and pYI19, respectively (46). These plasmids encode the same bacteriocin with respect to immunity to the bacteriocin activity. Bacteriocin 31 (Bac31), encoded on pYI17, is representative of the class 3 bacteriocins and is active against *E. faecalis* and *E. hirae*, as is the membrane-active class II bacteriocin of lactic acid bacteria (46). The Bac31 determinant consists of the structural gene *bacA* and the immunity gene *bacB*.

In this report, we describe the cloning and genetic analysis of the bacteriocin 41 determinant encoded on *E. faecalis* pheromone-responsive conjugative plasmid pYI14, which is a representative class 4 bacteriocin. We also describe the identification of the two functional domains that are required to produce the active bacteriocin by extracellular complementation of the two factors.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, media, and reagents. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *E. faecalis* strains were grown in Todd-Hewitt broth (THB; Difco Laboratories) at 37°C, unless otherwise noted. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth. The following antibiotic concentrations were used for the selection of *E. faecalis*: erythromycin, 12.5 μg ml^{-1}; streptomycin, 250 μg ml^{-1}; kanamycin, 250 μg ml^{-1}; spectinomycin, 250 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; rifampin, 25 μg ml^{-1}; fusidic acid, 25 μg ml^{-1}. The antibiotic concentrations used for the selection of *E. coli* were as follows: ampicillin, 100 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; chloramphenicol 50 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}. All antibiotics were obtained from Sigma Chemical Co. X-Gal (5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside) was obtained from Wako Pure Chemical Industries, Ltd., and was used at 40 μg ml^{-1}.

Conjugation experiments. Broth mating and solid-surface mating were performed as previously described (48, 49), with a donor/recipient ratio of 1:10. Broth matings (in THB) were carried out for 4 h, and solid-surface matings (on THB agar plates) were carried out overnight (16 h) at 37°C. Transfer frequencies were calculated as the number of transconjugants per donor cell (at the end of mating). Pheromone induction and detection of cell aggregation were performed as previously described (11, 12).

Soft-agar assay for bacteriocin production and immunity. The bacteriocin production assay was performed as described previously (22). The test for immunity to the bacteriocin was performed essentially as described previously (22).

Plasmid DNA methodology. Recombinant plasmids were generated in *E. coli* DH5α. Transformation of bacterial cells with plasmid DNA was achieved by electrotransformation as described previously (13). Plasmid DNA was purified from *E. coli* (38) or from *E. faecalis* as previously described (14). DNA fragments were purified from an agarose gel after electrophoresis with a Gene Clean II kit (Bio 101, Inc.). Recombinant DNA methodology, analyses of plasmid DNA with restriction enzymes, and agarose gel electrophoresis were carried out by standard methods (38). Restriction enzymes were purchased from New England BioLabs, Roche, Nippon Gene, and Takara Co., and reactions were carried out under the conditions recommended by the manufacturers. DNA ligations were performed with a DNA ligation kit from Takara. To end fill the endonuclease-digested DNA fragment for ligation, a DNA-blunting kit and Klenow enzyme were obtained from Takara and used according to the manufacturer's protocol (45).

Determination of the pYI14 restriction map. pYI14 plasmid DNA was digested with EcoRI, BamHI, KpnI, SphI, or XbaI or double digested with a combination of two of these restriction enzymes. Agarose gel electrophoresis analysis of the digested DNAs was performed to determine the cleavage sites within the plasmid. To determine the order of the EcoRI fragments of pYI14, a relational clone set was constructed as previously described (14, 46). After agarose gel electrophoresis of plasmid pYI14 DNA partially digested with EcoRI, fragments greater than 7 kb in size were eluted and used for cloning. The cloning vectors used were pBluescript-SK(+) and pAM401, and the host strain was *E. coli* DH5α.

DNA sequence analysis. Nucleotide sequence analysis was carried out as previously described (14). A deletion kit (Nippon Gene) was used. BamHI-E, BamHI-F, EcoRI-H, and the 2.1-kb fragment between BamHI-F and EcoRI-H were individually cloned into the pBluescript vector. The clones were used to construct a series of deletional clones. The resulting constructs were sequenced in both orientations with the *Taq* Dye primer and the *Taq* Big Dye terminator cycle sequencing kit (Applied Biosystems), a model 377 DNA sequencer, and a

310 gene analyzer (ABI Prism). A database search was performed with the BLASTn and tBLASTx programs of the National Center for Biotechnology Information, Bethesda, MD (1).

Generation of transposon (Tn5, mini-Tn7) insertion mutants. Insertion of Tn5 (Km^r) into the cloned plasmid DNA was performed as described elsewhere (47). Target plasmid pHT1100(pAM401 containing EcoRI fragments A and H) was introduced into *E. coli* K-12 TH688 (with Tn5 in the *thr* locus) (42) by electrotransformation. Transformants were spread onto selective plates containing kanamycin and chloramphenicol, and the plates were left at room temperature for 10 days. The bacteria that grew on the selective plates were pooled, and the plasmid DNA was then isolated and used to transform *E. coli* DH5α. The transformants were selected on plates containing kanamycin and chloramphenicol for the selection of Tn5-borne kanamycin resistance and plasmid-borne chloramphenicol resistance, respectively. The transformants were purified and examined to determine the location of Tn5 within the plasmid. The precise locations of Tn5 insertions were determined by DNA sequence analysis with a synthetic primer that hybridized to the end of Tn5. A GPS kit (NEB) was used to generate mini-Tn7 insertion mutants with plasmid pHT1100 according to the manufacturer's instructions.

PCR amplification and primers. PCR amplification was performed with the thermostable DNA polymerase Takara *Taq* (Takara Bio Inc.) and a Perkin-Elmer 9600 thermal cycler. PCR conditions varied according to the primers used and the size of the anticipated product. The custom primers used in this study were obtained from Invitrogen (Tokyo, Japan) and are listed in Table 1. Each of the amplified PCR products was trimmed by the appropriate restriction enzyme, purified with a QIAquick-spin column (Qiagen), and cloned into plasmid pAM401.

Nucleotide sequence accession number. The nucleotide sequence reported in this article is available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB271686.

RESULTS

Bacteriocinogenic *E. faecalis* strain and the pheromone-responsive bacteriocin plasmid. Four strains that were active against *E. faecalis* and were classified as class 4 bacteriocinogenic strains were isolated from clinical urine samples and were designated YI712, YI714, YI715, and YI716. YI712 harbored plasmid pYI12 (72 kb). YI714 and YI715 harbored plasmids pYI14 (61 kb) and pYI141 (48 kb) and plasmids pYI15 (61 kb) and pYI151 (48 kb), respectively. YI716 did not carry any plasmid. Each strain was used as a donor in mating experiments with plasmid-free recipient strain *E. faecalis* FA2-2 (Rif^r Fus^r) to determine whether these plasmids conferred bacteriocinogenic activity on the host. After incubating the broth mating cultures for 4 h, appropriately diluted mixtures were plated on an agar plate containing rifampin (25 μg/ml) and fusidic acid (25 μg/ml) to select for the recipient strains. After overnight incubation of the plates, a total of approximately 500 *E. faecalis* FA2-2 colonies were obtained from each mating and examined for bacteriocin production. Approximately 1 in 500 cells obtained from the mating experiments with each of the strains described above expressed bacteriocin activity against *E. faecalis* FA2-2. The bacteriocinogenic transconjugants of YI712, YI714, and YI715 harbored pYI12 (72 kb), pYI14 (61 kb), and pYI15 (61 kb), respectively. The same EcoRI restriction profiles were obtained for pYI14 and pYI15, implying that the two plasmids were identical. Each plasmid transferred between *E. faecalis* FA2-2 and *E. faecalis* OG1-10 at a frequency of about 10^{-3} per donor cell by broth mating. *E. faecalis* FA2-2(pYI12), FA2-2(pYI14), and FA2-2(pYI15) did not exhibit bacteriocin activity against *E. faecalis* OG1-10(pYI12), OG1-10(pYI14), or OG1-10(pYI15). These results imply that plasmids pYI12, pYI14, and pYI15 encoded the same bacteriocin with respect to the immunity character-

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant features or sequence (5'-3')[a]	Reference(s), source, or generated plasmid(s)
Strains		
E. faecalis		
FA2-2	*rif fus*	7
JH2SS	*spc str*	44
OG1-10	*str*, derivative of OG1	12
OG1X	*str*, protease-negative derivative of OG1-10	23
YI712	pYI12(Bac)	This study
YI714	pYI14(Bac), pYI141 (48 kb); clinical isolate	This study
YI715	pYI15(Bac), pYI151 (48 kb); clinical isolate	This study
E. coli DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*(argF-lacZYA)U169*	Bethesda Research Laboratories
Plasmids		
pAM401	*E. coli-E. faecalis* shuttle plasmid; *cat tet*	50
pLZ12-Km	*E. coli-Streptococcus* shuttle plasmid; *aphA*	19
pBlueScript SKII(+)	*E. coli* cloning vector; Amp[r]	Stratagene
pPD1	Bac21, 59-kb conjugative plasmid from strain 39-5	14, 47
pMG326	pMW119 containing a 16.7-kbp EcoRI-SalI fragment of pPD1; pheromone-regulatory region	14, 41
pYI12	Bac41, 72-kb conjugation plasmid from YI712	This study
pYI14	Bac41, 61-kb conjugative plasmid from YI714	This study
pYI15	Bac41, 61-kb conjugative plasmid from YI715	This study
pHT1100	EcoRI-relational clone of pYI14; pAM401 containing EcoRI fragments A and H	This study
pHT1101	EcoRI-relational clone of pYI14; pAM401 containing EcoRI fragment A	This study
pHT1102	EcoRI-relational clone of pYI14; pAM401 containing EcoRI fragments H and M	This study
pMG1103	Derivative of pHT1100 with BamHI E fragment deleted	This study
pMG1104	Derivative of pHT1100 with BamHI F fragment deleted	This study
pMG1106	Derivative of pHT1100; BamHI site at 4.1 kbp blunted with Klenow enzyme	This study
pMG1108	Derivative of pHT1100; BamHI site at 6.3 kbp blunted with Klenow enzyme	This study
pMG1109	Derivative of pHT1100; KpnI site at 4.6 kbp blunted with DNA-blunting kit (Takara)	This study
pMG1105-*n*	Tn5 insertional derivatives of pHT1100	This study
pMG1107-*n*	Mini-Tn7 insertional derivatives of pHT1100 created with GPS kit (New England BioLabs)	This study
pMG1110	*bacL₁* and *bacL₂*; pAM401 containing 2,932-bp EcoRI fragment amplified by PCR	This study
pMG1111	*bacA*; pAM401 containing 2,836-bp SalI fragment amplified by PCR	This study
pMG1112	*bacI*; pAM401 containing 777-bp BamHI fragment amplified by PCR	This study
pMG1113	*bacI* and ORF13; pAM401 containing 1,513-bp BamHI fragment amplified by PCR	This study
pMG1114	pLZ12-Km containing 10-kbp BglII fragment mapped from 1.7 kbp to 11.7 kbp	This study
pMG1115	Derivative of pMG1114; EcoRI fragment (8.5 kbp to vector region) deleted	This study
pMG1116	Derivative of pMG1114; three HindIII fragments (4.6- to 6.6-kbp region) deleted	This study
Oligonucleotides		
B9P2842F	ccg gaa tTC TAG CAA CCG AAA ACC ACG TTG G	pMG1110
B9P5773R	gcg gaa tTC ATT GCG CAG CAA ATC ATT GC	pMG1110
B9P6180F	aac gcg tcg ACA GGA ATT GAG ACA TAC GCT	pMG1111
B9P9015R	aac gcg tcg acT TCG TCA AAT CCA TTT CCC CTA	pMG1111
B9P8823F	ggc gga tcc GCA GCA GAA TTA GCA GGA GCG	pMG1112, pMG1113
B9P9599R	gcc gga tcc CAA AAG TCA TAC ATG ACC TCC	pMG1112
B9P10335R	gcc gga tcc CTG TAT AAA TCC ATA CTA CAC	pMG1113

[a] Underlining indicates the following restriction endonuclease recognition sequences: GAATTC; EcoRI, GTCGAC; SalI, GGATCC; BamHI. Lowercase letters indicate incorporated tag sequences.

istic. *E. faecalis* FA2-2 strains carrying pYI12, pYI14, or pYI15 were tested for bacteriocin production against the indicator strains *S. aureus* FDA209P, *E. faecalis* FA2-2 and OG1-10, *Enterococcus faecium* BM4105RF, *E. hirae* ATCC 9790, *Entero-*

coccus durans ATCC 49135, *Enterococcus raffinosus* JCM8733, *Enterococcus gallinarum* BM4174, *S. agalactiae*, *S. pyogenes*, *Listeria monocytogenes*, and *Listeria denitrificans*. Each of the three bacteriocinogenic strains only showed bacteriocin activity against
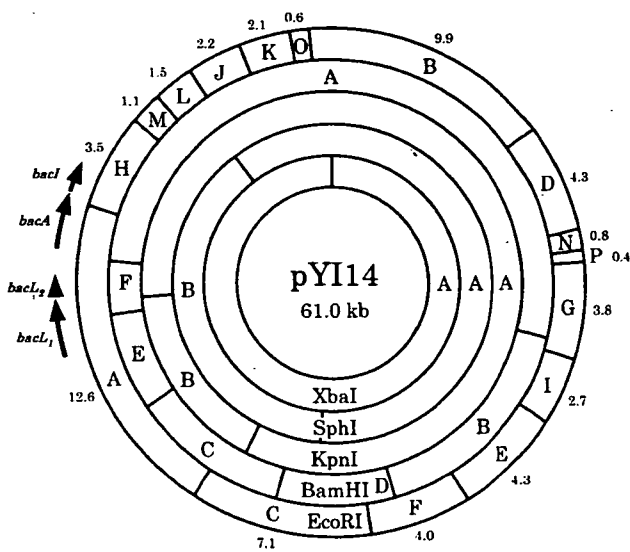
FIG. 1. Physical map of pYI14 showing the locations of bacteriocin 41 determinants $bacL_1$, $bacL_3$, $bacA$, and $bacI$. Each value is the size of the fragment in kilobases.

E. faecalis. Plasmid pYI14, isolated from strain YI714, was used as the representative plasmid encoding the bacteriocin.

The donor *E. faecalis* OG1-10(pYI14) and recipient *E. faecalis* FA2-2 formed a mating aggregate in the mating mixture. When OG1-10(pYI14) cells were exposed to *E. faecalis* FA2-2 culture filtrate (pheromone) for 4 h at 37°C, the OG1-10(pYI14) cells showed aggregation. Agarose gel electrophoresis of the EcoRI restriction fragments of pYI14 DNA was carried out, and the DNA was transferred to a membrane for Southern hybridization. The membrane was hybridized with a DNA probe containing the pheromone response genes of the pheromone-responsive plasmid pPD1 or plasmid pMG326, which contains the putative surface exclusion protein gene and the N-terminal region of the aggregation substance gene of pPD1 (14, 41). Each probe hybridized to specific pYI14 EcoRI fragments (data not shown). These results indicated that plasmid pYI14 was a pheromone-responsive plasmid.

Restriction map of pYI14. To determine the order of the EcoRI fragments, a relational clone set was obtained. The order of EcoRI fragments was determined to be A-H-M-L-J-K-O-B-D-N-P-G-I-E-F-C (Fig. 1). Each clone was digested with BamHI, KpnI, SphI, and XbaI, and the cleavage sites were determined (Fig. 1). Restriction sites within the EcoRI A and H fragments were also confirmed by sequencing (see the supplemental material).

Bacteriocin activity of the cloned DNA fragment. To examine the bacteriocin activity of the relational clones, each clone was introduced into *E. faecalis* OG1-10 and the resulting transformant was examined for bacteriocin activity. *E. faecalis* OG1-10 carrying plasmid pHT1100, which contained the EcoRI A and H fragments (16.1 kb), exhibited the bacteriocin activity (Fig. 2). *E. faecalis* OG1-10 carrying either the EcoRI A (12.6 kb) or HM (4.6 kb) fragments (plasmids pHT1101 and pHT1102, respectively) did not exhibit bacteriocin activity (Fig. 2). *E. faecalis* OG1-10 carrying the EcoRI HM fragments

showed resistance to the bacteriocin activity of *E. faecalis* OG1-10(pYI14). These results indicated that the bacteriocin determinant of pYI14 is located on the EcoRI A and H (AH) fragments and the immunity gene (i.e., the gene for resistance to its own bacteriocin) is located on the EcoRI H fragment.

DNA sequence analysis. The EcoRI AH fragments were sequenced, and computer analysis was used to identify open reading frames (ORFs) within the sequence. Fifteen ORFs (ORF1 to ORF15) were located in the region spanning map positions 0 to 12 kbp, as indicated by the numerical scale shown in Fig. 2, where position 0 is the BamHI site located between BamHI fragments E and C and position 12 kbp is the EcoRI site located between the EcoRI H and M fragments. (Fig. 1 and 2 and Table 2; see Fig. S1 in the supplemental material). Figure 2 shows the ORFs that have a deduced ribosome-binding site in the 20-base region upstream of the predicted start codon and the potential promoters for initiation of transcription.

Generation of Tn5 or mini-Tn7 insertion mutants. To examine the location of the bacteriocin determinant, mutants with altered bacteriocin expression were generated by Tn5 or mini-Tn7 insertion into pHT1100. The precise locations of Tn5 or mini-Tn7 insertions in the ORFs were determined by DNA sequence analysis (see Fig. S1 in the supplemental material), and the results are shown in Fig. 2 and Table 3. Tn5 insertions into ORF7, ORF8, and ORF11 resulted in defective bacteriocin activity in *E. faecalis* OG1-10. Insertion of mini-Tn7 into the C-terminal region of ORF11 also resulted in defective bacteriocin activity in *E. faecalis* OG1-10. *E. faecalis* OG1S carrying each of the insertion mutants showed resistance to the bacteriocin activity of *E. faecalis* OG1-10(pYI14), indicating that the mutant plasmids retained immunity to the bacteriocin.

Generation of deletion mutants by end filling after cleavage with a restriction enzyme. Mutant pHT1100 plasmids with BamHI fragment deletions within were also generated to examine the location of the bacteriocin determinant as described in Materials and Methods (Fig. 2) (47). Deletion mutant plasmids pMG1103 and pMG1104 possessed deletions of the 4.1-kbp BamHI E fragment between map positions 0 kb and 4.1 kb and the 2.2-kbp BamHI F fragment between map positions 4.1 kb and 6.3 kb, respectively. Plasmid pMG1103, which had a deletion in the amino-terminal region of ORF7 and had lost the six ORFs located upstream of ORF7, did not exhibit bacteriocin activity but retained immunity to the bacteriocin. Plasmid pMG1104, which had deletions within the carboxyl-terminal region of ORF7, ORF8, and ORF9 and the amino-terminal region of ORF10, did not exhibit bacteriocin activity but retained immunity to the bacteriocin. These results implied that the gene for immunity is located downstream of ORF11.

Generation of four-nucleotide insertion (deletion) mutants. Mutants with changes in ORF7 and ORF10 were generated to obtain mutants with in-frame changes in the determinant by blunt ending the recessed 3' terminus of the BamHI site or the prominent 3' terminus of the KpnI cleavage site within pHT1100 DNA that had been partially digested with these enzymes prior to ligation (Fig. 2) (45). Blunt ending the BamHI and KpnI sites resulted in the insertion of four nucleotides (5'-GATC-3') with the Klenow enzyme in the case of the BamHI site and the deletion of four nucleotides (5'-GTAC-3') with the T4 DNA polymerase DNA-blunting kit
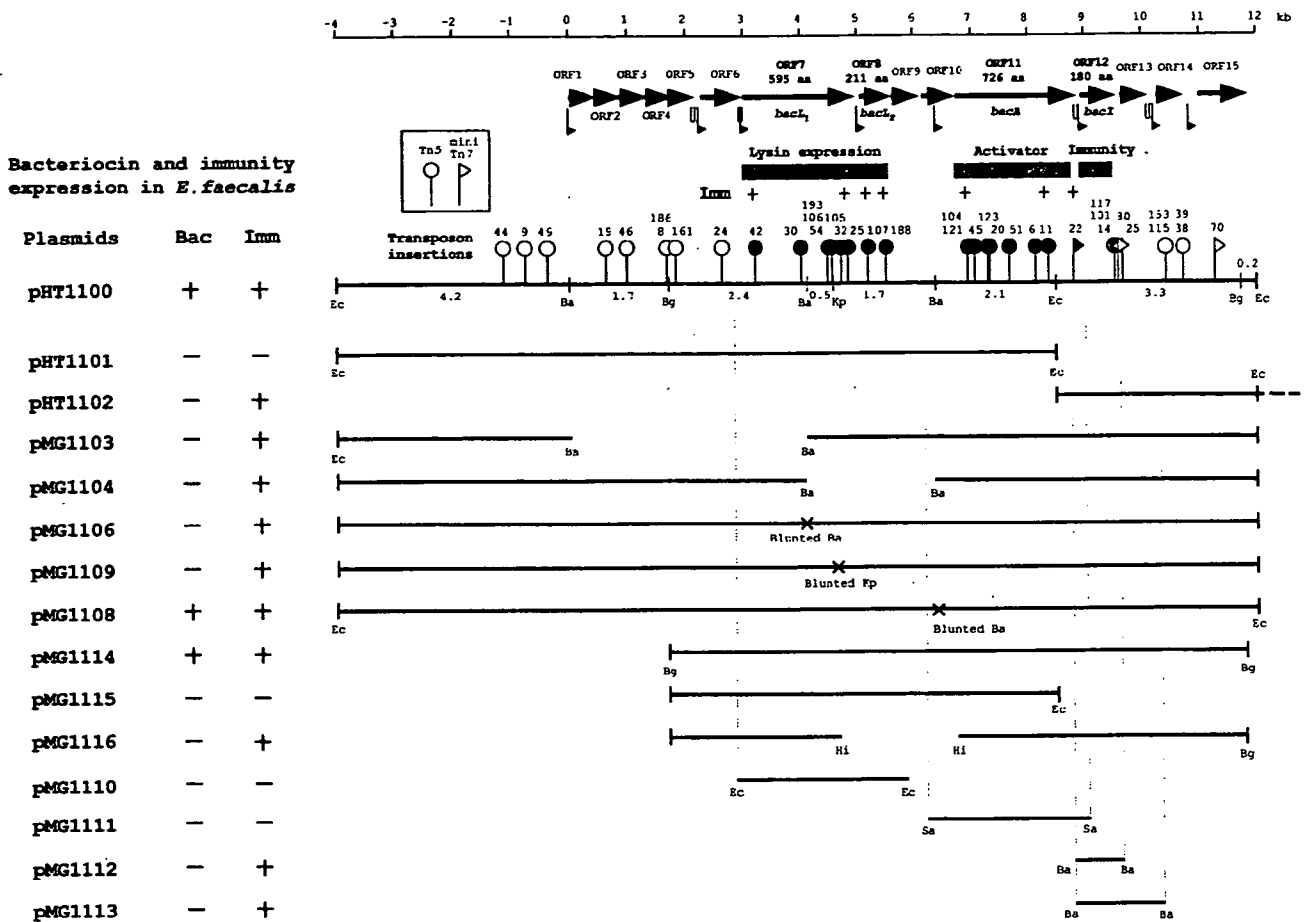
FIG. 2. Physical maps of the 16.1-kb region containing EcoRI fragments A (12.6 kb) and H (3.5 kb) in pYI14 (which is carried on pHT1100), transposon insertions, and subclones. The zero position of the numerical scale (top horizontal line) indicates the BamHI endonuclease recognition site located between the BamHI C and E fragments, and it runs in a clockwise direction on the physical map of Fig. 1. Thick horizontal arrows indicate the predicted ORFs and the direction of ORF transcription. The flags and hairpins below the ORFs indicate the potential promoter regions and inverted repeat sequences. The horizontal lines under the map represent the cloned pYI14 DNA fragments in the derivative plasmids listed on the left. Small vertical bars at ends of the lines represent the endonuclease recognition sites for cloning. The dotted vertical lines represent the ends of the amplified PCR fragment of pYI14 DNA used to clone the bacteriocin determinant. The endonuclease recognition sites incorporated for the cloning of the PCR products are indicated. Abbreviations of the endonuclease recognition sites: Eco, EcoRI; Ba, BamHI; Kp, KpnI; Bg, BglII; Hi, HindIII; Sa, SalI. Bac +, normal bacteriocin expression; Bac −, no bacteriocin expression; Imm +, resistance to bacteriocin 41; Imm −, sensitive to bacteriocin 41. The vertical lines with circular or triangular heads on the pHT1100 map show the points of transposon insertion. The circular heads indicate Tn5, and the triangular heads indicate mini-Tn7 and its orientations. The heads represent the levels of bacteriocin 41 expression in *E. faecalis* strains as follows: open heads, normal bacteriocin expression; black heads, no bacteriocin expression; gray heads, weak bacteriocin expression (Fig. 3A). The values on the insertions indicate the numbers of insertions and correspond to those shown in Table 3 (see also Fig. S1 in the supplemental material). The cross marks on the clones indicate the mutated endonuclease recognition sites (a four-base insertion or deletion). aa, amino acids.

(Takara) in the case of the KpnI site. The pMG1106 and pMG1109 mutants that resulted from the blunt ending of the BamHI site and KpnI sites in ORF7 did not exhibit bacteriocin activity but retained the immunity activity, indicating that ORF7 is essential for bacteriocin expression. The pMG1108 mutant, which resulted from the end filling of the BamHI site in ORF10, expressed both bacteriocin and immunity activity, suggesting that ORF10 is not essential for bacteriocin expression.

Subcloning of the bacteriocin determinant and generation of the derivative mutants. The 10.0-kb BglII fragment that is located between 1.7 kb and 11.7 kb on the map was cloned into shuttle vector pLZ12-Km (19) (Fig. 2), and the cloned plasmid

was designated pMG1114. pMG1114 expressed both bacteriocin activity and immunity, indicating that the bacteriocin determinant was located within the 10.0-kb BglII fragment. Deletion mutants pMG1115 and pMG1116 were generated from pMG1114. pMG1115 had a deletion of the 3.3-kbp EcoRI/BglII fragment between 8.4 kb and 11.7 kb on the map, which contains the C-terminal region of ORF11. pMG1115 did not express either the bacteriocin or immunity, indicating that ORF11 is necessary for bacteriocin activity. pMG1116 had a deletion of two HindIII fragments totaling 1.4 kb that were located between 5.2 kb and 6.6 kb on the map and contains the C-terminal region of ORF7 and all of ORF8, ORF9, and ORF10. pMG1116 did not express the bacteriocin but ex-

TABLE 2. ORFs encoded on the BamHI/EcoRI 11,952-bp-spanning region

ORF	Gene	Map location (bp)	Gene/protein size (base pairs/amino acids)	Homology	% Identity/similarity (amino acids)	Function
1		136–588	453/150	pcfS (E. faecalis pCF10)	98/100	Ssb[a]
2		602–754	153/50	EFB0044 (E. faecalis V583 pTEF2)	100/100	
3		766–1344	579/192	pcfT (E. faecalis pCF10)	89/90	Thermonuclease
4		1350–1670	321/106	pcfU (E. faecalis pCF10)	93/97	
5		1827–2009	183/60	Efae03001107 (E. faecium)	50/67	
6		2204–2920	717/238	Lipoprotein (E. faecalis V583)	31/45	
7	bacL₁	3058–4845	1,788/595	Lysozyme (B. subtilis bacteriophage B103)	37/52 (1–151)	Lysin (bacteriocin 41)
				Lysin (S. agalactiae prophage lambda Sa1)	46/63 (160–309)	
				Muramidase (L. plantarum WCFS1)	24/41 (318–577)	
8	bacL₂	5031–5666	636/211			Lysin expression
9		5689–6120	432/143	ORF50 (S. pneumoniae bacteriophage MM1)	31/51	Holin
10		6123–6650	528/175	EF0637 (E. faecalis V583)	27/45	
11	bacA	6693–8873	2,181/726	ybfG (B. subtilis)	41/56	Lysin activator
				ykuG (B. subtilis)	40/55	
12	bacI	8981–9523	543/180			Immunity
13		9590–10165	576/191			
14		10308–10640	333/110	EFB0057 (E. faecalis V583 pTEF2)	98/100	
15		11080–11781	702/233			

[a] Ssb, single-stranded binding protein.

pressed immunity. Analysis of the insertion mutants and deletion mutants showed that ORF7, ORF8, ORF11, and ORF12 are necessary for bacteriocin expression.

Extracellular complementation of nonbacteriocinogenic mutants. Extracellular complementation experiments to express bacteriocin activity were performed with ORF7 or ORF8 and ORF11 mutant strains on soft agar plates containing the indicator strain. OG1-10(pMG1106) and OG1-10(pMG1109), which were ORF7 mutants prepared by blunt ending, were streaked in proximity to streaks of either OG1-10 carrying the pHT1100 derivatives of the Tn5 insertion mutants in ORF11 or OG1-10(pHT1101) with a deletion in ORF11. This experiment showed that there was complementation of the bacteriocin activity at the streak junction. When OG1-10(pHT1101) was streaked in proximity to streaks of OG1-10 carrying the pHT1100 derivatives of the Tn5 insertion mutants in ORF7 or ORF8 and the end-filled mutants of ORF7, complementation of the bacteriocin activity was observed at the streak junction. These results indicated that the mutants fell into one of two complementation groups. Representative results are shown in Fig. 3 and Table 4. The OG1-10(pHT1100), OG1-10(pHT1101), and OG1-10(pMG1106) strains were inoculated in proximity in soft agar containing the indicator strain (Fig. 3B). Bacteriolysis was observed around the wild-type strain and also between OG1-10(pHT1101) and the wild-type strain or OG1-10(pMG1106), respectively. Bacteriolysis was also observed surrounding OG1-10(pHT1101). Figure 3C shows the complementation activity that resulted from cross-streaking of OG1-10(pMG1106) and OG1-10(pHT1101) on soft agar containing the indicator strain. Bacteriolysis was observed at the junction of the two strains. Based on these observations, the two complementation substances were tentatively designated L (lysin) and A (activator). OG1-10(pHT1101) and the ORF11 mutants were presumed to be defective in bacteriocin component A synthesis and tentatively assigned an L⁺ A⁻ phenotype. The ORF11 gene was designated bacA. OG1-10(pMG1106) and the ORF7 and ORF8 mutants

were presumed to be defective in bacteriocin component L synthesis and tentatively assigned an L⁻ A⁺ phenotype. The ORF7 and ORF8 genes were designated bacL₁ and bacL₂, respectively.

Cloning of component L, component A, and the immunity genes. The PCR product of each ORF was cloned to analyze its function in bacteriocin expression (Fig. 2). Cloned pMG1110, pMG1111, pMG1112, and pMG1113 contained ORF7/8 (bacL₁ and -L₂), ORF11 (bacA), ORF12, and ORF12/13, respectively (Fig. 2). Each of the individually cloned fragments did not express bacteriocin activity. pMG1112 and pMG1113, which contained ORF12 and ORF12/13, expressed immunity to the bacteriocin activity, indicating that ORF12 was the immunity gene, and it was designated bacI.

Extracellular complementation between cloned L and A components. Cross streaks of strains carrying the two cloned fragments were made on bacteriocin assay plates. When OG1-10(pMG1110), which contained ORF7 (bacL₁) and ORF8 (bacL₂), was streaked across a preexisting streak of OG1S (pMG1111), which contained ORF11 (bacA), a large area of bacteriolysis was observed around the two crossed strains (Table 4). Growth of the two strains was markedly inhibited. These data indicated that the product of each strain complemented to produce an active bacteriocin, but the two strains have no immunity to the bacteriocin; therefore, growth of the strains was inhibited by the bacteriocin.

DNA sequence analysis of ORFs located in the region containing the bacteriocin 41 determinant. A homology search of the 15 ORFs contained in the 12-kbp region was performed by BLAST against the protein databases, and the results are shown in Table 2 (1). ORF7 (bacL₁), ORF8 (bacL₂), ORF11 (bacA), and ORF12 (bacI) were essential for the expression of bacteriocin 41. bacL₁ encoded a 595-amino-acid protein. Computer analysis suggested that the deduced bacL₁-encoded protein had a signal peptide sequence and that a potential signal peptidase processing site corresponding to the L-K-A sequence was located at positions 19 to 21 (Fig. 4A). Comparison

TABLE 3. Transposon insertion mutants of pHT1100 and bacteriocin expression

Insertion no. in Fig. 2	Plasmid(s)[a]	Transposon	Map position (kb)[b]	Insertion location	Bac[c]	Imm[d]
	pYI14				++	+
	pHT1100				++	+
44	pMG1105-44	Tn5	−1.2	Upstream of ORF1	++	+
9	pMG1105-9	Tn5	−0.8	Upstream of ORF1	++	+
49	pMG1105-49	Tn5	−0.4	Upstream of ORF1	++	+
19	pMG1105-19	Tn5	0.7	ORF2	++	+
46	pMG1105-46	Tn5	1.0	ORF3	++	+
8, 186	pMG1105-8, -186	Tn5	1.7	Between ORF4 and ORF5	++	+
161	pMG1105-161	Tn5	1.9	ORF5	++	+
24	pMG1105-24	Tn5	2.6	ORF6	++	+
42	pMG1105-42	Tn5	3.2	ORF7 (*bacL₁*)	−	+
30	pMG1105-30	Tn5	4.0	ORF7 (*bacL₁*)	−	+
54, 105, 193	pMG1105-54, -105, -193	Tn5	4.5	ORF7 (*bacL₁*)	−	+
105	pMG1105-105	Tn5	4.6	ORF7 (*bacL₁*)	−	+
32	pMG1105-32	Tn5	4.7	ORF7 (*bacL₁*)	−	+
25	pMG1105-25	Tn5	4.8	ORF7 (*bacL₁*)	−	+
107	pMG1105-107	Tn5	5.2	ORF8 (*bacL₂*)	−	+
188	pMG1105-188	Tn5	5.5	ORF8 (*bacL₂*)	−	+
104, 121	pMG1105-104, -121	Tn5	6.9	ORF11 (*bacA*)	−	+
45	pMG1105-45	Tn5	7.1	ORF11 (*bacA*)	−	+
123	pMG1105-123	Tn5	7.3	ORF11 (*bacA*)	−	+
20	pMG1105-20	Tn5	7.3	ORF11 (*bacA*)	−	+
51	pMG1105-51	Tn5	7.7	ORF11 (*bacA*)	−	+
6	pMG1105-6	Tn5	8.1	ORF11 (*bacA*)	−	+
11	pMG1105-11	Tn5	8.4	ORF11 (*bacA*)	−	+
22	pMG1107-22	Mini-Tn7	8.7	ORF11 (*bacA*)	−	+
14, 101, 117	pMG1105-14, -101, -117	Tn5	9.5	ORF12 (*bacI*)	+	±
80	pMG1107-80	Mini-Tn7	9.5	Between ORF12 and ORF13	++	+
25	pMG1107-25	Mini-Tn7	9.6	ORF13	++	+
115, 153	pMG1105-115, -153	Tn5	10.4	ORF14	++	+
38, 39, 163	pMG1105-38, -39, -163	Tn5	10.7	Between ORF14 and ORF15	++	+
87	pMG1107-87	Mini-Tn7	11	Between ORF14 and ORF15	++	+
74	pMG1107-74	Mini-Tn7	11	Between ORF14 and ORF15	++	+
76	pMG1107-76	Mini-Tn7	11.1	Between ORF14 and ORF15	++	+
69	pMG1107-69	Mini-Tn7	11.1	ORF15	++	+.
59	pMG1107-59	Mini-Tn7	11.2	ORF15	++	+
70	pMG1107-70	Mini-Tn7	11.3	ORF15	++	+
40	pMG1107-40	Mini-Tn7	11.4	ORF15	++	+
80	pMG1107-80	Mini-Tn7	11.4	ORF15	++	+
83	pMG1107-83	Mini-Tn7	11.5	ORF15	++	+

[a] The host strain of the derivative was *E. faecalis* OG1S (OG1-10).
[b] The map position is the distance from the junction between EcoRI fragments A and H. Minus values indicate the opposite direction.
[c] Bac, bacteriocin expression. Symbols: ++, normal bacteriocin expression; +, weak bacteriocin expression (Fig. 3A); −, no bacteriocin expression.
[d] Imm, immunity expression. Symbols: +, positive expression; −, no expression; ±, weak expression.

of the primary structure of the deduced amino acid sequence of the BacL₁ protein showed significant homology with the cell wall lytic enzymes found in gram-positive bacteria (Fig. 4A) (32). Of the 595 amino acid residues of the BacL₁ protein, the N-terminal 151 amino acid residues showed a high level of homology with the lysozyme encoded on *Bacillus subtilis* bacteriophage B103 (accession number Q37896) (37). The 150-amino-acid sequence from residue 160 to residue 309, which is located in the center of the *bacL₁*-encoded protein, showed a high level of homology with the N-terminal amino acid residues of the lysin encoded on the *S. agalactiae* prophage lambda Sa1 (accession number NP 687631) (43), and the C-terminal 260 amino acid residues showed a high level of homology with the C-terminal amino acid residues of the muramidase of *Lactobacillus plantarum* WCFS1 (accession number CAD64901) (30). The *bacL₁*-encoded protein harbored a three-repeat structure of an almost identical amino acid sequence (Fig. 4B). The three-repeat structure located at the C terminus of the

bacL₁-encoded protein corresponded to the homologous C-terminal region of the *L. plantarum* WCS1 muramidase, which is thought to be a choline-binding region (28, 51). The repeat structure was composed of three copies of an almost identical 74-amino-acid sequence. The first copy was located between amino acid residues 333 and 406, the second copy was located between amino acid residues 424 and 497, and the third copy was located between amino acid residues 520 and 593. *bacL₂* encoded a 211-amino-acid protein and did not show any significant homology with other reported proteins. There was no obvious leader peptide with hydrophobic residues at the N-terminal peptide of the deduced *bacL₂*-encoded protein. *bacA* encoded a 726-amino-acid protein and showed a significant degree of homology with *ybfG* and *ykuG* of *B. subtilis*, but the function of these proteins is unknown (Fig. 5) (accession numbers CAB12014 for YbfG and CAA10870 for YkuG, respectively). The *bacA* protein had a putative signal peptide sequence, and a potential signal peptidase processing site corresponding to

A

1   2   3

1; pHT1100

2; pMG1105-14

3; pMG1106

L+A+I+   L+A+I±   L-A+I+

B

1   2   3

1; pHT1100

2; pHT1101

3; pMG1106

L+A+I+   L+A-I+   L-A+I+

C

2

1; pHT1101
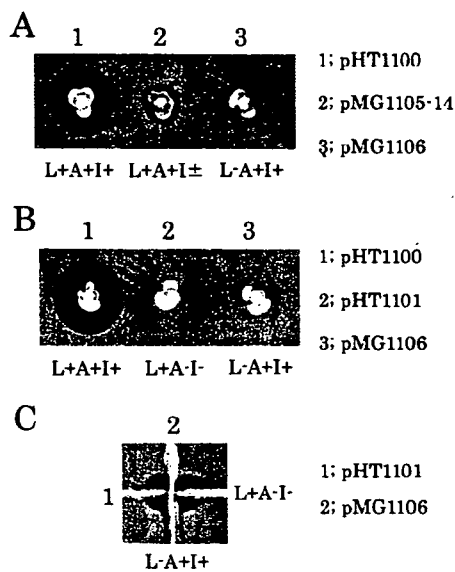
2; pMG1106

L+A-I-

1

L-A+I+

FIG. 3. Bacteriocin expression assay by the soft-agar method with *E. faecalis* OG1-10 carrying the representative pYI14 bacteriocin derivatives (A) and complementation assays (B and C). The indicator strain was *E. faecalis* OG1-10. The strains used are shown in Fig. 2 and Table 3. (A) 1, OG1-10(pHT1100) wild type; 2, OG1-10(pMG1105-14, a transposant of pHT1100::Tn5) (Tn5 inserted in the C-terminal region of ORF12); 3, OG1-10(pMG1106) in-frame *bacL$_1$* mutant. (B) 1, OG1-10(pHT1100); 2, OG1-10(pHT1101) *bacA* and *bacI* deletion mutant; 3, OG1-10(pMG1106). (C) 1; OG1-10(pHT1101), 2; OG1-10(pMG1106). L, *bacL$_1$* and *bacL$_2$* expression; A, *bacA* expression; I, immunity expression; +, positive expression; −, no expression; ±, weak expression.

the V-S-G sequence was located at positions 19 to 21 (Fig. 5). The *bacA* protein contained a 60-amino-acid sequence corresponding to the putative peptidoglycan-binding domain, which was located between amino acids 81 and 140 in the *bacA*-encoded protein, suggesting that the BacA protein could be directed to the bacterial cell surface.

DISCUSSION

Bacteriocin 41 of strain YI714 was encoded on *E. faecalis* pheromone-responsive plasmid pYI14 (61 kbp) and was only active against *E. faecalis*. The EcoRI AH fragments of pYI14,

which conferred the bacteriocin activity, were cloned and used for genetic analysis of the bacteriocin determinant. Transposon insertion and deletion mutant analysis of the EcoRI AH fragments and further subcloning of the bacteriocin determinant showed that a 6.6-kb fragment of pYI14 was the minimum-size fragment required for bacteriocin expression. The 6.6-kb region contained six ORFs, which were designated *bacL$_1$*, *bacL$_2$*, ORF9, ORF10, *bacA*, and *bacI*. All of the ORFs were oriented in the same direction and in that order. The insertion mutants were classified into one of two complementation classes for component L and component A. Each class showed extracellular complementation to produce the active bacteriocin. A series of PCR products containing the L-encoding region for component L, the A-encoding region for component A, and the immunity-encoding region for resistance to the bacteriocin were subcloned into *E. faecalis* OG1-10. The subclones for the L-encoding, A-encoding, and immunity-encoding regions contained *bacL$_1$* and *bacL$_2$*, *bacA*, and *bacI*, respectively. The subclone containing *bacL$_1$* and *bacL$_2$* produced an L component capable of extracellular complementation with the A component for expression of bacteriocin activity, indicating that *bacL$_1$* and *bacL$_2$* were required for component L. These results indicated that of the ORFs within the 6.6-kb region, *bacL$_1$*, *bacL$_2$*, and *bacA* are essential for the production of the active bacteriocin, and *bacI* is the immunity gene for resistance to the bacteriocin that is produced.

Tn5 insertions into *bacL$_1$* or *bacL$_2$* of the bacteriocin determinant did not result in a detectable polar effect on the expression of the downstream *bacA* or *bacI* gene, and insertion into *bacA* also did not result in a polar effect on the expression of *bacI*. Both component determinants and *bacI* were expressed when each of the determinants was cloned into vector plasmid pAM401 in either orientation within an *E. faecalis* OG1-10 background. These results suggested that a significant amount of transcription of the *bacL$_1$* and *bacL$_2$*, *bacA*, and *bacI* genes can occur from different promoters.

In the complementation experiment between the L$^+$ A$^-$ and L$^-$ A$^+$ strains, bacteriocin activity was observed around the L$^+$ A$^-$ strain. When the wild-type L$^+$ A$^+$ and mutant L$^+$ A$^-$ strains were inoculated in proximity to the bacteriocin assay, bacteriolysis was observed around the L$^+$ A$^-$ strain. The complementation experiment between the wild-type L$^+$ A$^+$ and mutant L$^-$ A$^+$ strains did not show any bacteriocin activ-

TABLE 4. Extracellular *trans*-complementation analysis of bacteriocin 41 activity[a]

Plasmid(s)	Genotype	Phenotype[b]	pMG1105-42, pMG1105-25	pMG1106, pMG1109	pMG1105-107, pMG1105-188	pMG1105-104, pMG1105-11	pHT1101	pMG1110	pMG1111
pMG1105-42, pMG1105-25	*bacL$_1$ L$_2$$^+$ A$^+$ I$^+$*	L$^-$A$^+$I$^+$	NT						
pMG1106, pMG1109	*bacL$_1$ L$_2$$^+$ A$^+$ I$^+$*	L$^-$A$^+$I$^+$	C$^-$	NT					
pMG1105-107, MG1105-188	*bacL$_1$$^+$ L$_2$A$^+$ I$^+$*	L$^-$A$^+$I$^+$	C$^-$	C$^-$	NT				
pMG1105-104, pMG1105-11	*bacL$_1$$^+$ L$_2$$^+$ A I$^+$*	L$^+$A$^-$I$^+$	C$^+$	C$^+$	C$^+$	NT			
pHT1101	*bacL$_1$$^+$ L$_2$$^+$ A I*	L$^+$A$^-$I$^-$	C$^+$	C$^+$	C$^+$	C$^-$	NT		
pMG1110	*bacL$_1$$^+$ L$_2$$^+$ A I*	L$^+$A$^-$I$^-$	C$^+$	C$^+$	C$^+$	C$^-$	C$^-$	NT	
pMG1111	*bacL$_1$ L$_2$A$^+$ I*	L$^-$A$^+$I$^-$	C$^-$	C$^-$	C$^-$	C$^+$	C$^+$	C$^+$	NT

[a] C$^+$; bacteriocin activity was detected at the intersection of the two strains by the soft-agar assay, C$^-$; no bacteriocin activity, NT; not tested.
[b] L, expression of lytic protein(s); A, activator expression; I, immunity expression.

A

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                                        putative processing site

                        signal peptide        ↓
ORF7 (BacL1)        1   MNYSQKAIDL CKKYSNFSLK AVAGRNGIL- -SIGYGHFTN EKHPIKPGMV ITESQATQIL RDDLNEHAAL ISKLLAIKAT   78
                        * :  **  *  *  :  :  **  *         :******:  :  * :   *  ** ** :* ***::    : * * :  *
Lysozyme            1   MQISQAGINL IKSFEGLQLK AYKAVPTEKH YTIGYGHYGS DVSPRQ---V ITAKQAEDML RDDVQAFVDG VNKALKVSVT   77


ORF7 (BacL1)       79   QNQFDALVSF SHSKGLGFLP SSDIMHFTNN KEFNSAAREM KLYVYDIGSI KLPKLVERRN AETALYLEGA SGHEETTNHA   158
                        ********** :  ***   ** :: :  *      ** :      * :       ** ** * **  *      *
Lysozyme           78   QNQFDALVSF AYNVGLGAFR SSSLLEYLNE GRTALAAAEF PRWNKSGGKV YQG-LVNRRA QEQALFNSGT PKNV------  150


ORF7 (BacL1)      159   -RIGFDVMIR WMEQKKAQHI TYSMDYRLGP NSYDCSSAVY FALKEAGFID PSTFPGNTDS LFGQLERVGW SQVPLVGGKY  247
                        *  :   *  :*  : : ********* ** :******:** ***: ** *  : :  **:   * : *:  :    :
Lysin (lambda Sa1)  1   MVINIEQAIA WMASRKGK-V TYSMDYRNGP SSYDCSSSVY FALRSAGASD -NGWAVNTEY EHDWLIKNGY VLI-AENTNW   77


ORF7 (BacL1)      250   HVQRGDIFIW GIRGNSGGEL GHTGIFIDDK DNIIHCTCGW DGNKCSINGI SVDNHDQVWV ASGRPPVTIY RFGGASKPYP  317
                        : ******** * ** *  *  ****:*: *  ****** :*   : * :*** ::*   :*:* * * *:
Lysin (lambda Sa1) 80   NAQRGDIFIW GKRGASAGAF GHTGMFV-DP DNIIHCNYGY ------NSI TVNNHDEIWG YNGQPYVYAY RYARKQSNAK  149


ORF7 (BacL1)  318  GDSSGSKGDS -VNPSAGVFY PSMRLPVSGD TDPNSPALDY YEAGQAIVYD SYVFANGYAW ISYVAGSGLR RYVAVGPDDG RTDTVWGTGF LN    408
                   ** **      :  :   :  :   :  : **   * : *:   *  **  * : *:: **  :    **  :   *:
Muramidase    554  GDEVGSVAKP DVVATSGSYR FTKTTAIKSS PATSATTVGS YNAGDTVYYN GKVTTNGQTW LRYMSYSGAQ HYVQISGEST STNVDKPQVT PQ    637


ORF7 (BacL1)  409  NTPSGSGSNT GSALSGVFYP SMRLPVSGDT DPNSPALAYY EAGQAIIYDS YVFANGYAWI SYIAGSGLRR YVAVGPDDGR TDTVWGTGFF DN    500
                        **  :   :        *:*:: *   **  : *:: * *** *: *:: ** :  *     *    .  -
Muramidase    638  ---------- ----SGSYRF TQTTAIKNTP AGNAPSVGTY SAGDTVYYNA KVTANGQTWL RYLSYSGAQH YVAI---SGN AAT------- --    710


ORF7 (BacL1)  501  GGDPGSQAHP NSIGLVPKAG NFVPNRKLPV SADTDPNSAA LDYYEAGQSI GYDSYIFANG YAWISYIAGS GLRRYVAVGP DDGRTDTVWG KGFFN  595
                   *:  *:   ** *    ** :    :  :: : *  *:::: :*: : * * * *:  *: * * ***
Muramidase    711  -GNTTSKPVT NSQG----AF RFVTTTNIRT APST--RASV VGEYNPGETV YYNGTVQAEG YTWLRYLSRS GATHYVA               781
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B

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ORF7 (BacL1)  321  SGSKGDSVNP -SAGVFYPSM RLPVSGDTDP NSPALDYYE AGQAIVYDSY VFANGYAWIS YVAGSGLRRY VAVGPDDGRT DTVWGTGFLN NTP    411
                   ***        *******   ***** *** ***** ***  ********  *** ***** ********** *******  *****
ORF7 (BacL1)  412  SGSGSNTGSA -LSGVFYPSM RLPVSGDTDP NSPALAYYE AGQAIIYDSY VFANGYAWIS YIAGSGLRRY VAVGPDDGRT DTVWGTGFFD NGGDPGS  506
                   *  *        ****** *  ***** *** ***** ***  ********  *** *****  ********** **********  ****** **
ORF7 (BacL1)  507  QAHPNSIGLV PKAGNFVPNR KLPVSADTDP NSAALDYYE AGQSIGYDSY IFANGYAWIS YIAGSGLRRY VAVGPDDGRT DTVWGKGFFN       595
```

FIG. 4. Comparison of the amino acid sequence of the predicted BacL₁ protein (ORF7) of bacteriocin 41 with the amino acid sequence of the cell wall lytic enzymes of gram-positive bacteria (A) and the repeat sequences found in the BacL₁ protein (B). Lysozyme, *B. subtilis* bacteriophage B103 (accession number Q37896); lysin, *S. agalactiae* prophage lambda Sa1 (accession number NP 687631); muramidase, *L. plantarum* WCFS1 (accession number CAD64901).

ity. These results suggested that the activator of component A modified component L, that the activated component L possessed the bacteriocin activity, and also that an excess of component A existed in the extracellular medium.

The β-hemolysin/bacteriocin (cytolysin) determinant encoded on pAD1 consists of the eight genes *cylR2*, *cylR1*, *cylL_L*, *cylL_S*, *cylM*, *cylB*, *cylA*, and *cylI* (2, 8, 9, 17, 18, 39). CylL_L and CylL_S are the cytolysin structural subunits. The CylL_L and CylL_S proteins are modified posttranslation by CylL_M (2), and the modified CylL_L and CylL_S proteins are secreted via CylL_B, which is the ATP-binding exporter (16). The extracellular cytolysin precursors CylL_L and CylL_S are converted to the active cytolysin by CylA (2, 22). In an early study of the β-hemolysin/bacteriocin (cytolysin) determinant (22), two functional domains within the operon were identified and it was found that one region encodes the toxin precursor L component, which is now known to be encoded byCylL₁, CylL₂, CylM, and CylB, and the other region encodes an activator A component, which is now known to be encoded by CylA and CylI (2, 8, 9, 17, 18, 39). In the complementation experiment between the A component-producing strain or the wild-type strain and the L component-producing strain on blood agar plates, the β-hemolysis zone occurred around or along the L component-producing strain (22), indicating that the A component activates the L component extracellularly and that the activated L component possesses the β-hemolysin/bacteriocin activity and an excess of extracellular A component is present

in the culture medium of the wild-type strain (24). These observations are similar to the extracellular complementation observed between the L component-producing strain and the A component-producing strain for bacteriocin 41.

The deduced amino acid sequence encoded by *bacL_L* showed a high degree of homology with the cell wall lytic enzymes and murein hydrolases of lysozyme, lysine, and the muramidase of gram-positive bacteria (32). These enzymes cleave glycan strains either between the *N*-acetylmuramic acid and *N*-acetylglucosamine or at the alternative acetylglucosamine-muramic acid glycoside linkage (34). Sequence alignments of the murein hydrolases of the gram-positive bacteria show that most of these enzymes display a domain structure. In general, these enzymes harbor an N-terminal signal peptide, followed by a second domain containing the enzymatic activity. In addition, these proteins harbor repeat structures or cell wall-targeting structures that flank either the N- or C-terminal side of the enzymatic domain (40). The repeated domains direct the murein hydrolase to its receptor on the cell surface of gram-positive bacteria (51). Murein hydrolase is usually synthesized as a preproenzyme, and after cleavage of the N-terminal signal peptide, the soluble proenzyme is secreted into the extracellular environment. The repeated domains or cell wall-targeting domains direct the proenzyme to its receptor on the bacterial cell surface. Proteolytic cleavage or activation of the proenzyme generates the mature enzyme (32).

Although the mechanism of activation or the precise mode of action of the *bacL_L*-encoded protein is not known, analysis

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                putative processing site

           signal peptide        ↓                                                                          81
ORF11(BacA)  MDEMVLGTQ KWLNKTYGNV SGFNKVPENG KTGWPTIYGL RRALQKEMGI QELSDNFGPT TERYFKEKVE KQLNERFGAG IGNIVKIMQG GFWCKGINPY  99
ybfG    .    MVDEMVLITQ QWLNDTYSGK HGYNPVEESG KTGWDTIYGL TRALQIELGI SEPADNFGPT TQRLFKPLKR QAPDSKP--- S-NMNFILQG ALWCKGFNP-  95
YkuG    '    MDEMVLGTQ KWLNETYKGK SGYNSIEENG KTGWKTMYAL TRALQLELGI TQTSDSFGPT TLRKLKELGP ISTSTNS--- KKNIVKIIQG ALYCKGYGP-  95
             .*****  ** ***  **  .  *.*  . * * **** *.* *  **** *.**  .  .* ****  * *  .   * ** .* **  . *. .**  *** .*


             putative peptidoglycan binding domain    140
ORF11(BacA)  VSGTEAVDGL MTGLTTLAIK KFQEMAGLAP S-GYMNAMLM KALLDMSAFA LVPGGDKNIR SMQQSLNAKY N--RYFGLLP CDGVYQRDTN SALIYALQAE  196
YbfG         ----GGFTGV FYEKTENAVK EFQKAAGLTT QDGIVTTLIM KALLDMSAFK LVSGGDSRIR QIQQNLNRDY N--DYIGLMP CDGLYGRDTN KALIYALQKE  189
YkuG         ----GGLTGT FGQGTKEAIA EMQLHMGLSK TDGVVTPKVF KALLNMDSYI LLNGASEKVR SIQQWLNNKY YNRENFYFMP CDGLYSRDTQ KSLVYAIQYE  191
              *       *  *  *.  *  **.  .*  .. ****  *  .. *.  *   .*  .** ** *   .* ***.* ***. .*.**.*  *


ORF11(BacA)  MGMDENTANG FYGPGTTAKT PTLTVG---S TGNFVKILQW ALYVNG-FNQ SAVFSGSFTS YIAAEVENFR LFMNLPPYNT SADMTVIKGL LSSAGNTDRA  292
YbfG         EGMSTSVANG FFGNGTTSLC PTLTPGD--S RTGFVLIVQY ALYCNGKSFD PGEFDGKYGV GVVSAVKAFQ EFMCLP-QTG YADMPTIKAL LSSSGDTTRT  286
YkuG         EGLSDSIANG NFGPTTQRLI PVLRIGETDE KNSFIHLFQA ALIFNG---Y NVPFDGVYSE SVRSKVKAFQ SFAKLQ-QSG TADFQTWASL LVSTGDPNRK  287
              *.   ***  .* * .  * *   *  .  *  .  ** **  * *   . .. *.*.  * *  * .  ** ****  * *  * .'


ORF11(BacA)  ASACDMATQL TKQQAQLIKD NGYSIVGRYL TGSVGVGANK KDKNLTLEEI QAITSVGLSI FPIYQDGGWE ESYFNEGNGL RDGSLAHNAA FKLGFPYGAT  392
YbfG         ASACDTATII TAEKAQTLRN NGYKTVGRYL TGNVRTSSGL TSKALTSKEL AVILDAGLKV EPIYQDGGYE SSYFVKDQGT RDAYSAASAA RRLGFPSGTT  386
YkuG         GVACDSITQI TSDRAESLKR AGYKIVGRYL TNAPGSTLNK KIQ---PGEL ETILKSGLNV FPIYQTYGGA TNYFNKEQGK KDAFAAYKAA KEYGFKNNTV  384
              ***  * . *  * *.   .  **  .* **  *   *   *.  . .  *  . *  **  ** * *  ****  *  *. .* .* * .  *    ** *.'


ORF11(BacA)  IYFAVDVDIL DGNIPGTVLP YIKKVK---- --ESLDANGM YKTGIYGTRN VCQQAIDAGF VEHCFVSDMS TGFSGNLGFP MPKEWAFDQF YEHSELG---  483
YbfG         IFFAVDFDAY DYEVTDKIIP YFQEIKSAFT KMQTFSTAPK YEIGVYGPRN ICIRTSEAGL TKYSFVANMS TGFSGNLGYP MPNNWAFDQF YEGTIGSGSG  486
YkuG         IYFAVDYDAY GNDLNNNIIP HFEGIN---- EIMNGFLGST YKIGIYAPRN VCTIVSKKGL AFASFVSGMS TGFSGNLGYP LPYNWAFDQI STITVGNGSG  480
              ****** *   .   ..*   .   .  * *.* ** .*  *     .**. ** ********.*  .* *****  .


ORF11(BacA)  -EPIDKVAVS GRDHGTKAFS TTIG------ NLIQLETIKL LNALGKNFTI KDVGIKLDTP TQIISSPTLD VYFKSSASWT HKVDDSGMSI SIKNGKIDTK  576
YbfG    .    SIGIDKDGYS GRDSGASNVN PPSDPVYDAR LRTLTDILST IPALENLTSL ANAMFEFDTT ETIFTSPELD IILSTSLLAT IPSEGSPNTI TITNGKPG--  584
YkuG         MIEIDNDICS GLDNGVNTIN IVPSE----- NKKFFDQIDV LYETAEKYAQ MQSDLNNGVK KTQLANELVA QYLRKDDYKG WKWVPTAGQI DPIYREWAVK  575
              **   * * *  *        .   .  . *. .  * *. * .*    *      .   *


ORF11(BacA)  VY-------- VNPIKESLNS YKDLLKNYN- ---------- ENQVDEMLNK LAPVIK---- -----NGYIE TGFCARNNLI GTKLVIKK-- ----EIGDSE  642
YbfG         ---------- -AYITGLLGD TQTSLTASQ- ---------- IDSYQNLLNS LSLSVR---- -----NGYLE VYVNPTAESL NIQIKIYTPD I---PVGDNV  650
YkuG         RLGEDLVNGI VDPISKTVIG TQHLMATYNA IIYSGGYSQT LRDFAGWTGD LLTTIQDRKL HAQEFNSPYD AAMKIIGNYM QFSLDDLFSD VDAINLANKT  675
              *                                       .  . . *            *   .             *


ORF11(BacA)  NKGTLQLEIE LYPKPLLPTD IKIPQPDYDK AYRDIKNGHV PQLNVEVILK GVLIG-ALAV VIIIGIASGA AELAGAITAF FAALA                  726
YbfG         TTG-LTTTIT FKIKTYKGVP VTSPESELAL DWPSYDQYLF PVVGVAALLL IGNMGSDLTN NKGVKVATAL SAMLLAIFAY YTS                    732
YkuG         SVGANAQPLN IAIRDYYSNN DCMNRFTQFV NNRFDGSLDK IFSEAEYYLN TNLDPVVVPI RLAFKRAFDV EDYSEEIGKI TARSI                  760
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FIG. 5. Comparison of the amino acid sequence of the predicted BacA protein (ORF11) of bacteriocin 41 with those of the predicted proteins encoded by the genomic DNA of *B. subtilis*. The accession numbers are CAB12014 for YbfG and CAA10870 for YkuG, respectively.

of the deduced amino acid sequence of the *bacL₁*-encoded protein suggests that the protein exhibits a domain structure. The domain structure is composed of an N-terminal signal peptide followed by a second domain containing the enzymatic activity and a third domain with the three amino acid sequence repeat structures. The *bacL₁*-encoded protein might be synthesized as a preproenzyme, and after signal peptide cleavage, the soluble proprotein encoded by *bacL₁* would be secreted into the extracellular environment. The repeat domains might function to direct the proprotein encoded by *bacL₁* to its receptor on the bacterial cell surface, and the proprotein encoded by *bacL₁* might be activated by the *bacA* protein, resulting in the generation of the mature BacL₁ protein. As described above, *bacL₂* was also essential for the expression of the L component. The deduced *bacL₂*-encoded protein was a 211-amino-acid protein with no leader peptide. The sequence data implied that the *bacL₂*-encoded protein might modify the *bacL₁*-encoded protein inside the bacterial cell. Bacteriocin 41 only showed bacteriocin activity against *E. faecalis*, which suggested that the *bacL₁*-encoded protein of bacteriocin 41 was highly specific for the glycan strand of the *E. faecalis* cell wall.

Recently, another group reported the discovery of a novel cell wall-degrading bacteriocin, which has been named enterolysin A (EnlA), in an *E. faecalis* strain isolated from fish (36). The bacteriocin gene *enlA* encodes a 343-amino-acid preprotein with a *sec*-dependent signal peptide of 27 amino acids. The mature EnlA protein consists of 316 amino acids and is homologous to the catalytic domains of a variety of cell wall-

degrading proteins. It might be that bacteriocin 41 belongs to the same group of enterococcal cell wall-degrading bacteriocins as EnlA, although the details of the mechanism of expression of EnlA, including the immunity factor, have not been clearly elucidated (15). However, our results imply that the mechanism of bacteriocin 41 expression is more complex than the EnlA expression system and that they are divergent systems.

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JAC

Investigation of isolation rates of *Pseudomonas aeruginosa* with and without multidrug resistance in medical facilities and clinical laboratories in Japan

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Objectives: To perform a large-scale investigation of *Pseudomonas aeruginosa* strains with and without drug resistance in Japan.

Methods: We distributed questionnaires to assess isolation rates of *P. aeruginosa* with and without drug resistance at medical facilities and clinical laboratories throughout Japan during the period January 2003 through June 2006. Completed questionnaires were obtained from 339 medical facilities and 4 clinical laboratories.

Results: The total number of *P. aeruginosa* strains isolated at the medical facilities was 549 746 and that at clinical laboratories was 640 232. Strains resistant to carbapenems, fluoroquinolones (ciprofloxacin or levofloxacin) and amikacin were defined as multidrug-resistant (MDR) strains, and strains resistant to two of these drugs were defined as two-drug-resistant (TDR) strains. The percentage of MDR at medical facilities and clinical laboratories was 2.4% and 1.1%, respectively, and that of TDR isolates was 6.4% and 4.2%, respectively. MDR and TDR isolates were found nationwide. No MDR isolates were found at approximately one-third of the medical facilities each year. The percentages of MDR and TDR isolates increased significantly from 2003 to 2005. *P. aeruginosa* strains were obtained mainly from the respiratory and urinary tracts, and the percentages of MDR and TDR isolates were particularly increased in the urinary tract during these years.

Conclusions: MDR *P. aeruginosa* was prevalent nationwide in Japan. The incidence was low, except in a limited number of facilities, but it increased significantly.

Keywords: nationwide surveillance, retrospective questionnaire, laboratory-based surveillance

Introduction

Pseudomonas aeruginosa has intrinsic resistance to many antimicrobial agents, and only a few antimicrobial agents show potent antibacterial activity against this bacterium. The emergence of multidrug-resistant (MDR) *P. aeruginosa* strains is a serious problem.[1–3] Nosocomial outbreaks of *P. aeruginosa* infection, particularly by MDR strains, have become problematic in hospitals in various countries,[4–8] including in Japan.[3,9,10] Many cases of MDR *P. aeruginosa* infection have been reported in Japan. However, there have been few nationwide investigations of the prevalence *P. aeruginosa* infection at medical facilities. We investigated isolation rates of *P. aeruginosa* strains

with and without drug resistance in cooperation with various medical facilities and clinical laboratories throughout Japan. This is the first surveillance study of clinically isolated *P. aeruginosa* with and without drug resistance in Japan.

Materials and methods

Methods and subjects

Information was gathered by means of a questionnaire. Questionnaires were sent on 27 July 2006 to 538 medical facilities and 4 clinical laboratories across Japan, including all 350 facilities with

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500 or more beds and 188 regional core hospitals with 120 or more but <500 beds and 4 large clinical laboratories. The regional core hospitals were selected by considering their geographic locations across the country. The four clinical laboratories had numerous branch laboratories (a total of 300 sites) covering all areas of Japan, and they are commissioned by clinics and hospitals to analyse clinical samples, including pathogenic bacterial samples.

Completed questionnaires were returned by 339 medical facilities (63% response rate) and the 4 clinical laboratories (100% response rate) as of 6 October 2006. The average number of beds in these medical facilities was 577 ± 230 (median 550; range 120–1505). The period of investigation was January 2003 through June 2006.

Questionnaires

The questionnaire enquired about (i) the number of beds, (ii) the total number of *P. aeruginosa* strains isolated each year with or without TDR or MDR, (iii) the number of patients with TDR or MDR strains and (iv) the tissue sources of the isolated strains.

Clinical isolation of P. aeruginosa strains

P. aeruginosa strains were isolated from inpatients and outpatients with suspected *P. aeruginosa* infection and subjected to drug-susceptibility testing. Repeat testing of single patients was assumed when repeat examinations were ordered. Strains isolated for analysis in this study were not from the environment, carriers, non-symptomatic patients or healthy staff.

Drug-resistant strains

Strains that were resistant to carbapenems, fluoroquinolones (ciprofloxacin or levofloxacin) and amikacin were defined as MDR strains. Strains that were resistant to two of these drugs were defined as two-drug-resistant (TDR) strains. Drug resistance was assessed by determining the MIC in culture medium containing the drugs or by determining the diameter of the growth inhibition zone (DGIZ) on culture agar with the use of drug-containing discs. Breakpoints were determined in accordance with the criteria for MDR strains specified by the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections of the Japanese Ministry of Health, Labour and Welfare. MIC breakpoints for carbapenems, amikacin, ciprofloxacin and levofloxacin were ≥ 16, ≥ 32, ≥ 4 and ≥ 8 mg/L, respectively; DGIZ breakpoints for these drugs were ≤ 13, ≤ 14, ≤ 15 and ≤ 13 mm, respectively.

Drug susceptibility tests for bacteria, including *P. aeruginosa*, are performed in Japan in accordance with the standards published by the Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Statistical analysis

Chronological trends in the proportions of TDR and MDR isolates were assessed by the Mantel–Haenszel χ^2 test. The numbers of isolates from various tissue sources were analysed by the χ^2 test. P values of <0.0001 were considered statistically significant.

Results

P. aeruginosa isolates and infections at medical facilities

During the study period, a total of 549 746 *P. aeruginosa* strains were isolated at the 339 medical facilities. The numbers of TDR and MDR isolates were 35 030 (6.4%) and 13 296 (2.4%), respectively. As shown in the upper half of Table 1, the total numbers of isolates, including TDR and MDR strains, as well as the adjusted numbers (number of isolates/1000 beds), increased gradually from 2003 to 2005. The percentages of TDR and MDR strains also increased significantly during the period ($P < 0.0001$). In addition, the number of patients with TDR or MDR strains (and the number of patients per 1000 beds/year) increased gradually from 2003 to 2005. The reason for the lack of increase in the first half of 2006 was unclear. Investigation of the entire year may yield different results.

We also analysed the number of patients with MDR per 1000 beds per month. The numbers ranged from 0 to 110.7 in 2003, 0 to 100.5 in 2004, 0 to 150.5 in 2005 and 0 to 106.7 in the first half of 2006. The median values for these years were 2.8, 3.6, 4.6 and 4.0, respectively. The 90 percentile values were 22.9, 25.0, 25.6 and 25.3, respectively. Some of the 339 medical facilities reported that no MDR *P. aeruginosa* strains were isolated in a given year. The number of these facilities was 103 (30.4%) in 2003, 93 (27.4%) in 2004, 93 (27.4%) in 2005 and 127 (37.5%) in the first half of 2006. Forty-eight facilities (14.2%) reported isolation of no MDR *P. aeruginosa* strains during the study period. The number of patients with MDR per 1000 beds per month was less than two in 90.4%, 89.0%, 87.9% and 89.3% of the facilities in 2003–06, respectively, whereas two or more patients with MDR strains were identified in ~10% of the facilities during the study period, suggesting that MDR *P. aeruginosa* was prevalent in a limited number of hospitals. We then investigated whether high incidence of MDR occurred geographically. No significant difference between geographic areas was found (data not shown).

P. aeruginosa isolated at clinical laboratories

Completed questionnaires from the four clinical laboratories were also analysed. The number of *P. aeruginosa* strains isolated during the study period was 640 232, and the numbers of TDR and MDR isolates were 26 913 (4.2%) and 6768 (1.1%), respectively. The data for each year are shown in the lower half of Table 1. The numbers increased markedly from 2003 to 2005, but the percentages of TDR and MDR did not. It could not be determined whether the percentages increased in 2006, given that data were obtained for only half of the year.

Comparison of the percentages of TDR and MDR isolates from the laboratories with those from the medical facilities showed lower percentages from the laboratories.

Tissue sources of P. aeruginosa

The tissue sources and percentages of the total *P. aeruginosa* strains isolated at the 339 medical facilities for the study periods and those of TDR and MDR strains are shown in Figure 1a. The percentages for each year were similar to those for the entire study period (data not shown). These results indicated that *P. aeruginosa*, including TDR and MDR strains, affected mainly the respiratory and urinary tracts. However, it is notable that the percentages of TDR and MDR isolates in the urinary tract were significantly greater than that of the total isolates ($P < 0.0001$) and the percentages of MDR

Table 1. Isolation of P. aeruginosa with or without multidrug-resistance in medical facilities and clinical laboratories

Strains	2003	2004	2005	2006 (half a year)
Medical facilities				
P. aeruginosa				
total numbers (nos.)[a]	145 148	160 784	168 762	75 052
nos. of isolates per 1000 beds/year	742	822	862	767
TDR strains				
total nos.	8615	10 236	11 125	5054
nos. of isolates per 1000 beds/year	44.0	52.3	56.9	51.7
rate (%)[b]	5.9	6.4	6.6	6.7
patient nos.[c]	3912	5624	4737	2342
nos. of patients per 1000 beds/year	20.0	23.3	24.2	23.9
MDR strains				
total nos.	2941	3894	4437	2024
nos. of isolates per 1000 beds/year	15.0	19.9	22.7	20.7
rate (%)	2.0	2.4	2.6	2.7
patient nos.	1234	1681	1969	888
nos. of patients per 1000 beds/year	6.3	8.6	10.1	9.1
Clinical laboratories				
P. aeruginosa				
total nos.	154 055	167 472	210 311	108 394
TDR strains				
total nos.	5899	7290	9108	4616
rate (%)	3.8	4.4	4.3	4.3
MDR strains				
total nos.	1436	1937	2284	1111
rate (%)	0.9	1.2	1.1	1.0

[a]Numbers of P. aeruginosa isolated from the 339 medical facilities and the 4 clinical laboratories that answered the questionnaire.
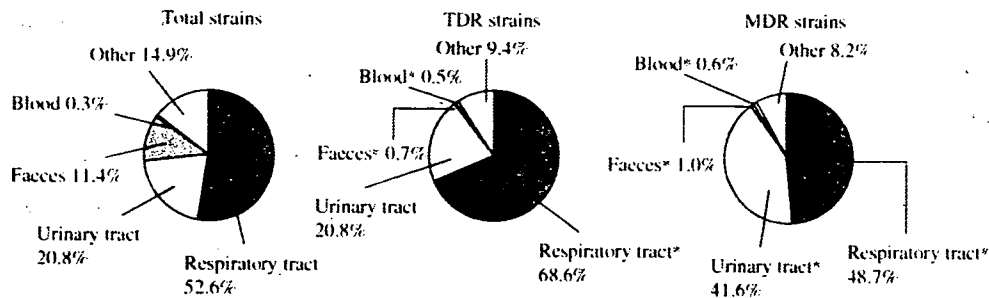[b]The ratio of the numbers of TDR or MDR P. aeruginosa to the total numbers of isolated P. aeruginosa (%).
[c]The numbers of patients with TDR or MDR P. aeruginosa.

(a) Medical facilities
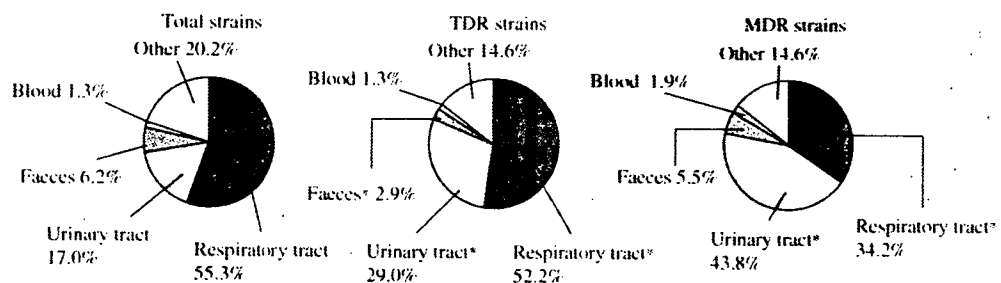
(b) Clinical laboratories

Figure 1. Tissue sources of total P. aeruginosa strains, TDR strains and MDR strains isolated during the study period at medical facilities (a) and clinical laboratories (b). An asterisk indicates a significant difference between numbers of TDR and total isolates or between MDR and total isolates. TDR, two-drug-resistant; MDR, multidrug-resistant.

isolates in the urinary tract surpassed those in the respiratory tract.

The tissue sources and percentages of *P. aeruginosa* isolated at the four clinical laboratories during the study period were also analysed (Figure 1b). The results showed that the percentage of MDR isolates in the urinary tract was significantly greater than that of the total and TDR strains ($P < 0.0001$).

Discussion

It was not practical to send questionnaires to all medical facilities and laboratories in Japan. Therefore, we selected all large-scale facilities and many regional core hospitals, as well as highly respected laboratories, by considering their geographic locations across the country. Data collected by these laboratories may provide information for various clinics and small-scale hospitals, and may shed light on different aspects of *P. aeruginosa* prevalence. By analysing questionnaires completed by 339 medical facilities and 4 laboratories, we believe that we obtained accurate information regarding the present state of *P. aeruginosa* prevalence in Japan. However, the use of retrospective data implies inherent biases.

Our survey showed that MDR *P. aeruginosa* was prevalent nationwide but the incidence was low, except in a limited number of facilities. The survey also showed that MDR strains were isolated from the urinary tract as well as the respiratory tract, suggesting the importance of management of patient's urine in the prevention and control of nosocomial MDR *P. aeruginosa* infection.[3,9]

The samples from clinical laboratories may provide information on the prevalence of *P. aeruginosa* infection at clinics and small-scale hospitals. The percentages of TDR and MDR *P. aeruginosa* isolates from the laboratories were lower than those from the medical facilities. Although the exact reasons are unclear, this may be related to differences in the use of antibiotics, the severity of cases, scales of nosocomial outbreaks and populations of inpatients and outpatients.

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Transparency declarations

None to declare.

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CASE REPORT

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Molecular epidemiology of outbreaks and containment of drug-resistant *Pseudomonas aeruginosa* in a Tokyo hospital

Abstract We witnessed outbreaks of multidrug-resistant (MDR) and drug-resistant *Pseudomonas aeruginosa* at a hospital in Tokyo, Japan, during the period September 2004 through May 2005. The first outbreak occurred in September and October 2004. Three isolates of MDR *P. aeruginosa* were identified from urine samples obtained from three nonambulatory immunodeficient patients in one ward. After 3 weeks, another outbreak of *P. aeruginosa* occurred in the hematology ward on the same floor of the hospital. During the outbreaks, environmental surveys were conducted twice in each of the two wards, at 2-week intervals, to identify the sources of the pathogens. A total of 23 *P. aeruginosa* isolates, including 11 from environmental sources, were analyzed for chromosomal DNA typing by pulsed-field gel electrophoresis, for O-antigen serotyping, and for other typing. Results revealed two causative clones, as well as environmental contamination by *P. aeruginosa* clones on the surfaces of urine volume-measuring devices in rooms where urine is handled, which may have been sources of the pathogens during the outbreaks. To prevent further outbreaks, we performed the following: (a) environmental surface monitoring for drug-resistant *P. aeruginosa*, (b) active surveillance of specimens, (c) strict isolation of infected patients or carriers of MDR *P. aeruginosa*, (d) rigorous contact precautions, and (e) disinfection with 70% alcohol on the surfaces of apparatuses contaminated by MDR or drug-resistant *P. aeruginosa* and in the rooms where urine is handled. As a result, the outbreaks were contained.

Key words Multidrug resistant · *Pseudomonas aeruginosa* · Environment · Survey · Infection control

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Introduction

Nosocomial infection caused by *Pseudomonas aeruginosa* has been recognized as an important problem in hospitals in recent years because of the danger posed to immunocompromised patients.[1] Nosocomial *P. aeruginosa* is usually multidrug-resistant (MDR), which is not well-defined internationally,[2] but is defined in Japan as resistance to imipenem (IPM; minimum inhibitory concentration [MIC], ≥ 16 µg/ml), amikacin (AMK; MIC, ≥ 32 µg/ml), and ciprofloxacin (CPFX; MIC, ≥ 4 µg/ml).[3]

Recently, we experienced outbreaks of *P. aeruginosa* at the 925-bed International Medical Center Hospital in Tokyo. We successfully controlled these outbreaks within several months. Here, we report how we controlled the outbreaks and determined the cause, the method of transmission, the patterns of drug resistance, and genotyping by pulsed-field gel electrophoresis (PFGE) of the causative *P. aeruginosa* isolates from clinical and environmental sources in the hospital.
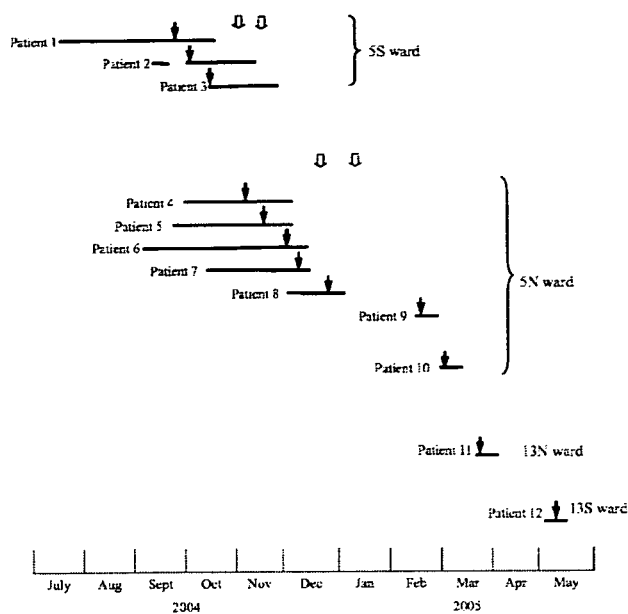
Methods

The first outbreak occurred in one ward (5S, the south part of the fifth floor) in September and October 2004. Three isolates of MDR *P. aeruginosa* were obtained from urine taken from three nonambulatory immunodeficient patients. The three patients were hospitalized during overlapping periods (Fig. 1). After 3 weeks, another outbreak of *P. aeruginosa* occurred in the hematology ward (5N, the north part of the fifth floor) on the same floor in the hospital. The

Fig. 1. Time course of *Pseudomonas aeruginosa* O11 detection in the hospital. The *parallel lines* indicate the periods of hospitalization. *Black arrows*, day of *P. aeruginosa* O11 detection; *white arrows*, day of environmental investigation for multidrug-resistant *P. aeruginosa*

causative isolates were not MDR *P. aeruginosa*, but they were AMK-sensitive and IPM- and CPFX-resistant. *P. aeruginosa* was consecutively isolated from three urine samples, one sputum sample, and one pharynx swab from five different patients in the ward. Each of these five patients had a serious underlying disease but was ambulatory. Two more patients were infected with *P. aeruginosa* 1 month later (Fig. 1). Two patients in other wards (13S and 13N, the south and north parts of the thirteenth floor, respectively) were found to have sporadic infections with *P. aeruginosa* during the same period or several weeks later (Fig. 1). The infections were contained by May 2005.

To determine the cause of the outbreaks and to prevent additional cases, an epidemiologic investigation was initiated by the hospital's infection-control team. Environmental surveys were conducted twice in each of the two wards (5S and 5N), at 2-week intervals, to identify the source(s) of the pathogens. Multiple samples from environmental surfaces were tested to detect *P. aeruginosa* on NAC agar medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with or without ceftazidime at a final concentration of 16 μg/ml. A total of 23 *P. aeruginosa* isolates, including 11 from environmental sources, were analyzed for chromosomal DNA typing with a counter-clamped homogeneous electric field system (CHEF Mapper; Bio-Rad Laboratories, Hercules, CA, USA), for O-antigen serotyping with control serum (Denka Seiken, Tokyo, Japan), and for antibiotic resistance by a microdilution method according to the guidelines of the Clinical Laboratory Standards Institute.[4] DNA sequences of the quinolone resistance-determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC*, and *parE* genes were determined as described previously.[5] Resistance genes other than quinolone-resistance genes were detected by

polymerase chain reaction with three primer pairs designed to amplify the sequences of the metallo β-lactamase bla_{IMP-1} gene,[6] aminoglycoside 6′-acetyltransferase $aac(6′)$-Iae gene,[5] and the aminoglycoside 2″-adenylyltransferase $aadB$ gene.[7]

The minimum inhibitory concentrations (MICs) of eight antibiotics tested against *P. aeruginosa* isolates are shown in Table 1. Five of the 12 clinical isolates and 3 of the 11 environmental isolates were MDR *P. aeruginosa* and were resistant to all antibiotics tested, except for gentamicin (GM) and polymixin B (PL-B). Six clinical isolates and 3 environmental isolates were resistant to IPM and CPFX but sensitive to AMK (Table 1). The remaining 6 isolates (IMCJ nos. 333, 262, 264, 268, 326, and 330) were each sensitive to four to eight antibiotics tested.

The five clinical and three environmental isolates of MDR *P. aeruginosa* carried the bla_{IMP-1} and $aac(6′)$-Iae genes, and showed mutations in T83I and S87L in the QRDRs of *gyrA* and *parC*, respectively (Table 1). Among these eight MDR isolates, two (IMCJ nos. 335 and 361) had an additional D87G mutation in the QRDR of *gyrA*. Six other clinical and two other environmental isolates carried the bla_{IMP-1} and $aadB$ genes, and showed mutations in T83I, E468D, and S87W in the QRDRs of *gyrA*, *gyrB*, and *parC*, respectively (Table 1). The presence of these genes and their alterations in the QRDRs in *gyrA*, *gyrB*, and *parC* are consistent with their resistance phenotypes. These genetic analyses of drug-resistant genes in MDR *P. aeruginosa* could provide useful information about the evolution of nosocomial pathogens.

Of the total 23 isolates, 18 expressed the O11 antigen, 4 expressed the O1 antigen, and 1 expressed the O6 antigen (Table 1). Notably, all clinical isolates and 6 environmental isolates expressed the O11 antigen (Table 1).

The PFGE patterns of the isolates are shown in Fig. 2. Among the 23 *P. aeruginosa* isolates, 13 different PFGE patterns were detected. Three clinical isolates (IMCJ nos. 254–256) and 3 environmental isolates (IMCJ nos. 260, 261, and 267) from the 5S ward were of the same pattern (PA1; Fig. 2 and Table 1). Six clinical isolates (IMCJ nos., 257, 270, 321–323, and 332) and 2 environmental isolates (IMCJ nos. 324 and 331) in the hematology ward (5N) were of the same pattern (PE). Taken together, the data indicate that the first outbreak was caused by one strain of MDR *P. aeruginosa*, but that the second outbreak was caused by a separate strain of drug-resistant *P. aeruginosa*. Three other MDR *P. aeruginosa* isolates, obtained from patients 10 (ward 5N), 11 (ward 13N), and 12 (ward 13S), had PFGE patterns (shown in lanes 8, 10, and 9, respectively, in Fig. 2) that differed from those of isolates obtained from patients 1, 2, and 3 (ward 5S) and from the isolates obtained from patients 4, 5, 6, 7, 8, and 9 (ward 5N), indicating that the infections in patients 10, 11, and 12 were sporadic.

In the environmental survey, three isolates of MDR *P. aeruginosa* (IMCJ260, IMCJ267, and IMCJ261: see Table 1) were obtained from wet surfaces in ward 5S; from a rack for urinals, from a urine volume-measuring device in a room for the handling of urine, and from a bath drain in a bathing room, respectively. Three *P. aeruginosa* isolates,

Table 1. Origins and characteristics of the *Pseudomonas aeruginosa* isolates from clinical and environmental sources

Strain	Source (specimen)	Date isolated	Ward	O serotype	MICs against[a]								PCR for resistance genes			Mutations in QRDRs[b]			PFGE type (lane in Fig. 2)
					PIPC	CAZ	IPM	AMK	GM	TOB	CPFX	PL-B	bla_IMP-1	aac(6')-Iae	aadB	GyrA	GyrB	ParC	
IMCJ254	Patient 1 (urine)	30-Sept	5S	O11	256	>256	16	128	8	256	>32	4	+	+	−	T83I		S87L	PA1 (2)
IMCJ255	Patient 2 (urine)	30-Sept	5S	O11	>256	>256	32	64	8	256	>32	4	+	+	−	T83I		S87L	PA1 (3)
IMCJ256	Patient 3 (urine)	18-Oct	5S	O11	>256	256	16	64	4	128	>32	4	+	+	−	T83I		S87L	PA1 (4)
IMCJ257	Patient 4 (urine)	8-Nov	5N	O11	>256	>256	>64	2	32	4	>32	4	+	−	+	T83I		S87W	PE (14)
IMCJ270	Patient 5 (sputum)	12-Nov	5N	O11	>256	>256	>64	1	32	2	>32	4	+	−	+	T83I	E468D	S87W	PE (15)
IMCJ321	Patient 6 (urine)	24-Nov	5N	O11	256	>256	>64	1	32	2	>32	4	+	−	+	T83I	E468D	S87W	PE (16)
IMCJ322	Patient 7 (pharynx)	8-Dec	5N	O11	>256	>256	>64	4	128	16	>32	4	+	−	+	T83I	E468D	S87W	PE (17)
IMCJ323	Patient 8 (urine)	16-Dec	5N	O11	>256	>256	>64	8	256	16	>32	4	+	−	+	T83I	E468D	S87W	PE (18)
IMCJ332	Patient 9 (urine)	2-Feb	5N	O11	>256	>256	>64	2	128	8	>32	4	+	−	+	T83I	E468D	S87W	PE (21)
IMCJ333	Patient 10 (pharynx)	28-Feb	5N	O11	>256	>256	16	2	4	8	>32	4	−	−	−	T83I		S87L	PA2 (8)
IMCJ335	Patient 11 (urine)	23-Mar	13N	O11	>256	>256	>64	64	8	128	>32	4	+	+	−	T83I + D87G		S87L	PA4 (10)
IMCJ361	Patient 12 (urine)	11-May	13S	O11	>256	>256	>64	64	8	64	>32	4	+	+	−	T83I + D87G		S87L	PA3 (9)
Environmental																			
IMCJ260	Rack for urinal	28-Oct	5S	O11	256	>256	64	64	8	256	>32	4	+	+	−	T83I		S87L	PA1 (5)
IMCJ261	Bath drain	28-Oct	5S	O11	>256	>256	>64	64	8	256	>32	4	+	+	−	T83I		S87L	PA1 (6)
IMCJ264	Sink in P1 room	28-Oct	5S	O1	8	2	4	16	8	2	0.5	4	−	−	−				PD (13)
IMCJ262	Sink in staff room	28-Oct	5S	O1	2	0.5	1	1	0.5	0.5	0.064	2	−	−	−				PG (23)
IMCJ267	Urine volume-measuring device	12-Nov	5S	O11	256	>256	32	64	>256	>256	>32	4	+	+	−	T83I		S87L	PA1 (7)
IMCJ268	Sink in staff room	12-Nov	5S	O1	1	1	1	2	0.5	0.25	0.064	2	−	−	−				PH (24)
IMCJ324	Sink in lavatory	21-Dec	5N	O11	>256	>256	>64	4	256	16	>32	4	+	+	−	T83I	E468D	S87W	PE (19)
IMCJ325	Urine volume-measuring device	21-Dec	5N	O11	>256	>256	>64	4	64	16	>32	4	+	−	−	T83I		S87L	PF (22)
IMCJ326	Sink in staff room	21-Dec	5N	O6	>256	32	8	16	128	128	0.19	4	−	−	−				PB (11)
IMCJ330	Sink in lavatory	11-Jan	5N	O1	>256	>256	>64	4	2	2	0.064	4	−	−	−				PC (12)
IMCJ331	Urine volume-measuring device	11-Jan	5N	O11	>256	>256	>64	4	>256	16	>32	4	+	−	+	T83I	E468D	S87W	PE (20)
IMCJ2, S1[c]				O11	>128	>28	128	128	16	64	32	2	+	+	−	T83I		S87L	PA (1)

[a] PIPC, piperacillin; CAZ, ceftazidime; IPM, imipenem; AMK, amikacin; GM, gentamicin; TOB, tobramycin; CPFX, ciprofloxacin; PL-B, polymixin B

[b] T83I, Thr at position 83 of GyrA changed to Ile (ACC→ATC); S87L, Ser at position 87 of ParC changed to Leu (TCG→TTG); E468D, Glu at position at 468 changed to Asp (GAG→GAT); S87W, Ser at position 87 of ParC changed to Trp (TCG→TGG); D87G, Asp at position 87 changed to Gly (GAC→GGC). The numbering of the amino acids is based on that of *P. aeruginosa* PAO1 (Genbank accession no. NC_002516).

[c] Nosocomial strain that caused outbreaks among hospitals in an area of Japan[3]

Fig. 2. Pulsed-field gel electrophoresis (PFGE) patterns and dendrogram for *P. aeruginosa* isolates from clinical and environmental sources. The isolates corresponding to each lane are listed in Table 1
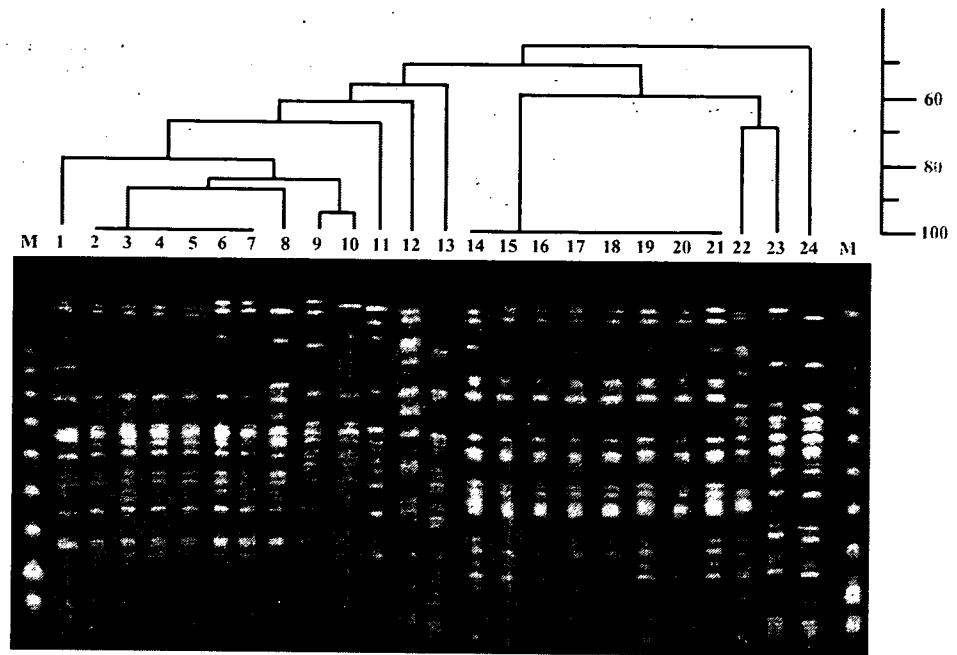
resistant to IPM and CPFX, but sensitive to AMK, were obtained in ward 5N. One isolate (IMCJ324) was from a sink in a lavatory, and two (IMCJ325 and IMCJ331) were obtained on different days from the surfaces of a urine volume-measuring device in a room for the handling of urine. As mentioned above, PFGE analysis revealed that three of the MDR *P. aeruginosa* isolates and two of three isolates of the IPM- and CPFX-resistant but AMK-sensitive *P. aeruginosa* were causative pathogens of the outbreaks. The data pointed to environmental contamination by drug-resistant *P. aeruginosa* in the bathing room, the lavatory, and the rooms for the handling of urine, which may have been sources of the pathogens during the outbreaks.

During the two outbreaks, we took the following steps: (a) environmental surface monitoring as described, (b) active surveillance for drug-resistant *P. aeruginosa* obtained from the samples of patients, (c) strict isolation of infected patients or carriers of MDR *P. aeruginosa*, (d) rigorous contact precautions, especially during the handling of urine and urinary catheters, and (e) disinfection with 70% alcohol on the surfaces of apparatuses contaminated by MDR or drug-resistant *P. aeruginosa* and in rooms where urine is handled. As a result, the outbreaks were contained. Each patient with MDR *P. aeruginosa* was isolated in a single room during the two outbreaks. However, if a single room is not available, an area in a ward that is separate from other patients could be used for patients with MDR *P. aerugi-nosa*. Inadequate use of antimicrobial agents against *P. aeruginosa* was not found in the wards where the outbreaks occurred, although prior-approval programs, including pre-approved indications for antibiotics against *P. aeruginosa*, such as carbapenems and aminoglycosides, were not performed, but were in the planning stage in this hospital.

However, sporadic MDR *P. aeruginosa* will continue to be isolated from inpatients who may bring the pathogens into the hospital. In fact, we have reported outbreaks of MDR *P. aeruginosa* in hospitals in a prefecture in Japan.[3] Analysis indicated that the *P. aeruginosa* IMCJ.S1 strain responsible for the outbreaks underwent clonal expansion.[3] The PFGE patterns of MDR *P. aeruginosa* isolates from the first outbreak described herein had 80% similarity to the pattern of *P. aeruginosa* IMCJ.S1. In addition, MDR isolates from the sporadic cases in the present study (patients 10, 11, and 12) had close similarity to the IMCJ.S1 and the strains associated with the first outbreak. These results suggest that some dominant MDR *P. aeruginosa* strains may be prevalent in Japan.

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Use of Protein Antigens for Early Serological Diagnosis of Leprosy[▽]

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Leprosy is a chronic and debilitating human disease caused by infection with the *Mycobacterium leprae*
bacillus. Despite the marked reduction in the number of registered worldwide leprosy cases as a result of the
widespread use of multidrug therapy, the number of new cases detected each year remains relatively stable.
This indicates that *M. leprae* is still being transmitted and that, without earlier diagnosis, *M. leprae* infection
will continue to pose a health problem. Current diagnostic techniques, based on the appearance of clinical
symptoms or of immunoglobulin M (IgM) antibodies that recognize the bacterial phenolic glycolipid I, are
unable to reliably identify early-stage leprosy. In this study we examine the ability of IgG within leprosy patient
sera to bind several *M. leprae* protein antigens. As expected, multibacillary leprosy patients provided stronger
responses than paucibacillary leprosy patients. We demonstrate that the geographic locations of the patients
can influence the antigens they recognize but that ML0405 and ML2331 are recognized by sera from diverse
regions (the Philippines, coastal and central Brazil, and Japan). A fusion construct of these two proteins
(designated leprosy IDRI diagnostic 1 [LID-1]) retained the diagnostic activity of the component antigens.
Upon testing against a panel of prospective sera from individuals who developed leprosy, we determined that
LID-1 was capable of diagnosing leprosy 6 to 8 months before the onset of clinical symptoms. A serological
diagnostic test capable of identifying and allowing treatment of early-stage leprosy could reduce transmission,
prevent functional disabilities and stigmatizing deformities, and facilitate leprosy eradication.

Cases in which *Mycobacterium leprae* infection manifests to
cause leprosy present as a bacteriologic, clinical, immunologic,
and pathological spectrum ranging from the extremes observed
in paucibacillary (PB) and multibacillary (MB) patients (21,
24). PB patients have one or a few skin lesions and a low or
absent bacterial index (BI; a measure of the number of acid-
fast bacilli in the dermis, expressed on a logarithmic scale) and
demonstrate specific cell-mediated immunity against *M. leprae*,
but they have low or absent titers of *M. leprae*-specific antibod-
ies and a granulomatous dermatopathology. In marked con-
trast, MB patients have multiple symmetric skin lesions and a
high BI and demonstrate high titers of anti-*M. leprae* antibod-
ies but an absence of specific cell-mediated immunity and a
dermatopathology largely devoid of functional lymphocytes
(21). Despite the implementation of a WHO-directed eradica-
tion program over the last 20 years, the worldwide annual rate
of new case detection for leprosy remains stable at approxi-
mately 300,000 (17, 18, 26, 27). Earlier and objective diagnosis
of leprosy could interrupt transmission and, in the long term,
help further reduce the number of new cases and facilitate
eradication.

There is no single diagnostic laboratory test for leprosy, and

diagnosis remains essentially clinical. Clinical diagnosis of lep-
rosy is dependent upon recognition of disease symptoms and is
therefore only possible once the disease has manifested. WHO
experts have listed diagnostic criteria as one or more of the
following: hypopigmented or reddish skin patches with definite
loss of sensation; thickened peripheral nerves; acid-fast bacilli
on skin smears/biopsy specimens (WHO Expert Committee on
Leprosy, 1998). Pure neuritic leprosy forms, however, present
with no skin lesion. Confounding WHO's implementation of a
global leprosy eradication strategy is that the number of
trained leprologists has diminished. This is inadvertently in-
creasing the likelihood that a clinical diagnosis is delayed or
even missed, especially in regions where leprosy has been con-
trolled (1, 13, 16, 25).

The presence of serum immunoglobulin M (IgM) antibody to
phenolic glycolipid I (PGL-I) correlates with BI in leprosy pa-
tients and has been used to support disease symptoms as a means
to categorize leprosy patients. Enzyme-linked immunosorbent as-
say (ELISA) and rapid lateral flow test formats have been devel-
oped for the detection of anti-PGL-I antibody (3, 4, 8, 19, 22, 23,
28). In one study, a lateral flow assay correctly diagnosed 97.4%
of MB patients, with a specificity of 86.2% (4). Patients toward
the PB end of the leprosy spectrum have low or no BI, however,
and the majority of these patients are not identified by PGL-I-
based tests (4, 7, 19). In addition, false-positive results in areas of
endemicity are relatively high (>10%) (4, 7, 19). Consequently,
none of these PGL-I-based tests has been widely implemented in