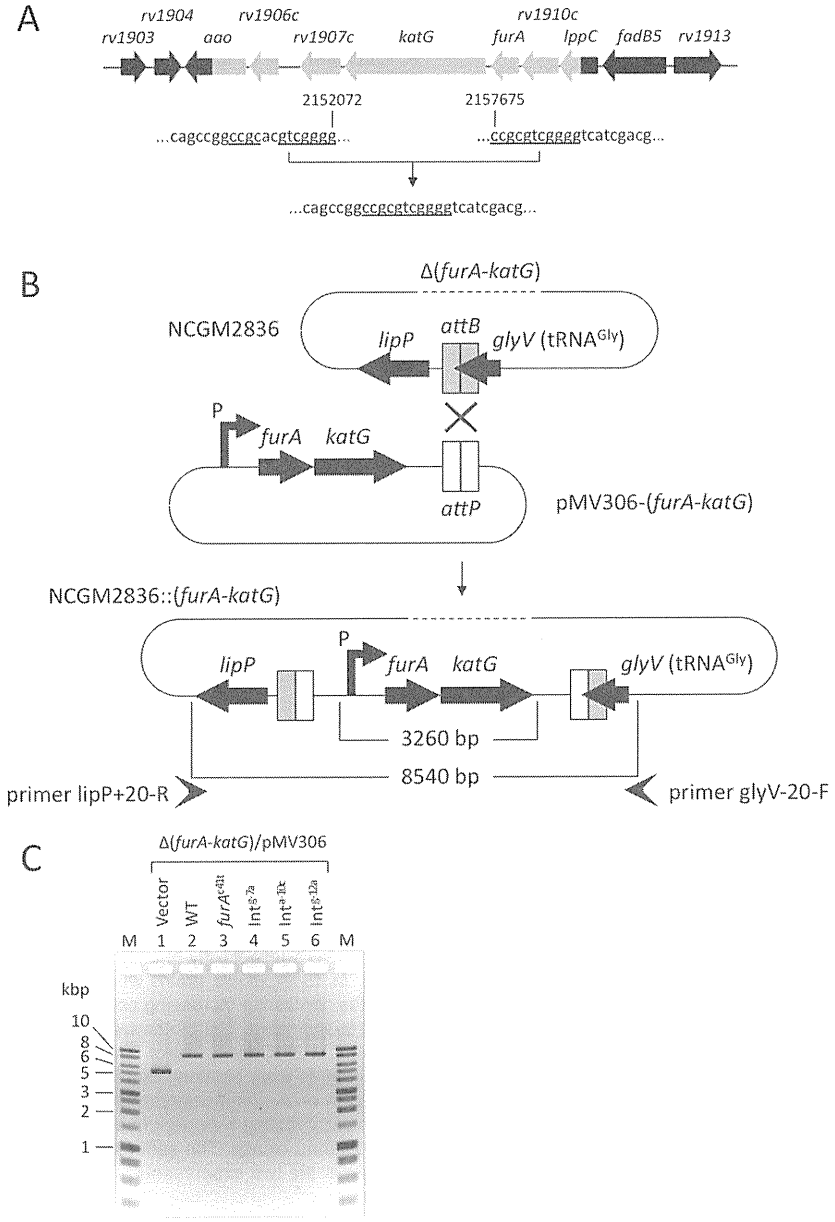

Fig. 1. A. Maps of the deleted regions adjacent to *furA–katG* in INH^r *M. tuberculosis* strain NCGM2836. Bold arrows indicate the open reading frames annotated in the H37Rv genome sequence (http://tuberculist.epfl.ch/). The grey bold arrows correspond to the deleted regions, with the end sequences and H37Rv genome co-ordinates given below. Underlined sequences are possible substrates for recombination.
B. Schematic representation of the construction of complementary strains. An integration-proficient vector pMV306 containing *furA–katG* with the Int$^{g\text{-}7a}$, Int$^{a\text{-}10c}$, Int$^{g\text{-}12a}$ or *furA*^{c41t} mutation was used to transform NCGM2836. Site-specific integration between *attB* and *attP* sites generated complementary isogenic strains.
C. The complementary isogenic strains were examined for the correct chromosomal structures by PCR using locus-specific primers (glyV-20-F/lipP+20-R). M, size marker.

and NCGM2836/Vector did not. NCGM2836/WT and NCGM2836/*furA*^{c41t} showed similar levels of activity as H37Rv, whereas NCGM2836/Int$^{g\text{-}7a}$, NCGM2836/Int$^{a\text{-}10c}$ and NCGM2836/Int$^{g\text{-}12a}$ showed significantly lower levels of activity than NCGM2836/WT.

These experiments using complementary strains demonstrated that the mutations Int$^{g\text{-}7a}$, Int$^{a\text{-}10c}$ and Int$^{g\text{-}12a}$ were associated with reductions in KatG expression and INH oxidase activities, but that the *furA*^{c41t} mutation was not.

INH susceptibility of the complementary strains

The complementary strains NCGM2836/Int$^{g\text{-}7a}$, NCGM2836/Int$^{a\text{-}10c}$ and NCGM2836/Int$^{g\text{-}12a}$ showed low levels of INH resistance (Fig. 3). NCGM2836/WT was resistant to 0.05 μg ml^{-1} but susceptible to 0.1 μg ml^{-1} INH, whereas NCGM2836/Vector was resistant to 1.0 μg ml^{-1} INH. NCGM2836/*furA*^{c41t} showed the same INH susceptibility as NCGM2836/WT, being resistant to 0.05 μg ml^{-1} but susceptible to 0.1 μg ml^{-1} INH. NCGM2836/Int$^{g\text{-}7a}$ and NCGM2836/Int$^{a\text{-}10c}$ were resistant to 0.2 μg ml^{-1} but susceptible to 0.4 μg ml^{-1} INH, whereas NCGM2836/Int$^{g\text{-}12a}$ was resistant to 0.1 μg ml^{-1} but susceptible to 0.15 μg ml^{-1} INH.

Three other INH susceptibility tests showed similar results (Table 2). The three complementary strains with mutations in the *furA–katG* intergenic region showed low levels of INH resistance. NCGM2836/Int$^{g\text{-}7a}$ and

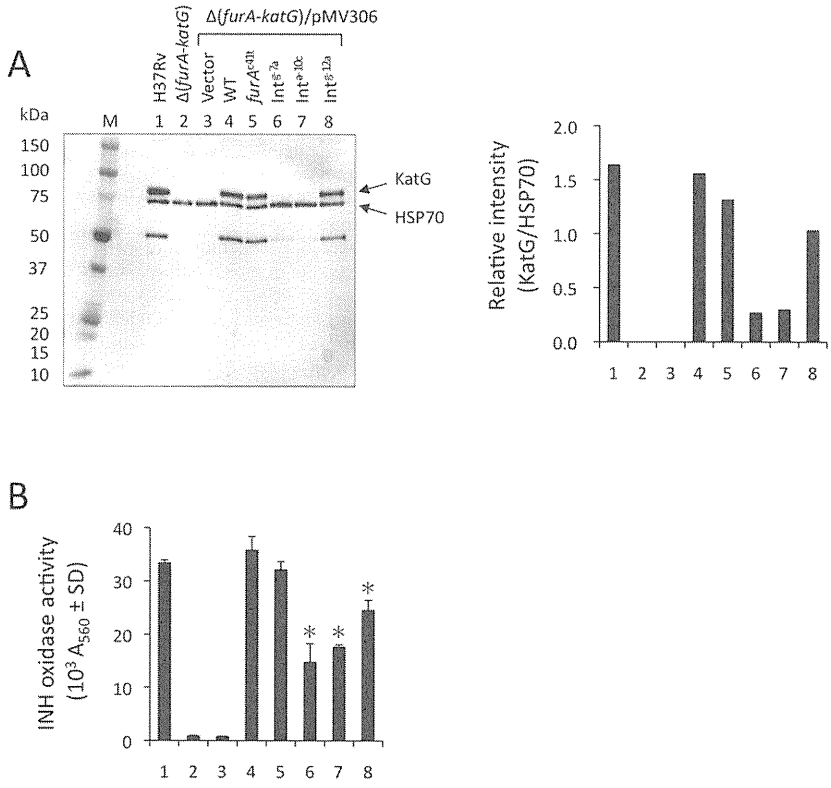

Fig. 2. KatG expression of complementary strains with mutations in *furA–katG*.
A. KatG and HSP70 expression were analysed in complementary strains by SDS-5%–20%-gradient-PAGE and Western blotting. The intensity of KatG was normalized relative to HSP70. This experiment was repeated three times with similar results. M, size marker.
B. INH oxidase activity of KatG in complementary strains. Each transformant was incubated in triplicate, and INH oxidase activity was measured. Data are shown as the means ± SD (*$P < 0.01$).

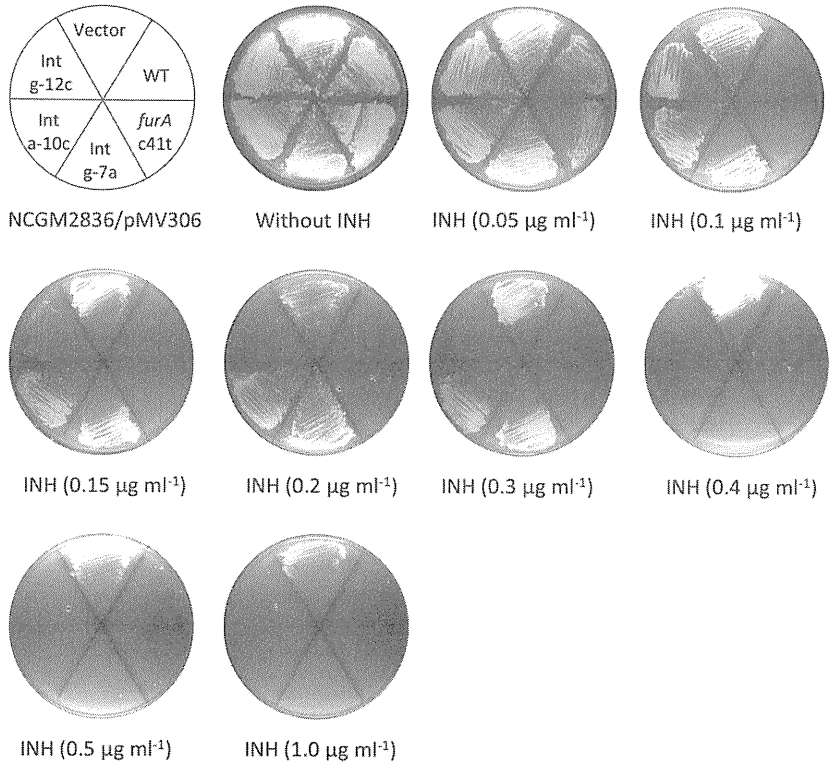

Fig. 3. INH susceptibility of complementary strains. *M. tuberculosis* strains were streaked on plates containing increasing concentrations of INH as indicated. The plates were incubated at 37°C for 2 weeks. Two clones of transformants were obtained by picking colonies in each complementary strain. INH susceptibility testing was performed twice using different clones. The same MICs were obtained in the two experiments.

Table 2. INH susceptibility of *M. tuberculosis* strain NCGM2836 Δ(*furA–katG*) transformants.

				Proportion method				Dilution method [MIC (μg ml^{-1})]	
	Genotype			7H10 agar[a]		Egg-based Ogawa medium[b]			
Strain	*furA*	Int[e]	*katG*	0.2[f]	1.0[g]	0.2[f]	1.0[g]	7H10 agar[c]	7H9 broth[d]
H37Rv	wt	wt	wt	S	S	S	S	0.05	0.125
Δ(*furA–katG*)/Vector	Deletion			R	R	R	R	> 1.0	> 32
Δ(*furA–katG*)/WT	wt	wt	wt	S	S	S	S	0.1	0.125
Δ(*furA–katG*)/*furA*^{c41t}	c41t	wt	wt	S	S	S	S	0.1	0.25
Δ(*furA–katG*)/Int^{g-7a}	wt	g-7a	wt	R	S	R	S	0.4	2.0
Δ(*furA–katG*)/Int^{a-10c}	wt	a-10c	wt	R	S	R	S	0.4	2.0
Δ(*furA–katG*)/Int^{g-12a}	wt	g-12a	wt	S	S	S	S	0.15	0.5

a. An agar proportion method using Middlebrook 7H10 agar medium recommended by Clinical and Laboratory Standards Institute. The susceptibility testing was performed twice with the same results.
b. A proportion method using egg-based Ogawa medium modified by WHO protocol and recommended by the Japanese Society of Tuberculosis. The susceptibility testing was performed twice with the same results.
c. A dilution method using Middlebrook 7H10 agar medium. The susceptibility testing was performed twice with the same results.
d. A microdilution method using Middlebrook 7H9 broth medium. The susceptibility testing was performed twice with the same results.
e. Int: intergenic region.
f. 0.2: resistant to INH (0.2 μg ml^{-1}) and susceptible to INH (1.0 μg ml^{-1}).
g. 1.0: resistant to INH (1.0 μg ml^{-1}).

NCGM2836/Int^{a-10c} were more resistant than NCGM2836/Int^{g-12a}. H37Rv and NCGM2836/WT were susceptible to INH, whereas NCGM2836/Vector was resistant. NCGM2836/*furA*^{c41t} was susceptible to INH (0.2 μg ml^{-1}) by proportion methods, with MICs of 0.1 μg ml^{-1} as determined by the 7H10 agar dilution method and 0.25 μg ml^{-1} as determined by the 7H9 broth dilution method. Although *furA*^{c41t} showed twofold higher INH resistance than NCGM2836/WT by the 7H9 broth dilution method, this difference was not significant relative to the error associated with this type of experiment. NCGM2836/Int^{g-7a} and NCGM2836/Int^{a-10c} had identical INH susceptibility profiles, i.e. they were resistant to INH (0.2 μg ml^{-1}) and their MICs were 0.4 and 2.0 μg ml^{-1} respectively. NCGM2836/Int^{g-12a} was susceptible to INH (0.2 μg ml^{-1}), with MICs of 0.15 and 0.5 μg ml^{-1} respectively. These data indicated that Int^{g-7a} and Int^{a-10c} mutations confer resistance to INH (0.2 μg ml^{-1}) and that the Int^{g-12a} mutation causes a slight decrease in INH susceptibility.

Regulation of katG *expression by the* furA–katG *intergenic region*

A polypurine sequence (GGAAGGAA) was identified in the *furA–katG* intergenic region, which is complementary to the 3′ end of the 16S rRNA sequence in *M. tuberculosis* (Fig. 4A) (Sala *et al.*, 2008). All three mutations in the intergenic region identified in INH^r clinical isolates, i.e. Int^{g-7a}, Int^{a-10c} and Int^{g-12a}, were located within this polypurine sequence (Fig. 4A). A reporter assay using *furA–katG′–′lacZ* gene fusions (Fig. 4B) showed that these mutations significantly repressed *lacZ* expression (Fig. 4C and D). These results suggested that the intergenic region plays a critical role in *katG* expression and that these mutations are responsible for INH resistance by decreasing *katG* expression.

Reduced katG *expression in INH^r clinical isolates with a mutation in the* furA–katG *intergenic region*

The four clinical isolates with mutations in the *furA–katG* intergenic region (Table 1) were tested for their levels of *katG* expression by Western blotting (Fig. 5A) and KatG activity by INH oxidation assays (Fig. 5B). The INH^r Δ(*furA–katG*) mutant NCGM2836 did not produce *katG*, whereas H37Rv, INH^s NCGM2898 and INH^r NCGM2828 with $P_{inhA}{}^{c-15t}$ expressed *katG* at similar levels. The isolates containing the Int^{g-7a} (NCGM2874 and NCGM2875) and Int^{a-10c} (NCGM2930) mutations expressed little *katG*. An isolate with Int^{g-12a} (NCGM2934) expressed *katG*, but at a level lower than that of INH^s controls (68% of H37Rv and 76% of INH^s NCGM2898). The levels of INH oxidase activities were correlated with those of *katG* expression (Fig. 5B). Four isolates with Int^{g-7a}, Int^{a-10c} and Int^{g-12a} mutations showed significantly lower levels of INH oxidase activity than the two INH^s controls and the one INH^r isolate with the $P_{inhA}{}^{c-15t}$ mutation; these levels were 21%, 21%, 29% and 57%, respectively, when compared to H37Rv (Fig. 5B). These results suggested that these mutations in the *furA–katG* intergenic region decrease the levels of *katG* expression and INH oxidase activity. Reduced *katG* expression is not a general feature of INH^r isolates, because an INH^r NCGM2828 with $P_{inhA}{}^{c-15t}$ expressed *katG* at similar levels to INH^s isolates.

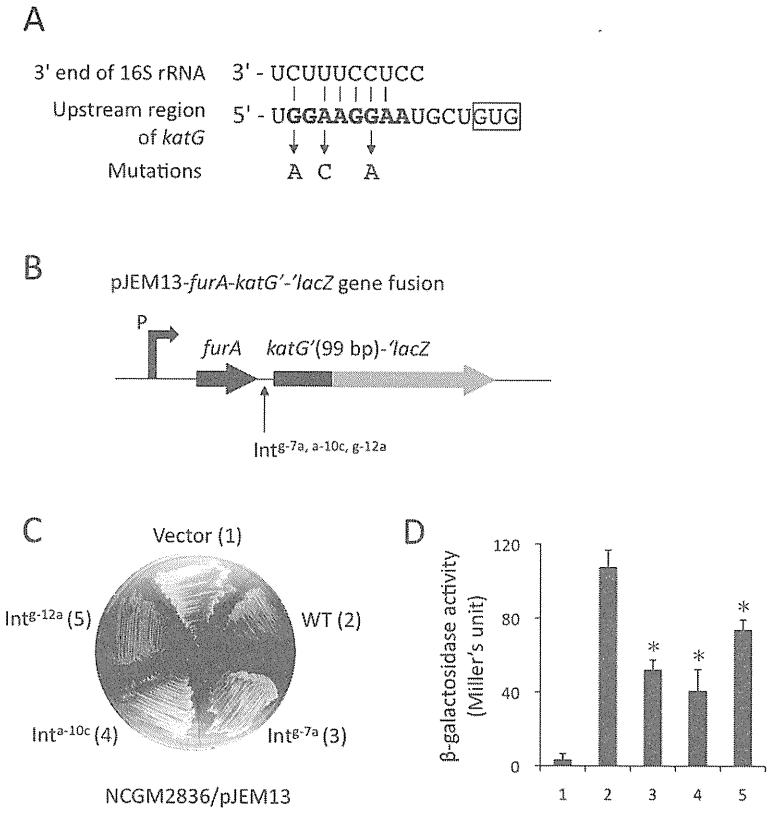

Fig. 4. β-Galactosidase activity of the *furA–katG'–'lacZ* fused gene carrying the $Int^{g\text{-}7a}$, $Int^{a\text{-}10c}$ or $Int^{g\text{-}12a}$ mutation.
A. Complementarities between the 3′ end of 16S rRNA and the polypurine sequence in the *furA–katG* intergenic region. The polypurine sequence is shown in bold and the translational initiation codon of *katG* is boxed.
B. Schematic representation of the construction of *furA–katG'–'lacZ* gene fusion. A fragment from 138 bp upstream of the first nucleotide of *furA* to the 99th base of *katG* with $Int^{g\text{-}7a}$, $Int^{a\text{-}10c}$ or $Int^{g\text{-}12a}$ mutation was fused to the *lacZ* gene. pJEM13-derived vectors were used to transform the NCGM2836 Δ(*furA–katG*) strain.
C. β-Galactosidase activity was monitored by streaking strains on 7H10 plates containing X-gal. The plates were incubated at 37°C for 2 weeks. Two clones of transformants were obtained by picking colonies in each strain. Duplicate experiments with different clones yielded similar results.
D. Quantitative β-galactosidase assay. Each transformant was incubated in triplicate, and β-galactosidase activity was measured. Data are shown as the means ± SD (*$P < 0.01$).

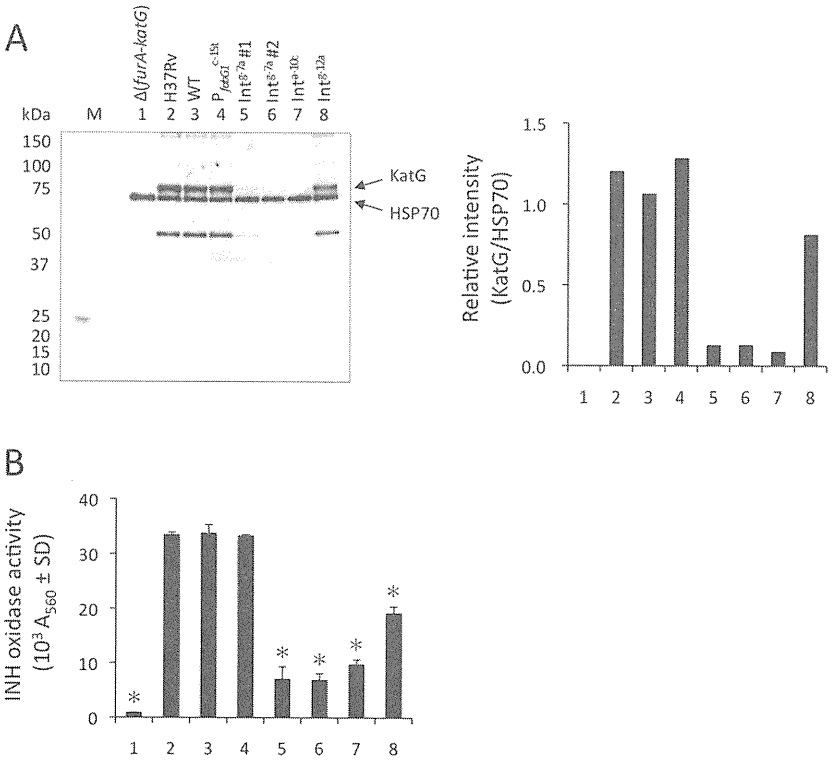

Fig. 5. KatG expression of clinical isolates with $Int^{g\text{-}7a}$, $Int^{a\text{-}10c}$ or $Int^{g\text{-}12a}$ mutation.
A. KatG and HSP70 expression in clinical isolates were analysed by SDS-5%–20%-gradient-PAGE and Western blotting. The intensity of KatG was normalized relative to HSP70. This experiment was repeated twice with similar results. M, size marker.
B. INH oxidase activity of KatG in clinical isolates. Each transformant was incubated in triplicate, and INH oxidase activity was measured. Data are shown as the means ± SD (*$P < 0.01$).

Reduced katG *expression in INH^r clinical isolates with* furA^{c41t} *mutation*

We also tested 18 clinical isolates with *furA*^{c41t} mutation and the one clinical isolate with a frameshift of *furA* (*furA*$^{\Delta 34c}$) (Table 1) for their levels of *katG* expression by Western blotting (Fig. 6A) and for KatG activity by INH oxidation assays (Fig. 6B). The INH^r Δ(*furA–katG*) mutant did not produce *katG*. H37Rv, INH^s NCGM2898 and INH^r NCGM2828 with $P_{inhA}^{c\text{-}15t}$ expressed *katG* at similar levels. All 18 INH^r isolates with *furA*^{c41t} tested produced smaller quantities of KatG than did the INH^s isolates and the INH^r isolates with $P_{inhA}^{c\text{-}15t}$, whereas the *furA*$^{\Delta 34c}$ mutant produced similar amounts of KatG as those of INH^s isolates (Fig. 6A). Quantification of these findings showed that the levels of KatG production in INH^r isolates with *furA*^{c41t} were 20–48% those of H37Rv and 23–55% those of NCGM2898. All of the clinical isolates with *furA*^{c41t} without *katG* mutations showed significantly lower levels of INH oxidase activity than did the two INH^s controls and the one INH^r isolate with $P_{inhA}^{c\text{-}15t}$, but these levels were only 64–87% those of the INH^s controls (Fig. 6B). Four of the five isolates with *furA*^{c41t} and *katG* mutations showed no INH oxidase activity, whereas the fourth, NCGM2881, had significantly lower activity, corresponding to 28% of the activity in INH^s controls (Fig. 6B). The *furA*$^{\Delta 34c}$ mutant with two *katG* mutations (Table 1) did not show any INH oxidase activity (Fig. 6B).

FurA^{A14V} *binding to the region upstream of* furA–katG

FurA is a negative transcriptional regulator of the *furA–katG* gene (Zahrt *et al.*, 2001). In addition, FurA may regulate other genes involved in pathogenesis (Pym *et al.*, 2001). In *Pseudomonas aeruginosa* Fur (PaFur), the loop between the N-terminal helices H1 and H2 is thought to be involved in DNA recognition (Pohl *et al.*,

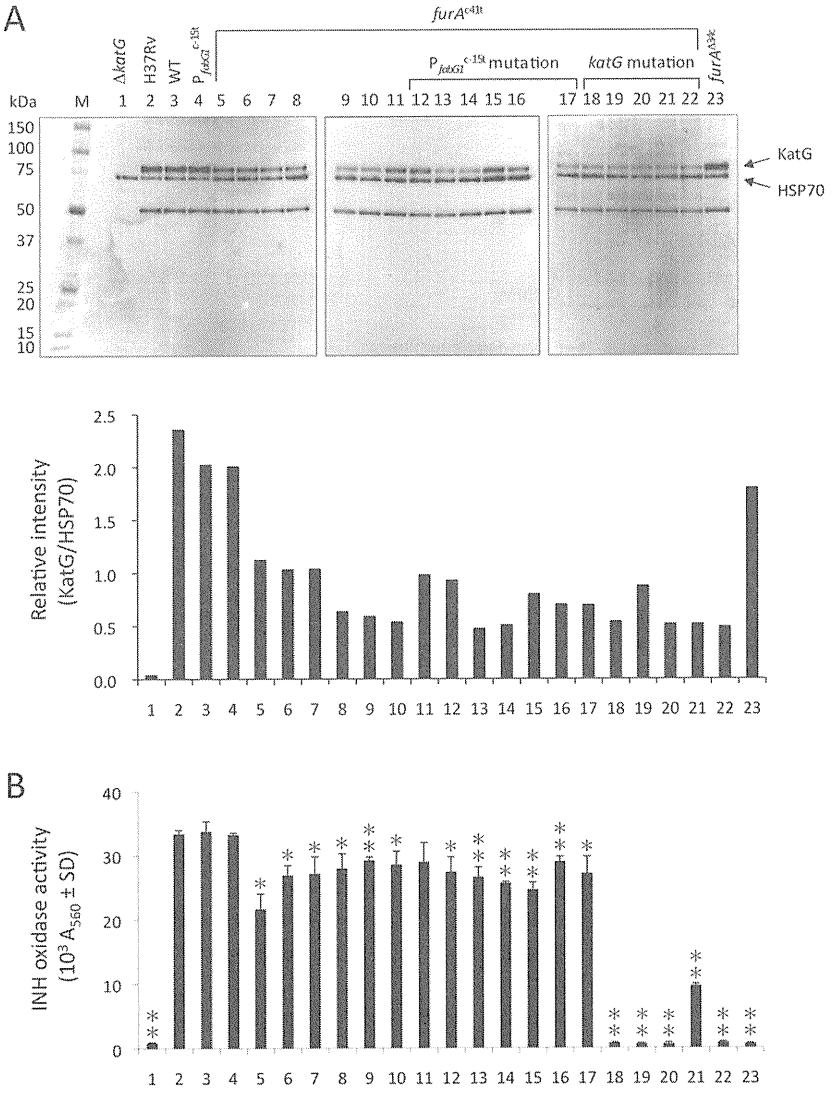

Fig. 6. KatG expression of clinical isolates with the *furA*^{c41t} mutation.
A. KatG and HSP70 expression in clinical isolates were analysed by SDS-5%–20%-gradient-PAGE and Western blotting. Relative intensity of KatG was normalized against HSP70. This experiment was repeated twice with similar results. M, size marker.
B. INH oxidase activity of KatG in clinical isolates. Each transformant was incubated in triplicate, and INH oxidase activity was measured. Data are shown as the means ± SD (*$P < 0.05$, **$P < 0.01$).

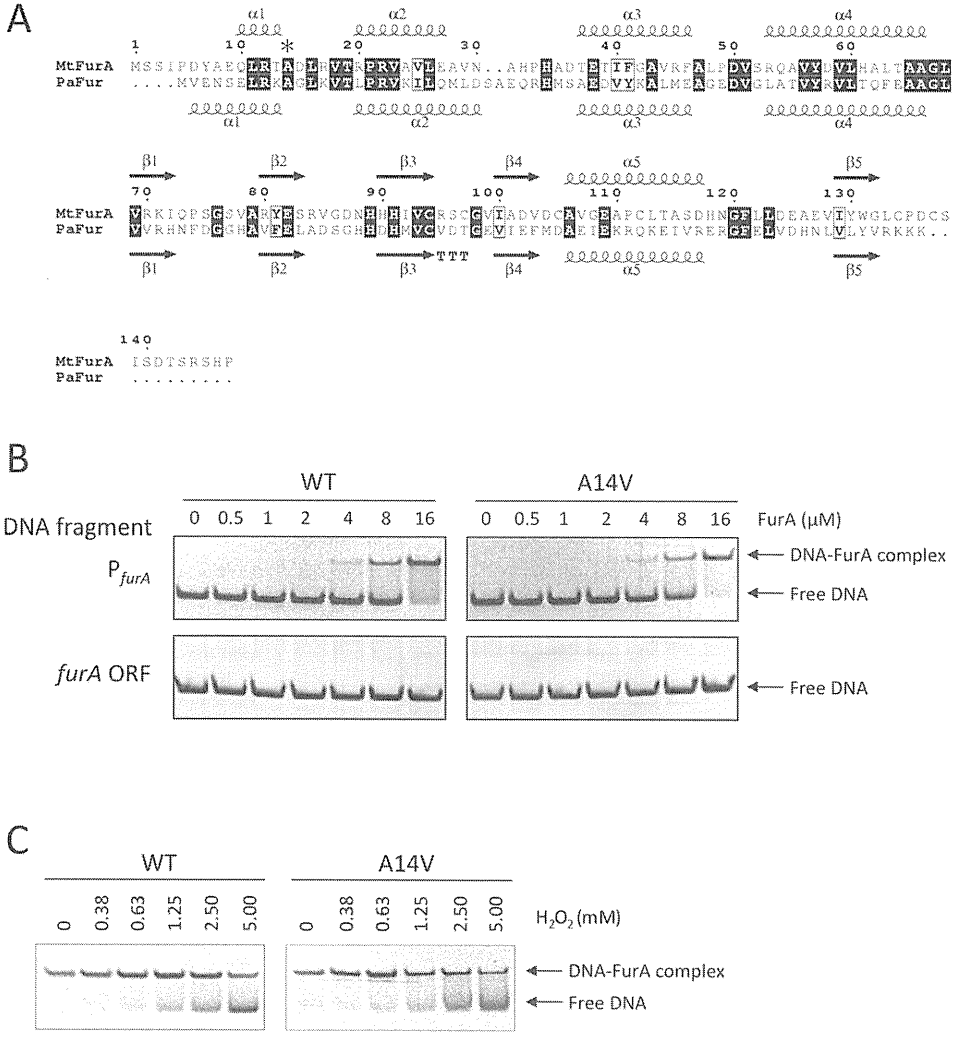

Fig. 7. Binding of FurA^{A14V} to the P_{furA}.
A. Sequence alignment of FurA (MtFurA) and a ferric uptake regulator of *P. aeruginosa* (PaFur). Secondary structures of MtFurA and PaFur are shown above and below the alignment respectively. Conserved residues are highlighted in black, and residues with highly conservative substitutions are boxed. The asterisk indicates the amino acid residue at the 14th position in MtFurA that was found to be mutated to valine in this study.
B. Effects of H_2O_2 on the binding of FurA^{A14V}. Purified FurA or FurA^{A14V} was mixed with DNA fragments and analysed by electrophoresis on 7.5% polyacrylamide gels, which were stained with ethidium bromide. This experiment was repeated three times with similar results.
C. Purified FurA or FurA^{A14V} was incubated with H_2O_2 at various concentrations for 10 min, mixed with DNA fragments and analysed by electrophoresis. This experiment was repeated three times with similar results.

2003). PaFur^{A10G} mutant fails to protect the *pvdS* promoter region (Barton *et al.*, 1996), suggesting that residue Ala-10, located at the C-terminal end of the helix H1, is critical for DNA recognition. As shown by the structure-based alignment (Fig. 7A), the A14V mutation in *M. tuberculosis* FurA (MtFurA) was located at the C-terminal end of helix H1. We therefore performed electrophoretic mobility shift assays (EMSA) to determine whether the FurA^{A14V} mutant affects DNA binding ability (Fig. 7B). EMSA showed that FurA^{A14V} as well as FurA bound to the upstream region of the *furA–katG* (P_{furA}) in a specific, protein dose-dependent manner.

Treatment of FurA with hydrogen peroxide (H_2O_2) has been reported to inhibit its binding to P_{furA} (Sala *et al.*, 2003). Since H_2O_2 treatment may remove iron from the FurA and/or oxidize cysteine residues, we assessed whether H_2O_2 treatment of FurA^{A14V} affects DNA binding (Fig. 7C). Treatment of FurA and FurA^{A14V} with H_2O_2 inhibited the binding of P_{furA} in a H_2O_2 dose-dependent manner. H_2O_2 had similar effects on binding of P_{furA} to FurA and FurA^{A14V}.

Since the binding of FurA to P_{furA} has been reported strong in the presence of Ni^{2+} ions (Sala *et al.*, 2003), we assessed whether its binding to FurA and FurA^{A14V} dif-

fered in the presence or absence of Ni^{2+}, Mn^{2+} or Zn^{2+}. We found that Ni^{2+}, Mn^{2+} and Zn^{2+} did not affect the binding pattern of P_{furA} to FurA and $FurA^{A14V}$ (Fig. S2). In the presence of Ni^{2+}, almost all the P_{furA} added to the binding buffer bound to FurA (Fig. S2). In the absence of Ni^{2+} or in the presence of Mn^{2+} or Zn^{2+}, however, the binding of FurA to P_{furA} was incomplete (Fig. S2). Similar results were observed when we assessed the binding of P_{furA} to $FurA^{A14V}$ (Fig. S2).

Discussion

To our knowledge, this is the first report showing that downregulation of *katG* expression causes INH resistance in clinical isolates of *M. tuberculosis*. These isolates had mutations in the *furA–katG* intergenic region. We constructed these mutations in isogenic strains and demonstrated that mutations in the *furA–katG* intergenic region (g-7a, a-10c and g-12a) are associated with INH resistance. The mechanism responsible for this downregulation involves a decrease in translational efficiency caused by base substitution in the *furA–katG* intergenic region. The polypurine sequence (GGAAGGAA), complementing the 3′ end of the 16S rRNA sequence, in the *furA–katG* intergenic region may act as a ribosome binding site in *M. tuberculosis* (Sala *et al.*, 2008). $Int^{a\text{-}10c}$ had an A to C mutation at the third nucleotide, and $Int^{g\text{-}7a}$ had a G to A mutation at the sixth nucleotide of the polypurine sequence (Fig. 4A). We found that these mutations in the polypurine sequence play a critical role in *katG* expression and showed that the mutations are responsible for INH resistance by decreasing *katG* expression (Figs 2–4). In the polypurine sequence, A at the third nucleotide and G at the sixth nucleotide are especially important for *katG* expression. Using reporter assays, we found that these two mutations significantly repressed *lacZ* expression to 48% and 38%, respectively, of that in the wild-type control. Similar *lacZ* repression was observed when the polypurine sequence was completely substituted with a polypyrimidine sequence (CCTCCCTC), suggesting that the polypurine sequence is necessary for the full expression of *katG* (Sala *et al.*, 2008). The $Int^{g\text{-}7a}$ and $Int^{a\text{-}10c}$ mutations conferred resistance to INH (0.2 μg ml^{-1}) and the $Int^{g\text{-}12a}$ mutation caused a slight decrease in INH susceptibility. We confirmed that the Δ(*furA–katG*) mutant, with no *katG* production and no INH oxidase activity, was highly resistant to INH. In contrast, the isogenic strains with $Int^{g\text{-}7a}$ and $Int^{a\text{-}10c}$ had low but detectable production and activity, with low-level resistance to INH. The levels of *katG*, and probably the levels of INH activated by *katG*, may therefore correlate with the levels of INH susceptibility.

It is unclear whether the $furA^{c41t}$ mutation is associated with INH resistance. Of the 18 INH^r clinical isolates with $furA^{c41t}$ mutations, seven had no other mutations, suggesting an association between the mutation and INH resistance. A role of FurA in INH resistance was suggested, and two strains carrying $FurA^{L68P}$ or $FurA^{C97Y}$ mutations were reported previously (Pym *et al.*, 2001). These isolates had additional mutations in *katG*. Therefore, the involvement of these both FurA mutants in *katG* expression and INH resistance is unclear. We found that the results of drug susceptibility testing with complementary strains differed from those with the clinical isolates, and EMSA showed no differences in the binding patterns of FurA and $FurA^{A14V}$, suggesting that this mutation is not associated with INH resistance. One explanation for the discrepancy is that these clinical isolates may have an unknown additional mutation(s) associated with INH resistance. RFLP analysis showed that 67% of the INH^r isolates carrying $furA^{c41t}$ belonged to cluster II, suggesting that these isolates expanded in a clonal manner. Therefore, these INH^r isolates carrying $furA^{c41t}$ may have unknown mutation(s). Alternatively, $furA^{c41t}$ may be associated with INH resistance, but the effect of the mutation may be observed in clinical isolates but not in complementary strains. Similar discrepancies between the drug MICs of clinical isolates and the complementary strains were reported in ethambutol-resistant clinical isolates and laboratory mutants harbouring an *embB* mutation (Safi *et al.*, 2010).

We found that FurA will not function as a negative regulator in *M. tuberculosis* under some culture conditions. For example, FurA did not effectively repress *katG* expression when *M. tuberculosis* isolates were cultured in MycoBroth (modified Middlebrook 7H9-ADC) for 2 weeks, since the $furA^{\Delta 34c}$ mutant, which did not produce functional FurA, expressed KatG at the same level as INH^s isolates with *furA*. Deletion of *furA* has been shown to result in derepression of *katG* in *Mycobacterium smegmatis* (Zahrt *et al.*, 2001). In addition, complementation of the *M. tuberculosis* Δ(*furA–katG*) strain with *katG*, but not *furA* resulted in high levels of KatG production and catalase activity (Pym *et al.*, 2001). These discrepancies in the function of FurA may have been due to differences in host strains used or phases of culture examined. In addition, the $furA^{\Delta 34c}$ mutant also had mutations in *katG* [g368a (G123E), g895a (G299S) and g1388t (R463L)] and in *inhA* (t-8c), suggesting that the Δ34c mutation may not be involved in INH resistance. $katG^{g1388t}$ (R463L) is a neutral mutation and $P_{inhA}{}^{t\text{-}8c}$ is responsible for INH resistance (Zhang and Telenti, 2000).

The mutations of *katG*, the regulatory region of *fabG1–inhA*, and *inhA* have been associated with INH resistance, although a significant population of INH^r clinical isolates in Japan showed no mutations in these regions (Ando *et al.*, 2010). The mutations $Int^{g\text{-}7a}$, $Int^{a\text{-}10c}$, $Int^{g\text{-}12a}$ and $furA^{c41t}$ characterized in the present

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.

Acknowledgements

We thank Tomofumi Iwata for technical assistance, Drs Juliano Timm and Brigitte Gicquel (Institut Pasteur, France) for providing the pJEM13 vector, and Dr William R. Jacobs Jr (Howard Hughes Medical Institute, USA) for providing the pMV306 vector. This study was supported by Health Sciences Research grants (H21-SHINKO-IPPAN-016) and Grant for International Health Research (21A-105) from the Ministry of Health, Labour and Welfare of Japan, and by Grant-in-Aid for Scientific Research (C) (22590411) and Grant-in-Aid for Young Scientists (B) (22790423) from the Ministry of Education, Culture, Sports, Science and Technology.

References

Alland, D., Steyn, A.J., Weisbrod, T., Aldrich, K., and Jacobs, W.R., Jr (2000) Characterization of the *Mycobacterium tuberculosis iniBAC* promoter, a promoter that responds to cell wall biosynthesis inhibition. *J Bacteriol* **182:** 1802–1811.

Ando, H., Kondo, Y., Suetake, T., Toyota, E., Kato, S., Mori, T., and Kirikae, T. (2010) Identification of *katG* mutations associated with high-level isoniazid resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **54:** 1793–1799.

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K.S., Wilson, T., *et al.* (1994) *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263:** 227–230.

Bardou, F., Raynaud, C., Ramos, C., Laneelle, M.A., and Laneelle, G. (1998) Mechanism of isoniazid uptake in *Mycobacterium tuberculosis*. *Microbiology* **144:** 2539–2544.

Barton, H.A., Johnson, Z., Cox, C.D., Vasil, A.I., and Vasil, M.L. (1996) Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. *Mol Microbiol* **21:** 1001–1017.

Bernstein, J., Lott, W.A., Steinberg, B.A., and Yale, H.L. (1952) Chemotherapy of experimental tuberculosis. V. Isonicotinic acid hydrazide (nydrazid) and related compounds. *Am Rev Tuberc* **65:** 357–364.

Caminero, J.A. (2010) Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding. *Int J Tuberc Lung Dis* **14:** 382–390.

Espinal, M.A., Laszlo, A., Simonsen, L., Boulahbal, F., Kim, S.J., Reniero, A., *et al.* (2001) Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. *N Engl J Med* **344:** 1294–1303.

Fujiki, A. (2001) *TB Bacteriology Examination to Stop TB.* Tokyo: The Research Institute of Tuberculosis.

Gouet, P., Courcelle, E., Stuart, D.I., and Metoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* **15:** 305–308.

Kong, D., and Kunimoto, D.Y. (1995) Secretion of human interleukin 2 by recombinant *Mycobacterium bovis* BCG. *Infect Immun* **63:** 799–803.

Lee, A.S., Lim, I.H., Tang, L.L., Telenti, A., and Wong, S.Y. (1999) Contribution of *kasA* analysis to detection of isoniazid-resistant *Mycobacterium tuberculosis* in Singapore. *Antimicrob Agents Chemother* **43:** 2087–2089.

Lee, A.S., Teo, A.S., and Wong, S.Y. (2001) Novel mutations in *ndh* in isoniazid-resistant *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother* **45:** 2157–2159.

NCCLS (2003) *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard.* NCCLS document M24-A, Wayne, PA: NCCLS.

Otsuka, Y., Parniewski, P., Zwolska, Z., Kai, M., Fujino, T., Kirikae, F., *et al.* (2004) Characterization of a trinucleotide repeat sequence $(CGG)_5$ and potential use in restriction fragment length polymorphism typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* **42:** 3538–3548.

Pohl, E., Haller, J.C., Mijovilovich, A., Meyer-Klaucke, W., Garman, E., and Vasil, M.L. (2003) Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. *Mol Microbiol* **47:** 903–915.

Pym, A.S., Domenech, P., Honore, N., Song, J., Deretic, V., and Cole, S.T. (2001) Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*. *Mol Microbiol* **40:** 879–889.

Safi, H., Fleischmann, R.D., Peterson, S.N., Jones, M.B., Jarrahi, B., and Alland, D. (2010) Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **54:** 103–108.

Sala, C., Forti, F., Di Florio, E., Canneva, F., Milano, A., Riccardi, G., and Ghisotti, D. (2003) *Mycobacterium tuberculosis* FurA autoregulates its own expression. *J Bacteriol* **185:** 5357–5362.

Sala, C., Forti, F., Magnoni, F., and Ghisotti, D. (2008) The *katG* mRNA of *Mycobacterium tuberculosis* and *Mycobac-*

terium smegmatis is processed at its 5′ end and is stabilized by both a polypurine sequence and translation initiation. *BMC Mol Biol* **9:** 33.

Sekiguchi, J., Miyoshi-Akiyama, T., Augustynowicz-Kopec, E., Zwolska, Z., Kirikae, F., Toyota, E., *et al.* (2007) Detection of multidrug resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* **45:** 179–192.

Stover, C.K., Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., *et al.* (1991) New use of BCG for recombinant vaccines. *Nature* **351:** 456–460.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Timm, J., Lim, E.M., and Gicquel, B. (1994) *Escherichia coli*-mycobacteria shuttle vectors for operon and gene fusions to *lacZ*: the pJEM series. *J Bacteriol* **176:** 6749–6753.

Vilcheze, C., Weisbrod, T.R., Chen, B., Kremer, L., Hazbon, M.H., Wang, F., *et al.* (2005) Altered NADH/NAD^+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. *Antimicrob Agents Chemother* **49:** 708–720.

Vilcheze, C., Wang, F., Arai, M., Hazbon, M.H., Colangeli, R., Kremer, L., *et al.* (2006) Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. *Nat Med* **12:** 1027–1029.

Wei, C.J., Lei, B., Musser, J.M., and Tu, S.C. (2003) Isoniazid activation defects in recombinant *Mycobacterium tuberculosis* catalase-peroxidase (KatG) mutants evident in InhA inhibitor production. *Antimicrob Agents Chemother* **47:** 670–675.

WHO (2003) *Guidelines for Surveillance of Drug Resistance in Tuberculosis*. Geneva: WHO.

Zahrt, T.C., Song, J., Siple, J., and Deretic, V. (2001) Mycobacterial FurA is a negative regulator of catalase-peroxidase gene *katG*. *Mol Microbiol* **39:** 1174–1185.

Zhang, Y., and Telenti, A. (2000) Genetics of drug resistance in *Mycobacterium tuberculosis*. In *Molecular Genetics of Mycobacteria*. Hatful, G.F., and Jacobs, W.R., Jr (eds). Washington, DC: ASM Press, pp. 235–254.

Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358:** 591–593.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Journal of Medical Microbiology (2011), 60, 184–188 DOI 10.1099/jmm.0.024729-0

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

Hiroki Ando,[1] Satoshi Mitarai,[2] Yuji Kondo,[3] Toshinori Suetake,[3] Seiya Kato,[2] Toru Mori[2] and Teruo Kirikae[1]

Correspondence
Teruo Kirikae
tkirikae@ri.ncgm.go.jp

[1]Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan

[2]Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3-1-24 Matsuyama, Kiyose, Tokyo 204-8533, Japan

[3]Third Department, Research and Development Laboratory, Nipro Corporation, 3023 Noji, Kusatsu, Shiga 525-0055, Japan

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.

Received 22 July 2010
Accepted 1 November 2010

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.

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.

ACKNOWLEDGEMENTS

We thank the Tuberculosis Research Committee (Ryoken), Japan, for supporting the collection of clinical MDR *M. tuberculosis* isolates. This study was supported by the Ministry of Health, Labour and Welfare of Japan with health sciences research grants (H21-SHINKO-IPPAN-016) and a grant for international health research (21A-105), and by the Ministry of Education, Culture, Sports, Science and Technology with a grant-in-aid for scientific research (C) (22590411) and a grant-in-aid for young scientists (B) (22790423).

REFERENCES

Ando, H., Mitarai, S., Kondo, Y., Suetake, T., Sekiguchi, J. I., Kato, S., Mori, T. & Kirikae, T. (2010). Pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates in Japan. *Clin Microbiol Infect* **16**, 1164–1168.

Aubry, A., Veziris, N., Cambau, E., Truffot-Pernot, C., Jarlier, V. & Fisher, L. M. (2006). Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. *Antimicrob Agents Chemother* **50**, 104–112.

Aziz, M., Laszlo, A., Raviglione, M. C., Rieder, H. L., Espinal, M. A. & Wright, A. (2003). *Guidelines for Surveillance of Drug Resistance in Tuberculosis.* Geneva: World Health Organization.

Caminero, J. A. (2010). Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding. *Int J Tuberc Lung Dis* **14**, 382–390.

Conde, M. B., Efron, A., Loredo, C., De Souza, G. R., Graca, N. P., Cezar, M. C., Ram, M., Chaudhary, M. A., Bishai, W. R. & other authors (2009). Moxifloxacin versus ethambutol in the initial treatment of tuberculosis: a double-blind, randomised, controlled phase II trial. *Lancet* **373**, 1183–1189.

Fujiki, A. (2001). *TB Bacteriology Examination to Stop TB.* Tokyo: The Research Institute of Tuberculosis.

Guillemin, I., Jarlier, V. & Cambau, E. (1998). Correlation between quinolone susceptibility patterns and sequences in the A and B subunits of DNA gyrase in mycobacteria. *Antimicrob Agents Chemother* **42**, 2084–2088.

Johnston, J. C., Shahidi, N. C., Sadatsafavi, M. & Fitzgerald, J. M. (2009). Treatment outcomes of multidrug-resistant tuberculosis: a systematic review and meta-analysis. *PLoS ONE* **4**, e6914.

Sekiguchi, J., Miyoshi-Akiyama, T., Augustynowicz-Kopec, E., Zwolska, Z., Kirikae, F., Toyota, E., Kobayashi, I., Morita, K., Kudo, K. & other authors (2007a). Detection of multidrug resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* **45**, 179–192.

Sekiguchi, J., Nakamura, T., Miyoshi-Akiyama, T., Kirikae, F., Kobayashi, I., Augustynowicz-Kopec, E., Zwolska, Z., Morita, K., Suetake, T. & other authors (2007b). Development and evaluation of a line probe assay for rapid identification of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* strains. *J Clin Microbiol* **45**, 2802–2807.

Sotgiu, G., Ferrara, G., Matteelli, A., Richardson, M. D., Centis, R., Ruesch-Gerdes, S., Toungoussova, O., Zellweger, J. P., Spanevello, A. & other authors (2009). Epidemiology and clinical management of XDR-TB: a systematic review by TBNET. *Eur Respir J* **33**, 871–881.

TRC (2007). Drug-resistant *Mycobacterium tuberculosis* in Japan: a nationwide survey, 2002. *Int J Tuberc Lung Dis* **11**, 1129–1135.

Van Deun, A., Martin, A. & Palomino, J. C. (2010a). Diagnosis of drug-resistant tuberculosis: reliability and rapidity of detection. *Int J Tuberc Lung Dis* **14**, 131–140.

Van Deun, A., Maug, A. K., Salim, M. A., Das, P. K., Sarker, M. R., Daru, P. & Rieder, H. L. (2010b). Short, highly effective, and inexpensive standardized treatment of multidrug-resistant tuberculosis. *Am J Respir Crit Care Med* **182**, 684–692.

Wang, J. Y., Hsueh, P. R., Jan, I. S., Lee, L. N., Liaw, Y. S., Yang, P. C. & Luh, K. T. (2006). Empirical treatment with a fluoroquinolone delays the treatment for tuberculosis and is associated with a poor prognosis in endemic areas. *Thorax* **61**, 903–908.

Yew, W. W., Lange, C. & Leung, C. C. (2010). Treatment of tuberculosis update 2010. *Eur Respir J* (in press). doi: 10.1183/09031936.00033010

Yoon, Y. S., Lee, H. J., Yoon, H. I., Yoo, C. G., Kim, Y. W., Han, S. K., Shim, Y. S. & Yim, J. J. (2005). Impact of fluoroquinolones on the diagnosis of pulmonary tuberculosis initially treated as bacterial pneumonia. *Int J Tuberc Lung Dis* **9**, 1215–1219.

Zhang, Y. & Telenti, A. (2000). *Genetics of Drug Resistance in Mycobacterium tuberculosis.* Washington, DC: American Society for Microbiology.

Acknowledgements

We thank the members of the TB Task Force of Greater Lisbon and its Fast and Faster Track TB Programmes for their kind cooperation. This work was presented in part at the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, September 2007.

Transparency Declaration

This study was supported by grants SDH.IC.I.01.17-TB and TB Faster Track for Greater Lisbon, from the Fundação Calouste Gulbenkian. D. Machado and L. Rodrigues were supported by grants PTDC/BIA-MIC/BIC-01/2008 and SFRH/BD/24931/2005 from Fundação para a Ciência e a Tecnologia (FCT, Portugal). The authors declare that they have no conflicts of interest.

References

1. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* 2003; 16: 319–354.
2. Tortoli E. The new mycobacteria: an update. *FEMS Immunol Med Microbiol* 2006; 48: 159–178.
3. Griffith DE, Aksamitt T, Brown-Elliott BA *et al.* An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* 2007; 175: 367–416.
4. Martín-Casabona N, Bahrmand AR, Bennedsen J *et al.* Non-tuberculous mycobacteria: patterns of isolation a multi-country retrospective survey. *Int J Tuberc Lung Dis* 2004; 8: 1186–1193.
5. Piersimoni C, Scarparo C. Pulmonary infections associated with non-tuberculous mycobacteria in immunocompetent patients. *Lancet Infect Dis* 2008; 8: 323–334.
6. Primm TP, Lucero CA, Falkinham JO III. Health impacts of environmental mycobacteria. *Clin Microbiol Rev* 2004; 17: 98–106.
7. Siddiqi S, Rüsch-Gerdes S. MGIT™ Procedure Manual for BACTEC™ MGIT 960™ TB System (Also applicable for Manual MGIT). Mycobacteria Growth Indicator Tube (MGIT) Culture and Drug Susceptibility Demonstration Projects. Foundation for Innovative New Diagnostics Ed. 2006. Available at: http://www.finddiagnostics.org/resource-centre/reports_brochures/071130_mait_manual.html (last accessed 3 July 2009).
8. Kent PT, Kubica GP. *Public health mycobacteriology: a guide for the level III laboratory*. Atlanta, GA: US Department of Health and Human Services, Centers for Disease Control, 1985.
9. Richter E, Rüsch-Gerdes S, Hillemann D. Evaluation of the GenoType Mycobacterium assay for identification of mycobacterial species from cultures. *J Clin Microbiol* 2006; 44: 1769–1775.
10. Lane DJ. 16S/23S rRNA sequencing. In: Stachebrandt E, Goodfellow M, eds. *Nucleic acid techniques in bacterial systematics*. New York, NY, John Wiley and Sons, 1991; 115–175.
11. Gitti Z, Neonakis I, Fanti G, Kontos F, Maraki S, Tselentis Y. Use of the GenoType Mycobacterium CM and AS assays to analyze 76 nontuberculous mycobacterial isolates from Greece. *J Clin Microbiol* 2006; 44: 2244–2246.
12. Mäkinen J, Marjamäki M, Marttila H, Soini H. Evaluation of a novel strip test, GenoType Mycobacterium CM/AS, for species identification of mycobacterial cultures. *Clin Microbiol Infect* 2006; 12: 481–483.
13. Roth A, Reischl U, Streubel A *et al.* Novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S-23S rRNA gene spacer and restriction endonucleases. *J Clin Microbiol* 2000; 38: 1094–1104.
14. Ruiz P, Gutierrez J, Zerolo FJ, Casal M. GenoType Mycobacterium assay for identification of mycobacterial species isolated from human clinical samples by using liquid medium. *J Clin Microbiol* 2002; 40: 3076–3078.
15. Russo C, Tortoli E, Menichella D. Evaluation of the new GenoType Mycobacterium assay for identification of mycobacterial species. *J Clin Microbiol* 2006; 44: 334–339.
16. Direcção Geral de Saúde. *Programa Nacional de Luta contra a Tuberculose (PNT). Ponto da situação epidemiológica e de desempenho, ano 2007*, Lisboa, Portugal, DGS 2008; 1–17
17. Tabarsi P, Baghaei P, Farnia P *et al.* Nontuberculous mycobacteria among patients who are suspected for multidrug-resistant tuberculosis – need for earlier identification of nontuberculosis mycobacteria. *Am J Med Sci* 2009; 337: 182–184.

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

H. Ando[1], S. Mitarai[2], Y. Kondo[3], T. Suetake[3], J.-I. Sekiguchi[1,2], S. Kato[2], T. Mori[2] and T. Kirikae[1]

1) *Department of Infectious Diseases, Research Institute, International Medical Centre of Japan,* 2) *Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo and* 3) *Third Department, Research and Development Laboratory, Nipro Corporation, Shiga, Japan*

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

Original Submission: 30 April 2009; **Revised Submission:** 7 July 2009; **Accepted:** 13 July 2009
Editor: G. Greub
Article published online: 14 October 2009

Clin Microbiol Infect 2010; **16**: 1164–1168
10.1111/j.1469-0691.2009.03078.x

Corresponding author and reprint requests: T. Kirikae, International Medical Centre of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan
E-mail: tkirikae@ri.imcj.go.jp

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.

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	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 nonsense 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.

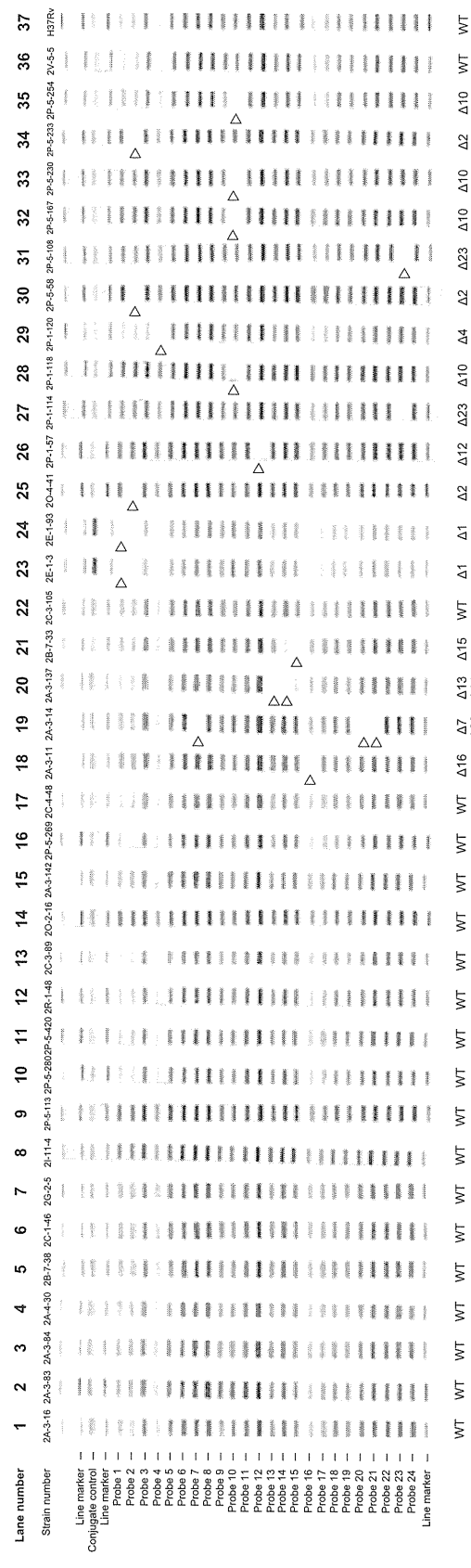

FIG. 1. Line probe assay (LiPA) patterns of all *Mycobacterium tuberculosis* isolates tested in this study. Positions of the oligonucleotides, conjugate control lines and (in blue) are shown. A negative signal is indicated by an open triangle. LiPA patterns are shown in lanes 1–37. WT, wild-type *pncA*.