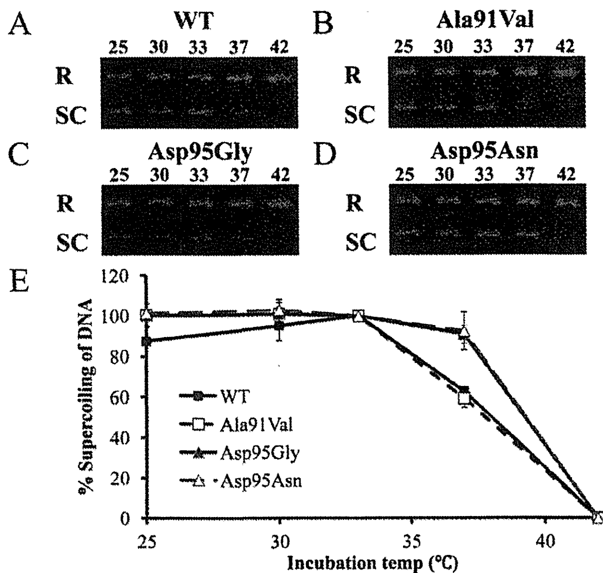

FIG 7 Temperature-dependent DNA supercoiling activity of DNA gyrases. Relaxed pBR322 (0.3 μg) was incubated with WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) at the temperatures (in °C) indicated above the lanes. The proportion of supercoiled DNA compared to that of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

ACKNOWLEDGMENTS

We thank Haruka Suzuki, Yukari Fukushima, and Aiko Ohnuma for their technical support.

This work was supported by grants from the U.S.-Japan Cooperative

Downloaded from http://aac.asm.org/ on January 30, 2012 by NATL INST OF INFECTIOUS DISEAS

Medical Science Program, the Global Center of Excellence Program, the Establishment of International Collaboration Centers for Zoonosis Control, and the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and in part by grants from the Japan Initiative for Global Research Network on Infectious Diseases MEXT to Y.S. and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to Y.S. and C.N.

REFERENCES

1. **An DD, et al.** 2009. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob. Agents Chemother. 53:4835–4839.
2. **Aubry A, et al.** 2006. Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. Antimicrob. Agents Chemother. 50: 104–112.
3. **Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau E.** 2004. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. Antimicrob. Agents Chemother. 48: 1281–1288.
4. **Cambau E, Perani E, Guillemin I, Jamet P, Ji B.** 1997. Multidrug-resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae.* Lancet 349:103–104.
5. **Cambau E, Carthagena L, Chauffour A, Ji B, Jarlierbl V.** 2006. Dihydropteroate synthase mutations in the folP1 gene predict dapsone resistance in replaced cases of leprosy. Clin. Infect. Dis. 42:238–241.
6. **Cambau E, et al.** 2002. Molecular detection of rifampin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. Clin. Infect. Dis. 34:39–45.
7. **Campbell PJ, et al.** 2011. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis.* Antimicrob. Agents Chemother. 55:2032–2041.
8. **Champoux JJ.** 2001. DNA topoisomerases: structure, function, and mechanism. Annu. Rev. Biochem. 70:369–413.
9. **Cui Z, Wang J, Lu J, Huang X, Hu Z.** 2011. Association of mutation patterns in gyrA/B genes and ofloxacin resistance levels in *Mycobacterium tuberculosis* isolates from east China in 2009. BMC Infect. Dis. 11:78–82.
10. **Feuerriegel S, et al.** 2009. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob. Agents Chemother. 53:3353–3356.
11. **Goto M, et al.** 2006. Guideline for the treatment of Hansen's disease in Japan (second edition), Japanese leprosy. Nihon Hansenbyo Gakkai Zasshi 75:191–226. (In Japanese.)
12. **Honore N, Cole ST.** 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae.* Antimicrob. Agents Chemother. 37:414–418.
13. **Hooper DC.** 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin. Infect. Dis. 31:S24–S28.
14. **Ichihara N.** 1984. Phase I study on DL-8280 (OFX). Chemotherapy 32: 118–149.
15. **Kai M, et al.** 1999. Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. FEMS Microbiol. Lett. 177:231–235.
16. **Kim H, et al.** 2011. Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. Antimicrob. Agents Chemother. 55:3661–3667.
17. **Kyaw UK, Aye DKS.** 2006. A case of multi-drug resistant leprogy-relapse or re-infection, Myanmar J. Curr. Med. Pract. 10:41–43.
18. **Levy L, Ji B.** 2006. The mouse foot-pad technique for cultivation of *Mycobacterium leprae.* Lepr. Rev. 77:5–24.
19. **Maeda S, et al.** 2001. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob. Agents Chemother. 45:3635–3639.
20. **Matrat S, Cambau E, Jarlier V, Aubry A.** 2008. Are all the DNA gyrase mutations found in *Mycobacterium leprae* clinical strains involved in resistance to fluoroquinolones? Antimicrob. Agents Chemother. 52: 745–747.
21. **Matrat S, et al.** 2007. Expression and purification of an active form of the *Mycobacterium leprae* DNA gyrase and its inhibition by quinolones. Antimicrob. Agents Chemother. 51:1643–1648.
22. **Matsuoka M.** 2010. The history of *Mycobacterium leprae* Thai-53 strain. Lepr. Rev. 81:137.
23. **Matsuoka M, et al.** 2007. The frequency of drug resistance mutations in Mycobacterium leprae isolates in untreated and replaced leprosy patients from Myanmar, Indonesia and the Philippines. Lepr. Rev. 78:343–352.
24. **Matsuoka M, Kashiwabara Y, Namisato M.** 2000. A Mycobacterium leprae isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. Int. J. Lepr. Other Mycobact. Dis. 68:452–455.
25. **Matsuoka M, Kashiwabara Y, Liangfen Z, Goto M, Kitajima S.** 2003. A second case of multidrug resistant Mycobacterium leprae isolated from a Japanese patient with relapsed lepromatous leprosy. Int. J. Lepr. Other Mycobact. Dis. 71:240–243.
26. **Matsuoka M, et al.** 2010. Possible mode of emerging drug resistant leprosy cases revealed in Mexican samples' analysis. Jpn. J. Infect. Dis. 63: 412–416.
27. **Nakashima M, et al.** 1995. Pharmacokinetics and tolerance of DU-6859a, a new fluoroquinolone, after single and multiple oral doses in healthy volunteers. Antimicrob. Agents Chemother. 39:170–174.
28. **Nakashima M, et al.** 1995. Single- and multiple-dose pharmacokinetics of AM-1155, a new 6-fluoro-8-methoxy quinolone, in humans. Antimicrob. Agents Chemother. 39:2635–2640.
29. **Norman G, Joseph G, Ebenezer G, Rao PSSS, Job CK.** 2003. Secondary rifampin resistance following multi-drug therapy–a case report. Int. J. Lepr. Other Mycobact. Dis. 71:18–21.
30. **Ohnishi N, et al.** 2005. Safety, pharmacokinetics and influence on the intestinal flora of BAY 12-8039 (moxifloxacin hydrochloride) after oral administration in healthy male subjects. Jpn. Pharmacol. Ther. 33: 1029–1045.
31. **Pan XS, Yague G, Fisher LM.** 2001. Quinolone resistance mutations in *Streptococcus pneumoniae* GyrA and ParC proteins: mechanistic insights into quinolone action from enzymatic analysis, intracellular levels, and phenotypes of wild-type and mutant proteins. Antimicrob. Agents Chemother. 45:3140–3147.
32. **Ramaswamy S, Musser JM.** 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis 1998 update. Tuber. Lung Dis. 79:3–29.
33. **Scollard DM, et al.** 2006. The continuing challenges of leprosy. Clin. Microbiol. Rev. 19:338–381.
34. **Sun Z, et al.** 2008. Comparison of *gyrA* gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. Int. J. Antimicrob. Agents 31:115–121.
35. **Williams DL, Spring L, Harris E, Roche P, Gillis TP.** 2000. Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. Antimicrob. Agents Chemother. 44:1530–1537.
36. **World Health Organization.** 2004. Multidrug therapy against leprosy. Report WHO/CDS/CPE/CEE/2004.46. World Health Organization, Geneva, Switzerland.
37. **World Health Organization.** 2011. Leprosy update, 2011. Wkly. Epidemiol. Rec. 86:389–399.
38. Reference deleted.
39. **Yin X, Yu Z.** 2010. Mutation characterization of gyrA and gyrB genes in levofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates from Guangdong province in China. J. Infect. 61:150–154.
40. **You E-Y, Kang TJ, Kim S-K, Lee S-B, Chae G-T.** 2010. Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. J. Infect. 50:6–11.

Downloaded from http://aac.asm.org/ on January 30, 2012 by NATL INST OF INFECTIOUS DISEAS

AAC Accepts, published online ahead of print on 17 January 2012
Antimicrob. Agents Chemother. doi:10.1128/AAC.05831-11
Copyright © 2012, American Society for Microbiology. All Rights Reserved.

AAC Accepts published online ahead of print

1 Mutation Analysis of Mycobacterial *rpoB* Genes and Rifampicin Resistance Using
2 Recombinant *Mycobacterium smegmatis*
3
4 Noboru Nakata*, Masanori Kai, and Masahiko Makino
5
6 Department of Mycobacteriology, Leprosy Research Center, National Institute of
7 Infectious Diseases, Tokyo, Japan
8
9 Running title, Mycobacterial *rpoB* Genes and Rifampicin Resistance
10
11 *Corresponding author. Mailing address: Department of Mycobacteriology, Leprosy
12 Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho,
13 Higashimurayama, Tokyo 189-0002, Japan. Phone: 81 (42) 391 8211; fax: 81 (42) 394
14 9092; E-mail: n-nakata@nih.go.jp
15
16

AAC Accepts published online ahead of print

17 **ABSTRACT**

18 Rifampicin is a major drug used to treat leprosy and tuberculosis. Rifampicin resistance
19 of *Mycobacterium leprae* and *Mycobacterium tuberculosis* results from mutation in the
20 *rpoB* encoding the β subunit of RNA polymerase. Molecular diagnosis for rifampicin
21 resistance in these two mycobacteria would be clinically valuable, but the relation
22 between the mutations and susceptibility to rifampicin must be clarified before its use.
23 Analysis of responsible mutations for rifampicin resistance using clinical isolates
24 presents some limitations. Each clinical isolate has its own genetic variations in some
25 loci other than *rpoB*, which might affect rifampicin susceptibility. For this study, we
26 constructed recombinant strains of *Mycobacterium smegmatis*, carrying the *M. leprae or*
27 *M. tuberculosis rpoB* with or without mutation, and disrupting their own *rpoB* on the
28 chromosome. Rifampicin and rifabutin susceptibilities of the recombinant bacteria were
29 measured to examine the influence of the mutations. Results confirmed that several
30 mutations detected in clinical isolates of these two pathogenic mycobacteria can confer
31 rifampicin resistance, but they also suggested that some mutations detected in *M. leprae*
32 isolates or rifampicin-resistant *M. tuberculosis* isolates are not involved in rifampicin
33 resistance.

34

AAC Accepts published online ahead of print

35 **INTRODUCTION**

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

AAC Accepts published online ahead of print

59 19), but some other mutations in the 81 bp region have not yet been confirmed
60 completely as responsible for rifampicin resistance.

61 We have established a method to determine the mutations responsible for dapsone
62 resistance of *M. leprae* using recombinant *Mycobacterium smegmatis* (16). In the
63 present study, we assessed the applicability of rifampicin resistance to analysis. Then we
64 analyzed *rpoB* mutations conferring rifampicin resistance of *M. leprae* and *M.*
65 *tuberculosis*.

AAC Accepts published online ahead of print

66 MATERIALS AND METHODS

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

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

86 **Disruption of the *rpoB* gene on the *M. smegmatis* chromosome.** *M. smegmatis*
87 mc²155 cells were transformed with plasmids carrying the *M. leprae* or *M. tuberculosis*
88 *rpoB* with or without a point mutation. Recombinants were selected on LB medium
89 containing kanamycin. Allelic exchange mutants were constructed using the

AAC Accepts published online ahead of print

90 temperature-sensitive mycobacteriophage method as described in an earlier report (2).
91 Using the *M. smegmatis* mc^{2}155 genome sequence (accession number CP000480), the
92 upstream and downstream flanking DNA sequences were used to generate a deletion
93 mutation in the *rpoB* gene (MSMEG_1367). To disrupt the *rpoB* gene, DNA segments
94 from 1119 bp upstream through 21 bp downstream of the initiation codon of *M.*
95 *smegmatis rpoB* and from 39 bp upstream through 941 bp downstream of the
96 termination codon were cloned directionally into the cosmid vector pYUB854, which
97 contains a res-hyg-res cassette and a cos sequence for lambda phage assembly. Plasmids
98 thus produced were digested with *Pac*I and ligated to the PH101 genomic DNA excised
99 from the phasmid phAE87 by *Pac*I digestion. The ligated DNA was packaged
100 (GigaPackIII Gold Packaging Extract; Stratagene, La Jolla, CA). The resultant mixture
101 was used for transduction of *E. coli* STBL2 (Life Technologies Inc., Carlsbad, CA) to
102 yield cosmid DNA. After *E. coli* was transduced and the transductants were plated on
103 hygromycin-containing medium, phasmid DNA was prepared from the pooled
104 antibiotic-resistant transductants and electroporated into *M. smegmatis* mc^{2}155.
105 Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M.*
106 *smegmatis* transformant carrying the *M. leprae* or *M. tuberculosis rpoB* gene was
107 infected by the produced temperature-sensitive phage at 37°C for allelic exchange, and
108 kanamycin-resistant and hygromycin-resistant colonies were isolated. Two colonies for
109 each point mutation were subjected to subsequent tests.

110 **Drug susceptibility testing**. The MIC values for *M. smegmatis* recombinant
111 clones were determined by culture on Middlebrook 7H10 agar plates containing
112 two-fold serial dilutions of rifampicin (0.25–32 μg/ml) or rifabutin (0.0625–8 μg/ml).
113 The MIC value for each strain was defined as the lowest concentration of the drug

AAC Accepts published online ahead of print

114 necessary to inhibit bacterial growth.

115

AAC Accepts published online ahead of print

116 **RESULTS**

117 **Construction of recombinant *M. smegmatis* strains.** In our previous study, we
118 sequenced the *rpoB* regions of *M. leprae* clinical samples isolated in Vietnam and
119 detected several mutations (11). In addition to these mutations, we detected some
120 mutations (GGC→GGG at codon 507, ACC→ACA at codon 508, and GGC→GTC at
121 codon 547) in clinical specimens from Vietnam and other countries (unpublished data).
122 We prepared plasmids with mutations in the *M. leprae* and *M. tuberculosis rpoB* genes.
123 Each plasmid has one of 40 mutations (12 for *M. leprae rpoB* and 28 for *M. tuberculosis*
124 *rpoB*) presented in Table 2. Mutated sequences were confirmed by sequencing. Plasmids
125 carrying the *M. leprae* or *M. tuberculosis rpoB* with or without a point mutation were
126 introduced individually into *M. smegmatis*. The *M. smegmatis* transformants were
127 subjected to allelic exchange to disrupt the *rpoB* gene on their own chromosome (Fig. 1).
128 Isolation of *rpoB*-disrupted mutants carrying the pNN301-*rpoB* constructs was
129 unsuccessful. Consequently, the recombinant strains with pMV261-*rpoB* constructs
130 were used for subsequent tests. PCR analysis confirmed that the *M. smegmatis rpoB*
131 sequences in the recombinant strains with pMV261-*rpoB* constructs were replaced by
132 hygromycin resistance gene sequences (data not shown). All strains showed comparable
133 growth rates to that of the wild type *M. smegmatis*.

134 **Drug susceptibility**. Rifampicin susceptibilities and rifabutin susceptibilities of the
135 recombinant *M. smegmatis* strains were tested. The MIC values of rifampicin and
136 rifabutin for the recombinant *M. smegmatis* strains and the fold increases in MIC
137 compared to the wild type sequences are presented in Table 2. It should be noted that the
138 MIC values for the *M. smegmatis* strains might be shifted from those for *M. leprae* or *M.*
139 *tuberculosis* because of their differences in cell wall permeability and other factors. The

AAC Accepts published online ahead of print

140 MIC value of rifampicin for the recombinant *M. smegmatis* with the wild type sequence
141 of the *M. leprae rpoB* or *M. tuberculosis rpoB* was 1 μg/ml. Most strains that have a
142 mutation at codon 511, 513, 516, 522, 526, 531, or 533 showed rifampicin resistance. In
143 contrast, strains that have a mutation at codon 507, 508, 517, 521, 523, or 532 showed
144 comparable levels of MIC value of rifampicin to those of the wild type sequence. The
145 MIC values of rifabutin for the recombinant *M. smegmatis* strains with the wild type
146 sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* were 0.25 μg/ml. Generally,
147 rifabutin was more efficacious than rifampicin in terms of concentration.
148

AAC Accepts published online ahead of print

149 **DISCUSSION**

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

160 We tested 2 silent mutations and 10 mutations that change amino-acid residues for
161 *M. leprae*. Codons 516, 526, 531, and 533 in the *M. leprae rpoB* are known as
162 responsible codons for rifampicin resistance. However, it remains unclear whether or
163 not mutations that have not been reported previously can confer rifampicin resistance.
164 Our results show that not all mutations in the *rpoB* gene detected in *M. leprae* clinical
165 samples confer rifampicin resistance. *M. leprae* is not cultivable. Therefore, it has been
166 very difficult to analyze the mutation-susceptibility relation. Using recombinant *M.*
167 *smegmatis*, however, we can analyze it in a few weeks. We also tested 1 silent mutation
168 and 24 mutations that change amino acids, 2 deletions, and 1 insertion for *M.*
169 *tuberculosis*. Some mutations did not confer rifampicin resistance, which is inconsistent
170 with susceptibility of the *M. tuberculosis* clinical isolates reported previously. Most
171 mutations at codon 516, 526, or 531 showed rifampicin resistance. It is interesting that
172 the strains with mutation GAC516→CAC for D516H were not rifampicin resistant. All

AAC Accepts published online ahead of print

173 other mutations at codon 516 showed rifampicin resistance. Mutation GAC516→CAC
174 in *M. tuberculosis* was reported in a strain with multiple mutations and should not be
175 involved in rifampicin resistance.

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

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

196

AAC Accepts published online ahead of print

197 **ACKNOWLEDGMENTS**

198 This work was supported by grants from the Ministry of Health, Labor and Welfare

199 (Emerging and Re-Emerging Infectious Diseases).

200

AAC Accepts published online ahead of print

201 REFERENCES

202 1. **Bahrmand, A. R., L. P. Titov, A. H. Tasbiti, S. Yari, and E. A. Graviss.** 2009.
203 High-level rifampin resistance correlates with multiple mutations in the rpoB
204 gene of pulmonary tuberculosis isolates from the Afghanistan border of Iran. J
205 Clin Microbiol **47:**2744-50.

206 2. **Bardarov, S., S. Bardarov Jr, M. S. Pavelka Jr, V. Sambandamurthy, M.**
207 **Larsen, J. Tufariello, J. Chan, G. Hatfull, and W. R. Jacobs Jr.** 2002.
208 Specialized transduction: an efficient method for generating marked and
209 unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis*
210 BCG and *M. smegmatis*. Microbiology **148:**3007-3017.

211 3. **Cambau, E., P. Bonnafous, E. Perani, W. Sougakoff, B. Ji, and V. Jarlier.**
212 2002. Molecular detection of rifampin and ofloxacin resistance for patients who
213 experience relapse of multibacillary leprosy. Clin Infect Dis **34:**39-45.

214 4. **Cavusoglu, C., A. Turhan, P. Akinci, and I. Soyler.** 2006. Evaluation of the
215 Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance
216 in *Mycobacterium tuberculosis* isolates. J Clin Microbiol **44:**2338-42.

217 5. **Chikamatsu, K., K. Mizuno, H. Yamada, and S. Mitarai.** 2009.
218 [Cross-resistance between rifampicin and rifabutin among multi-drug resistant
219 *Mycobacterium tuberculosis* strains]. Kekkaku **84:**631-3.

220 6. **Dickinson, J. M., and D. A. Mitchison.** 1987. In vitro activity of new
221 rifamycins against rifampicin-resistant M. tuberculosis and MAIS-complex
222 mycobacteria. Tubercle **68:**177-82.

223 7. **Franzblau, S. G., and R. C. Hastings.** 1988. In vitro and in vivo activities of
224 macrolides against Mycobacterium leprae. Antimicrob Agents Chemother

AAC Accepts published online ahead of print

225 **32:**1758-62.

226 8. **Hauck, Y., M. Fabre, G. Vergnaud, C. Soler, and C. Pourcel.** 2009.
227 Comparison of two commercial assays for the characterization of rpoB
228 mutations in *Mycobacterium tuberculosis* and description of new mutations
229 conferring weak resistance to rifampicin. J Antimicrob Chemother **64:**259-62.

230 9. **Honore, N., and S. T. Cole.** 1993. Molecular basis of rifampin resistance in
231 *Mycobacterium leprae*. Antimicrob Agents Chemother **37:**414-8.

232 10. **Huitric, E., J. Werngren, P. Jureen, and S. Hoffner.** 2006. Resistance levels
233 and rpoB gene mutations among in vitro-selected rifampin-resistant
234 *Mycobacterium tuberculosis* mutants. Antimicrob Agents Chemother **50:**2860-2.

235 11. **Kai, M., N. H. Nguyen Phuc, H. A. Nguyen, T. H. Pham, K. H. Nguyen, Y.**
236 **Miyamoto, Y. Maeda, Y. Fukutomi, N. Nakata, M. Matsuoka, M. Makino,**
237 **and T. T. Nguyen.** 2011. Analysis of drug-resistant strains of *Mycobacterium*
238 *leprae* in an endemic area of Vietnam. Clin Infect Dis **52:**e127-32.

239 12. **Kapur, V., L. L. Li, S. Iordanescu, M. R. Hamrick, A. Wanger, B. N.**
240 **Kreiswirth, and J. M. Musser.** 1994. Characterization by automated DNA
241 sequencing of mutations in the gene (rpoB) encoding the RNA polymerase beta
242 subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York
243 City and Texas. J Clin Microbiol **32:**1095-8.

244 13. **Luna-Herrera, J., M. V. Reddy, and P. R. Gangadharam.** 1995. In-vitro and
245 intracellular activity of rifabutin on drug-susceptible and multiple drug-resistant
246 (MDR) tubercle bacilli. J Antimicrob Chemother **36:**355-63.

247 14. **Maeda, S., M. Matsuoka, N. Nakata, M. Kai, Y. Maeda, K. Hashimoto, H.**
248 **Kimura, K. Kobayashi, and Y. Kashiwabara.** 2001. Multidrug resistant

AAC Accepts published online ahead of print

249 *Mycobacterium leprae* from patients with leprosy. Antimicrob Agents
250 Chemother **45:**3635-3639.

251 15. **McClure, W. R., and C. L. Cech.** 1978. On the mechanism of rifampicin
252 inhibition of RNA synthesis. J Biol Chem **253:**8949-56.

253 16. **Nakata, N., M. Kai, and M. Makino.** 2011. Mutation analysis of the
254 *Mycobacterium leprae* folP1 gene and dapsone resistance. Antimicrob Agents
255 Chemother **55:**762-6.

256 17. **Ramaswamy, S., and J. M. Musser.** 1998. Molecular genetic basis of
257 antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update.
258 Tuber Lung Dis **79:**3-29.

259 18. **Rattan, A., A. Kalia, and N. Ahmad.** 1998. Multidrug-resistant *Mycobacterium*
260 *tuberculosis*: molecular perspectives. Emerg Infect Dis **4:**195-209.

261 19. **Rigouts, L., O. Nolasco, P. de Rijk, E. Nduwamahoro, A. Van Deun, A.**
262 **Ramsay, J. Arevalo, and F. Portaels.** 2007. Newly developed primers for
263 comprehensive amplification of the rpoB gene and detection of rifampin
264 resistance in *Mycobacterium tuberculosis*. J Clin Microbiol **45:**252-4.

265 20. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L.**
266 **Matter, K. Schopfer, and T. Bodmer.** 1993. Detection of rifampicin-resistance
267 mutations in *Mycobacterium tuberculosis*. Lancet **341:**647-50.

268 21. **Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M.**
269 **Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis.** 1994.
270 Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob
271 Agents Chemother **38:**2380-6.

272 22. **Yang, B., H. Koga, H. Ohno, K. Ogawa, M. Fukuda, Y. Hirakata, S.**

AAC Accepts published online ahead of print

273 **Maesaki, K. Tomono, T. Tashiro, and S. Kohno.** 1998. Relationship between
274 antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and *rpoB*
275 mutations of *Mycobacterium tuberculosis*. J Antimicrob Chemother **42:**621-8.
276
277
278

AAC Accepts published online ahead of print

279 **FIGURE LEGENDS**

280

281 Figure 1. Construction of recombinant *M. smegmatis* strains for rifampicin
282 susceptibility testing.

283

284 Figure 2. Mutations introduced into the *M. leprae rpoB* gene or *M. tuberculosis rpoB*
285 gene and rifampicin susceptibility. The consensus amino acid sequence of the *M. leprae*
286 RpoB and *M. tuberculosis* RpoB between codons 506 and 565 is shown. The *M. leprae*
287 *rpoB* sequence and codons are shown above the consensus amino acid sequence. The *M.*
288 *tuberculosis rpoB* sequence and codons are shown below the consensus sequence.
289 Mutated codons that gave rise to rifampicin resistance are surrounded by ovals. Mutated
290 codons that showed comparable levels of rifampicin susceptibility to those of the wild
291 type sequences are surrounded by rectangles.

AAC Accepts published online ahead of print

292 Table 1 Primers used for this study

	Primer	Sequence[a]	Application
293	**for *M. smegmatis***		
294	MSRBUF	GCCTTAAGGAGGAGAAGGACGAGGCCAC	*rpoB* disruption, upstream forward
295	MSRBUR	GCTCTAGACAAGATGCATCCTTCCAGCA	*rpoB* disruption, upstream reverse
296	MSRBDF	GCAAGCTTTCGCGCAACGAATCCGCGTC	*rpoB* disruption, downstream forward
297	MSRBDR	GCACTAGTAGCGCACGCAGCTTCTTCTG	*rpoB* disruption, downstream reverse
298	MSRBF	TGGTCAAGCAGTTCCTCAAC	detection of *rpoB* disruption, forward
299	MSRBR	CGTTGTTGACGATGATCTCG	detection of *rpoB* disruption, reverse
300			
301	**for *M. leprae***		
302	MLRBWTF	GCGGATCCGTGCTGGAAGGATGCATCTT	cloning of *M. leprae rpoB*, forward
303	MLRBWTR	GCGTTAACCTAAGCCAGATCTTCTATGG	cloning of *M. leprae rpoB*, reverse
304	MLRBWTF1	CAGTTCATGGATCAGAACAACCCTC	introduction of point mutation at codons 507 and 508
305	MLRBWTF2	TGTCGGCGCTGGGCCCGGGTGGTTT	introduction of point mutation at codon 526
306	MLRBWTF3	TTCGCACTACGGCCGGATGTGCCCG	introduction of point mutation at cidib 547
307	MLRBWTR1	CGACAGCTGGCTGGTGCCGAAGAAT	introduction of point mutation at codons 513, 516, and 517
308	MLRBWTR2	GCCGGCGCTTGTGGGTCAGGCCCGA	introduction of point mutation at codons 531, 532, and 533
309	MLRB507GGG	CGACAGCTGGCTGGTCCCGAAGAAT	introduction of point mutation GGC507→GGG
310	MLRB507AGC	CGACAGCTGGCTGGTGCTGAAGAAT	introduction of point mutation GGC507→AGC
311	MLRB508ACA	CGACAGCTGGCTTGTGCCGAAGAAT	introduction of point mutation ACC508→ACA
312	MLRB513GTG	GTGTTCATGGATCAGAACAACCCTC	introduction of point mutation CAG513→GTG
313	MLRB516AAT	CAGTTCATGAATCAGAACAACCCTC	introduction of point mutation GAT516→AAT
314	MLRB517CAT	CAGTTCATGGATCATAACAACCCTC	introduction of point mutation CAG517→CAT
315	MLRB526TAC	GCCGGCGCTTGTAGGTCAGGCCCGA	introduction of point mutation CAC526→TAC
316	MLRB531TTG	TGTTGGCGCTGGGCCCGGGTGGTTT	introduction of point mutation TCG531→TTG
317	MLRB531TGG	TGTGGGCGCTGGGCCCGGGTGGTTT	introduction of point mutation TCG531→TGG
318	MLRB532TCG	TGTCGTCGCTGGGCCCGGGTGGTTT	introduction of point mutation GCG532→TCG
319	MLRB533CCG	TGTCGGCGCCGGGCCCGGGTGGTTT	introduction of point mutation CTG533→CCG
320	MLRB547ATC	GGGTGCACGTCACGGATCTCTAGCC	introduction of point mutation GTC547→ATC
321			
322	**for *M. tuberculosis***		
323	MTRBWTF	GCGAATTCTTGGCAGATTCCCGCCAGAG	cloning of *M. tuberculosis rpoB*, forward
324	MTRBWTR	GCAAGCTTTTACGCAAGATCCTCGACAC	cloning of *M. tuberculosis rpoB*, reverse

[a] Restriction sites are underlined

325