	–	–
2C-3-89	Wild type	S	+	G419A[b]	R140H
2O-2-16	Wild type	S	+	G419A[b]	R140H
2A-3-142	Wild type	S	+	G419A[b], G493A[b]	R140H, A165T
2P-5-269	Wild type	S	+	G419A[b], G493A[b]	R140H, A165T
2C-4-48	Wild type	S	+	G419A[b], C450A[b], G493A[b]	R140H, G150G, A165T
2A-3-11	Δ16	R	–	T175C	S59P
2A-3-14	Δ7, Δ20, Δ21	R	–	Δ59[b], G232A[b]	Frameshift, G78S
2A-3-137	Δ13, Δ14	R	–	C153A, G493A[b]	H51Q, A165T
2B-7-33	Δ15	R	–	C161T	P54L
2C-3-105	Δ33	R	–	A410C	H137P
2E-1-3	Δ1, Δ28	R	–	T-7C, A340G[b]	T114A
2E-1-93	Δ1, Δ28	R	–	T-7C, A340G[b]	T114A
2O-4-41	Δ2	R	–	C8A	A3E
2P-1-57	Δ12	R	–	Δ(129–130)[b], G493A[b]	Frameshift, A165T
2P-1-114	Δ23	R	–	261::AC[b]	Frameshift
2P-1-118	Δ10	R	–	T100G	Y34D
2P-1-120	Δ4	R	–	T26G	V9G
2P-5-58	Δ2	R	–	C8A, G419A[b]	A3E, R140H
2P-5-108	Δ23	R	–	261::AC	Frameshift
2P-5-167	Δ10	R	–	T100G	Y34D
2P-5-230	Δ10	R	–	C102A[b]	Y34[c]
2P-5-233	Δ2	R	–	C8A	A3E
2P-5-254	Δ10	R	–	T100G, G400T[b]	Y34D, A134S
2V-5-5	Δ31, Δ32	R	–	Δ(374–389)[b]	Frameshift

[a]Δ indicates a negative signal at a probe.
[b]Mutation not previously reported.
[c]Ochre mutation.

The *pncA* genes of all isolates tested were sequenced (Table 1). One or more *pncA* mutations were identified in 24 isolates, and 12 isolates had no mutation. Among the 24 isolates with *pncA* mutations, we found 20 different mutations, of which 11 have not been previously reported. Of these 11 novel mutations, four were frameshift mutations (Δ59, Δ(129–130), 261::AC, Δ(374–389)), five were mutations causing an amino acid substitution (G232A, A340G, G400T, G419A, G493A), one was a non-sense mutation (C102A), and one was a silent mutation (C450A) (Table 1). The G419A and G493A mutations are not associated with pyrazinamide resistance, because the isolates with these mutations were pyrazinamide-sensitive. It is unknown whether G232A, A340G and G400T are associated with pyrazinamide resistance, because isolates with these mutations had an additional mutation in *pncA* that conferred pyrazinamide resistance. As shown in Table 1, the isolate with the C102A mutation (strain 2P-5-230) was resistant to pyrazinamide and exhibited no PZase activity. The C102A mutation changed the 34th amino acid of PZase into a stop codon, suggesting that the C102A mutation is associated with pyrazinamide resistance. The results of the LiPA were fully consistent with the DNA sequencing results (Table 1). The LiPA correctly identified pyrazinamide susceptibility and resistance in all strains in which a mutation occurred.

We found that 53% of MDR *M. tuberculosis* isolates (19 of 36) obtained in Japan were resistant to pyrazinamide. Although the number of MDR isolates detected in this study was small, the results suggest that the majority of MDR *M. tuberculosis* isolates in Japan are resistant to pyrazinamide. Mphahlele *et al.* [16] reported that 52% of South African MDR *M. tuberculosis* isolates are resistant to PZA. Thus, a majority of MDR *M. tuberculosis* isolates in other countries may also be resistant to pyrazinamide. Clinical trials of the LiPA for *in vitro* diagnosis in Japan started in April 2009.

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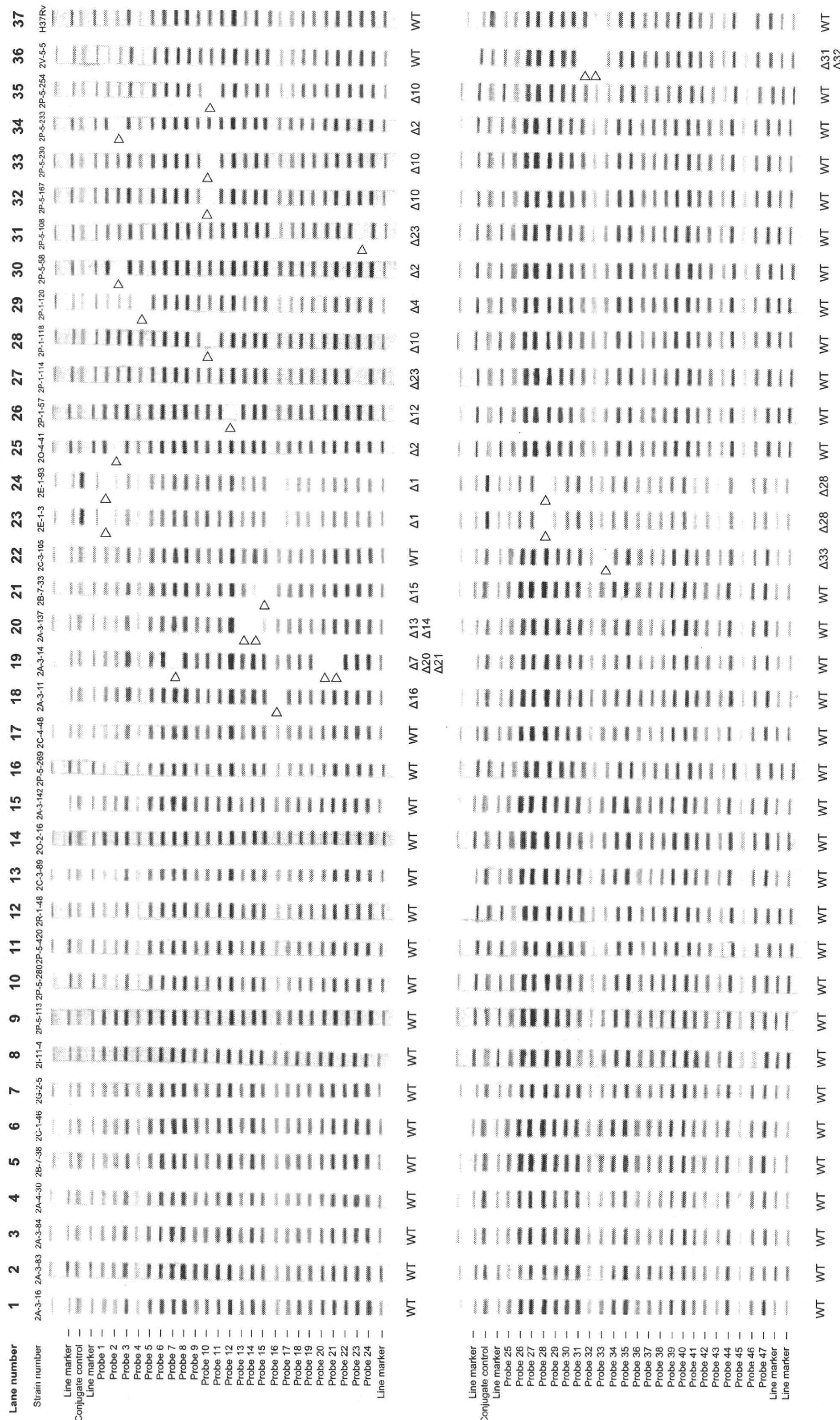

FIG. 1. Line probe assay (LiPA) patterns of all *Mycobacterium tuberculosis* isolates tested in this study. Positions of the oligonucleotides, conjugate control lines and (in blue) are shown. A negative signal is indicated by an open triangle. LiPA patterns are shown in lanes 1–37. WT, wild-type *pncA*.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Location of 47 oligonucleotide probes designed to cover *Mycobacterium tuberculosis pncA*.

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Speciation and susceptibility of *Nocardia* isolated from ocular infections

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Abstract

Twenty *Nocardia* spp. isolated from ocular infections were identified by 16S rRNA gene sequencing and susceptibility was determined using the E-test (AB Biodisk, Sweden). Species distribution among the 20 isolates was as follows: *Nocardia levis* (*n* = 7), *Nocardia farcinica* (*n* = 3), *Nocardia abscessus* (*n* = 2), *Nocardia brasiliensis* (*n* = 2), *Nocardia amamiensis* (*n* = 2), *Nocardia puris* (*n* = 1), *Nocardia beijingensis* (*n* = 1), *Nocardia otitidiscaviarum* (*n* = 1) and *Nocardia thailandica* (*n* = 1). All isolates were sensitive to amikacin. Eighteen (90%) isolates were sensitive to tobramycin, 11 (55%) to ciprofloxacin and gatifloxacin, and seven (35%) to azithromycin and clarithromycin. Molecular methods are useful for the identification and for the detection of *Nocardia* species that have not so far been reported in human infections.

Keywords: *N. amamiensis*, *N. thailandica*, *N. levis*, *N. puris*, ocular infections

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Identification of *katG* Mutations Associated with High-Level Isoniazid Resistance in *Mycobacterium tuberculosis*▽†

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Isoniazid (INH) is an effective first-line antituberculosis drug. KatG, a catalase-peroxidase, converts INH to an active form in *Mycobacterium tuberculosis*, and *katG* mutations are major causes of INH resistance. In the present study, we sequenced *katG* of 108 INH-resistant *M. tuberculosis* clinical isolates. Consequently, 9 novel KatG mutants with a single-amino-acid substitution were found. All of these mutants had significantly lower INH oxidase activities than the wild type, and each mutant showed various levels of activity. Isolates having mutations with relatively low activities showed high-level INH resistance. On the basis of our results and known mutations associated with INH resistance, we developed a new hybridization-based line probe assay for rapid detection of INH-resistant *M. tuberculosis* isolates.

Isoniazid (INH) is an effective drug used in the treatment of tuberculosis and has been in common use to treat tuberculosis since its introduction in 1952 (4). However, the emergence of INH-resistant (Inh^r) *Mycobacterium tuberculosis* is jeopardizing the continued utility of INH (10).

Drug resistance in *M. tuberculosis* is caused by mutations in restricted regions of the genome (36). Mutations in *katG*, the upstream region of the *fabG1-inhA* operon ($P_{fabG1\text{-}inhA}$), and *inhA* are responsible for INH resistance (36). The *katG* gene encodes the bifunctional catalase-peroxidase enzyme that converts INH to an active form (35).

Previously, we developed a DNA sequencing-based method to detect mutations in regions associated with INH resistance in *M. tuberculosis*, including *katG* and $P_{fabG1\text{-}inhA}$ (28). Consequently, five novel mutations in *katG* associated with INH resistance were found (28). In the present study, we cloned 21 *katG* mutants, including 15 novel mutants, and compared their INH oxidase activities. Certain *katG* mutations were shown to cause high-level INH resistance, which suggests the possibility of determining the degree of INH resistance, such as high- or low-level resistance, by detecting these *katG* mutations. Furthermore, to detect these mutations in ordinary-scale clinical laboratories without sequencing, we developed a new hybridization-based line probe assay (LiPA) for INH resistance in *M. tuberculosis* isolates, which can be applied easily in clinical use.

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MATERIALS AND METHODS

Bacterial strains and plasmids. One hundred eight Inh^r *M. tuberculosis* isolates were obtained from single patients at the International Medical Center of Japan and National Hospital Organization Tokyo National Hospital from 2003 to 2008. INH-susceptible (Inh^s) *M. tuberculosis* strains H37Rv and IMCJ 2751 were used. The IMCJ 2751 isolate has a *katG(G1388T)* [KatG(R463L)] neutral mutation. The *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* TOP10F′ (Invitrogen, Carlsbad, CA) was used as the host for cloning. *E. coli* UM262 (17) was used as the host for expression of *katG* derived from clinical isolates and H37Rv.

Drug susceptibility testing. All clinical isolates, H37Rv, and IMCJ 2751 were tested for drug susceptibility. Strains were analyzed by an agar proportion method with egg-based Ogawa medium (Vit Spectrum-SR [Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan] or Wellpack [Japan BCG Laboratory, Tokyo, Japan]), which is based on a slightly modified WHO protocol (3) and is recommended by the Japanese Society of Tuberculosis (3, 12). The medium contained INH (0.2 μg/ml and 1.0 μg/ml), rifampin (RIF) (40 μg/ml), ethambutol (EB) (2.5 μg/ml), kanamycin (KM) (20 μg/ml), *p*-aminosalicylic acid (PAS) (0.5 μg/ml), streptomycin (SM) (10 μg/ml), ethionamide (TH) (20 μg/ml), enviomycin (EVM) (20 μg/ml), cycloserine (CS) (30 μg/ml), and levofloxacin (LVFX) (1.0 μg/ml). The results of drug susceptibility testing are shown in Table S1 in the supplemental material.

Isolation of genomic DNA. Genomic DNA from *M. tuberculosis* was extracted as described previously (22).

DNA sequencing of INH resistance-related genes. The *furA-katG* operon and its upstream region were amplified by PCR with primers −129*furA* (5′-GCTCATCGGAACATACGAAG-3′) and *katG*+50 (5′-GTGCTGCGGCGGGTTGTGGTTGATCGGCGG-3′). The *fabG1-inhA* operon and $P_{fabG1\text{-}inhA}$ were also amplified, using primers −200*fabG1* (5′-TTCGTAGGGCGTCAATACAC-3′) and *inhA*+40 (5′-CCGAACGACAGCAGCAGGAC-3′). PCR products were used as templates for direct DNA sequencing. DNA sequences were compared with the H37Rv sequence using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan).

Construction of plasmids. The coding regions of *katG* from H37Rv, IMCJ 2751, and Inh^r clinical isolates with *katG* mutations were amplified by PCR with the primers *katG*-F-ccc (5′-CCCGAGCAACACCCACCCATTACAGAAAC-3′) and *katG*-R (5′-TCAGCGCACGTCGAACC-3′) and cloned into pTrcHis2-TOPO (Invitrogen) using the TA cloning method. The pTrcHis2-TOPO vector encodes a C-terminal peptide containing a c-*myc* epitope and a 6×His tag. However, the expressed recombinant KatG protein did not have any additional amino acid residues, such as the c-*myc* epitope and the 6×His tag, because the

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
TOP10F′	F′ [*lacI*^{q} Tn*10* (Tet^{r})] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^{r}) *endA1 nupG*	Invitrogen
UM262	*katG*::Tn*10 recA pro leu rpsL hsdM hsdR endl lacY*	17
Plasmids		
pTrcHis2-TOPO	TA cloning and expression vector; Ap^{r} Km^{r}	Invitrogen
p*katG*-wt	pTrcHis2-TOPO carrying *katG*	This study
p*katG*-1	p*katG*-wt carrying G1388T (neutral mutation)	This study
p*katG*-2	p*katG*-1 carrying C379G	This study
p*katG*-3	p*katG*-1 carrying C694T	This study
p*katG*-4	p*katG*-wt carrying A398C	This study
p*katG*-5	p*katG*-1 carrying T1147C	This study
p*katG*-6	p*katG*-1 carrying 1297::C, Δ1305C	This study
p*katG*-7	p*katG*-1 carrying a290g	This study
p*katG*-8	p*katG*-1 carrying C1465A	This study
p*katG*-9	p*katG*-wt carrying G944C	This study
p*katG*-10	p*katG*-1 carrying T1259C	This study
p*katG*-11	p*katG*-wt carrying G944C, G1159C	This study
p*katG*-12	p*katG*-1 carrying G368A, G895A	This study
p*katG*-13	p*katG*-1 carrying G1255C	This study
p*katG*-14	p*katG*-1 carrying C195T (silent mutation), T527C	This study
p*katG*-15	p*katG*-wt carrying Δ(478–479)	This study
p*katG*-16	p*katG*-1 carrying G944C	This study
p*katG*-17	p*katG*-wt carrying Δ371G	This study
p*katG*-18	p*katG*-1 carrying C1894T	This study
p*katG*-19	p*katG*-wt carrying C945A	This study
p*katG*-20	p*katG*-1 carrying Δ(571–576)	This study
p*katG*-21	p*katG*-1 carrying G1624C	This study

katG-R reverse primer included the native stop codon. The DNA sequences of all clones were confirmed by sequencing.

RFLP. IS*6110*-probed restriction fragment length polymorphism (RFLP) was performed as described previously (22). Patterns with more than 70% similarity were postulated to form a cluster.

Immunoblotting. Proteins separated by SDS-PAGE were transferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The proteins on the membranes were detected using primary antibodies specific for KatG (28). KatG was visualized with horseradish peroxidase-conjugated secondary antibodies.

Enzyme assays. KatG mediates free-radical formation from INH oxidation in the presence of H_2O_2. The activities of KatG were detected spectrophotometrically by following the reduction of nitroblue tetrazolium (NBT) at A_{560} (28, 32). Peroxidase activity was monitored spectrophotometrically by following the oxidation of 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at A_{405} (21). Catalase activity was measured spectrophotometrically by following the degradation of H_2O_2 at A_{240} (21). The catalase activity is shown as values subtracted from that of the vector control. All assays were carried out at 25°C. The absorbance was read 200 s after the initiation of the reaction.

LiPA. The line probe assay (LiPA) was performed as described previously (1, 29). In brief, 41 oligonucleotide probes were designed to cover mutations in the *furA-katG* (35 probes for *katG* and 2 for *furA*), $P_{fabG1\text{-}inhA}$ (2 probes), and *fabG1* (2 probes) regions (Table 2). These probes were immobilized on two strips. Six regions, located within $P_{fabG1\text{-}inhA}$ (477 bp), *fabG1* (209 bp), *furA* (256 bp), and *katG* (612 bp, 698 bp, and 907 bp), were amplified by nested PCR. Immobilized probes on the two strips were hybridized with the biotinylated PCR products and then incubated with streptavidin labeled with alkaline phosphatase. The color development was performed by incubation with 5-bromo-4-chloro-3′-indolylphosphatase *p*-toluidine and NBT.

RESULTS

Drug susceptibility profiles. As shown in Table S1 in the supplemental material, among 108 Inh^{r} isolates, 65 (60%) were resistant to INH at 0.2 μg/ml but susceptible to INH at 1.0 μg/ml. The remaining 43 (40%) were resistant to INH at 1.0 μg/ml. Among the 108 isolates, 44 (41%) were resistant to INH but susceptible to other antituberculosis drugs. Thirteen (12%) were multidrug-resistant (MDR) isolates and five (5%) were extensively drug resistant (XDR).

IS*6110*-probed RFLP. The results of IS*6110*-probed fingerprinting of the 108 Inh^{r} isolates are shown in Fig. S1 in the supplemental material. Five clusters were detected, consisting of a total of 63 isolates (58%), including 12 (11%) in cluster I, 22 (20%) in cluster II, 12 (11%) in cluster III, 12 (11%) in cluster IV, and 5 (5%) in cluster V. These observations suggested that the majority of Inh^{r} isolates in Japan expanded in a clonal manner.

Correlation between drug susceptibility and IS*6110*-probed RFLP. With regard to the degree of INH resistance, the proportions of high-level Inh^{r} isolates, i.e., isolates resistant to INH (1.0 μg/ml), were 1 (8%) in cluster I, 8 (36%) in cluster II, 4 (33%) in cluster III, 4 (33%) in cluster IV, and 5 (100%) in cluster V. These results indicated that the majority of isolates belonging to cluster I were resistant to INH (0.2 μg/ml) and susceptible to INH (1.0 μg/ml) and that those belonging to cluster V were highly resistant to INH. Six of 13 MDR isolates (46%) and 1 of 5 XDR isolates (20%) belonged to the clusters, but other MDR and XDR isolates did not belong to any clusters, indicating that they emerged sporadically in Japan.

Mutations in *furA-katG*, *fabG1-inhA*, and their upstream regions. We sequenced the *furA-katG* operon, the *fabG1-inhA* operon, and their upstream regions in all Inh^{r} isolates tested. Of the 108 isolates, 105 had at least one mutation (see Table S1

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TABLE 2. Locations of 41 oligonucleotide probes designed to cover a mutation(s) associated with INH resistance

Probe	Amino acid (nucleotide) region covered by probe
inhA-1	(−17 to −3)[a]
inhA-2	95–100
fabG1-1	202–206
fabG1-2	230–235
furA-1	12–17
furA-2	6–12
katG-1	45–51
katG-2	63–68
katG-3	92–97
katG-4	94–99
katG-5	105–111
katG-6	123–127
katG-7	132–137
katG-8	135–140
katG-9	140–145
katG-10	157–163
katG-11	170–174
katG-12	174–179
katG-13	178–183
katG-14	190–194
katG-15	228–236
katG-16	247–252
katG-17	256–261
katG-18	271–277
katG-19	294–299
katG-20	313–318
katG-21	323–327
katG-22	326–330
katG-23	383–387
katG-24	389–391
katG-25	417–422
katG-26	457–462
katG-27	479–482
katG-28	486–490
katG-29	522–528
katG-30	539–543
katG-31	553–558
katG-32	565–569
katG-33	591–596
katG-34	631–635
katG-35	707–712

[a] Nucleotide position relative to the initiation codon of *fabG1*.

in the supplemental material), while the remaining 3 had no mutations in the regions sequenced. Of the 105 isolates with mutations, 64 had mutations in the *furA-katG* operon, 62 had mutations in *fabG1-inhA* operon, and 21 had mutations in both regions. Of the 64 with mutations in the *furA-katG* operon, six had a large-scale deletion adjacent to the *furA-katG* operon (Fig. 1; see also Table S1 in the supplemental material). As shown by genetic maps (Fig. 1), these isolates had large-scale deletions, ranging in size from 2.3 to 34.4 kb. The remaining 58 isolates did not have large-scale deletions.

Twenty-eight different mutations were found among the 58 isolates with mutations in the *furA-katG* operon (see Table S1 in the supplemental material). Twenty-three were in *katG*, two were in *furA*, and three were in the intergenic region. Seven different mutations were found among the 62 isolates with mutations in the *fabG1-inhA* operon (see Table S1 in the supplemental material). Three were in the upstream region, two were in *fabG1*, and two were in *inhA*. Of the 28 different mutations found in the *furA-katG* operon, 22 were novel (2 in *furA*, 3 in the intergenic region of the *furA-katG* operon, and 17 in *katG*). Of the seven different mutations found in the *fabG1-inhA* operon, four were novel: one in the upstream region of the *fabG1-inhA* operon, two in *fabG1*, and one in *inhA* (see Table S1 in the supplemental material).

Correlation between INH resistance and mutations. We recently reported 5 novel mutations in *katG* (28). Including these mutations, 280 different mutations in *katG* were found in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) when articles were searched by the keywords "*katG*," "mutation," and "tuberculosis." In addition, six mutations in the upstream region of the *fabG1-inhA* operon, including C−15T, and seven in *inhA* cause INH resistance (27, 28, 36). In this study, we found an additional 17 novel mutations in *katG*. One was a silent mutation (C195T [A65A]), while the other 16 caused amino acid substitutions. These mutations and amino acid substitutions are shown in Table 3. Furthermore, several novel mutations were detected in the present study: one in *fabG1* (G609A [L203L]), one in *furA* (C41T [A14V]), and three in the intergenic region of the *furA-katG* operon (G−7A, A−10C, and G−12A).

We will report elsewhere that these mutations in *furA* and the intergenic region are associated with INH resistance induced by downregulation of *katG* expression (H. Ando and T. Kirikae, unpublished results), and those in *fabG1* are also associated with INH resistance induced by upregulation of *inhA* expression (Ando et al., unpublished). In the present study, we examined whether novel mutations in *katG* are associated with INH resistance.

Correlation between mutations and IS*6110*-probed RFLP. As shown in Fig. S1 and Table S1 in the supplemental material, all isolates belonging to cluster I detected in the IS*6110*-probed RFLP analysis, 11 (50%) in cluster II, and 8 (67%) in cluster III had a C−15T mutation in the *inhA* promoter region. All isolates in cluster IV had a C41T mutation in *furA*. All isolates in cluster V had a G944C/G945A (S315T/R) mutation. Isolates harboring *katG* mutations, except those with the G944C/G945A (S315T/R) mutation, did not cluster in the IS*6110*-probed RFLP.

Enzymatic activity of the novel KatG mutants. We cloned a wild-type (WT) *katG* gene (p*katG*-wt) from H37Rv, a *katG* gene carrying a G1388T neutral mutation (p*katG*-1) from IMCJ 2751, and 20 *katG* genes harboring mutations causing amino acid substitutions (p*katG*-2 to -21) from Inh^r isolates (Tables 1 and 3). Among the mutants, 15 were novel and 6 had been reported previously (the *katG*-1, -7, -9, -13, -16, and -19 mutants) (Table 3). These *katG* genes were expressed in *katG*-deficient *E. coli* UM262. As shown in Fig. 2, *E. coli* isolates with *katG*-wt expressed KatG (lanes 1 and 15), whereas *E. coli* isolates with an empty vector did not (lanes 2 and 16). *E. coli* isolates carrying *katG* mutants other than the *katG*-15 (lane 8) and *katG*-17 (lane 24) mutants expressed KatG proteins at levels similar to those observed for *E. coli* isolates carrying p*katG*-wt. *E. coli* isolates with *katG*-15 (lane 8) and *katG*-17 (lane 24), which had a frame shift mutation (Table 3), did not express *katG*.

INH oxidase, peroxidase, and catalase activities were assessed using these clones (Table 4). Of the cloned mutants, one with KatG(R463L) from IMCJ 2751 showed levels of these activities similar to those observed for the wild type, and the KatG(R463L) mutation was not associated with INH resis-

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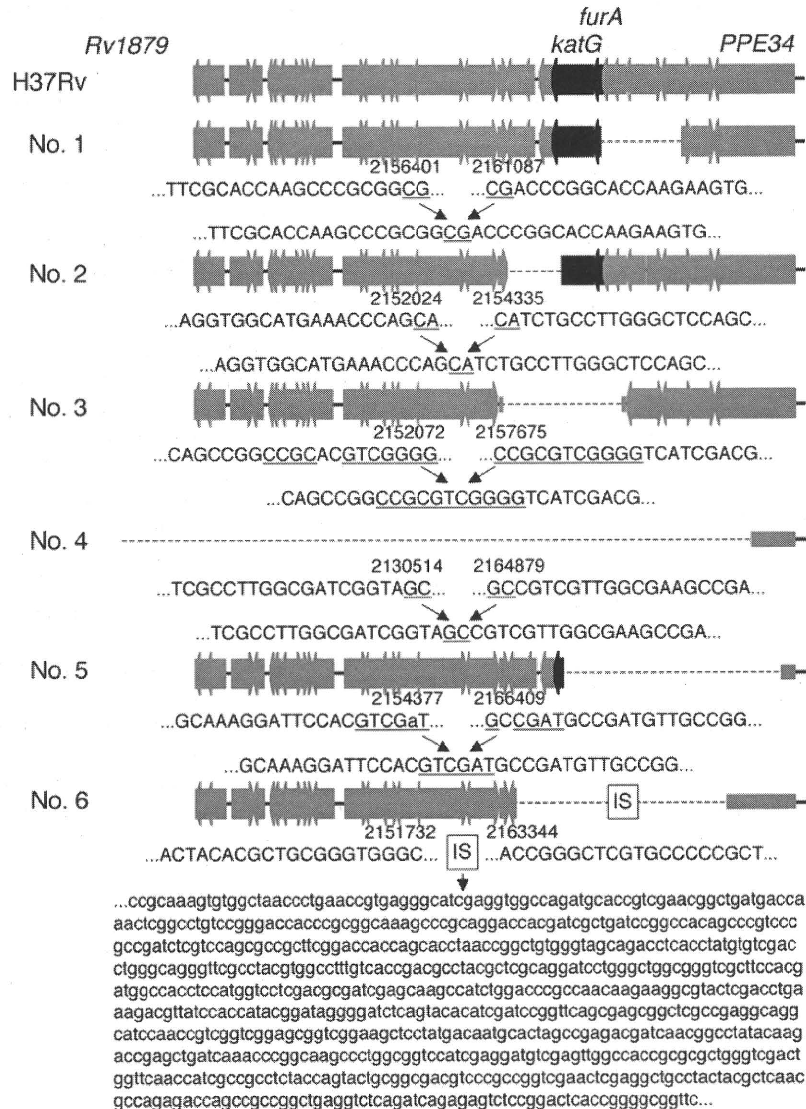

FIG. 1. Maps of large-scale deleted regions adjacent to *katG* in six Inh^r *M. tuberculosis* isolates. Bold arrows indicate the open reading frames annotated in the H37Rv genome sequence (http://genolist.pasteur.fr/TubercuList/). The dotted lines correspond to the deleted regions, with the end sequences and H37Rv genome coordinates given below. Underlined sequences are possible substrates for recombination. The box labeled "IS" represents the 750-bp fragment of IS*6110*. Numbers 1 to 6 represent the names of the isolates and correspond to the numbers shown in Table S1 in the supplemental material. A nucleotide shown in lowercase in region 5 indicates a mutation.

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tance (Table 4). With regard to INH oxidase activity, *E. coli* isolates with *katG*-2 to -8 showed 1/3 to 1/17 less activity than those with *katG*-wt. *E. coli* isolates carrying *katG*-9 to -13 showed reduced activity compared to those carrying *katG*-2 to -8. *E. coli* isolates carrying *katG*-14 to -21 showed no activity (i.e., levels similar to those observed for vector controls). These results indicated that the degree of INH oxidase activity is correlated with that of INH resistance. *E. coli* isolates with *katG*-wt and *katG*-1 showed the highest levels of INH oxidase activity, and *M. tuberculosis* isolates with these genes were sensitive to INH. *E. coli* isolates carrying *katG*-2 to -8 showed slightly weaker activities, and *M. tuberculosis* isolates with these genes were resistant to INH at 0.2 μg/ml but susceptible to INH at 1.0 μg/ml. *E. coli* isolates with *katG*-9 to -21 showed weak or no activity, and *M. tuberculosis* isolates with these genes were resistant to INH at 1.0 μg/ml.

The peroxidase and catalase activities of *E. coli* isolates with mutations were correlated well with each other and also with INH oxidase activity (Table 4). However, in *E. coli* isolates carrying some clones, peroxidase/catalase activities were different from INH oxidase activity, i.e., *E. coli* isolates with *katG*-16 and -9 showed weak activity.

Development of a LiPA for detection of INH resistance. To detect novel mutations associated with INH resistance, we developed a new LiPA based on the reverse hybridization principle (25). Forty-one oligonucleotide probes were designed for the LiPA to detect mutations containing the *furA-katG* operon, the *fabG1-inhA* operon, $P_{fabG1\text{-}inhA}$, and *fabG1* (Table

TABLE 3. *katG* mutations found in Inh[r] isolates

Clone	Mutation(s)	
	Nucleotide	Amino acid
katG-1[a]	G1388T	R463L
katG-2[c]	C379G[b]	Q127E[b]
katG-3[c]	C694T[b]	P232S[b]
katG-4	A398C[b]	N133T[b]
katG-5[c]	T1147C[b]	S383P[b]
katG-6[c]	1297::C[b], Δ1305C[b]	KQT433-435QAD[b]
katG-7[c]	A290G	H97R
katG-8[c]	C1465A[b]	R489S[b]
katG-9	G944C	S315T
katG-10[c]	T1259C[b]	M420T[b]
katG-11	G944C, G1159C[b]	S315T, D387H[b]
katG-12[c]	G368A[b], G895A	G123E[b], G299S
katG-13[c]	G1255C	D419H
katG-14[c]	C195T[b], T527C[b]	A65A[b], M176T[b]
katG-15	Δ(478–479)[b]	Frame shift[b]
katG-16[c]	G944C	S315T
katG-17	Δ371G[b]	Frame shift[b]
katG-18[c]	C1894t[b]	R632C[b]
katG-19	C945A	S315R
katG-20[c]	Δ(571–576)[b]	Δ(191W-192E)[b]
katG-21[c]	G1624C[b]	D542H[b]

[a] *katG*-1 carrying a G1388T (R463L) neutral mutation was cloned from the Inh[s] strain IMCJ 2751.
[b] These mutations have not previously been reported. Other mutations were previously reported in references 36 (G1388T), 7 (A290G), 36 (G944C), 7 (G895A), 6 (G1255C), and 36 (C945A).
[c] This clone also had a G1388T neutral mutation.

2). As shown in Fig. S2 in the supplemental material, the LiPA could detect all mutations found in this study.

DISCUSSION

The results of RFLP and sequence analysis in the present study indicated that there are several predominant strains of Inh[r] *M. tuberculosis* with different genetic backgrounds in Japan (see Fig. S1 and Table S1 in the supplemental material). These strains had *katG(G944C)* (S315T), an *inhA* promoter mutation, *fabG1(G609A)* (L203L), and *furA(C41T)* (A14V) (see Table S1 in the supplemental material). Inh[r] isolates were reported to expand clonally in several regions, including northwestern Russia (20), the Netherlands (30), San Francisco, CA (13), Venezuela (2), and Sierra Leone (15). These clonal Inh[r] strains had a KatG(S315T) or *inhA* promoter mutation. Gagneux et al. (13) reported that the strains carrying the KatG(S315T) or *inhA* promoter mutation were more likely to spread than those carrying other mutations; our results were consistent with these previous findings. In addition, strains with *fabG1(G609A)* (L203L) and *furA(C41T)* (A14V) mutations were also more likely to spread in Japan.

Of Inh[r] isolates, a smaller number (22%) had S315T/R mutations in Japan (Table S1). The prevalences of the KatG(S315T) mutation in *M. tuberculosis* strains from around the world differ, especially with regard to the prevalence of tuberculosis. In regions where the prevalence of tuberculosis is low or intermediate, the mutation has been reported relatively infrequently: it occurred in 26% to 30% of 95 isolates from Singapore (16) and Madrid (23) and rarely in isolates from Scotland (11) and Finland (19). In contrast, the S315T mutation accounted for INH resistance in 52% to 64% of strains in Africa (8, 14, 31), 79% in Peru (9), 91% in Russia (18), and 58% in New York, NY. (23).

We found four KatG mutations (D419H, M420T, D542H, and R632C) that are associated with high-level INH resistance, and we also found three KatG mutations (H97R, N133T, and P232S) that are associated with low-level INH resistance (Table 4). The S315 mutation is known to confer high-level INH resistance (24, 26, 33). KatG is a functional homodimer, and each monomer is composed of two domains that are mainly α-helical. The N-terminal domain contains a heme binding site, whereas the C-terminal domain lacks this feature (34). The high-level INH resistance-associated mutations D419H and M420T are located in the region connecting the N-terminal and C-terminal domains (5). The interdomain interactions between the N-terminal and C-terminal domains of the two monomers are essential for forming the functional homodimer (5). The changes in the interdomain interactions due to the D419H and M420T mutations may result in loss of enzymatic activities of KatG. D542H and R632C are located in the 16th

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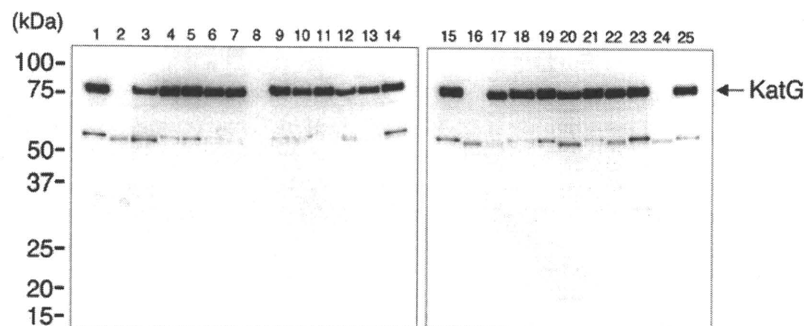

FIG. 2. Western blot of whole-cell extracts from *katG*-deficient *E. coli* strain UM262 transformed with the empty vector, pTrcHis2-TOPO, or recombinant plasmids expressing various KatG mutations as follows: lanes 1 and 15, WT; lanes 2 and 16, empty vector; lane 3, R463L and D542H; lane 4, S315T and R463L; lane 5, Q127E and R463L; lane 6, P232S and R463L; lane 7, G123E, G299S, and R463L; lane 8, frame shift mutation from position 160; lane 9, S315T and D387H; lane 10, R463L and R489S; lane 11, S315R; lane 12, M420T and R463L; lane 13, A65A, M176T, and R463L; lane 14, H97R and R463L; lane 17, Δ(191W-192E) and R463L; lane 18, N133T; lane 19, R463L; lane 20, R463L and R632C; lane 21, S315T; lane 22, D419H and R463L; lane 23, S383P and R463L; lane 24, frame shift mutation from position 124; lane 25, in-frame insertion and deletion and R463L. The positions of molecular mass markers are shown on the left.

TABLE 4. Enzymatic activities of KatG mutants detected in this study

Plasmid	Amino acid mutation(s)		Mean activity ± SD[a]			Additional mutation associated with INH resistance	INH resistance level[b]
	Not previously reported	Previously reported	INH oxidase (10^3 A_{560} units)	Peroxidase (10^2 A_{405} units)	Catalase (10^2 A_{240} units)		
pTrcHis2-TOPO[c]			4.84 ± 0.17	6.89 ± 0.70	0.00 ± 0.24		
p*katG*-wt			177.16 ± 18.50	286.08 ± 0.43	142.26 ± 0.16		S
p*katG*-1		R463L	162.00 ± 11.31	289.62 ± 1.40	141.85 ± 0.13		S
p*katG*-2	Q127E	R463L	60.18 ± 0.95	256.07 ± 7.80	143.21 ± 0.35	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-3	P232S	R463L	54.47 ± 0.36	62.25 ± 0.05	76.63 ± 0.52		0.2
p*katG*-4	N133T		40.67 ± 6.31	36.00 ± 0.26	100.61 ± 5.55		0.2
p*katG*-5	S383P	R463L	38.49 ± 0.04	42.24 ± 3.64	107.65 ± 4.13	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-6	KQT433-435QAD[d]	R463L	20.02 ± 0.48	106.47 ± 1.17	142.81 ± 0.11	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-7		H97R, R463L	17.42 ± 0.35	26.80 ± 0.44	27.86 ± 2.01		0.2
p*katG*-8	R489S	R463L	10.40 ± 0.16	27.34 ± 0.27	14.83 ± 0.93	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-9		S315T	8.83 ± 0.04	102.00 ± 2.54	71.26 ± 1.71		1.0
p*katG*-10	M420T	R463L	8.42 ± 0.14	21.02 ± 0.37	46.45 ± 0.20		1.0
p*katG*-11	D387H	S315T	7.93 ± 0.08	34.75 ± 0.61	35.71 ± 0.41		1.0
p*katG*-12	G123E	G299S, R463L	6.87 ± 0.66	6.02 ± 0.17	−0.70 ± 1.42	$P_{fabG1\text{-}inhA}$T−8C	1.0
p*katG*-13		D419H, R463L	6.30 ± 0.52	7.67 ± 0.01	4.49 ± 0.39		1.0
p*katG*-14	M176T[e]	R463L	5.14 ± 0.01	4.67 ± 0.07	1.06 ± 0.30	$P_{fabG1\text{-}inhA}$C−15T	1.0
p*katG*-15	Frame shift[f]		5.02 ± 0.24	4.01 ± 0.57	−1.75 ± 1.16		1.0
p*katG*-16		S315T, R463L	3.83 ± 0.18	84.41 ± 0.17	117.07 ± 7.56		1.0
p*katG*-17	Frame shift[g]		3.30 ± 0.69	4.59 ± 0.09	2.07 ± 1.51		1.0
p*katG*-18	R632C	R463L	3.26 ± 0.13	1.56 ± 0.08	−7.41 ± 0.76		1.0
p*katG*-19		S315R	3.19 ± 0.76	3.24 ± 0.02	−2.36 ± 0.71		1.0
p*katG*-20	Δ(191W-192E)[h]	R463L	2.78 ± 0.09	2.09 ± 0.04	2.61 ± 1.86		1.0
p*katG*-21	D542H	R463L	1.63 ± 0.49	0.32 ± 0.17	−7.00 ± 0.69		1.0

[a] Mean (n = 3) ± SD.
[b] The INH susceptibility levels for clinical isolates with *katG* mutations are shown, as follows: S, INH sensitive; 0.2, resistant to INH (0.2 μg/ml) and susceptible to INH (1.0 μg/ml); and 1.0, resistant to INH (1.0 μg/ml).
[c] A vector control.
[d] 1297::C and Δ1305C.
[e] This isolate had an additional A65A silent mutation.
[f] Δ(478–479).
[g] Δ371G.
[h] Δ(571–576).

and 19th α-helices in the C-terminal domain, respectively, and showed no enzymatic activities, although the functional role of the C-terminal domain in KatG remains unclear (5, 34). The mutations associated with low-level INH resistance, H97R, N133T, and P232S, are located adjacent to the INH binding pocket (5). They may weakly affect the binding affinity of INH. The S315T mutation located at the INH binding pocket could block binding of INH without interfering with catalysis (5).

The new LiPA was able to distinguish high-level INH resistance (resistant to 1.0 μg/ml) from low-level INH resistance (resistant to 0.2 μg/ml and sensitive to 1.0 μg/ml) in clinical isolates without sequencing. Thus, we were able to determine the degree of INH resistance using this LiPA. This assay would be useful in clinical application in combination with culture-based drug susceptibility tests. We have recently developed a LiPA to detect a *pncA* mutation(s) for rapid detection of pyrazinamide-resistant *M. tuberculosis* (29), which was shown to be readily usable in clinical applications (1). The whole procedure takes only 9 h, and the estimated cost per sample is $35. The clinical trials for *in vitro* diagnosis are in progress (from April 2009 to March 2010) in Japan. The trials will reveal the specificity of the LiPA. It will be beneficial especially in developing countries where the laboratories are scarcely equipped because of the high cost of setting them up.

Assessment of INH oxidase activities of *M. tuberculosis* isolates may provide useful information about INH resistance. The INH oxidase activities of KatG mutants showed good correlations with the degree of INH resistance (Table 4). Other enzymatic activities of KatG mutants, i.e., peroxidase and catalase activities, were also correlated with the degree of INH resistance (Table 4). However, the activities of the S315T mutant were not, i.e., this mutant showed catalase-peroxidase activities but no INH oxidase activity (Table 4). Other S315 mutants, such as the S315R (Table 4) and S315N (32) mutants, have lost all three kinds of enzymatic activity. Thus, the Inh^r isolates with KatG(S315T), retaining catalase-peroxidase activities, may have a survival advantage, and this may explain the global spread of strains with the KatG(S315T) mutation.

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