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Analysis of a Novel Class 1 Integron Containing Metallo-β-Lactamase Gene VIM-2 in *Pseudomonas aeruginosa*

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Carbapenems such as imipenem are stable to most β-lactamases. Recently, increased numbers of carbapenemase producing Gram-negative bacterial strains have been isolated because of the increased use of cabapenems. In this respect, control of these infectious carbapenemase producing Gram-negative bacteria and understanding their resistance mechanism are becoming more important. These carbapenem-hydrolyzing β-lactamase genes have been reported to exist mostly as gene cassettes in an integron. This implies that antibiotic resistance genes may be transferred to other bacteria via the integron. In the present study, we identified and analyzed an integron containing VIM-2 type metallo-β-lactamase gene in a carbapenemase producing *Pseudomonas aeruginosa*. In addition, the possibility of resistance spread by integron located in a plasmid was tested. Among glucose non-fermenting Gram-negative bacilli with reduced imipenem susceptibility (MIC≥8 μg/ml) isolated from Korean patients, *P. aeruginosa* 1082 showed resistance to most β-lactams, cephalosporin, and aminoglycoside. We found that *P. aeruginosa* 1082 was inhibited by EDTA in EDTA double disk synergy test which means that this strain produces metallo-β-lactamase. Class 1 integron containing *bla*$_{VIM-2}$ (carbapenem resistance gene), *qacF (*quaternary ammonium compound resistance gene), *aacA4* (aminoglycoside resistance gene), *catB3* (chloramphenicol resistance gene), *bla*$_{OXA-30}$ (extended-spectrum β-lactam resistance gene), and *aadA1* (aminoglycoside resistance gene) gene cassettes was detected in *P. aeruginosa* 1082. The size of the integron was 5,246 bp and the structure and arrangement of the integron was a novel one in comparison with other integrons found in other *P. aeruginosa.* The integron could be transferred to *Escherichia coli* JM109 from *P. aeruginosa* 1082 possibly via self-transferable plasmid DNA. The integron and a *bla*$_{VIM-2}$ gene were detected in the plasmid DNA of the transconjugants whose imipenem resistance was slightly increased as a result of accepting the integron from the donor strain.

***Keywords*:** *bla*$_{VIM-2}$, Class 1 integron, *P. aeruginosa*

Pseudomonas aeruginosa is mostly a nosocomial pathogen that is becoming increasingly multidrug resistant (Bonomo and Szabo, 2006). *Pseudomonas* spp. are frequently isolated from clinical specimens and thought to be one of the most antibiotic-resistant infectious microorganisms. Several antimicrobial agents, however, are available for the treatment of infectious diseases caused by these microorganisms (Cardoso *et al.*, 2008). Because the prevalent use of antibiotics has broadened the spectrum of antibiotic resistance of *P. aeruginosa*, the need of more powerful therapeutic agents for the treatment of theses multidrug-resistant microorganisms is becoming more urgent. Even though carbapenems are routinely used among other bacterial strains for the effective treatment of these strains, the emergence of strains with acquired carbapenemases, particularly Ambler class B metallo-β-lactamases (MBLs), has made the treatment using carbapenems ineffective (Walsh *et al.*, 2005).

There are two major groups of MBLs, IMP, and VIM, and they share only 30 to 40% amino acid homology. The genes encoding IMP and VIM are referred as *bla*$_{VIM}$ and *bla*$_{IMP}$, respectively. These genes are usually carried as mobile gene cassettes inserted in integrons which are sometimes located in plasmids along with other resistance determinants such as aminoglycoside modifying enzymes (Arakawa *et al*., 1995; Lauretti *et al*., 1999; Juan *et al.*, 2008). The *bla*$_{IMP-1}$ gene is located in plasmids or on the chromosome while *bla*$_{VIM-2}$ is located mainly in plasmids (Poirel *et al.*, 2000). The fact that the involved integrons are frequently located in plasmids or transposons certainly contributes to the global dissemination of these resistance genes (Arakawa *et al.*, 1995; Jones *et al.*, 1997; Fluit and Schmitz, 1999; Lauretti *et al.*, 1999; Poirel *et al.*, 2001). In fact, several outbreaks by MBL-producing *P. aeruginosa* strains have been described worldwide (Senda *et al.*, 1996; Mendes *et al.*, 2004; Lagatolla *et al.*, 2006; Mendes *et al.*, 2007; Pena *et al.*, 2007). Most of these integrons belong to class 1, but their structures vary greatly (Lee *et al.*, 2002). Since MBLs need Zn^{2+} binding in their catalytic domain to hydrolyze the β-lactam ring, they are inhibited by chelating agents such as EDTA (Nordmann and Poirel, 2002; Prats *et al.*, 2002).

Integrons are genetic structures capable of capturing gene cassettes. Class 1 integrons, which are most commonly isolated integrons from antibiotic-resistant clinical isolates, possess two conserved segments (5'-CS and 3'-CS) located

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Table 1. Primers used in this work

Primer name	Sequence (5'→3')	Use
INT/5'CS	CTTCTAGAAAACCGAGGATGC	Amplification of Class 1 integron
INT/3'CS	CTCTCTAGATTTTAATGCGGATG	Amplification of Class 1 integron
VIM-2F'	ATGTTCAAACTTTTGAGTAAG	Amplification and sequencing of bla_{VIM-2}
VIM-2R'	CTACTCAACGACTGAGCG	Amplification and sequencing of bla_{VIM-2}

on either side of the integrated genes (Recchia and Hall, 1995). 5'-CS supplies the integrase gene (*intI1*), the integration site (*attI1*) and a strong promoter that ensures expression of the integrated gene cassettes. 3'-CS carries additional antimicrobial resistance genes, such as *qacEΔ1* (encoding low-level resistance to quaternary ammonium compounds) and *sul1* (encoding resistance to sulphonamides) (Rodriguez *et al.*, 2008). Gene cassettes are discrete mobile units comprising a gene, usually an antibiotic resistance gene, and an integrase-specific recombination site that is a member of a family of sites known as 59 base elements (Recchia and Hall, 1995). Cassettes can exist either free as a circularized form or integrated at the *attI1* site as a result of a single site-specific recombination event between the *attI1* site in an integron and a 59 base element in a cassette catalyzed by the integrase (Stokes *et al.*, 1997).

In this study, we analyzed the genetic structure of a class 1 integron containing VIM-2 type metallo-β-lactamase gene in *P. aeruginosa* 1082 isolated from Korean patients and compared it with other known integrons. The possibility of resistance spread by the integron located in a transferable plasmid was also tested.

Materials and Methods

Bacterial strains and antimicrobial susceptibility test

P. aeruginosa 1082 was isolated from the catheterized urine of patients in a tertiary care hospital in Korea. The species was identified by the Vitek system (bioMerieux, USA), and confirmed to be *P. aeruginosa* by 16S rDNA sequencing. Susceptibility tests of *P. aeruginosa* 1082 against various antimicrobial agents (ampicillin, piperacillin, cefazolin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamycin, tobramycin, and amikacin) were performed by the disk diffusion methods (Gaydos and Harrington, 1982). Minimal Inhibitory Concentrations (MICs) of the same antibiotics against *P. aeruginosa* 1082 were determined by the use of microbroth dilution method (NCCLS, 2000). Production of metallo-β-lactamase was screened by EDTA-disk synergy tests (Lee *et al.*, 2001).

Polymerase chain reaction for class 1 integron and bla_{VIM-2}

Following plasmid DNA extraction, the presence of a class 1 integron and bla_{VIM-2} was tested by PCR amplification using specific primers (Lauretti *et al.*, 1999) (Table 1). PCR for a class 1 integron was carried out using 1 μl of plasmid DNA, 10 pmol of each primer, 0.25 mM/L of dNTP's, 3.75 mM of $MgCl_2$, and 1.25 U of i-maxII *Taq* polymerase (Intron, Korea) in a total volume of 20 μl. PCR reactions were subjected to an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58.5°C and 3.5 min of extension at 72°C followed by a final extension step at 72°C for 10 min. PCR of bla_{VIM-2} was performed using 3 μl of plasmid DNA, 10 pmol of each primer, 0.5 mM/L of dNTP's, 7.5 mM of $MgCl_2$, and 2.5 U of i-maxII *Taq* polymerase (Intron) in a total volume of 50 μl. PCR reactions were subjected to an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 45 sec of denaturation at 94°C, 40 sec of annealing at 57°C and 1 min of extension at 72°C followed by a final extension step at 72°C for 7 min. Amplified PCR products were sequenced on both strands using the primers used for the PCR (Macrogen, Korea).

Analysis of class 1 integron in *P. aeruginosa* 1082

The PCR amplicon of class 1 integron in *P. aeruginosa* 1082 was inserted into pCR2.1-TOPO vector (Invitrogen, USA) and transformed into *Escherichia coli* TOP10. The transformants were screened on LB agar containing 50 μg/ml of ampicillin and X-Gal. Plasmids from cloned strains were used for full sequencing of the class 1 integron (Macrogen, Korea). Full sequencing was performed by using a primer walking method. Resulting sequences were then compared with those available at GenBank (www.ncbi.nih.gov/BLAST). Multiple sequence alignments were performed using the program CLUSTAL X 1.81.

Conjugation

Transfer of resistance genes in a plasmid from rifampin-sensitive *P. aeruginosa* 1082 to rifampin-resistant *E. coli* JM109 was attempted by a liquid conjugation assay at 37°C (Poirel *et al.*, 1999). Transconjugant selection was performed on Mueller-Hinton agar plates containing rifampin (100 μg/ml) and ampicillin (100 μg/ml).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been registered in the GenBank nucleotide sequence database under the following accession numbers: for the identification of *P. aeruginosa* 1082, DQ294293; for the integron in *P. aeruginosa* 1082, DQ287356.

Results

Characterization of *P. aeruginosa* 1082 and its Metallo-β-lactamase production

P. aeruginosa 1082 was isolated from the catheterized urine of patients in a Korean hospital. It was indentified based on chemical reactions using the ViTek system (Table 2) and was verified by 16S rDNA nucleotide sequence (Please refer to GenBank DQ294293, data not shown). The result of

Table 2. Identification of *P. aeruginosa* by biochemical assays using the Vitek system

Biochemical reaction	Results
Oxidase	Positive
Glucose fermentation	Positive
Glucose oxidation	Positive
Acetamide	Positive
Esculin	Negative
Plant indican	Negative
Urease	Positive
Malonate	Positive
Polymyxin B	Negative
Adonitol	Negative
ONPG fermentation	Negative
L-Arabinose	Negative
Arginine decarboxylase	Positive
Lysine decarboxylase	Negative
Ornithine decarboxylase	Negative
TSI	Positive
Lactose	Negative
Citrate	Positive
Tryptophan	Negative
Inositol	Negative
HS2	Negative
Rhamnose	Negative

EDTA-disk synergy test was positive. Imipenem could not inhibit the growth of *P. aeruginosa* 1082 indicating the production of carbapenemase by this strain. The activity of carbapenemase was, however, blocked by metal chelating EDTA resulting in growth inhibition between the imipenem disk and the EDTA disk. This result strongly suggests the production of metallo-β-lactamase by *P. aeruginosa* 1082 (Fig. 1). MICs by microbroth dilution test also revealed that *P. aeruginosa* 1082 was resistant to all the antimicrobial agents tested including the carbapenems: ampicillin (>256 μg/ml), piperacillin (>128 μg/ml), cefazolin (>256 μg/ml), cefotaxime (>128 μg/ml), ceftazidime (64 μg/ml), cefepime (>64 μg/ml), azteronam (32 μg/ml), imipenem (256 μg/ml), meropenem (128 μg/ml), gentamicin (>64 μg/ml), tobramycin (>64 μg/ml), and amikacin (>64 μg/ml) (Table 3). This strain is multidrug resistant especially carbapenem resistant.

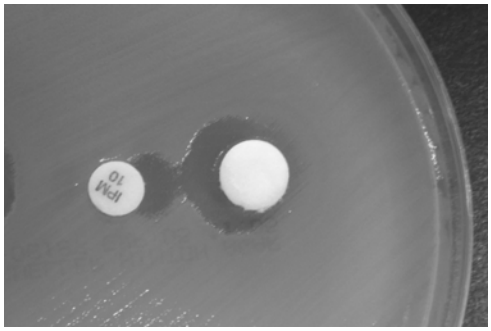

Fig. 1. EDTA-disk synergy test. Disk on the left : imipenem (10 μg), disk on the right : EDTA (10 μl of 0.5 M)

Analysis of Class 1 integron in *P. aeruginosa* 1082

The amplicons of the class 1 integron and bla_{VIM-2} in a plasmid from *P. aeruginosa* 1082 was detected by PCR with specific primers (Fig. 2). We cloned these PCR products into pCR2.1-TOPO vector (Invitrogen) and transformed into *E. coli* TOP10. Plasmid DNAs were extracted from the obtained transformants and subjected to the nucleotide sequence analysis. The nucleotide sequence analysis of the 5,246 bp PCR product revealed that this integron belonged to class 1 according to the nucleotide sequence homology of integrase genes, and it contained four gene cassettes known to be responsible for antibiotic resistances (Please refer to Gen-

Table 3. MICs of β-lactams and aminoglycosides against *P. aeruginosa* 1082 and MIC interpretive standards for *P. aeruginosa*

Antibiotics	MICs (μg/ml)			
	P. aeruginosa 1082	NCCLS[a] Interpretive standards		
		Susceptible	Intermediate	Resistant
Ampicillin[b]	>256	NA	NA	NA
Piperacillin	>128	≤64	NA	≥128
Cefazolin[b]	>256	NA	NA	NA
Cefotaxime	>128	≤8	16~32	≥64
Ceftazidime	64	≤8	16	≥32
Cefepime	>64	≤8	16	≥32
Aztreonam	32	≤8	16	≥32
Imipenem	256	≤4	8	≥16
Meropenem	128	≤4	8	≥16
Gentamicin	>64	≤4	8	≥16
Tobramycin	>64	≤4	8	≥16
Amikacin	>64	≤16	32	≥64

[a] National Committee for Clinical Laboratory Standards
[b] Antibiotics without interpretive standard of susceptibility for *P. aeruginosa* in NCCLS guideline
NA, Not applicable

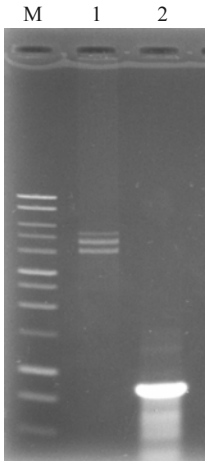

Fig. 2. Amplification of an integron and *bla*$_{VIM-2}$ gene in *P. aeruginosa* 1082. Lanes: M, DNA molecular weight marker (1 kb DNA ladder); 1, class 1 integron (5,246 bp); 2, *bla*$_{VIM-2}$ (801 bp)

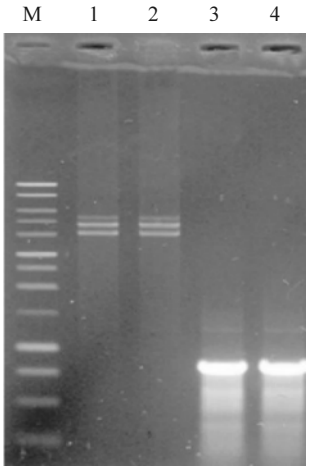

Fig. 4. Amplification of the integron and *bla*$_{VIM-2}$ gene of *P. aeruginosa* 1082 and transconjugant. Lanes: M, DNA molecular weight marker (1 kb DNA ladder); 1, class 1 integron in *P. aeruginosa* 1082; 2, class 1 integron in the transconjugant; 3, *bla*$_{VIM-2}$ in *P. aeruginosa* 1082; 4, *bla*$_{VIM-2}$ in the transconjugant.

Bank accession no. DQ287356). Schematic structure of this integron is depicted in Fig. 3. The sequencing data showed that the 5'-CS element of this integron contained an *intI1* integrase gene with its own promoter region (P_I), 2 putative promoters (P1 and P2), and an *attI1* recombination site. The 3'-CS element contained the *qacEΔ1* at both ends. The gene cassettes of *bla*$_{VIM-2}$ (carbapenem resistance gene), *qacF (*quaternary ammonium compound resistance gene), *aacA4* (aminoglycoside resistance gene), *catB3* (chloramphenicol resistance gene), *bla*$_{OXA-30}$ (extended-spectrum β-lactam resistance gene), and *aadA1* (aminoglycoside resistance gene) were arrayed serially in the middle of the integron structure. All gene cassettes have a core site (GTTRRRY) in 5'-CS end and an inverse core site (RYYYAAC) in 3'-CS end except the last gene cassette *aadA1* which has the deleted core site (Fig. 3). The sequence of *bla*$_{VIM-2}$ was identical to those of *Pseudomonas putida* and *Achromobacter xylosoxidans* reported previously (Lee *et al*., 2002; Shin *et al*., 2005).

Conjugative transfer of resistance

Since most plasmid-mediated MBLs confer resistance to carbapenem, this study was continued by performing conjugation assays using rifampin-resistant *E. coli* JM109 as a recipient strain and Mueller-Hinton agar plates containing ampicillin (100 μg/ml) and rifampin (100 μg/ml) to select transconjugants. The plasmid containing the integron was readily transferred from rifampin-sensitive *P. aeruginosa* 1082 to *E. coli* JM109. Class 1 integron and *bla*$_{VIM-2}$ in transconjugant *E. coli* JM109 were detected by PCR (Fig. 4). Resistance of the transconjugants to the tested antimicrobial agents was lower than that of *P. aeruginosa* 1082, but was higher than that of the original strain (Table 4). Carbapenem resistance also seemed to be transferred from *P. aeruginosa* 1082 to *E. coli* JM109 recipients even though the resistance did not increased substantially. This discrepancy might be due to differences in other factors involved in antibiotic resistance between *P. aeruginosa* and *E. coli.*

Discussion

The VIM-2 enzyme was first described in a strain of *P. aeruginosa* isolated from a blood culture of a woman treated with imipenem in Marseilles, France in 1996 (Poirel *et al*., 2000). In Korea, VIM-2 is the most frequently detected MBL since the first identification of VIM-2 in *P. aeruginosa* isolated in 1995. *P. aeruginosa* isolates with *bla*$_{VIM-2}$ were widespread in Korean hospitals being detected in nine of 29 hospitals surveyed with an overall prevalence rate of 11.9% among the imipenem-resistant *P. aeruginosa* isolates (Lee *et al*., 2002; Shin *et al*., 2005).

In this study, *P. aeruginosa* 1082 identified by ViTek system and 16S rDNA sequencing harboring the *bla*$_{VIM-2}$ gene was isolated from a patient in a hospital and the nucleotide sequence of the gene was registered in GenBank (GenBank

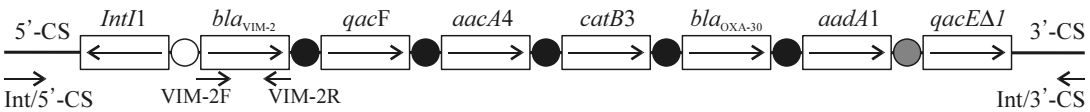

Fig. 3. Schematic structure of the *bla*$_{VIM-2}$ gene cassette containing integron of *P. aeruginosa* 1082. Arrows inside the boxes indicate transcriptional orientation. The *attI*1 recombination site is represented by a white circle, 59 bp elements by black circles, and 59 bp element without core site (GTTRRRY) by a gray circle. Primers used for sequencing are shown under the integron structures (see Table 1 for primer sequences).

Table 4. MICs of β-lactams and aminoglycosides against *P. aeruginosa* 1082, transconjugant, and *E. coli* JM109

	MICs (μg/ml)		
Antibiotics	*P. aeruginosa* 1082	*E. coli* JM109	Transconjugant
Ampicillin	>256	0.25	>256
Piperacillin	>128	1	>128
Cefazolin	>256	≤0.03	128
Cefotaxime	>128	≤0.25	0.5
Ceftazidime	64	0.25	0.5
Cefepime	>64	≤0.03	0.125
Aztreonam	32	0.125	0.125
Imipenem	256	0.06	0.125
Meropenem	128	≤0.06	≤0.06
Gentamicin	>64	0.125	0.25
Tobramycin	>64	0.06	1
Amikacin	>64	0.06	1

accession no. DQ294293). The isolate exhibited synergy with EDTA in the imipenem-EDTA double disk synergy test indicating MBL production and showed relatively high resistance to various antimicrobial agents tested (Table 3). According to integron analysis, *P. aeruginosa* 1082 has a novel arrayed class 1 integron containing bla_{VIM-2} gene (Fig. 5), and the MBL should be expressed at high levels because of the location of the bla_{VIM-2} gene cassette being in the first position in the integron (Fig. 4). This cassette was deposited in GenBank (GenBank accession no. DQ287356). The MBL gene cassette was located upstream of an array of gene cassettes containing *qac*F, *aacA*4, *catB*3, bla_{OXA-30}, and *aad*A1 which was located upstream of *qacE*Δ1 usually found in the 3' region of class 1 integrons.

In earlier studies, bla_{IMP} has been found located on a conjugative plasmid (Watanabe *et al.*, 1991), but bla_{VIM-2} has not previously been found on self-transferable elements (Poirel *et al.*, 2000). Usually, the bla_{VIM-1} gene is located on the chromosome (Lauretti *et al.*, 1999). On the other hand, acquired MBL genes in *P. aeruginosa* are often carried on plasmids and are usually non-transferable by conjugation at least to *E. coli* (Ito *et al.*, 1995; Iyobe *et al.*, 2000). The class 1 integron containing bla_{VIM-2} in our isolate, however, could be transferred to *E. coli* JM109 by conjugation. Con-

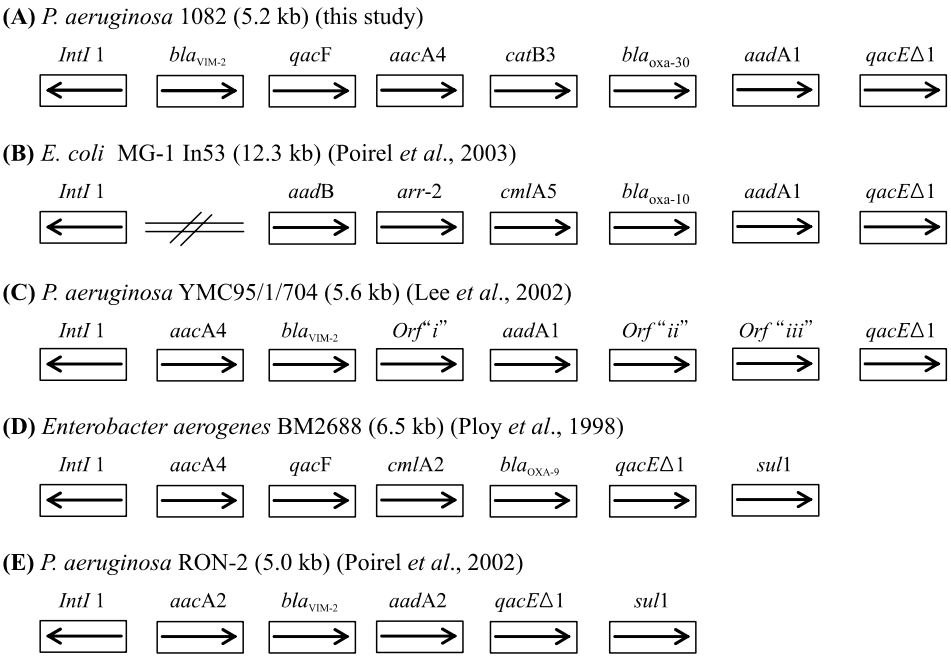

Fig. 5. Compared structures of the class 1 integrons in *P. aeruginosa* 1082 with those of other bacterial integrons. The intI1 integrase gene that encodes an integrase is part of the 5'-CS; the 3'-CS located downstream of the integrated gene cassette(s) includes the sulphonamide resistance gene *sul*1. Inserted gene cassettes are indicated by boxes, with arrows indicating their transcriptional orientation.

jugation experiments were performed as described previously (Lee *et al*., 2002) with rifampin-resistant *E. coli* JM109 as a recipient. PCR of the class 1 integron and bla_{VIM-2} in recipient strains demonstrated that the class 1 integron carrying bla_{VIM-2} was transferred from *P. aeruginosa* 1082 to *E. coli* JM109. This suggests a horizontal spread of the integron itself as previously reported (Yu *et al.*, 2006). The MIC test of the transconjugant showed that the MICs of the recipient is lower than those of *P. aeruginosa* 1082, but it is higher than those of the reference strain *E. coli* JM109. This results suggest that the different levels of resistance to β-lactams between these two strains was probably due to the differences in the expression of other resistance mechanisms (efflux system, cell permeability, and exposure time to antibiotic agents).

Although integrons themselves are not mobile, several class 1 integrons have been found in Tn21 and Tn21-related transposons (Heikkila *et al.*, 1993; Sundstrom *et al.*, 1993; Fluit and Schmitz, 1999; Toleman *et al.*, 2003) which enables the integrons to be transposed. These findings raise the possibility that the class 1 integron described in this study may also be a part of a transposon. This increases the threat of the bla_{VIM-2} gene being disseminated among diverse genera of bacteria.

Acknowledgements

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