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

Noboru Nakata, Masanori Kai, and Masahiko Makino

Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

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* mc^{2}155 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* mc^{2}155 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* mc^{2}155 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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Address correspondence to Noboru Nakata, n-nakata@nih.go.jp.
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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.

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

Mutation	Rifampin		Rifabutin		Reference(s)
	MIC (μg/ml)	Fold increase[a]	MIC (μg/ml)	Fold increase	
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-*

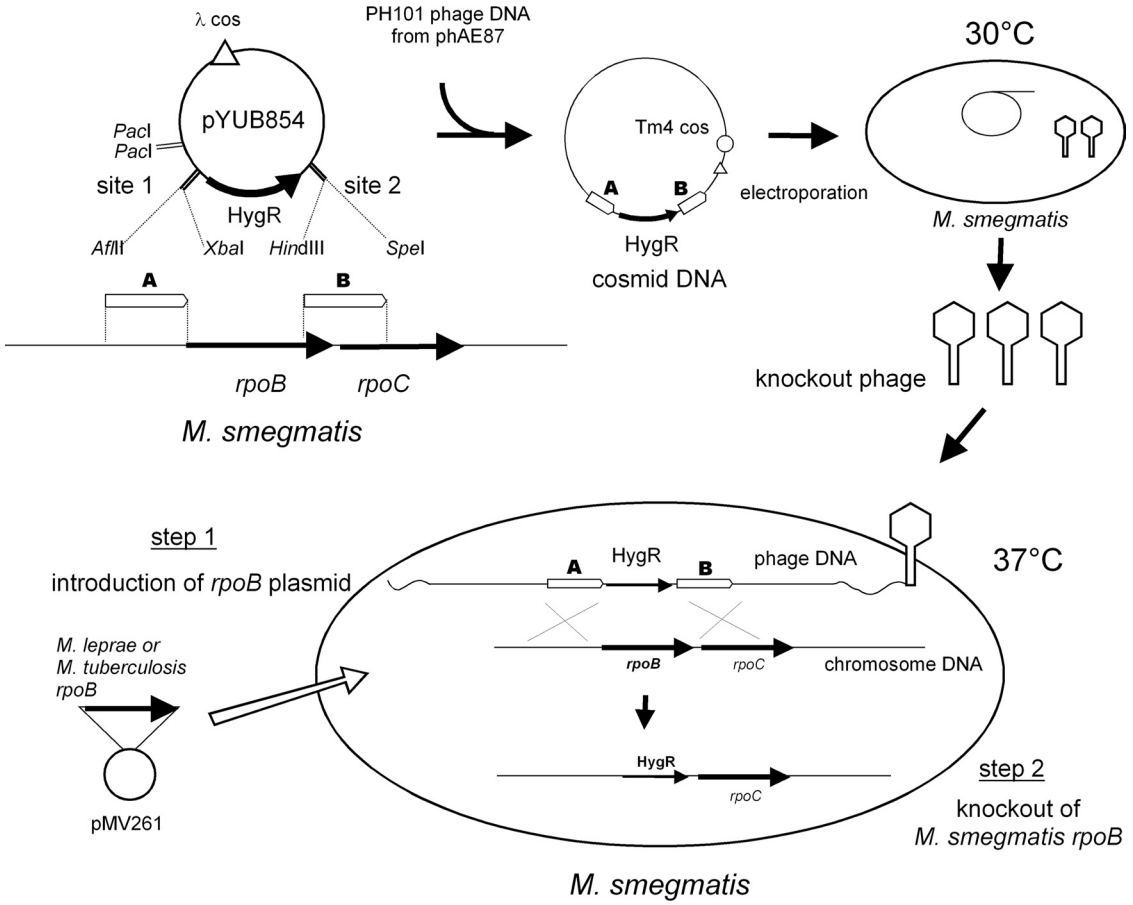

FIG 1 Construction of recombinant *M. smegmatis* strains for rifampin susceptibility testing.

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

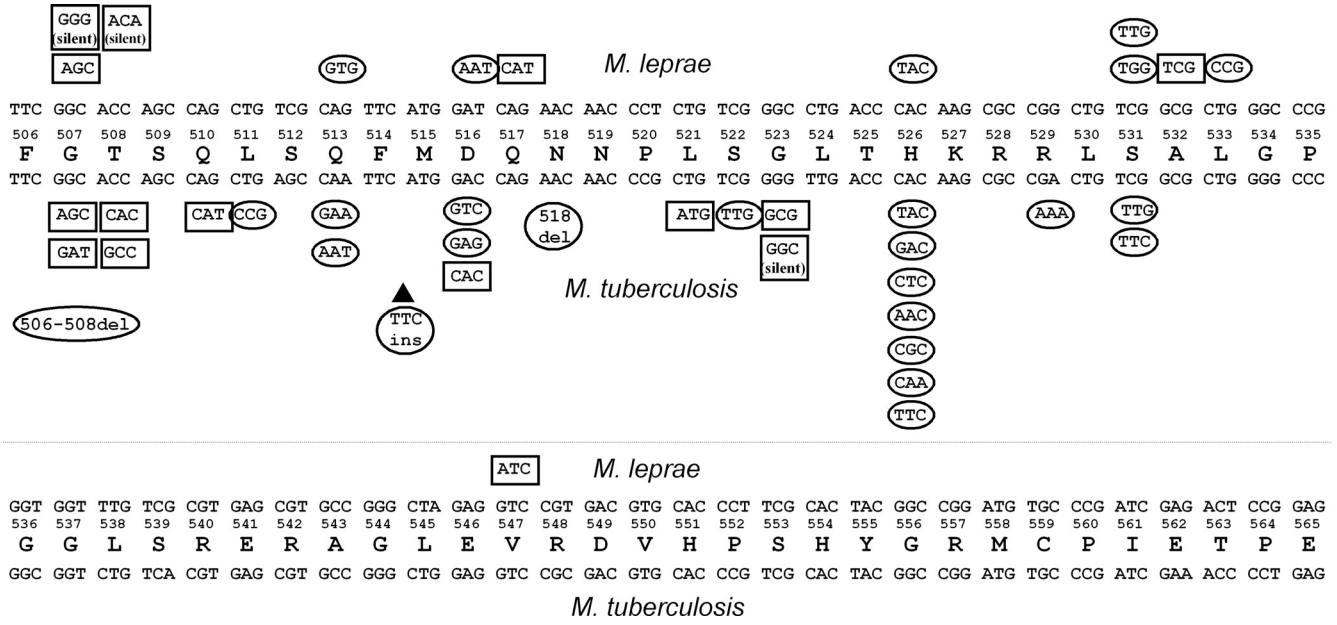

FIG 2 Mutations introduced into the *M. leprae rpoB* gene or *M. tuberculosis rpoB* gene and rifampin susceptibility. The consensus amino acid sequence of *M. leprae* RpoB and *M. tuberculosis* RpoB between codons 506 and 565 is shown. The *M. leprae rpoB* sequence and codons are shown above the consensus 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

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

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