PCR Typing of Genetic Determinants for Metallo-β-Lactamases and Integrases Carried by Gram-Negative Bacteria Isolated in Japan, with Focus on the Class 3 Integron

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From January 2001 to December 2002, 587 strains of gram-negative bacterial isolates demonstrating resistance to ceftazidime and a combination of sulbactam and cefoperazone were subjected to a disk diffusion screening test using sodium mercaptoacetic acid; 431 strains (73.4%) appeared to produce metallo-β-lactamase (MBL). Of these 431 strains, 357 were found by PCR to carry genes for IMP-1 type MBL (bla_{IMP-1}), while only 7 and 67 strains carried the IMP-2 gene (bla_{IMP-2}) and the VIM-2 gene (bla_{VIM-2}), respectively. Neither VIM-1 nor SPM-1 type MBL genes were found among the strains tested. Of 431 strains, 427 carried the *intI1* gene, and 4 strains carrying both the *intI1* and *intI3* genes were reidentified as *Pseudomonas putida* harboring bla_{IMP-1}. Of these four *P. putida* strains, three strains and one strain, respectively, were separately isolated from two hospitals located in the same prefecture, and the three strains showed very similar pulsed-field gel electrophoresis patterns. Of 357 bla_{IMP-1} carriers, 116, 53, 51, 47, and 30 strains were identified as *Pseudomonas aeruginosa*, *Alcaligenes xylosoxidans*, *P. putida/fluorescens*, *Serratia marcescens*, and *Acinetobacter baumannii*, respectively. Four strains carrying bla_{IMP-2} were reidentified as *P. putida*. Sixty-three *P. aeruginosa* strains and four *P. putida* strains carried bla_{VIM-2}. Of 427 *intI1*-positive strains, 180, 53, 51, 47, and 35 were identified as *P. aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, *S. marcescens*, and *A. baumannii*, respectively. In the present study, it was confirmed that strains carrying bla_{IMP-1} with a class 1 integron are the most prevalent type in Japan, although several *intI3* carriers have also been identified sporadically in this country.

Since metallo-β-lactamases (MBLs) can hydrolyze a very wide range of broad-spectrum β-lactams, MBL-producing gram-negative bacteria usually demonstrate consistent resistance to a variety of broad-spectrum β-lactams, including oxy-iminocephalosporins, cephamycins, and carbapenems, which are the last resort for control of infections caused by gram-negative bacteria. Thus, MBL-producing gram-negative bacteria have been recognized to be among the most important nosocomial pathogens (3), and further proliferation of these strains in clinical settings will pose a serious global problem in the future (8). For this reason, aggressive surveillance of MBL producers with respect to the classification of the genetic determinant for MBLs as well as the integron will be extremely important.

At least three major groups of plasmid-mediated MBLs—the IMP, VIM, and SPM types—have been recognized worldwide, and their genetic determinants are often associated with integrons (5, 9). IMP-1-producing *Serratia marcescens* was initially identified in Japan in 1991 (14), and in 1997 VIM-1 and SPM-1 producers were also isolated, in Italy (10) and Brazil (23), respectively. After these reports, variants of these MBL types have been reported from almost every global region. For the IMP and VIM types of MBLs, at least 12 and 6 variants, respectively, have been published or reported to date (http://www.lahey.org/studies/other.stm#table 1). Moreover, genes

for these MBLs are usually located in integrons that successfully accumulate many antibiotic-resistant gene cassettes as a gene cluster (22).

At least nine genetically different integrons have been identified in various bacterial species (5), and class 1 (9), class 2 (16), and class 3 (1) integrons are often found in pathogenic gram-negative bacilli including *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Acinetobacter* spp., *Escherichia coli*, *S. marcescens*, *Citrobacter freundii*, and *Salmonella* spp. (15). Among these integrons, those in class 1 and class 3 have been reported to carry genetic determinants for MBLs. An IMP-1 type MBL associated with a class 1 integron was initially found in an *S. marcescens* clinical isolate in Japan (14), and integron-associated IMP-1 type MBLs were subsequently found in various gram-negative bacterial species such as *Klebsiella pneumoniae*, *C. freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *E. coli*, *Proteus vulgaris*, *Acinetobacter* spp., *Alicaligenes* spp., and *P. putida* (2, 20, 21).

In 1993, a class 3 integron-mediating IMP-1 was first identified by Arakawa et al. in an *S. marcescens* strain isolated in Japan (1). The organization of a class 1 integron that carries the gene for IMP-1 type MBL was characterized for *P. aeruginosa* (9). Although various class 1 integrons that convey MBL genes have been reported from a variety of gram-negative bacterial species isolated from different geographical regions, only a few isolates carrying class 3 integrons have been reported to date. Recently, *intI3*, a genetic determinant for class 3 integrons, was identified in a class A β-lactamase (GES-1)-producing *K. pneumoniae* strain isolated in Portugal (EMBL accession no. AY219651). This finding may suggest the poten-

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TABLE 1. PCR primers for detection of MBL and integrase genes

Gene	Primer name (sequence)	Expected size of amplicon (bp)	Reference or source
MBL genes			
bla_{IMP-1}	F1 (5′-ACC GCA GCA GAG TCT TTG CC-3′)	587	This study
	R1 (5′-ACA ACC AGT TTT GCC TTA CC-3′)		
bla_{IMP-2}	F2 (5′-GTT TTA TGT GTA TGC TTC C-3′)	678	This study
	R2 (5′-AGC CTG TTC CCA TGT AC-3′)		
bla_{VIM-1}	F3 (5′-AGT GGT GAG TAT CCG ACA G-3′)	261	24
	R3 (5′-ATG AAA GTG CGT GGA GAC-3′)		
bla_{VIM-2}	F4 (5′-ATG TTC AAA CTT TTG AGT AAG-3′)	801	17[a]
	R4 (5′-CTA CTC AAC GAC TGA GCG-3′)		
bla_{SPM-1}	F5 (5′-GCG TTT TGT TTG TTG CTC-3′)	786	This study
	R5 (5′-TTG GGG ATG TGA GAC TAC-3′)		
Integrase genes			
intI1	F6 (5′-GCA TCC TCG GTT TTC TGG-3′)	457	This study
	R6 (5′-GGT GTG GCG GGC TTC GTG-3′)		
intI2	F7 (5′-CAC GGA TAT GCG ACA AAA AGG T-3′)	789	This study
	R7 (5′-GTA GCA AAC GAG TGA CGA AAT G-3′)		
intI3	F8 (5′-ATC TGC CAA ACC TGA CTG-3′)	922	This study
	R8 (5′-CGA ATG CCC CAA CAA CTC-3′)		
Coamplification of the intI3–bla_{IMP-1} region	F9 (5′-GGT CTT GTA GGC TGT AAT TG-3′)	609	This study
	R9 (5′-TTG TGG CTT GGA ACC TTT AC-3′)		

[a] Since the nucleotide sequence appearing in reference 17 has a typographical error, the accurate sequence is shown in this table.

tial for a future worldwide dissemination of bacteria carrying class 3 integrons in addition to the widely dispersal of class 1 integrons. In the present study, we characterized the types of MBLs and integrons found in various gram-negative bacteria isolated in Japanese clinical environments.

MATERIALS AND METHODS

Bacterial strains. From January 2001 to December 2002, 978 strains belonging to gram-negative bacterial species were submitted to the reference laboratory for antibiotic resistance at the Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo, Japan, for typing and/or characterization of β-lactamases, including extended-spectrum β-lactamases (ESBLs), AmpC or CMY type class C cephamycinases, and class B MBLs. Of these strains, 587 demonstrating high-level resistance to both ceftazidime and sulbactam-cefoperazone (MICs, ≥128 μg/ml) were selected for screening of MBL production, because, from our experience, most MBL-producing gram-negative bacteria demonstrate very high levels of resistance to these agents. In the case of *Acinetobacter baumannii*, however, strains for which MICs of ceftazidime and sulbactam-cefoperazone were ≥16 μg/ml were also selected for screening in order to prevent overlooking of MBL producers. This is because several MBL-producing *Acinetobacter* strains were found to demonstrate low-level resistance to these agents in our preliminary study, and no data were available on the distribution of MICs of ceftazidime and sulbactam-cefoperazone for MBL-producing *Acinetobacter* spp. *Stenotrophomonas maltophilia* and *Chryseobacterium* spp., which produce chromosomal MBLs, were excluded from this study. Strains to be tested were collected from 108 Japanese hospitals, with only one strain selected from each patient.

Screening of MBL producers. Strains selected by the criteria described above were subjected to a screening test for MBL production by using disks containing an MBL inhibitor (2). Two Kerby-Bauer disks containing 30 μg of ceftazidime and one disk containing 3 mg of sodium mercaptoacetic acid (SMA) (Eiken Chemical Co. Ltd., Tokyo, Japan) were used in the screening test. When the strains produce MBL together with a large amount of ESBL, AmpC cephalosporinase, or CMY type enzymes as well as bacterial membrane alterations, no evident expansion of the growth inhibition zone around the ceftazidime disk tends to appear. For such strains, a disk containing 10 μg of imipenem or meropenem was used instead of the ceftazidime disk. However, disks containing 10 μg of imipenem or meropenem are not suitable for screening all strains that produce MBL only, because the MICs of imipenem and meropenem for several MBL producers are lower than 8 μg/ml, as reported in previous studies (2, 20). Appearance of a large growth inhibition zone around the disks containing imi-

penem or meropenem sometimes leads to an incorrect judgment. Several samples of the SMA-test results are shown in Fig. 1.

PCR detection of genes for MBLs and integrases. Although PCR analysis can predict only the approximate types of genes, it is suitable for testing a large number of samples at the same time. In the present study, therefore, we used this method for the rough classification of genetic determinants for MBLs and integrases. PCR analyses for the detection of MBL genes were carried out for all strains for which the screening test using SMA disks gave positive results. PCR amplification for the detection of genes for MBL and integrases was performed according to the method reported by Senda et al. (20), using each positive-control strain to avoid false-negative results. The PCR primers for bla_{VIM-1} and bla_{VIM-2} were used with the same PCR conditions as those for the other types of MBL genes. The five primer sets used in this study for amplifying MBL genes are shown in Table 1. PCR amplification of integrase genes was also performed as described above, by using three primer sets shown in Table 1.

Sequencing analyses of PCR amplicons. Sequencing analyses on both strands were performed on five amplicons in each bla_{IMP-1}-, bla_{IMP-2}-, or bla_{VIM-2}-positive strain and on five amplicons in the intI1-positive strain. All four amplicons from intI3-positive strains were sequenced from both sides. PCR amplicons were purified by use of the Qiaquick PCR purification kit (Qiagen K.K., Tokyo, Japan) prior to the labeling reaction. DNA sequences were determined by using BigDye Primer Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, Calif.) and an ABI model 377 DNA sequence analyzer (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Nucleotide and amino acid sequences were analyzed by the GENETYX program (version 11.0, available at http://www.sdc.co.jp/genetyx/; SDC Co. Ltd., Tokyo, Japan) and were submitted to the DNA Data Bank of Japan (DDBJ) database to check the identity or similarity of each sequence by using FASTA (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html).

PCR analysis of the relationship between the intI3 and bla_{IMP-1} genes. A PCR primer set (Table 1) was made for coamplification of the intI3 and bla_{IMP-1} genes. By this PCR analysis, a fragment containing the 5′ regions of both the intI3 and bla_{IMP-1} genes is able to be amplified, so that the distance and relationship between the intI3 and bla_{IMP-1} genes can be measured.

Reidentification of bla_{VIM-2}- or intI3-positive strains of *P. putida/fluorescens*. It is sometimes difficult to distinguish *P. putida* from *Pseudomonas fluorescens* by the routine identification protocol that depends on biochemical profiles. Therefore, four strains of *P. putida/fluorescens* carrying bla_{VIM-2} were reidentified by a sequencing analysis of their 16S rRNA according to the method described previously (19). Four intI3-positive strains of *P. putida/fluorescens* were also reidentified in the same manner.

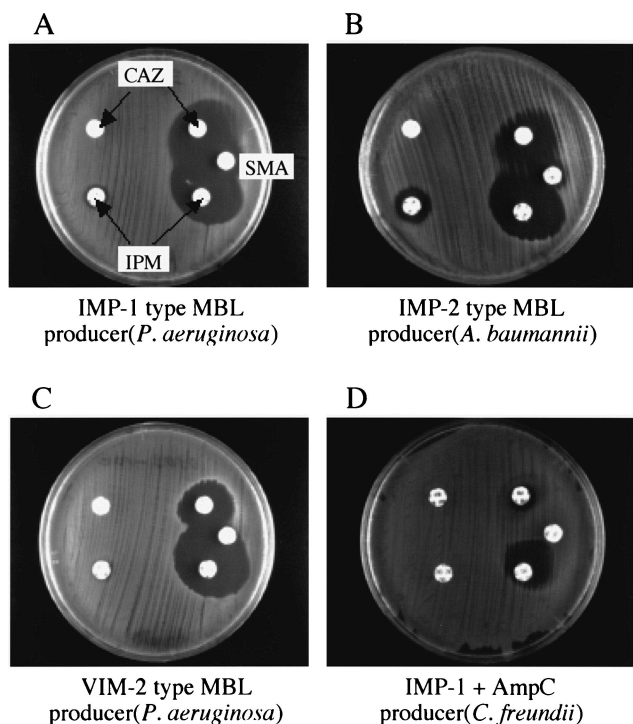**Pulsed-field gel electrophoretic (PFGE) analysis of intI3-positive strains.**

FIG. 1. Inhibition test for detection of MBL producers by use of SMA. Sample results of inhibition tests using SMA for detection of strains producing IMP-1, IMP-2, or VIM-2 type MBLs are shown. For MBL-producing strains, a clear expansion of the growth inhibition zone appears between the disks containing ceftazidime and SMA, respectively (A to C). In strains producing MBL as well as ESBLs or AmpC or CMY type cephalosporinases, the expansion of the growth inhibition zone is usually unclear. For such strains, the use of disks containing imipenem or meropenem instead of ceftazidime is recommended. By this substitution of disks, a clear growth inhibition zone appears between the disks containing imipenem and SMA, respectively (D). The MIC of imipenem or meropenem for MBL producers is sometimes lower than 32 μg/ml, but that of ceftazidime is usually higher than 64 μg/ml. Thus, disks containing 30 μg of ceftazidime are suitable for the first testing to detect MBL producers. IPM, imipenem; CAZ, ceftazidime.

Chromosomal DNAs prepared from four strains of *P. putida* were embedded in agarose gel plugs (InCert; Bio-Whittaker Molecular Applications, Rockland, Maine). Plugs were mounted in wells of agarose and electrophoresed after treatment with reaction mixtures according to the method described previously (20).

RESULTS

Screening of MBL production by using an SMA disk. Of 587 strains tested, 431 (73.4%) appeared to produce MBL, as determined by the screening tests using disks containing a thiol compound (2), SMA. *P. aeruginosa* ($n = 116$), *Alcaligenes xylosoxidans* ($n = 53$), *P. putida/fluorescens* ($n = 51$), *S. marcescens* ($n = 47$), *A. baumannii* ($n = 30$), *K. pneumoniae* ($n = 23$), and *E. coli* ($n = 17$) were the main species of SMA test-positive strains (Table 2). One hundred fifty-six strains were SMA negative even when a combination of disks containing imipenem and SMA, respectively, were used together. Since many of these strains were susceptible to meropenem, they were speculated to be either producers of class A enzymes

such as ESBLs, hyperproducers of chromosomal class C enzymes, or producers of plasmid-mediated AmpC and CMY type cephamycinases; some of them may also acquire altered membrane permeability to β-lactams. Further characterization of these strains will be performed in the next study.

PCR typing of MBL genes. Of 431 SMA-positive strains, 357 appeared to carry bla_{IMP-1} or one of its mutants such as bla_{IMP-3} or bla_{IMP-6}, which are indistinguishable from bla_{IMP-1} with the PCR primers and conditions used in this study. Of these 357 strains, 116, 53, 51, 47, 30, and 23 were identified as *P. aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, *S. marcescens*, *A. baumannii*, and *K. pneumoniae*, respectively (Table 3). Sixty-seven and seven strains appeared to carry bla_{VIM-2} and bla_{IMP-2}, respectively, and all these strains harbored the

TABLE 2. Results of screening tests using SMA disks

Bacterial species	No. of strains testing:		Total
	Positive	Negative	
Pseudomonas aeruginosa	180	48	228
Pseudomonas putida/fluorescens	55	1	56
Alcaligenes xylosoxidans	53	0	53
Serratia marcescens	47	9	56
Acinetobacter baumannii	35	13	48
Klebsiella pneumoniae	23	16	39
Escherichia coli	17	40	57
Enterobacter cloacae	5	8	13
Burkholderia cepacia	5	2	7
Citrobacter freundii	3	8	11
Klebsiella oxytoca	2	4	6
Providencia rettgeri	2	0	2
Alcaligenes faecalis	1	0	1
Morganella morganii	1	2	3
Acinetobacter lwoffii	1	1	2
Enterobacter aerogenes	1	3	4
Proteus spp.	0	1	1
Total	431	156	587

TABLE 3. Number of strains of each MBL type among SMA test-positive strains

Bacterial species	No. of strains of type:			Total no. of strains
	IMP-1	IMP-2	VIM-2	
Pseudomonas aeruginosa	116	1	63	180
Pseudomonas putida/fluorescens	51	0	4[a]	55
Alcaligenes xylosoxidans	53	0	0	53
Serratia marcescens	47	0	0	47
Acinetobacter baumannii	30	5	0	35
Klebsiella pneumoniae	23	0	0	23
Escherichia coli	17	0	0	17
Enterobacter cloacae	5	0	0	5
Burkholderia cepacia	5	0	0	5
Citrobacter freundii	3	0	0	3
Klebsiella oxytoca	2	0	0	2
Providencia rettgeri	2	0	0	2
Alcaligenes faecalis	1	0	0	1
Morganella morganii	1	0	0	1
Acinetobacter lwoffii	0	1	0	1
Enterobacter aerogenes	1	0	0	1
Total	357	7	67	431

[a] These strains were reidentified as *P. putida*.

TABLE 4. Combination of MBL and *intI* genes among all SMA
test-positive strains

Type of MBL	No. of strains with the following type of integrase gene:		Total no. of strains
	intI1	*intI1 + intI3*	
IMP-1	353	4[a]	357
IMP-2	7	0	7
VIM-2	67	0	67
Total	427	4	431

[a] Reidentified as *P. putida.*

intI1 gene (Tables 3 and 4). No isolate carrying two or more
different types of MBL genes together was found among the
strains tested. Of 67 *bla*$_{VIM-2}$-positive strains, 63 were *P. aerugi-
nosa* and the remaining 4 were reidentified as *P. putida* (Table
3) by 16S rRNA sequencing. As for seven *bla*$_{IMP-2}$-positive
strains, one was *P. aeruginosa* and five were *A. baumannii*
(Table 3). All SMA-positive strains were found to carry one of
the MBL genes tested in this study, although no strain harbor-
ing *bla*$_{VIM-1}$ or *bla*$_{SPM-1}$ was detected among them.

PCR typing of integrase genes. Of 431 SMA-positive strains,
427 appeared to carry the *intI1* gene. Among these 427 strains,
180, 53, 51, 47, 35, and 23 were identified, respectively, as *P.
aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, *S. marcescens*,
A. baumannii, and *K. pneumoniae*. Only four strains appeared
to carry both the *intI1* and *intI3* genes, and these were reiden-
tified as *P. putida* (Table 4) by sequencing of 16S rRNA as well
as by the conventional identification protocol depending on the
characteristic biochemical properties of each bacterial species.
Of 51 *P. putida/fluorescens* strains harboring only the *intI1*
gene, 47 and 4 appeared to carry *bla*$_{IMP-1}$ and *bla*$_{VIM-2}$, respec-
tively (Table 5). All four *P. putida* strains positive for both the
intI1 and *intI3* genes carried the IMP-1 type MBL gene. The
four *intI1*-positive strains carrying the VIM-2 type MBL gene
were also reidentified as *P. putida* (Table 5).

Sequencing analyses of *bla* and *intI* genes. The nucleotide
sequences of 15 amplicons, consisting of 5 *bla*$_{IMP-1}$, 5 *bla*$_{IMP-2}$,
and 5 *bla*$_{VIM-2}$ amplicons, were consistent with the types pre-
dicted by the preceding PCR analyses, at least within the se-
quenced areas. Similarly, the nucleotide sequences of nine
amplicons, consisting of five *intI1* and four *intI3* genes, also
coincided with the results predicted by the PCR analyses.

Relationships of the *intI3* and *bla*$_{IMP-1}$ genes. According to
the result of a PCR analysis using a set of primers for ampli-

TABLE 5. Combination of MBL and *intI* genes
in *P. putida/fluorescens*

Type of MBL	No. of strains with the following type of integrase gene:		Total no. of strains
	intI1	*intI1 + intI3*	
IMP-1	47	4[a]	51
VIM-2	4[a]	0	4
Total	51	4	55

[a] Reidentified as *P. putida.*

fication of a 609-bp fragment in the *intI3–bla*$_{IMP-1}$ region (Ta-
ble 1), the *intI3* gene was suggested to be located adjacent to
the *bla*$_{IMP-1}$ gene in the configuration reported previously (1)
in all four *P. putida* strains carrying both the *intI3* and *bla*$_{IMP-1}$
genes.

***P. putida* strains carrying both the *intI1* and *intI3* genes.** Of
four *P. putida* strains carrying both the *intI1* and *intI3* genes,
three were isolated at hospital A and one was isolated at
hospital B; both these hospitals are located in Mie Prefecture.
Table 6 shows the clinical associations of these four strains.
The *Spe*I-digested genomic DNA patterns of the four clinical
isolates were classified into two types (Fig. 2). The three strains
isolated in hospital A, NCB 01-121, NCB 02-182, and NCB
02-204, demonstrated very similar PFGE patterns, suggesting a
clonal lineage.

DISCUSSION

Since 1988, transferable carbapenem resistance has been
found in several *P. aeruginosa* strains isolated in Toyama Pre-
fecture, Japan (12, 25). In 1991, an IMP-1 type MBL, initially
characterized in a strain of *S. marcescens*, gave high-level re-
sistance to various broad-spectrum β-lactams including imi-
penem (6, 14). This strain was isolated in a hospital in Aichi
Prefecture and had the *intI1* gene just upstream of the *bla*$_{IMP-1}$
gene cassette on the chromosome. In 1993, several *S. marc-
escens* strains harboring a plasmid-mediated *bla*$_{IMP-1}$ gene
were also identified in Aichi Prefecture (1, 6). Several of these
strains were found to carry a novel integron-like element that
was classified as a class 3 integron (5). The newly identified
class 3 integrase, IntI3, has about 60.9% identity to the previ-
ously identified IntI1 at the amino acid sequence level (1). The
genomic organization of a class 1 integron was characterized in
a *bla*$_{IMP-1}$-carrying strain (9) which was isolated in Japan. The
emergence and development of carbapenem resistance
through the acquisition of genes for MBLs has since become a
matter of general concern, especially in gram-negative bacteria
(13, 21).

At least three genetically different clusters of MBLs have
been found to date: IMP-1 to IMP-12 (4), VIM-1 to VIM-6
(EMBL accession no. AY165025), and SPM-1 (23) have been
published or registered with the EMBL/GenBank database.
Almost all of their genetic determinants are associated with
class 1 integrons, and only a few strains have been found to
carry class 3 integrons to date. In the present study, almost all
MBL genes were found to correlate with class 1 integrons. To
our knowledge, only *S. marcescens* strain AK9373 and a small
number of strains isolated in Aichi Prefecture carry the *intI3*
gene (N. Shibata, Y. Arakawa, H. Kurokawa, Y. Doi, and K.
Shibayama, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., ab-
str. C524, 2001). Recently however, a class 3 integron mediat-
ing a gene for a GES-1 type class A β-lactamase, a kind of
ESBL, was submitted from Portugal to EMBL/GenBank (ac-
cession no. AY219651). Because *intI* genes are usually accom-
panied by genes for aminoglycoside-modifying enzymes such as
aacA4 or *aadA1*, these gene cassettes in integrons may be
derived from some nonpathogenic bacteria such as aminogly-
coside-producing actinomycetes, which are widely distributed
in the natural environment. Therefore, the two strains possess-
ing class 3 integrons found in Japan (1) and Portugal may have

TABLE 6. Clinical associations of *P. putida* isolates carrying both the *intI1* and *intI3* genes[a]

Strain no.	Age of patient (yr)	Sex of patient	Disease	Specimen type	Hospital	Date of isolation (mo/yr)
NCB 01-121	77	Male	Prostatic cancer	Urine	A	6/2001
NCB 02-182	79	Male	Cerebral infarction	Urine	A	5/2002
NCB 02-190	66	Female	Cerebral infarction	Sputum	B	5/2002
NCB 02-204	76	Female	Gallbladder cancer	Biliary tract drainage tube	A	6/2002

[a] All these strains were isolated in Mie Prefecture.

been generated independently of each other by the integration of separate β-lactamase gene cassettes in clinical environments, where the strong influence of antimicrobial agents has continued.

In the present study, we confirmed that class 1 is the most abundant type of integron in Japan, although four *P. putida* strains carrying both class 1 and class 3 integrons have been newly identified. Although the reason why these four strains carry two types of integron is not well understood at present, analyses of the organization and function of class 1 and class 3 integrons in these strains may elucidate their biological advantage in the future. In any case, since *intI3* has already been identified in at least three gram-negative bacterial species (*S. marcescens*, *K. pneumoniae*, and *P. putida*) isolated from geographically different areas, the emergence and proliferation of class 3 integrons that carry various gene cassettes responsible for multiple antimicrobial resistance may become not so much a local problem as a global issue.
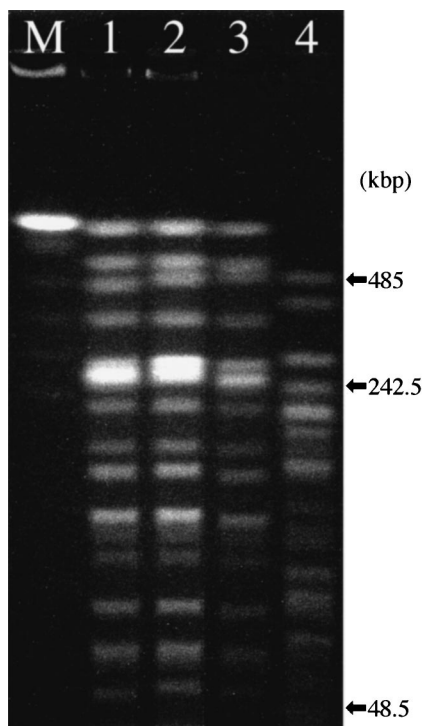

FIG. 2. PFGE of four *P. putida* strains carrying both the *intI1* and *intI3* genes. Lanes: M, size marker for PFGE; 1, *P. putida* strain NCB 01-121; 2, strain NCB 02-182; 3, strain NCB 02-204; 4, strain NCB 02-190. Clinical associations of the strains subjected to this PFGE analysis are listed in Table 6.

It was confirmed that the IMP-1 type of MBL is the most common MBL in Japan at present, although IMP-3 (7) and IMP-6 (27) have also been identified in Japan. Since very few amino acid substitutions exist among IMP-1, IMP-3, and IMP-6, the latter two MBLs are fundamentally variants of IMP-1. It is difficult to confirm whether all the bla_{IMP-1}-positive strains found in this study carry genuine bla_{IMP-1}, since the primer sets used in this study cannot distinguish bla_{IMP-3} and bla_{IMP-6} from bla_{IMP-1}. Strains producing IMP-3 or IMP-6 usually demonstrate susceptibility or low-level resistance to imipenem (MICs, 1 to 16 μg/ml) (7, 27). However, almost all bla_{IMP-1}-positive strains identified in this study, especially in *P. aeruginosa* and *S. marcescens*, demonstrated high-level resistance to imipenem (MICs, >32 μg/ml), as was reported previously (6, 18, 20). In any event, the bla_{IMP-1}-positive strains detected in this study appeared to produce MBLs belonging to the IMP-1 complex, consisting mainly of IMP-1 and probably small amounts of its variants, IMP-3 and IMP-6. Detailed typing of the MBLs produced by the 357 bla_{IMP-1}-positive strains will be continued in the next study.

IMP-2 was initially found in *A. baumannii* in Italy (18), and its variant IMP-8 was also identified in *K. pneumoniae* in Taiwan (26). In the present study, one strain of *P. aeruginosa* and five strains of *A. baumannii* were found to carry the bla_{IMP-2} gene in Japan. IMP-2 is not a variant of IMP-1, since many amino acid substitutions are found between them (18). One has to wonder why IMP-2 has been found in such widely separated geographical regions as Europe and the Far East. The increased frequency of international travel and transportation may well be involved in the transmission of IMP-2 producers, although they may have emerged independently in each area. Precise characterization of the genetic organization of each integron is necessary to determine their origins and relationships.

The present study suggests that VIM-2 producers are also increasing in Japan, especially in *Pseudomonas* spp. VIM-2 was initially identified in France in 1996 (17). VIM-2 producers have occasionally caused outbreaks in many countries recently, including Korea (11), where VIM-2 producers are reportedly increasing (28). It is not well understood why VIM-2 producers are becoming predominant in Korea, a country adjacent to Japan, where IMP-1 producers are very common. A similar observation was made in the isolation of ESBL producers. Strains producing TEM- or SHV-derived ESBLs have been increasing in Korea, while such ESBL producers still remain rare in Japan, though producers of CTX-M type enzymes, including Toho-1, are becoming common in Japan. These discrepancies may depend on differences in the administration of antimicrobial agents.

In the present study, 16 gram-negative bacterial species were found to carry genes for at least three types of MBLs. Indeed, although *P. aeruginosa*, *A. xylosoxidans*, *P. putida/fluorescens*, and *S. marcescens* are the most frequent carriers of MBL genes among these 16 bacterial species, bla_{IMP-1} genes have already been transmitted into various bacterial species belonging to the family *Enterobacteriaceae*, such as *E. coli*, *E. cloacae*, *E. aerogenes*, *C. freundii*, *K. pneumoniae*, *Providencia rettgeri*, and *Morganella morganii*, through the mediation of class 1 integrons, which are usually carried on transferable plasmids. Isolation of MBL producers belonging to these bacterial species is still very rare even in Japan, which may be due to the instability of plasmids carrying MBL and integrase genes in these strains. However, the frequency of isolation of MBL producers belonging to these species is also likely to increase in the future, as it already has in *P. aeruginosa* and *S. marcescens*, if the gene cassettes or gene clusters locating in integron structures are transposed to stable resident plasmids or chromosomes in each bacterial species.

In hospital A, three strains of *P. putida* carrying *intI1*, *intI3*, and bla_{IMP-1} have been isolated from different patients for more than 12 months. Since these strains demonstrated very similar PFGE patterns, it is suggested that they are a clonal lineage and have been existing in the hospital for nearly 1 year. Since *P. putida* can grow at temperatures below 10°C and tends to be isolated from damp materials and environments such as plant surfaces, mops, sponges, and sinks, these strains might have survived in such nosocomial environments. Indeed, *P. putida* sometimes causes opportunistic infections such as respiratory and urinary tract infections, but its virulence is not as strong as that of *P. aeruginosa*. However, it can fully work as a reservoir of integrons and MBL genes. Thus, we should pay continuous attention even to such low-virulence bacterial species when they carry special genetic determinants such as MBL genes.

The bla_{IMP-1} genes move together with class 1 or class 3 integrons in many nosocomial gram-negative bacilli. Furthermore, genes for bla_{IMP-2} and bla_{VIM-2} have also been dispersing into various non-glucose-fermenting bacteria such as *P. putida* and *A. baumannii*. Hence, we should take special precautions against the further global dissemination of integron-associated MBL genes among a variety of nosocomial gram-negative bacterial species.

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