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Characterization of a Trinucleotide Repeat Sequence $(CGG)_5$ and Potential Use in Restriction Fragment Length Polymorphism Typing of *Mycobacterium tuberculosis*

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The genomes of 28 bacterial strains, including mycobacterial species *Mycobacterium tuberculosis* and *Mycobacterium bovis*, were analyzed for the presence of a special class of microsatellite, that of trinucleotide repeat sequences (TRS). Results of a search of all 10 possible TRS motifs (i.e., CCT, CGG, CTG, GAA, GAT, GTA, GTC, GTG, GTT, and TAT) with five or more repeating units showed that $(CGG)_5$ was highly represented within the genomic DNA of *M. tuberculosis* and *M. bovis*. Most of the $(CGG)_5$ repeats in the genome were within the open reading frames of two large gene families encoding PE_PGRS and PPE proteins that have the motifs Pro-Glu (PE) and Pro-Pro-Glu (PPE). $(CGG)_5$-probed Southern hybridization showed that some mycobacterial species, such as *Mycobacterium marinum*, *Mycobacterium kansasii*, and *Mycobacterium szulgai*, possess many copies of $(CGG)_5$ in their genomes. Analysis of clinical isolates obtained from Tokyo and Warsaw with both IS*6110* and $(CGG)_5$ probes showed that there is an association between the fingerprinting patterns and the geographic origin of the isolates and that $(CGG)_5$ fingerprinting patterns were relatively more stable than IS*6110* patterns. The $(CGG)_5$ repeat is a unique sequence for some mycobacterial species, and $(CGG)_5$ fingerprinting can be used as an epidemiologic method for these species as well as IS*6110* fingerprinting can. If these two fingerprinting methods are used together, the precise analysis of *M. tuberculosis* isolates will be accomplished. $(CGG)_5$-based fingerprinting is particularly useful for *M. tuberculosis* isolates with few or no insertion elements and for the identification of other mycobacterial species when informative probes are lacking.

DNA fingerprinting of the inserted IS*6110* element specific for the *Mycobacterium tuberculosis* complex is a powerful epidemiological tool for visualizing DNA restriction fragment length polymorphisms (RFLP) of *M. tuberculosis* (26). The major limitation of IS*6110*-based RFLP typing is the difficulty of discriminating genetic polymorphisms of *M. tuberculosis* isolates with only a few copies of the element. In addition, there are two reports (1, 29) that described IS*6110*-based RFLP as unstable, although other studies have confirmed a high degree of stability (5, 15). Yeh and colleagues (29) indicated that genotypes with IS*6110* were relatively unstable because they changed rapidly compared with those based on another marker. Alito et al. (1) reported that a multidrug-resistant outbreak strain changed rapidly, according to IS*6110* RFLP, over a period of a few years.

A number of alternative typing methods for *M. tuberculosis* isolates that use genetic markers, such as polymorphic GC-rich repetitive sequences (PGRS) (19), tandem repeat sequences of 10 bp found in PPE family proteins (10), the direct repeat (9), a $(GTG)_5$ repeat (28), IS*1547* (6), *katG* (30), and tandem repeats of 40 to 100 bp (14, 24), have been reported.

Trinucleotide repeat sequences (TRS) comprise a class of microsatellites that are involved in human neurodegenerative diseases (27). Studies in *Escherichia coli* showed that these TRS, such as $(CTG)_n$ and $(CGG)_n$, may effect genetic instability during DNA replication, transcription, and repair processes (17). $(GAA)_{12}$ has been found in a plasmid of *Mycoplasma gallisepticum* (12, 13), and it positively regulates gene expression in this plasmid. It is not as well known whether bacterial genomes possess tandem repeat sequences. The types, lengths, and distribution of such sequences may serve as valuable markers for phylogenetic or epidemiologic studies of various bacteria.

In the present study, we searched for all possible TRS in various bacterial strains and found that *M. tuberculosis* and *Mycobacterium bovis* possess many $(CGG)_5$ repeats. We also analyzed *M. tuberculosis* clinical isolates obtained from Japan and Poland with $(CGG)_5$-based DNA fingerprinting and show that this method is useful for the genetic analysis of clinical isolates of *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains. The sources of mycobacterial strains used in this study are listed in Table 1. Clinical isolates were obtained from the International Medical

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TABLE 1. Mycobacterial strains used in this study

Strain	Property or origin[a]	Source or reference
M. tuberculosis H37Rv	ATCC 27294	ATCC
M. tuberculosis H37Ra	ATCC 25177	ATCC
M. abscessus	Clinical isolate (IMCJ 268)	IMCJ
M. avium	ATCC 25291	ATCC
M. bovis BCG	Japanese strain 172	Japan BCG Laboratory
M. chelonae	JCM 6390 (ATCC 14472)	JCM
M. fortuitum	Clinical isolate (IMCJ 531)	IMCJ
M. gastri	GTC 610 (ATCC 15754)	GTC
M. intracellulare	JCM 6384 (ATCC 13950)	JCM
M. kansasii	JCM 6379 (ATCC 12478)	JCM
M. marinum	GTC 616 (ATCC 927)	GTC
M. nonchromogenicum	JCM 6364 (ATCC 19530)	JCM
M. peregrinum	Clinical isolate (IMCJ 460)	IMCJ
M. scrofulaceum	JCM 6381 (ATCC 19981)	JCM
M. simiae	GTC 620 (ATCC 25275)	GTC
M. smegmatis	ATCC 19420	ATCC
M. szulgai	JCM 6383 (ATCC 35799)	JCM
M. terrae	GTC 623 (ATCC 15755)	GTC
M. xenopi	Clinical isolate (IMCJ 788)	IMCJ

[a] JCM, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Saitama, Japan; GTC, Gifu Type Culture Collection, Department of Microbiology-Bioinformatics, Regeneration and Advanced Medical Science, Gifu University, Graduate School of Medicine, Bacterial Genetic Resources, Gifu, Japan.

Center of Japan (IMCJ) in Tokyo, Japan, in 2001 and from the National Research Institute of Tuberculosis and Lung Diseases in Warsaw, Poland, in 2000. These clinical isolates were obtained from different patients. Drug susceptibility testing was performed by conventional culture on solid media with a proportion method (Wellpack; Japan BCG Laboratory, Tokyo, Japan) or by a microdilution method with Vit spectrum SR (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan). The antituberculosis drugs tested and the concentrations used were as follows: isoniazid, 0.2 and 1.0 μg/ml; rifampin, 40 μg/ml; ethambutol, 2.5 μg/ml; streptomycin, 10 μg/ml; *para*-aminosalicylic acid, 0.5 μg/ml; cycloserine, 30 μg/ml; ethionamide, 20 μg/ml; kanamycin, 20 μg/ml; enviomycin, 20 μg/ml; and levofloxacin, 1.0 μg/ml. Drug resistance is defined as resistance to at least one drug. Serial cultures were made from *M. tuberculosis* strain H37Rv and a clinical isolate from Japan (IMCJ 541) and were passaged weekly over 9 weeks.

Genome sequence. The genome sequences of 28 bacterial strains were downloaded from the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html), The Institute for Genomic Research website (http://www.tigr.org/CMR), the Sanger Center (http://www.sanger.ac.uk), and the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Isolation and restriction enzyme digestion of mycobacterial DNA. Chromosomal DNA of the mycobacterial strains and *M. tuberculosis* clinical isolates were prepared as described previously (16, 26) with slight modifications. Briefly, for isolation of genomic DNA, *M. tuberculosis* strains were grown on egg-based Ogawa solid medium (Kyokuto Pharmaceutical Co., Ltd.) for 3 to 5 weeks. All bacterial cells from one slant were transferred to 400 μl of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA [pH 8.0]), and the solution was heated at 80°C for 20 min to kill the bacteria. Fifty microliters of lysozyme (10 mg/ml) was added, and the tube was incubated overnight at 37°C. Seventy microliters of sodium dodecyl sulfate (10%) and 5 μl of proteinase K (10 mg/ml) were added, and the mixture was incubated for 10 min at 65°C. A 100-μl volume of 5 M NaCl and the same volume of an *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB)-NaCl solution (4.1 g of NaCl and 10 g of CTAB per 100 ml) were added together. The tubes were vortexed and incubated for 10 min at 65°C. An equal volume of chloroform-isoamylalcohol (24:1) was added, the mixture was centrifuged for 5 min at 12,000 × *g*, and the aqueous supernatant was carefully transferred to a fresh tube. The total DNA was precipitated in isopropanol and was redissolved in 20 μl of 0.1× TE buffer. All restriction enzymes used in this study, AatII, AfaI, AluI, EcoRI, HinfI, MluI, NruI, NsbI, PstI, PvuII, SacI, Sau3AI, SalI, SmaI, XhoI, and XspI, were purchased from Takara Bio Inc. (Shiga, Japan). Chromosomal DNA was digested overnight with each restriction enzyme (1 U/μg of DNA) under the conditions specified by the manufacturer. The digested fragments were separated by electrophoresis on horizontal 1% agarose gel at 15 V for 20 h (14-cm gel) in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). A 1-kb DNA ladder and λ DNA restricted with HindIII (Promega Corp., Madison, Wis.) were used as size markers. The gels were then stained with ethidium bromide, and the results were recorded photographically.

Southern blotting. Gels were depurinated in 0.25 M HCl for 30 min and then denatured in 0.5 M NaOH and 1.5 M NaCl for 30 min. DNA fragments were transferred to an N^+ Hybond membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) overnight, and the DNA was fixed to the membrane by UV irradiation.

The IS*6110* probe used in this study was a 245-bp DNA fragment amplified by PCR as described previously (26). Briefly, oligonucleotides INS1 (5′-CGTGAG GGCATCGAGGTGGC-3′) and INS2 (5′-GCGTAGGCGTCGGTGACAAA-3′) were used to amplify a 245-bp fragment from purified chromosomal *M. bovis* BCG DNA by PCR. The 15-mer oligonucleotide $(CGG)_5$, 5′-CGGCGGCGGC GGCGG-3′, was synthesized (Nippn TechnoCluster, Inc., Tokyo, Japan). These probes were labeled with horseradish peroxidase by the ECL direct system (Amersham Biosciences). Hybridization and detection were performed according to the recommendations of the manufacturer. Autoradiographs were obtained by exposing the membrane to X-ray film.

Analysis. IS*6110*- and $(CGG)_5$-based fingerprinting patterns were analyzed with Molecular Analyst Fingerprinting Plus software, version 1.6 (Bio-Rad Laboratories, Inc., Hercules, Calif.). To facilitate the comparison of the fingerprinting patterns, normalization was carried out with the use of molecular weight standards and the IS*6110*- or $(CGG)_5$-fingerprinting patterns of two clinical isolates, IMCJ 541 and a Poland-derived isolate, no. 28 (P 28), on each gel. Each dendrogram was calculated with the unweighted pair group method with average linkage according to the supplier's instructions.

RESULTS

Presence of TRS in mycobacterial strains and other bacterial species. To detect TRS among bacterial genomes and to determine the types of TRS and their repeat sizes, we searched for all 10 possible TRS motifs (i.e., CCT, CGG, CTG, GAA, GAT, GTA, GTC, GTG, GTT, and TAT) of five or more repeating units with the BLASTN algorithm (2). Among 28 bacterial strains, the numbers of TRS displayed large variation, with values ranging from zero to 38 (shown in the extreme right column in Table 2). *M. tuberculosis* strains H37Rv and CDC1551 and *M. bovis* possessed markedly more TRS copies than other species examined. The majority of the other species possessed fewer than 10 copies. Five strains, *Listeria innocua*, *Listeria monocytogenes*, *Staphylococcus aureus* N315, *Thermoplasma acidophilum*, and *Thermoplasma volcanium*, did not possess any TRS. The types of TRS varied (Table 2). $(CCT)_5$ did not exist in any of the bacteria examined in this study. CGG repeats, predominantly $(CGG)_5$, existed with high frequency in the genomes of *M. tuberculosis* strains H37Rv and CDC1551 and *M. bovis*; the frequencies of the appearance of CGG with five or more repeats were one per 150 to 200 kb. *Neisseria meningitidis* MC58 and *Pseudomonas aeruginosa* possessed six copies of $(CGG)_5$ with a frequency of one copy per 380 kb and five copies with a frequency of one copy per 1,250 kb, respectively. Few $(CGG)_5$ repeats were found in *E. coli* K12-MG1655, *E. coli* O157:H7 EDL933, *E. coli* O157:H7 VT2-Sakai, *N. meningitidis* serogroup A Z2491, *Salmonella enterica*, and *S. enterica* serovar Typhimurium. There were no $(CGG)_5$ repeats in *Clostridium acetobutylicum*, *Clostridium perfringens*, *Helicobacter pylori* 26695, *H. pylori* J99, *L. innocua*, *L. monocytogenes*, *Mycobacterium leprae*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Mycoplasma pulmonis*, *Rickettsia conorii*, *Rickettsia prowazekii*, *S. aureus* Mu50, *S. aureus* N315, *T. acidophilum*, *T. volcanium*, and *Yersinia pestis*. Other possible repeats of CTG, GAA, GAT, GTA, GTC, GTG, GTT, and TAT were found sporadically among various bacterial strains.

TABLE 2. Distribution among bacterial genomes of TRS with five or more repeats

Microorganism (genome size [bp]) (GenBank accession no.)	No. of triplet repeats (no. of TRS copies per genome)										Total no. of TRS copies
	CCT	CGG	CTG	GAA	GAT	GTA	GTC	GTG	GTT	TAT	
C. acetobutylicum ATCC 824 (3940880) (NC_003030)			5 (1)	5 (1)					5 (1)	5 (2) 6 (2)	7
C. perfringens 13 (3031430) (NC_003366)										5 (3) 6 (1)	4
E. coli K12-MG1655 (4639221) (NC_000913)		5 (2)									2
E. coli O157:H7 EDL933 (5528445) (NC_002655)		5 (1)							5 (1)		2
E. coli O157:H7 VT2-Sakai (5498450) (NC_002695)		5 (1)							5 (1)		2
H. pylori 26695 (1667867) (NC_000915)						8 (1)			5 (1)		2
H. pylori J99 (1643831) (NC_000921)				5 (1)		8 (1)		5 (1)	5 (1)		4
L. innocua CLIP 11262 (3011208) (NC_003212)											0
L. monocytogenes EGD-e (2944528) (NC_003210)											0
M. bovis AF2122/97 (4345492) (NC_002945)		5 (22)			5 (1)		5 (1)	5 (4)			28
M. leprae TN (3268203) (NC_002677)				21 (1)	5 (2)	5 (3) 9 (1)		5 (1) 6 (1)			9
M. tuberculosis CDC1551 (4403836) (NC_002755)		5 (32) 6 (1)			5 (1)		5 (1)	5 (3)			38
M. tuberculosis H37Rv lab strain (4411529) (NC_000962)		5 (27) 6 (1) 7 (1)			5 (1)		5 (1)	5 (3)			34
M. genitalium G-37 (580074) (NC_000908)				5 (1) 6 (1) 16 (1)		5 (1) 7 (1) 8 (1) 9 (1) 10 (1) 11 (1) 16 (1)			11 (1)		11
M. pneumoniae M129 (816394) (NC_000912)						5 (1) 7 (1)					2
M. pulmonis UAB CTIP (963879) (NC_002771)									5 (2)	6 (1)	3
N. meningitidis MC58 (2272351) (NC_003112)		5 (6)									6
N. meningitidis serogroup A Z2491 (2184406) (NC_003116)		5 (2) 6 (1)									3
P. aeruginosa PA01 (6264403) (NC_002516)		5 (5)						5 (2) 14 (1)			8
R. conorii Malish 7 (1268755) (NC_003103)										5 (1)	1
R. prowazekii Madrid E (1111523) (NC_000963)								5 (1)		5 (1)	2
S. enterica serovar Typhi CT18 (4809037) (NC_003198)		5 (2) 6 (1)	10 (1)					5 (1)			5
S. enterica serovar Typhimurium LT2 SGSC1412 (4857432) (NC_003197)		5 (4)	5 (2)								6
S. aureus Mu50 (2878040) (NC_002758)					5 (1)						1
S. aureus N315 (2160837) (NC_002745)											0
T. acidophilum DSM 1728 (1564906) (NC_002578)											0
T. volcanium GSS1 (1584804) (NC_002689)											0
Y. pestis CO92 (4653728) (NC_003143)			5 (1) 6 (1)					5 (2)			4

However, only a few copies of these TRS were found. For example, one copy of $(CTG)_5$ was found in *C. acetobutylicum*, one $(CTG)_{10}$ was found in *S. enterica* serovar Typhi, two $(CTG)_5$ repeats were found in *S. enterica* serovar Typhimurium, and one $(CTG)_5$ and one $(CTG)_6$ repeat were found in *Y. pestis*. Relatively large TRS with 21 or 16 repeats were detected in *M. leprae* and *Mycoplasma genitalium*, respectively. *M. genitalium* possessed three types of TRS repeats (GAA, GTA, and GTT) and different numbers of repeats [$(GAA)_5$, $(GAA)_6$, and $(GAA)_{16}$; $(GTA)_5$, $(GTA)_7$, $(GTA)_8$, $(GTA)_9$, $(GTA)_{10}$, $(GTA)_{11}$, and $(GTA)_{16}$; and $(GTT)_{11}$].

Positions of $(CGG)_5$, $(CGG)_6$, and $(CGG)_7$ in the genome. The *M. tuberculosis* and *M. bovis* genomes consist of 4.4 and 4.3 Mb, respectively. All $(CGG)_5$, $(CGG)_6$, and $(CGG)_7$ repeats in both *M. tuberculosis* strains H37Rv and CDC1551 were located between 0.05 and 4.0 Mb (Table 3). These repeats appeared to be distributed randomly. In strain H37Rv, one $(CGG)_7$ was located at 0.05 Mb, and one $(CGG)_6$ was located at 2.4 Mb. Five $(CGG)_5$ repeats were between 0.1 and 1.0 Mb, six were between 1.0 and 2.0 Mb, eight were between 2.0 and 3.0 Mb, and eight were between 3.0 and 4.4 Mb. In strain CDC1551, one $(CGG)_6$ repeat was located at 0.05 Mb. Six $(CGG)_5$ repeats were between 0.1 and 1.0 Mb, 6 were between 1.0 and 2.0 Mb, 11 were between 2.0 and 3.0 Mb, and 9 were between 3.0 and 4.4 Mb. In *M. bovis*, four $(CGG)_5$ repeats were located between 0.26 and 1.0 Mb, five were between 1.0 and 2.0 Mb, seven were between 2.0 and 3.0 Mb, and six were between 3.0 and 4.3 Mb (Table 3). Almost all of the $(CGG)_5$, $(CGG)_6$, and $(CGG)_7$ repeats in *M. tuberculosis* and *M. bovis* were located within the open reading frame (ORF), with the exception of six $(CGG)_5$ repeats that were located between 1.1 and 3.96 Mb in strain CDC1551. Among these, the four $(CGG)_5$ repeats at 1.09, 3.74, 3.76, and 3.96 Mb were in the putative ORF with authentic frameshift or point mutation (Table 3).

In strain H37Rv, the genes containing $(CGG)_5$ and $(CGG)_6$ encoded the PPE and PE_PGRS families of proteins. A gene

TABLE 3. Position of $(CGG)_5$, $(CGG)_6$, and $(CGG)_7$ within the genome in three mycobacterial strains

Strain and position (bp)	No. of repeats	Gene no.	Product	Domain	Translation
M. tuberculosis					
55532	7	Rv0050	Probable penicillin-binding protein, PonA	ORF	poly(Pro)
261808	5	Rv0218	Hypothetical protein	ORF	poly(Ala)
340616	5	Rv0280	PPE family protein	ORF	poly(Ala)
362891	5	Rv0297	PE_PGRS family protein	ORF	poly(Gly)
672720	5	Rv0578c	PE_PGRS family protein	ORF	poly(Gly)
968964	5	Rv0872c	PE_PGRS family protein	ORF	poly(Gly)
1091589	5	Rv0977	PE_PGRS family protein	ORF	poly(Gly)
1189183	5	Rv1067c	PE_PGRS family protein	ORF	poly(Gly)
1189430	5	Rv1068c	PE_PGRS family protein	ORF	poly(Gly)
1191358	5	Rv1068c	PE_PGRS family protein	ORF	poly(Gly)
1213387	5	Rv1087	PE_PGRS family protein	ORF	poly(Gly)
1631645	5	Rv1450c	PE_PGRS family protein	ORF	poly(Gly)
2357161	5	Rv2098c	PE_PGRS family protein	ORF	poly(Gly)
2357267	6	Rv2098c	PE_PGRS family protein	ORF	poly(Gly)
2387312	5	Rv2126c	PE_PGRS family protein	ORF	poly(Gly)
2423539	5	Rv2126c	PE_PGRS family protein	ORF	poly(Gly)
2639030	5	Rv2356c	PPE family protein	ORF	poly(Ala)
2639330	5	Rv2356c	PPE family protein	ORF	poly(Ala)
2639442	5	Rv2356	PPE family protein	ORF	poly(Ala)
2802267	5	Rv2490c	PE_PGRS family protein	ORF	poly(Gly)
2922778	5	Rv2591	PE_PGRS family protein	ORF	poly(Gly)
3528969	5	Rv3159c	PPE family protein	ORF	poly(Ala)
3752989	5	Rv3347c	PPE family protein	ORF	poly(Ala)
3766907	5	Rv3350c	PPE family protein	ORF	poly(Ala)
3802146	5	Rv3388	PE_PGRS family protein	ORF	poly(Gly)
3803514	5	Rv3388	PE_PGRS family protein	ORF	poly(Gly)
3969420	5	Rv3532	PPE family protein	ORF	poly(Ala)
3972241	5	Rv3533c	PPE family protein	ORF	poly(Ala)
4029032	5	Rv3587c	Hypothetical protein	ORF	poly(Pro)
M. tuberculosis CDC1551					
55478	6	MT0056	Penicillin-binding protein	ORF	poly(Pro)
261924	5	MT0228	Hypothetical protein	ORF	poly(Ala)
340680	5	MT0292	PPE family protein	ORF	poly(Ala)
362955	5	MT0311	PE_PGRS family protein	ORF	poly(Gly)
674173	5	MT0607	PE_PGRS family protein	ORF	poly(Gly)
927976	5	MT0855	PE_PGRS family protein	ORF	poly(Gly)
968979	5	MT0894	PE_PGRS family protein	ORF	poly(Gly)
1091604	5	MT1004	Putative; PE_PGRS family protein, authentic frame shift	ORF	poly(Gly)
1189231	5	MT1096.1	PE_PGRS family protein	ORF	poly(Gly)
1189478	5	MT1096.1	PE_PGRS family protein	ORF	poly(Gly)
1191406	5	MT1097	PE_PGRS family protein	ORF	poly(Gly)
1213545	5	MT1118.1		UTR	poly(Gly)
1631528	5	MT1497.1	PE_PGRS family protein	ORF	poly(Gly)
2359430	5	MT2159	PE family-related protein	ORF	poly(Gly)
2359536	5	MT2159	PE family-related protein	ORF	poly(Gly)
2385890	5	MT2184 ?	Conserved hypothetical protein ?	Terminator ?	poly(Gly)
2422232	5	MT2220	PE_PGRS family protein	ORF	poly(Gly)
2633780	5	MT2423	PPE family protein	ORF	poly(Ala)
2634080	5	MT2423	PPE family protein	ORF	poly(Ala)
2636362	5	MT2425	PPE family protein	ORF	poly(Ala)
2636662	5	MT2425	PPE family protein	ORF	poly(Ala)
2636774	5	MT2425	PPE family protein	ORF	poly(Ala)
2797756	5	MT2564	PE_PGRS family protein	ORF	poly(Gly)
2918923	5	MT2668.1	PE_PGRS family protein	ORF	poly(Gly)
3524456	5	MT3247	PPE family protein	ORF	poly(Ala)
3526605	5	MT3248	PPE family protein	ORF	poly(Ala)
3745224	5	MT3453	Putative; PPE family protein, authentic frame shift	ORF	poly(Ala)
3759134	5	MT3458	Putative; PPE family protein, authentic frame shift	ORF	poly(Ala)
3793024	5	MT3495	PE_PGRS family protein	ORF	poly(Gly)
3794392	5	MT3495	PE_PGRS family protein	ORF	poly(Gly)
3961566	5	MT3636	Putative; PPE family protein, authentic point mutation	ORF	poly(Gly)
3964387	5	MT3637	PPE family protein	ORF	poly(Ala)
4021174	5	MT3693	Hypothetical protein	ORF	poly(Pro)
M. bovis					
262035	5	Mb0224	Probable conserved transmembrane protein	ORF	poly(Ala)
341620	5	Mb0288	PPE family protein	ORF	poly(Ala)
363940	5	Mb0305	PE_PGRS family protein	ORF	poly(Gly)
673964	5	Mb0593c	PE_PGRS family protein	ORF	poly(Gly)
1092029	5	Mb1002	PE_PGRS family protein	ORF	poly(Gly)
1189891	5	Mb1096c	PE_PGRS family protein	ORF	poly(Gly)
1192527	5	Mb1097c	PE_PGRS family protein	ORF	poly(Gly)

Continued on following page

TABLE 3—*Continued*

Strain and position (bp)	No. of repeats	Gene no.	Product	Domain	Translation
1214721	5	Mb1116	PE_PGRS family protein	ORF	Poly(Gly)
1627966	5	Mb1485c	PE_PGRS family protein	ORF	Poly(Gly)
2339003	5	Mb2125c	Conserved hypothetical protein PE_PGRS family protein	ORF	Poly(Gly)
2339109	5	Mb2125c	Conserved hypothetical protein PE_PGRS family protein	ORF	Poly(Gly)
2367710	5	Mb2150c	Conserved hypothetical protein PE_PGRS family protein	ORF	Poly(Gly)
2604931	5	Mb2376c	PPE family protein	ORF	Poly(Ala)
2607401	5	Mb2377c	PPE family protein	ORF	Poly(Ala)
2607513	5	Mb2377c	PPE family protein	ORF	Poly(Ala)
2769065	5	Mb2517c	PE_PGRS family protein	ORF	Poly(Gly)
3706526	5	Mb3380c	PPE family protein	ORF	Poly(Ala)
3720437	5	Mb3385c	PPE family protein	ORF	Poly(Ala)
3755777	5	Mb3420	PE_PGRS family protein	ORF	Poly(Gly)
3912639	5	Mb3562	PPE family protein	ORF	Poly(Ala)
3915460	5	Mb3563c	PPE family protein	ORF	Poly(Ala)
3972250	5	Mb3618c	Probable conserved membrane protein	ORF	Poly(Pro)

containing $(CGG)_7$, PonA, encoded a penicillin-binding protein (Table 3). In strain CDC1551, the genes containing $(CGG)_5$ encoded the PPE, PE_PGRS, and PE families of proteins. A gene containing $(CGG)_6$ encoded a penicillin-binding protein (Table 3). In *M. bovis*, all genes containing $(CGG)_5$ encoded PPE and PE_PGRS family proteins, with the exception of two genes that encoded probable conserved membrane proteins (Table 3). In all three strains, the $(CGG)_5$ in the PPE genes translated to poly(Ala), and the $(CGG)_5$ and $(CGG)_6$ in the PE_PGRS and PE genes translated to poly(Gly). In both *M. tuberculosis* strains, the $(CGG)_6$ and $(CGG)_7$ in genes encoding penicillin-binding proteins translated to poly(Pro) (Table 3). In *M. bovis*, the two $(CGG)_5$ repeats in genes encoding probable conserved membrane proteins translated to poly(Ala) and poly(Pro) (Table 3). Most of the $(CGG)_5$ repeats within the PPE genes were located in the N-terminal PPE domain of the genes (data not shown). All $(CGG)_5$ and $(CGG)_6$ repeats within the PE_PGRS genes consisting of PE and PGRS domains were located in the PGRS domain (data not shown). Two $(CGG)_5$ repeats within the PE family-related gene (MT2159) in strain CDC1551 were located in the C-terminal domain of the genes (data not shown).

Genomic stability. To examine whether $(CGG)_5$ repeats in the genome are stable, two *M. tuberculosis* strains (H37Rv and IMCJ 541) were analyzed for $(CGG)_5$- and IS*6110*-probed fingerprints. The fingerprint patterns among culture periods were identical for strain H37Rv (Fig. 1A). These findings were confirmed with strain IMCJ 541 (Fig. 1B). The data indicate that $(CGG)_5$ repeats are stable in the genome for at least a few months. In the IS*6110*-probed fingerprints, the patterns did not change during the 9 weeks of culture of strain H37Rv or strain IMCJ 541 (data not shown), indicating that IS*6110* inserts are also stable over a few months.

Comparison of fingerprints between *M. tuberculosis* strains H37Rv and H37Ra. The virulent *M. tuberculosis* strain H37Rv and its avirulent derivative strain H37Ra were originally derived from the same strain, H37 (22, 23). It was reported that there are distinct differences between these strains with respect to IS*6110*-probed fingerprint patterns (3, 11). We investigated whether differences exist between these strains with respect to $(CGG)_5$-probed fingerprint patterns. DNA derived from the H37Rv and H37Ra strains were digested with 16 restriction enzymes as described in Materials and Methods. Unexpectedly, the patterns of $(CGG)_5$-based hybridization showed no differences between the H37Rv and H37Ra strains (Fig. 2A). For example, the $(CGG)_5$-based RFLP patterns of PvuII-digested fragments of H37Rv were identical to those of H37Ra (Fig. 2A, PvuII). However, the IS*6110*-based RFLP patterns of H37Rv were markedly different from those of H37Ra, which were analyzed with the use of the same blot of PvuII-digested fragments used in the $(CGG)_5$-based RFLP analysis (Fig. 2B). In the IS*6110*-based RFLP patterns, H37Rv showed 9 bands, and H37Ra showed 11 bands. Strain H37Rv but not H37Ra showed one band of 5.1 kb. Strain H37Ra but not H37Rv showed three bands of 1.1, 2.3, and 3.0 kb.

IS*6110*- and $(CGG)_5$-probed DNA fingerprinting of *M. tuberculosis* clinical isolates. To assess the potential usefulness of $(CGG)_5$ as an epidemiologic marker for *M. tuberculosis*, 109 clinical isolates obtained from Tokyo (76 isolates) and Warsaw (33 isolates) and the H37Rv and H37Ra strains were analyzed by the IS*6110*- and $(CGG)_5$-probed fingerprint methods. For IS*6110*-probed hybridization, DNA of these isolates was digested with PvuII according to a standardized protocol (26). For $(CGG)_5$-probed hybridization, DNA of the isolates was digested with AluI. When DNA of the H37Rv and H37Ra strains was digested with AatII, EcoRI, MluI, NruI, NsbI, PstI, PvuII, SacI, SalI, or XhoI, relatively higher-molecular-weight DNA fragments were visualized by the probe with a minimum size of 1 to 3.5 kb and a maximum size of more than 10 kb (Fig. 2A). When digested with AfaI, AluI, HinfI, Sau3AI, SmaI, or XspI, DNA fragments of sizes of 0.5 to 8 kb were visualized. When DNA of five clinical isolates selected at random were digested with AluI, clear $(CGG)_5$ fingerprint patterns with 10 to 14 copies of DNA fragments of 0.75 to 8 kb were detected (data not shown). Although we used AluI for this fingerprinting method, other enzymes may also be used.

IS*6110* fingerprint patterns obtained from clinical isolates and the corresponding dendrogram are shown in Fig. 3A. IS*6110* copies were detected in 110 of 111 isolates. One isolate from Japan had no copy. As indicated in Fig. 3A, 10 of 111 isolates (9.0% of tested isolates), including 8 isolates from Japan and 2 from Poland, possessed fewer than 6 copies of IS*6110*, which was insufficient to distinguish polymorphisms. Except for these 10 isolates with fewer than 6 copies of IS*6110*, the IS*6110* fingerprint patterns of 101 isolates showed $\geq$28% similarity; 98 patterns were found (Fig. 3A). Five clusters with

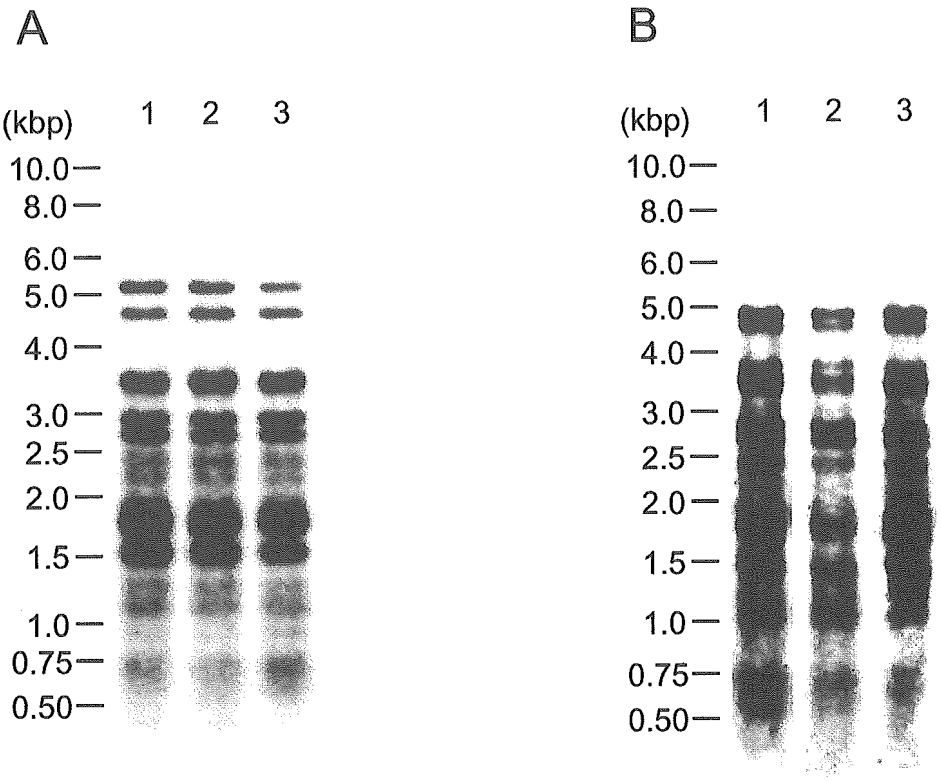

FIG. 1. (CGG)$_5$ fingerprinting of *M. tuberculosis* H37Rv (A) and clinical isolate IMCJ 541 (B), which were cultured and serially passaged weekly. The bacteria were harvested at 0 (lane 1), 3 (lane 2), and 9 (lane 3) weeks after culture.

≥44% similarity, including clusters Ia, IIa, IIIa, IVa, and Va, were detected (Fig. 3A). Cluster Ia was composed of seven Poland-derived isolates. Cluster IIa was composed of two H37 variants and 11 Japan- and 6 Poland-derived isolates. Cluster IIIa was composed of three Japan- and seven Poland-derived isolates. Cluster IVa was composed of four Japan- and five Poland-derived isolates. Cluster Va was composed predominantly of Japan-derived isolates (46 isolates from Japan and 2 from Poland). The majority of Japan-derived isolates (61%) and Poland-derived isolates (76%) belonged to cluster Va and to clusters Ia to IVa, respectively.

(CGG)$_5$ fingerprint patterns and the corresponding dendrogram are shown in Fig. 3B. (CGG)$_5$ copies were detected in all clinical isolates tested. The copy number ranged from 8 to 16, with a mean of 13.0 ± 1.5 per isolate. The number of (CGG)$_5$ copies of Japan- and Poland-derived isolates ranged from 8 to 16, with a mean of 12.9 ± 1.5 per isolate and from 11 to 15, with a mean of 13.2 ± 1.3 per isolate, respectively. A total of

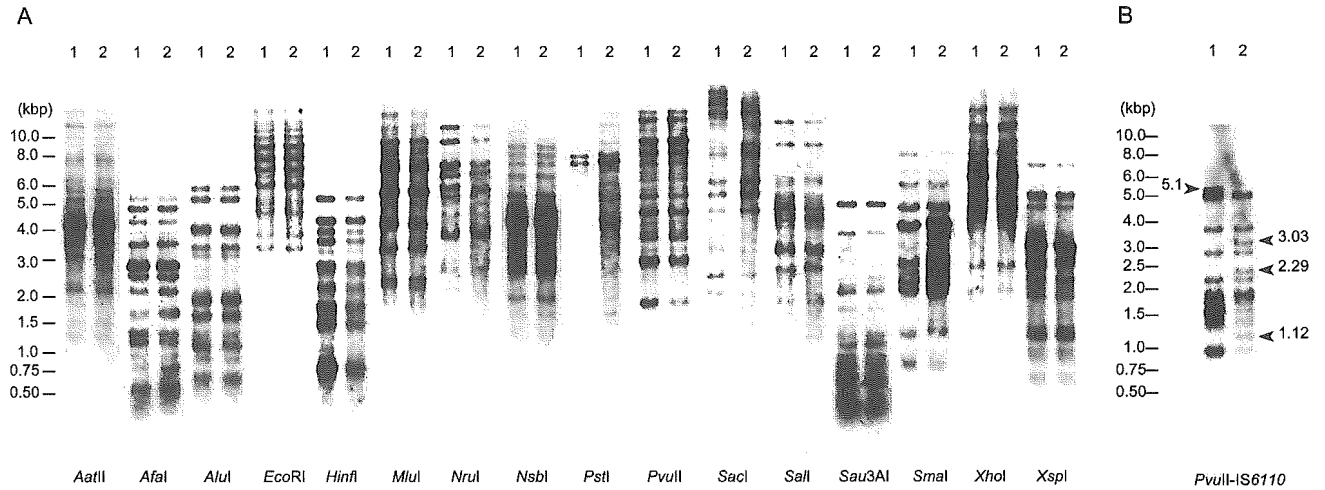

FIG. 2. (CGG)$_5$ (A) and IS*6110* (B) fingerprinting of *M. tuberculosis* strains H37Rv (lane 1) and H37Ra (lane 2). Genomic DNA was digested with 16 restriction enzymes. The digested fragments were separated by electrophoresis.

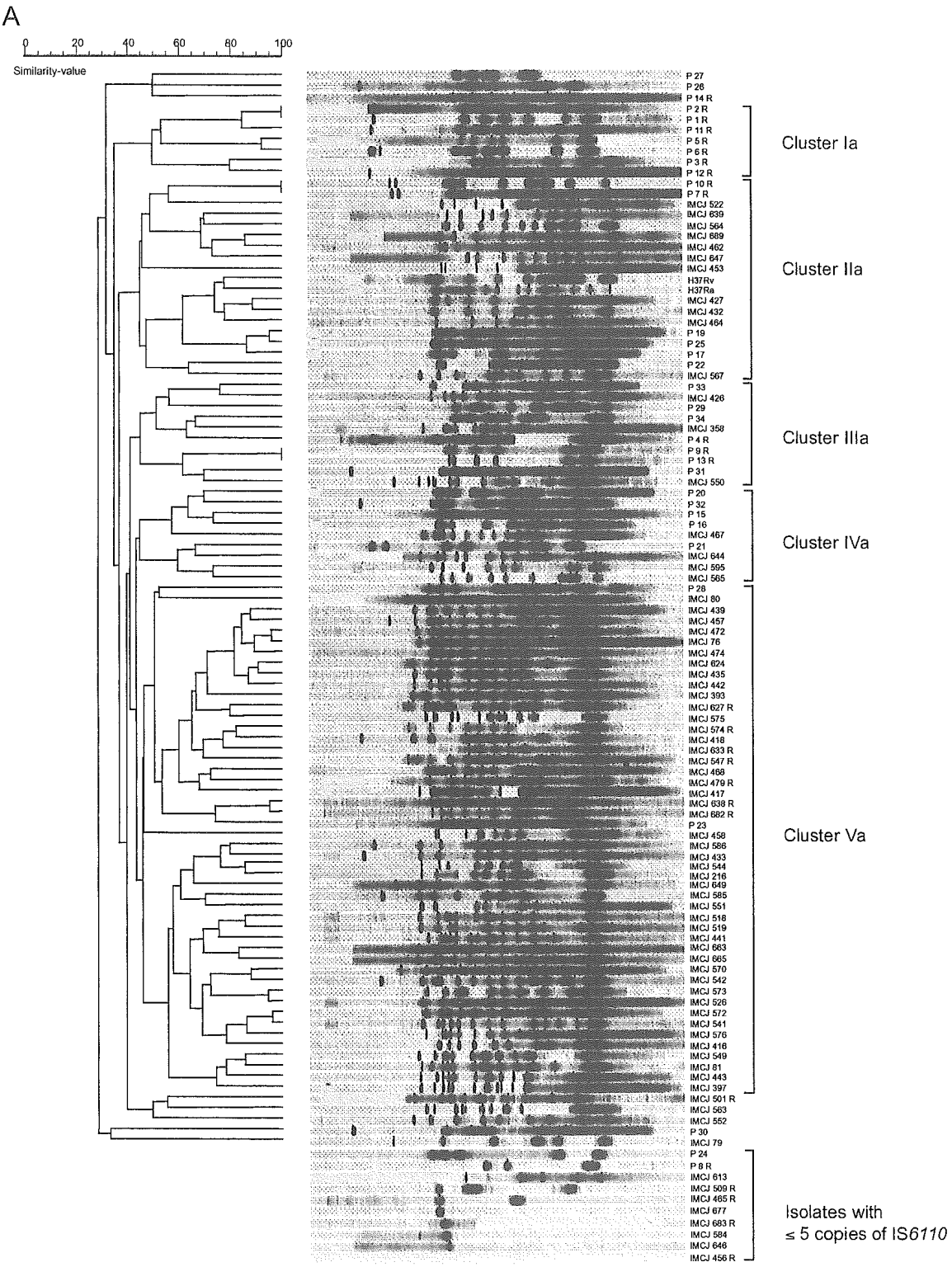

FIG. 3. IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from Japan and Poland and the respective corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. The scale depicts the similarity of patterns calculated as described in Materials and Methods. In IS*6110*-probed DNA fingerprint patterns, five clusters showing a similarity of more than 44% were designated clusters Ia, IIa, IIIa, IVa, and Va. Isolates with five or less than five copies are indicated in panel A. In $(CGG)_5$-probed DNA fingerprint patterns, four clusters showing a similarity of more than 70% were designated clusters Ib, IIb, IIIb, and IVb. The isolates are named according to their origin as IMCJ (Japan) or P (Poland); the suffix R indicates drug resistance For example, IMCJ 627 R is a Japan-derived drug-resistant isolate.

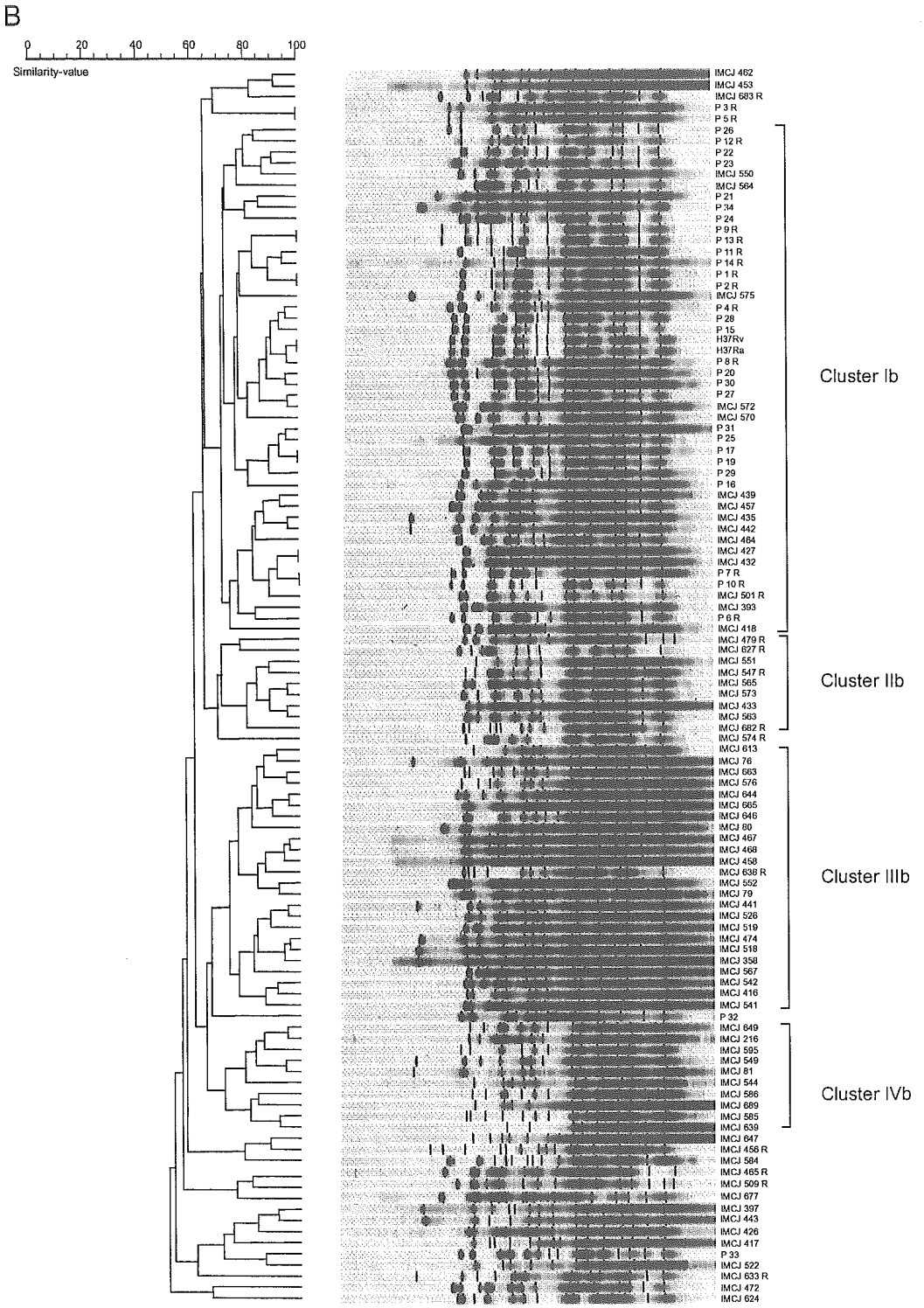

FIG. 3—*Continued.*

104 (CGG)$_5$ fingerprint patterns were found with ≥50% similarity (Fig. 3B). Four clusters with ≥70% similarity, including clusters Ib to IVb, were detected (Fig. 3B). Cluster Ib was composed of two H37 variants and 15 Japan- and 29 Poland-derived isolates. Clusters IIb, IIIb, and IVb were composed of 9, 24, and 10 Japan-derived isolates, respectively. Over half of the Japan-derived isolates (57%) and the majority of the Poland-derived isolates (88%) belonged to clusters IIb to IVb and to cluster Ib, respectively (Fig. 3B).

Both the IS*6110* and (CGG)$_5$ fingerprint analyses showed an association between fingerprint pattern and geographic origin, indicating a correlation between them. Ten isolates that were

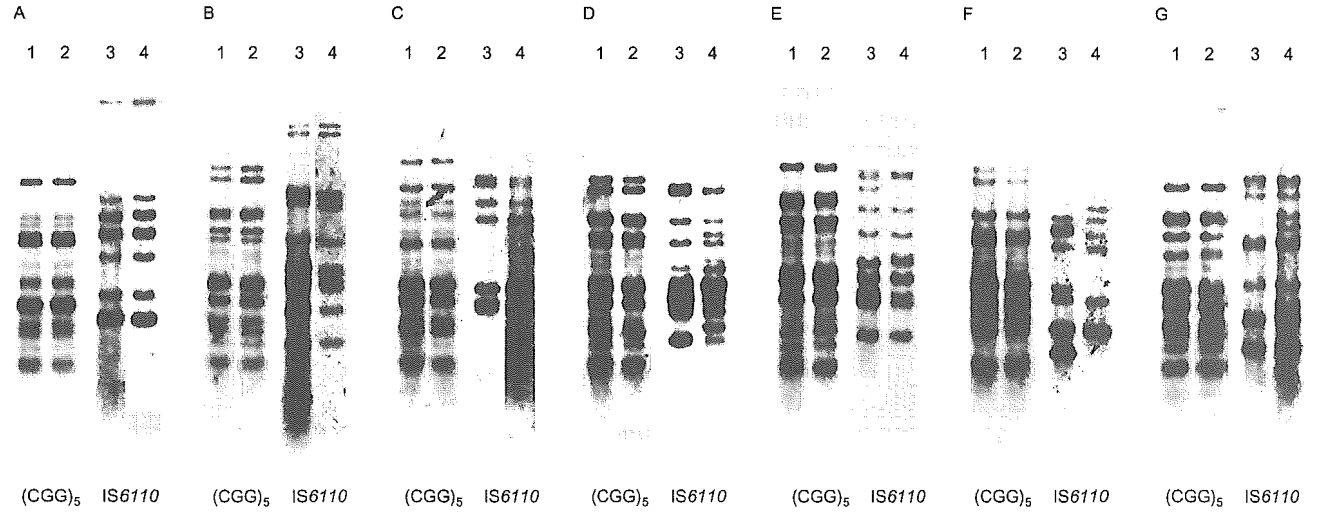

FIG. 4. $(CGG)_5$- and IS*6110*-probed DNA fingerprinting patterns of *M. tuberculosis* isolates that shared identical $(CGG)_5$ fingerprinting. (A) Lanes 1 and 3, P1; lanes 2 and 4, P2. (B) Lanes 1 and 3, P 7; lanes 2 and 4, P 10. (C) Lanes 1 and 3, P 9; lanes 2 and 4, P 13. (D) Lanes 1 and 3, H37Rv; lanes 2 and 4, H37Ra. (E) Lanes 1 and 3, IMCJ 427; lanes 2 and 4, IMCJ 432. (F) Lanes 1 and 3, P 3; lanes 2 and 4, P 5. (G) Lanes 1 and 3, P 17; lanes 2 and 4, P 19.

indistinguishable by IS*6110* RFLP because of the presence of few copies of the marker could be analyzed by $(CGG)_5$ marker. Three and seven pairs of isolates were identical to each other in the IS*6110* and $(CGG)_5$ fingerprint patterns, respectively (Fig. 4). The three pairs P 1 and P 2, P 7 and P 10, and P 9 and P 13 were identical to each other in the IS*6110* and $(CGG)_5$ fingerprint patterns (Fig. 4A to C, respectively). The four pairs H37Rv and H37Ra, IMCJ 427 and IMCJ 432, P 3 and P 5, and P 17 and P 19 were identical to each other in the $(CGG)_5$ fingerprint pattern but different in the IS*6110* fingerprint pattern (Fig. 4D, E, F, and G, respectively). The data suggest that the $(CGG)_5$ fingerprint patterns are more stable than the IS*6110* patterns.

Occurrence of $(CGG)_5$ among various mycobacterial strains. We investigated the presence of $(CGG)_5$ repeat sequences in mycobacterial species. $(CGG)_5$ hybridization patterns from various mycobacterial species are shown in Fig. 5. Bands ranging from 0 to 20 in number were seen. *Mycobacterium szulgai* possessed 20 bands. *M. bovis* BCG, *Mycobacterium marinum*, and *Mycobacterium kansasii* possessed 16 bands. *Mycobacterium nonchromogenicum*, *Mycobacterium terrae*, *Mycobacterium gastri*, *Mycobacterium simiae*, *Mycobacterium smegmatis*, and *Mycobacterium intracellulare* possessed 14, 12, 8, 5, 5, and 3 bands, respectively. *Mycobacterium peregrinum* possessed two bands. *Mycobacterium fortuitum* and *Mycobacterium chelonae* possessed one band. *Mycobacterium scrofulaceum*, *Mycobacterium avium*, *Mycobacterium xenopi*, and *Mycobacterium abscessus* showed no bands.

DISCUSSION

In this study, we found that various bacterial strains contain TRS in their genomes. In humans, TRS are associated with hereditary neurologic and neuromuscular disorders, including myotonic dystrophy, Huntington's disease, Fragile X syndrome, and Friedreich's ataxia (27). These diseases result from TRS expansion such as $(CTG)_n$, $(CGG)_n$, and $(GAA)_n$ (27). The TRS sizes associated with these diseases are usually quite large. For example, 80 to 3,000 repeats of CTG have been found in myotonic dystrophy, 230 to 2,000 repeats of CGG have been found in Fragile X syndrome, and 200 to 900 repeats of GAA have been found in Friedreich's ataxia (21). These expanded TRS can form hairpin structures or intramolecular triplex structures that result in genetic instability (21). The TRS sizes found in bacteria were relatively small. The largest size TRS identified was 21 repeats of GAA in *M. leprae*. The most frequently identified TRS was five repeats of CGG in *M. tuberculosis* and *M. bovis*. TRS found in bacteria are not likely to be linked to genetic instability because of the lower repeat number.

The $(CGG)_5$ TRS found in two strains of *M. tuberculosis* (H37Rv and CDC1551) and in one strain of *M. bovis* existed in genes encoding PE protein families, including a PE_PGRS subfamily and PPE protein families comprising 88 to 101 and 61 to 69 kinds of proteins, respectively, which occupy approximately 8% of the genome (4, 7, 8). The functional properties of $(CGG)_5$ in these genes are unknown, but $(CGG)_5$ should not play an important role in the development of the variations among different strains. $(CGG)_5$ in the PPE genes was located in the conserved N-terminal domain PPE but not in the C-terminal variable domain containing the major polymorphic tandem repeats with the consensus sequence of GCCGGT GTTG (10, 18). $(CGG)_5$ in the PE_PGRS genes was within the C-terminal variable domain containing the PGRS with the consensus sequence of CGGCGGCAA (18, 19). $(CGG)_5$ in the PE_PGRS genes did not comprise part of the consensus sequence of PGRS. $(CGG)_5$ was contained in 13 and 12 PE_PGRS genes in H37Rv and CDC1551, respectively. Among these genes, deletion or insertion was detected at one site of Rv1068c, two sites of Rv1087, and two sites of Rv1450c compared with their orthologs, MT1097, MT1118.1, and MT1497.1, respectively (data not shown). However, $(CGG)_5$ was not near these sites, indicating that it did not directly affect the deletion and insertion of PE_PGRS genes. $(CGG)_5$ in

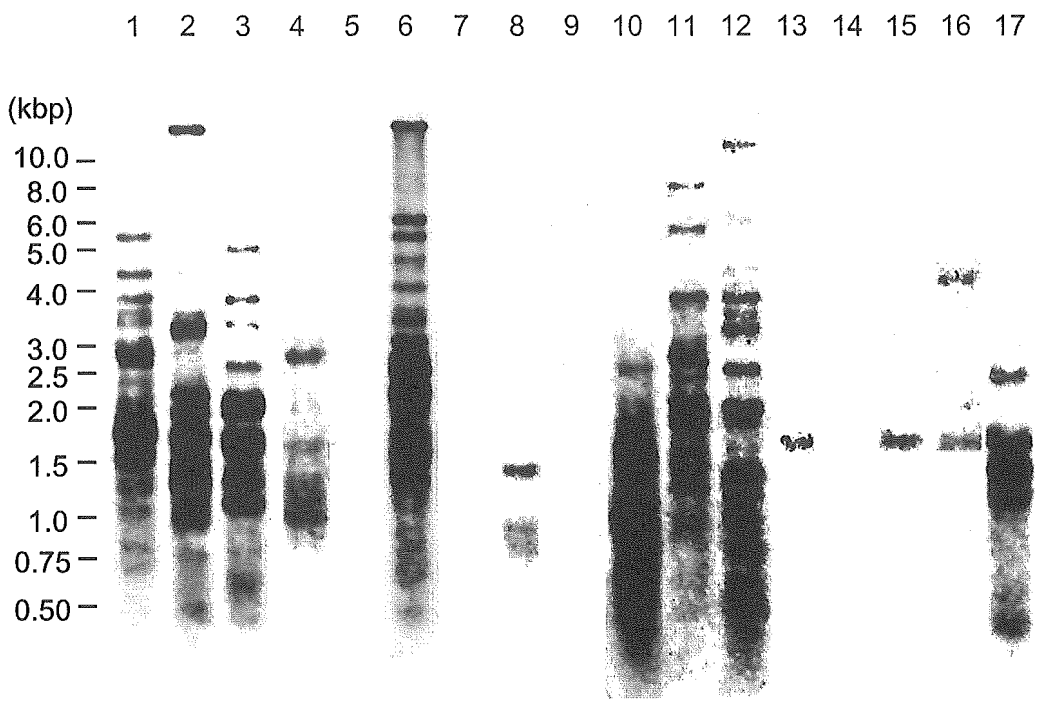

FIG. 5. (CGG)$_5$-probed fingerprinting of AluI-digested DNA from various mycobacterial species. Lane 1, *M. bovis* BCG; lane 2, *M. marinum*; lane 3, *M. kansasii*; lane 4, *M. simiae*; lane 5, *M. scrofulaceum*; lane 6, *M. szulgai*; lane 7, *M. avium*; lane 8, *M. intracellulare*; lane 9, *M. xenopi*; lane 10, *M. gastri*; lane 11, *M. terrae*; lane 12, *M. nonchromogenicum*; lane 13, *M. fortuitum*; lane 14, *M. abscessus*; lane 15, *M. chelonae*; lane 16, *M. peregrinum*; lane 17, *M. smegmatis*.

PPE, PE, and PE_PGRS genes translated to neutral-charged amino acids of poly(Ala) and poly(Gly), respectively, with no special substitution, indicating that these regions do not participate in the formation of unique structures within these proteins. Thus, the (CGG)$_5$ sequences in these genes will likely not have characteristic properties regarding function.

It is unclear whether TRS in bacteria, particularly (CGG)$_5$ in *M. tuberculosis* and *M. bovis*, participate in their pathogenesis. There was no difference between virulent strain H37Rv and the derived avirulent strain H37Ra in (CGG)$_5$-probed fingerprinting (Fig. 2). No correlation was found between the virulency of mycobacterial species and the numbers of bands in (CGG)$_5$-probed fingerprinting or copies of (CGG)$_5$ (Table 2 and Fig. 5). For example, *M. leprae* had no (CGG)$_5$ repeats (Table 2). Some rare etiologic agents of nontuberculous mycobacteria, such as *M. smegmatis* and *M. szulgai* (20), did possess several copies of (CGG)$_5$ in their genomes (Fig. 5), whereas some common etiologic agents, such as *M. avium*, *M. xenopi*, and *M. abscessus* (20), possessed no (CGG)$_5$ repeats (Fig. 5). These results indicate that (CGG)$_5$ repeats do not participate directly in the virulency of mycobacterial species.

Whereas fingerprinting analysis showed that both (CGG)$_5$ and IS*6110* were sufficiently stable epidemiologic markers, (CGG)$_5$ appeared to be more stable than IS*6110* (Fig. 1). We were unable to find any differences between strains H37Rv and H37Ra in (CGG)$_5$-probed fingerprinting by extensive studies with various restriction enzymes. However, four different bands were detected between these strains with PvuII-IS*6110* fingerprinting (Fig. 2B). Lari et al. (11) compared H37Rv and H37Ra strains maintained at their institution by IS*6110* fingerprinting with EcoNI, PstI, and PvuII and found different patterns between these strains. Bifani et al. (3) compared the PvuII-IS*6110* fingerprints of 15 and 3 different catalogued variants of H37Rv and H37Ra, respectively. Ten distinct fingerprint patterns, making up nine H37Rv variants and one H37Ra variant, were identified. A discrepancy between IS*6110*- and (CGG)$_5$-probed fingerprints of laboratory strains was observed in three pairs of clinical isolates (Fig. 4). In these cases, each isolate was identical in (CGG)$_5$ fingerprinting pattern but differed in its IS*6110* fingerprinting pattern. Our recent epidemiological case report of intrafamilial tuberculosis transmission showed that two clinical isolates from a father and son were identical in (CGG)$_5$-probed fingerprinting patterns, whereas one different band was detected between them by IS*6110*-probed fingerprinting (25). Collectively, IS*6110*-probed fingerprint patterns changed more rapidly than did (CGG)$_5$-probed patterns, suggesting that there are different mechanisms by which these patterns change. In other terms, although (CGG)$_5$-probed fingerprinting will hardly detect a few mutations in a clone of *M. tuberculosis*, it will easily detect an origin among the clones. The (CGG)$_5$-probed fingerprinting combined with IS*6110*-probed fingerprinting will provide more powerful information about tuberculosis epidemiology.

We collected and analyzed the isolates in this study in Japan and Poland. If isolates could be collected worldwide, it would provide more exact epidemiological data. In conclusion, the (CGG)$_5$ repeat is a useful probe for DNA fingerprinting of *M. tuberculosis*, because all strains tested here possessed more than eight copies. In addition, (CGG)$_5$-probed fingerprinting will be a useful tool for the investigation of *M. bovis*, *M. marinum*, *M. kansasii*, and *M. szulgai*.

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Laboratory and Epidemiology Communications

Further Acquisition of Drug-Resistance in Multidrug-Resistant Tuberculosis during Chemotherapy

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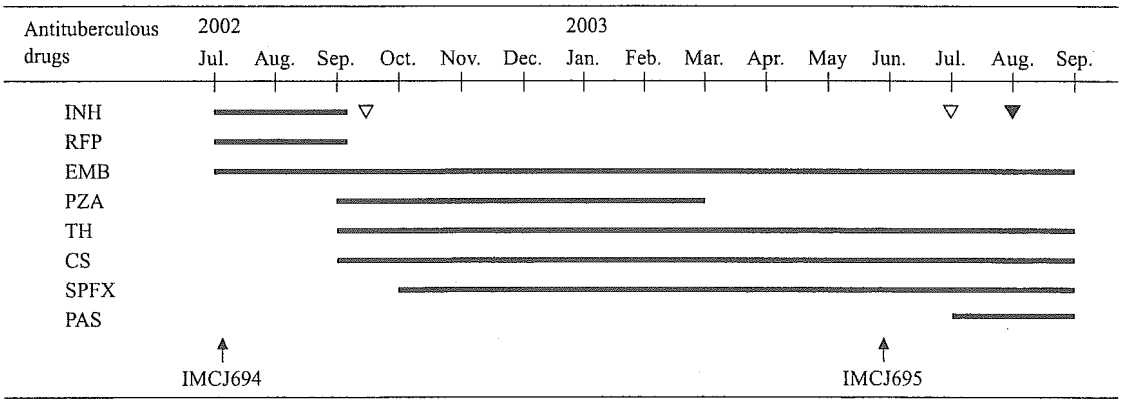
Multidrug-resistant tuberculosis (MDR-TB) resulting from failure to control primary tuberculosis (1) poses a serious clinical problem. Understanding how an organism can acquire resistance to multiple drugs is essential to prevent the emergence of a multidrug-resistant organism in an individual receiving antituberculous chemotherapy.

A 41-year-old man visited a doctor complaining of a left chest pain. A chest radiograph revealed pneumothorax and his sputum was positive for acid-fast bacilli (AFB). He was referred to a hospital in Tokyo in July 2002. The patient had a history of antituberculous chemotherapy from 1992 to 1997 in South Korea with isoniazid (INH), rifamipicin (RFP), ethambutol (EMB), and streptomycin (SM) for 1 month, and then with INH, RFP, EMB, and cycloserine (CS) for 5 years. After the chemotherapy, he still continued to have a productive cough.

Four-drug chemotherapy consisting of INH, RFP, pyrazinamide (PZA), and EMB was started in July 2002 (Table 1). Table 2 shows the drug sensitivity to various drugs (Vit Spectrum-SR™; Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). The drug sensitivity to PZA was not tested at that time. The isolate was found to be already resistant to INH, RFP, SM, and levofloxacin (LVFX). Therefore, in September 2002, the drugs were immediately changed to PZA, EMB, CS, and ethionamide (TH) and then to five drugs (PZA, EMB, CS, TH, and sparfloxacin [SPFX]) in October 2002. Because the sputa smears remained AFB positive and the symptoms did not improved, the patient received right thoracoplasty in December 2002. The drugs were changed to the four drugs EMB, CS, TH, and SPFX in March 2003. Drug sensitivity testing was conducted in June 2003 (Tables 1 and 2). The isolates were resistant to EMB and TH. The PZA sensitivity test that measures *M. tuberculosis* pyrazinamidase (PZase), which converts PZA to an active form, was conducted in August 2003, and was negative for both July 2002 and June 2003 isolates; i.e., they were resistant to PZA. The drugs were changed to EMB, CS, *p*-aminosalicylic acid (PAS), and SPFX in July 2003. The sputum smear was still AFB-positive. He again received pulmonary resection at the left S6 segment in August 2003. After the resection, the smear turned AFB-negative, and he was discharged from the hospital in September 2003.

To determine whether isolates in July 2002 and in June 2003 came from a single clone, chromosomal DNA was analyzed by restriction fragment length polymorphism (RFLP) (2) using a IS*6110* probe (3) and a trinucleotide

Table 1. Timetable of chemotherapy



INH, Isoniazid; RFP, Rifampicin; EMB, Ethambutol; PZA, Pyrazinamide; TH, Ethionamide; CS, Cycloserine; SPFX, sparfloxacin; PAS, *p*-aminosalicylic acid. ▽: The results of susceptibility testing of antituberculous drugs except for PZA were reported. ▼: The PZase activity was determined. ↑: Isolation date.

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Table 2. Patterns of antimicrobial susceptibility of MDR-TB isolates

Month of isolation	Isolate No.	Antibiotics [μg/ml]										
		INH [0.2, 1.0]	RFP [40]	EMB [2.5]	SM [10]	KM [20]	TH [20]	PAS [0.5]	CS [30]	EVM [20]	LVFX [1.0]	PZA[1)]
July 2002	IMCJ694	R, S	R	S	R	S	S	S	S	S	R	ND
June 2003	IMCJ695	R, S	R	R	R	S	R	S	S	S	R	ND

Abbreviations are in Table 1. R, Resistance; S, Sensitive; RFP, Rifampicin; SM, Streptomycin; KM, Kanamycin; EVM, Enviomycin; LVFX, Levofloxacin; ND, not determined.
1): Isolates IMCJ694 and IMCJ695 were negative for PZase activity, which was measured in August 2003.

Table 3. Analysis of MDR-TB isolates for mutations conferring resistance to isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin, kanamycin, and levofloxacin

Isolate No. (Month of isolation)	INH		RFP	EMB	PZA	SM	SM, KM	LVFX
	katG mutation	*inhA* promoter mutation	*rpoB* mutation	*embB* mutation	*pncA* mutation	*rpsL* mutation	*rrs* mutation	*gyrA* mutation
IMCJ694 (July 2002)	L48Q (CTG→CAG), R463L[1)] (CGG→CTG)	−15C→T	S531L (TCG→TTG)	WT	P54L (CCG→CTG)	K43R (AAG→AGG)	WT	D94G (GAC→GGC) S95T[1)] (AGC→ACC)
IMCJ695 (June 2003)	L48Q (CTG→CAG), R463L[1)] (CGG→CTG)	−15C→T	S531L (TCG→TTG)	M306V (ATG→GTG)	P54L (CCG→CTG)	K43R (AAG→AGG)	WT	D94G (GAC→GGC) S95T[1)] (AGC→ACC)

Abbreviations are in Tables 1 and 2. L48Q, Leu48Gln; R463L, Arg463Leu; S531L, Ser531Leu; M306V, Met306Val; P54L, Pro54Leu; K43R, Lys43Arg; D94G, Asp94Gly; S95T, Ser95Thr.
WT: wild type. Corresponding with nucleotide sequences of a laboratory strain of *M. tuberculosis* H37Rv.
1): Natural polymorphism with no association with drug-resistance (5).
Amino acid numbering based on the GenBank database under accession no. NC_000962.

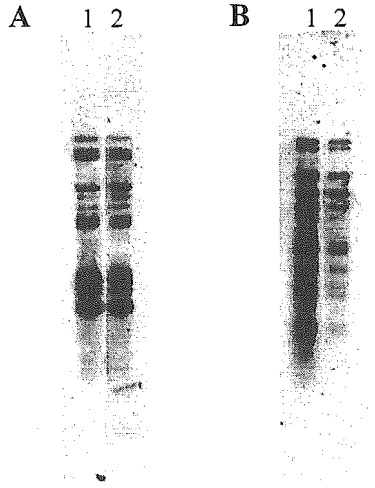

Fig. 1. IS*6110* and $(CGG)_5$ restriction fragment length polymorphism (RFLP) typing.
*Pvu*II- or *Alu*I-digested genomic DNA was analyzed by Southern blot hybridization with a peroxidase-labeled DNA probe for IS*6110* (A) and $(CGG)_5$ (B), respectively. Lane 1, *M. tuberculosis* isolate obtained in July 2002; Lane 2, *M. tuberculosis* isolate obtained in June 2003.

repeat sequence $(CGG)_5$ probe (4) . The IS*6110* patterns (Fig. 1A) and $(CGG)_5$ patterns (Fig. 1B) were identical between July 2002 and June 2003 isolates. The results indicate that these isolates were of the same origin.

Drug resistance in *M. tuberculosis* is caused by mutations in restricted regions of its genome (5). We PCR-amplified eight drug resistance-associated regions, including *rpoB* for RFP, *katG* and the *inhA* promoter for INH, *emb*B for EMB, *pncA* for PZA, *rpsL* and *rrs* for SM and KM, and *gyrA* for LVFX, and sequenced them (J. Sekiguchi et al., unpublished data). As shown in Table 3, the July 2002 isolate had eight mutations among these regions, including Ser531Leu in *rpoB*, Leu48Gln and Arg463Leu in *katG*, nucleotide substitution C to T in the *inhA*-promoter region, Pro54Leu in *pncA*, Lys43Arg in *rpsL*, and Asp94Gly and Ser95Thr in *gyrA*. Of these mutations, Arg463Leu in *katG* and Ser95Thr in *gyrA* are known to be natural polymorphisms with no association with drug resistance (5). The other six mutations are known to be associated with resistance to RFP, INH, PZA, SM, and LVFX, respectively. The profile of the mutations was well correlated with that of the drug susceptibility (Tables 2 and 3). The June 2003 isolates showed nine mutations. Among them, eight mutations were identical to those of the July 2002 isolates. In addition to these mutations, the June 2003 isolates had the mutation Met306Val in *embB*. This explains the EMB-resistance of the isolate. The isolate was also resistant to TH. However, mutations that may be associated with TH-resistance were not found in the present study. In general, mutations that play a role in resistance to EMB were not identified.

The patient had been treated with ineffective drugs against MDR-TB organisms, such as INH and RFP from July to September 2002, PZA from September 2002 to March 2003, TH at least in March 2003, and EMB from March to July 2003. SPFX that was given from October 2002 to July 2003 may also have been ineffective, given that SPFX, like LVFX, is a fluoroquinolone and the organisms were resistant to LVFX. Insufficient information about the drug resistance of the bacteria resulted in the use of ineffective drugs. PZA was given from September 2002 to March 2003 without monitor-

ing PZA susceptibility or PZase activity. PZA susceptibility testing or a PZase assay should be added to routine mycobacterial examination. The genetic diagnosis system is useful for rapidly diagnosing drug-resistant *M. tuberculosis*.

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Detection of the Antiseptic- and Disinfectant-Resistance Genes *qacA*, *qacB*, and *qacC* in Methicillin-Resistant *Staphylococcus aureus* Isolated in a Tokyo Hospital

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Use of antiseptics and disinfectants is essential in infection control practices in hospital and other health care settings. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates resistant to antiseptics and disinfectants have been reported in Australia and the United Kingdom in the last decade (1). On the other hand, few methicillin-sensitive *S. aureus* (MSSA) isolates resistant to antiseptics and disinfectants were reported. The isolates are mainly mediated by plasmids encoding the *qacA*, *qacB*, and *qacC* genes that confer resistance to organic cations by means of a multidrug efflux pump (1).

We conducted MRSA surveillance in October 2003 in a hospital with 24 wards and 925 beds in Tokyo (2); 241 MRSA isolates were obtained from 72 inpatients. Sixty-five of them were derived each from a single patient. They were evaluated by restriction fragment length polymorphism (RFLP) of genomic DNA using pulsed-field gel electrophoresis (PFGE). We examined here whether or not these isolates had the antiseptic- and disinfectant-resistant genes *qacA*, *qacB*, and *qacC*.

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Table 1. Primers used for the multiplex PCR-RFLP assay

Target gene (Accession no.)	Primer (Direction)	Nucleotide sequence	Position[1)]	Product size [bp]
qacA/*qacB* (X56628/U22531)	qacA/B-F (Forward)	5′-TCCTTTTAATGCTGGCTTATACC-3′	924-946	220
	qacA/B-R (Reverse)	5′-AGCCKTACCTGCTCCAACTA-3′	1143-1124	
qacC (M37889)	qacC-F (Forward)	5′-GGCTTTTCAAAATTTATACCATCCT-3′	73-97	249
	qacC-R (Reverse)	5′-ATGCGATGTTCCGAAAATGT-3′	321-302	

[1)]: Primer positions were based on the nucleotide position from the start codon of the gene.
The mismatch sequence not to produce a *Alu*I site into the amplified fragment is underlined.

To detect these genes simultaneously, we developed a multiplex PCR-RFLP-based method using two primers to amplify the three genes. The nucleotide sequences of the primers are listed in Table 1. MRSA isolates were suspended in 100 μl of H_2O. The suspension was heated at 100°C for 10 min and centrifuged for 12,000 rpm for 5 min. One microliter of the supernatant was subjected to the multiplex PCR. The PCR was performed with 1.25 U of *Ex Taq*™ DNA polymerase (TaKaRa Bio, Shiga, Japan) and the two pairs of primers: qacA/B-F and qacA/B-R, which are expected to produce 220 bp of *qacA* or *qacB*; and qacC-F and qacC-R, which are expected to produce 249 bp of *qacC*. In the PCR, the same pair of PCR primers was used for the *qacA* and *qacB*, based on the fact that they are highly homologous to each other. After PCR, the products were digested with 5U of *Alu*I at 37°C for 90 min. The *qacB* was expected to be digested into two fragments of 176 bp and 44 bp; the *qacA* and *qacC* were not expected to be digested. Ten microliters of the *Alu*I-treated PCR products were subjected to electrophoresis in 15-25% polyacrilamide gel and stained with ethidium bromide. As shown in Fig. 1, bands with expected sizes of DNA fragments derived from the genes were detected. Standard PCR with the same primers as those used in the multiplex PCR and the DNA sequence of the PCR products was done. The sequencing showed that PCR products amplified by using the primers of qacA/B-F and qacA/B-R were *qacA* or *qacB*, and that those by qacC-F and qacC-R were *qacC*. These data confirmed that these three genes were detected by the multiplex PCR-RFLP-based method.

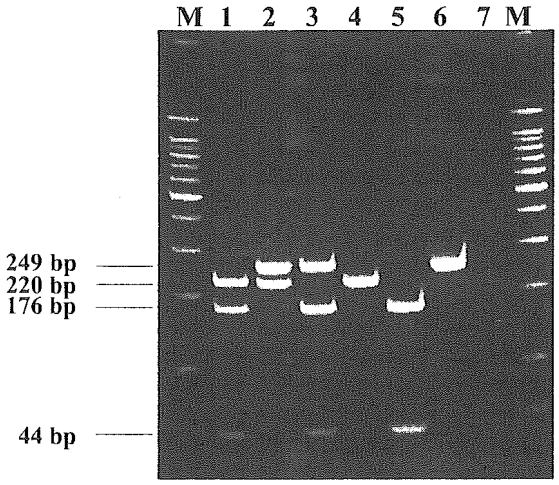

Fig. 1. Multiplex PCR-RFLP patterns after *Alu*I-digestion. Lanes, M: 100 bp ladder as molecular weight markers; Lane 1, both *qacA*- and *qacB*-positive; 2, both *qacA*- and *qacC*-positive; 3, both *qacB*- and *qacC*-positive; 4, *qacA*-positive; 5, *qacB*-positive; 6, *qacC*-positive; 7, *qac*-negative.

Chromosomal DNA was typed by using RFLP-based PFGE (2). Plasmid DNA typing was done by using agarose gel electrophoresis. The minimum inhibitory concentrations (MICs) of acriflavin (AF), benzalkonium chloride (BKC), benzethonium chloride (BTC), and alkyldiaminoethylglycine hydrochloride (AEG) were determined by using a twofold dilution method in Muller-Hinton broth. Southern blotting was performed to detect the *qacA*, *gacB*, and *qacC* genes by an ECL direct nucleic acid labeling and detection system (Amersham Biosciences Corp., Piscataway, N. J., USA). The Southern blotting failed to detect the *qacA* and *qacB* separately because of their similarities.

The 65 MRSA isolates tested were similar in MICs of AEG (25.0 or 12.5 μg/ml), whereas they were different in MICs of AF, BTC, and BKC. Among them, 34, 34, and 34 isolates showed relatively high resistance to AF, BKC, and BTC (MICs: ≥100, ≥6.25, and ≥6.25 μg/ml), respectively (Table 2). All of the 34 isolates resistant to AF (MIC: ≥100 μg/ml) had cross-resistance to BKC (MIC: ≥6.25 μg/ml), and to BTC (MIC: ≥6.25 μg/ml) (Table 2). In Table 2, all the isolates are classified into 42 types according to the parameters shown. One isolate of type No. 31 was slightly resistant to AF, BKC, and BTC (MICs: 50.0, 3.13, and 3.13 μg/ml, respectively). All of 30 isolates sensitive to AF (MIC: ≤25.0 μg/ml) were sensitive to both BKC and BTC (MICs: 0.78-3.13 μg/ml and 1.56-3.13 μg/ml).

The profiles of plasmid typing are shown in Fig. 2A. Plasmids of 17 different sizes, ranging from 2.4 kb to 300 kb, were detected. The isolates were classified into 14 plasmid patterns (Table 3). Of 65 isolates, 55 had one to seven plasmids. As summarized in Table 2, isolates with plasmid pattern α, β, or γ represented 48% of the total, and these isolates had both 35 kb and 50 kb plasmids.

The results of the multiplex PCR-RFLP assay are summarized in Table 2. Among the 65 MRSA isolates, 32 (including isolates of types Nos. 1, 2, 4-7, 10, 11, 13, 14, 16, 17, 20, and 21) were positive for *qacA*. The type No. 31 isolate was positive for *qacB*. Seven isolates of types Nos. 35 and 36 were positive for *qacC*.

To determine whether *qacA*, *qacB*, and *qacC* were on bacterial genome or plasmids, Southern blotting was conducted. The *qacA*, *qacB*, and *qacC* were detected only in plasmid DNA (Fig. 2B), not in chromosomal DNA (data not shown). As summarized in Table 2, all of 32 isolates with PCR-positive for *qacA* had two plasmids of 35 kb and 50 kb plasmids encoding *qacA* (Lanes 1-4 in Fig. 2B and Table 2).

Table 2. Genotypic and phenotypic characterization of the 65 MRSA clinical isolates

Type	No. of isolate	Ward	PFGE[1)] pattern	Plasmid pattern	Multiplex PCR-RFLP	Southern blot on Plasmid (kbp)		MICs (μg/ml)			
						qacA/B	*qacC*	AEG	AF	BKC	BTC
1	9	7N, 8N, 9S, 10S, 11N, 11S, 12S, 15	A1	α	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
2	1	8S	A1	β	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
3	1	12N	A1	ζ	–	–	–	25.0	25.0	1.56	1.56
4	1	8S	A28	β	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
5	3	7S, 12N	A29	α	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
6	1	15	A9	α	*qacA*	35, 50	–	25.0	400.0	6.25	12.50
7	1	12S	A21(Y2)	α	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
8	1	11S	A30	η	–	–	–	25.0	12.5	1.56	1.56
9	1	12N	A2(M1)	θ	–	–	–	25.0	12.5	1.56	1.56
10	2	7N	A18(M2)	γ	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
11	1	12S	A31	γ	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
12	1	12S	A32	–	–	–	–	25.0	12.5	1.56	1.56
13	3	7N, 11S, 16	A3	α	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
14	2	9N	A3	β	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
15	1	9N	A3	ζ	–	–	–	25.0	12.5	1.56	1.56
16	3	4S, 11S	A4	α	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
17	1	12S	A20	α	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
18	1	8N	AT	–	–	–	–	25.0	25.0	1.56	1.56
19	1	8N	Y5	–	–	–	–	25.0	12.5	1.56	1.56
20	2	4S, 11N	Y4(A6)	α	*qacA*	35, 50	–	25.0	100.0	6.25	6.25
21	1	7N	Y1(Y)	δ	*qacA*	35, 50	–	25.0	200.0	6.25	6.25
22	1	13S	AU1	κ	–	–	–	25.0	25.0	1.56	1.56
23	1	12S	AU2	κ	–	–	–	25.0	12.5	1.56	1.56
24	1	7N	AU3	κ	–	–	–	25.0	12.5	1.56	1.56
25	1	16	AU4	–	–	–	–	25.0	12.5	1.56	1.56
26	1	15	AU5	α	*qacA*	35, 50	–	25.0	100.0	12.50	12.50
27	1	14	AV1	λ	–	–	–	25.0	12.5	1.56	1.56
28	1	5N	AV2	μ	–	–	–	25.0	12.5	0.78	1.56
29	1	15	AV3	ν	–	–	–	25.0	12.5	0.78	1.56
30	2	5N	AB	β	*qacA*	35, 50	–	25.0	100.0	6.25	6.25
31	1	9S	AW	ε	*qacB*	10, 45	–	25.0	50.0	3.13	3.13
32	1	14	AE1(AE)	–	–	–	–	25.0	12.5	0.78	1.56
33	1	5N	AE2	κ	–	–	–	25.0	25.0	1.56	1.56
34	1	12S	AX	μ	–	–	–	25.0	25.0	1.56	1.56
35	1	13S	J8	ι	*qacC*	–	3.0	25.0	6.3	3.13	3.13
36	6	13S	J7(R2)	ι	*qacC*	–	3.0	25.0	12.5	3.13	3.13
37	1	7N	J5	θ	–	–	–	25.0	25.0	1.56	1.56
38	1	8N	AY	–	–	–	–	25.0	6.3	1.56	1.56
39	1	9S	AZ	κ	–	–	–	12.5	6.3	1.56	1.56
40	1	8N	BA	ξ	–	–	–	12.5	12.5	1.56	1.56
41	1	4S	BB	–	–	–	–	25.0	12.5	1.56	1.56
42	1	4S	BC	θ	–	–	–	12.5	12.5	1.56	1.56

[1)]: The PFGE patterns were reported in ref (1).

One with PCR-positive for *qacB* had two plasmids of 10 kb and 45 kb encoding *qacB* (Lane 5 in Fig. 2B and Table 2). Seven with PCR-positive for *qacC* had a 3 kb plasmid encoding *qacC* (Lane 9 in Fig. 2C and Table 2).

Based on PFGE typing, plasmid typing, multiplex PCR-RFLP, Southern blotting, and MICs, the isolates were classified into 42 types (Table 2). The presence of *qacA* corresponded exactly to resistance to AF, BKC, and BTC; i.e., all isolates harboring *qacA* were resistant to AF, BKC, and BTC, whereas all isolates not harboring *qacA*, *qacB*, and *qacC* were sensitive to these disinfectants. An isolate harboring *qacB* (isolate type No. 31) was sensitive to AF, BKC, and BTC, although the MICs were slightly higher than those of isolates without *qacA*, *qacB*, and *qacC*. Seven isolates harboring *qacC* (isolate types Nos. 35 and 36) were also sensitive to AF, BKC, and BTC, and the MICs of BKC and BTC were slightly higher than those of isolates without theses genes.

Of 65 MRSA, 34 (52.3%) were resistant to AF, BKC, and BTC, and had both 50 kb and 35 kb plasmids encoding *qacA*. Of these 34 isolates, 28 showed PFGE pattern A1 and its closely related patterns (cluster A: patterns A1 to A30). Previous studies (2-5) showed that the MRSA isolates showing PFGE pattern A1 were widely spread in a hospital in Tokyo. These isolates were resistant to disinfectants and had the 50 kb and 35 kb plasmids encoding *qacA*, indicating that the antiseptic and disinfectant resistance is mediated by *qacA*. Acquisition of resistance may be one of the most important

Table 3. Plasmid typing pattern classified by plasmid size

Plasmid pattern	Plasmid size
α	2.4, 35, 50
β	35, 50
γ	2.6, 35, 50
δ	3.5, 35, 50
ε	10, 45
ζ	2.4
η	2.4, 2.6, 3.2, 4.0, 5.5, 40, 180
θ	40, 180
ι	3.0, 40, 170
κ	33, 170
λ	3.5, 33, 170
μ	2.4, 33, 170
ν	40, 300
ξ	33, 48

factors in MRSA survival and wide transmission in hospital.

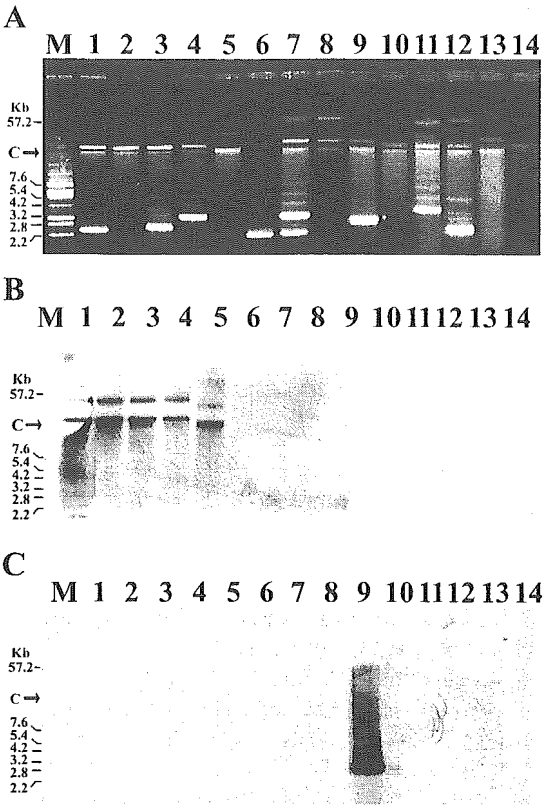

Fig. 2. Plasmid patterns of MRSA isolates (A) and Southern blotting hybridized with *qacA/B* (B) and with *qacC* (C). Lane M: Marker plasmids derived from *Escherichia coli* V517. Lanes 1 to 14 are corresponding to plasmid patterns, α to ξ, listed in Table 2.

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Laboratory and Epidemiology Communications

Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Serratia marcescens* in a Long-Term Care Facility for Patients with Severe Motor and Intellectual Disabilities

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Assessing the risk of nosocomial infection is necessary for optimizing the quality of patient care and the practice of infection control in long-term care facilities for patients with severe motor and intellectual disabilities (SMID). We conducted a molecular epidemiological study of pathogens in December 2002 and August 2003 in two wards of such a facility having three wards. Among the 39 inpatients in the wards, 20 had tracheotomy or were cared for with mechanical ventilators. The isolates were tested for chromosomal DNA typing by using a contour-clamped homogeneous electric field system (CHEF Mapper™: Bio-Rad Laboratories, Hercules, Calif., USA).

In December 2002, 14 of 20 patients carried at least one methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, or *Serratia marcescens* strain (Table 1). MRSA was isolated from 11 specimens from 9 patients, including eight patients' sputa, one patient's abscess, and one patient's eye mucus. Among these, two were obtained on different days from an abscess of patient P5 and two others from different sites of patient P7. *P. aeruginosa* was obtained from nine patients' sputa and *S. marcescens* from five patients' sputa. Three patients, P1, P4, and P6, carried MRSA, *P. aeruginosa*, and *S. marcescens* in the same specimen, and the other three patients, P3, P7, and P5, carried MRSA and *P. aeruginosa*.

The survey was repeated in August 2003. Eighteen patients carried at least one MRSA, *P. aeruginosa*, or *S. marcescens* strain (Table 1). MRSA strains were isolated from six patients, including four patients' sputa and two patients' urine. *P. aeruginosa* was isolated from 13 patients' sputa, and *S. marcescens* from three patients' sputa. No patient simultaneously carried MRSA, *P. aeruginosa*, and *S. marcescens* strains. Only one patient, P15, had both MRSA and *P. aeruginosa*, and two patients, P1 and P11, had *P. aeruginosa* and *S. marcescens*. Nine patients, P1, P2, P3, P4, P7, P8, P11, P13, and P14, carried MRSA, and either *P. aeruginosa* or *S. marcescens* both in December 2002 and in August 2003.

The PFGE patterns of these MRSA isolates are shown in Fig. 1A. From a total of 17 isolates, 12 different PFGE patterns were detected. Band-based cluster analysis of these patterns (Molecular Analyst™: Bio-Rad) revealed a cluster consisting of patterns A1, A3, and A16 (Fig. 1B) (patterns

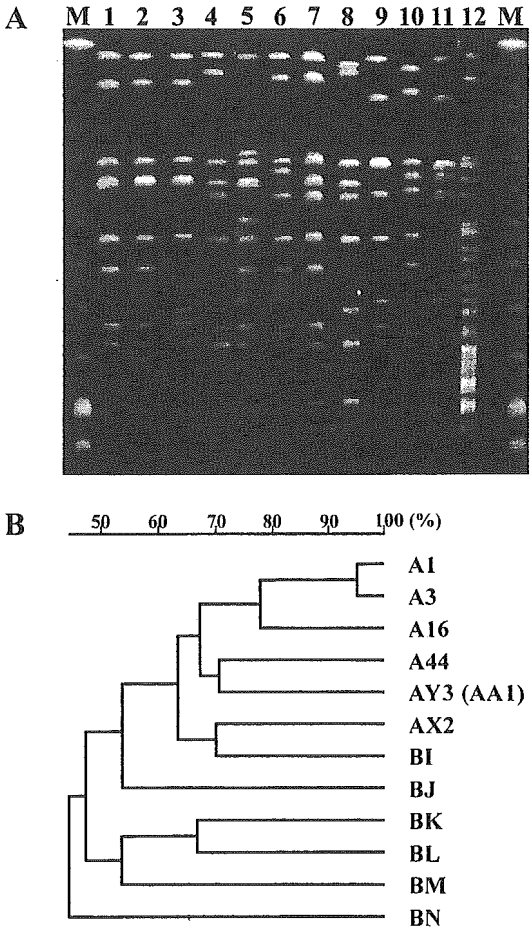

Fig. 1. Molecular analysis of MRSA isolate. A: pulsed-field gel electrophoresis of *Sma*I-digested genomic DNA from MRSA isolates. M: low range PFG Marker. Lanes 1 to 12 corresponding to the following PFGE pattern; 1: A1, 2: A3, 3: A16, 4: A44, 6: AY3, 7: BJ, 8: BI, 9: BK, 10: BL, 11: BM, 12: BN. B: cluster analysis of MRSA isolates based on PFGE patterns of *Sma* I-digested genomic DNA.

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