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bla$_{VIM-2}$ Cassette-Containing Novel Integrons in Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* and *Pseudomonas putida* Isolates Disseminated in a Korean Hospital

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We investigated the phenotypic and genetic properties of metallo-β-lactamase-producing *Pseudomonas* isolates collected at a tertiary-care hospital in Korea since 1995. The prevalence of imipenem resistance among *Pseudomonas aeruginosa* isolates reached 16% in 1997, when 9% of the resistant organisms were found to produce VIM-2 β-lactamase, a class B enzyme previously found only in *P. aeruginosa* isolates from Europe. VIM-2-producing isolates of *Pseudomonas putida* were also detected. Resistance was transferable from both these species to *P. aeruginosa* PAO4089Rp by filter mating, although the resistance determinant could not be found on any detectable plasmid. Serotyping showed that many of the VIM-2-producing *P. aeruginosa* isolates belonged to serotypes O:11 and O:12, and pulsed-field gel electrophoresis of *Xba*I-digested genomic DNA revealed that many had identical profiles, whereas the *P. putida* isolates were diverse. Sequencing showed that the *bla*$_{VIM-2}$ genes resided as cassettes in class 1 integrons. In contrast to previous VIM-encoding integrons, the integron sequenced from a *P. aeruginosa* isolate had *bla*$_{VIM}$ located downstream of a variant of *aacA4*. *bla*$_{VIM}$ also lay in a class 1 integron in a representative *P. putida* strain, but the organization of this integron was different from that sequenced from the *P. aeruginosa* strain. In conclusion, the metallo-β-lactamase produced by these imipenem-resistant *Pseudomonas* isolates was VIM-2, and the accumulation of producers reflected clonal dissemination as well as horizontal spread. Strict measures are required in order to control a further spread of resistance.

Carbapenems such as imipenem are stable to most β-lactamases. Nevertheless, the first isolate of *Pseudomonas aeruginosa* with transferable imipenem resistance due to a metallo-β-lactamase production was reported in Japan in 1991 (26). The enzyme produced by this isolate almost certainly was IMP-1, which subsequently has been repeatedly found in Japan, not only in *P. aeruginosa* and *Acinetobacter baumannii* (23), but also in *Serratia marcescens* (14) and other members of *Enterobacteriaceae* (5). Rasmussen and Bush (18) predicted that the increasing use of carbapenems would select for more carbapenem-hydrolyzing organism on a wider scale, and this prediction is now proving correct. Several close relatives of IMP-1 have been reported during the past 3 years: IMP-2 from an isolate of *A. baumannii* in Italy (19), IMP-3 (MET-1) from *Shigella flexneri* in Japan (6), and IMP-4 from *Acinetobacter* and *Citrobacter* spp. from Hong Kong, China (3).

Descriptions of IMP-5 to IMP-8 enzymes are understood to be in press. IMP-1 to IMP-4 have at least 80% homology to each other, but in 1999, a new type of acquired metallo-β-lactamase, VIM-1, was reported to have been found in an isolate of *P. aeruginosa* collected in Italy (8). Soon afterwards, isolates of *P. aeruginosa* with a related enzyme, VIM-2, were reported in France (16, 17). Since then, outbreaks of VIM β-lactamase-producing *P. aeruginosa* have been reported in Greece (25) and Italy (4).

Although the VIM and IMP metallo-β-lactamase have only 30 to 40% amino acid homology, both are often encoded by mobile gene cassettes inserted into integrons (1, 8, 17), which are sometimes located on plasmids. Most of these integrons belong to class 1, but their structures vary among isolates.

In the present study, we determined the phenotypes and genetic properties of metallo-β-lactamase-producing *Pseudomonas* strains collected at one hospital in Korea since 1995.

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MATERIALS AND METHODS

Bacterial strains and susceptibility testing. Nonduplicate isolates of *Pseudomonas* spp. were from clinical specimens from patients in a tertiary-care hospital in Seoul, Korea. The species were identified by conventional methods (7) and with the ID 32 GN system (bioMerieux, Marcy-l'Etoile, France). Imipenem resistance was initially detected by a disk diffusion method (13), and the resistant isolates were kept in skim milk at −76°C until used for further testing. The MICs of antimicrobial agents were determined by an agar dilution method (12). The agents and β-lactamase inhibitors used were ampicillin and cephalothin (Sigma, St. Louis, Mo.), cefoxitin and imipenem (Merck/Sharp & Dohme, Rahway, N.J.),

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TABLE 1. Trend of isolation of imipenem-resistant *Pseudomonas* spp. and detection of bla_{VIM-2}-positive isolates

Yr	*P. aeruginosa*			*P. putida*		
	No. of isolates (% imipenem resistance)	No. of bla_{VIM-2} isolates		No. of isolates (% imipenem resistance)	No. of bla_{VIM-2} isolates	
		Tested	Positive (%)		Tested	Positive (%)
1994	1,939 (4)	1	0 (0)	38 (8)	0	NA[a]
1995	966 (3)	20	5 (25.0)	6 (0)	0	NA
1996	2,189 (6)	8	0 (0)	37 (16)	0	NA
1997	2,078 (16)	130	15 (11.5)	17 (6)	1	1 (100)
1998	2,934 (15)	147	12 (8.2)	35 (29)	5	5 (100)
1999	3,129 (15)	187	11 (5.9)	35 (17)	3	3 (100)
Total	13,235 (11)	493	43 (8.7)	168 (15)	9	9 (100)

[a] NA, not applicable.

meropenem (Sumitomo, Tokyo, Japan), cefotaxime (Aventis, Frankfurt, Germany), ceftazidime and clavulanic acid (GlaxoSmithKline, Greenford, United Kingdom), piperacillin and tazobactam (Wyeth, Pearl River, N.Y.), aztreonam (Bristol-Myers Squibb, Princeton, N.J.), amikacin and kanamycin (Dong-A Pharmaceutical, Seoul, Korea), gentamicin (Chong Kun Dang Pharmaceutical, Seoul, Korea), streptomycin (Meiji Seika, Tokyo, Japan), tobramycin (Daewoong-Lilly, Seoul, Korea), and ciprofloxacin (Bayer Korea, Seoul, Korea). Metallo-β-lactamase production was screened with modified Hodge and EDTA disk synergy tests (9).

Conjugation. The filter mating method (20) was used to determine the transferability of the carbapenem resistance determinant using the rifampin-resistant mutant *P. aeruginosa*, PAO4089Rp (26) as a recipient. Mueller-Hinton agar containing 100 μg of rifampin per ml and 2 μg of imipenem per ml was used to select transconjugants.

Serotyping, plasmid preparation, PFGE of genomic DNA, and hybridization. O serotypes of the *P. aeruginosa* isolates were determined by a slide-agglutination method of the International Antigenic Typing Scheme. Plasmids were isolated by alkaline lysis (21). For pulsed-field gel electrophoresis (PFGE), *Xba*I-digested genomic DNA was prepared according to the instruction of Bio-Rad (Hercules, Calif.) and fragments were separated for 20 h at 6 V/cm at 11°C using a CHEF-DR II System (Bio-Rad), with initial and final pulse times of 0.5 and 30 s, respectively. DNA fingerprints were compared visually and were interpreted as recommended by Tenover et al. (24).

For Southern hybridization, plasmid or genomic DNA was transferred from electrophoresis gels to nylon membranes using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). A DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) was used to label the bla_{VIM-2} probe and for hybridization.

β-Lactamase assays and isoelectric focusing. The β-lactamase activities of cell sonicates from overnight Luria-Bertani broth cultures were determined using a spectrophotometer (UV-1601PC; Shimadzu Corp., Tokyo, Japan) with a 100 μM solution of β-lactams in 50 mM phosphate buffer (pH 7.0) at 30°C. The wavelengths used were 235 nm for benzylpenicillin (Sigma) and 297 nm for both imipenem and meropenem (11). The protein content of the sonicates was determined with a commercial assay reagent (Bio-Rad).

The isoelectric points of β-lactamases were determined by loading cell sonicates to precast pH 3 to 10 gels (Novel Experimental Technology, San Diego, Calif.). These were electrophoresed on a ThermoFlow Electrophoresis Temperature Control System (Novel Experimental Technology). After electrophoresis, the gels were overlaid with filter papers soaked in 0.7 mg of nitrocefin per ml in a 30 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid–NaOH buffer (pH 7.0) (8). Sonicates of *Escherichia coli* and *P. aeruginosa* VR-143/97 (8) producing TEM-1 (pI 5.4) and VIM-1 (pI 5.3) β-lactamases, respectively, were used as controls. Imipenem-hydrolyzing bands were confirmed by observation for growth when the gel was overlaid with Mueller-Hinton agar containing *E. coli* ATCC 25922 and 2 μg of imipenem per ml and then incubated overnight at 37°C. The gels were overlaid with a filter paper soaked in 20 mM EDTA for 5 min before the imipenem-containing agar was added so that inhibition of imipenem-hydrolyzing activities could be observed.

Detection of metallo-β-lactamase genes by PCR. Previously reported PCR primers were used to determine the presence of bla_{IMP} (2) and bla_{VIM-2} (17). PCR was carried out with 1 μl of heat-extracted template DNA, 20 pmol of each primer, and PreMix (Bioneer, Cheongwon, Korea) containing 1 U of *Taq* DNA polymerase in a total volume of 20 μl. A thermal cycler (Eppendorf-Netheler-Hinz, Hamburg, Germany) was used under the following conditions: 94°C for 5 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s, followed by 72°C for 7 min. The bands were confirmed by hybridization with a bla_{VIM-2} probe.

Sequencing the bla_{VIM}-containing integron. PCR products were obtained by the hot-start method in a total volume of 100 μl, with 5 μl of heat-extracted template DNA, 20 pmol of primers INT/5′CS and INT/3′CS (19), and 3 U of LA *Taq* DNA polymerase (Takara, Shiga, Japan). The reaction conditions consisted of a predenaturation at 94°C for 12 min and then 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 5 min. The extension time was increased by 5 s at each cycle, as described by Levesque and Roy (10).

The PCR products were ligated in the pGEM-T Easy Vector (Promega, Madison, Wis.) and transformed into *E. coli* DH5α by electroporation using an *E. coli* Pulser (Bio-Rad) (22). Successful clones were selected by the observation of decreased imipenem disk zones. Plasmids from cloned strains were used to sequence bla_{VIM} and the other cassettes in the integrons, using the dideoxy-chain termination method with an ABI 3700 Autosequencer and the reagents supplied by its manufacturer (Perkin-Elmer, Foster City, Calif.). Primers INT/5′CS and INT/3′CS were those reported previously (19). The nucleotides of custom-designed primers were as follows: INT7-R, 5′-GTT CTT CTA CGG CAA GGT GC-3′ (bp 261 to 280); VIM-2-F, 5′-ATT GGT CTA TTT GAC CGC GTC-3′ (bp 55 to 75); VIM-2-R, 5′-TGC TAC TCA ACG ACT GAG CG-3′, (bp 784 to 803); VIM-2b-F, 5′-CAA CAA CAC TAC CCG GAA GC-3′, (bp 679 to 698), VIM-2a-R, 5′-ATC TGG TAA AGC CGG ACC TC-3′ (bp 127 to146); AAC6-F, 5′-ACT GAG CAT GAC CTT GCA AT-3′ (bp 4 to 23), AAC6-R, 5′-CTG GCG TGT TTG AAC CAT GT-3′ (bp 470 to 489); AACA7-F, 5′-AGC ATT GGG CTC GTG GTC G-3′ (bp 323 to 341); AACA7-R, 5′-GAC ACC TCC GTG AAT CCA G-3′ (bp 410 to 428); AADA1-F, 5′-TGA TTT GCT GGT TAC GGT GAC-3′ (bp 144 to 164); AADA1-R, 5′-CAC TAC ATT TCG CTC ATC G-3′ (bp 543 to 561); ORF1-F, 5′-GAC TTC TGG GCG TTC ATT GG-3′ (bp 257 to 276); ORF2a-R (5′-TGG CGA CAA GGT GAA AGA C-3′ (bp 486 to 504); ORF2b-R, 5′-TGG CTA TCA GCA CTG CGT TGA G-3′ (bp 1002 to 1023); ORF2c-F, 5′-ATC CAG CTC AAC GCA GTG CTG AT-3′ (bp 1007 to 1029); ORF3-R, 5′-AGA AGC CAA ACG CCC ATC GTA-3′ (bp 1 to 21 upstream from *orf "iii"*) (Fig. 2). The determination of the sequences was repeated with more than two clones from independent amplicons. Also, the sequences on both strands were determined.

Nucleotide sequence accession numbers. The nucleotide sequences reported have been assigned the GenBank accession nos. as follows: for the integron from *P. aeruginosa* YMC 95/1/704, AY029772; for *P. putida* YMC 97/8/322, AF327064.

RESULTS

Prevalence of imipenem resistance and metallo-β-lactamase producer. In 1995, 3% of *P. aeruginosa* isolates at the hospital were imipenem resistant, and among the resistant isolates examined, five had metallo-β-lactamases. Of eight imipenem-resistant *P. aeruginosa* collected in 1996, none had metallo-β-lactamases. In 1997, however, the prevalence of imipenem resistance rose to 16% and among the total of 130 imipenem-resistant isolates 11.5% had metallo-β-lactamases (Table 1). The prevalence rate of imipenem resistance in *P. aeruginosa* then stabilized, as did the proportion with metallo-β-lactamases. Metallo-β-lactamase-producing *P. putida* isolates were

TABLE 2. Antimicrobial susceptibilities of bla_{VIM-2}-positive clinical isolates and their transconjugants

Antimicrobial agent[a]	*P. aeruginosa*				*P. putida*	
	CI[b] (n = 43)			TC[b] (n = 6)	CI (n = 9)	TC (n = 2)
	MIC range[c]	MIC_{50}[c]	MIC_{90}[c]	MIC range	MIC range	MIC range
Imipenem	8–>128	16	128	16–32	16–128	16–32
Meropenem	8–>128	16	128	8–32	32–128	8–16
Piperacillin	64–>256	256	>256	8–128	64–>256	256
Piperacillin-tazobactam[d]	32–>128	128	>128	8–128	32–>256	128
Cefotaxime	>128	>128	>128	64–>128	>128	>128
Cefotaxime-clavulanic acid[d]	>128	>128	>128	32–>128	>128	>128
Ceftazidime	32–>128	128	>128	8–64	32–>128	128
Aztreonam	4–64	16	32	2–4	16–32	4
Amikacin	2–>128	>128	16	4	16–>128	4
Gentamicin	8–>128	>128	128	64	32–>128	64
Kanamycin	64–>128	>128	>128	>128	16–>128	>128
Streptomycin	128–>128	>128	>128	>128	>128	128
Tobramycin	2–>128	>128	32	16–32	16–>128	32
Ciprofloxacin	0.12–>128	32	16	0.12–0.25	16	0.12

[a] MICs of ampicillin, cephalothin, and cefoxitin for all of the strains were >128 μg/ml.
[b] Abbreviations: CI, clinical isolates; TC, transconjugants.
[c] Values are in micrograms per milliliter; MIC_{50} and MIC_{90}, MICs for 50 and 90% of strains, respectively.
[d] Piperacillin-tazobactam: tazobactam at a fixed concentration of 4 μg/ml; cefotaxime- clavulanic acid: a 2:1 combination.

first detected in 1997. PCR showed that all of the metallo-β-lactamase producers had bla_{VIM-2} but none had bla_{IMP-1}.

Isoelectric focusing of extracts of the bla_{VIM-2}-positive isolates detected a β-lactamase with a pI of ca. 5.3, which was no longer apparent if the gels were overlaid with EDTA. A biological assay showed that this pI 5.3 band inactivated imipenem, allowing the growth of the *E. coli* indicator strain in an agar overlay. Almost all of the bla_{VIM-2}-positive isolates also had other β-lactamase bands with pIs of ca. 7 to >8.

Among the total of 52 bla_{VIM-2}-positive *P. aeruginosa* and *P. putida* isolates collected, 35 (67%) were from urines, 10 (19%) were from sputa, and 2 (4%) were from blood samples; among the 9 *P. putida* isolates all except one were from urine. Most of the source patients had underlying malignancy, recent brain surgery, indwelling urinary catheters, prior therapy with various antimicrobial agents, and/or admission to intensive care units (data not shown).

Conjugative transfer of resistance. Carbapenem resistance was transferred from 6 of the 43 *P. aeruginosa* isolates and 2 of the 9 *P. putida* isolates with bla_{VIM-2} to a *P. aeruginosa* recipient. However, repeated attempts failed to detect the presence of plasmids capable of hybridizing with the bla_{VIM-2} probe, while the fragments of the *Xba*I-digested genomic DNA of clinical isolates showed hybridization. Resistances to ciprofloxacin and amikacin were not transferred to the transconjugants.

Susceptibilities of isolates and transconjugants. MIC ranges of imipenem for bla_{VIM-2}-positive clinical isolates and transconjugants of *P. aeruginosa* were 8 to >128 and 16 to 32 μg/ml, respectively; those for *P. putida* isolates and transconjugants were 16 to 128 and 16 to 32 μg/ml, respectively (Table 2). MICs of imipenem and meropenem for the *P. aeruginosa* recipient were 0.25 and 0.5 μg/ml, respectively. MICs of all of the other β-lactams for clinical isolates were ≥32 μg/ml, except for aztreonam, whose MICs were as low as 4 μg/ml for some isolates.

A slight reduction in the MICs of piperacillin was achieved in the presence of tazobactam (4 μg/ml), but the MICs of cefotaxime were not reduced by clavulanic acid. Most of the VIM-2-producing *P. aeruginosa* isolates were also resistant to gentamicin, tobramycin, and ciprofloxacin.

Serotype and PFGE profile. PFGE of *Xba*I-digested genomic DNA from bla_{VIM-2}-positive *P. aeruginosa* showed that 26 isolates gave an identical pattern, E1 (Fig. 1). Among these, 11 strains were isolated from 1997 to 1999: 6 serotype O:11 isolates were from two each of pediatric and neurosurgery patients and from one each of gynecology and rehabilitation patients; 5 O polyagglutinating (OPA) isolates were from two internal medicine patients and from one each of pediatric, rehabilitation, and neurosurgery patients. Among the six isolates with PFGE pattern C2, five were serotype O:12 and were isolated from four ICU patients in 1997 and one pediatric patient in 1995. Among the six isolates of *P. aeruginosa* that were gene transfer positive, four had PFGE pattern C2 with serotype O:12. The remaining two serotype OPA isolates had PFGE patterns A and D1. Eight different PFGE profiles were seen among the nine bla_{VIM-2}-positive *P. putida* isolates.

Biochemical detection of VIM β-lactamases. Cell sonicates of all bla_{VIM-2}-positive isolates hydrolyzed imipenem in spectrophotometric assays; this activity was inhibited by EDTA, but not by clavulanic acid. Using extracts of a representative *P. aeruginosa* strain, relative hydrolysis rates of 100 μM benzylpenicillin, imipenem, and meropenem were in a 100:21:7 ratio. With extracts of a *P. putida* isolate this ratio was 100:2:<1, reflecting a relatively higher background penicillinase activity. Imipenem-hydrolyzing activities of the *P. aeruginosa* and *P. putida* extracts were 96 and 61 U per mg of protein, respectively (data not shown).

Sequences of bla_{VIM-2} and flanking genes. PCR products of the integron carrying bla_{VIM-2} were detected from 41 of the 43 *P. aeruginosa* and all of the 9 *P. putida* isolates. The sizes of the integrons carrying the bla_{VIM-2} varied among the isolates, from ca. 3 to 6 kb, but in 23 of 50 isolates they were ca. 5 kb. The sizes in the 25 *P. aeruginosa* strains with PFGE pattern E1 were ca. 4 kb in 6 isolates, 5 kb in 15 isolates, and 6 kb in 4 isolates. The first isolates of *P. aeruginosa* and *P. putida* were chosen to analyze the nucleotide sequence of the bla_{VIM} allele and its

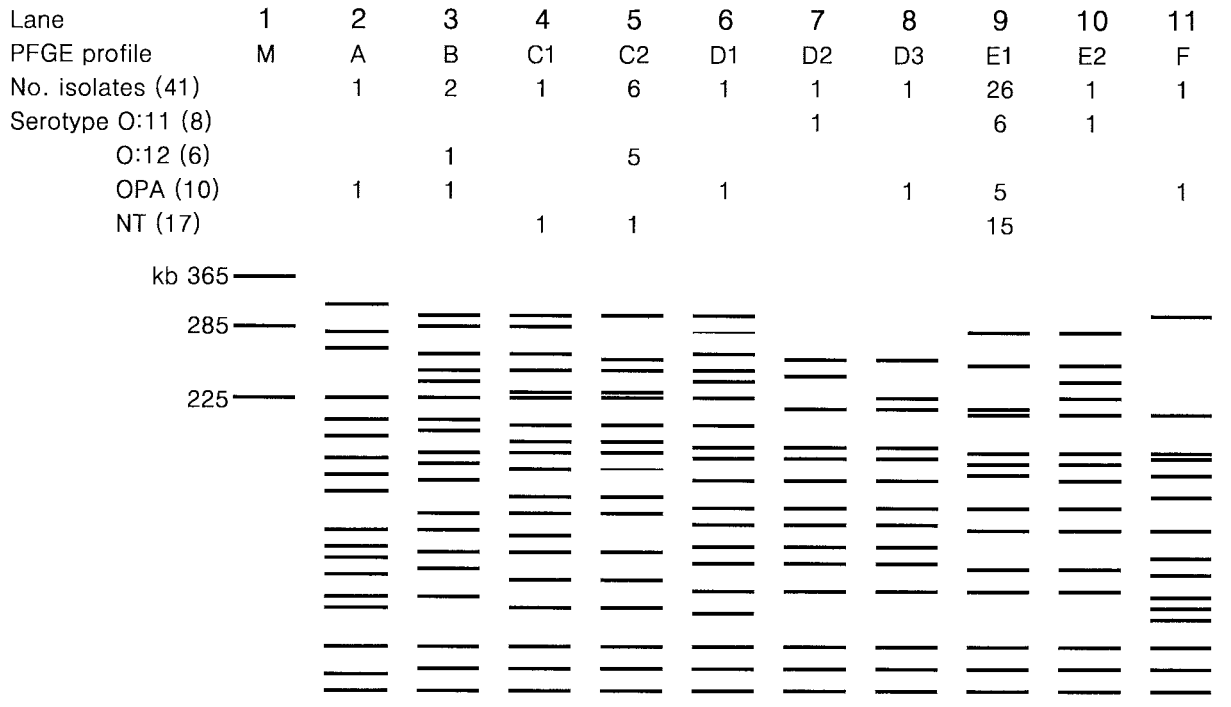

FIG. 1. Schematic presentation of PFGE patterns of *Xba*I-digested genomic DNA of bla_{VIM-2}-positive isolates of *P. aeruginosa*. M in lane 1 is a size marker (a chromosome of *Saccharomyces cerevisiae*). The numbers of isolates with serotypes O:11, O:12, and OPA are shown under each PFGE profile, and total numbers are in parentheses. OPA, O polyagglutination; NT, serotype not tested.

flanking genes. Analysis revealed sequences of bla_{VIM-2} in both cases. The structure of the integron from the *P. aeruginosa* isolate was *aacA4* [Asp171→Val variant of *aac(6′)-II*], followed immediately downstream by bla_{VIM-2}; in addition *aadA1* was present. The integron from the *P. putida* isolate differed in that the first cassette was bla_{VIM-2} followed by *aacA7* and *aadA1* (Fig. 2).

DISCUSSION

The frequency of imipenem resistance among *P. aeruginosa* isolates at the hospital rose to 16% in 1997, thereafter stabilizing. Approximately 9% of the resistance isolates were metallo-β-lactamase-producers, and all these had bla_{VIM-2} (Table 1). In another study in 1999 (K. Lee, H. S. Lee, S. H. Kang, M. H. Lee, W. K. Song, S. J. Kim, and Y. Chong, Abstr. 7th Western Pacific Cong. Chemother. Infect. Dis., abstr. FP2.8, 2000), it was found that bla_{VIM-2}-positive *P. aeruginosa* isolates were widespread in other Korean hospitals too, being detected in 9 of 29 hospitals surveyed, with an overall prevalence rate of 11.9% among imipenem-resistant *P. aeruginosa* isolates. As reported here, bla_{VIM-2} has also spread to *P. putida* in a Korean hospital.

It is striking that the metallo-β-lactamase found in Korea is not the IMP-1 type which is scattered in Japan, but the VIM type, which was previously reported only from Europe (8, 17). Moreover, although VIM-1 and VIM-2 β-lactamase-producing *P. aeruginosa* isolates were collected in 1996 and 1997 in Europe, it is now clear that VIM-2 producers have existed in Korea since 1995 and have become much more widespread than has been observed elsewhere. Most of the present bla_{VIM-2}-positive isolates were *P. aeruginosa*, but nine were *P. putida.* The *P. aeruginosa* isolates were mostly from urines and sputa, indicating that these were important sources of the spread. PFGE of *Xba*I-digested genomic DNA showed that many of these organisms clustered to patterns C2 and E1. Serotyping showed that all members of the former group tested were serotype O:12, whereas those of the latter were either serotype O:11 or serotype OPA. These data imply a major role for clonal spread. It is interesting that VIM-producing *P. aeruginosa* isolates reported in Greece (25) were also serotype O:12, which is otherwise a rare type. The occurrence of bla_{VIM-2} in different PFGE and serotypes, as well as in two different *Pseudomonas* species in the present study, indicated horizontal spread, too. Only two of nine *P. putida* isolates shared an identical PFGE profile, suggesting a much greater role for horizontal spread in the latter species (data not shown). Moreover, an ongoing study has revealed the presence of bla_{VIM-2} in *Acinetobacter* and *Serratia* spp. from Korea.

In earlier studies, bla_{IMP} has been found to be located on conjugative plasmids (26), but bla_{VIM-2} has not previously been found on self-transferable elements (17), while the bla_{VIM-1} gene was located on the chromosome in the sole producer studied (8). Initial attempts, using an *E. coli* recipient, to transfer bla_{VIM-2} from the present isolates were unsuccessful. Nevertheless, we were able to transfer the resistance from six *P. aeruginosa* and two *P. putida* isolates by using *P. aeruginosa* PAO4089Rp as a recipient. Repeated attempts to detect bla_{VIM} on plasmids were unsuccessful with both the isolates and the transconjugants. Rather, fragments of genomic DNA separated by PFGE hybridized with a gene probe for bla_{VIM-2}, suggesting a chromosomal location. We cannot, however, ex-

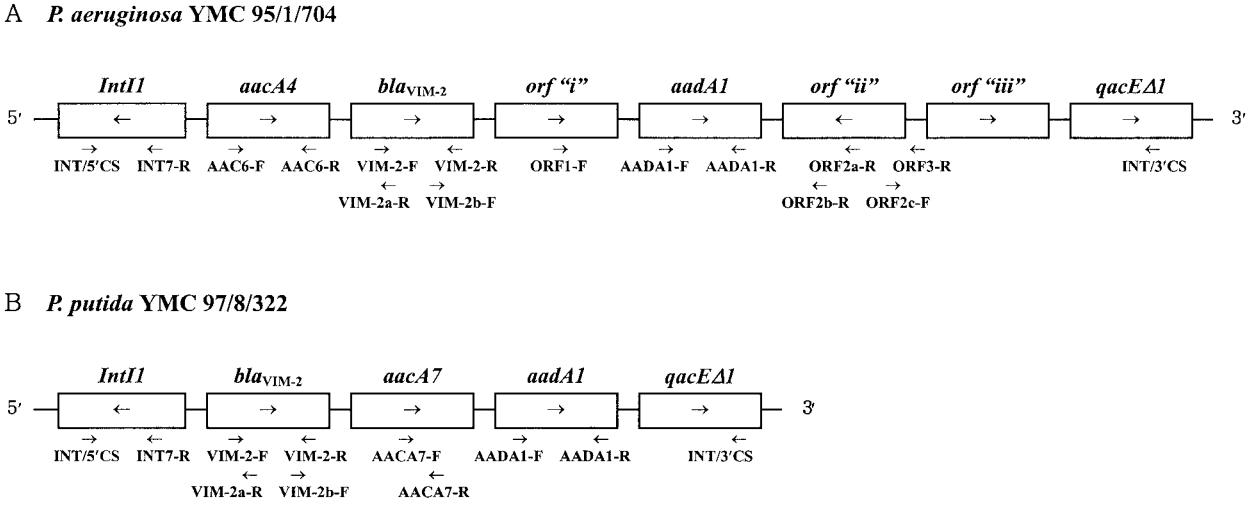

FIG. 2. Schematic structure (not to scale) of integrons in isolates of *P. aeruginosa* YMC 95/1/704 and *P. putida* YMC 97/8/322, which had approximate sizes of 5.6 and 3 kb, respectively. The ORFs are boxed. The names of unknown *orfs* "*i*," "*ii*," and "*iii*" are arbitrary. Arrows indicate their transcriptional orientation. *orf* "*ii*" had a reversed orientation. Primers used for sequencing are shown under the integron structures (see text for primer sequences).

clude possible carriage by a large plasmid that associated strongly with the chromosome. MICs of imipenem for the present isolates of *P. aeruginosa* ranged from 8 to >128 μg/ml, with an MIC_{50} of 16 μg/ml. Variations in the level of resistance may depend on the amount of VIM enzyme or on the permeability of the isolates. Not all VIM-2-producing isolates therefore would be categorized as imipenem resistant when using the NCCLS resistance breakpoint of ≥16 μg/ml. This situation recapitulates that seen with some extended-spectrum β-lactamase-producing isolates of *E. coli* and *Klebsiella pneumoniae*, which appear susceptible to extended-spectrum cephalosporins at NCCLS breakpoints (12).

Most of our VIM-2-producing *P. aeruginosa* isolates were also resistant to most aminoglycosides and ciprofloxacin, creating a serious problem for the choice of therapy. However, carbapenemase-producing *P. aeruginosa* isolates are susceptible to aztreonam because there is poor hydrolysis (18).

It was reported elsewhere that the pI of VIM-2 β-lactamase was 5.6 rather than 5.3 (17); in our experience, however, it was difficult to obtain sharp metallo-β-lactamase bands from cell sonicates as reported also for *Stenotrophomonas maltophilia* (15) and repeated analysis found that the pI of VIM-2 was indistinguishable from that of the VIM-1 control, which was run in parallel.

Our initial attempts directly to sequence the PCR products of integrons were unsuccessful due to the presence of another integron. Therefore, we used cloned strains. The nucleotide sequences of the bla_{VIM-2} alleles from our *P. aeruginosa* and *P. putida* strains were identical to each other and to that of the original VIM-2 producer, *P. aeruginosa* COL-1 (17). The structure of the integron in our *P. aeruginosa* strain was different from that in strain COL-1, which contained only the VIM-2 cassette (17), and was more similar to those in strains RON-1 and RON-2 (16), which also contained other cassettes besides bla_{VIM-2}. However, the first cassette at the 5′ end of the integron from our *P. aeruginosa* strain was different from previously described *aacA7* (strain RON-1) or *aacA29a* (RON-2) (16) in that it was a variant of *aacA4*, conferring resistance to gentamicin, tobramycin, and kanamycin, but not to amikacin. Also, downstream of the bla_{VIM-2}, *aadA1* was present rather than *aacC1* and *aacA4* (strain RON-1) or *aacA29b* (RON-2). Furthermore, unknown *orf* "*i*," *orf* "*ii*," and *orf* "*iii*" occupied a large space between bla_{VIM-2} and *qacEΔ1*. Strangely, repeated sequencing showed that the unknown *orf* "*ii*" lay in a reversed orientation, which would preclude its expression under the single promoter of the integron structure. The integron from the *P. putida* strain was different in that the first cassette was bla_{VIM-2} and that *aacA7* was present along with *aadA1* and *qacEΔ1*.

In conclusion, the metallo-β-lactamase produced by these *P. aeruginosa* isolates, collected in Korea since 1995, was VIM-2 and this enzyme and its contingent resistance have also reached *P. putida.* This is the first report of VIM-2 β-lactamase outside Europe and the first report for *P. putida* worldwide. The bla_{VIM-2} gene resided on two novel class 1 integrons. The persistence and spread of VIM-2-mediated resistance reflected clonal as well as horizontal spread. Stricter measures are required to control its further spread in Korea and elsewhere.

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REFERENCES

1. **Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, and M. Ohta.** 1995. A novel integron-like element carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **39:** 1612–1615.
2. **Chong, Y., K. Lee, R. Okamoto, and M. Inoue.** 1997. Characteristics of extended-spectrum β-lactam hydrolyzing activity of *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from clinical specimens. Korean J. Infect. Dis. **29:**477–485.
3. **Chu, Y.-W., M. Afzal-Shah, E. T. S. Houang, M.-F. I. Palepou, D. J. Lyon, N. Woodford, and D. Livermore.** 2001. IMP-4, a novel metallo-β-lactamase

from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. Antimicrob. Agents Chemother. **45:**710–714.

4. **Cornaglia, G., A. Mazzariol, L. Lauretti, G. M. Rossolini, and R. Fontana.** 2000. Hospital outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing VIM-1, a novel transferable metallo-β-lactamase. Clin. Infect. Dis. **31:**1119–1125.
5. **Hirakata, Y., K. Izumikawa, T. Yamaguchi, H. Takemura, H. Tanaka, R. Yoshida, J. Matsuda, M. Nakano, K. Tomono, S. Maesaki, M. Kaku, Y. Yamada, S. Kamihara, and S. Kohno.** 1998. Rapid detection and evaluation of clinical characteristics of emerging multiple-drug-resistant gram-negative rods carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **42:**2006–2011.
6. **Iyobe, S., H. Kusadokoro, J. Ozaki, N. Matsumura, S. Minami, S. Haruta, T. Sawai, and K. O'Hara.** 2000. Amino acid substitutions in a variant of IMP-1 metallo-β-lactamase. Antimicrob. Agents Chemother. **44:**2023–2027.
7. **Kiska, D. L., and P. H. Gilligan.** 1999. Pseudomonas, p. 517–525. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.
8. **Lauretti, L., M. L. Riccio, A. Mazzariol, G. Cornaglia, G. Amicosante, R. Fontana, and G. M. Rossolini.** 1999. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **43:**1584–1590.
9. **Lee, K., Y. Chong, H. B. Shin, Y. A. Kim, D. Yong, and J. H. Yum.** 2001. Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. Clin. Microbiol. Infect. **7:**88–91.
10. **Levesque, C., and P. H. Roy.** 1993. PCR analysis of integrons, p. 590–594. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
11. **Livermore, D. M., and J. D. Williams.** 1996. β-Lactams: mode of action and mechanisms of bacterial resistance, p. 502–578. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, Md.
12. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
13. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for antimicrobial disk susceptibility tests, 6th ed., M2-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
14. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo-β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
15. **Payne, D. J., R. Cramp, J. H. Bateson, J. Neale, and D. Knowles.** 1994. Rapid identification of metallo- and serine β-lactamases. Antimicrob. Agents Chemother. **38:**991–996.
16. **Poirel, L., T. Lambert, S. Tuerkoglue, E. Ronco, J.-L. Gaillard, and P. Nordmann.** 2001. Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the bla_{VIM-2} carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. Antimicrob. Agents Chemother. **45:**546–552.
17. **Poirel, L., T. Naas, D. Nicolas, L. Collet, S. Bellais, J.-D. Cavallo, and P. Nordmann.** 2000. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. Antimicrob. Agents Chemother. **44:**891–897.
18. **Rasmussen, B., and K. Bush.** 1997. Carbapenem-hydrolyzing β-lactamases. Antimicrob. Agents Chemother. **41:**223–232.
19. **Riccio, M. L., N. Franceschini, L. Boschi, B. Caravelli, G. Cornaglia, R. Fontana, G. Amicosante, and G. M. Rossolini.** 2000. Characterization of the metallo-β-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of bla_{IMP} allelic variants carried by gene cassettes of different phylogeny. Antimicrob. Agents Chemother. **44:**1229–1235.
20. **Rice, L. B., and R. A. Bonomo.** 1996. Genetic and biochemical mechanisms of bacterial resistance to antimicrobial agents. p. 453–501. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, Md.
21. **Sambrook, J., and D. W. Russell (ed.).** 2001. Molecular cloning, a laboratory manual, 3rd ed., p. 1.32. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning, a laboratory manual, 3rd ed., p. 15.14. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Takahashi, A., S. Yomoda, I. Kobayashi, T. Okubo, M. Tsunoda, and S. Iyobe.** 2000. Detection of carbapenemase-producing *Acinetobacter baumannii* in a hospital. J. Clin. Microbiol. **38:**526–529.
24. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
25. **Tsakris, A., S. Pournaras, N. Woodford, M.-F. I. Palepou, G. S. Babini, J. Douboyas, and D. M. Livermore.** 2000. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. J. Clin. Microbiol. **38:**1290–1292.
26. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**147–151.