II. 研究成果の刊行に関する一覧表

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研究成果の刊行に関する一覧表

雑　誌

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III. 研究成果の刊行物・別冊

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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).

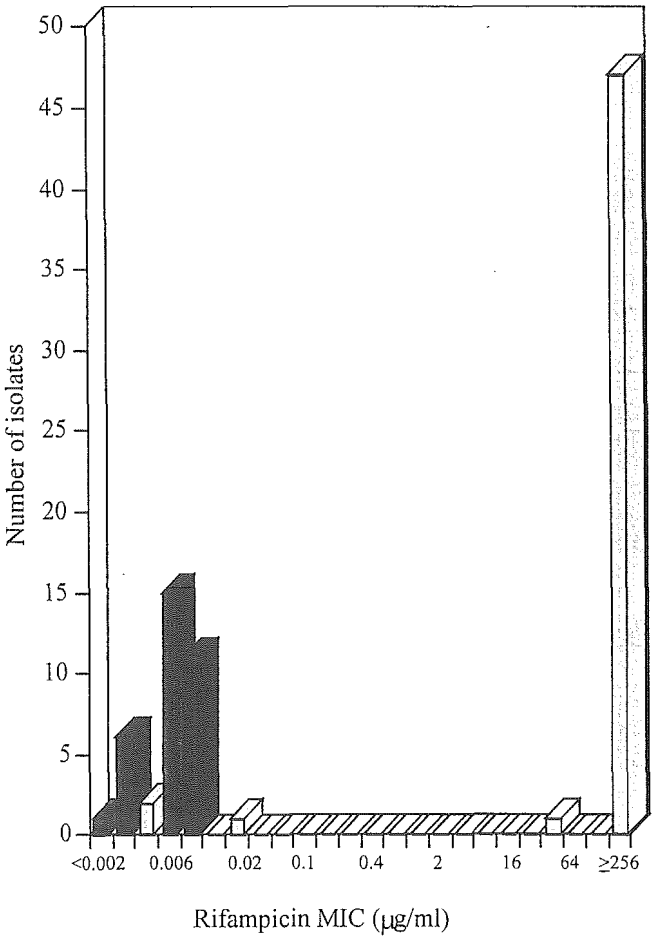

Fig. 1. Distribution of rifampicin minimum inhibitory concentrations (*MICs*) for 84 methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in Tokyo and Chubu hospitals. *Gray bars* represent MRSA isolates obtained from tuberculosis wards; *black bars* represent MRSA isolates from other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

Table 2. Resistance to rifampicin and mutations in the *rpoB* gene of *S. aureus* in tuberculosis wards

Hospital[a]	Specimen or reference strain	No. of isolates	Rifampicin MIC (μg/ml)	PFGE genotype[b]	*rpoB* gene		Type no. assigned
					Nucleotide changes[c]	Amino acid changes[d]	
T	Sputum	1	48	A14	TCT→CCT	Ser464→Pro	1
T	Sputum	1	>256	A2(M1)	CAA→CTA	Gln468→Leu	2
T	Sputum	1	>256	F6	GAC→AAC	Asp471→Asn[e]	3
T	Sputum	1	>256	F2	GCT→GAT, ATT→CTT	Ala477→Asp, Ile527→Leu[e]	4
T	Sputum	2	>256	F4	GCT→GAT, ATT→CTT	Ala477→Asp, Ile527→Leu[e]	5
C	Gastric juices	1	>256	M2(A18)	GCT→GAT	Ala477→Asp	6
C	Sputum	12	>256	A2(M1)	GCT→GAT	Ala477→Asp	7
C	Sputum	1	>256	AO	GCT→GAT	Ala477→Asp	8
C	Sputum	1	>256	M7	GCT→GAT	Ala477→Asp	9
T	Sputum	1	>256	G2	GCT→GAT	Ala477→Asp	10
T	Sputum	1	>256	J1	GTT→TTT, TCA→TTA	Val453→Phe[e], Ser486→Leu	11
T	Arterial blood	1	>256	J1	TCA→TTA	Ser486→Leu	12
	Sputum	9					
	Nasal cavity	1					
T	Nasal cavity	1	>256	J2	TCA→TTA	Ser486→Leu	13
T	Sputum	1	>256	J4	TCA→TTA	Ser486→Leu	14
T	Thorax drain	1	>256	R1	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	15
	Sputum	1					
T	Sputum	3	>256	J7(R2)	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	16
	Arterial blood	1					
	Urine	1					
T	Sputum	1	>256	J8	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	17
T	Sputum	1	>256	A1	CAT→TAT	His481→Asp	18
T	Sputum	1	>256	A2(M1)	CAT→TAT	His481→Asp	19
T	Urine	1	>256	S	CAT→GAT	His481→Asp	20
T	Sputum	1	>256	A22	CAT→TAT	His481→Asp	21
T	Sputum	1	0.015	AU1	No change	No change	22
C	Sputum	2	0.005	A3	No change	No change	23
	ATCC29213		0.005	–	No change	No change	
	N315		0.004	–	–	–	

MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis
[a]T, Tokyo hospital; C, Chubu district hospital
[b]Data from references 8–13
[c]Base changes are underlined
[d]The numbering of the amino acids is based on that of *S. aureus* N315 (GenBank accession no. NC-002745)
[e]Novel mutation

J1, and had mutation Ser486→Leu; and 5 isolates from Tokyo (type 16) were resistant to RFP (MIC, >256μg/ml), showed PFGE pattern J7(R2), and had two mutations of Ala 473→Thr and His 481→Asp, indicating that there was clonal expansion of these RFP-resistant MRSA strains in tuberculosis wards in both hospitals. Sixteen isolates of types 1–4, 6, 8–11, 13, 14, and 17–21 were resistant to PFP, but showed different genotypes (PFGE patterns and *rpoB* mutations), indicating that individual strains of RFP-resistant MRSA existed in tuberculosis patients. Collectively, these results suggest that there were two types of transmission mode of MRSA isolates: some were transmitted within tuberculosis wards and the others were brought from outside the wards.

In conclusion, MRSAs obtained from tuberculosis wards in two hospitals in Japan had resistance to RFP and mutation(s) in the particular regions of *rpoB*. It is difficult to conclude that RFP-resistant MRSA isolates were emerging in the wards during RFP therapy. Nevertheless, the present results strongly suggest an emergence of such MRSAs in tuberculosis wards in Japan. It is necessary to monitor PFP resistance in both tuberculosis and other wards.

The DNA sequences of part of the *rpoB* of MRSA reported here were registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB195713, AB195714, and AB195715.

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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) $\geq$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 $\leq$ 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 $\geq$ 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™ 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™ 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}ZΔ*M15* Tn*10*(Tet^r) Amy Cam^r]	Stratagene
E. coli DH5α	F$^-$ 80d*lacZ*YA-*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 ≤ 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 ≥ 2 μg/mL) (Table 3). Seventeen clinical isolates (53.1%) were highly resistant to RIF (MIC ≥ 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.

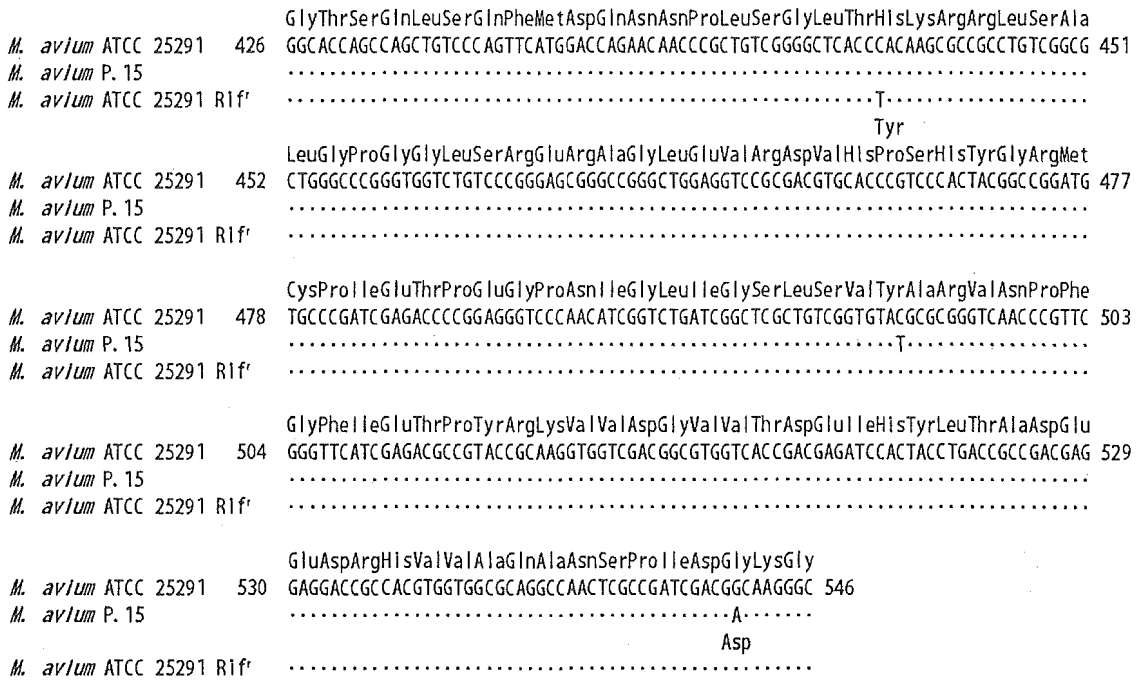

Fig. 1. Alignment of the nucleotide sequence and corresponding amino acid sequence of the 81 bp core region (amino acids 426–452) and downstream region (amino acids 453–546) of *Mycobacterium avium* strains ATCC 25291, P.15 and ATCC 25291 Rif^r. Numbers indicate the amino acid positions of *M. avium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