in GyrB to FQ resistance utilizing recombinant technology and *in vitro* assay.

On the basis of reports on *M. tuberculosis*, we selected target amino acid substitutions at position 464, 502 and 504 in *M. leprae* GyrB, equivalent to position 461, 499 and 501 in *M. tuberculosis*, to reveal the significance of these amino acid substitutions for reduced FQ sensitivity, and conducted the FQ-inhibited supercoiling assay and FQ-mediated DNA cleavage assay using recombinant DNA gyrase.

Methods

Drugs and kits

Ofloxacin (OFX), ciprofloxacin (CIP) and levofloxacin (LVX) were purchased from LKT Laboratories, Inc. (St. Paul, MN); moxifloxacin (MXF) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada); sitafloxacin (SIT) was from Daiichi-sankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan); ampicillin and kanamycin were purchased from Meiji Seika Pharma Ltd. (Tokyo, Japan). Oligonucleotide primers were synthesized by Life Technologies Corp. (Carlsbad, CA). Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA). The supercoiling assay kit and supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom).

Bacterial strains and plasmid

The Thai-53 strain of *M. leprae* [19], maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strains TOP-10 (Life Technologies Corp.), Rosetta-gami 2, and BL21 (DE3) pLysS (Merck KGaA, Darmstadt, Germany) were used for cloning and protein expression. pET-20b (+) (Merck KGaA) vector was used to construct expression plasmids for *M. leprae* DNA gyrases.

Construction of expression plasmids

Wild-type (WT) recombinant GyrA and GyrB expression plasmids were constructed as described previously [14,16]. Mutations were introduced into the WT *gyrB* gene by PCR using pairs of complementary primers containing the mutations of interest (Table 1). All PCR reactions were carried out in a thermal cycler (Life Technologies Corp.) under the following conditions: pre-denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 10 s, annealing at 50–60°C for 15 s, and extension at 68°C for 1 to 3 min, and then a final extension at 68°C for 5 min. The *gyrB* C-terminal cassettes with base substitutions were digested with *Pml* I and *Xho* I, ligated into WT *gyrB* expression plasmid, and digested with the same restriction endonucleases to obtain mutant *gyrB* expression plasmid (Figure 2). The nucleotide sequences of the DNA gyrase genes in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI Prism 3130xl genetic analyzer (Life Technologies Corp.) according to the manufacturer's protocol.

Expression and purification of recombinant DNA gyrase subunits

Recombinant DNA gyrase subunits were expressed and purified as previously described [13,14,16,20]. Briefly, expression plasmids carrying the *gyrA* and *gyrB* of *M. leprae* were transformed into *E. coli* Rosetta-gami 2 and BL21 (DE3) pLysS, respectively. The transformants were grown in Luria-Bertani (LB) medium in the presence of 100 μg/mL Ampicillin to the log phase and the expression of DNA gyrase was induced with the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Osaka, Japan), followed by further incubation at 14°C for 16 h. The harvested *E. coli* were lysed by sonication (Sonifier 250; Branson, Danbury, CT) and the recombinant DNA gyrase subunits in supernatants after centrifugation (10,000× g for 30 min) were purified by Ni-NTA Agarose resin (Life Technologies Corp.) column chromatography and dialyzed against DNA gyrase dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA). The purified protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling assay and inhibition by FQs

ATP-dependent and FQ-inhibited DNA supercoiling assays were performed according to previous reports [13,14,16,20]. DNA supercoiling activity was examined with reaction mixture consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (0.3 μg), and GyrA and GyrB subunits (50 ng each) in a total volume of 30 μl. Reactions were run at 30°C for 1.5 h followed by stopping with the addition of 30 μl chloroform/iso-amyl alcohol (24:1 mixture) and 3 μl of 10× DNA loading solution. The total reaction mixtures were subjected to electrophoresis on 1% agarose gels in 1× Tris-borate-EDTA (TBE) buffer and stained by ethidium bromide (0.7 μg/ml). The extent of supercoiled DNA was quantified with ImageJ (http://rsbweb.nih.gov/ij) and the inhibitory effects of FQs on DNA gyrase were assessed by determining the drug concentration required to inhibit the supercoiling activity of the DNA gyrase by 50% (IC_{50}s) in the

Table 1. Nucleotide sequences of primers used in this study.

Primer name	Primer sequence (Nucleotide Position)
k-54	5′-CGTAAAGCACGTGAGTTAGTGCGTCGAAAAAGTGCC-3′ (1270–1305)
k-55	5′-GGCTCGAGCTAATGATGATGATGATGGACATCCAGGAAACGAACATCC-3′ (2013–2037)
D464N_Fw	5′-A GTG GAA GGT **AAT** TCG GCT GGT G
D464N_Rv	5′-C ACC AGC CGA **ATT** ACC TTC CAC T
N502D_Fw	5′-A GTG CTA AAG **GAC** ACC GAA GTT C
N502D_Rv	5′-G AAC TTC GGT **GTC** CTT TAG CAC T
E504V_Fw	5′-A AAG AAC ACC **GTA** GTT CAA GCA A
E504V_Rv	5′-T TGC TTG AAC **TAC** GGT GTT CTT T

Mutated codons are indicated in bold face.
doi:10.1371/journal.pntd.0001838.t001

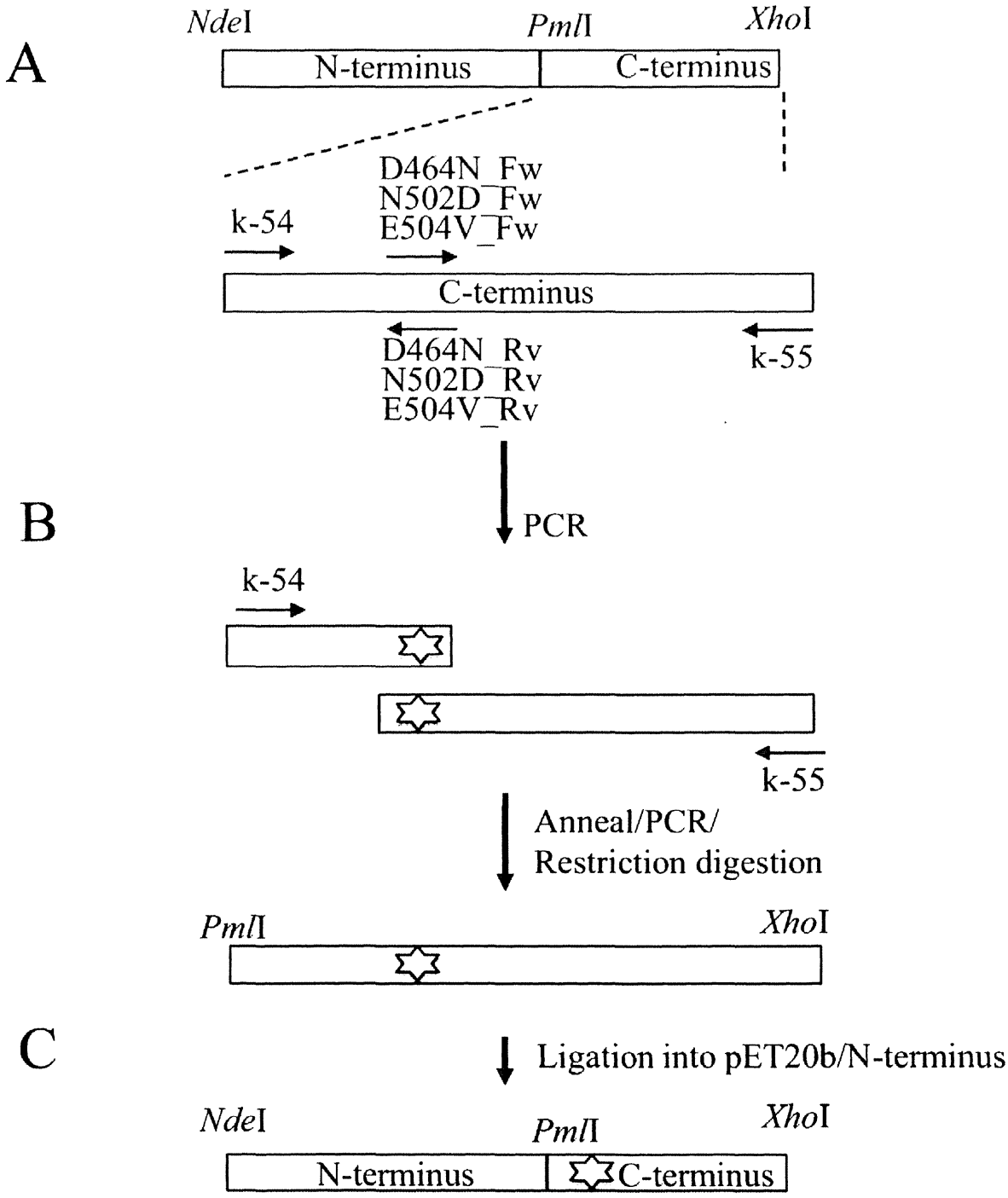

Figure 2. Construction of WT and mutant DNA gyrase expression plasmid. (A) Primer pairs k-54+D464N_Rv, N502D_Rvor E504V_Rv (Table 1) were used for amplifying the DNA fragment encoding N-terminus half (amino acid 424 to 467, 505 or 507, respectively) of C-terminus region of GyrB carrying Asp464Asn, Asn502Asp and Glu504Val, respectively. Primer pairs k-55+D464N_Fw, N502D_Fw or E504V_Fw (Table 1) were used for amplifying the DNA fragment encoding the C-terminus half (amino acid 461, 499 or 501 to 678, respectively) of the C-terminus region of GyrB carrying Asp464Asn, Asn502Asp and Glu504Val, respectively. (B) To complete the C-terminus region encoding cassette, DNA fragments encoding the N-terminus half and C-terminus half of the C-terminus region of GyrB were annealed and reamplified by PCR using the primer pair of k-54 and k-55. (C) The mutated *gyrB*-C cassettes were digested with *Pml*I and *Xho*I restriction endonucleases and ligated into the expression plasmid containing the WT*gyrB*N-terminus region DNA fragment digested by the same enzymes.
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presence or absence of serial two-fold increases in the concentrations of OFX, MXF, SIT, CIP and LVX. Enzymatic assays were performed at least three times to confirm the reproducibility.

FQ-mediated DNA cleavage assay

DNA cleavage assays were also carried out as described in previous reports [13,14,16,20,21]. Briefly, the reaction mixture

(total volume 30 µl) contained DNA gyrase assay buffer, purified DNA gyrase subunits, supercoiled pBR322 DNA (0.3 µg) and increasing concentrations of OFX, MXF, SIT, CIP and LVX. After incubation for 2 h at 30°C, cleavage reactions were stopped by adding 3 µl of 2% SDS and 3 µl proteinase K (1 mg/ml). After subsequent incubation for 30 min at 30°C, proteinase K reactions were stopped by the addition of 3 µl of 0.5 mM EDTA, 30 µl chloroform/iso-amyl alcohol (24:1 mixture) and 3 µl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 0.8% agarose gels in 1× TBE buffer, followed by ethidium bromide staining. The extent of DNA cleavage was quantified with ImageJ (http://rsbweb.nih.gov/ij) and the FQ concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined.

Results

Construction and purification of recombinant WT and mutant DNA gyrase subunits

The WT GyrA and GyrB expression plasmids constructed in our previous work [14] were used. DNA fragments with mutations causing amino acid substitutions at position 464, 502 and 504 in GyrB were amplified from WT GyrB expression plasmid [14] and introduced into expression vector pET-20b (+). Recombinant GyrA and GyrB were expressed as C-terminus hexa-histidine tagged protein for ease of purification, as the His-tag has been shown not to interfere with the catalytic functions of GyrA and GyrB [13–16,20,22]. Expressed recombinant WT and mutant DNA gyrase subunits were purified as 0.4 to 1.7 mg soluble His-tagged protein with molecular weights of 80 kDa and 75 kDa for GyrA and GyrB, respectively, from 500 ml cultures. The purity of recombinant proteins was confirmed by SDS-PAGE (Figure S1). All of the recombinant proteins were obtained with high purity (>90–95%).

ATP-dependent DNA supercoiling activities of WT and mutant DNA gyrases

Combinations of WT GyrA and WT or mutant GyrBs (GyrB-Asp464Asn, GyrB-Asn502Asp or GyrB-Glu504Val) were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Figure S2). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Figure S2 A–D, lane 3), while neither subunit alone exhibited DNA supercoiling activity (Figure S2 A–D, lane 4, 5). In addition, no supercoiling activity was observed when ATP was omitted from the reaction condition (Figure S2 A–D, lane 6). Consequently, ATP-dependent DNA supercoiling activities were confirmed with WT and three mutant DNA gyrases.

IC_{50}s of five FQs for WT and mutant DNA gyrases

FQs-inhibited DNA supercoiling activities were assessed for the determination of IC_{50}s. Figure 3 shows a representative result of the inhibitory effect of OFX and the results for the other FQs are presented in Figure S3. Results show the dose-dependent inhibition of five FQs against WT and mutant DNA gyrases, as summarized in Table 2. The five FQs inhibited the DNA supercoiling activities of WT DNA gyrase at low concentration (Table 2).

CC_{25}s of five FQs for WT and mutant DNA gyrases

DNA cleavage assay was performed in the presence of increasing concentrations of FQs to estimate CC_{25}s. Figure 4 presents the results of a representative DNA cleavage assay using

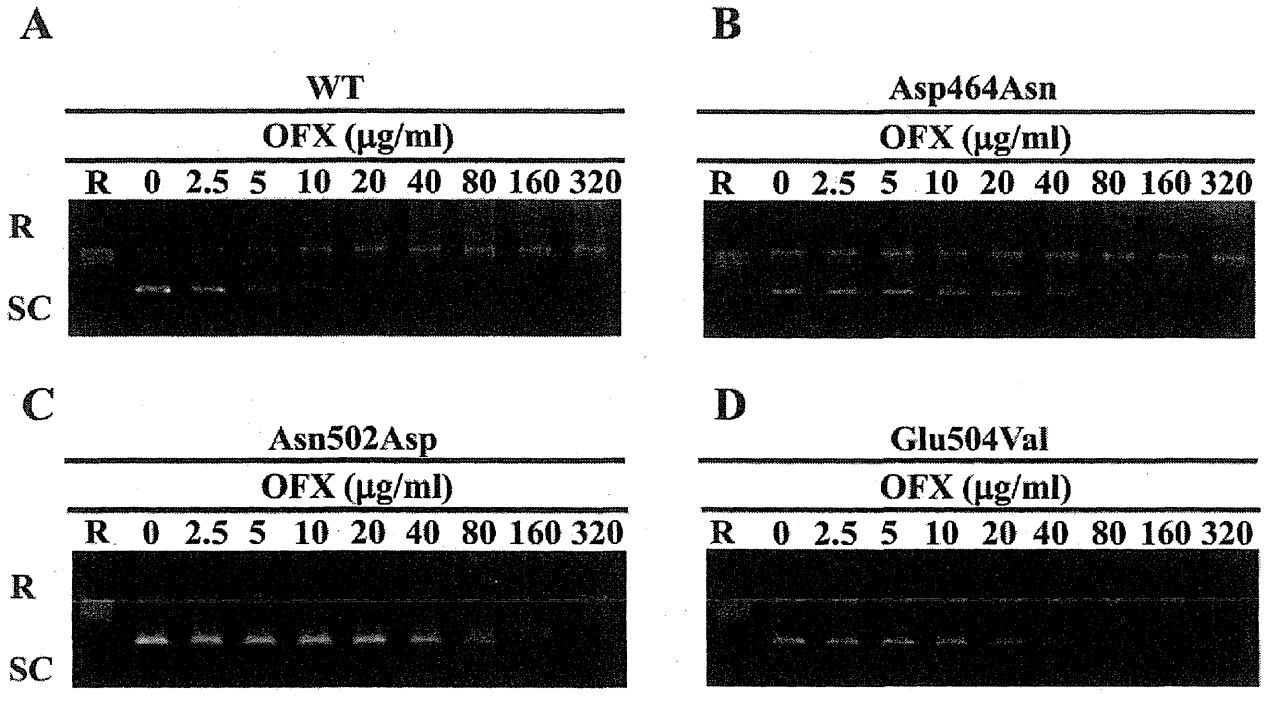

Figure 3. OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 mg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. FQ-inhibited supercoiling activity assay was performed in combination of WTGyrA+WTGyrB (A), GyrB-Asp464Asn (B), GyrB-Asn502Asp (C) and GyrB-Glu504Val (D). R and SC denote relaxed and supercoiled pBR322 DNA, respectively.
doi:10.1371/journal.pntd.0001838.g003

Table 2. IC_{50}s and CC_{25}s of FQs against WT and mutant DNA gyrases.

Drug	IC_{50} (μg/ml)				CC_{25} (μg/ml)			
	WT	Asp464Asn	Asn502Asp	Glu504Val	WT	Asp464Asn	Asn502Asp	Glu504Val
OFX	5.7±0.8	53.9±9.0	106.6±25.1	34.6±4.3	2.4±0.2	32.7±6.3	78.2±12.6	30.0±7.9
MXF	1.7±0.3	4.1±0.4	17.8±2.6	13.9±0.6	0.6±0.0	3.3±0.9	15.3±2.6	9.6±1.7
SIT	0.5±0.1	1.8±0.3	1.6±0.6	1.7±0.2	0.2±0.0	0.9±0.0	1.0±0.2	0.7±0.1
CIP	2.3±0.3	11.3±2.7	257.9±46.1	49.3±9.4	0.9±0.2	6.5±0.6	42.5±13.6	24.7±0.5
LVX	4.5±0.3	32.9±3.2	46.8±1.1	19.9±2.9	1.4±0.1	18.6±4.9	51.7±10.6	9.3±0.7

doi:10.1371/journal.pntd.0001838.t002

OFX, and Figure S4 shows those using other FQs. Table 2 shows the CC_{25}s of FQs for WT and mutant DNA gyrases. Highest CC_{25}s of FQs were observed for GyrB-Asn502Asp DNA gyrase.

Discussion

We focused on amino acid substitutions at position 464, 502 and 504 in GyrB in *M. leprae* equivalent to 461, 499 and 501, respectively, in *M. tuberculosis*, as amino acid substitutions at these positions in *M. tuberculosis* are known to contribute to FQ resistance [13 16,20,22,23]. We carried out a FQ-mediated supercoiling activity inhibition assay and a DNA cleavage assay using recombinant WT and mutant DNA gyrases at 30°C, the optimal temperature of *M. leprae* growth [24], and calculated IC_{50}s and CC_{25}s of five FQs, including OFX, MXF, SIT, CIP and LVX. All FQs inhibited DNA supercoiling activities of WT DNA gyrase at low concentration (Table 2). In strong contrast, three mutant DNA gyrases showed reduced sensitivity to all five FQs. GyrB-Asn502Asp DNA gyrase exhibited the lowest FQ sensitivity among the three mutant DNA gyrases. IC_{50}s of OFX, MXF, SIT, CIP and LVX for GyrB-Asp464Asn, Asn502Asp and Glu504Val DNA gyrases were 2.4- to 9.5-fold, 3.2- to 112.1-fold and 3.4- to 21.4-fold higher than those for WT DNA gyrase (Figure 3, 5, S3 and Table 2). A similar tendency was observed in the DNA cleavage assay. Namely, CC_{25}s of OFX, MFX, SIT, CIP and LVX for GyrB-Asp464Asn, Asn502Asp and Glu504Val DNA gyrases were 4.5- to 13.6-fold, 5.0- to 47.2-fold and 3.5- to 27.4-fold higher than for WT DNA gyrase (Figure 4, 5, S4, Table 2). These results suggested the contribution of these amino acid substitutions in GyrB to reduced FQ sensitivity and the possible emergence of *M. leprae* with mutant GyrB, although previously identified Asp to Asn amino acid substitution in GyrB at position 205 [8] was revealed not to have an effect on FQ susceptibility [13]. It is noteworthy that mutant DNA gyrases exhibited a similar

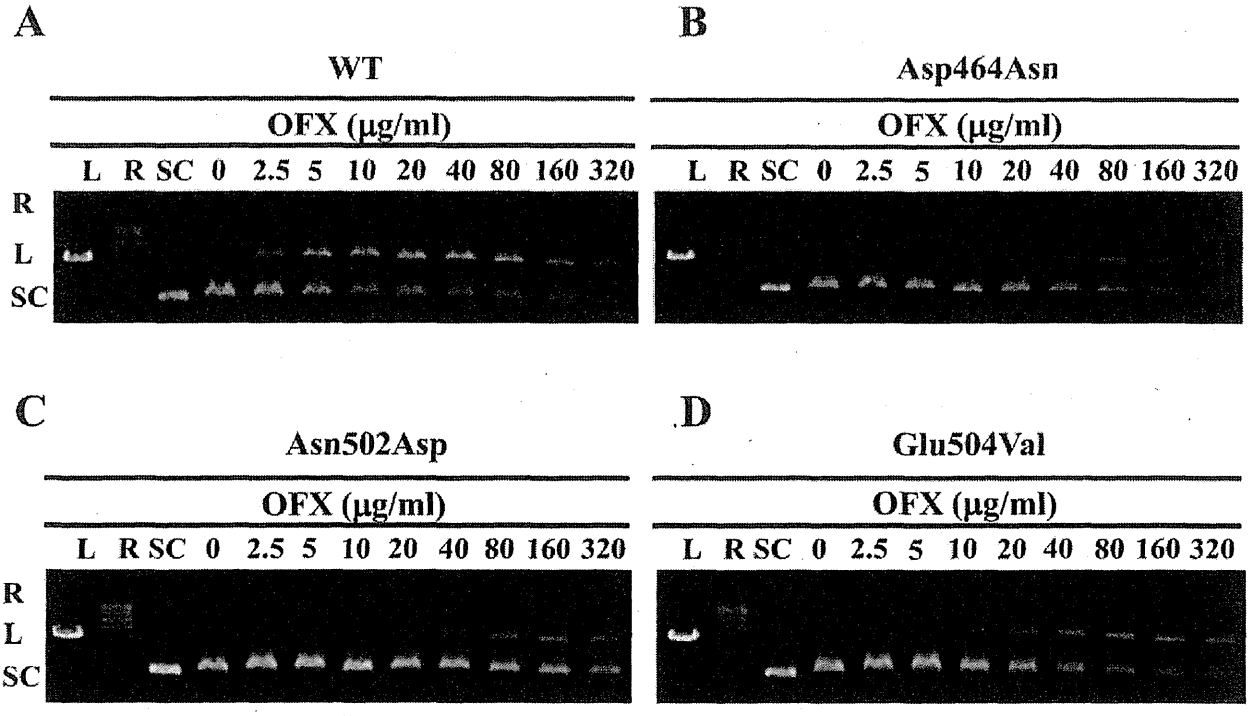

Figure 4. OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 mg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed in combination of WT GyrA+WT GyrB (A), GyrB-Asp464Asn (B), GyrB-Asn502Asp (C) and GyrB-Glu504Val (D). R, L and SC denote relaxed, linear and supercoiled pBR322 DNA, respectively.
doi:10.1371/journal.pntd.0001838.g004

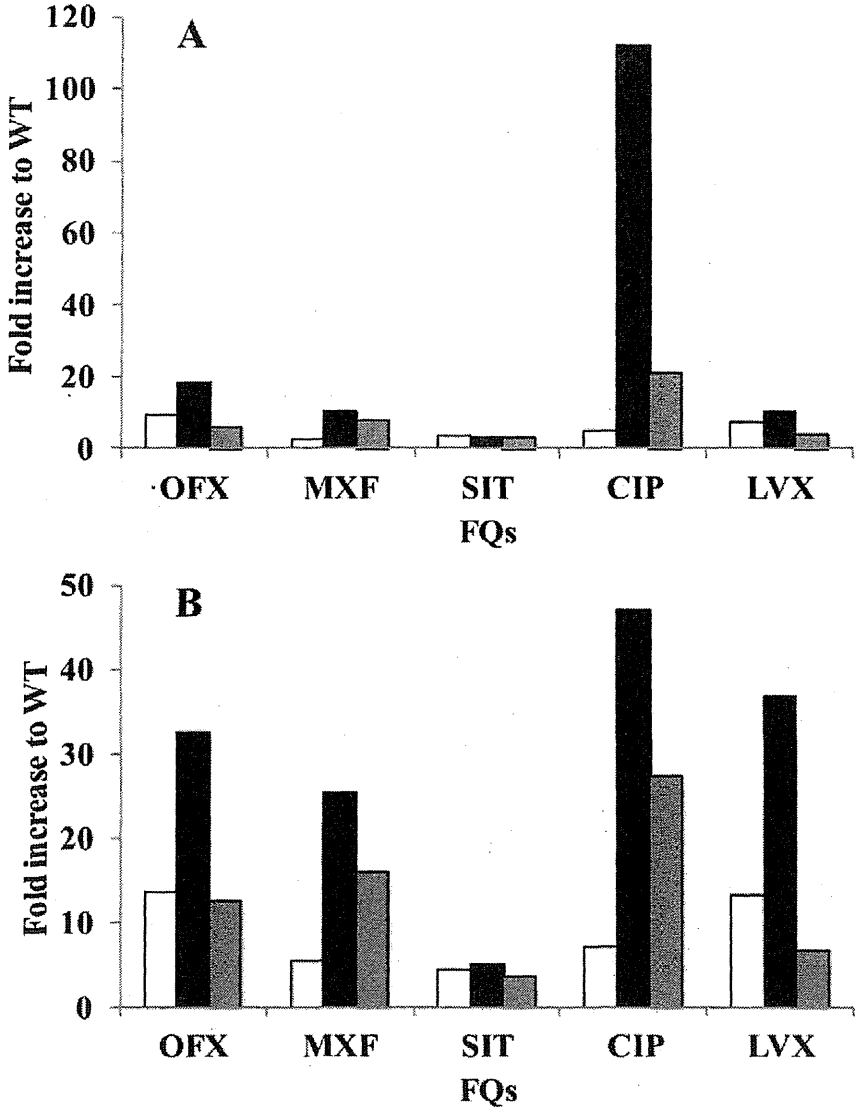

Figure 5. Increased IC_{50}s and CC_{25}s of FQs for mutant DNA gyrases. IC_{50}s and CC_{25}s were calculated by the quinolone-inhibited supercoiling assay and FQ-mediated cleavage assay, respectively. Fold increase of each FQ for mutant DNA gyrases was plotted. (A) IC_{50}s, (B) CC_{25}s. Open, closed and hatched bar denotes the value for GyrB- GyrB-Asp464Asn, GyrB-Asn502Asp and GyrB-Glu504Val DNA gyrase, respectively.
doi:10.1371/journal.pntd.0001838.g005

sensitivity pattern to those reported for mutant GyrB in *M. tuberculosis*. *M. leprae* GyrB-Asn502Asp DNA gyrase had lower FQ sensitivity than GyrB-Asp426Asn and GyrB-Glu504Val DNA gyrase, as has been shown in *M. tuberculosis* [15 18]. The high homology of the entire GyrB and full sequence match in QRDR between *M. leprae* and *M. tuberculosis* might lead to a similar tendency of FQ sensitivity. It is interesting that the Asp to Asn amino acid substitution in *E. coli* at position equivalent to 464 in *M. leprae* showed enhancing effect on CIP resistance [25] where Glu to Asp or Ala amino acid substitution in *Streptococcus pneumonia* at position equivalent to 504 in *M. leprae* showed little or reducing effect on CIP resistance, respectively [26]. Overall or QRDR structure of GyrB might affect the acquisition of FQ resistance.

IC_{50}s of FQs were 8 to 40 times higher than the minimum inhibitory concentrations (MICs) in *M. tuberculosis* [17,18,22]. This non-proportionality presumably reflects basic differences in the cell-permeating properties and the accumulation of different FQs [22]. We investigated the inhibitory effects of OFX, GAT, MXF, LVX and SIT against WT and mutant DNA gyrases. IC_{50}s of OFX for WT DNA gyrase was 5.7 μg/ml (Table 2) and it seemed reasonable that OFX has been used by a single application of 400 to 600 mg for leprosy patients with a single lesion and two or three doses of 400 to 600 mg in combination with first-line drugs, DDS and RIF [27] for the treatment of patients with MDR leprosy. On the contrary, IC_{50}s of OFX for GyrB-Asp464Asn, Asn502Asp and Glu504Val showed 9.5, 18.7 and 6.1 fold higher concentration comparing to WT DNA gyrase, respectively, and OFX seems not to have the ability to inhibit *M. leprae* with DNA gyrase with these mutations. On the other hand, the order of inhibitory activity was SIT>MXF>CIP>LVX>OFX. Namely, SIT most effectively inhibited WT and mutant DNA gyrases among five FQs. IC_{50}s of SIT for WT was 0.5 μg/ml and the increase was 3.6-, 3.2- and 3.4-fold for GyrB-Asp464Asn, GyrB-Asn502Asp and GyrB-Glu504Val DNA gyrases, respectively. In addition, the maximum

serum concentration (Cmax) of OFX, SIT, CIP and LVX in 100 mg dosage was determined in clinical trials to be 0.95, 1.00, 1.33 and 1.22 μg/ml, respectively [28–31], and that of MFX in 400 mg dose to be 4.13 [32]. SIT might strongly inhibit *M. leprae* carrying GyrB-Asp464Asn, Asn502Asp and Glu504Val DNA gyrase as well as that carrying GyrA-Ala90Val, Asp95Gly, and Asp95Asn [14,16,20]. Thus, SIT is a promising candidate for the treatment of leprosy caused by OFX-resistant *M. leprae* with these problematic gyrases. Although SIT is now only approved in Japan and mild gastrointestinal disorders as adverse reactions have been reported, our data in this study might encourage the use of SIT for OFX-resistant leprosy.

In conclusion, we revealed the contribution of Asp464Asn, Asn502Asp and Glu504Val amino acid substitution to reduced sensitivity to FQ in *M. leprae* by an *in vitro* assay. This suggested the possible emergence of FQ-resistant *M. leprae* carrying GyrB with these amino acid substitutions in the future. Hence we would like to propose the analysis of these amino acid substitutions in GyrB to detect FQ-resistant leprosy. Additionally, effectiveness of sitafloxacin to the mutant DNA gyrases suggested the potential use of this FQ for the treatment of ofloxacin resistant cases.

Supporting Information

Figure S1 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were over expressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M: Protein marker (NEB), 1: WTGyrA, 2: WTGyrB, 3: GyrB-Asp464Asn, 4: GyrB-Asn502Asp, 5: GyrB-Glu504Val. 300 ng of each protein was loaded on 5–20% gradient polyacrylamide gel.
(TIF)

Figure S2 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A), DNA gyrases bearing GyrB-Asp464Asn (B), Asn502Asp (C) and Glu504Val (D) were analyzed. Relaxed pBR322 (0.3 mg) was incubated with GyrA (50 ng) or GyrB (50 ng) or both. Lanes: 1: relaxed pBR322 alone, 2: relaxed pBR322 and ATP, 3: relaxed pBR322, ATP, GyrA and GyrB, 4: relaxed pBR322, ATP and GyrA, 5: relaxed pBR322, ATP and GyrB, 6: relaxed pBR322, GyrA and GyrB.
(TIF)

Figure S3 Inhibitory activities of (A) MXF, (B) SIT, (C) CIP and (D) LVX on supercoiling activities against *M. leprae* WT and mutant DNA gyrases. Relaxed pBR322 DNA (0.3 mg) was incubated with 50 ng each of GyrA and GyrB in the absence or presence of the indicated concentration (in mg/ml) of three FQs. The reactions were stopped, and the DNA products were analyzed by electrophoresis on 1% agarose gel. R and SC denote relaxed and supercoiled pBR322 DNA, respectively.
(TIF)

Figure S4 DNA cleavage activity of (A) MXF, (B) SIT, (C) CIP and (D) LVX against *M. leprae*WT and mutant DNA gyrases. Supercoiled pBR322 DNA (0.3 mg) was incubated with 50 ng each of GyrA and GyrB in the absence or presence of the indicated concentration (in mg/ml) of three FQs. The reactions were stopped, and the processed DNA products were analyzed by electrophoresis on 1% agarose gel. R, L and SC denote relaxed, linear and supercoiled pBR322 DNA, respectively.
(TIF)

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Author Contributions

Conceived and designed the experiments: TM MM CN YS. Performed the experiments: KY HK CN. Analyzed the data: KY CN YS. Contributed reagents/materials/analysis tools: KY TM MM. Wrote the paper: KY TM MM CN YS.

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Amino Acid Substitutions at Position 95 in GyrA Can Add Fluoroquinolone Resistance to *Mycobacterium leprae*

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Amino acid substitutions at position 89 or 91 in GyrA of fluoroquinolone-resistant *Mycobacterium leprae* clinical isolates have been reported. In contrast, those at position 94 in *M. tuberculosis*, equivalent to position 95 in *M. leprae*, have been identified most frequently. To verify the possible contribution of amino acid substitutions at position 95 in *M. leprae* to fluoroquinolone resistance, we conducted an *in vitro* assay using wild-type and mutant recombinant DNA gyrases. Fluoroquinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay revealed the potent contribution of an amino acid substitution of Asp to Gly or Asn at position 95 to fluoroquinolone resistance. These results suggested the possible future emergence of quinolone-resistant *M. leprae* isolates with these amino acid substitutions and the usefulness of detecting these mutations for the rapid identification of fluoroquinolone resistance in leprosy.

Leprosy is a chronic human infectious disease caused by *Mycobacterium leprae* which may cause severe disabilities due to damage to the peripheral nerves (33). The World Health Organization (WHO) reported the global number of registered new cases in 2010 to be 228,474, while during 2009 it was 244,796 (37). Although the number of new cases detected globally fell by 16,322 (6.7%) during this period, new leprosy cases are still detected every year, mainly in Asia, Latin America, and Africa (21, 37). In the 1980s, the WHO introduced multidrug therapy (MDT), composed of dapsone (DDS), rifampin (RIF), and clofazimine (36). Recently, fluoroquinolones (FQs), especially ofloxacin (OFX), have been recommended for the treatment of leprosy with a single lesion. The emergence of multidrug-resistant (MDR) leprosy, resistant to both DDS and RIF owing to therapeutic failure or low compliance, has been reported (17, 29), and FQs are thought to be important. For appropriate treatment, early assessment of drug susceptibility is essential; however, *M. leprae* cannot be cultivated on artificial media and a drug susceptibility test depending on *in vitro* growth is not available. Consequently, antibiotic susceptibility tests have relied on the mouse footpad leprosy model, requiring 8 to 12 months because of the slow growth of *M. leprae* (18). Recently, genetic analysis of drug-resistant *M. leprae* substantiated the correlation of DDS, RIF, and OFX resistance with mutations in *folP1*, encoding dihydropteroate synthetase (5, 15, 19, 23–25, 35); *rpoB* (4, 6, 12, 19, 23–25, 33), encoding the beta subunit of RNA polymerase; and *gyrA*, encoding the A subunit of DNA gyrase (4, 19, 24, 26, 40), respectively. Among these, data for *folP1* in *M. tuberculosis* are not available as DDS is not used for the treatment of tuberculosis. Mutations in *rpoB* observed in *M. leprae* showed good agreement with those obtained from RIF-resistant *M. tuberculosis*. In contrast, the distribution of mutations in *gyrA* of FQ-resistant *M. tuberculosis* was distinct from that in *gyrA* of OFX-resistant *M. leprae* (Fig. 1). Namely, amino acid substitutions at position 94 in GyrA were found in approximately half of FQ-resistant *M. tuberculosis* isolates, whereas no amino acid substitutions at position 95, equivalent to position 94 in *M. tuberculosis*, have been reported in *M. leprae*, and 11 cases with amino acid substitutions at position 91, equivalent to position 94 in *M. tuberculosis*, were reported from a total of six countries (4, 19, 24, 26, 40). Thus, elucidation of the contribution of amino acid substitutions at position 95 of GyrA in *M. leprae* to FQ resistance is important for the gene-based detection of fluoroquinolone resistance.

FQs inhibit type II DNA topoisomerases, DNA gyrase, and topoisomerase IV, which play crucial roles in DNA replication during cell division (8). As *M. leprae* has only DNA gyrase, this is the sole target of FQs. DNA gyrase, consisting of two GyrA and two GyrB subunits, catalyzes the negative supercoiling of the circular bacterial chromosome by cleaving double strands and passing the enwrapped DNA, followed by resealing the double strands (8, 13). To reveal the significance of amino acid substitution at position 95 to FQ resistance, we conducted the FQ-mediated supercoiling activity inhibition assay and DNA cleavage assay using recombinant DNA gyrases having an amino acid substitution in GyrA at position 95, Asp to Gly (GyrA-Asp95Gly) or Asp to Asn (GyrA-Asp95Asn). These mutations are frequently found in FQ-resistant *M. tuberculosis* strains (1, 7, 9, 10, 32, 34, 39) but not in FQ-resistant *M. leprae* strains.

MATERIALS AND METHODS

Materials. The Thai-53 strain of *M. leprae* (22), maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strains TOP-10 (Life Technologies Corp., Carlsbad, CA), Rosetta-gami 2, and BL21(DE3)(pLysS) (Merck KGaA, Darmstadt, Germany) were used for cloning and protein expression. GyrA and GyrB expression plasmids were constructed on the basis of pET-20b(+) (Merck KGaA). OFX and gatifloxacin (GAT) were purchased from LKT Laboratories, Inc. (St. Paul, MN); moxifloxacin (MXF) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichisankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Ampicillin was purchased from Meiji Seika Pharma, Ltd. (Tokyo, Ja-

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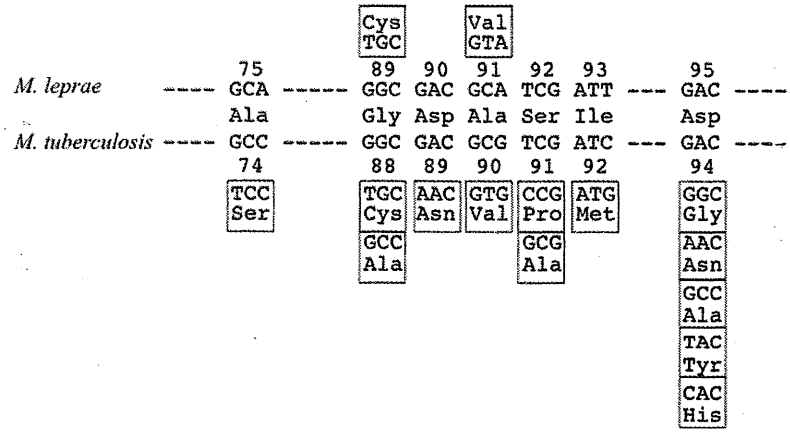

FIG 1 Nucleotide substitutions encoding the quinolone resistance-determining region in *gyrA* of WT and FQ-resistant *M. leprae* and *M. tuberculosis*. Nucleotide sequences encoding the quinolone resistance-determining region of WT *M. leprae* and *M. tuberculosis* GyrA were aligned with the amino acid sequence at the corresponding positions indicated by the numbers. Altered amino acids and the corresponding nucleotide substitutions of *M. leprae* and *M. tuberculosis* are placed above and below WT sequences, respectively.

pan). Oligonucleotide primers were synthesized by Life Technologies Corp. Restriction enzymes were obtained from New England BioLabs, Inc. (Ipswich, MA). The supercoiling assay kit and supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom).

Construction of recombinant wild-type (WT) and mutant DNA gyrase expression plasmids. DNA gyrase expression vectors were constructed basically as previously described (16), and Fig. 2 presents an overview of the procedure. The sequences of the primers used in the study

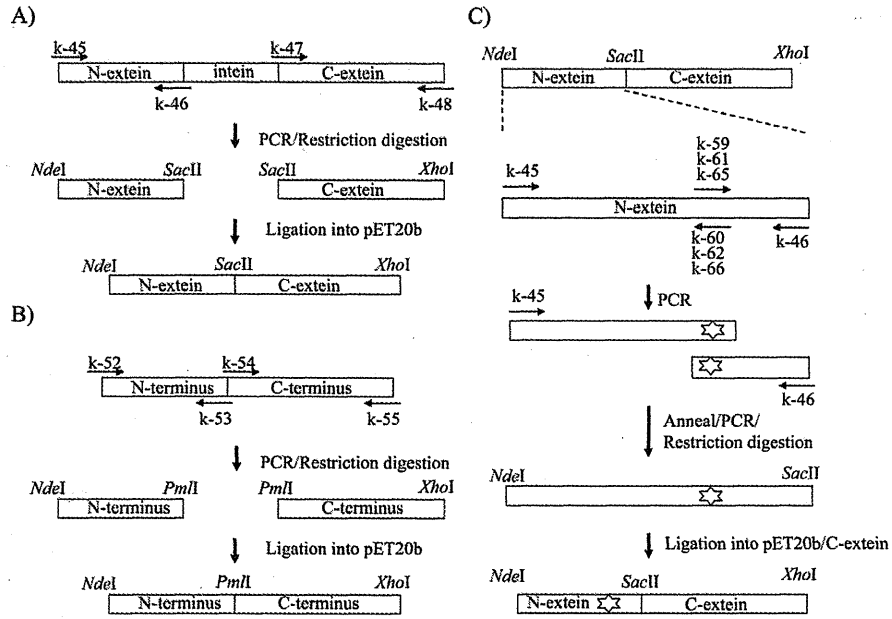

FIG 2 Construction of WT and mutant DNA gyrase expression plasmid. (A) DNA fragments encoding N-extein (amino acids 1 to 130) and C-extein of GyrA (amino acids 125 to 830) were amplified by PCR with primer pairs k-45/k-46 and k-47/k-48 (Table 1), respectively. Similarly, those encoding the N-terminal (amino acids 1 to 428) and C-terminal (amino acids 424 to 679) regions of GyrB were amplified with primer pairs k-52/k-53 and k-54/k-55 (Table 1), respectively. PCR products encoding N-extein and C-extein of GyrA were digested by NdeI-SacII and SacII-XhoI, respectively, and introduced simultaneously into NdeI-XhoI-digested plasmid pET-20b (+). (B) DNA fragments encoding the N-terminal and C-terminal regions of GyrB were digested by NdeI-PmaCI and PmaCI-XhoI, respectively, and introduced into pET20b as described above. (C) Primer pairs consisting of primer k-45 and primer k-60, k-62, or k-66 (Table 1) were used for amplifying the DNA fragment encoding the N-terminal portion (amino acids 1 to 94) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. Primer pairs consisting of primer k-46 and primer k-59, k-61, or k-65 (Table 1) were used for amplifying the DNA fragment encoding the C-terminal portion (amino acids 88 to 130) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. To complete the N-extein-encoding cassette, DNA fragments encoding the N-terminal and C-terminal regions of N-extein of GyrA were annealed and reamplified by PCR using the primer pair k-45/k-46. The mutated *gyrA*-N cassettes were digested with NdeI and SacII restriction endonucleases and ligated into the expression plasmid containing WT *gyrA* C-extein DNA fragment digested by the same enzymes.

TABLE 1 Nucleotide sequences of primers used in PCR

Primer name	Primer sequence (nucleotide positions)[a]
k-45	5′-GGCATATGACTGATATCACGCTGCCACCAG-3′ (1–25)
k-46	5′-ATAACGCATCGCCGCGGGTGGGTCATTACC-3′ (361–390)
k-47	5′-CACCCGCGGCGATGCGTTATACCGAGGCTCGGCTTACTCC-3′ (371–410)
k-48	5′-GGCTCGAGTTAATGATGATGATGATGATGACCGACACCGCCGTCGG-3′ (2471–2490)
k-52	5′-GGCATATGGCTGCCCAGAGGAAG-3′ (1–18)
k-53	5′-CTAACTCACGTGCTTTACGTGCAGCTATTC-3′ (1259–1288)
k-54	5′-CGTAAAGCACGTGAGTTAGTGCGTCGAAAAAGTGCC-3′ (1270–1305)
k-55	5′-GGCTCGAGCTAATGATGATGATGATGATGGACATCCAGGAAACGAACATCC-3′ (2013–2037)
k-59	5′-GCACGGCGAC**GTG**TCGATTTATG-3′ (261–283)
k-60	5′-CATAAATCGA**CAC**GTCGCCGTGC-3′ (261–283)
k-61	5′-CATCGATTTAT**GGC**ACGTTAGTGC-3′ (272–295)
k-62	5′-GCACTAACGT**GCC**ATAAATCGATG-3′ (272–295)
k-65	5′-CATCGATTTAT**AAC**ACGTTAGTGC-3′ (272–295)
k-66	5′-GCACTAACGT**GTT**ATAAATCGATG-3′ (272–295)

[a] Six-histidine tag codons are underlined, and mutated codons are shown in bold type.

are shown in Table 1. All PCRs were carried out in a thermal cycler (Applied Biosystems) under the following conditions: predenaturation at 98°C for 2 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 50 to 60°C for 15 s, and extension at 68°C for 1 to 2.5 min; and then a final extension at 68°C for 2 min. The nucleotide sequences of the DNA gyrase genes in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit (Life Technologies Corp.) and an ABI Prism 3130*xl* genetic analyzer (Life Technologies Corp.) according to the manufacturer's protocol.

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described (2, 3, 16, 20, 21, 31). Expression plasmids carrying the *gyrA* (WT and mutants) and WT *gyrB* genes of *M. leprae* were transformed into *E. coli* Rosetta-gami 2 and BL21(DE3)(pLysS), respectively. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Tokyo, Japan), followed by further incubation at 14°C for 16 h. The recombinant DNA gyrase subunit in the supernatant of the sonication lysate (by Sonifier 250; Branson, Danbury, CT) was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Life Technologies Corp.) column chromatography. The protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling activities and inhibition by FQs. ATP-dependent and quinolone-inhibited DNA supercoiling assays were carried out as previously described (2, 3, 16, 20, 21, 31) with the following modifications. DNA supercoiling activity was examined with a reaction mixture (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and purified GyrA and GyrB (50 ng each) subunits. Reactions were performed at 30°C for 1.5 h and stopped by adding an equal volume of chloroform-isoamyl alcohol (24:1 mixture) and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer, followed by ethidium bromide (0.7 μg/ml) staining. Supercoiling activity was evaluated by tracing the brightness of the bands with the software ImageJ (http://rsbweb.nih.gov/ij). Gyrase bearing an Ala91Val amino acid substitution in GyrA was used as a positive control for all assays (20). The inhibitory effect of FQs on DNA gyrases was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s). All enzyme assays were performed at least three times to confirm reproducibility.

Quinolone-mediated DNA cleavage assay. DNA cleavage assays were carried out as previously described (16, 20, 21, 31). The reaction mixture (total volume, 30 μl) contained DNA gyrase reaction buffer, recombinant DNA gyrase subunits (50 ng), supercoiled pBR322 DNA (300 ng), and 2-fold serially increasing concentrations of FQs. After incubation for 2 h at 30°C, 3 μl of 2% SDS and 3 μl proteinase K (1 mg/ml) were added to the reaction mixture. After subsequent incubation for 30 min at 30°C, reactions were stopped by the addition of 3 μl of 0.5 mM EDTA, 30 μl chloroform-isoamyl alcohol (24:1 mixture), and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 0.8% agarose gels in 1× TBE buffer, followed by ethidium bromide staining. The extent of DNA cleavage was quantified with ImageJ, and the quinolone concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined.

Temperature sensitivity of *M. leprae* DNA gyrase. The reactions with mixtures (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and recombinant DNA gyrase subunits

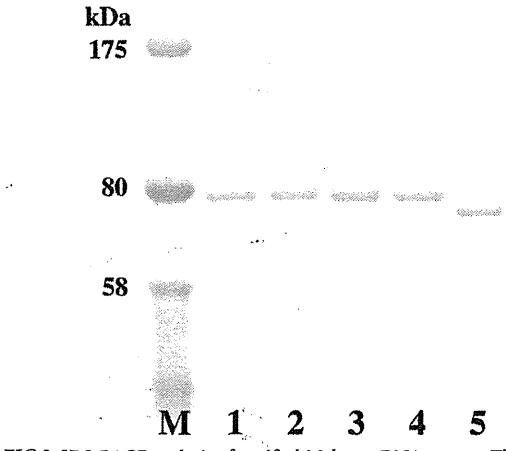

FIG 3 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were overexpressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M, protein marker (NEB); 1, WT GyrA; 2, GyrA-Ala91Val; 3, GyrA-Asp95Gly; 4, GyrA-Asp95Asn; 5, WT GyrB. Three hundred nanograms of each protein was loaded onto a 5 to 20% gradient polyacrylamide gel.

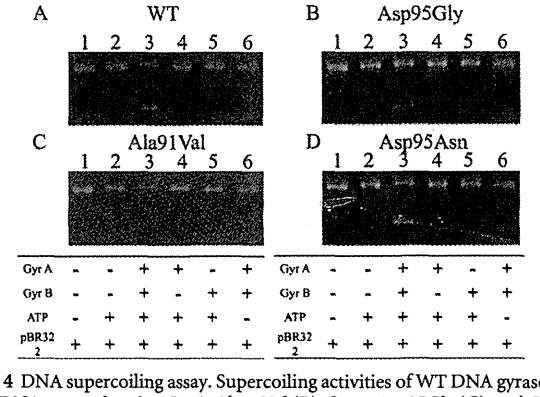

FIG 4 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A) and DNA gyrases bearing GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) were analyzed. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) or GyrB (50 ng), or both. Lanes: 1, relaxed pBR322 alone; 2, relaxed pBR322 and ATP; 3, relaxed pBR322, ATP, GyrA, and GyrB; 4, relaxed pBR322, ATP, and GyrA; 5, relaxed pBR322, ATP, and GyrB; 6, relaxed pBR322, GyrA, and GyrB.

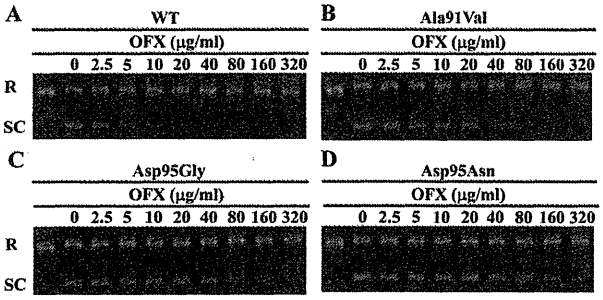

FIG 5 OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. Quinolone-inhibited supercoiling activity assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R and SC, relaxed and supercoiled pBR322 DNA, respectively.

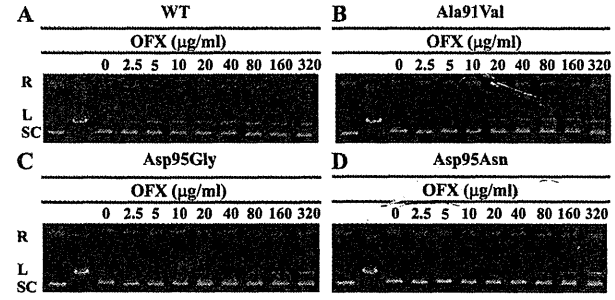

FIG 6 OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R, L, and SC, relaxed, linear, and supercoiled pBR322 DNA, respectively.

(50 ng) were run at 25, 30, 33, 37, and 42°C for 1.5 h. Supercoiling activities of recombinant DNA gyrases were evaluated at each reaction temperature as described above.

RESULTS

Construction and purification of recombinant His-tagged GyrA and GyrB proteins. DNA fragments, including the *gyrA* and *gyrB* genes, were successfully amplified from *M. leprae* Thai-53 strain DNA and inserted in frame downstream of a T7 promoter in pET-20b (+). GyrA and GyrB were expressed as C-terminal hexahistidine-tagged proteins for ease of purification, as the His tag has been shown not to interfere with the catalytic functions of GyrA and GyrB (2, 3, 16, 20, 21, 31). Expressed recombinant WT and mutant DNA gyrase subunits were purified as 0.3 to 1.5 mg soluble His-tagged 80-kDa protein of GyrA and 75-kDa protein of GyrB from 500-ml cultures. The purity of the recombinant proteins was confirmed by SDS-PAGE (Fig. 3). All of the recombinant proteins were obtained with high purity (>95%).

DNA supercoiling activities. Combinations of GyrA WT, Ala91Val, Asp95Gly, or Asp95Asn and WT GyrB subunits were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Fig. 4). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Fig. 4A to D, lane 3), while neither subunit alone exhibited DNA supercoiling activity (Fig. 4A to D, lanes 4 and 5). In addition, no supercoiling activity was observed when ATP was omitted from the reaction mixture (Fig. 4A to D, lane 6).

Inhibition of DNA gyrase activities by FQs. The IC_{50}s of FQs were determined using the quinolone-inhibited DNA supercoiling assay (Fig. 5). Representative data showing the inhibitory effects of OFX against DNA gyrase are shown in Fig. 5, and data for other FQs are presented in Fig. S1 in the supplemental material. IC_{50}s of each FQ against WT and mutant DNA gyrases are summarized in Table 2. Each FQ showed dose-dependent inhibition, with IC_{50}s ranging from 0.4 to 262.3 μg/ml. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher IC_{50}s to quinolones (Table 2; Fig. 5; see Fig. S1 in the supplemental material) than WT gyrase (Table 2). These DNA gyrases also showed higher resistance than DNA gyrase bearing GyrA-Ala91Val, which was simultaneously analyzed as a positive control for resistance to FQs. Inhibitory effects of FQs were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

FQ-mediated DNA-cleavable complex formation. The CC_{25}s of FQs were determined. Figure 6 shows the result of a DNA cleavage assay using OFX, and Fig. S2 in the supplemental material presents the results using GAT, MXF, and SIT. Table 2 summarizes the CC_{25}s of each DNA gyrase. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher CC_{25}s to quinolones than WT gyrase (Table 2). These DNA gyrases also showed higher CC_{25}s than gyrase bearing GyrA-Ala91Val (Table 2). Effects on cleavable complex formation were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

Temperature sensitivity of *M. leprae* DNA gyrase. Figure 7 shows the effects of temperature on DNA gyrase activities. The highest DNA supercoiling activities were observed at 33°C in all DNA gyrases. WT and GyrA-A91V DNA gyrases showed reduced DNA supercoiling activities at 37°C, whereas Gyr-Asp95Gly and Asp95Asn DNA gyrases maintained activities comparable to those at 33°C. No supercoiling activities were observed in any of the DNA gyrases at 42°C.

TABLE 2 IC_{50}s and CC_{25}s of FQs against WT and mutant DNA gyrases[a]

Drug	IC_{50}				CC_{25}			
	WT	Ala91Val	Asp95Gly	Asp95Asn	WT	Ala91Val	Asp95Gly	Asp95Asn
OFX	6.8 ± 0.8	39.4 ± 15.5 (5.8)	161.2 ± 44.2 (23.7)	262.3 ± 105.8 (38.6)	7.3 ± 0.5	75.5 ± 16.8 (10.1)	240.5 ± 30.7 (32.1)	269.5 ± 76.5 (35.9)
GAT	1.0 ± 0.1	3.1 ± 0.7 (3.1)	7.5 ± 1.6 (7.5)	13.8 ± 1.6 (13.8)	1.1 ± 0.2	4.3 ± 0.2 (3.9)	15.6 ± 3.6 (14.2)	13.5 ± 3.1 (12.3)
MXF	1.5 ± 0.3	5.2 ± 1.0 (3.5)	21.5 ± 4.7 (14.3)	34.7 ± 3.1 (23.1)	1.0 ± 0.1	4.5 ± 1.0 (4.5)	25.5 ± 3.7 (25.5)	20.8 ± 5.0 (20.8)
SIT	0.4 ± 0.0	1.0 ± 0.2 (2.5)	2.2 ± 0.5 (5.5)	3.9 ± 0.6 (9.8)	0.3 ± 0.0	0.9 ± 0.0 (3.0)	2.2 ± 0.6 (7.3)	2.3 ± 0.4 (7.7)

[a] IC_{50}s and CC_{25}s are in μg/ml, and data in parentheses represent the fold increase compared to WT.

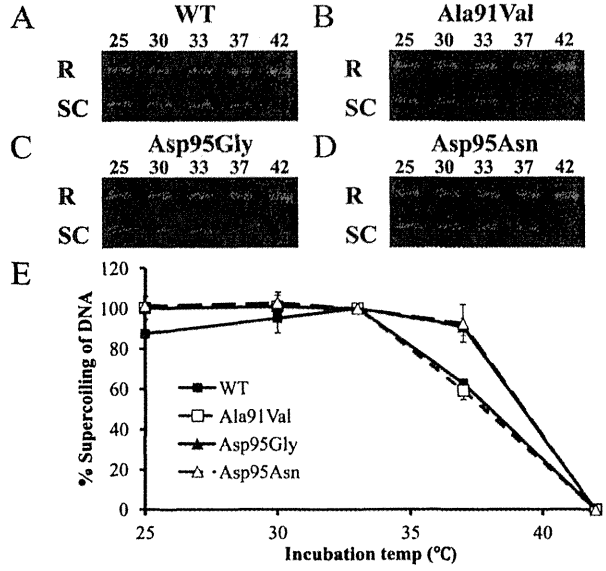

FIG 7 Temperature-dependent DNA supercoiling activity of DNA gyrases. Relaxed pBR322 (0.3 μg) was incubated with WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) at the temperatures (in °C) indicated above the lanes. The proportion of supercoiled DNA compared to that of WT DNA gyrase at 33°C is plotted for each incubation temperature.

DISCUSSION

Mutations in the *gyrA* gene of quinolone-resistant *M. leprae* clinical isolates have predominantly been reported at codon 91, and a smaller number have been reported at codon 89 (4, 19, 24, 26, 40). Amino acid substitutions at other positions have not been reported, in strong contrast to the substitutions reported in *M. tuberculosis*, with predominant mutations in codon 94 (1, 7, 9, 10, 32, 34, 39), equivalent to codon 95 in *M. leprae* (Fig. 1). This study aimed to obtain basic data for the rapid detection of FQ-resistant leprosy by elucidating the correlation between mutations at codon 95 and quinolone resistance.

To explain the discrepancy described above, we first hypothesized that amino acid substitution at position 95 in GyrA of *M. leprae* has less of an influence on FQ resistance. Hence, we carried out a quinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay at 30°C, the optimal temperature of *M. leprae* growth, using recombinant DNA gyrases and calculated IC_{50}s and CC_{25}s of four FQs, OFX, MXF, GAT, and SIT. The DNA gyrase bearing GyrA-Ala91Val, used as a control, exhibited resistance, having approximately 2- to 10-fold higher IC_{50}s and CC_{25}s of FQs than WT DNA gyrase, as has been reported previously (20, 21). Interestingly, DNA gyrases bearing GyrA-Asp95Gly or -Asp95Asn showed resistance, having approximately 5- to 40-fold higher IC_{50}s and CC_{25}s of FQs than WT DNA gyrase (Table 2). Namely, amino acid substitution from Asp to Gly or Asn at position 95 added higher resistance to DNA gyrase than that from Ala to Val at position 91. This was similar to the observation in *M. tuberculosis* (2, 3). These results suggested that a possible property of Asp95Gly and Asp95Asn amino acid substitutions in GyrA is to give higher FQ resistance to DNA gyrase in *M. leprae*.

We then hypothesized that amino acid substitutions at position 95 place a disadvantage on the enzymatic property of DNA gyrases, especially lower or abolished activity at higher temperatures, and thus, we conducted a DNA supercoiling assay at various temperatures: 25, 30, 33, 37, and 42°C. DNA supercoiling activities of WT and GyrA-Ala91Val DNA gyrase showed a similar temperature dependence, with the highest activity being at 25 to 33°C, reduced activity occurring at 37°C, and activity being completely abolished at 42°C. In contrast, DNA gyrases bearing GyrA-Asp95Gly or -Asp95Asn maintained their activities even at 37°C. Our hypothesis was rejected by these data.

The influence of the clear usage of FQs for the treatment of leprosy and tuberculosis might solve this question. For leprosy patients with a single lesion, a single application of 400 to 600 mg of OFX is used. For the treatment of MDR leprosy, two or three doses of 400 to 600 mg in combination with first-line drugs DDS and RIF (11) are applied. In contrast, for tuberculosis, OFX is taken twice daily at 400 mg each time with first-line drugs such as isoniazid and rifampin for several months (11, 36). The maximum serum concentration (C_{max}) of OFX has been reported to show a dose-dependent increase. The C_{max}s achieved with administration of 100 mg, 300 mg, and 600 mg of OFX in humans were 1.00, 2.81, and 6.81 μg/ml, respectively (14). The blood concentration of OFX is low in leprosy patients and is maintained at a high level in tuberculosis patients because of the treatment regimen. Thus, *M. leprae* carrying DNA gyrase with lower resistance, such as GyrA-Ala91Val, might be predominantly selected for various reasons in leprosy patients, whereas GyrA-Asp94Gly or -Asp94Asn is predominantly found in *M. tuberculosis*-infected patients (1, 7, 9, 10, 32, 34, 39); however, the possible emergence in the future of highly FQ-resistant *M. leprae* having an amino acid substitution at position 95 cannot be rejected, especially when MDR leprosy is treated by repeated administration of FQs.

We investigated the inhibitory effects of OFX, GAT, MXF, and SIT against WT and mutant DNA gyrases. IC_{50}s of OFX for WT and GyrA-Ala91Val, -Asp95Gly, and -Asp95Asn DNA gyrases were 6.8, 39.4, 161.2, and 262.3 μg/ml, respectively (Table 2). The order of FQ inhibitory activity was SIT > GAT > MXF > OFX. OFX does not have the ability to inhibit *M. leprae* with DNA gyrase carrying GyrA-Asp95Gly or -Asp95Asn. The IC_{50} of SIT was the lowest of the four quinolones, with IC_{50}s of 0.4, 1.0, 2.2, and 3.9 μg/ml for WT, A91V, D95G, and D95N gyrases, respectively. As the C_{max}s of OFX, GAT, MXF, and SIT at the 100-mg dosage were determined in clinical trials to be 1.00, 0.87 to 5.41, 4, and 0.3 to 1.9 μg/ml, respectively (14, 27, 28, 30), SIT might strongly inhibit *M. leprae* carrying GyrA-Ala91Val DNA gyrase and be a promising candidate for the treatment of the majority of cases of FQ-resistant leprosy.

In conclusion, we revealed the contribution of the GyrA-Asp95Gly and -Asp95Asn amino acid substitutions to FQ resistance in *M. leprae* by an *in vitro* assay. This suggested the possible emergence in the future of FQ-resistant *M. leprae* carrying GyrA with these amino acid substitutions, although further analysis is needed to clarify a direct relationship to *in vivo* resistance. Hence, we would like to propose analysis for these amino acid substitutions to detect FQ-resistant leprosy.

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Short communication

Sensitivities of ciprofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates to fluoroquinolones: role of mutant DNA gyrase subunits in drug resistance

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ABSTRACT

Minimum inhibitory concentrations of sitafloxacin, gatifloxacin, moxifloxacin, sparfloxacin, levofloxacin and ciprofloxacin against 59 ciprofloxacin-resistant clinical isolates of *Mycobacterium tuberculosis* from Japan were determined. The isolates were most susceptible to sitafloxacin and gatifloxacin. To understand better the basis for drug resistance, nucleotide sequences encoding the *gyrA* and *gyrB* quinolone resistance-determining region were determined. Predicted amino acid sequences revealed distinct mutational patterns likely to be responsible for fluoroquinolone resistance. Double *gyrA* mutations as well as mutations in both *gyrA* and *gyrB* correlated with increased resistance to all fluoroquinolones.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a serious global health problem as one-third of the world's population is infected, resulting in 9 million new cases and nearly 2 million deaths in 2010 [1]. The World Health Organization (WHO) recommends a four-drug combination therapeutic strategy termed 'directly observed treatment, short-course (DOTS)' to prevent the spread of drug-resistant TB; nevertheless, an increasing number of multidrug-resistant TB (MDR-TB) isolates, resistant to more than two drugs including rifampicin and isoniazid, have arisen in some Eastern European and Western Asian countries [1]. Accordingly, broad-spectrum fluoroquinolones (FQs) have been used to treat MDR-TB [2]. Unfortunately, their increasing use has generated large numbers of FQ-resistant *M. tuberculosis* strains [1] and thus researchers have focused on elucidating the mechanism of acquired resistance.

FQs target type II DNA topoisomerases, including DNA gyrase and topoisomerase IV. DNA gyrase mediates the supercoiling of double-stranded DNA during DNA replication, and topoisomerase IV separates or deconcatenates replicated chromosomes. Amino acid substitutions in putative FQ-binding regions in DNA gyrase, topoisomerase IV, or both, confer resistance upon several bacterial species [3]. These regions are highly conserved in bacteria and are referred to as the quinolone resistance-determining regions (QRDRs) [3]. *Mycobacterium tuberculosis* lacks topoisomerase IV [4], thus leaving DNA gyrase as the apparent sole target of FQs.

Single missense mutations in *gyrA* have been associated with FQ resistance [5–9], and strains carrying two missense mutations in both *gyrA* and *gyrB* generally exhibit higher-level resistance [8,9]. These findings suggest that amino acid substitutions in GyrA and GyrB impart FQ resistance to *M. tuberculosis*.

Interestingly, the rate and mode of mutations vary with respect to geographical origin [5–9]; however, similar studies have not, to our knowledge, been conducted in Japan. Japan suffers from the unhappy distinction of nearly 30% of its MDR-TB being extensively drug-resistant TB (XDR-TB) [10], which, in addition to being resistant to rifampicin and isoniazid, is also resistant to any FQ and any injectable agent. Therefore, the aim of the present study was to compare the antimicrobial activities of FQs against Japanese ciprofloxacin (CIP)-resistant clinical isolates and to identify QRDR mutations imparting FQ resistance.

2. Materials and methods

2.1. Bacterial strains

The 59 *M. tuberculosis* clinical isolates studied came from 11 hospitals in Japan and were resistant to ≥4 μg/mL CIP using the

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proportion method on Ogawa medium (2% phosphate) [20 g of KH_2PO_4, 5.0 g of sodium glutamate, 1.0 g of magnesium citrate, 30 g of starch, 2.0 g of sodium pyruvate, 40 mL of glycerol, 40 mL of malachite green (2% solution), 2000 mL of homogenised whole egg and 1000 mL of distilled water]. *Mycobacterium tuberculosis* H37Rv was used as a quinolone-susceptible strain.

2.2. Drugs

Sitafloxacin (STFX) and levofloxacin (LVX) were provided by Daiichi Pharmaceuticals Co., Ltd. (Tokyo, Japan). Gatifloxacin (GFLX) and sparfloxacin (SPFX) were from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and Kyorin Pharmaceutical Inc. (Tokyo, Japan), respectively. Moxifloxacin (MXF) and CIP were from Bayer Yakuhin, Ltd. (Osaka, Japan). Drugs were dissolved in 0.1 M NaOH at 50 mg/mL (except for MXF, which was dissolved in distilled water to 10 μg/mL) and used as stock solutions.

2.3. Minimum inhibitory concentration (MIC) determination

Isolates were cultured in Middlebrook 7H9 medium (Becton Dickinson, Franklin Lakes, NJ) containing 0.05% Tween 80, 10% oleic–albumin–dextrose–catalase (OADC) and 0.2% glycerol at 37 °C for 7 days and then diluted with the same medium to an optical density at 540 nm of 0.2. Middlebrook 7H11 agar (Becton Dickinson) plates (15 mL volume) without or with drugs diluted serially to yield final concentrations from 0.39–12.5, 0.78–6.25, 0.78–12.5, 1.56–12.5, 3.13–25 and 6.25–50 μg/mL, respectively, for STFX, GFLX, MXF, SPFX, LVX and CIP were inoculated with 0.1 mL of 100-fold-diluted bacterial suspensions described above. Bacterial growth was assessed after 3 weeks at 37 °C under 5% CO_2. The MIC was defined as the lowest concentration that inhibited visible growth.

2.4. Sequence analysis

Chromosomal DNA was extracted from a colony by mechanical disruption [11]. DNA fragments encoding GyrA and GyrB QRDRs were amplified and were sequenced using the primer pairs TBgyrA1N (5′-AGCGCAGCTACATCGACTATGCG-3′)/TBgyrA2N (5′-CTTCGGTGTACCTCATCGCCGCC-3′) and TBgyrB1N (5′-TCGGCGCAAGCCCGTATCGCGGC-3′)/TBgyrB2N (5′-CATCAGCACGATCTTGTGGTAGC-3′). Reaction mixtures (50 μL) contained 1.25 U of LA *Taq*™ DNA polymerase (Takara Bio Inc., Shiga, Japan), LA PCR buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.5 mM of each primer and 10 ng of DNA. Polymerase chain reaction (PCR) was carried out using a Takara PCR Thermal Cycler PERSONAL (Takara Bio Inc.) for 35 cycles of denaturation for 5 s at 98 °C, annealing for 10 s at 55 °C and extension for 30 s at 72 °C; and final extension for 3 min at 72 °C. PCR products were separated by 1% agarose gel electrophoresis in TAE buffer [40 mM Tris–acetate, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.3] extracted from an excised gel block into supernatant by centrifugation at 20 000 × *g* for 5 min and were directly sequenced using a BigDye® Terminator v.1.1 Cycle Sequencing Kit and a Model 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA).

3. Results

3.1. Characterisation of Mycobacterium tuberculosis isolates

MICs to six FQs for all 59 CIP-resistant strains were determined (Table 1). Most of the CIP-resistant isolates (52/59) were inhibited by STFX and GFLX at 3.13 μg/mL. MXF and SPFX inhibited 35/59 and 32/59 strains, respectively, at the same concentration, in contrast to LVX that only inhibited 12/59 strains. These tests verified that all strains were CIP-resistant.

3.2. Quinolone resistance-determining region mutations

Sequence analysis found eight single-point and six double-point mutational patterns (Table 1). All isolates harboured QRDR mutations. Substitution at amino acid 94 was most common (39/59) followed by amino acid 90 (17/59).

3.3. Minimum inhibitory concentration as a function of single GyrA quinolone resistance-determining region mutations

Table 2 summarises the drug susceptibility assays of GyrA and GyrB mutants. A strain with Asp → Val substitution at amino acid 94 (Asp94Val) exhibited susceptibility to each drug with the exception of LVX and CIP, both with MIC_{50} values (MICs inhibiting >50% of tested strains) of 6.25 μg/mL. Four strains with a Ser91Pro substitution exhibiting low CIP resistance were significantly inhibited by the five other FQs. Strains with Ala90Val or Asp94Ala substitution showed slightly diminished susceptibility to CIP and LVX but were susceptible to the other FQs. In contrast, MIC_{50} values for the Asp94Tyr mutant were 0.78, 1.56, 3.13, 3.13, 6.25 and 12.5 μg/mL, respectively, for STFX, GFLX, MXF, SPFX, LVX and CIP. The Asp94Asn mutant was less susceptible than the aforementioned mutants, but more so than the Asp94Gly and Gly88Cys mutants, the latter of which was the least susceptible. In addition, variation in MICs for strains with the same mutation was found.

3.4. Minimum inhibitory concentration as a function of multiple quinolone resistance-determining region mutations

Strains with double mutations tended to exhibit lower susceptibility than the corresponding single mutant. An isolate harbouring both Ala90Val and Asp94Ala amino acid substitutions (isolate ID55) (Table 1) exhibited lower susceptibility to CIP than single Ala90Val or Asp94Ala mutants. Similarly, Ala90Val + Ser91Pro and Ala90Val + Asp94Val mutants exhibited lower susceptibility to CIP than single mutants at each position (Table 2). Two *gyrB* mutations linked to a *gyrA* mutation were identified as follows: GyrA-Ala90Val + GyrB-Thr539Asn and GyrA-Asp94Gly + GyrB-Ser486Phe, of which the former *gyrB* mutation was associated with reduced susceptibility and the latter was not (Table 2).

4. Discussion

Treating MDR-TB has come to rely increasingly on FQs, as emphasised by the WHO recommendation [1] that each MDR clinical isolate registered should be routinely tested for ofloxacin (OFX) or CIP susceptibility. Because FQs are widely used for treating other infectious diseases, their efficacy for treating TB may be compromised. To counteract this possibility, it is hoped that CIP-resistant strains will be cured with newer FQs [2]. To our knowledge, the present study is the first to investigate drug sensitivities of CIP-resistant clinical isolates from patients in Japan, a country with a high rate of XDR-TB amongst MDR-TB [9]. Drug susceptibility patterns have been previously analysed in several geographic locations (Table 3). In Hong Kong, STFX, GFLX and MXF MIC_{50} values were lower than those of OFX, LVX and SPFX ($n = 32$) [6]. A study in Italy ($n = 17$) reported median MICs and the results can be summarised as OFX > CIP and LVX > SPFX > GFLX and MXF [7]. MXF and GFLX were shown to be more active than OFX in Belgium ($n = 22$) [8]. A similar effectiveness of CIP, OFX, LVX and MXF was demonstrated in Taiwan ($n = 6$) [9]. All the data showed similarity and the main conclusion drawn from these studies and the present study is that STFX,

Table 1
Minimum inhibitory concentrations (MICs) of six quinolones and amino acid substitutions in GyrA and GyrB for ciprofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates in Japan.

Isolate ID	MIC (μg/mL)						Amino acid substitution in QRDR					
							GyrA				GyrB	
	STFX	GFLX	MXF	SPFX	LVX	CIP	Gly88	Ala90	Ser91	Asp94	Ser486	Thr539
1	12.5	3.13	12.5	6.25	12.5	>50	Cys					
2	≤0.39	≤0.78	0.78	1.56	3.13	6.25		Val				
3	≤0.39	≤0.78	1.56	1.56	3.13	6.25		Val				
4	0.78	0.78	1.56	1.56	3.13	6.25		Val				
5	0.78	0.78	1.56	3.13	3.13	6.25		Val				
6	≤0.39	1.56	1.56	1.56	6.25	6.25		Val				
7	0.78	1.56	3.13	3.13	6.25	6.25		Val				
8	0.78	1.56	1.56	1.56	3.13	12.5		Val				
9	0.78	≤0.78	0.78	3.13	6.25	12.5		Val				
10	≤0.39	≤0.78	1.56	3.13	6.25	12.5		Val				
11	≤0.39	1.56	3.13	3.13	6.25	12.5		Val				
12	6.25	3.13	3.13	3.13	6.25	12.5		Val				
13	0.78	1.56	3.13	3.13	6.25	25		Val				
14	0.78	1.56	3.13	1.56	3.13	6.25			Pro			
15	1.56	1.56	3.13	1.56	3.13	6.25			Pro			
16	3.13	3.13	3.13	1.56	3.13	6.25			Pro			
17	1.56	1.56	3.13	1.56	6.25	6.25			Pro			
18	≤0.39	≤0.78	1.56	1.56	3.13	6.25				Ala		
19	0.78	≤0.78	1.56	1.56	3.13	6.25				Ala		
20	1.56	≤0.78	1.56	1.56	3.13	6.25				Ala		
21	≤0.39	1.56	1.56	3.13	3.13	12.5				Ala		
22	≤0.39	≤0.78	1.56	3.13	6.25	12.5				Ala		
23	0.78	1.56	1.56	3.13	6.25	12.5				Ala		
24	≤0.39	1.56	3.13	3.13	6.25	12.5				Ala		
25	3.13	≤0.78	3.13	3.13	6.25	12.5				Ala		
26	3.13	1.56	3.13	3.13	6.25	25				Ala		
27	1.56	3.13	6.25	6.25	12.5	25				Ala		
28	3.13	1.56	3.13	6.25	6.25	12.5				Gly		
29	1.56	3.13	6.25	6.25	12.5	12.5				Gly		
30	1.56	1.56	3.13	3.13	6.25	25				Gly		
31	3.13	1.56	3.13	3.13	6.25	25				Gly		
32	0.78	3.13	6.25	6.25	12.5	25				Gly		
33	0.78	3.13	6.25	6.25	12.5	25				Gly		
34	0.78	3.13	6.25	6.25	12.5	25				Gly		
35	1.56	3.13	6.25	6.25	12.5	25				Gly		
36	3.13	1.56	6.25	6.25	12.5	25				Gly		
37	3.13	3.13	6.25	6.25	12.5	25				Gly		
38	3.13	3.13	6.25	6.25	12.5	25				Gly		
39	6.25	3.13	12.5	6.25	12.5	25				Gly		
40	3.13	3.13	6.25	6.25	12.5	>50				Gly		
41	6.25	6.25	12.5	6.25	12.5	>50				Gly		
42	3.13	6.25	12.5	12.5	12.5	>50				Gly		
43	1.56	1.56	3.13	6.25	6.25	12.5				Asn		
44	1.56	1.56	3.13	6.25	6.25	25				Asn		
45	1.56	3.13	6.25	6.25	6.25	25				Asn		
46	3.13	6.25	12.5	12.5	25	25				Asn		
47	>12.5	6.25	12.5	12.5	25	>50				Asn		
48	≤0.39	1.56	3.13	3.13	6.25	12.5				Tyr		
49	0.78	≤0.78	3.13	3.13	6.25	12.5				Tyr		
50	1.56	3.13	6.25	3.13	12.5	12.5				Tyr		
51	0.78	1.56	3.13	6.25	6.25	25				Tyr		
52	3.13	3.13	6.25	6.25	6.25	>50				Tyr		
53	0.78	≤0.78	1.56	1.56	6.25	6.25				Val		
54	>12.5	>6.25	>12.5	>12.5	>25	>50		Val	Pro			
55	6.25	6.25	12.5	>12.5	>25	>50		Val		Ala		
56	3.13	6.25	6.25	12.5	25	25		Val		Val		
57	0.78	3.13	12.5	12.5	12.5	25		Val				Asn
58	3.13	3.13	12.5	12.5	12.5	25		Val				Asn
59	1.56	1.56	3.13	3.13	6.25	25				Gly	Phe	
No. of strains with MIC ≤3.13 μg/mL	52	52	35	32	12	0						

STFX, sitafloxacin; GFLX, gatifloxacin; MXF, moxifloxacin; SPFX, sparfloxacin; LVX, levofloxacin; CIP, ciprofloxacin; QRDR, quinolone-resistance determining region.

GFLX and MXF can inhibit the growth of OFX- or CIP-resistant *M. tuberculosis* in vitro. As the maximum serum concentrations (C_{max}) of OFX [12], GFLX [13], STFX [14] and MFX [15] for a 100 mg dosage were determined in clinical trials to be 1.0, 1.1, 3.1 and 4.0 μg/mL, respectively, STFX, GFLX and MFX might inhibit the OFX- or CIP-resistant *M. tuberculosis* in therapeutic use.

This study demonstrated that all CIP-resistant clinical isolates harboured *gyrA* mutations, similar to the data from Belgium (96%) [8], Italy (89%) [7] and Vietnam (83%) [5]. However, a significantly lower correlation was reported by investigators in Hong Kong (58%) [6] and Taiwan (50%) [9]. Use of a relatively high CIP MIC (≥4 μg/mL) for defining resistance might be a

Table 2
Amino acid substitutions in GyrA and GyrB and associated minimum inhibitory concentrations (MICs).

Amino acid substitution in QRDR		No. of isolates	MIC (μg/mL)											
			STFX		GFLX		MXF		SPFX		LVX		CIP	
GyrA	GyrB		Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}	Range	MIC_{50}
Asp94Val		1	0.78	0.78	≤0.78	≤0.78	1.56	1.56	1.56	1.56	6.25	6.25	6.25	6.25
Ser91Pro		4	0.78–3.13	1.56	1.56–3.13	1.56	3.13	3.13	1.56	1.56	3.13–6.25	3.13	6.25	6.25
Ala90Val		12	≤0.39–6.25	0.78	≤0.78–3.13	≤0.78	0.78–3.13	1.56	1.56–3.13	3.13	3.13–6.25	6.25	6.25–12.5	6.25
Asp94Ala		10	≤0.39–3.13	0.78	≤0.78–3.13	≤0.78	1.56–6.25	1.56	1.56–6.25	3.13	3.13–12.5	6.25	6.25–25	12.5
Asp94Tyr		5	≤0.39–3.13	0.78	≤0.78–3.13	1.56	3.13–6.25	3.13	3.13–6.25	3.13	6.25–12.5	6.25	12.5 to >50	12.5
Asp94Gly		15	0.78–6.25	3.13	1.56–6.25	3.13	3.13–12.5	6.25	3.13–12.5	6.25	6.25–12.5	12.5	12.5 to >50	25
Asp94Asn		5	1.56 to >12.5	1.56	1.56–6.25	3.13	3.13–12.5	6.25	6.25–12.5	6.25	6.25–25	6.25	12.5 to >50	25
Gly88Cys		1	12.5	12.5	3.13	3.13	12.5	12.5	6.25	6.25	12.5	12.5	>50	>50
Ala90Val + Asp94Val		1	3.13	3.13	6.25	6.25	6.25	6.25	12.5	12.5	25	25	25	25
Ala90Val + Asp94Ala		1	6.25	6.25	6.25	6.25	12.5	12.5	>12.5	>12.5	>25	>25	>50	>50
Ala90Val + Ser91Pro		1	>12.5	>12.5	>6.25	>6.25	>12.5	>12.5	>12.5	>12.5	>25	>25	>50	>50
Asp94Gly	Ser486Phe	1	1.56	1.56	1.56	1.56	3.13	3.13	3.13	3.13	6.25	6.25	25	25
Ala90Val	Thr539Asn	2	0.78–3.13	0.78	3.13	3.13	12.5	12.5	12.5	12.5	12.5	12.5	25	25

QRDR, quinolone-resistance determining region; STFX, sitafloxacin; GFLX, gatifloxacin; MXF, moxifloxacin; SPFX, sparfloxacin; LVX, levofloxacin; CIP, ciprofloxacin; MIC_{50}, MIC inhibiting >50% of tested strains.

Table 3
Amino acid substitutions in GyrA and GyrB and MIC_{50} values (μg/mL).

Amino acid substitution in QRDR		These data (n = 59)							Italy (n = 17) [7]							Taiwan (n = 6) [9]					Hong Kong (n = 31) [6]							Belgium (n = 22) [8]			
GyrA	GyrB	N^a	STFX	GFLX	MXF	SPFX	LVX	CIP	N^a	GFLX	MXF	SPFX	LVX	CIP	OFX	N^a	MXF	LVX	CIP	OFX	N^a	STFX	GFLX	MXF	SPFX	LVX	OFX	N^a	GFLX	MXF	OFX
Asp94Val		1	0.78	≤0.78	1.56	1.56	6.25	6.25	0							0					0							0			
Asp89Asn		0							0							0					0							1	1	2	4
Ser91Pro		4	1.56	1.56	3.13	1.56	3.13	6.25	2	0.5	1	1	2	4	4	0					4	1	2	1	0.5	2	4	0			
Ala90Val		12	0.78	≤0.78	1.56	3.13	6.25	6.25	9	1	1	2	4	4	8	1	1	4	8	16	5	0.5	1	1	2	4	8	10	0.5	0.5	4
Asp94Ala		10	0.78	≤0.78	1.56	3.13	6.25	12.5	1	0.5	1	2	4	4	4	0					3	0.5	2	1	2	4	8	3	0.25	0.5	4
Asp94Tyr		5	0.78	1.56	3.13	3.13	6.25	12.5	0							0					5	2	2	2	4	8	16	3	1	2	8
Asp94His		0							0							0					3	1	4	2	4	8	16	0			
Asp94Gly		15	3.13	3.13	6.25	6.25	12.5	25	4	1	1	2	4	4	8	4	1	2	4	4	11	2	4	2	4	8	16	4	1	2	8
Asp94Asn		5	1.56	3.13	6.25	6.25	6.25	25	1	1	1	2	2	4	8	0					1	4	4	2	2	4	8	1	1	4	16
Gly88Cys		1	12.5	3.13	12.5	6.25	12.5	>50	0							0					0							0			
	Asn538Asp	0							0							1	1	1	4	8	0							0			
	Asn533Thr	0							0							0					0							1	0.5	0.5	1
Ala90Val + Asp94Val		1	3.13	6.25	6.25	12.5	25	25	0							0					0							0			
Ala90Val + Asp94Ala		1	6.25	6.25	12.5	>12.5	>25	>50	0							0					0							0			
Ala90Val + Asp94Asn		0							0							0					0							1	>4	>8	>32
Ala90Val + Ser91Pro		1	>12.5	>6.25	>12.5	>12.5	>25	>50	0							0					0							0			
Asp94Gly	Ser486Phe	1	1.56	1.56	3.13	3.13	6.25	25	0							0					0							0			
Ala90Val	Thr539Asn	2	0.78	3.13	12.5	12.5	12.5	25	0							0					0							0			
Asp94His	del678–9	0							0							0					0							1	1	2	4

MIC_{50}, MIC inhibiting >50% of tested strains; STFX, sitafloxacin; GFLX, gatifloxacin; MXF, moxifloxacin; SPFX, sparfloxacin; LVX, levofloxacin; CIP, ciprofloxacin; OFX, ofloxacin.
[a] Number of isolates.

reason for the 100% correlation between CIP resistance and *gyrA* mutations. The current data regarding GyrA QRDR mutations and their influence on MIC values of FQs for isolates with a variety of mutations are generally consistent with findings by others (Table 3). Variations in MICs in strains with the same mutations were also observed, similar to studies by others. Mechanisms other than amino acid substitution in DNA gyrase subunits, such as permeability change, might cause this phenomenon. The present study uniquely contributes data on the effect of Asp94Val, Gly88Cys, Ala90Val + Asp94Val, Ala90Val + Asp94Ala and Ala90Val + Ser91Pro mutations on resistance to FQs. The Gly88Cys mutation in particular contributed to high-level resistance. Others have also reported drug sensitivity data for GyrA QRDR double mutants, namely Ala90Val + Ser91Pro, Ala90Val + Asp94Gly and Ala90Val + Asp94Asn mutants highly resistant to OFX. Note, however, that the findings reported here not only supported the former studies' conclusions but also added an insight into cross-resistance of isolates with these double mutations in *gyrA* to a wide range of FQs. Although no single *gyrB* mutations conferring drug resistance have been identified in this study, we report here that only one of the two *gyrB* mutations found in this study appeared to clearly contribute to increased resistance beyond the level conferred by the *gyrA* mutation alone.

In conclusion, these data suggest that MXF, STFX and GFLX more potently inhibit CIP-resistant *M. tuberculosis* compared with other FQs, as has been shown in previous studies. These data provide a foundation upon which to base new therapeutic strategies for more effectively treating MDR-TB. In addition, the data enhance previously available information on the contribution of amino acid substitutions in DNA gyrase to help improve gene-based methods for predicting FQ susceptibility, which enables the appropriate choice of a FQ for the treatment of MDR-TB.

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NOTE *Bacteriology*

Mycobacterium pseudoshottsii Isolated from 24 Farmed Fishes in Western Japan

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ABSTRACT. Mycobacteria isolated from epizootics of farmed fishes in western Japan were examined for the first time using multigenotypic analysis. By analysis of the sequences of the internal transcribed spacer between the 16S and 23S rRNA genes (ITS) region and the partial 16S rRNA, *hsp65* and *rpoB* genes, *M. pseudoshottsii* was identified as the causative agent in these infections. Prior to this study, only *M. marinum* has been known as the causative agent of lethal mycobacterial disease in marine fishes in Japan.
KEY WORDS: lethal fish infection, *Mycobacterium pseudoshottsii*, mycolactone.

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Mycobacterium (M.) marinum, M. salmoniphilum, M. fortuitum, M. chelonae and *M. abscessus* are the most commonly identified mycobacterial fish pathogens [1, 4]. In particular, *M. marinum* is found in a wide range of saltwater species [2]. Molecular and phylogenetic analyses have facilitated the worldwide recovery of novel mycobacterial species, strains and isolates, such as *M. shottsii* [9] and *M. pseudoshottsii,* from wild marine fishes [3, 10].

M. pseudoshottsii, a slow-growing, photochromogenic mycobacterium, was initially isolated in 2005 from striped bass [10]. Its biochemical reactions, growth characteristics and mycolic acid profiles resemble those of *M. shottsii*, a nonpigmented mycobacterium that was isolated during the same epizootic outbreak [10]. However, the sequences of the 16S rRNA gene and the gene encoding the 65 kDa heat shock protein (hsp65) revealed that the isolate was unique [9, 10]. Initially, *M. pseudoshottsii* was found only in wild Chesapeake Bay striped bass; however, both the range of host species and the area of disease distribution have expanded to a variety of fishes and locations [13, 15]. In Japan, molecular and genotypic examinations of piscine-related nontuberculous mycobacteria (NTM) are rare. Here, we report on the genotypic analysis of mycobacteria isolated from infected fishes raised on farms in western Japan.

Twenty-four isolates were recovered from moribund yellowtails (*Seriola quinqueradiata*) , greater amberjack (*Seriola dumerili*), striped jack (*Pseudocaranx dentex*), sevenband grouper (*Epinephelus septemfasciatus*), and yellowtail amberjack (*Seriola lalandi*) at fish farms in the western part of Japan from 1999 to 2008 (Table 1). The diseased fish generally showed lethargy, anorexia, emaciation and abdominal distension with ascites. Sometimes, mass culling of the same fish group at a farm was needed because of mass mortality. In some cases, skin ulceration and eye corneal ulceration were observed. White nodules were often found in several internal organs especially in enlarged spleens and kidneys. Isolation was attempted with the affected organ, kidneys, spleen, liver and gills of each fish. These tissues were aseptically dissected, homogenized in phosphate buffered saline, inoculated on 2% Ogawa egg slant (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) or homogenized with 4% NaOH for 10 min and inoculated on 1% Ogawa egg slant (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Incubation was performed at 23 to 25°C for 2 to 3 months. Subculture were performed for colony purification with 2% Ogawa egg slant and/or Middlebrook 7H11 agar supplemented with 10% OADC enrichment (Becton, Dickinson and Company, Fukushima, Japan).

Multigenotypic analysis was used to identify the resulting isolates. One loopful of mycobacterial colonies on Ogawa egg slant or 7H11 agar was suspended in 400 μl sterilized phosphate-buffered saline supplemented with 0.05% Tween 80 and was stored at –80°C until DNA was extracted. A frozen bacterial suspension was crushed in a bead-beating instrument (Magnalizer; Roche Diagnostics Japan, Tokyo, Japan) at 3,000 rpm for 90 sec with zirconia beads (diameter, 2 mm). Total genomic DNA was purified from the crashed suspension using a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics Japan, Tokyo, Japan) and was stored at –20°C.

An approximately 1,500-bp fragment of the 16S rRNA gene, the partial sequences of the *hsp65* and *rpoB* genes and the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region) were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, U.S.A.) with the primers listed in Table 2. The amplicons of the isolates were sequenced using an ABI

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Table 1. Origin of the mycobacterial strains used in this study

Strain	Host fish	Isolation date	Site isolated	Location of fish farm (Prefecture)
MF01	Yellow tail (*Seriola quinqueradiata*)	Oct/19/2004	Kidney	Kagoshima
MF06	Yellow tail (*Seriola quinqueradiata*)	Sep/08/2008	Kidney	Kagoshima
MF09	Yellow tail (*Seriola quinqueradiata*)	Jul /19/2001	Kidney	Oita
MF10	Yellow tail (*Seriola quinqueradiata*)	Oct/31/2001	Kidney	Oita
MF12	Yellow tail (*Seriola quinqueradiata*)	Aug/19/2008	Kidney	Ehime
MF14	Yellow tail (*Seriola quinqueradiata*)	Aug/29/2008	Kidney	Ehime
MF31	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Spleen	Kagoshima
MF32	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF33	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF34	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF35	Yellow tail (*Seriola quinqueradiata*)	Feb/02/2005	Kidney	Kagoshima
MF36	Yellow tail (*Seriola quinqueradiata*)	Feb/02/2005	Kidney	Kagoshima
MF44	Yellow tail (*Seriola quinqueradiata*)	Jul /19/2001	NC[a)]	Oita
MF45	Yellow tail (*Seriola quinqueradiata*)	Oct/01/2001	NC	Oita
MF46	Yellow tail (*Seriola quinqueradiata*)	NC/– /2004	NC	Ehime
MF02	Greater amberjack (*Seriola dumerili*)	Jan/06/2005	Kidney	Kagoshima
MF05	Greater amberjack (*Seriola dumerili*)	Nov/15/2005	Kidney	Kagoshima
MF07	Greater amberjack (*Seriola dumerili*)	NC/– /2006	Miliary nodule	Miyazaki
MF40	Greater amberjack (*Seriola dumerili*)	Jan/05/2004	NC	Kagoshima
MF13	Sevenband grouper (*Epinephelus septemfasciatus*)	Aug/18/2008	Kidney	Ehime
MF15	Sevenband grouper (*Epinephelus septemfasciatus*)	Oct/– /2008	Kidney	Ehime
MF04	Striped jack (*Pseudocaranx dentex*)	Nov/15/2005	Kidney	Kagoshima
MF08	Striped jack (*Pseudocaranx dentex*)	Sep/06/1999	Kidney	Oita
MF11	Yellowtail amberjack (*Seriola lalandi*)	Aug/09/2007	Spleen	Oita

a) Not clear.

Table 2. Primers used in this study

Primer	Sequence (positions)	PCR target (fragment size)	Reference
8F16S	5'-AGAGTTTGATCCTGGCTCAG- 3' (8–27)	16S rRNA gene (app. 1,500 bp)	12
1047R16S	5'-TGCACACAGGCCACAAGGGA- 3' (1,047–1,028)		
830F16S	5'-GTGTGGGTTTCCTTCCTTGG- 3' (830–849)		
1542R16S	5'-AAGGAGGTGATCCAGCCGCA- 3' (1,542–1,523)		
ITSF	5'-TTGTACACACCGCCCGTC- 3' (16S, 1,390–)	16S-23S ITS region (app. 340 bp)	11
ITSR	5'-TCTCGATGCCAAGGCATCCACC- 3' (23S, 44–)		
TB11	5'-ACCAACGATGGTGTGTCCAT- 3' (398–417)	*hsp65* (439bp)	16
TB12	5'-CTTGTCGAACCGCATACCCT- 3' (836–817)		
MF	5'-CGACCACTTCGGCAACCG- 3'	*rpoB* (342 bp)	5
MR	5'-TCGATCGGGCACATCCGG- 3'		

Prism 310 PCR Genetic Analyzer (Applied Biosystems) [6] and compared to the sequences of six strains of mycobacteria: "*M. ulcerans* subsp. *shinshuense*" ATCC33728 [6], *M. ulcerans* ATCC19423 (type strain), *M. ulcerans* Agy99 [14], *M. marinum* ATCC 927 (type strain), *M. marinum* clinical isolate strain 112509 (the preceding 5 strains originated in humans) and *M. pseudoshottsii* JCM15466 (type strain). The JCM strain was distributed by the Microbe Division of the Riken BioResource Center (BRC; Saitama, Japan). Isolate and reference sequences were deposited into the DNA Data Bank of Japan (DDBJ) under accession numbers AB548704 to AB548734 and AB642161 to AB642165.

The sequences of the 1,475-bp fragment of 16S rRNA gene from the piscine isolates showed almost complete identity with the *M. pseudoshottsii* reference strain (99.93–100% identity). Only a single mismatch was found at nucleotide position 487 or 488 in 9 of 24 piscine isolates compared with the DNA sequence of *M. pseudoshottsii* JCM15466. However, conserved mismatches with the 5 strains that originated in humans were found at nucleotide positions 95, 969, 1,007 and 1,215 (Table 3). *M. ulcerans* Agy99 had sum upped 3-base pair insertion (TTT) at nucleotide position 1,449–1,451. Similarly, the ITS regions of the piscine isolates and the *M. pseudoshottsii* reference strain were either identical or differed at position 57, while conserved mismatches with the strains originating in humans were at nucleotide positions 30 and 62. All of the sequences of *hsp65* and *rpoB* gene fragments from the iso-