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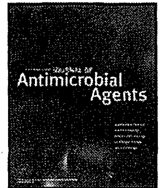

Short communication

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.
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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]						
GyrA	GyrB	*N*[a]	STFX	GFLX	MXF	SPFX	LVX	CIP	*N*[a]	GFLX	MXF	SPFX	LVX	CIP	OFX
Asp94Val		1	0.78	≤0.78	1.56	1.56	6.25	6.25	0						
Asp89Asn		0							0						
Ser91Pro		4	1.56	1.56	3.13	1.56	3.13	6.25	2	0.5	1	1	2	4	4
Ala90Val		12	0.78	≤0.78	1.56	3.13	6.25	6.25	9	1	1	2	4	4	8
Asp94Ala		10	0.78	≤0.78	1.56	3.13	6.25	12.5	1	0.5	1	2	4	4	4
Asp94Tyr		5	0.78	1.56	3.13	3.13	6.25	12.5	0						
Asp94His		0							0						
Asp94Gly		15	3.13	3.13	6.25	6.25	12.5	25	4	1	1	2	4	4	8
Asp94Asn		5	1.56	3.13	6.25	6.25	6.25	25	1	1	1	2	2	4	8
Gly88Cys		1	12.5	3.13	12.5	6.25	12.5	>50	0						
	Asn538Asp	0							0						
	Asn533Thr	0							0						
Ala90Val + Asp94Val		1	3.13	6.25	6.25	12.5	25	25	0						
Ala90Val + Asp94Ala		1	6.25	6.25	12.5	>12.5	>25	>50	0						
Ala90Val + Asp94Asn		0							0						
Ala90Val + Ser91Pro		1	>12.5	>6.25	>12.5	>12.5	>25	>50	0						
Asp94Gly	Ser486Phe	1	1.56	1.56	3.13	3.13	6.25	25	0						
Ala90Val	Thr539Asn	2	0.78	3.13	12.5	12.5	12.5	25	0						
Asp94His	del678–9	0							0						

Amino acid substitution in QRDR		Taiwan (*n* = 6) [9]					Hong Kong (*n* = 31) [6]							Belgium (*n* = 22) [8]			
GyrA	GyrB	*N*[a]	MXF	LVX	CIP	OFX	*N*[a]	STFX	GFLX	MXF	SPFX	LVX	OFX	*N*[a]	GFLX	MXF	OFX
Asp94Val		0					0							0			
Asp89Asn		0					0							1	1	2	4
Ser91Pro		0					4	1	2	1	0.5	2	4	0			
Ala90Val		1	1	4	8	16	5	0.5	1	1	2	4	8	10	0.5	0.5	4
Asp94Ala		0					3	0.5	2	1	2	4	8	3	0.25	0.5	4
Asp94Tyr		0					5	2	2	2	4	8	16	3	1	2	8
Asp94His		0					3	1	4	2	4	8	16	0			
Asp94Gly		4	1	2	4	4	11	2	4	2	4	8	16	4	1	2	8
Asp94Asn		0					1	4	4	2	2	4	8	1	1	4	16
Gly88Cys		0					0							0			
	Asn538Asp	1	1	1	4	8	0							0			
	Asn533Thr	0					0							1	0.5	0.5	1
Ala90Val + Asp94Val		0					0							0			
Ala90Val + Asp94Ala		0					0							0			
Ala90Val + Asp94Asn		0					0							1	>4	>8	>32
Ala90Val + Ser91Pro		0					0							0			
Asp94Gly	Ser486Phe	0					0							0			
Ala90Val	Thr539Asn	0					0							0			
Asp94His	del678–9	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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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-

Table 3. Alignment of the 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences from 24 piscine isolates and 6 reference strains[a)]

Strain	Prefecture/ Country	Nucleotide sequence positions																			
		16S rRNA[b)]								ITS region				*hsp65*[c)]						*rpoB*[d)]	
		95	487–8	492	969	1007	1215	1247	1288	30	57	62	83	455	571	637	639	647	797	92	143
M. shinshuense ATCC 33728	Nagano/Japan	T	GG	G	A	G	T	G	G	G	G	T	A	C	T	C	C	A	T	C	C
M. ulcerans ATCC 19423^T	NC[e)]/ Australia	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C	T	C
M. ulcerans Agy99	NC / Ghana	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C	T	C
M. marinum ATCC 927^T	NC / USA	T	GG	A	A	G	T	A	A	G	G	T	A	C	C	C	T	G	C	C	G
M. marinum 112509	Tokyo/Japan	T	GG	A	A	G	T	A	A	G	G	T	G	C	C	C	T	G	C	C	G
M. pseudoshottsii JCM 15466^T	NC / USA	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF01 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF06 (yellow tail)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF09 (yellow tail)	Oita/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF10 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF12 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF14 (yellow tail)	Ehime/Japan	C	AA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF31 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF32 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF33 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF34 (yellow tail)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF35 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF36 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF44 (yellow tail)	Oita/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF45 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF46 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF02 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF05 (greater amberjack)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF07 (greater amberjack)	Miyazaki /Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF40 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF13 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF15 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF04 (striped jack)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF08 (striped jack)	Oita/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF11 (yellowtail amberjack)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C

a) Only nucleotide differences are noted. Nucleotide positions were based on the b) *E. coli* 16S rRNA gene (accession No. J01859). c) *M. tuberculosis hsp65* gene. (accession No. M15467) and d) *rpoB* gene (accession No. AF057454). e) Not clear.

lates showed complete identity with those of the *M. pseudoshottsii* sequences (Table 3). A conserved mismatch between piscine and human isolates in *hsp65* gene fragments was only found at nucleotide position 637. The results showed that the 24 piscine isolates were all identified as *M. pseudoshottsii* rather than *M. marinum*.

A lethal case of *M. marinum* in cultured yellowtails, which was identified using biological, biochemical and 16S rRNA sequence analyses, has been reported in Japan [17]. In our study, *M. pseudoshottsii* was identified as an additional source of atypical piscine mycobacteriosis and (the bacteria) had been distributed in farmed fisheries in the west part of Japan since 1999. Further studies are needed to develop an easier method to distinguish *M. pseudoshottsii* from *M. marinum* because both strains might have not been differentiated before in Japan. Their differences in susceptibility to antimicrobial agents and in capacity for human pathogenesis should be elucidated. In addition, *M. pseudoshottsii* produces a unique plasmid-encoded toxic macrolide, mycolactone F [7], suggesting that *M. pseudoshottsii* provides a reservoir in aquatic environments for the horizontal transfer of the plasmid-borne genes that encode mycolactone F. Interestingly the potency of mycolactone F with regard to apoptosis in a mammalian cell line was significantly less than that of mycolactone A/B, which is produced by *M. ulcerans*, a causative agent of Buruli ulcer [17]. Further molecular, biochemical and drug susceptibility studies are needed to understand the possible role of mycolactone F in mycobacteriosis and to fully characterize piscine mycobacterial infections in Japan.

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Mutation Analysis of Mycobacterial *rpoB* Genes and Rifampin Resistance Using Recombinant *Mycobacterium smegmatis*

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Rifampin is a major drug used to treat leprosy and tuberculosis. The rifampin resistance of *Mycobacterium leprae* and *Mycobacterium tuberculosis* results from a mutation in the *rpoB* gene, encoding the β subunit of RNA polymerase. A method for the molecular determination of rifampin resistance in these two mycobacteria would be clinically valuable, but the relationship between the mutations and susceptibility to rifampin must be clarified before its use. Analyses of mutations responsible for rifampin resistance using clinical isolates present some limitations. Each clinical isolate has its own genetic variations in some loci other than *rpoB*, which might affect rifampin susceptibility. For this study, we constructed recombinant strains of *Mycobacterium smegmatis* carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without mutation and disrupted their own *rpoB* genes on the chromosome. The rifampin and rifabutin susceptibilities of the recombinant bacteria were measured to examine the influence of the mutations. The results confirmed that several mutations detected in clinical isolates of these two pathogenic mycobacteria can confer rifampin resistance, but they also suggested that some mutations detected in *M. leprae* isolates or rifampin-resistant *M. tuberculosis* isolates are not involved in rifampin resistance.

Leprosy and tuberculosis persist as important global public health concerns. Rifampin, a major drug used to treat these two infectious diseases, has a molecular mechanism of activity involving the inhibition of DNA-dependent RNA polymerase (15). In *Escherichia coli*, this enzyme is a complex oligomer comprised of four subunits, α, β, β′, and σ, encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively. Rifampin binds to the β subunit of RNA polymerase and results in transcription inhibition (15). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, reportedly result in resistance to rifampin in several mycobacterial species, including *Mycobacterium leprae* and *Mycobacterium tuberculosis* (9, 21). The former has not yet been cultured on artificial media; it requires 11 to 14 days to double in experimentally infected mice. Therefore, it is difficult to determine the rifampin susceptibilities of *M. leprae* isolates. The standardized method using a mouse footpad takes more than half a year to determine the rifampin susceptibility of *M. leprae* isolates and requires 5×10^3 *M. leprae* bacilli (3), which require almost a year to prepare. *In vitro* drug susceptibility testing for *M. leprae* using a radioactive reagent requires more (10^7) *M. leprae* cells (7). In contrast, mutations in the *rpoB* gene of *M. leprae* can be detected in a few days or less. It would be very helpful if mutations responsible for rifampin resistance could be determined without performing mouse footpad testing. The main mutations that confer rifampin resistance to *M. tuberculosis* are located in the 81-bp core region of the *rpoB* gene, encompassing codons 507 to 533, known as the rifampin resistance-determining region (RRDR) (17, 18). About 95% of rifampin-resistant *M. tuberculosis* strains have a mutation in this region (18, 20). Four mutations, D516V, H526Y, H526D, and S531L, are most commonly associated with the high-level rifampin resistance of *M. tuberculosis* strains (4, 10, 19), but some other mutations in the 81-bp region have not yet been confirmed completely as being responsible for rifampin resistance.

We have established a method to determine the mutations responsible for the dapsone resistance of *M. leprae* using recombinant *Mycobacterium smegmatis* strains (16). In the present study, we assessed the applicability of the determination of rifampin resistance for analysis. We then analyzed *rpoB* mutations conferring rifampin resistance to *M. leprae* and *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5α was used for DNA cloning. *M. smegmatis* mc²155 was used as a mycobacterial host to produce strains for drug susceptibility testing. Plasmids pYUB854 and phAE87 were kindly provided by W. R. Jacobs, Jr. (Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY). *M. smegmatis* mc²155 and its transformants were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.5% bovine serum albumin (fraction V), 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80.

Site-directed mutagenesis. The wild-type *rpoB* genes of *M. leprae* and *M. tuberculosis* were amplified from *M. leprae* Thai-53 and *M. tuberculosis* H37Rv by PCR and cloned into pMV261. Site-directed mutagenesis was performed by using PCR with DNA polymerase (Takara PrimeStar HS; Takara Bio Inc., Kyoto, Japan) and the primers presented in Table 1. PCR products were purified and phosphorylated with T4 kinase and ATP and were then ligated to make them circular. The ligation mixture was used to transform *E. coli* DH5α cells, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants. The mutated sequences were then confirmed by sequencing. The inserts of the plasmids were also cloned into pNN301 (16). Mutations introduced into the *M. leprae rpoB* or *M. tuberculosis rpoB* gene are listed in Table 2.

Disruption of the *rpoB* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc²155 cells were transformed with plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allel-

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TABLE 1 Primers used for this study

Primer	Sequence[a]	Application
M. smegmatis		
MSRBUF	GCCTTAAGGAGGAGAAGGACGAGGCCAC	*rpoB* disruption, upstream forward
MSRBUR	GCTCTAGACAAGATGCATCCTTCCAGCA	*rpoB* disruption, upstream reverse
MSRBDF	GCAAGCTTTCGCGCAACGAATCCGCGTC	*rpoB* disruption, downstream forward
MSRBDR	GCACTAGTAGCGCACGCAGCTTCTTCTG	*rpoB* disruption, downstream reverse
MSRBF	TGGTCAAGCAGTTCCTCAAC	Detection of *rpoB* disruption, forward
MSRBR	CGTTGTTGACGATGATCTCG	Detection of *rpoB* disruption, reverse
M. leprae		
MLRBWTF	GCGGATCCGTGCTGGAAGGATGCATCTT	Cloning of *M. leprae rpoB*, forward
MLRBWTR	GCGTTAACCTAAGCCAGATCTTCTATGG	Cloning of *M. leprae rpoB*, reverse
MLRBWTF1	CAGTTCATGGATCAGAACAACCCTC	Introduction of point mutation at codons 507 and 508
MLRBWTF2	TGTCGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation at codon 526
MLRBWTF3	TTCGCACTACGGCCGGATGTGCCCG	Introduction of point mutation at codon 547
MLRBWTR1	CGACAGCTGGCTGGTGCCGAAGAAT	Introduction of point mutation at codons 513, 516, and 517
MLRBWTR2	GCCGGCGCTTGTGGGTCAGGCCCGA	Introduction of point mutation at codons 531, 532, and 533
MLRB507GGG	CGACAGCTGGCTGGTCCCGAAGAAT	Introduction of point mutation GGC507→GGG
MLRB507AGC	CGACAGCTGGCTGGTGCTGAAGAAT	Introduction of point mutation GGC507→AGC
MLRB508ACA	CGACAGCTGGCTTGTGCCGAAGAAT	Introduction of point mutation ACC508→ACA
MLRB513GTG	GTGTTCATGGATCAGAACAACCCTC	Introduction of point mutation CAG513→GTG
MLRB516AAT	CAGTTCATGAATCAGAACAACCCTC	Introduction of point mutation GAT516→AAT
MLRB517CAT	CAGTTCATGGATCATAACAACCCTC	Introduction of point mutation CAG517→CAT
MLRB526TAC	GCCGGCGCTTGTAGGTCAGGCCCGA	Introduction of point mutation CAC526→TAC
MLRB531TTG	TGTTGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TTG
MLRB531TGG	TGTGGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TGG
MLRB532TCG	TGTCGTCGCTGGGCCCGGGTGGTTT	Introduction of point mutation GCG532→TCG
MLRB533CCG	TGTCGGCGCCGGGCCCGGGTGGTTT	Introduction of point mutation CTG533→CCG
MLRB547ATC	GGGTGCACGTCACGGATCTCTAGCC	Introduction of point mutation GTC547→ATC
M. tuberculosis		
MTRBWTF	GCGAATTCTTGGCAGATTCCCGCCAGAG	Cloning of *M. tuberculosis rpoB*, forward
MTRBWTR	GCAAGCTTTTACGCAAGATCCTCGACAC	Cloning of *M. tuberculosis rpoB*, reverse
MTRBWTF1	AATTCATGGACCAGAACAACCCGCT	Introduction of point mutation at codons 507, 508, 510, 511, 512, and 513 and deletion of codons 506-508
MTRBWTF2	CTGTCGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation at codons 522, 523, 526, and 531
MTRBWTR1	GGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of mutation at codons 514, 516, 518, 519, and 521; deletion of codon 518; and insertion of TTC between codons 514 and 515
MTRBWTR2	TCGGCGCTTGTGGGTCAACCCCGAC	Introduction of point mutations TCG531→TTC and TCG531→TTG
MTRB507AGC	GGCTCAGCTGGCTGGTGCTGAAGAA	Introduction of point mutation GGC507→AGC
MTRB507GAT	GGCTCAGCTGGCTGGTATCGAAGAA	Introduction of point mutation GGC507→GAT
MTRB508CAC	GGCTCAGCTGGCTGTGGCCGAAGAA	Introduction of point mutation ACC508→CAC
MTRB508GCC	GGCTCAGCTGGCTGGCGCCGAAGAA	Introduction of point mutation ACC508→GCC
MTRB510CAT	GGCTCAGATGGCTGGTGCCGAAGAA	Introduction of point mutation CAG510→CAT
MTRB511CCG	GGCTCGGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CTG511→CCG
MTRB513AAT1	TGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→AAT
MTRB513AAT2	ATTTCATGGACCAGAACAACCCGCT	Introduction of point mutation CAA513→AAT
MTRB513GAA	CGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→GAA
MTRB516GAG	AATTCATGGAGCAGAACAACCCGCT	Introduction of point mutation GAC516→GAG
MTRB516CAC	AATTCATGCACCAGAACAACCCGCT	Introduction of point mutation GAC516→CAC
MTRB516GTC	AATTCATGGTCCAGAACAACCCGCT	Introduction of point mutation GAC516→GTC
MTRB521ATG	AATTCATGGACCAGAACAACCCGAT	Introduction of point mutation CTG521→ATG
MTRB522TTG	TCGGCGCTTGTGGGTCAACCCCAAC	Introduction of point mutation TCG522→TTG
MTRB523GCG	TCGGCGCTTGTGGGTCAACGCCGAC	Introduction of point mutation GGG523→GCG
MTRB523GGC	TCGGCGCTTGTGGGTCAAGCCCGAC	Introduction of point mutation GGG523→GGC
MTRB526CTC	TCGGCGCTTGAGGGTCAACCCCGAC	Introduction of point mutation CAC526→CTC
MTRB526TAC	TCGGCGCTTGTAGGTCAACCCCGAC	Introduction of point mutation CAC526→TAC
MTRB526GAC	TCGGCGCTTGTCGGTCAACCCCGAC	Introduction of point mutation CAC526→GAC
MTRB526TTC	TCGGCGCTTGAAGGTCAACCCCGAC	Introduction of point mutation CAC526→TTC
MTRB526AAC	TCGGCGCTTGTTGGTCAACCCCGAC	Introduction of point mutation CAC526→AAC
MTRB526CGC	TCGGCGCTTGCGGGTCAACCCCGAC	Introduction of point mutation CAC526→CGC
MTRB526CAA	TCGGCGCTTTTGGGTCAACCCCGAC	Introduction of point mutation CAC526→CAA
MTRB529AAA	TTTGCGCTTGTGGGTCAACC	Introduction of point mutation CGA529→AAA
MTRB531TTC	CTGTTCGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTC
MTRB531TTG	CTGTTGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTG
MTRB506d	GGCTCAGCTGGCTGAACTCCTTGAT	Introduction of mutation 506-508del
MTRBin514TTC	AATTCTTCATGGACCAGAACAACCC	Introduction of mutation 514insTTC
MTRBd518	AATTCATGGACCAGAACCCGCTGTC	Introduction of mutation 518del

[a] Restriction sites are underlined.

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TABLE 2 Rifampin and rifabutin susceptibilities of the recombinant *M. smegmatis* strains

	Rifampin		Rifabutin		
Mutation	MIC (μg/ml)	Fold increase[a]	MIC (μg/ml)	Fold increase	Reference(s)
M. leprae					
Wild type	1		0.25		
GGC507→GGG (silent)	1	1	0.25	1	This study
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	3
ACC508→ACA (silent)	1	1	0.25	1	This study
CAG513→GTG (Q513V)	32	32	8	32	3
GAT516→AAT (D516N)	32	32	2	8	14
CAG517→CAT (Q517H)	1	1	0.25	1	11
CAC526→TAC (H526Y)	32	32	8	32	14
TCG531→TTG (S531L)	32	32	4	16	3, 14
TCG531→TGG (S531W)	32	32	8	32	14
GCG532→TCG (A532S)	1	1	0.25	1	11
CTG533→CCG (L533P)	32	32	4	16	14
GTC547→ATC (V547I)	1	1	0.25	1	This study
M. tuberculosis					
Wild type	1		0.25		
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	1
GGC507→GAT (G507D)	0.5	0.5	0.125	0.5	1
ACC508→CAC (T508H)	0.5	0.5	0.125	0.5	1
ACC508→GCC (T508A)	1	1	0.25	1	1
CAG510→CAT (Q510H)	1	1	0.25	1	22
CTG511→CCG (L511P)	16	16	1	4	1, 12
CAA513→AAT (Q513N)	8	8	0.5	2	1
CAA513→GAA (Q513E)	32	32	2	8	1
GAC516→GAG (D516E)	8	8	0.5	2	12
GAC516→CAC (D516H)	1	1	0.25	1	1
GAC516→GTC (D516V)	32	32	2	8	12, 21, 22
CTG521→ATG (L521M)	1	1	0.125	0.5	21
TCG522→TTG (S522L)	>32	>32	8	32	21
GGG523→GCG (G523A)	1	1	0.125	0.5	1
GGG523→GGC (silent)	1	1	0.25	1	1
CAC526→CTC (H526L)	32	32	4	16	12, 22
CAC526→TAC (H526Y)	>32	>32	8	32	12, 22
CAC526→GAC (H526D)	>32	>32	8	32	12, 22
CAC526→TTC (H526F)	>32	>32	4	16	1
CAC526→AAC (H526N)	32	32	2	8	8
CAC526→CGC (H526R)	32	32	8	32	12, 22
CAC526→CAA (H526Q)	8	8	0.5	2	1
CGA529→AAA (R529K)	32	32	4	16	22
TCG531→TTC (S531F)	32	32	4	16	1
TCG531→TTG (S531L)	32	32	8	32	21, 22
506-508del[b]	16	16	0.5	2	5
514insTTC[c]	>32	>32	8	32	12 22
518del[d]	32	32	2	8	22

[a] Fold increase in MIC compared to the wild-type sequence.
[b] Deletion of codons 506 to 508.
[c] Insertion of TTC between codons 514 and 515.
[d] Deletion of codon 518.

ic-exchange mutants were constructed by using a temperature-sensitive mycobacteriophage method described in a previous report (2). Using the *M. smegmatis* mc^{2}155 genome sequence (GenBank accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *rpoB* gene (MSMEG_1367). To disrupt the *rpoB* gene, DNA segments from 1,119 bp upstream through 21 bp downstream of the initiation codon of *M. smegmatis rpoB* and from 39 bp upstream through 941 bp downstream of the termination codon were cloned directionally into the cosmid vector pYUB854, which contains a *res-hyg-res* cassette and a *cos* sequence for lambda phage assembly. The plasmids thus produced were digested with PacI and ligated into PH101 genomic DNA excised from the phage-plasmid hybrid (phasmid) phAE87 by PacI digestion. The ligated DNA was packaged (GigaPackIII Gold packaging extract; Stratagene, La Jolla, CA). The resultant mixture was used for the transduction of *E. coli* STBL2 cells (Life Technologies Inc., Carlsbad, CA) to yield cosmid DNA. After *E. coli* was transduced and the transductants were plated onto hygromycin-containing medium, phasmid DNA was prepared from the pooled antibiotic-resistant transductants and electroporated into *M. smegmatis* mc^{2}155. Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M. smeg-*

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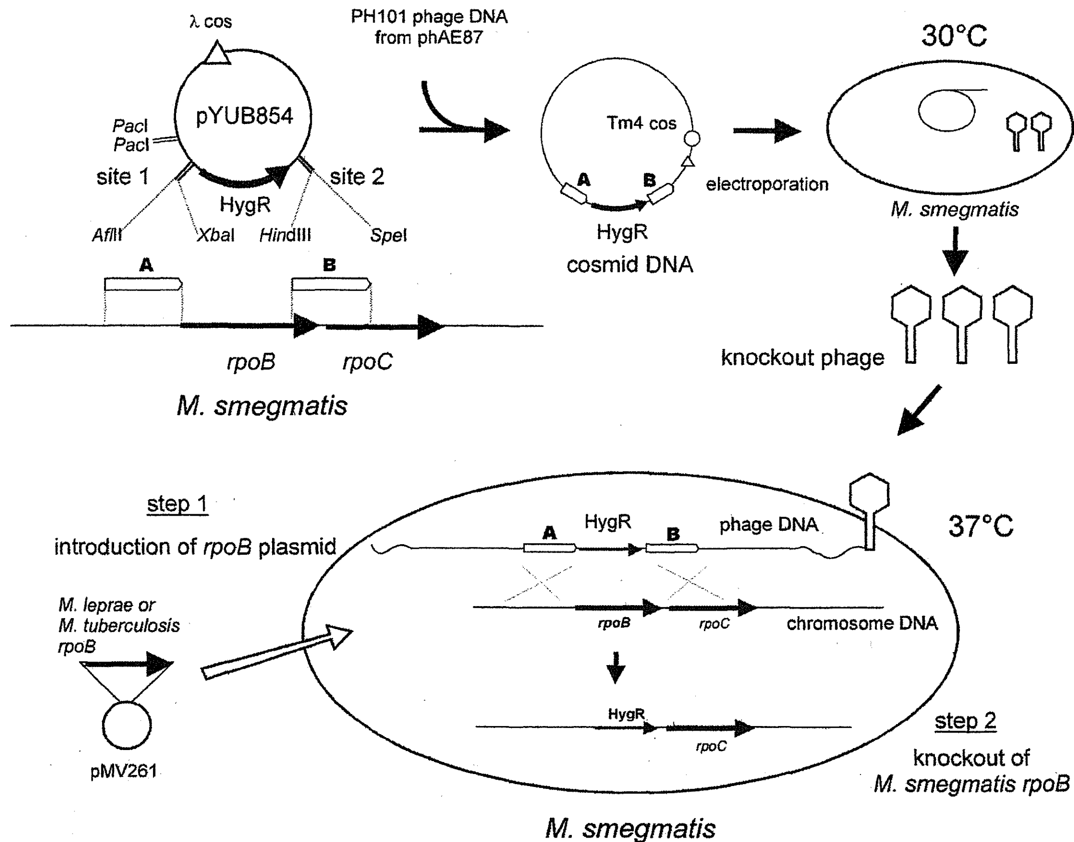

FIG 1 Construction of recombinant *M. smegmatis* strains for rifampin susceptibility testing.

matis transformant carrying the *M. leprae* or *M. tuberculosis rpoB* gene was infected with the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin-resistant and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

Drug susceptibility testing. The MIC values for *M. smegmatis* recombinant clones were determined by culture on Middlebrook 7H10 agar plates containing 2-fold serial dilutions of rifampin (0.25 to 32 μg/ml) or rifabutin (0.0625 to 8 μg/ml). The MIC value for each strain was defined as the lowest concentration of the drug necessary to inhibit bacterial growth.

RESULTS

Construction of recombinant *M. smegmatis* strains. In our previous study, we sequenced the *rpoB* regions of *M. leprae* clinical samples isolated in Vietnam and detected several mutations (11). In addition to these mutations, we detected some mutations (GGC→GGG at codon 507, ACC→ACA at codon 508, and GTC→ATC at codon 547) in clinical specimens from Vietnam and other countries (our unpublished data). We prepared plasmids with mutations in the *M. leprae* and *M. tuberculosis rpoB* genes. Each plasmid has one of 40 mutations (12 for *M. leprae rpoB* and 28 for *M. tuberculosis rpoB*) presented in Table 2. The mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation were introduced individually into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *rpoB* gene on their own chromosome (Fig. 1). The isolation of *rpoB*-disrupted mutants carrying the pNN301-*rpoB* constructs was unsuccessful. Consequently, the recombinant strains with pMV261-*rpoB* constructs were used for subsequent tests. PCR analysis confirmed that the *M. smegmatis rpoB* sequences in the recombinant strains with pMV261-*rpoB* constructs were replaced by hygromycin resistance gene sequences (see Fig. S1 in the supplemental material). All strains showed growth rates comparable to that of wild-type *M. smegmatis.*

Drug susceptibility. The rifampin susceptibilities and rifabutin susceptibilities of the recombinant *M. smegmatis* strains were tested (see Fig. S2 in the supplemental material). The MIC values of rifampin and rifabutin for the recombinant *M. smegmatis* strains and the fold increases in MIC compared to the wild-type sequences are presented in Table 2. It should be noted that the MIC values for the *M. smegmatis* strains might be shifted from those for *M. leprae* or *M. tuberculosis* because of their differences in cell wall permeability and other factors. The MIC value of rifampin for the recombinant *M. smegmatis* strain with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene was 1 μg/ml. Most strains that had a mutation at codon 511, 513, 516, 522, 526, 531, or 533 showed rifampin resistance. In contrast, strains that had a mutation at codon 507, 508, 517, 521, 523, or 532 showed MIC values of rifampin comparable to those for the wild-type sequence. The MIC values of rifabutin for the recombinant

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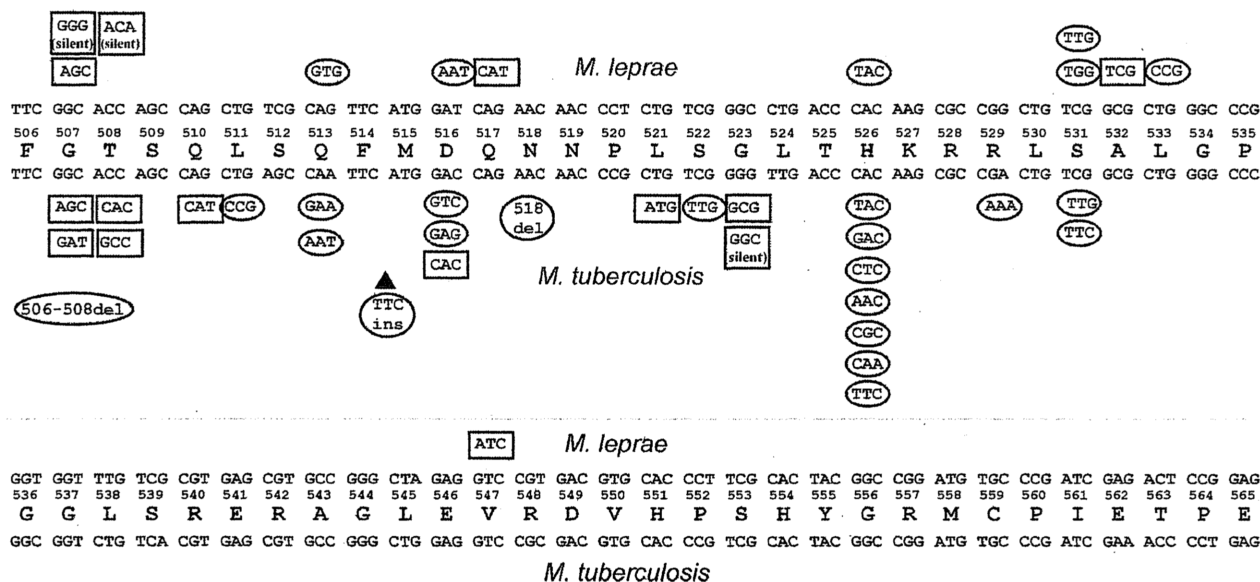

FIG 2 Mutations introduced into the *M. leprae rpoB* gene or *M. tuberculosis rpoB* gene and rifampin susceptibility. The consensus amino acid sequence of *M. leprae* RpoB and *M. tuberculosis* RpoB between codons 506 and 565 is shown. The *M. leprae rpoB* sequence and codons are shown above the consensus amino acid sequence. The *M. tuberculosis rpoB* sequence and codons are shown below the consensus sequence. Mutated codons that gave rise to rifampin resistance are surrounded by ovals. Mutated codons that showed levels of rifampin susceptibility comparable to those of the wild-type sequences are surrounded by rectangles.

M. smegmatis strains with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene were 0.25 μg/ml. Generally, rifabutin was more efficacious than rifampin in terms of concentration.

DISCUSSION

To functionally replace the *rpoB* gene of *M. smegmatis* with the *M. leprae* or *M tuberculosis* counterpart, we used a method established in our previous study (16). Because *rpoB* is a necessary gene for bacterial growth, this genetic locus cannot be disrupted without compensating for its activity. Therefore, we first introduced the *rpoB* gene of *M. leprae* or *M. tuberculosis* into *M. smegmatis* using vector plasmids of two types before disrupting the *rpoB* gene on the *M. smegmatis* chromosome. One vector was pMV261, a multicopy shuttle plasmid. The other was a single-copy integrative shuttle plasmid, pNN301. However, the isolation of *rpoB*-disrupted mutants carrying pNN301-*rpoB* constructs was unsuccessful, probably because of insufficient RpoB expression.

We tested 2 silent mutations and 10 mutations that change amino acid residues for *M. leprae* (Fig. 2). Codons 516, 526, 531, and 533 in the *M. leprae rpoB* gene are known to be codons responsible for rifampin resistance. However, it remains unclear whether or not mutations that have not been reported previously can confer rifampin resistance. Our results show that not all mutations in the *rpoB* gene detected in *M. leprae* clinical samples confer rifampin resistance. *M. leprae* is not cultivable. Therefore, it has been very difficult to analyze the mutation-susceptibility relationship. Using recombinant *M. smegmatis*, however, we can analyze it in a few weeks. We also tested 1 silent mutation, 24 mutations that change amino acids, 2 deletions, and 1 insertion for *M. tuberculosis*. Some mutations did not confer rifampin resistance, which is inconsistent with the susceptibility of the *M. tuberculosis* clinical isolates reported previously. Most mutations at codon 516, 526, or 531 showed rifampin resistance. It is interesting that the strains with the mutation GAC516→CAC for D516H were not rifampin resistant. All other mutations at codon 516 showed rifampin resistance. The mutation GAC516→CAC in *M. tuberculosis* was reported for a strain with multiple mutations and should not be involved in rifampin resistance.

Rifabutin, a spiropiperidyl rifampin, is a rifamycin derivative that is more active than rifampin against slow-growing mycobacteria, including *M. tuberculosis* and *M. avium-M. intracellulare* complex strains, *in vitro* and *in vivo*. It is also active against some rifampin-resistant strains of *M. tuberculosis* (6, 13). Our results indicate that some mutations (e.g., GAT516→AAT of *M. leprae* and GAC516→GAG of *M. tuberculosis*) show weak resistance to rifabutin.

Molecular methods designed to detect drug resistance have some limitations. In some cases, the identified mutations are not related to the acquisition of resistance. Caution is necessary when considering mutations, especially if the mutation detected in clinical isolates is not reported very often. For example, Q510H and L521M mutations were detected in rifampin-resistant *M. tuberculosis* isolates (21, 22), but our results suggest that these mutations are not responsible for rifampin resistance (Table 2). The method used for this study can directly assess the influence of designated mutations in *rpoB*. If the mutations can confer rifampin resistance, we can eliminate the possibility that genetic variation in some region other than *rpoB* on the chromosome of the clinical isolates is responsible for the resistance. Bahrmand et al. previously reported the high-level rifampin resistance of *M. tuberculosis* isolates with multiple mutations within the *rpoB* gene (1). Our method might also be useful for analyzing multiple mutations

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detected in the *rpoB* gene of clinical isolates to determine the contribution of each single mutation to rifampin resistance.

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CASE REPORT

Mycobacterium shigaense sp. nov., a novel slowly growing scotochromogenic mycobacterium that produced nodules in an erythroderma patient with severe cellular immunodeficiency and a history of Hodgkin's disease

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ABSTRACT

A novel slow-growing scotochromogenic mycobacterium was isolated from skin biopsies from a patient with a history of Hodgkin's disease and severe cellular immunodeficiency as an opportunistic pathogen. Clinical characterization of these lesions revealed papules and nodules with pathological granuloma formation. Genotypic analysis using 16S rRNA misidentified this isolate as *Mycobacterium simiae*. However, multiple gene analysis using the internal transcribed spacer between the 16S and 23S rRNA genes, and the *rpoB* and *hsp65* genes revealed the presence of a novel mycobacterium. The antimicrobial susceptibility of this isolate was completely different from that of *M. simiae.* On the basis of these findings, we propose naming this new species *Mycobacterium shigaense* sp. nov., and conclude that multiple gene analysis is required for the appropriate diagnosis and treatment of non-tuberculous mycobacterial infections.

Key words: cellular immunodeficiency, *Mycobacterium shigaense* sp.nov., non-tuberculous mycobacteria, opportunistic infection.

INTRODUCTION

Non-tuberculous mycobacteria (NTM) have been well recognized as causative agents of human diseases. Recently, a number of new species have been added to the NTM. Some of these cause opportunistic infections in immunocompromised patients, not only those with AIDS, but also non-AIDS associated infections. Here, we report an additional new species of mycobacterium that caused cutaneous infection occurring as an opportunistic infection.

CASE REPORT

A 55-year-old Japanese male with a history of treatment of neck-oriented Hodgkin's disease in 2000, presented with erythema accompanied by generalized itching in 2005. The lesion persisted and worsened after treatment with a low dose of oral corticosteroids, resulting in erythroderma and scattered cutaneous nodules on the body trunk in 2007. On physical examination, he presented with itchy erythema over more than 90% of the total body surface (erythroderma) (Fig. 1a); scattered nodules on his chest, back and extremities (Fig. 1b); and multiple papules (Fig. 1c) on his back. He also presented with high-grade fever and slight lymphadenopathy of the neck and axilla. Cytomegalovirus (CMV) retinitis was diagnosed by ophthalmologists and several positive values of CMV antigen were detected at the end stage. His symptoms progressed, and after 2008 he was treated with ganciclovir or valganciclovir. Laboratory tests showed elevated levels of white blood cells ($10.2 \times 10^3/mm^3$; normal range [NR], $3.0–8.0 \times 10^3$) composed of 79.8% segmented neutrophils (NR, 40–74), 3.9% eosinophils (NR, 0–7), 11% lymphocytes (NR, 15–48) and 0% atypical lymphocytes, lactate dehydrogenase (295 IU/L; NR, 100–210), C-reactive protein (1.9 mg/dL; NR, <0.3), immunoglobulin E (188 498 IU/mL; NR, <400) and soluble interleukin-2 receptor (7470 U/mL; NR, 135–483). The platelet counts, liver and renal functions, serum immunoglobulin levels, complement values and angiotensin-converting enzyme were all within normal ranges. Antibodies against human T-lymphotropic virus-1 and HIV-1 were negative. Phenotypic analysis of peripheral lymphocytes revealed an increase in CD3 (95%; NR, 60–78%), T-cell receptors (TCR)-$\alpha\beta$ (94%) and -$\gamma\delta$ (1%), CD4 (93%; NR, 28–47) CD8 (3%; NR, 25–42) and CD19 (1%; NR, 6–16). Southern blot of peripheral lymphocytes revealed no monoclonal band. A tuberculin skin test for purified protein derivative was

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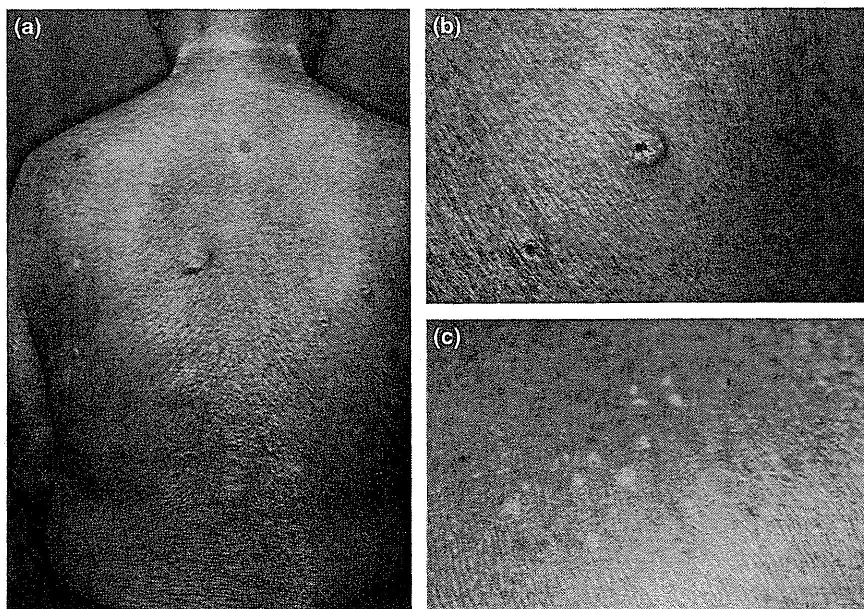

Figure 1. (a) Itchy erythema covered more than 90% of the body surface. (b) Scattered cutaneous nodules on the trunk. (c) Multiple papules on the back.

negative. A systemic investigation using computed tomography, endoscopy and Gallium scintigraphy revealed no abnormalities or internal malignancies, including a recurrence of Hodgkin's disease.

Skin biopsies were taken from the erythema, nodules and papules. A biopsy specimen from the erythema showed only lymphocytic infiltration (primarily CD4 T cells) around superficial dermal vessels (Fig. 2a). The lymphocytes were histologically normal, and Southern blot analysis of the biopsy specimen revealed no monoclonal band. The papules were histologically diagnosed as molluscum contagiosum (MC), because numerous basophilic inclusion bodies were observed in keratinocytes which located in the upper dermis. The nodular lesions showed dense infiltration of histiocytes in the superficial dermis, which formed granulomatous lesions (Fig. 2b). Ziehl–Neelsen staining of repeated biopsy specimens from these nodules showed multiple copies of banded acid-fast bacilli (Fig. 2c).

From these findings, we initially diagnosed an opportunistic mycobacterium infection in a patient of cellular immunodeficiency and administrated 400 mg of oral clarithromycin and 400 mg of isoniazid daily. The nodules improved within a few weeks. Multiple biopsies and histological investigations with Ziehl–Neelsen staining failed to detect any bacilli. The medicines were administrated for 12 months. New lesions of MC sometimes occurred after cessation of drug therapy, but no nodules were found. Oral prednisolone was administrated for the erythroderma. The erythroderma often recurred after healing of the mycobacterial infection, but because none of the skin biopsies from the erythroderma and peripheral blood showed atypical cells, the origin of the erythroderma is unknown.

While oral corticosteroids were effective, a daily low dose was needed to control the erythroderma. In October 2009, he complained of abdominal pain. At the time, he was almost blind due to CMV retinitis. Computed tomography showed a mass in the small

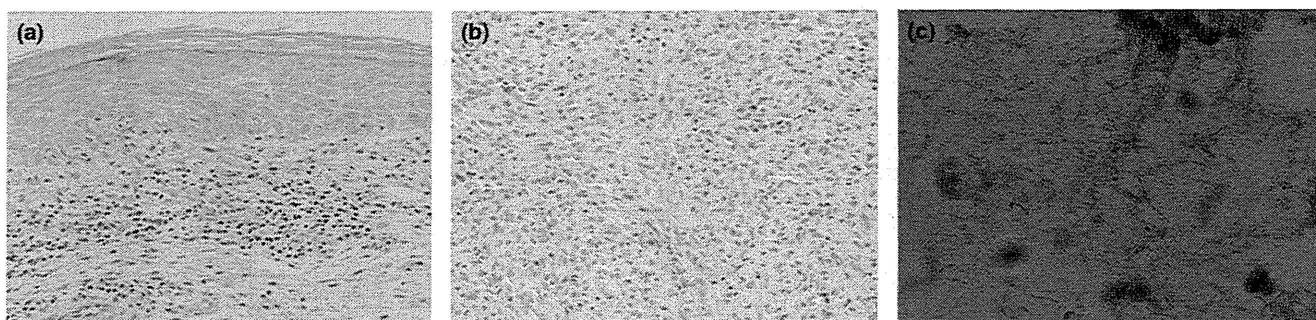

Figure 2. (a) Histological examination of erythema. Lymphocytic infiltration around superficial dermal vessels (hematoxylin–eosin [HE], original magnification ×100). (b) Histological examination of nodular lesions. Dense infiltrated histiocytes formed granulomatous lesions in the dermis (HE, ×200). (c) Ziehl–Neelsen staining of a skin biopsy from a nodule (oil immersion, ×1000).

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intestine and perforation of the gastrointestinal tract. Although a surgical resection of the mass in the small intestine was performed, he died of sepsis in November 2009. An autopsy was not performed. However, a histopathological examination of the mass revealed dense atypical lymphocytic infiltration without Hodgkin's cells, and Southern blot analysis showed a monoclonal band of TCR-αβ cells. Therefore, we concluded that he died not of a recurrence of Hodgkin's disease, but of non-Hodgkin T-cell lymphoma (NHL) with severe immunodeficiency.

A skin biopsy from the nodules confirmed multiple copies of acid-fast bacilli with Ziehl–Neelsen staining, although polymerase chain reaction (PCR) tests targeting *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium leprae* were all negative. The sequencing and genotypic analysis of DNA from the biopsy specimens using the first one-third of the16S rRNA gene showed the highest similarities to *Mycobacterium simiae* (99.54% identity with a 2-bp difference) and *Mycobacterium interjectum* (98.61% identity with a 6-bp difference) when compared with the Ribosomal Differentiation of Micro-organisms (RIDOM) database.[1]

The mycobacterium was isolated from the skin biopsy using the BBL MGIT tube (Becton Dickinson, Franklin Lakes, NJ, USA) and designated *Mycobacterium* sp. UN-152. Phenotypic characteristics were analyzed after sub-culturing on 2% Ogawa egg slant medium (Table 1).[2] The strain was scotochromogenic with an intense yellow color in both light and dark conditions and had a banded appearance after Ziehl–Neelsen staining (Fig. 3), however, usual strains of *M. simiae* are photochromogenic. The strain was slow-growing, had a smooth colonial morphology, and was positive for 3-day arylsulfatase activity, 68°C and semi-quantitative catalase activity

Table 1. Phenotypic differentiation between isolate *Mycobacterium* sp. UN-152 and genotypically similar species of mycobacteria

Characteristics	Isolate UN152 of *Mycobacterium* sp.	*Mycobacterium simiae* ATCC 25275^T	*Mycobacterium interjectum* ATCC 51457^T
Growth† in 7 days	+	+	+
Growth† at:			
25°C	+	+	+
30°C	+	+	+
37°C	+	+	+
42°C	–	+	–
Colony morphology	Smooth	Smooth	Smooth
Colony pigmentation			
In the dark	+	–	+
Photoactivity	–	+	–
Growth† supplemented with:			
PNB (500 μg/mL)	+	+	+
NaCl (5%)	+	+	+
TCH (1 μg/mL)	+	+	+
TCH (10 μg/mL)	+	+	+
Iron uptake	–	–	–
Niacin	–	+	–
Tween-80 hydrolysis (5, 10 days)	–	–	–
Urease	+	+	+
Nitrate reduction	–	–	–
Semi-quantitative catalase	+	+	+
68°C catalase	+	+	+
Arylsulfatase (3 day)	+	–	–
Pyrazinamidase	+	+	+
MPB64 production	–	–	–

†Bacterial growth was examined on 2% Ogawa slants.

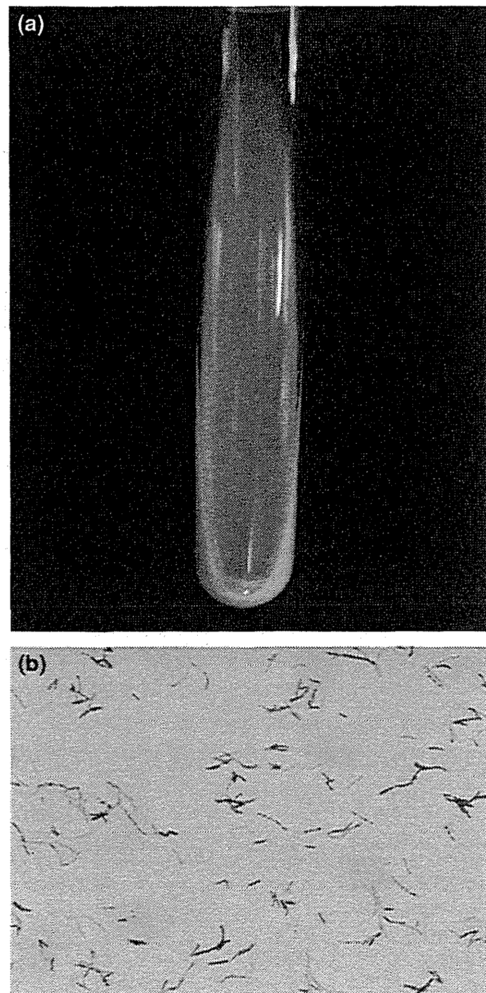

Figure 3. (a) Scotochromogenic colonies of *Mycobacterium* sp. UN-152 sub-cultured on 2% Ogawa egg medium. (b) Ziehl–Neelsen staining of *Mycobacterium* sp. UN-152 sub-cultured on 2% Ogawa egg medium (oil immersion, ×1000).

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and urease activity, but was negative for niacin activity, which suggested that this isolate was phenotypically different from *M. simiae*.

DNA–DNA hybridization to identify the species (DDH Mycobacteria Kyokuto Pharmaceutical Industrial, Tokyo, Japan) produced no matches with any of the 18 mycobacteria species included in the panel with *M. simiae*.[3] Further genotypic analysis was performed in an attempt to identify this isolate. Sequence analysis targeting fragments of the 16S rRNA gene, the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region), and the *rpoB* and *hsp65* genes was performed (Table 2). Amplified PCR products were sequenced using an ABI Prism 310 PCR Genetic Analyzer (Applied Biosystems, Foster City, CA).[8] The sequences of isolate UN-152 were compared to those from the *M. simiae* (ATCC25275^T) type strain and the *M. simiae* clinical isolate 51808 from Japan.[9] We also performed a similarity search using BLAST to find identical and/or closely-related species of mycobacteria.[10] Phylogenetic analyses were performed using the neighbor joining method with Kimura's two-parameter distance correction model with 1000 bootstrap replications in the MEGA version 4.0.2 (Build#: 4028) software package.[11]

The sequence of the first one-third of the 16S rRNA gene from a sub-culture was identical with that from the previously examined skin biopsy. There were only four sites of a point difference between the sequence of UN-152 and that of *M. simiae* (99.7%

Table 2. Primers used in this study

Primer	Sequence	Target (amplified fragment size)	Reference
8F16S	5′-AGAGTTTGATCCTGGCTCAG-3′	16S rRNA gene (~1500 bp)	4
1047R16S	5′-TGCACACAGGCCACAAGGGA-3′		
830F16S	5′-GTGTGGGTTTCCTTCCTTGG-3′		
1542R16S	5′-AAGGAGGTGATCCAGCCGCA-3′		
ITSF	5′-TTGTACACACCGCCCGTC-3′	16S-23S ITS region (~340 bp)	5
ITSR	5′-TCTCGATGCCAAGGCATCCACC-3′		
MF	5′-CGACCACTTCGGCAACCG-3′	*rpoB* gene (351 bp)	6
MR	5′-TCGATCGGGCACATCCGG-3′		
TB11	5′-ACCAACGATGGTGTGTCCAT-3′	*hsp65* gene (441 bp)	7
TB12	5′-CTTGTCGAACCGCATACCCT-3′		

Table 3. DNA sequence similarities between isolate *Mycobacterium* sp. UN-152 and highly similar species of mycobacteria

Species†	% identity 16S rRNA (1471 bp)	ITS (280 bp)	*rpoB* (315 bp)	*hsp65* (401 bp)
Mycobacterium sp. UN-152	100	100	100	100
Mycobacterium simiae ATCC 25275^T	99.7	88.4	90.2	94.0
M. simiae 051808	99.7	88.4	90.8	94.0
Mycobacterium sherrisii ATCC BAA-832^T	99.5	ND	ND‡	93.0
Mycobacterium triplex ATCC 700071^T	99.1	85.7	ND	94.3
Mycobacterium cookii CIP 105396^T	ND	ND	95.9	93.3

†Sequence data (accession number in parenthesis) of three species were taken from database: *M. sherrisii* (AY353699, AY365190), *M. triplex* (U57632, GQ153291, AF334028) and *M. cookii* (AF547824, AY544904). ‡Not determined.

Table 4. Antibiotic susceptibility tests

Antibiotics	Minimal inhibitory concentration (μg/mL) Isolate UN152 of *Mycobacterium* sp.	*Mycobacterium simiae* isolate	*M. simiae* (ATCC 25275T)
Streptomycin (SM)	8	16	4
Ethambutol (EB)	>128	32	44
Kanamycin (KM)	4	8	4
Isoniazid (INH)	>32	32	4
Rifampicin (REF)	0.03	>32	>32
Levofloxacin (LVFX)	0.5	2	1
Clarithromycin (CAM)	1	8	2
Ethionamide (TH)	>16	4	4
Amikacin (AMK)	4	8	4

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