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Relationship between Rifampin MICs for and *rpoB* Mutations of *Mycobacterium tuberculosis* Strains Isolated in Japan

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We analyzed the relationship between rifampin MICs and *rpoB* mutations of 40 clinical isolates of *Mycobacterium tuberculosis*. A point mutation in either codon 516, 526, or 531 was found in 13 strains requiring MICs of ≧64 μg/ml, while 21 strains requiring MICs of ≦1 μg/ml showed no alteration in these codons. However, 3 of these 21 strains contained a point mutation in either codon 515 or 533. Of the other six strains requiring MICs between 2 and 32 μg/ml, three contained a point mutation in codon 516 or 526, while no alteration was detected in the other three. Our results indicate that the sequencing analysis of a 69-bp fragment in the *rpoB* gene is useful in predicting rifampin-resistant phenotypes.

The recent interest in drug-resistant *Mycobacterium tuberculosis* is due, in part, to the increased incidence of infection caused by multidrug-resistant *M. tuberculosis* in AIDS patients (4, 5, 14). When strains are resistant to isoniazid (INH) and rifampin (RFP) (4, 14), these patients often die within a few months (5). In Japan, the number of patients with tuberculosis (TB) has diminished since the introduction of INH and RFP treatment. However, approximately 50,000 new TB cases were still diagnosed every year during the last decade, indicating unsatisfactory control of the disease. In addition, the rates of resistance for first-line anti-TB drugs such as INH and RFP in 1992 were 1.5 and 0.7%, respectively, in previously untreated patients and 17.9 and 15.1%, respectively, in previously treated patients (23).

Molecular diagnostic technology allows rapid detection of *M. tuberculosis* in clinical specimens (2, 13, 17, 22). However, since the drug susceptibility test is performed after the growth of the organism, results of such tests are available only after several weeks. Although a new method using a ready-made DNA probe reduces the time required for detecting drug-resistant *M. tuberculosis*, this method is based on the culture of the microorganism (10). The molecular and genetic mechanisms of INH, RFP, quinolone, and streptomycin resistance in *M. tuberculosis* have been identified (1, 6, 8, 11, 12, 16, 18–21, 25, 27). For example, deletions and other mutations in the catalase-peroxidase gene (*katG*) have been found in INH-resistant strains of *M. tuberculosis* (16, 27), and point mutations have been observed in the RNA polymerase β subunit gene (*rpoB*) in RFP-resistant *M. tuberculosis* strains (20, 21). However, few reports have described the relationship between the MICs of anti-TB agents and genetic alterations (3). In this study, we examined the relationship between MICs of RFP and alterations in the *rpoB* gene of *M. tuberculosis* strains in order to elucidate the exact alterations or amino acid substitutions producing the resistant phenotype.

Forty clinical isolates of *M. tuberculosis* were recovered in our hospital or kindly provided by five affiliated hospitals located throughout western Japan. Thirty-two strains were isolated from non-AIDS patients with TB treated with RFP previously, 7 strains were isolated from untreated patients with TB, and 1 strain was isolated from an AIDS patient who had never been treated. The patients were not related to each other. The strains were suspected to be RFP resistant on the basis of a direct susceptibility test using 1% Ogawa egg medium with RFP (Eiken Chemistry, Tokyo, Japan) performed in our laboratories. The standard strain of *M. tuberculosis*, H37Rv, was employed as a control.

RFP susceptibility testing of all strains was performed by the broth microdilution method with Middlebrook 7H9 broth containing ADC supplement (Difco, Detroit, Mich.). Briefly, 100 μl of serial twofold dilutions of RFP solution (Daiichi Seiyaku, Tokyo, Japan), ranging from 0.125 to 1024 μg/ml, was dispensed into each well of a 96-well microplate (Cell Wells 25850; Corning Glass Works, Corning, N.Y.). A few colonies grown on Ogawa egg medium were inoculated into 15 ml of 7H9 broth and incubated at 37°C for 1 week. Then, the bacterial concentration was adjusted to a McFarland no. 0.5 standard corresponding to 5×10^7 CFU/ml by dilution with threefold-concentrated 7H9 broth. Finally, 100 μl of the twofold-concentrated bacterial suspensions containing 5×10^6 CFU was inoculated into each well, with a final RFP concentration ranging from 0.063 to 512 μg/ml. When successful, the growth in RFP-free wells was observed after 7 or 10 days of incubation at 37°C, and the MIC represented the lowest concentration of RFP that completely inhibited bacterial growth and showed a negative result in the nitrate reduction test. The strains with MICs of 2 to >512 μg/ml were considered to have an RFP-resistant phenotype.

M. tuberculosis DNA was extracted from bacterial cells maintained on Ogawa egg medium by using phenol and chloroform as described previously (13). Ten nanograms of DNA was used for the following PCRs. The DNAs extracted from all isolates were initially used for the specific PCR for the *M. tuberculosis* targeting protein antigen b (Pab) gene (13, 17) to confirm the identification of all tested strains. The PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

Primers TR1 and TR2b, reported by Telenti et al. (20), were used for PCR-direct sequencing analysis to compare alterations in the 69-bp region in the *rpoB* gene of strains isolated in Japan with those from other countries. The PCR was per-

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TABLE 1. The relationship between MICs of RFP and genetic alterations in the *rpoB* gene

MIC of RFP (μg/ml)	No. of strains (total, 40)	No. of strains containing alterations (total, 20)	Position(s) of amino acid substitution(s)[a] (no. of strains)
>512	2	2	Asp-526 (1), Trp-531 (1)
512	6	6	Leu-531 (3), Pro-526 (2), Arg-526 (1)
256	1	1	Tyr-516 and Asn-526 (1)[b]
128	3	3	Leu-531 (2), Tyr-516 (1)
64	1	1	Leu-531 (1)
32	1	0	
16	1	1	Leu-526 (1)
8	2	1	Gly-526 (1)
4	2	1	Val-516 (1)
2	0	0	
1	2	1	Pro-533 (1)
0.5	1	1	Pro-533 (1)
0.25	1	0	
0.125	1	0	
0.063	16	2	Val-515 (1), Leu-521 (1)[c]

[a] *E. coli* numbering system for β subunit of the RNA polymerase.
[b] Double point mutations were observed in one strain.
[c] Silent mutation.

formed with 2 μM TR1 with the 5′ end labeled with 100 mM dATP by T4 polynucleotide kinase (10 U/μl; Takara Shuzo, Otsu, Japan), 2 μM TR2b, and 100 μl of the PCR mixture (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 10 μg of gelatin per ml, 0.25 mM [each] deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase) (Takara Shuzo). The amplification was performed with a Perkin-Elmer PJ 2000 DNA thermal cycler. Then, PCR products were purified with phenol and chloroform, and this was followed by ethanol precipitation. After elimination of the unincorporated primers and each deoxynucleoside by filtration through SUPREC-02 microconcentrators (Takara Shuzo), the DNAs were resuspended in 20 μl of buffer containing 5 U of λ exonuclease (Pharmacia LKB Biotechnology, Uppsala, Sweden) for denaturation. Then, cycle sequencing was performed with an AutoCycle Sequencing Kit (Pharmacia LKB Biotechnology) according to the manufacturer's instructions, using the fluorescent TR1 primer synthesized by Pharmacia, with the following cycle sequence program: 25 cycles of 95°C for 36 s, 50°C for 36 s, and 72°C for 84 s. Five microliters of each aliquot of reaction mixture was electrophoresed in ReadyMix gel (Pharmacia) and sequenced by an A.L.F. DNA Sequencer II (Pharmacia). Similarly, the sequences of antisense strands of all strains tested were determined under the same conditions as described above, with the exception of phosphorated TR2b and the fluorescent RP10 antisense sequence primer (5′-GATCAGACCGATGTTGGG CC-3′).

The PCR products of the Pab gene were obtained from all strains tested, indicating that all strains were identified as *M. tuberculosis* (data not shown). Table 1 summarizes the MICs of RFP for 40 strains of *M. tuberculosis* and the alterations in the *rpoB* gene. The growth of 21 strains was inhibited at RFP concentrations between 0.063 and 1 μg/ml, indicating the susceptibility of these strains. On the other hand, 13 strains were highly resistant to RFP, with MICs for these strains of ≧64 μg/ml. The MICs for the remaining 6 strains ranged between 2 and 32 μg/ml. Genetic alterations were present in 20 of the 40 strains examined, while no alteration was observed in the other 20 strains. A point mutation was confirmed in all 20 strains, and a double point mutation was detected in one strain, but neither insertion nor deletion was observed. The present results were comparable to those reported previously (8, 20, 25) from studies using the same *rpoB* codon numbering systems of the *Escherichia coli rpoB* sequence (Fig. 1). Consequently, seven strains (35%) showed a point mutation in codon 526, seven strains (35%) showed a point mutation in codon 531, three strains (15%) showed a point mutation in codon 516, two strains (10%) showed a point mutation in codon 533, and one strain (5%) showed a mutation in codon 515 with a GTG (Val) substitution which had never been reported before. Another double point mutation in two noncontiguous codons, 516 and 526, was observed, and one strain (5%) showed a silent mutation in codon 521 due to a substitution by TTG. Furthermore, similar patterns were observed in codon 531, as six of seven strains had a similar point mutation from TCG (Ser) to TTG (Leu). These results were highly consistent with those reported previously with regard to the position and frequency of alteration related to the RFP-resistant *M. tuberculosis* phenotype. The correlation between MICs of RFP and point mutations in either codon 516, 526, or 531 was analyzed. All 13 strains requiring MICs of ≧64 μg/ml contained a point mutation in either codon 516, 526, or 531, while all 21 strains requiring MICs of ≦1 μg/ml showed no alterations in these codons. Of the other six strains requiring MICs between 2 and 32 μg/ml, three strains contained a point mutation in either codon 516 or 526, while no alterations were detected in the other three strains. In addition, all seven strains containing a point mutation in codon 531 showed a highly resistant phenotype, requiring MICs of ≧64 μg/ml, and were also resistant to both INH and ethambutol but displayed various susceptibilities to streptomycin (data not shown). On the other hand, the MICs for three strains with a point mutation in either codon 515 or 533 were all ≦1 μg/ml.

The molecular basis of the resistance of *M. tuberculosis* to anti-TB agents such as INH, RFP, streptomycin, and quinolones has been analyzed in the last few years. The early availability of results of drug susceptibility testing with important

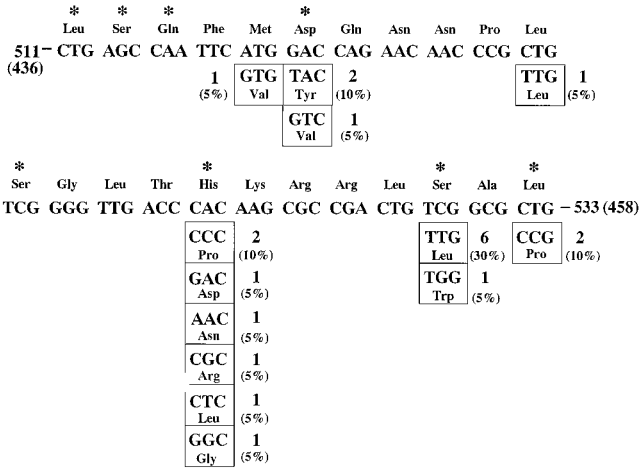

FIG. 1. Substitution of nucleic and amino acids in 69-bp region of *rpoB* in all strains containing alterations. The codon numbering system used for *E. coli* was applied, and codon numbers for *M. tuberculosis* are shown in parentheses. The positions of amino acid substitutions reported previously are indicated above the alignment (*). The numbers appearing underneath amino acids represent the number of isolates, and the frequencies are indicated in parentheses. A total of 21 point mutations were observed in 20 strains. One strain showed double mutations in codons 516 and 526, while another strain showed a silent point mutation in codon 521.

anti-TB agents based on the molecular method may be helpful in the management of TB, particularly in patients with AIDS. In this study, we evaluated the relationship between MICs of RFP and alterations in the *rpoB* gene of *M. tuberculosis* strains isolated in Japan. We also compared our results with those reported previously for *M. tuberculosis* (3, 8, 20, 21, 25). Our results confirmed these early findings by demonstrating the presence of several and frequent alterations related to codon position and amino acid substitution. Our study also extended the early findings by demonstrating several new results. First, substitutions occurring in codons 516, 526, and 531 were considered important to the acquisition of resistance to RFP. In particular, alterations in codon 531 were observed exclusively in strains requiring MICs of ≧64 μg/ml. In contrast, no alteration in these codons was observed in strains requiring MICs of ≦1 μg/ml. One of the mechanisms of RFP resistance is based on changes in RNA polymerase (20, 26), and our study suggests that these codons may play an important role in determining the resistance phenotype. Similar observations have been reported previously (3, 20, 25). For example, Bodmer et al. (3) reported that 20 of 22 strains containing a substitution in either codon 516, 526, or 531 showed an RFP-resistant phenotype and required MICs of ≧8 μg/ml.

The second major finding in our study was that the amino acid substitution in codon 526 observed in seven strains was not tyrosine. This is in contrast to previous studies demonstrating that the major amino acid substitution in codon 526 was tyrosine, with a frequency of 32% (8) or 18.2% (25). Although our results were unlike those of previous reports, the seven strains were not related epidemiologically because they were obtained from four hospitals located in different areas and showed six different kinds of amino acid substitutions in codon 526. In this regard, Williams et al. (25) also reported that 11 RFP-resistant *M. tuberculosis* strains isolated in Japan had no substitution for tyrosine in this codon. These differences may reflect geographical genetic differences in RFP-resistant *M. tuberculosis* strains among different countries in the world (9). However, further evaluation using a large number of resistant strains isolated in Japan is necessary.

Third, our study demonstrated in two strains a change from Leu (CTG) to Pro (CCG) in codon 533. Although this alteration has been reported previously for RFP-resistant strains (8, 20, 25), the phenotypes of the two RFP-susceptible strains were associated with MICs between 0.5 and 1 μg/ml. Furthermore, mutation of Met (ATG) to Val (GTG) in codon 515, which has never been reported in the past, was observed in one strain with a susceptible phenotype (MIC, 0.063 μg/ml). The results of nucleic acid sequencing and the RFP MICs for the three strains mentioned above were repeatedly determined in our laboratory and another affiliated laboratory (data not shown). On the basis of these results, we believe that the change in codon 533 or 515 is not likely correlated with the RFP-resistant phenotype. In addition, no mutation was observed in three strains requiring MICs ranging from 2 to 32 μg/ml, indicating a possible alteration occurring outside the 69-bp region of the *rpoB* gene or a possible change in permeability of a membrane resulting from the RFP-resistant phenotype.

The rapid detection of drug-resistant *M. tuberculosis* in clinical specimens by molecular techniques may be a rational and specific method and may become popular in the near future (7, 15, 24). In this study, our results showed a relationship between susceptibility to RFP and alterations in the *rpoB* gene and may indicate the utility of sequencing the *rpoB* gene for rapid testing of RFP susceptibility. However, since the relationship between gene alteration and drug-resistant phenotype is still unclear, the discovery of a novel drug-resistant gene or further analysis of the relationship between MICs and gene alteration is necessary.

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