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3 Hansen 病

病原体名

らい菌/*Mycobacterium leprae*

微生物学，分類

抗酸菌で，人工培養に成功していない．

疾患名

Hansen 病/Hansen's disease，leprosy

らい（癩）については偏見を助長するものとして，少数の専門用語（らい菌，らい反応など）以外は使用しない．

病原体のリザーバー

ヒトの他にココノオビアルマジロ，一部のサルなど．

疫　学

日本人の新規患者は年間 0～1 人程度で，高齢者である．一方，在日外国人の新規患者は毎年 4 人程度で，約半数はブラジル人である．なお，世界ではインドやブラジルなどを中心に毎年約 25 万人の新規患者がいる[1,2]．

病原性

らい菌の増殖は遅く（世代時間：12～13 日），至適発育温度は 31℃前後で，毒力はきわめて弱い．らい菌はマクロファージ内で増殖し，皮膚症状が主で，病状は慢性に経過する．また，らい菌の膜表面にあるフェノール性糖脂質と末梢神経の Schwann 細胞表面のラミニン 2 との親和性が高いため，末梢神経の障害が起こる．

発症に大きく関与する感染の機会は，免疫能が完全でない乳幼児期に大量・頻回にらい菌を吸入すること（呼吸器感染）といわれている．発症に影響を与える因子としては，個々人のらい菌に対する特異的な細胞性免疫能の他，公衆衛生の程度，経済状態，栄養状態などの環境・社会的因子が論じられている．

感染予防策

(CDC) 標準予防策

関連法規

普通の感染症であり，現在は新規患者に関する関連法規はない．

臨床症状と経過

問診で知覚の異常（ケガをしても痛くない，頻回の火傷・熱傷，痛み・痒みのない皮疹），発展途上国出身者，高齢の日本人などの場合は鑑別の一つとして Hansen 病を頭の片隅に入れておく．

皮膚症状は環状紅斑や紅斑局面，結節など多彩で，皮疹部に一致して知覚（触覚，痛覚，温度覚）が低下していることが多い．

末梢神経も障害される．皮疹部に一致して触・痛・

表 1　らい菌検査の種類と特徴

らい菌検査の種類	サンプル	何がわかるか	長　所	短　所
皮膚スメア検査	皮膚スメア，滲出液（生検皮膚付着組織液）	菌の有無（抗酸菌染色）	準備が簡単（メスとスライドガラス），数時間で判定	菌種の同定不可能，菌少数の場合は見落としの可能性，採取の手技によって偽陰性の可能性あり
病理組織検査	生検組織（皮膚，末梢神経）	菌の有無と特異的な組織学的変化	病態の把握や治療効果判定	侵襲的検査法
PCR 法（遺伝子検査）	生検皮膚，メス刃付着組織液，滲出液	菌由来核酸成分の有無	短時間，高感度	死菌でも検出される可能性あり，検査中の汚染に注意

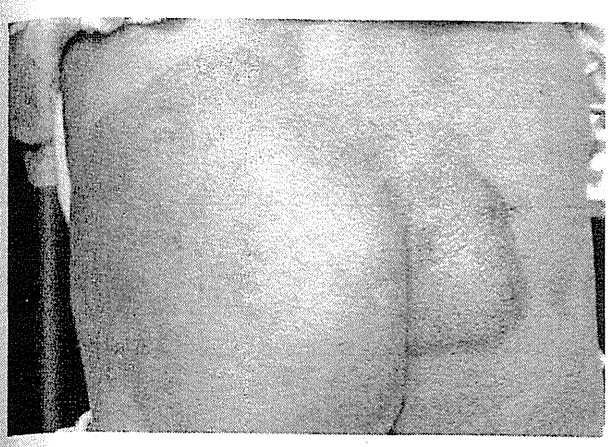

図 1　少菌型(PB，TT)Hansen 病の症例
中心治癒性の環状紅斑．中心部および皮疹部では知覚(触・痛・温度覚)低下．

温度覚の低下がみられ，外傷や火傷・熱傷などが頻発する．さらに，運動神経もおかされ，指趾などが屈曲することもある．

治療中，またその前後に急激な炎症反応(らい反応)が起こることがある．皮疹の増悪や再燃，急激な神経痛や運動障害などを起こす．

臨床検査

らい菌を検出することは診断に直結するため，以下の3検査の中から複数の検査を組み合せてらい菌を証明する(表1)．①皮膚スメア検査(皮疹部からメスで組織液を採取し，抗酸菌染色し，検鏡する)，②病理組織の抗酸菌染色，③PCR 法．レプロミン反応については，反応液は供給されておらず，実施されていない．

診断と病型

以下の4点を総合的に勘案して診断する[2]．①知覚障害のある皮疹，②神経障害(触・痛・温度覚低下，運動障害，神経肥厚)，③らい菌の証明，④病理所見．

鑑別すべき疾患は，サルコイドーシス，皮膚結核，非結核性抗酸菌症，環状肉芽腫，結節性紅斑など多数である．

患者ごとに皮疹の数や形態，神経肥厚や知覚・運動障害の程度，らい菌の数，病理組織所見に差異が大きいが，これはらい菌に対する生体の免疫能を反映するものであり，これを基に病型分類を行う．菌を検出しにくい少菌型(paucibacillary：PB)(図1)と，らい菌を検出できる多菌型(multibacillary：MB)(図2)に分類する(表2)．さらに Ridley-Jopling 分類を行う．

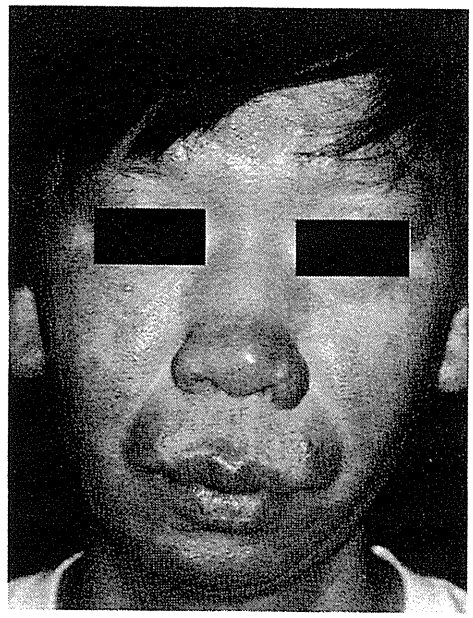

図 2　多菌型(MB，LL)Hansen 病の症例
紅色結節が多発し，表面は光沢を呈する．眉毛が薄くなっている．

治　療

治療の基本は，神経症状をコントロールしながら，らい菌を生体から排除することである．WHO の推奨する多剤併用療法(multidrug therapy：MDT)を基本に治療する．rifampicin(RFP)，diaphenylsulfone(DDS)，clofazimine(CLF)を，PB では前2剤を6ヵ月間，MB では3剤を1～3年間内服する．

なお，キノロン系薬，minocycline(MINO)，clarithromycin(CAM)などもらい菌に対して有効である．

らい反応の治療はステロイドなどの内服を行う．

Hansen 病の歴史

Hansen 病は有効な治療薬がない時代には病状が進行し，顔面，手足に皮疹および末梢神経障害による痛覚麻痺，変形，運動障害などを形成した．そのため外見上の問題と手足の不自由による就労の困難など，さらに宗教上の問題などから，昔から世界中で偏見・差別，排除の対象となった．日本では明治時代になって救済から隔離に進む，「癩予防ニ関スル件」(1907年)，「癩予防法」(1931年)，さらに Hansen 病に有効な治療薬が開発されていた1953年に至っても「らい予防法」として Hansen 病に関する法律が継続した．原則的に一般保険診療の対象にならず，療養所での医療が続いてきた．医学的進歩，人権思想の高まりとともに改変されるべき法律が1996年まで存続してしまった．医療関係者は単に医学の進歩を追求するのみならず，病気に関連する法律や社会的状況なども考慮して，病め

表2　Hansen 病の病型分類

菌数による分類	少菌型 paucibacillary(PB)	多菌型 multibacillary(MB)
免疫学的分類 (Ridley-Jopling 分類)	(I 群)　TT 型	B 群 BT 型　BB 型　BL 型　LL 型
らい菌に対する細胞性免疫能	良好	低下/なし
皮膚スメア検査	陰性	陽性
らい菌	少数/発見しがたい	多数
皮疹の数	少数	多数
皮疹の分布	左右非対称性	左右対称性
皮疹の性状	斑(環状斑)	紅斑(環状斑)，丘疹，結節
皮疹の表面	乾燥性，無毛	光沢，平滑
皮疹部の知覚障害	高度(触覚，痛覚，温度覚)	軽度 / 正常
病理所見	類上皮細胞性肉芽腫	組織球性肉芽腫
	巨細胞，神経への細胞浸潤	組織球の泡沫状変化
病理でのらい菌	陰性	陽性
主たる診断根拠	皮疹部の知覚障害	皮膚スメア検査等でのらい菌の証明
治療 multidrug therapy(MDT)	WHO/MDT：PB 6 ヵ月間 RFP，DDS	WHO/MDT：MB 1 ～ 3 年間 RFP，DDS，CLF

る人々へ，人間として最善を尽くすことが必要である．

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関連 URL

1) WHO のハンセン病欄．〈http://www.who.int/lep〉
2) 国立感染症研究所ハンセン病研究センター．〈http://www.nih.go.jp/niid/lrc/〉

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

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

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

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

MATERIALS AND METHODS

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

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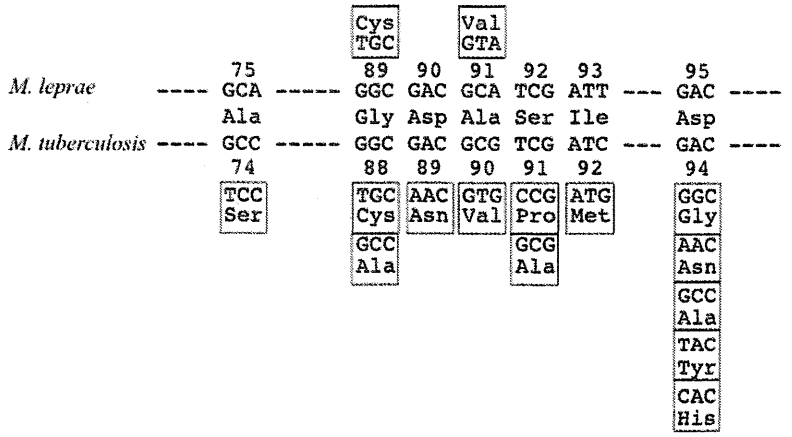

FIG 1 Nucleotide substitutions encoding the quinolone resistance-determining region in *gyrA* of WT and FQ-resistant *M. leprae* and *M. tuberculosis*. Nucleotide sequences encoding the quinolone resistance-determining region of WT *M. leprae* and *M. tuberculosis* GyrA were aligned with the amino acid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

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

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

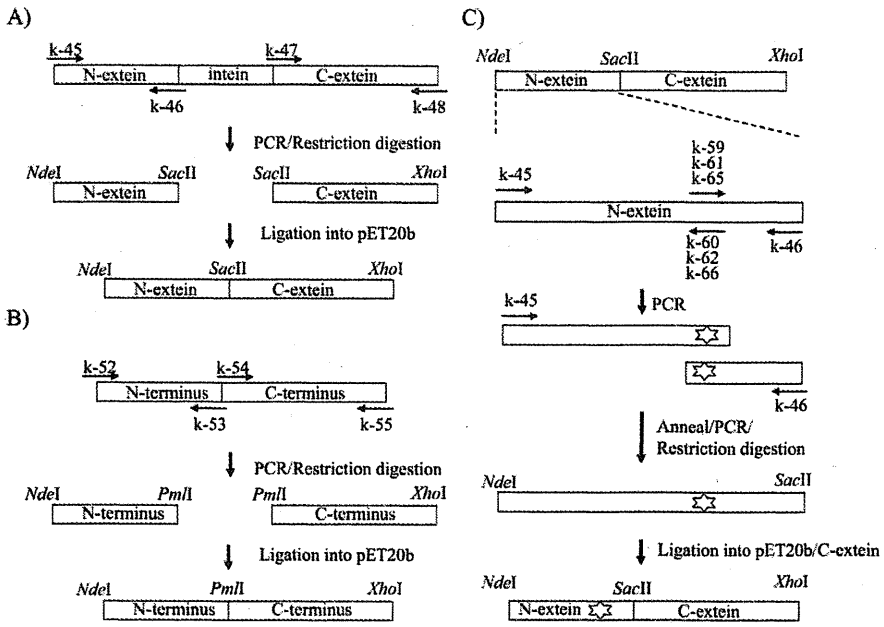

FIG 2 Construction of WT and mutant DNA gyrase expression plasmid. (A) DNA fragments encoding N-extein (amino acids 1 to 130) and C-extein of GyrA (amino acids 125 to 830) were amplified by PCR with primer pairs k-45/k-46 and k-47/k-48 (Table 1), respectively. Similarly, those encoding the N-terminal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

TABLE 1 Nucleotide sequences of primers used in PCR

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

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

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

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

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

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

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

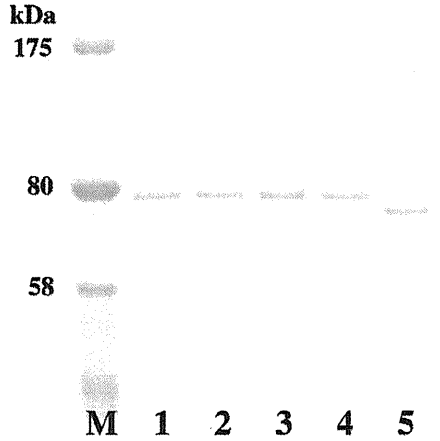

FIG 3 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were overexpressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M, protein marker (NEB); 1, WT GyrA; 2, GyrA-Ala91Val; 3, GyrA-Asp95Gly; 4, GyrA-Asp95Asn; 5, WT GyrB. Three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

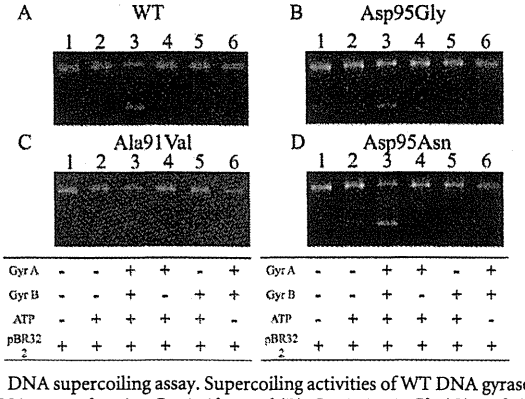

FIG 4 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A) and DNA gyrases bearing GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) were analyzed. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) or GyrB (50 ng), or both. Lanes: 1, relaxed pBR322 alone; 2, relaxed pBR322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

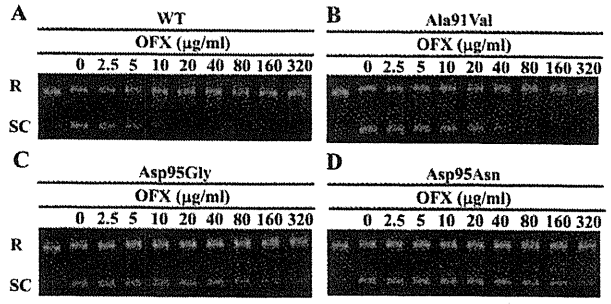

FIG 5 OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. Quinolone-inhibited supercoiling activity assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val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

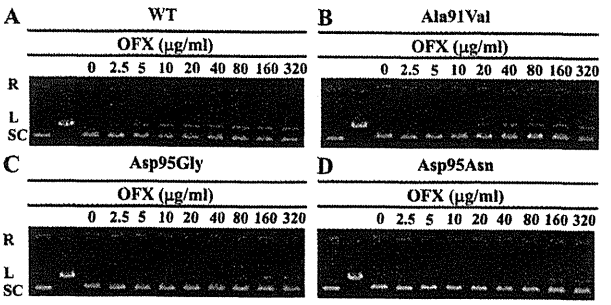

FIG 6 OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn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

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

RESULTS

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

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

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

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

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

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

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

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

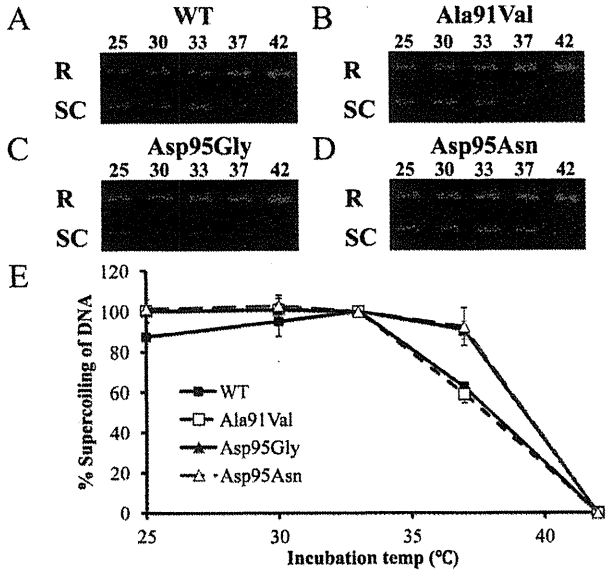

FIG 7 Temperature-dependent DNA supercoiling activity of DNA gyrases. Relaxed pBR322 (0.3 μg) was incubated with WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) at the temperatures (in °C) indicated above the lanes. The proportion of supercoiled DNA compared to that of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

DISCUSSION

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

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

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

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

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

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

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

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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

INTRODUCTION

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

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*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5α was used for DNA cloning. *M. smegmatis* mc^2155 was used as a mycobacterial host to produce strains for drug susceptibility testing. Plasmids pYUB854 and phAE87 were kindly provided by Professor W. R. Jacobs, Jr. (Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY). *M. smegmatis* mc^2155 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 by PCR from *M. leprae* Thai-53 and *M. tuberculosis* H37Rv and cloned into pMV261. Site-directed mutagenesis was performed 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, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants. Then the mutated sequences were 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* are listed in Table 2.

Disruption of the *rpoB* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc^2155 cells were transformed with plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allelic exchange mutants were constructed using the

90 temperature-sensitive mycobacteriophage method as described in an earlier report (2).
91 Using the *M. smegmatis* mc^2155 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^2155.
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

114 necessary to inhibit bacterial growth.

115

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

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