

## Class II Transposon-Borne Structure Harboring Metallo- $\beta$ -Lactamase Gene *bla*<sub>VIM-2</sub> in *Pseudomonas putida*

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Received 31 March 2006/Returned for modification 7 May 2006/Accepted 24 May 2006

**A plasmid-encoded class II transposon element was identified in a carbapenem-resistant *Pseudomonas putida* isolate. Tn1332, closely related to Tn1331, harbored the metallo- $\beta$ -lactamase gene *bla*<sub>VIM-2</sub> in addition to four other antibiotic resistance genes, *aacA4*, *aadA1*, *bla*<sub>OXA-9</sub>, and *bla*<sub>TEM-1</sub>, and two novel insertion sequences, *ISPPu17* and *ISPPu18*.**

*Pseudomonas putida* is a gram-negative aerobe rarely involved in human infections and considered, as opposed to *Pseudomonas aeruginosa*, to be a low-grade pathogen (22). Resistance to carbapenems in *P. putida* may be due to metallo- $\beta$ -lactamases (MBLs) such as IMP-1, IMP-12, VIM-1, VIM-2, and VIM-6 (4–7, 9, 14, 16). These MBL-encoding genes are part of gene cassettes located in class 1 integron structures (21).

The *bla*<sub>VIM-2</sub> gene was first identified from a *P. aeruginosa* isolate recovered in 1996 from Marseilles, France (13). Further studies showed that a single *bla*<sub>VIM-2</sub>-positive isolate had disseminated in the hematology unit where it had initially been identified (1). The *bla*<sub>VIM-2</sub> gene cassette was always identified throughout the world as part of class 1 integrons varying in size and structure and is now the most prevalent carbapenemase gene (10, 12, 21).

The aim of our study was to analyze the  $\beta$ -lactamase content of a carbapenem-resistant *P. putida* strain that was isolated from the same hospitalization unit as that mentioned above and that had carbapenemase activity. *P. putida* strain 9335 was isolated in January 2004 from bronchial aspirate and several blood cultures of a 59-year-old immunocompromised patient treated with an imipenem-containing regimen for 5 days. *P. putida* 9335 was identified using the API 32GN system (bioMérieux, Marcy-L'Étoile, France) and confirmed by 16S rRNA gene sequencing. *P. putida* 9335 was resistant to all  $\beta$ -lactams, including meropenem and imipenem (Table 1). It was also resistant to aminoglycosides, fluoroquinolones, sulfonamides, and chloramphenicol and remained susceptible only to colistin and rifampin.

Production of an MBL was revealed by using Etest strips with imipenem and EDTA (AB Biodisk, Solna, Sweden) (20). Whole-cell DNA of *P. putida* 9335 was extracted as described previously (11) and used as a template in PCR, followed by cloning experiments. Cloning of the *bla*<sub>VIM-2</sub> gene into *Escherichia coli* DH10B was performed by using HindIII-restricted genomic DNA of *P. putida* 9335 that was subsequently ligated into HindIII-restricted pBK-CMV phagemid (Stratagene, Amsterdam, The Netherlands), and the recombinant plasmid

p9335H expressing VIM-2 (Table 1) was selected as described previously (11).

The nucleotide sequence of the ca. 14-kb insert of plasmid p9335H was determined. It contained an 11,172-bp-long transposon termed Tn1332, closely related to Tn1331 and belonging to the Tn3 family, that had been identified previously in a *Klebsiella pneumoniae* isolate from Argentina (17). Tn1331 is closely related to Tn3, with an additional 3-kb fragment containing several antibiotic resistance genes, namely, *aacA4*, *aadA1*, and *bla*<sub>OXA-9</sub>, but does not possess any *attII* site (Fig. 1) (3). As reported for Tn1331, the *aadA1* and *bla*<sub>OXA-9</sub> gene cassettes are fused as a single gene cassette that may have arisen as a consequence of a recombination event involving two integrons (15). The first five amino acids of the leader peptide of  $\beta$ -lactamase TEM-1 were fused to the AAC(6')-Ib protein (2), with this fusion likely being the consequence of a 520-bp duplication, including part of *tnpR* and the 5' part of the *bla*<sub>TEM-1</sub> gene, during the genesis of Tn1331 (15, 17, 18). Compared to Tn1331, Tn1332 carried the *bla*<sub>VIM-2</sub> gene cassette that had been inserted between the *aacA4* and *aadA1*

TABLE 1. MICs of  $\beta$ -lactams for *P. putida* 9335, *P. putida* reference strain CIP104063, *E. coli* DH10B harboring recombinant plasmid p9335H, and *E. coli* reference strain DH10B

$\beta$ -Lactam	MIC ( $\mu$ g/ml) for:			
	<i>P. putida</i> 9335	<i>P. putida</i> CIP104063 <sup>b</sup>	<i>E. coli</i> DH10B(p9335H)	<i>E. coli</i> DH10B
Amoxicillin	>512	>512	>512	4
Ticarcillin	>512	>512	>512	4
Piperacillin	128	16	512	1
Piperacillin + TZB <sup>a</sup>	128	16	512	1
Cefuroxime	>512	>512	>512	2
Ceftazidime	256	2	32	0.06
Cefotaxime	>512	>512	64	0.12
Cefepime	128	2	0.25	0.06
Cefoxitin	>512	512	512	4
Moxalactam	>512	>512	>512	0.06
Aztreonam	256	8	0.25	0.12
Imipenem	64	0.5	1	0.06
Meropenem	64	1	2	0.12

<sup>a</sup> TZB, tazobactam at a fixed concentration of 4  $\mu$ g/ml.

<sup>b</sup> Expressing a wild-type  $\beta$ -lactam resistance profile.

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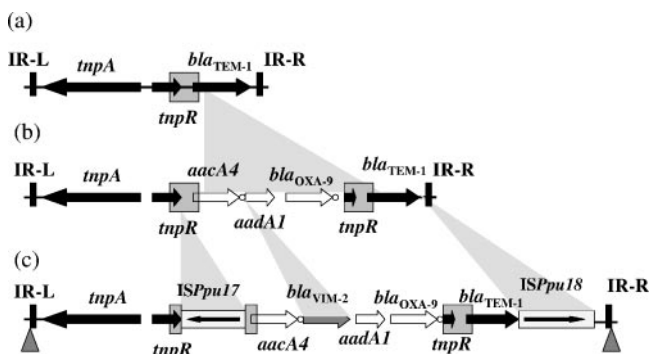


FIG. 1. Structure comparison of Tn3 (a), Tn1331 (20) (b), and Tn1332 (c). The arrows indicate the locations of the genes (*tnpA*, *tnpR*, *aacA4*, *bla<sub>VIM-2</sub>*, *aadA1*, *bla<sub>OXA-9</sub>*, and *bla<sub>TEM-1</sub>*). The white circles indicate 59-bp associated with genes (*aacA4*, *bla<sub>VIM-2</sub>*, and *bla<sub>OXA-9</sub>*). The left terminal inverted repeat (IR-L) and right terminal inverted repeat (IR-R) sequences of transposon Tn1332 are indicated as black vertical rectangles. The gray boxes represent the 520-bp *tnpR* repeat. The *IS<sub>Ppu17</sub>* and *IS<sub>Ppu18</sub>* elements are shown as boxes, with arrows indicating orientations of transcription of their *tnpA* genes. The target site duplication generated by Tn1332 transposition is indicated by gray triangles.

aminoglycoside resistance gene cassettes (Fig. 1). In addition, Tn1332 carried two novel insertion sequences, *IS<sub>Ppu17</sub>* and *IS<sub>Ppu18</sub>* (Fig. 1). *IS<sub>Ppu17</sub>*, an IS30 family member, is 1,066 bp long, and its transposase (321 amino acids) shares 44% identity with that of *IS<sub>Pu1</sub>* from *Photobacterium luminescens*. The inverted repeats (IRs) of *IS<sub>Ppu17</sub>* are 22 bp long, and transposition of *IS<sub>Ppu17</sub>* generated a 3-bp duplication at its insertion site. *IS<sub>Ppu18</sub>*, a member of the IS4 family, is 1,192 bp long and encodes a 326-amino-acid transposase which shares 94% pro-

tein identity with *IS<sub>Pre2</sub>* from *Pseudomonas resinovorans* and with *IS1384* located on a plasmid from *P. putida*. The IRs of *IS<sub>Ppu18</sub>* are 12 bp long, and transposition of *IS<sub>Ppu18</sub>* generated a 4-bp duplication.

Immediately upstream and downstream of the 38-bp-long IRs of Tn1332, a 5-bp duplication was identified that was the signature of the transposition process for *bla<sub>VIM-2</sub>* acquisition. The common promoter sequences present in the 5' conserved region of class 1 integrons and responsible for the expression of gene cassettes were absent (8). Thus, the promoter sequences enhancing *bla<sub>VIM-2</sub>* expression in Tn1332 might be the same as those described for Tn1331 that enhanced *bla<sub>TEM-1</sub>* gene expression, being part of the 520-bp direct repeats upstream of the *aacA4* gene (19). However, the presence of *IS<sub>Ppu17</sub>* in Tn1332 might also be the source of additional promoter sequences involved in expression of the *bla<sub>VIM-2</sub>* gene (Fig. 1).

Analytical isoelectric focusing was performed with  $\beta$ -lactamase extracts of cultures of *P. putida* 9335 and *E. coli* DH10B(p9335H), as described previously (11). Three identical pI values were visualized from both extracts that confirmed that all  $\beta$ -lactamase genes were expressed. Two bands were detected at pI values of 5.4 and 6.9, consistent with the expression of  $\beta$ -lactamases TEM-1 and OXA-9 (2), respectively, whereas a pI value of 5.6 corresponded to expression of VIM-2 (13; data not shown).

A pulsed-field gel electrophoresis analysis was performed as described previously (1), followed by an I-CeuI digestion and a Southern blot hybridization (13) analysis for determination of the precise genetic location of the *bla<sub>VIM-2</sub>* gene in *P. putida* 9335. Chromosomal DNAs from VIM-2-positive *P. aeruginosa* isolate COL-1 (13) and from the *P. putida* CIP104063 refer-

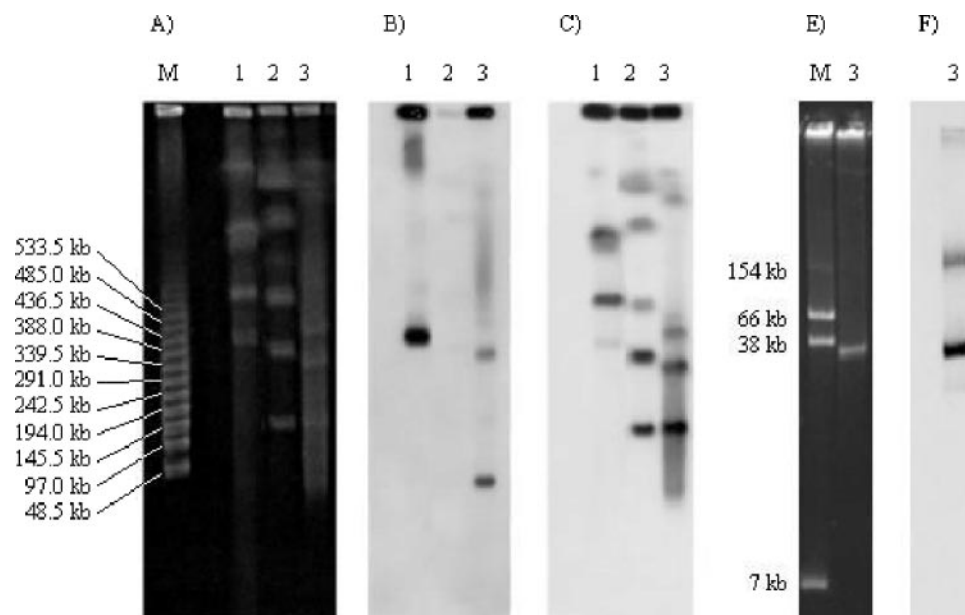


FIG. 2. (A) Pulsed-field gel electrophoresis profiles of I-CeuI digestion of whole-cell DNAs of *Pseudomonas* strains. Lane M, molecular size marker (band sizes are in kilobase pairs); lane 1, *P. aeruginosa* COL-1 (*bla<sub>VIM-2</sub>* positive) (13); lane 2, *P. putida* reference strain CIP104063 (*bla<sub>VIM-2</sub>* negative); lane 3, *P. putida* 9335 (*bla<sub>VIM-2</sub>* positive) (this work). Southern hybridization was performed with a specific internal probe for the *bla<sub>VIM-2</sub>* gene (B) and a probe for the 16S-23S rRNA gene (C). (E) Plasmid analysis. Lane M, *E. coli* 50192 (band sizes are in kb); lane 3, *P. putida* 9335. (F) Results of a Southern hybridization performed with probe for *bla<sub>VIM-2</sub>*.

ence strain were included. The membrane was successively hybridized with probes for *bla*<sub>VIM-2</sub> and 16S and 23S rRNA genes, as described previously (13). A strong hybridization signal was detected with the *bla*<sub>VIM-2</sub>-specific probe that did not cohybridize with the RNA-specific probe (Fig. 2). Plasmid content of the *P. putida* 9335 isolate identified a ca. 30-kb plasmid by a positive signal with the *bla*<sub>VIM-2</sub> probe after Southern blot hybridization (Fig. 2). However, attempts to transfer the  $\beta$ -lactam resistance marker into *E. coli* DH10B and *P. aeruginosa* PU21 by electroporation failed (13).

We report here another transposon structure likely at the origin of *bla*<sub>VIM-2</sub> acquisition that did not directly involve an integron structure. Surprisingly, whereas transfer of an identical *bla*<sub>VIM-2</sub>-carrying genetic structure from *P. aeruginosa* to *P. putida* could have been expected, it was not the case here. These findings underline the important genetic plasticity at the origin of acquisition and dissemination of MBL genes.

**Nucleotide sequence accession number.** The entire nucleotide sequence of TnI332 reported in this work has been assigned to the GenBank and EMBL databases under the accession number DQ174113. The two insertion sequences identified, IS*Ppu*17 and IS*Ppu*18, have been registered in the IS database (<http://www-is.biotoul.fr/>).

This work was funded by a grant from the Ministère de l'Éducation Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, France, and mostly by the European Community 6th PCRD, LSHM-CT-2005-018705. L.P. is a researcher from the INSERM, Paris, France.

#### REFERENCES

- Aubron, C., L. Poirel, N. Fortineau, L. Collet, and P. Nordmann. 2005. Nosocomial spread of *Pseudomonas aeruginosa* isolates expressing the metallo- $\beta$ -lactamase VIM-2 in an haematology unit of a French hospital. *Microb. Drug Resist.* **11**:254–259.
- Bojorquez, D., M. Belei, S. F. Delira, S. Sholly, J. Mead, and M. E. Tolmasey. 1998. Characterization of OXA-9, a  $\beta$ -lactamase encoded by the multiresistance transposon TnI331. *Cell. Mol. Biol. (Noisy-Le-grand)* **44**:483–491.
- Dery, K. J., B. Soballe, M. S. Witherspoon, D. Bui, R. Koch, D. J. Sherratt, and M. E. Tolmasey. 2003. The aminoglycoside 6'-N-acetyltransferase type Ib encoded by TnI331 is evenly distributed within the cell's cytoplasm. *Antimicrob. Agents Chemother.* **47**:2897–2902.
- Docquier, J. D., M. L. Riccio, C. Mugnaioli, F. Luzzaro, A. Endimiani, A. Toniolo, G. Amicosante, and G. M. Rossolini. 2003. IMP-12, a new plasmid-encoded metallo- $\beta$ -lactamase from a *Pseudomonas putida* clinical isolate. *Antimicrob. Agents Chemother.* **47**:1522–1528.
- Fiett, J., A. Baraniak, A. Mrówka, M. Fleischer, Z. Drulis-Kawa, L. Naumiuk, A. Samet, W. Hryniewicz, and M. Gniadkowski. 2006. Molecular epidemiology of acquired-metallo- $\beta$ -lactamase-producing bacteria in Poland. *Antimicrob. Agents Chemother.* **50**:880–886.
- Koh, T. H., G. C. Wang, and L.-H. Sng. 2004. IMP-1 and a novel metallo- $\beta$ -lactamase, VIM-6, in fluorescent pseudomonads isolated in Singapore. *Antimicrob. Agents Chemother.* **48**:2334–2336.
- Lee, K., J. B. Lim, J. H. Yum, D. Yong, Y. Chong, J. M. Kim, and D. M. Livermore. 2002. *bla*<sub>VIM-2</sub> cassette-containing novel integrons in metallo- $\beta$ -lactamase-producing *Pseudomonas aeruginosa* and *Pseudomonas putida* isolates disseminated in a Korean hospital. *Antimicrob. Agents Chemother.* **46**:1053–1058.
- Lévesque, C., L. Piche, C. Larose, and P. H. Roy. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob. Agents Chemother.* **39**:185–191.
- Lombardi, G., F. Luzzaro, J. D. Docquier, M. L. Riccio, M. Perilli, A. Coli, G. Amicosante, G. M. Rossolini, and A. Toniolo. 2002. Nosocomial infections caused by multidrug-resistant isolates of *Pseudomonas putida* producing VIM-1 metallo- $\beta$ -lactamase. *J. Clin. Microbiol.* **40**:4051–4055.
- Pallecchi, L., M. L. Riccio, J. D. Docquier, R. Fontana, and G. M. Rossolini. 2001. Molecular heterogeneity of *bla*<sub>VIM-2</sub>-containing integrons from *Pseudomonas aeruginosa* plasmids encoding the VIM-2 metallo- $\beta$ -lactamase. *FEMS Microbiol. Lett.* **195**:145–150.
- Poirel, L., M. Guibert, D. Girlich, T. Naas, and P. Nordmann. 1999. Cloning, sequence analyses, expression, and distribution of *ampC-ampR* from *Morganella morganii* clinical isolates. *Antimicrob. Agents Chemother.* **43**:769–776.
- Poirel, L., T. Lambert, S. Türkoglu, E. Ronco, J.-L. Gaillard, and P. Nordmann. 2001. Characterization of class 1 integrons from *Pseudomonas aeruginosa* that contain the *bla*<sub>VIM-2</sub> carbapenem-hydrolyzing  $\beta$ -lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob. Agents Chemother.* **45**:546–552.
- 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- $\beta$ -lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob. Agents Chemother.* **44**:891–897.
- Riccio, M. L., L. Pallecchi, J. D. Docquier, S. Cresti, M. R. Catania, L. Pagani, C. Lagatolla, G. Cornaglia, R. Fontana, and G. M. Rossolini. 2005. Clonal relatedness and conserved integron structures in epidemiologically unrelated *Pseudomonas aeruginosa* strains producing the VIM-1 metallo- $\beta$ -lactamase from different Italian hospitals. *Antimicrob. Agents Chemother.* **49**:104–110.
- Sarno, R., G. McGillivray, D. J. Sherratt, L. A. Actis, and M. E. Tolmasey. 2002. Complete nucleotide sequence of *Klebsiella pneumoniae* multiresistance plasmid pJHCMW1. *Antimicrob. Agents Chemother.* **46**:3422–3427.
- Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa. 2003. PCR typing of genetic determinants for metallo- $\beta$ -lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *J. Clin. Microbiol.* **41**:5407–5413.
- Tolmasey, M. E. 1990. Sequencing and expression of *aadA*, *bla*, and *tnpR* from the multiresistance transposon TnI331. *Plasmid* **24**:218–226.
- Tolmasey, M. E. 2000. Bacterial resistance to aminoglycosides and  $\beta$ -lactams: the TnI331 transposon paradigm. *Front. Biosci.* **5**:D20–D29.
- Tolmasey, M. E., and J. H. Crosa. 1993. Genetic organization of antibiotic resistance genes (*aac(6')-Ib*, *aadA*, and *oxa9*) in the multiresistance transposon TnI331. *Plasmid* **29**:31–40.
- Walsh, T. R., A. Bolmström, A. Qwarnström, and A. Gales. 2002. Evaluation of a new Etest for detecting metallo- $\beta$ -lactamases in routine clinical testing. *J. Clin. Microbiol.* **40**:2755–2759.
- Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann. 2005. Metallo- $\beta$ -lactamases: the quiet before the storm? *Clin. Microbiol. Rev.* **18**:306–325.
- Yang, C. H., T. Young, M. Y. Peng, and M. C. Weng. 1996. Clinical spectrum of *Pseudomonas putida* infection. *J. Formos. Med. Assoc.* **95**:754–761.