TABLE 4—*Continued*

| Gene | No. of isolates ($n$ = 138) | Isolate origin | Changes | | % Resistant (no. of isolates displaying resistance) | Other mutations |
|---|---|---|---|---|---|---|
| | | | Nucleotide | Amino acid (silent mutation) | | |
| | 4 | Japan | GAG→GCG | E378A | 0 | |
| | 1 | Japan | GTG→TTG | V492L* | 0 | |
| | 1 | Poland | CAG→CGG | Q497R | 100 | |
| | 2 | Japan | GCC→ACC | A680T* | 0 | |
| | 3 | Japan | GCC→GTC | A1007V* | 0 | |
| | 1 | Japan | GAC→AAC | D1024N | 0 | |
| | 2 | Japan | CTG→CTA and GAG→GCG | (L355L) and E378A | 0 | |
| | 1 | Japan | AAT→TAT and ATG→ATA | N296Y* and M306I | 100 | |
| | 2 | Japan | ATG→CTG, GAG→GCG, and CCC→CCA | M306L, E378A, and (P1075P) | 100 | |
| *pncA* | 89 | Japan | None | None | 0 | |
| | 30 | Poland | None | None | 0 | |
| | 2 | Japan | TCC→TCT | (S65S) | 0 | |
| | 1 | Japan | GCG→GAG | A3E* | 100 | |
| | 1 | Poland | CAG→CCG | Q10P | 100 | |
| | 1 | Japan | GAC→GCC | D12A | 100 | |
| | 2 | Japan | CAC→CAA | H51Q | 100 | |
| | 1 | Poland | CAC→CAG | H51Q | 100 | |
| | 3 | Japan | CCG→CTG | P54L* | 100 | |
| | 1 | Japan | TGC→TGG | C72W* | 100 | |
| | 2 | Japan | GGT→AGT | G132S | 100 | |
| | 2 | Japan | ATT→ACT | I133T | 100 | |
| | 1 | Poland | CGC→AGC | R148S | 100 | |
| | 1 | Japan | ATG→GTG | M175V* | 100 | |
| | 1 | Japan | GAC→AAC | D53N* | 100 | |
| *gyrA* | 7 | Japan | GAG→CAG | E21Q* | 0 | |
| | 11 | Poland | GAG→CAG | E21Q* | 0 | |
| | 95 | Japan | GAG→CAG and AGC→ACC | E21Q* and S95T | 0 | |
| | 22 | Poland | GAG→CAG and AGC→ACC | E21Q* and S95T | 0 | |
| | 1 | Japan | GAG→CAG, GAC→GGC, and AGC→ACC | E21Q*, D94G, and S95T | 100 | |
| | 1 | Japan | GAG→CAG, GCG→GTG, and AGC→ACC | E21Q*, A90V, and S95T | 100 | |
| | 1 | Japan | GAG→CAG, GCG→GTG, GAC→GCC, and AGC→ACC | E21Q*, A90V, D94A, and S95T | 100 | |
| *rpsL* | 87 | Japan | AAA→AAG | (K121K) | 11.5 (10) | |
| | 33 | Poland | AAA→AAG | (K121K) | 6.1 (2) | |
| | 2 | Japan | AAA→AAG | (K121K) | 100 | 516C→T in *rrs* |
| | 1 | Japan | AAA→AAG | (K121K) | 100 | 1,061C insertion* in *rrs* |
| | 1 | Japan | AAA→AAG | (K121K) | 100 | 1,400A→G in *rrs* |
| | 1 | Japan | AAA→AAG | (K121K) | 100 | 1,400A→G and 1539A→G in *rrs* |
| | 13 | Japan | AAG→AGG and AAA→AAG | K43R and (K121K) | 100 | |
| *rrs* | 33 | Poland | None | | 6.1 (2) | (K121K) in *rpsL* |
| | 87 | Japan | None | | 11.5 (10) | (K121K) in *rpsL* |
| | 13 | Japan | None | | 100 | K43R and (K121K) in *rpsL* |
| | 2 | Japan | 516C→T | | 100 | (K121K) in *rpsL* |
| | 1 | Japan | 1,061C insertion* | | 100 | (K121K) in *rpsL* |
| | 1 | Japan | 1,400A→G | | 100 | (K121K) in *rpsL* |
| | 1 | Japan | 1,400A→G and 1539A→G | | 100 | (K121K) in *rpsL* |

[a] *, mutation not previously reported.
[b] The codon numbering system of RpoB initially described by Telenti et al. (31) was used. The codon numbers of RpoB are designated on the basis of alignment of translated *E. coli rpoB* sequence with a portion of translated *M. tuberculosis* sequence and are not the positions of the actual *M. tuberculosis rpoB* codons.
[c] Nucleotide numbering based on nucleotide position relative to *mabA* start codon.

and H37Ra were positive for PZase activity (data not shown). The remaining 17 *M. tuberculosis* clinical isolates and *M. bovis* BCG were negative for PZase activity. All PZase-positive bacilli were sensitive to PZA, and all PZase-negative bacilli were resistant to PZA. These data were consistent with previously published results (15, 39).

(v) **STR resistance and *rpsL* and *rrs*.** STR resistance is related to mutations in *rpsL* and *rrs* (17, 19, 39). In the present

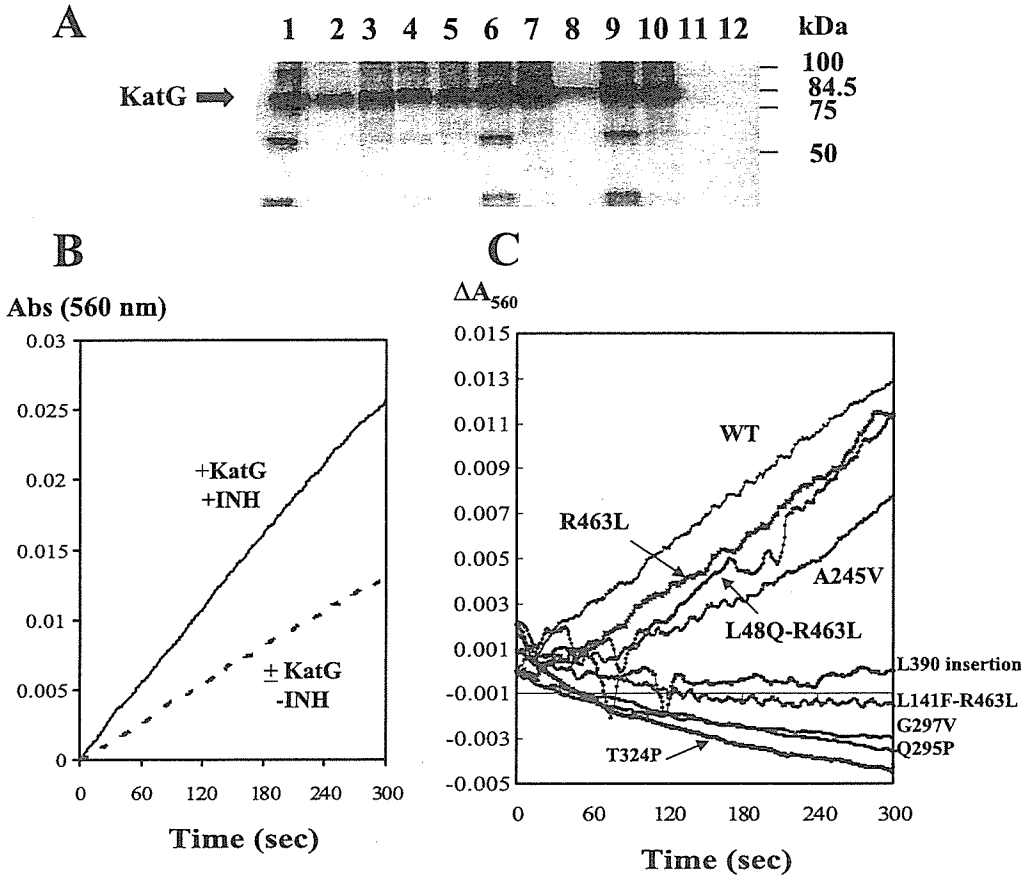


FIG. 2. (A) Western blot analysis of whole-cell protein extracts from KatG-deficient *E. coli* strain UM262 complemented with a control plasmid, pCRT7/NT, or recombinant plasmids expressing various KatG mutants. Lane 1, wild-type KatG; lane 2, $KatG^{Q295P}$; lane 3, $KatG^{T324P}$; lane 4, $KatG^{L48Q\text{-}R463L}$; lane 5, $KatG^{R463L}$; lane 6, $KatG^{G297V}$; lane 7, $KatG^{A245V}$; lane 8, $KatG^{L390\ insertion}$; lane 9, $KatG^{L141F\text{-}R463L}$; lane 10, purified KatG protein; lane 11, control plasmid pCRT7/NT; and lane 12, *E. coli* strain UM262. The positions of molecular mass markers are shown. (B) Time course of NBT reduction with and without the addition of 2.4 mM INH with wild-type KatG. Abs, absorbance. (C) Time course of net INH-dependent NBT reduction by wild-type KatG (WT) and eight KatG mutants. For each KatG sample tested, NBT reduction in the absence of INH was subtracted from that in the presence of INH to obtain the net INH-dependent NBT reduction over time. The concentration of KatG was determined by enzyme-linked immunosorbent assay.

study, all isolates, regardless of STR resistance status, had a silent mutation (K121K) in *rpsL*, and, therefore, the K121K mutation is not associated with STR resistance.

We found a novel insertional mutation in *rrs*. The insertion is the likely cause of STR resistance, because the isolate with the mutation was resistant to STR.

**(vi) FQ resistance and *gyrA*.** Mutations in the FQ resistance-determining region (QRDR) in *gyrA* are responsible for resistance in at least 96% of FQ-resistant *M. tuberculosis* isolates (17, 30, 39). In the present study, we found one novel mutation, E21Q, in *gyrA*, and all isolates tested, except the H37Rv strain, contained this mutation. However, some isolates were susceptible to FQs and others were resistant. Therefore, it is not clear that this mutation is associated with resistance to FQs. E21Q is located upstream of the QRDR.

**Catalase and INH oxidation activities of recombinant KatG mutants.** KatG, catalase-peroxidase, converts INH to its biologically active form (38). Some mutations in *katG* reduce or eliminate the enzymatic activity that is associated with INH resistance (9, 39). To measure catalase and INH oxidation activities, we expressed wild-type KatG and the A245V, Q295P, G297V, T324P, L48Q-R463L, L141F-R463L, R463L, and L390 insertion mutants of KatG in *katG*-deficient *E. coli* (Fig. 2A and Table 5). The catalase activities of these mutants were determined at various $H_2O_2$ concentrations. The $k_{cat}$, $K_m$, and $k_{cat}/K_m$ ratio values are shown in Table 5. Catalase activity was not detected for the $KatG^{Q295P}$, $KatG^{G297V}$, $KatG^{T324P}$, $KatG^{L141F\text{-}R463L}$, or $KatG^{L390\ insertion}$ mutants. The $k_{cat}$ of the $KatG^{L48Q\text{-}R463L}$ mutant was 26% lower than that of wild-type KatG. In contrast, the $KatG^{A245V}$ and $KatG^{R463L}$ mutants showed activities similar to that of wild-type KatG.

The INH oxidation activities of the mutants were determined in the presence of $H_2O_2$ by monitoring the free radical generation in the NBT reduction reaction. When wild-type KatG was tested in this assay, there was a significant background activity of NBT reduction in the absence of INH, whereas the NBT reduction was increased significantly in the presence of INH (Fig. 2B). We subtracted the background activity to obtain the net INH oxidation/NBT reduction. The net values are shown in Fig. 2C. The $KatG^{Q295P}$, $KatG^{G297V}$, $KatG^{T324P}$, $KatG^{L141F\text{-}R463L}$, and $KatG^{L390\ insertion}$ mutants did not show enhanced activity, whereas wild-type KatG and

TABLE 5. Catalase activity of wild-type and mutant KatG, KatG-mediated INH-converting activity, and INH susceptibility

| Recombinant KatG[a] | Catalase activity | | | KatG-mediated INH-converting activity[b] | INH susceptibility[c] |
|---|---|---|---|---|---|
| | $k_{cat}$ ($S^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ratio (mutant/wild type) | | |
| Wild type | 2,403 ± 440 | 60.1 ± 9.5 | 1.00 | + | S |
| A245V* | 2,666 ± 530 | 62.8 ± 10.8 | 1.06 | + | S |
| Q295P* | ND[d] | ND | NA[e] | − | R |
| G297V* | 188 ± 36 | 68.7 ± 7.0 | 0.07 | − | R |
| T324P* | ND | ND | NA | − | R |
| L48Q*-R463L | 1,776 ± 310 | 64.8 ± 12.5 | 0.69 | + | R |
| L141F*-R463L | ND | ND | NA | − | R |
| R463L | 2543 ± 450 | 64.0 ± 11.0 | 1.01 | + | S |
| L390 (CTA) insertion* | ND | ND | NA | − | R |

[a] *, novel mutation.
[b] Time courses of net KatG-mediated INH conversion are shown in Fig. 2C.
[c] INH susceptibilities shown are those of *M. tuberculosis* H37Rv strains having the *katG* gene of the wild type and those of clinical strains having the *katG* gene with the respective mutation(s). S, susceptible; R, resistant.
[d] ND, not detected.
[e] NA, not applicable.

the $KatG^{L48Q\text{-}R463L}$, $KatG^{A245V}$, and $KatG^{R463L}$ mutants of KatG showed significant enhancement of activity in the presence of INH.

Collectively, these results for the enzymatic activities of KatG mutants indicate that the Q295P, G297V, T324P, L141F, and L390 insertion mutants cause loss of enzymatic activity, whereas the A245V and R463L mutants have no effect on the enzymatic activity. The L48Q mutation has little effect on enzymatic activity; however, there was no isolate that carried only the L48Q mutation in KatG in the present study. These mutations and enzymatic activities, except for those of the L48Q-R463L mutant, correlated well with INH susceptibility (Table 5). The L48Q-R463L mutant also carried the −15C→T transition upstream of *mabA* (Table 4). Therefore, the INH resistance of this mutant is likely due to the −15C→T mutation, which is known to be related to INH resistance (10, 22, 39).

**Sequencing of *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. bovis* BCG.** PCR products amplified from *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. bovis* BCG were sequenced with the same sequencing primers as those for *M. tuberculosis* (Table 2). When the nucleotide sequences of BCG were compared with those of *M. bovis* AF2122/97 (GenBank accession no. NC_002945) (8), the sequences were identical. When the sequences of *M. bovis* BCG were compared with those of *M. tuberculosis* H37Rv, the sequences of *rpoB*, the promoter region of the *mabA-inhA* operon, and *rrs* were identical. The R463L (CGG→CTG at nt positions 1387 to 1389) and silent P29P (CCC→CCA at nt positions 85 to 87) mutations were found in *katG* of BCG. E378A (GAG→GCG at nt positions 1159 to 1161) in *embB*, H57D (CAC→GAC at nt positions 169 to 171) in *pncA*, K121K (AAA→AAG at nt positions 361 to 363 [silent]) in *rpsL*, and S95T (AGC→ACC at nt positions 283 to 285) in *gyrA* were identified in *M. bovis* BCG.

**Correlation between drug susceptibility and mutations in BCG.** We next compared the sequences of BCG and *M. tuberculosis* H37Rv and found four mutations, R463L in *katG*, E378A in *embB*, H57D in *pncA*, and S95T in *gyrA*, that caused amino acid substitutions in *M. bovis* BCG. R463L in *katG* is known not to be associated with INH resistance in *M. tuberculosis* (33) and may not be associated with INH resistance in *M. bovis*. E378A in *embB* is not associated with EMB resistance in *M. tuberculosis* (32, 39). H57D in *pncA* was reported previously and is characteristic of PZA resistance in *M. bovis* (39). S95T in *gyrA* is not associated with FQ resistance in *M. tuberculosis* (39). Therefore, in *M. bovis*, mutations except H57D in *pncA* may be polymorphisms not associated with drug resistance.

**Detection and sequencing of drug resistance-related genes of *M. tuberculosis* in sputa from tubercular patients.** We tested a total of 10 sputa from 10 tuberculosis-diagnosed patients. These patients had been received treatment with antitubercular drugs. Of these samples, six were positive for acid-fast bacilli (AFB; >101/field in two samples, 26 to 50/field in two samples, 1/field in one sample, and 1/several fields in one sample) under microscopic observation, and four were negative. Five of the six samples that were positive for AFB were positive for all eight genes tested by PCR. One sample which was positive for AFB (one/several fields) was positive for five genes (*rpoB*, *pncA*, *rpsL*, *rrs*, and *gyrA*) by PCR. The four AFB-negative samples yielded no PCR products and were negative by culture, suggesting that tuberculosis was not active in these patients who had received treatment.

PCR products (a total of 45) were subjected to DNA sequencing. No mutations were identified in 38 of the PCR products. The remaining seven PCR products contained nine mutations. The seven PCR products were obtained from one sputum sample. The sputum sample was cultured, and after several weeks, an isolate of *M. tuberculosis* was obtained and analyzed. We conducted PCR analysis of this isolate and detected the same nine mutations. This isolate was resistant to RIF, INH, EMB, STR, FQs, and PZA. These results indicate that our DNA sequencing-based method can be used to detect MDR strains of *M. tuberculosis* in sputa obtained from clinical tuberculosis patients.

## DISCUSSION

Our novel DNA sequencing-based method described here is useful for detection and diagnosis of drug-resistant strains of *M. tuberculosis*, especially MDR strains. Our method has

several advantages. First, it allows simultaneous detection of mutations in eight genes associated with resistance to six antituberculosis drugs. Second, the entire assay from DNA extraction to the DNA sequencing can be completed within 1 working day. Third, this method is sensitive enough to detect 1 ng of genomic DNA (i.e., $3 \times 10^5$ *M. tuberculosis* cells). We found that this method worked well even in positive sputum containing few bacilli, as determined by acid-fast staining. Fourth, our DNA sequencing-based method allows detection of both novel and known mutations. In the present study, we identified 25 novel mutations by PCR-based analysis.

This strategy does have a few disadvantages. The DNA sequencer and sequencing are costly, and the procedure is somewhat complicated. However, this issue may be addressed if DNA sequencing costs are reduced by new sequencing methods and equipment. Also, this strategy may not be able to detect very low numbers of bacilli in sputa. We did not have the opportunity to test AFB smear-negative but culture-positive samples. Among the smear- and culture-positive samples we tested, one sample with small numbers of bacilli (one/several fields) was negative for three of the eight genes tested, indicating that the sensitivity of this method is limited. However, the sensitivity and accuracy of our method are comparable to those of traditional drug susceptibility tests, and this is sufficient for use in the clinical setting.

The method described here was excellent for diagnosis of RIF-resistant *M. tuberculosis* isolates with 100% specificity, sensitivity, and test efficiency. RIF interferes with the synthesis of mRNA by binding to the β-subunit of RNA polymerase (RpoB) in bacterial cells (39). The RIF-binding site is a pocket in the upper wall of the main channel for double-stranded DNA entry just upstream of the polymerase catalytic center. The various RIF-resistant mutations are clustered around this pocket (39). Mutations in *rpoB* have been found in 95% to 100% of clinical RIF-resistant isolates of *M. tuberculosis* (39). Most of the mutations found in the 28 RIF-resistant isolates tested here were located between nucleotides 1276 and 1356 (codons 507 to 533) of *rpoB*, which is the 81-bp core region of this gene (Table 4) (31, 39). Two other mutations, V146F and E562A, were located outside of the 81-bp core region. Isolates with V146F were reported to show low-level resistance to RIF (MIC, ≤4 μg/ml) (39). It is not known whether the E562A mutation is involved in resistance because the isolate with this mutation also had another mutation in the 81-bp core region (39). The V146F mutation could not be detected by the DNA sequencing method described here. Although we were able to detect the E562A mutation by our sequencing method, we did not find this mutation in any of 138 isolates tested in the present study.

Our sequencing method is applicable for diagnosis of INH-resistant isolates with 89.5% sensitivity, 100% specificity, and 97.1% test efficiency (Table 6). The sensitivity of the two-temperature PCR for *katG* was lower than that of the PCR for *rpoB* or *mabA-inhA* (Fig. 1B), and, therefore, we will need to increase the sensitivity to detect *katG* mutations to assess INH resistance. The mode of INH action is one of the most complicated among all antibiotics. INH is a prodrug that requires activation of the bacterial catalase-peroxidase enzyme (KatG) (38) to generate a range of reactive radicals, which then affect multiple systems, including cell wall mycolic acid synthesis and lipid peroxidation and NAD metabolism, and cause DNA damage (39). Deficient efflux of INH radicals and defective antioxidative defenses may underlie the susceptibility of *M. tuberculosis* to INH (39). Mutations in *katG* are among the most frequently detected mutations in INH-resistant clinical isolates. Mutations in *inhA* and its promoter region, which is located upstream of the *mabA-inhA* operon, are also common (16, 17, 39). Our sequencing method should identify a majority of INH-resistant isolates. We are able to detect mutations in *katG* and the region upstream of *mabA* in 90% (34/38) of INH-resistant isolates. Ten different mutations (L48Q, L141F, M257T, Q295P, G297V, S315T, S315N, T324P, R463L, and V708P) were detected in *katG* of INH-resistant isolates, and 3 mutations (A65T, A245V, and V725A) were identified in INH-susceptible isolates. The L48Q, A65T, L141F, A245V, M257T, Q295P, G297V, T324P, V708P, V725A, and L390 insertion mutations are novel (Table 4).

To date, several mutations in *katG* in MDR isolates have been reported (9, 16, 17, 38, 39). Rouse et al. (24) reported previously that codons 104 and 108 encode amino acids located near the catalytic site of KatG and that the residues encoded by codons 270, 275, and 315 participate in binding the heme group

TABLE 6. The diagnostic performance of the DNA sequencing-based method in comparison with drug susceptibility testing[a]

| Drug susceptibility test result[b] | No. of isolates ($n$ = 138) | | % Sensitivity[c] | % Specificity[d] | % Test efficiency[e] |
|---|---|---|---|---|---|
| | Mutation positive | Mutation negative | | | |
| RIF | | | | | |
| Resistant | 28 | 0 | 100 | 100 | 100 |
| Susceptible | 0 | 110 | | | |
| INH | | | | | |
| Resistant | 34 | 4 | 89.5 | 100 | 97.1 |
| Susceptible | 0 | 100 | | | |
| EMB | | | | | |
| Resistant | 15 | 3 | 83.3 | 100 | 97.8 |
| Susceptible | 0 | 120 | | | |
| PZA | | | | | |
| Resistant | 17 | 0 | 100 | 100 | 100 |
| Susceptible | 0 | 121 | | | |
| STR | | | | | |
| Resistant | 18 | 12 | 60.0 | 100 | 91.3 |
| Susceptible | 0 | 108 | | | |
| OFX | | | | | |
| Resistant | 3 | 0 | 100 | 100 | 100 |
| Susceptible | 0 | 135 | | | |

[a] The diagnostic performance of the DNA sequencing-based method in comparison with drug susceptibility testing was determined after resolution of polymorphisms.

[b] Drug susceptibility for antituberculosis agents except for PZA was determined by the agar proportion method according to NCCLS (now CLSI) guidelines, and that for PZA was determined by PZase activity.

[c] Sensitivity: no. of drug-resistant isolates with mutations/(no. of drug-resistant isolates with mutations + no. of drug-resistant isolates without mutations).

[d] Specificity: no. of drug-susceptible isolates without mutations/(no. of drug-susceptible isolates with mutations + no. of drug-susceptible isolates without mutations).

[e] Test efficiency: (no. of drug-resistant isolates with mutations + no. of drug-susceptible isolates without mutations)/no. of all isolates tested.

of KatG (24). Mutations in these regions, therefore, are thought to cause loss of KatG enzymatic function (24). Yu et al. (36) reported that residue W321 of KatG was important for substrate binding and that residue Y229 was critical to protect the catalase activity of KatG (37). We compared the catalase and INH oxidation activities of eight KatG mutants identified in this study with those of the wild type. Although we were able to express all of the KatG mutants (Fig. 2A), we were unable to detect the catalase and INH oxidation activities of four KatG mutants, $KatG^{Q295P}$, $KatG^{T324P}$, $KatG^{L141F\text{-}R463L}$, and $KatG^{L390\ insertion}$ (Table 5 and Fig. 2C). The specific effects of these mutations on KatG function need to be analyzed further. The lack of activity or lower activity of these mutants, however, correlated quite well with the INH-resistant phenotype of their respective *M. tuberculosis* isolates. The enzymatic activity of $KatG^{L48Q\text{-}R463L}$ was not correlated with INH susceptibility (Table 5), and this mutation had little effect on the measured activities (Table 5 and Fig. 2C). The isolate carrying the L48Q-R463L mutations also had the −15C→T transition upstream of *mabA* (Table 4), which is known to be associated with INH resistance (10, 22, 39). The $KatG^{A245V}$ and $KatG^{R463L}$ mutants showed activities similar to those of wild-type KatG, and these results are consistent with the INH-susceptibility phenotypes of their respective isolates. Therefore, we concluded that the L48Q, A245V, and R463L mutations are merely polymorphisms that do not influence INH resistance.

We found mutations in the region upstream of *mabA* or in the regulatory region of the *mabA-inhA* operon in 12 of 38 INH-resistant isolates. Five of these isolates had no other mutations within *katG* (Table 4). Our present results support those of Morris et al. (16), who examined the *inhA* locus for sequence polymorphisms by single-strand conformation polymorphism analysis and DNA sequencing of 42 INH-resistant isolates. They found no alterations in the coding portion of *inhA*, but five isolates had mutations in the regulatory region of the *mabA-inhA* operon (16).

Mutations in *kasA*, which encodes β-ketoacyl ACP synthase (11), and *ndh*, which encodes NADH dehydrogenase (12), have been found in a small proportion of clinical isolates, and we plan to modify our sequencing method to analyze *ndh* and *kasA*.

Our method was sufficient for diagnosis of EMB-resistant isolates, although a limited portion (80%) of *embB* was sequenced. EMB inhibits polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (39). Three homologues of arabinosyltransferases, EmbC, EmbA, and EmbB, have been proposed to be the targets of EMB (32, 39). Mutations in *embB* are found in 47% to 69% of EMB-resistant isolates of *M. tuberculosis* (28, 32). Most EMB-resistant isolates with *embB* mutations exhibited high-level resistance (2, 27). The 35% of EMB-resistant isolates that do not have *embB* mutations showed decreased resistance to EMB (2). We were able to detect a majority (15/18 isolates) of EMB-resistant isolates with our sequencing-based analysis. In addition, we identified two novel mutations, D354A and N296Y, in EMB-resistant isolates in the present study.

*pncA* is known to be associated with PZA resistance (17, 39). In the present study, we sequenced the complete open reading frame of *pncA* and its promoter region. PZA enters the organism through passive diffusion and is converted to pyrazinoic acid by cytoplasmic PZase. Despite recent progress, the targets of pyrazinoic acid are still not known (39). All PZA-resistant *M. tuberculosis* isolates tested in the present study contained at least one mutation within *pncA* and showed no PZase activity (Table 4). Our results are consistent with those of previous studies that showed 72% to 95% of PZA-resistant clinical isolates of *M. tuberculosis* carried *pncA* mutations (25). All of the *pncA* mutations identified in the present study of PZA-resistant isolates caused amino acid substitutions. Among these mutations, 5A3E, D53N, P54L, C72W, and M175V were novel. The *pncA* mutations were highly diverse and scattered across the gene.

STR, an aminoglycoside, inhibits initiation of mRNA translation. The site of action is the small 30S subunit of the ribosome, especially ribosomal protein S12 and the 16S rRNA (17). *M. tuberculosis* becomes resistant when targets of STR in the ribosomes are mutated. The principal site of mutation is the *rpsL* gene, which encodes ribosomal protein S12 (6, 19, 27). The most frequently observed mutation in *rpsL* was K43R. In the present study, 13 of 30 STR-resistant isolates tested had the K43R mutation. Mutation of the *rrs* gene is also associated with STR resistance in *M. tuberculosis*. *M. tuberculosis* has only a single copy of the *rrs* gene, which encodes the 16S rRNA. Thus, the loops of 16S rRNA that interact with the S12 protein constitute an easily selected mutation site. Such *rrs* mutations are clustered in the highly conserved 530 loop and in the adjacent 915 region (6). In addition, a 1400A→G mutation of *rrs* was identified in both amikacin- and kanamycin-resistant clinical isolates of *M. tuberculosis* (1, 29). These isolates were resistant to STR, indicating that this mutation may contribute to STR resistance (1, 29). In the present study, one STR-resistant isolate had two mutations, 1400A→G and 1539A→G. Because the STR resistance of the isolate can be explained by the 1400A→G mutation, it is unclear whether the 1539A→G mutation is associated with STR resistance.

FQs are active in vitro against *M. tuberculosis* isolates (5) and are increasingly being used in combination with other agents to treat tuberculosis. The principal mechanism of resistance to FQs identified in other bacterial species is alteration of the target proteins DNA gyrase and topoisomerase IV. DNA gyrase is composed of two A and two B subunits, which are encoded by *gyrA* and *gyrB*, respectively (39). Mutations in *gyrA* are associated with high-level resistance of *M. tuberculosis* to FQs (39). *gyrB* mutations associated with resistance have only been identified in laboratory mutants of *M. tuberculosis* (39). Mutations associated with FQ resistance occur within a relatively restricted region of *gyrA*. We identified three mutations, A90V, D94GA, and D94G, in FQ-resistant isolates. We also identified a polymorphism, S95T, that is not associated with FQ resistance. The G88C, D89G, S91P, and D94A, -N, -H, or -Y mutations in *gyrA* have also been found in FQ-resistant isolates (4, 30). These mutations are presumed to be located in the FQ-binding region (4, 30).

Some researchers have described mutations that caused amino acid substitutions but not drug resistance (30, 33, 39). In the present study, we identified several novel mutations that cause amino acid substitutions but do not confer drug resistance. Except for these mutations and silent mutations, the drug resistance profiles of the isolates tested correlated quite

well with the various mutations that we identified (Table 6). The sensitivities of the DNA sequencing-based method (i.e., the ability to detect true drug resistance) were 100%, 89.5%, 83.3%, 100%, 60%, and 100% for the RIF-, INH-, EMB-, PZA-, STR-, and OFX-resistant strains, respectively. The specificities (i.e., the ability to detect true drug susceptibility) were 100% for all drugs tested. The test efficiencies (i.e., the ability to give the correct answer in all samples tested) were 100%, 97.1%, 97.8%, 100%, 91.3%, and 100% for the RIF-, INH-, EMB-, PZA-, STR-, and OFX-resistant strains, respectively. These results indicate that our DNA sequencing-based method is effective for detection of MDR strains. However, when novel mutations in drug resistance-related genes are detected by our method, it is essential to also perform drug susceptibility testing, because novel mutations are not necessarily associated with drug resistance. Of the 25 novel mutations we detected, we cloned 7 novel mutations in KatG. Significant information could be gained if all novel mutations were cloned. For practical purposes, it would be helpful to know the phenotypic manifestations of specific mutations.

In conclusion, we have shown the usefulness of our DNA sequencing strategy for drug susceptibility screening of various targets. Most MDR *M. tuberculosis* strains, which are defined as those strains resistant to both RIF and INH, are resistant to other antitubercular drugs. Our new sequencing-based method can rapidly and efficiently assess MDR of *M. tuberculosis*. The method can also be used to detect MDR *M. tuberculosis* in sputa from patients. Further studies will focus on the clinical application of this method for diagnosis of drug-resistant *M. tuberculosis*.

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# Development of information retrieval and web information integration system for nosocomial infection anecdotal research papers

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*Abstract*—**Sharing infectious information is very effective to correspond and prevent infection outbreak. In past, we have developed a pilot nosocomial infection anecdotal research database system on the web to access easily. This system is able to search target research papers using some categories, but are only able to list papers with abstract. Therefore, If we wanted to survey the a focused infection, we must read all retrieved documents and choose target data like number of patients or isolate numbers. In this article, we would like to suggest using natural language processing technique and extracting numeric information which is in documents in order to integrate web data. Consequently, we are able to develop web information integrated system which can extract numeric data in which retrieved research papers crossly.**

*Index Terms*—**nosocomial infection anecdotal research papers, web data integration system, numeric information.**

## I. Introduction

SHERING Infectious information is very effective when we adapt outbreak situation. For instance, we can know what infection disease is outbreak now around them, and how they corresponded the infection disease in past. Therefore we can settle this outbreak fast and adequately. In past, we have developed a pilot nosocomial infection anecdotal research database system to access easily [1][2]. This system contained more than 350 infection outbreak research papers with some categorize factors, which are date, hospital, pathogen, journal, study methods and so on, and we opened this system to the public on World Wide Web as "web-based database". So, user was able to highly structured abstracts. These categories which we decided as important factor for nosocomial infection research papers are very useful because we became possible focusing and retrieving these papers according to our demand. However, we are not able to survey in order to grasp crossover and keyword search, if we used this system. For instance, most of patients number is in body of a paper, and we are not able to treat the number as "data" which are calculated and analyzed usually. Therefore, we considered that we were able to make information integration system[3] for infection outbreak database, if we used selectable categories and numeric values.

In this article, we would like to suggest using natural language processing technique and extracting numeric information which is in documents in order to integrate web data and showing new retrieve information system.

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## II. Method

### A. Target

Our target database is "Hospital Infection Outbreak Database" website [4], and this site has database in which this system has 362 nosocomial infection anecdotal research papers. Most papers dealt with epidemiological investigations. A few outbreaks due to non-infectious origin were included, such as acute onset of diminished vision and hearing in dialysis patients, pseudo-outbreaks[2]. A webpage was created for the web-based database search. The website presents study background, instructions for use and search menu in English and Japanese. The search menu has category search interface and a user is able to select from a pull down menu of choices, which are pathogens, infection sites, modes of transmission, types of investigation and word/service. These infection articles naturally have some numeric values, which are various numbers, values, grade and so on, for example, total number of patient, number of relevant health care workers etc.

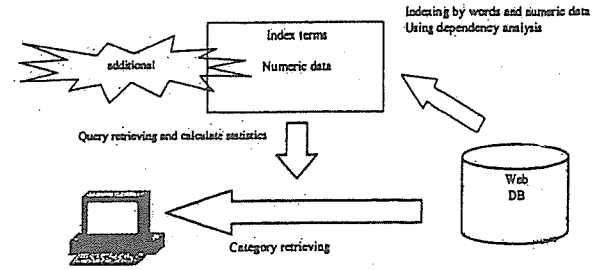


Fig.1. system concept

*B. Information integration technology and numeric information*

Information integration technology [3] has been focus of constant attention of web information beneficial use. If we are able to treat piecemeal data of web as integrated data, Value of web data was enhanced very much. In general, the information integration is three main processes [5]:

1. Collecting web pages where necessary information is described.
2. Extracting relevant information from the web pages
3. Relating the relevant information

Recently World Wide Web information integrated system is used agent technology. All web pages are not able to be processed by simple program because web data structure are very various. So, the agent technology is adopted in order to effect mutually.

Numeric information is very important in not only web pages but also any other documents. However, it is difficult that we treat numeric value correctly if the numeric values are in free text documents. When we want to extract useful information from free text data, we are able to use various natural language processing techniques. In particular, we considered that a dependency structure analysis [6] is useful when we would extract numeric value and relevant words, because dependency information informed relations of certain numeric value and corresponding word. Dependency structure analysis consists of two steps. In the first step, dependency matrix is constructed, in which each element corresponds to a pair of chunks and represents the probability of a dependency relation between them. The second step is to find the optimal combination of dependencies to form entire sentences.

*C. Tool developing*

We adapt a dependency analysis in order to extract numeric values and words to which the numeric values related. We developed extracting tool of numeric values and corresponding words. This tool was able to extract numeric information in free text documents. Next, we developed information retrieval system which targeted papers using query in order to narrow the search to user's wants and could do crossover survey based on numeric information. This system wrapped to the pilot nosocomial infection anecdotal research database system and added these new functions. For example, user could do keyword search on this database and this system could display some statistics (e.g. mean of patients numbers) of retrieved papers. This tool implemented on the *"Hospital Infection Outbreak Database"* website. Figure 1 shows concept of this tool.

## III. RESULT

The tools of dependency analysis extracted numeric values and corresponding words. number of numeric information in these reports was 2987 in these infection outbreak research papers. We could calculate numeric data in which we had deal with papers.

Table 1 shows one of instance of statistical data as follows;

Numeric information was appended each infection research papers and we could extract and calculate basic statistics. This tool gives all infection research papers its numeric data when it does indexing process.

Next, we implemented this function of dependency analysis and retrieving using keywords as integration information retrieval system is able to display search output with statistics. Users had been able to retrieve his and hers demanding information using keywords and get numeric statistics of these data. Of course, this system implemented on prior system as adding in. Figure 2, 3 show interfaces of this system. Section 1 is query field adding this time, section 2 is the prior system in figure 2. Section 3 in figure 3 is some statistics of numeric information (number of patients, week, Health care Workers (HCWs)).

TABLE I
STATISTIC OF NUMERIC VALUES AND CORRESPONDING WORDS

| corresponding words | numbers | Mean | S.D. | Median |
|---|---|---|---|---|
| patients | 366 | 33.364 | 79.433 | 10 |
| days | 174 | 15.293 | 22.464 | 9 |
| cases | 119 | 17.485 | 28.057 | 8 |
| months | 93 | 7.461 | 7.555 | 6 |
| weeks | 61 | 6.109 | 7.275 | 3 |
| isolates | 50 | 173.568 | 810.207 | 15 |
| years | 49 | 12.637 | 19.875 | 5 |
| hours | 35 | 102.531 | 207.594 | 48 |
| strains | 32 | 15.032 | 17.142 | 6 |
| infants | 30 | 47.793 | 127.376 | 9 |
| HCWs | 28 | 57.321 | 69.633 | 26.5 |

S.D.: Standard deviation

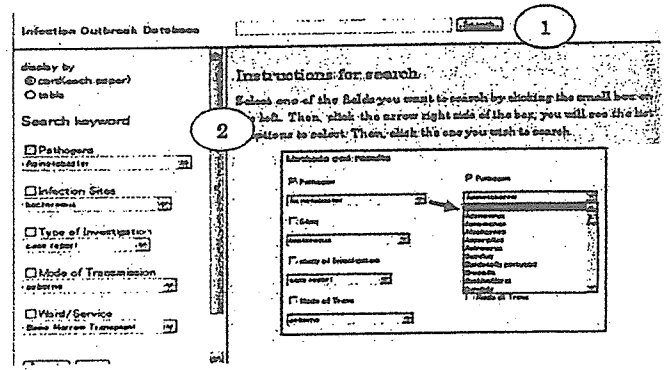


Fig.2. Interface of retrieval system (previous search)

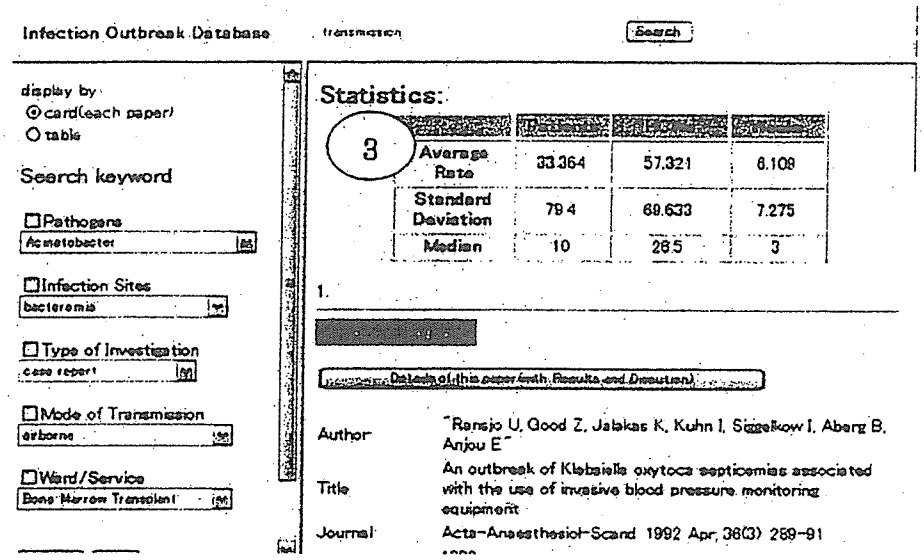


Figure3. Interface of retrieval system (result)

In this paper, we tried to extract relation between numeric data and corresponding words from the viewpoint of information integration. Concretely, we made the tool of dependence structure analysis and extracting relation numeric value and corresponding words. In addition, we made keyword search interface on prior epidemiological web database present essential information. We became possible the calculation of basic statistic in each retrieval result and we can expect radius of impact as numeric data if a similar outbreak is found using category and keyword.

However, all relation of extracted numeric data and corresponding words is not always correct when dependence analysis or syntax analysis, because all numeric data is not expression of typical numeric data. For example, an abstract of one research paper have three infants data if the infants which infect divide two category from a point of view and the research paper refer to these infants. In these cases, it is very difficult to distinct tellingly which numeric data is typical and dependent and we must use more advanced technique as semantic analysis. Currently semantic analysis had Almost all typical corresponding words (patients, cases, weeks and so on), however, are referred one times in each abstracts because this nosocomial outbreak investigation database collects typical cases.

Consequently, we developed web information integration system and were able to survey using the numeric information and display basic statistics in retrieved nosocomial infection anecdotal research papers.

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# Association of *rpoB* mutations with rifampicin resistance in *Mycobacterium avium*

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**Abstract**

The susceptibility of clinical isolates of *Mycobacterium avium* to rifampicin (RIF) was examined. All 32 clinical isolates tested, including 18 from Japan, 13 from Poland and 1 from Thailand, were resistant to RIF (minimum inhibitory concentrations (MICs) ≥32 μg/mL for 17 isolates and 2–16 μg/mL for 15 isolates), whereas the type strain of *M. avium* ATCC 25291 was susceptible to RIF (MIC ≤ 0.03 μg/mL). Mutations in nucleotides 1276–1356 of the *rpoB* gene, termed the 81 bp core region, are associated with RIF resistance in *Mycobacterium tuberculosis*. No mutations were found in this region in any of the *M. avium* clinical isolates tested. However, mutation of G → A to give a Gly544 → Asp substitution was identified within the *rpoB* gene downstream of the 81 bp region in all clinical isolates. A RIF-resistant strain (ATCC 25291 Rif$^r$; MIC ≥ 32 μg/mL) obtained by culturing the type strain in RIF-containing broth possessed a mutation C → T to give a His445 → Tyr substitution within the 81 bp region. When the *rpoB* gene of the ATCC 25291 Rif$^r$ strain and of a clinical isolate were inserted into *Mycobacterium smegmatis*, organisms with the ATCC 25291 Rif$^r$ sequence, but not those with the clinical isolate sequence, showed resistance to RIF. These results suggest that mutations of the 81 bp region of *rpoB*, as well as factors other than *rpoB* mutation, confer RIF resistance in *M. avium*.


*Keywords:* *Mycobacterium avium*; Rifampicin; *rpoB*

## 1. Introduction

*Mycobacterium avium* is a non-tuberculous mycobacterium associated with life-threatening infections in patients with chronic obstructive lung disease, immunocompromised individuals such as HIV-infected patients and, occasionally, in individuals without apparent predisposing conditions [1,2]. *Mycobacterium avium* infection in the absence of other diseases is occasionally associated with frequent exposure to environmental organisms such as those found in bath water [3,4].

According to guidelines proposed by the American Thoracic Society, four-drug chemotherapy consisting of rifampicin (RIF), clarithromycin, streptomycin and ethambutol, is used to treat against *M. avium* pulmonary disease [2]. However, results show variable success, ranging from 40% to 91% [5]. Most *M. avium* clinical isolates are thought to be resistant to RIF in vitro [6]. Agreement regarding the indications for susceptibility testing is lacking [7].

The mechanisms of RIF resistance in *M. avium* have yet to be defined [8–10]. RIF resistance in *Mycobacterium tuberculosis* as well as in a number of bacteria such as *Escherichia coli* and *Staphylococcus aureus* has been shown to result from a restricted set of mutations in the *rpoB* gene, which encodes the β subunit of RNA polymerase [11–14]. RIF acts to inhibit mRNA synthesis in bacteria by binding to the

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RNA polymerase β subunit (RpoB) [15,16]. The RIF binding site consists of a pocket located on the upper wall of the main channel for double-stranded DNA entry, upstream of the polymerase catalytic centre [14]. Various RIF resistance mutations are clustered around this pocket [17]. In RIF-resistant *M. tuberculosis*, mutations in the *rpoB* gene have been found in 95–100% of clinical isolates [13]. Most of the mutations are located between nucleotides 1276 and 1356 (codons 426–452), a region termed the 81 bp core region [18].

In the present study, we examined whether mutations in the *rpoB* gene of clinical isolates and of a RIF-resistant laboratory strain of *M. avium* exist and whether such mutations participate in RIF resistance.

## 2. Materials and methods

### 2.1. Strains and plasmid DNA

A total of 32 clinical isolates of *M. avium* were used in this study. Eighteen were obtained from the International Medical Center of Japan, Tokyo, 13 were from the National Research Institute of Tuberculosis and Lung Diseases in Warsaw, Poland, and 1 was provided by T. Chotpitayasunondh, Sirikit National Institute of Child Health, Bangkok, Thailand. These clinical isolates were each derived from a separate patient. *Mycobacterium avium* was identified with COBAS AMPLICOR™ *M. avium* Test (Roche Diagnostic, Tokyo, Japan). *Mycobacterium avium* ATCC 25291 and *Mycobacterium smegmatis* ATCC 700084 were obtained from American Type Culture Collection (Manassas, VA). *Escherichia coli* DH5α and XL2-Blue strains were from Toyobo Co., Ltd. (Osaka, Japan) and Stratagene (La Jolla, CA), respectively. The *E. coli* mycobacterial shuttle vector pGFM-11 [19] was provided by C. Locht, Pasteur Institute, Lille, France. pGFM-11 was digested with *Pst*I, resulting in deletion of the *gfp* gene, and the digested plasmid, named pM-11, was used as a vector for cloning and expression.

Plasmid DNA was isolated and purified with a NucleoSpin Plasmid Kit (BD Biosciences Clontech, Palo Alto, CA) for small-scale purification and a QIAGEN Large-Construct Kit (QIAGEN K.K., Tokyo, Japan) for large-scale purification. *Mycobacterium* spp. were grown and maintained at 37 °C in Ogawa egg-based medium (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan). *Escherichia coli* was cultured at 37 °C in Luria–Bertani (LB) broth (Nacalai Tesque, Inc., Kyoto, Japan) and maintained on LB agar plates (Nacalai Tesque).

### 2.2. Antimicrobial agents

RIF and kanamycin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nakalai Tesque, respectively. RIF was dissolved in methanol at 5 mg/mL and diluted in Middlebrook 7H9 broth medium (BD Diagnostic Systems, Sparks, MD) supplemented with 10% v/v BBL™ Middlebrook OADC (oleic acid, albumin, dextrose and catalase) enrichment (BD Diagnostic Systems) and 0.2% glycerol (7H9-OADC). Kanamycin was dissolved in sterilised distilled water at 50 mg/mL and stored at −20 °C.

### 2.3. Derivation of a RIF-resistant strain by serial passage

A RIF-resistant *M. avium* strain, ATCC 25291 Rif$^r$, was obtained by culturing the RIF-susceptible strain ATCC 25291 in the presence of RIF. Briefly, ATCC 25291 was grown on egg-based medium and then in 7H9-OADC at 37 °C until heavy growth was visible (ca. 2 weeks). The suspension was then inoculated into 7H9-OADC containing 1 μg/mL RIF. Every 3 or 4 days, the suspension was passaged six times in fresh 7H9-OADC containing RIF at two-fold increasing concentrations up to 32 μg/mL. Organisms growing in the medium containing 32 μg/mL RIF were plated onto Middlebrook 7H10 agar (BD Diagnostic Systems) supplemented with Middlebrook OADC enrichment (7H10-OADC agar) and 32 μg/mL RIF. Plates were incubated in a humidified incubator with 5% $CO_2$ at 37 °C until colonies formed. Organisms from a single colony, named *M. avium* ATCC 25291 Rif$^r$, were isolated and maintained in 7H9-OADC containing 32 μg/mL RIF.

### 2.4. RIF susceptibility testing

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of RIF. A serial two-fold dilution of RIF was prepared in 100 μL/well 7H9-OADC in 96-well plates (Nalge Nunc International, Rochester, NY). Clinical isolates of *M. avium* ATCC 25291 and ATCC 25291 Rif$^r$ grown on egg-based medium were inoculated into 7H9-OADC and grown for 2 weeks. The turbidity of the bacterial suspension was adjusted to McFarland value No. 1 ($OD_{530}$ 0.16) with 7H9-OADC. The adjusted bacterial suspension was diluted at 1:100, and then 100 μL/well was added to 7H9-OADC containing RIF in 96-well plates. The plates were cultured in 5% $CO_2$ at 37 °C. MICs were determined 7–14 days after culture when sufficient bacterial growth in the RIF-free control well was observed. As described in Section 3.1, the ATCC 25291 strain was sensitive to RIF. Therefore, the strain was used as a standard strain for the determination of MICs. Intermediate and high resistance to RIF were defined as MICs of 2–16 μg/mL and ≥32 μg/mL, respectively.

### 2.5. Bacterial DNA extraction and polymerase chain reaction (PCR) amplification of the rpoB gene

Genomic DNA of *M. avium* strains was extracted with DNAzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and was stored at −20 °C until used in PCR. *rpoB* genes from ATCC 25291, ATCC 25291 Rif$^r$ and all clinical isolates were amplified by PCR. The PCR

Table 1
Polymerase chain reaction (PCR) primers

| Primer | Sequences (5′ → 3′)[a] | Expected PCR product in *rpoB*[b] |
|---|---|---|
| MA-F1 | GCATCATGCATTTTGGCAGATTTCCGCCAGAGCAAGACGGA | 1–3510 |
| MA-R1a | GTTGGATGCATTTAAGCCAGGTCCTCGACGGACGCGGAT | |
| MA-F1 | GCATCATGCATTTTGGCAGATTTCCGCCAGAGCAAGACGGA | 1–1382 |
| MA-R1b | CGCTCCCGGGACAGACCA | |
| MA-F2 | AGGAGAAGCGCTACGACC | 887–2286 |
| MA-R2 | CTCCTCGGCGCCCAGCTT | |
| MA-F3a[c] | CGTGTTGACGTCCATCCACAT | 2217–3510 |
| MA-R1a | GTTGGATGCATTTAAGCCAGGTCCTCGACGGACGCGGAT | |
| MA-F3b[d] | ACGTGCTCACCTCGATCC | 2216–3510 |
| MA-R1a | GTTGGATGCATTTAAGCCAGGTCCTCGACGGACGCGGAT | |

[a] Underlined letters represent restriction enzyme sites.
[b] Numbers represents nucleotide positions of *rpoB*.
[c] The primer was designed for *rpoB* of *Mycobacterium avium* ATCC 25291 and ATCC 25291 Rif$^r$.
[d] The primer was designed for *rpoB* of *M. avium* P.15.

products were sequenced to detect mutations in *rpoB*. The complete DNA sequence of the *M. avium rpoB* has not been reported, although some of the genomic sequence of *M. avium* 104 strain is published in TIGR (The Institute for Genomic Research, Rockville, MD) database. The *M. avium rpoB* DNA sequence was identified by a BLAST search of the published genomic sequence of *M. avium* 104 against the *rpoB* DNA sequence of *M. tuberculosis* H37Rv (GenBank Accession No. NC_000962). PCR to amplify the *rpoB* gene was based on the obtained *M. avium rpoB* sequence. Briefly, the DNA template was amplified in 25 μL reaction solution containing 0.875 U Expand High Fidelity Enzyme Mix (Roche Applied Science, Mannheim, Germany), 0.2 mM deoxynucleotide triphosphate (Takara Bio Inc., Shiga, Japan), 0.3 μM primers, (1× Q-) Solution (QIAGEN Inc., Valencia, CA) and 1× PCR buffer (Roche Applied Science). A pair of PCR primers, MA-F2 and MA-R2, was designed to amplify 1400 bp of the 887–2286 region containing the 81 bp core region (nucleotides 1276–1356; codons 426–452) of *M. tuberculosis*, in which a mutation is known to be responsible for at least 96% of RIF-resistant *M. tuberculosis* isolates (Table 1) [13,14]. In addition to the MA-F2 and MA-R2 primer pair, two pairs (MA-F1 and MA-R1b; and MA-F3a and MA-R1a) for *M. avium* ATCC 25291 and ATCC 25291 Rif$^r$, and two pairs (MA-F1 and MA-R1b; and MA-F3b and MA-R1a) for *M. avium* P.15 were designed to amplify partially the sequence of *rpoB* gene (Table 1). PCR was carried out with a GeneAmp PCR System 9700 (Applied Biosystems Inc., Foster City, CA) and consisted of an initial 2 min denaturation at 94 °C followed by 30 cycles of 94 °C denaturation for 15 s, 60 °C annealing for 30 s, 72 °C elongation for 1 min and a final elongation at 72 °C for 7 min.

### 2.6. DNA sequencing of rpoB and analysis of sequence homology

PCR products of *rpoB* were purified with a Microcon YM-30 filter (Millipore Corp., Bedford, MA) and the sequencing reaction was performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Nucleotide and amino acid sequence homology were analysed with the GENETYX-WIN software system (Software Development, Co., Ltd., Tokyo, Japan).

### 2.7. PCR cloning of rpoB

The *rpoB* genes of *M. avium* ATCC 25291 and ATCC 25291 Rif$^r$ strains as well as a clinical isolate (P.15) were cloned and inserted into the *E. coli* mycobacterial shuttle vector pM-11 to construct pMA1, pMA2 and pMA3, respectively. PCR cloning primers with the *Eco*T22I site in both the 5′ and 3′ primers, MA-F1 and MA-R1a, were designed to amplify *rpoB* (Table 1). Genomic DNA of *M. avium* ATCC 25291, *M. avium* ATCC 25291 Rif$^r$ and *M. avium* P.15 was amplified in 50 μL reaction volume containing deoxynucleotide triphosphate (0.2 mM) (Takara Bio Inc.), 2.5 U Easy-A$^{TM}$ High Fidelity PCR Cloning Enzyme (Stratagene), 0.1 μM primers, 1× Q-Solution and 1× provided PCR buffer. The amplification was carried out in a GeneAmp PCR System 9700. The amplification reaction consisted of an initial 2 min denaturation at 95 °C followed by 30 cycles of 95 °C denaturation for 40 s, 60 °C annealing for 30 s, 72 °C elongation for 4 min and a final elongation at 72 °C for 7 min. PCR products were treated with *Eco*T22I, electrophoresed on 1% agarose gels (Wako Pure Chemical Industries, Ltd.) to separate the products, and purified from the gels with a Sephaglas$^{TM}$ BandPrep Kit (Amersham Biosciences). Purified PCR products were ligated into the *Pst*I site of the pGFM-11 vector with DNA Ligation Kit ver 2.1 I Solution (Takara Bio Inc.) according to the manufacturer's instructions. The plasmid was transformed into *E. coli* DH5α or *E. coli* XL2-Blue strains by heat shock. Transformants were plated onto LB agar containing 50 μg/mL kanamycin. The transformants were subcultured in LB broth containing 50 μg/mL kanamycin and the plasmid DNA was extracted from *E. coli* and purified with a NucleoSpin Plasmid Kit

Table 2
Bacterial strains and plasmids used for cloning

| Strains or plasmids | Characteristics | Reference or source |
|---|---|---|
| Strains | | |
| *Escherichia coli* XL2-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*$^q$Z$\Delta$*M15* Tn*10*(Tet$^r$) Amy Cam$^r$] | Stratagene |
| *E. coli* DH5$\alpha$ | F$^-$80d*laclac*ZYA-*arg*F)U169 *deo*R *rel*A1 *endA*1 *hsd*R17($r_K^-$,$m_K^+$) *pho*A *sup*E44 $^-$*thi*-1 *gry*A96 *rel*A1 | Toyobo Co. Ltd |
| *Mycobacterium avium* ATCC 25291 | Rifampicin-susceptible strain | |
| *Mycobacterium smegmatis* ATCC 700084 | Rifampicin-resistant strain (MIC = 16 μg/mL) | |
| Plasmids | | |
| pGFM-11 | Kanamycin-resistant, *E. coli*–mycobacterial shuttle vector | [19] |
| pM-11 | Kanamycin-resistant, pGFM-11 with deletion of 730 bp *Pst*I fragment (*gfp* gene) | This study |
| pMA1 | Kanamycin-resistant, pM-11 with 3.5 kbp *Eco*T22I fragment of *rpoB* gene of *M. avium* ATCC 25291 strain | This study |
| pMA2 | Kanamycin-resistant, pM-11 with 3.5 kbp *Eco*T22I fragment of *rpoB* gene of *M. avium* ATCC 25291 Rif$^r$ strain | This study |
| pMA3 | Kanamycin-resistant, pM-11 with 3.5 kbp *Eco*T22I fragment of *rpoB* gene of *M. avium* P.15 strain | This study |

according to the manufacturer's instructions. The *rpoB* insert was sequenced using the sequencing primers to ensure that the cloning procedure did not cause additional mutations to the cloned genes. pM-11 was used as a control. Plasmid DNA from each strain was prepared with a QIAGEN Large-Construct Kit, according to the manufacturer's instructions, to obtain a total of 400 μg plasmid DNA for electroporation. Plasmid DNA genotypes are listed in Table 2.

### 2.8. Electroporation into M. smegmatis

Electroporation of plasmid DNA pM-11, pMA1, pMA2 or pMA3 into *M. smegmatis* ATCC 700084 was performed as described by Parish and Stoker [20], in 0.2 cm gap width electroporation cuvettes (Bio-Rad Laboratories, Inc., Hercules, CA) in a Gene Pulser II Electroporation System (Bio-Rad Laboratories, Inc.) at room temperature, 2.5 kV, 25 μF and 1000 Ω. Transformants were incubated in 7H9-OADC agar at 37 °C for 4 h. After incubation, the organisms were plated onto 7H10-OADC agar containing 50 μg/mL kanamycin and incubated at 37 °C in 5% $CO_2$ until colonies formed. The colonies were inoculated into 7H9-OADC agar and cultured for 4 days. DNA was extracted from the transformants with DNAzol Reagent. The presence of plasmid DNA in the transformants was confirmed by DNA sequencing. RIF susceptibility of *M. smegmatis* with or without plasmid DNA pM-11, pMA1, pMA2 or pMA3 was determined.

## 3. Results

### 3.1. RIF susceptibility

RIF susceptibility of ATCC 25291 and ATCC 25291 Rif$^r$ strains and of 32 clinical isolates was examined (Table 3). ATCC 25291 was susceptible to RIF (MIC $\leq$ 0.03 μg/mL), whereas ATCC 25291 Rif$^r$ was resistant to RIF (MIC > 32 μg/mL). All clinical isolates were variably resistant to RIF (MIC $\geq$ 2 μg/mL) (Table 3). Seventeen clinical isolates (53.1%) were highly resistant to RIF (MIC $\geq$ 32 μg/mL) and 15 isolates (46.9%) showed intermediate resistance to RIF (MIC 2–16 μg/mL). The majority of isolates obtained from Japan (16/18; 88.8%) were highly resistant to RIF, 12/13 (92.3%) isolates obtained from Poland and one from Thailand showed intermediate resistance to RIF.

### 3.2. DNA sequencing of the 81 bp core region of rpoB

The 81 bp core region of *rpoB*, containing nucleotides 1276–1356 and corresponding to amino acid codons 426–452, is responsible for RIF resistance in *M. tuberculosis* [13]. The DNA sequence of this region was determined in the two laboratory strains and in all clinical isolates of *M. avium* tested. A C → T point mutation at nucleotide position 1333 was detected in the ATCC 25291 Rif$^r$ strain compared with the parent strain (Fig. 1). The mutation resulted in amino acid substitution His445 → Tyr. The DNA sequence of the 81 bp core region of *rpoB* was identical in all 32 clinical isolates tested and in the ATCC 25291 strain. When compared with the DNA sequence of *M. tuberculosis* H37Rv, *M. avium* ATCC 25291 and the clinical isolates tested showed six different nucleotides in this region. However, the amino acid sequence of this region in *M. tuberculosis* H37Rv was identical to those of *M. avium* ATCC 25291 and the clinical isolates.

### 3.3. DNA sequencing of the region downstream of the 81 bp core region

Because no mutations were found in the 81 bp core region in the clinical isolates, the DNA sequence of the region downstream of the 81 bp core region in *rpoB*, nucleotides 1357–1638 corresponding to amino acids 453–546, were determined in the laboratory strains and in all clinical

Table 3
Rifampicin susceptibility of *Mycobacterium avium* strains used in this study, and mutations detected in the region of the *rpoB* gene from 1357 to 1638 bp in clinical isolates of *M. avium*

| Strain | Origin | Rifampicin MIC (μg/mL)[a] | Codon (amino acid substitution, position) containing a mutation at position[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1407 | 1425 | 1479 | 1491 | 1495 | 1530 | 1548 | 1554 | 1563 | 1631 |
| ATCC 25291 | Demark | ≤0.03 | GTG (Val 469) | GGC (Gly 475) | TCG (Ser 493) | TAC (Tyr 497) | CGG (Arg 499) | TAC (Tyr 510) | GGC (Gly 516) | GTC (Val 518) | GAG (Glu 521) | GGC (Gly 544) |
| ATCC 25291 Rif$^r$ | Lab. | >32 | – | – | – | – | – | – | – | – | – | – |
| IMCJ.1 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.2 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.3 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.4 | Japan | >32 | – | – | – | – | – | TAT | GGA | – | GAA | GAC |
| IMCJ.5 | Japan | 32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.6 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.7 | Japan | 32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.8 | Japan | 2 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.9 | Japan | >32 | – | – | – | – | – | – | – | GTG | – | GAC |
| IMCJ.10 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.11 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.12 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.13 | Japan | 32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.14 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.15 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | GAA | GAC |
| IMCJ.16 | Japan | >32 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| IMCJ.17 | Japan | >32 | – | – | – | – | – | TAT | GGA | – | GAA | GAC |
| IMCJ.18 | Japan | 16 | – | – | TCA | – | – | – | GGA | – | – | GAC |
| P.2 | Poland | 2 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.3 | Poland | 4 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.4 | Poland | 8 | – | – | – | – | – | – | – | – | – | GAC |
| P.5 | Poland | 8 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.9 | Poland | 2 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.11 | Poland | 4 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.12 | Poland | 8 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.13 | Poland | 2 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.14 | Poland | 8 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.15 | Poland | 32 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.16 | Poland | 16 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.18 | Poland | 16 | – | – | – | TAT | – | – | – | – | – | GAC |
| P.19 | Poland | 16 | – | – | – | TAT | – | – | – | – | – | GAC |
| T.1 | Thailand | 8 | GTA | GGG | – | – | AGG | – | – | – | – | GAC |

–, same nucleotide as that of the ATCC 25291 strain.
[a] Minimum inhibitory concentrations (MICs) were determined by microdilution.
[b] Base changes are underlined.

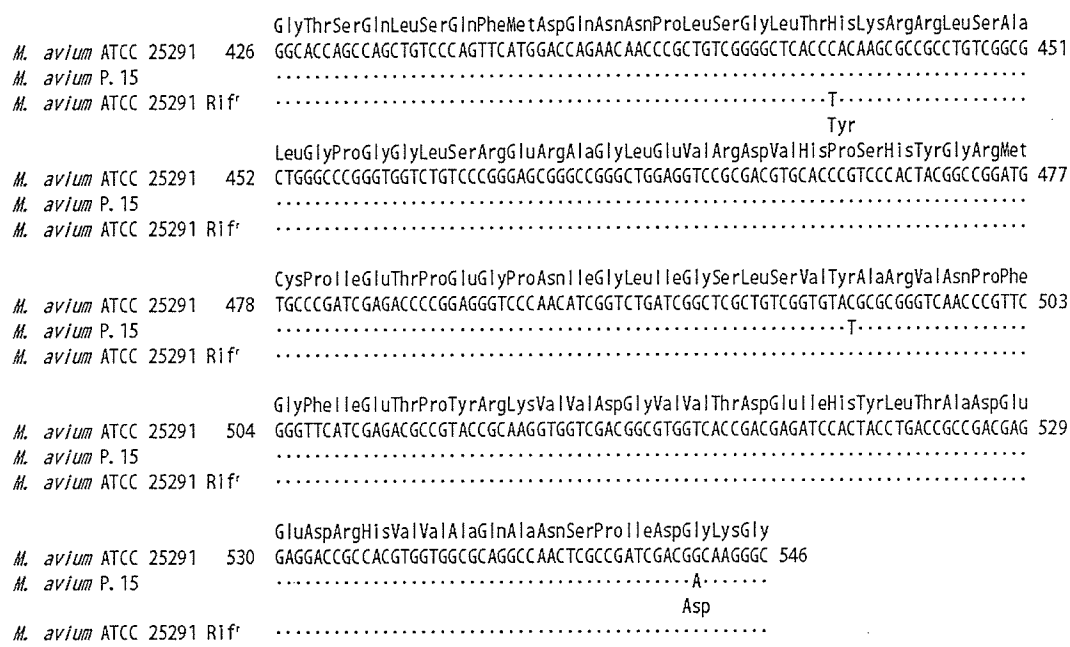


Fig. 1. Alignment of the nucleotide sequence and corresponding amino acid sequence of the 81 bp core region (amino acids 426–452) and downstream region (amino acids 453–546) of *Mycobacterium avium* strains ATCC 25291, P.15 and ATCC 25291 Rif$^r$. Numbers indicate the amino acid positions of *M. avium rpoB*.

isolates. No mutations were found in this region in the ATCC 25291 Rif$^r$ strain in comparison with the sequence of the parent strain. However, a G → A point mutation at nucleotide 1631 leading to amino acid substitution Gly544 → Asp was found in this region in all clinical isolates. In addition, point mutations at nine other positions, which did not lead to amino acid substitutions, were found in the clinical isolates but not in the ATCC 25291 Rif$^r$ strain. These mutations were G → A at nucleotide 1407, C → G at 1425, G → A at 1479, C → T at 1491, C → A at 1495, C → T at 1530, C → A at 1548, C → G at 1554 and G → A at 1563 (Table 3; Fig. 1). The number of these silent mutations varied among the isolates and ranged from one to three. Of 18 isolates obtained from Japan, 15 (83.3%) and 17 (94.4%) possessed mutations of G → A at 1479 and C → A at 1548, respectively, and 15 (83.3%) possessed both mutations. Two isolates obtained from Japan, IMCJ.4 and IMCJ.17, possessed three mutations of C → T at 1530, C → A at 1548 and G → A at 1563. One isolate, IMCJ.15 possessed three mutations of G → A at 1479, C → A at 1548 and G → A at 1563. Of 13 isolates obtained from Poland, 12 (92.3%) possessed a mutation of C → T at 1491. The isolate obtained from Thailand, T.1, possessed three mutations of G → A at 1407, C → G at 1425 and C → A at 1495.

### 3.4. DNA sequencing of the complete rpoB gene from M. avium ATCC 25291, ATCC 25291 Rif$^r$ and clinical isolate P.15

The sequence of the complete open reading frame of *rpoB* was determined in *M. avium* ATCC 25291 and ATCC 25291 Rif$^r$, and in the clinical isolate P.15. All showed the same size *rpoB* gene (3510 bp). The *M. avium* ATCC 25291 *rpoB* nucleotide sequence was 89% homologous to that of *M. tuberculosis* H37Rv (3519 bp), and the amino acid sequence was 94% homologous to that of *M. tuberculosis* H37Rv. There were two mutations in ATCC 25291 Rif$^r$ *rpoB* in comparison with the parent strain: one was a mutation of C → T at nucleotide 1333 that led to amino acid substitution His445 → Tyr, and the other was a silent mutation at nucleotide 570. In the clinical isolate 16 mutations were identified, of which one mutation of G → A at nucleotide 1631 led to the amino acid substitution Gly554 → Asp described above. The other 15 mutations did not lead to any amino acid substitutions.

### 3.5. RIF susceptibility of M. smegmatis transformed with the M. avium rpoB gene

The *rpoB* genes of *M. avium* ATCC 25291, ATCC 25291 Rif$^r$ and the clinical isolate P.15 were cloned into pM-11, as described in Section 2.7. The genes were expressed in *M. smegmatis* because expression was not successful in *M. avium* ATCC 25291 and because it was reported that pAL5000, the ancestor of pM-11, is unable to transform members of the *M. avium* complex [6,21]. MICs of RIF for the transformants were then determined (Table 4). *Mycobacterium smegmatis* ATCC 700084 was relatively resistant to RIF (MIC 16 μg/mL) in comparison with *M. avium* ATCC 25291. MICs for *M. smegmatis* ATCC 700084 organisms carrying pM-11, pMA1 (ATCC 25291) and pMA3 (P.15) were 16 μg/mL,

Table 4
Rifampicin minimum inhibitory concentrations (MICs) for transformants of *Mycobacterium smegmatis* strain ATCC 700084

| Strain | Plasmid[a] | Characteristic | Rifampicin MIC (μg/mL) |
|---|---|---|---|
| | None | Host strain | 16 |
| | pM-11 | Transformant harbouring cloning vector for *M. avium rpoB* gene | 16 |
| | pMA1 | Transformant harbouring the wild-type *rpoB* gene ligated to pM-11 | 16 |
| | pMA2 | Transformant harbouring *rpoB* gene with an amino acid substitution His445 → Tyr ligated to pM-11 | 64 |
| | pMA3 | Transformant harbouring *rpoB* gene with an amino acid substitution Gly544 → Asp ligated to pM-11 | 16 |

[a] Characteristics of plasmids are cited in Table 2.

identical to that of the parent strain. The MIC for the *M. smegmatis* ATCC 700084 strain carrying pMA2 (ATCC 25291 Rif$^r$) was significantly greater (64 μg/mL).

## 4. Discussion

It has been reported that *M. avium* isolates associated with human disease are more resistant to RIF than are isolates from natural sources [22]. In fact, all clinical isolates tested in this study, obtained from three geographically separate countries, were relatively resistant to RIF. On the other hand, the parental ATCC 25291 strain isolated from lesions of a hen [23] was quite sensitive to RIF. The number of *M. avium* and *Mycobacterium intracellulare* isolates that are significant in human disease is related to the number of isolates from environmental sources such as soil and house dust [22]. Unsuitable management of bath water occasionally causes waterborne *M. avium* infection [3,4]. These data suggest that some, but not all, strains of *M. avium* existing in the environment are able to infect humans and to cause chronic pulmonary disease. The virulence of *M. avium* strains is probably associated with drug resistance.

Mutation of the 81 bp core region of *rpoB* participates in RIF resistance in *M. avium*; a mutation was identified in the RIF-resistant laboratory Rif$^r$ strain. *Mycobacterium smegmatis* expressing the *rpoB* gene of the clinical isolate P.15 was resistant to RIF, but not more than the parent strain. Two RIF-resistant isolates from Belgium and the USA were reported to possess a mutation causing an amino acid substitution; however, whether they were clinical isolates was not mentioned [11].

Factors other than *rpoB* mutations may participate in RIF resistance in clinical isolates of *M. avium*. No mutations were identified in the 81 bp core region of *rpoB* in any clinical isolate in this study. A point mutation leading to an amino acid substitution was identified at nucleotide 1631 in the region downstream from the 81 bp core region in all clinical isolates. Nevertheless, the *M. smegmatis* strain expressing clinical isolate P.15 *rpoB* was not more resistant to RIF than the parent strain. These results indicate that mutations in *rpoB* are not associated with RIF resistance in the clinical isolates of *M. avium*. However, we cannot rule out the possibility that the *rpoB* gene of Japanese isolates that are more resistant to RIF than P.15 was associated with the RIF resistance. In addition, the level of RIF resistance of the clinical isolates differed. For example, most isolates obtained from Japan were highly resistant to RIF, whereas most isolates obtained from Poland or Thailand showed intermediate resistance (Table 3), suggesting that these isolates may possess different mechanisms of RIF resistance. A permeability barrier to RIF has been reported in *M. intracellulare* [8]. Efflux pumps may remove RIF in some *Mycobacterium* spp., but they appear to have little effect on accumulation of the drug [24]. Ribosylation of RIF has also been reported in fast-growing *Mycobacterium* strains such as *M. smegmatis* [25].

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NOTE

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# Emergence of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* in tuberculosis wards

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**Abstract** To assess whether the occurrence of rifampicin (RFP) resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) is related to treatment of tuberculosis, we determined the RFP susceptibility of MRSA isolates obtained from tuberculosis patients and screened for mutation(s) in the *rpoB* gene of these isolates. The MICs of RFP for 84 MRSA isolates obtained from two hospitals in Japan were determined. DNA was sequenced in the region 1318–1602 nucleotides (nt) of the *rpoB* gene, which includes RFP resistance-determining clusters I (1384–1464 nt, 462–488 amino acids). The majority of MRSA isolates from tuberculosis wards, i.e., 48 of 51 (94%) [33 of 34 in a Tokyo hospital (97%) and 15 of 17 in a Chubu hospital (88%)], were resistant to RFP. Meanwhile, no isolates of 33 from the other wards were resistant to RFP. All RFP-resistant MRSA isolates had a mutation(s), including novel mutation(s) such as Val453→Phe, Asp471→Asn, and Ile527→Leu, in *rpoB*. An emergence of RFP-resistant MRSAs in tuberculosis wards in Japan was strongly suggested.

**Key words** Rifampicin · Drug resistance · MRSA · *rpoB* · Tuberculosis

Rifampicin (RFP) is one of the first-line antituberculous agents and also a potent antimicrobial agent against methicillin-resistant *Staphylococcus aureus* (MRSA).[1,2] RFP acts by interacting in a specific manner with the β-subunit of the bacterial RNA polymerase encoded by the *rpoB* gene.[3] In MRSA infections, RFP is often used in combination with antibiotics with lower penetrability, such as vancomycin.[4,5] The combination therapy with RFP revealed strong activity and good tissue penetration that is required to reach deep-seated infections effectively.[4,5] In such a situation, there is a high risk of emergence of RFP-resistant MRSA. Most RFP-resistant MRSA organisms and other bacteria are known to have a mutation(s) in the particular regions, clusters I and II in the *rpoB* gene encoding the RNA polymerase β-subunit.[4–7]

In the present study, we examined RFP susceptibility of MRSA isolates obtained from inpatients with tuberculosis and screened for mutations in the *rpoB* gene of these isolates. A total of 84 MRSA isolates obtained from hospitals in Tokyo[8–12] and Chubu district[13] were analyzed. *S. aureus* ATCC29213 and ATCC700699 strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Of these isolates, 51 were obtained from tuberculosis wards in both hospitals (34 from a hospital in Tokyo during an MRSA outkreak in 2001[12] and MRSA surveillance studies done before and after the outbreak in 2000–2003[8–11] and 17 from a hospital in Chubu during an MRSA outbreak[13]), and 33 other isolates were from other wards in a Tokyo hospital.[8] All MRSA isolates were analyzed by pulsed-field gel electrophoresis (PFGE) as described previously.[8–13] Differences between tuberculosis wards and the other wards in the isolation numbers of MRSA were analyzed by Fisher's exact probability test. A $P$ value <0.05 was considered statistically significant.

The minimum inhibitory concentration (MIC) of RFP was determined by an E-test (AB BIODISK, Dalvagen, Sweden), and the result was interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards.[14] The staphylococcal breakpoint for resistance to RFP is defined as ≥4 μg/ml (susceptible is defined as ≤1 μg/ml).[14]

The distribution of RFP MICs for the MRSA isolates obtained from tuberculosis and other wards is shown in Fig. 1. The MICs of RFP ranged from ≤0.002 to ≥256 μg/ml.

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Among the 84 MRSA isolates, 48 were resistant to RFP with MIC ≥48 μg/ml. The other isolates were susceptible to RFP with MIC ≤0.015 μg/ml. The majority of MRSA isolates from tuberculosis wards, i.e., 48 of 51 (94%) [33 of 34 in a Tokyo hospital (97%) and 15 of 17 in a Chubu hospital (88%)], were resistant to RFP (Fig. 1, Table 1). Meanwhile, 0 of 33 isolates from the other wards were resistant to RFP ($\chi^2 = 72.47$, $P < 0.001$) (see Table 1).

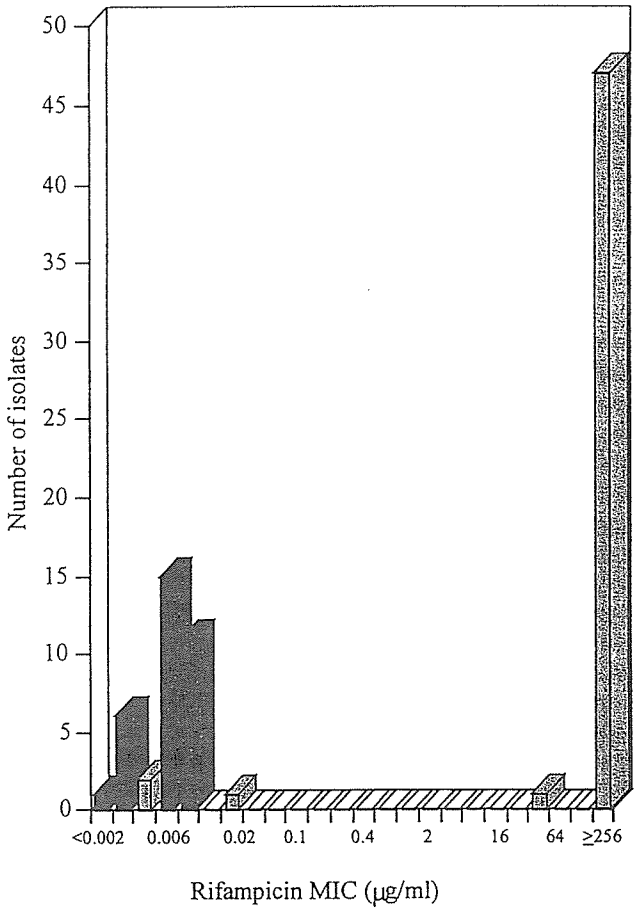


**Fig. 1.** Distribution of rifampicin minimum inhibitory concentrations (*MICs*) for 84 methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in Tokyo and Chubu hospitals. *Gray bars* represent MRSA isolates obtained from tuberculosis wards; *black bars* represent MRSA isolates from other wards

The DNA sequence of the region of 1318–1602 at nucleotide positions (nt) of *rpoB*, corresponding to codons 440–534 (amino acid number, aa number), which includes the RFP resistance-determining cluster I (1384–1464 nt, 462–488 aa)[4] and cluster II (1543–1590 nt, 515–530 aa)[4] of *S. aureus* were amplified by polymerase chain reaction (PCR) with the primers rpoB-F (5′-CCG TCG TTT ACG TTC TGT AGG-3′) and rpoB-R (5′-AAA GCC GAA TTC ATT TAC ACG-3′). PCR products were sequenced with the same primers by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, USA). Of 84 isolates analyzed, 32 had one mutation and 16 had two mutations in clusters I and II of *rpoB* (Table 2). A total of 64 mutations were identified, and all mutations resulted in amino acid substitution. Of them, 60 mutations were located in cluster I: 19 were Ala 477→Asp, 14 were Ser 486→Leu, 12 were His 481→Asp, 12 were Ala 473→Thr, 1 was Ser 464→Pro, 1 was Gln 468→Leu, and 1 was Asp 471→Asn. Three mutations were located in cluster II; all three were Ile 527→Leu. One was found in the region upstream from cluster I, i.e., Val453→Phe. All mutations except for the three mutations, Asp471→Asn, Ile527→Leu, and Val453→Phe, were already reported to be related to RFP resistance in *S. aureus*.[4–7] Type 3 isolates were resistant to RFP and had a single mutation of Asp471→Asn, indicating that the *rpoB* mutation was associated with RFP resistance. The mutations at 527 aa, Ile527→Phe or Ile527→Met, were known to be related to RFP resistance.[2] However, whether the mutation Ile527→Leu at the same position was associated with RFP resistance is unclear, because additional mutations known to be related to RFP resistance were present (see type 4 and 5 isolates, Table 2). The association of Val453→Phe with RFP resistance is also unclear because there was an additional mutation associated with RFP resistance (see type 11 isolates, Table 2). Nevertheless, three novel mutations of Asp471→Asn, Ile527→Leu, and Val453→Phe were identified in *S. aureus*.

Based on RFP susceptibility testing, PFGE genotyping, and DNA sequencing of *rpoB*, the MRSA isolates from tuberculosis wards were classified into 23 types (see Table 2). Among 84 isolates, 12 isolates from a Chubu district hospital (type 7) were resistant to RFP (MIC, >256 μg/ml), showed PFGE pattern A2(M1), and had a mutation of Ala 477→Asp; 11 isolates from a Tokyo hospital (type 12) were resistant to RFP (MIC, >256 μg/ml), showed PFGE pattern

**Table 1.** Frequency of rifampicin-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in tuberculosis wards

| Rifampicin susceptibility | No. (%) of isolates | | | |
|---|---|---|---|---|
| | Tuberculosis wards | | | Other wards |
| | Tokyo[a] ($n = 34$) | Chubu district[b] ($n = 17$) | Total ($n = 51$) | T ($n = 33$) |
| Resistant | 33 (97%) | 15 (88%) | 48 (94%) | 0 (0%) |
| Susceptible | 1 (3%) | 2 (12%) | 3 (6%) | 33 (100%) |

[a] Tokyo hospital
[b] Chubu district hospital