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bla_{IMP-9} and Its Association with Large Plasmids Carried by *Pseudomonas aeruginosa* Isolates from the People's Republic of China

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A novel plasmid-mediated metallo-β-lactamase (IMP-9) is described in seven isolates of *Pseudomonas aeruginosa* from Guangzhou, China, isolated in 2000. The gene was carried on a large (~450-kb) IncP-2 conjugative plasmid. This is the first report of carriage of bla_{IMP} genes on such large plasmids.

Acquired β-lactamase genes in *Pseudomonas aeruginosa* are often associated with transposons and integrons and carried on R plasmids, which can be classified into 13 incompatibility groups (P1 to 13) with a wide range of sizes (8 to 483 kb). The IncP2 plasmids are described as particularly common in *P. aeruginosa* (50% of the transmissible plasmids), are very large, and are distributed worldwide (13).

Carriage of metallo-β-lactamase (MBL) genes of the bla_{IMP} or bla_{VIM} type has been reported infrequently on plasmids in *P. aeruginosa*. A limited number of $bla_{IMP/VIM}$ genes, such as bla_{IMP-1}, $_{-3}$, $_{-10}$, and $_{-12}$ and bla_{VIM-2}, have been reported on plasmids with a size range of 31 to 56 kb in *P. aeruginosa* or *P. putida* (5, 10, 11, 15, 20, 24). Some studies have failed to find plasmids in carbapenem-resistant *P. aeruginosa* even though the resistance marker was transferred by conjugation (16).

The carbapenem resistance rates in *P. aeruginosa* isolates in the city of Guangzhou, China, have been reported to be 16 to 18% during the period 1998 to 2001, according to a multicenter surveillance of antibiotic resistance in nosocomial isolates from that area (25).

In this report, we describe the identification of a new variant of the IMP-type plasmid-mediated MBL gene in carbapenem-resistant isolates of *P. aeruginosa* from that area.

In the year 2000, a total of 301 clinically significant and nonduplicated *P. aeruginosa* isolates were collected from the 12 participating centers, and 54 isolates from 11 centers were

TABLE 1. Bacterial strains used in this study

Strain (characteristic[s])	Application	Reference or source
P. aeruginosa 101/1477 (IMP-1 producer)	Positive control	D. M. Livermore
P. aeruginosa NCTC 50814 (*met lys his* Rif)	Recipient	NCTC[a]
E. coli UB1637/R (*his lys trp* Rif)	Recipient	4
P. aeruginosa ATCC 27853	Quality control	ATCC[b]
E. coli ATCC 25922	Quality control	ATCC
Agrobacterium tumefaciens C58(pAtC58 [543 kb]/pTiC58 [214 kb])	Plasmid size	6
Rhizobium leguminosarum 3841 (147, 151, 350, 488, 684, and 870 kb)	Plasmid size	http://www.sanger.ac.uk/Projects/Microbes/
Rhizobium leguminosarum VF39 (150, 220, 400, 500, 700, and 900 kb)	Plasmid size	9
Rhizobium leguminosarum LRS39401 (150, 220, 400, 700, and 900 kb)	Plasmid size	9
P. aeruginosa PAO1 (IncP2 plasmid pBS31 [400 kb])	IncP test	C. M. Thomas
P. putida ML4262 (*trp his*) (IncP9 plasmid R2 [68 kb])	IncP test	C. M. Thomas
P. putida ML4262 (*trp his*) (IncP2 plasmid pBS228 [130 kb])	IncP test	C. M. Thomas
P. aeruginosa PU21 (*ilvB112 leu-1 str-1* Rif^r)	IncP test	G. A. Jacoby
P. aeruginosa PAO2003 (*arg-32 str-39 rec-2*)	IncP test	G. A. Jacoby
P. aeruginosa PAO2003(CAM) (camphor plasmid)	IncP test	G. A. Jacoby

[a] NCTC, National Collection of Type Cultures.
[b] ATCC, American Type Culture Collection.

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TABLE 2. Oligonucleotide primers used for PCR amplification and sequencing in this study

Gene and primer(s)	Oligonucleotide sequence	Reference
bla_{IMP}		
IMP-1	5′-CTACCGCAGCAGAGTCTTTG-3′	22a
IMP-2	5′-AACCAGTTTTGCCTTACCAT-3′	
IMP-13	5′-ATCCAAGCAGCAAGCGCGTTA-3′	3
IMP-15	5′-CGTGCTGCTGCAACGACTTGT-3′	
*aac*A4		
A-1	5′-GTAACATCGTTGCTGCTCCA-3′	This study
A-2	5′-GGTTCGAGCACTGGTTGAGT-3′	This study
A-3	5′-CGTTTGGATCTTGGTGACCT-3′	This study
catB8		
CB-1	5′-GTCATCACGATCTGGAAGCA-3′	This study
bla_{OXA-10}		
J-1	5′-GCTAATGCCGTACTCGAAAGA-3′	This study
Class I integron		
INT-5CS	5′-CTTCTAGAAAACCGAGGATGC-3′	21
INT-3CS	5′-CTCTCTAGATTTTAATGCGGATG-3′	
bla_{VIM}		
VIMF	5′-ATGGTGTTTGGTCGCATATC-3′	20
VIMB	5′-TGGGCCATTCAGCCAGATC-3′	

found to be resistant to imipenem by disk screening (zone diameter, ≤17 mm) (Clinical and Laboratory Standards Institute [formerly National Committee on Clinical and Laboratory Standards]); 29 of them were randomly selected as representative isolates from each of the 11 centers. The standard strains used in this study as quality controls or for conjugation and plasmid incompatibility testing are listed in Table 1. The PCR primers used are listed in Table 2.

By the Etest MBL (AB BIODISK, Solna, Sweden), 19 of the 29 *P. aeruginosa* isolates were positive for production of MBL. Seven of the 29 isolates screened with PCR primers IMP-1 and -2 were positive. No bla_{VIM} genes were detected in the 29 isolates. The discrepancy between the results of Etest MBL (19/29 positive) and PCR (7/19) could be explained by the presence of carbapenemases other than IMP- or VIM-type enzymes.

The bla_{IMP}-positive isolates were found to carry a new bla_{IMP} variant, named bla_{IMP-9}, as identified by direct sequencing of both strands of the PCR amplicon with primers IMP-13 and IMP-15 (3). It was 82 to 91% homologous to other bla_{IMP} alleles, and its product was 78 to 91% identical to the other IMP-type enzymes (http://www.ncbi.nlm.nih.gov/). In isolate 96, the bla_{IMP-9} gene was found to be carried on a gene cassette inserted between a 5′- and a 3′-conserved segment typical of *sul-1*-associated integrons, as determined by PCR mapping and sequencing with primers listed in Table 2 (EMBL/GenBank accession number AY033653). The integron cassette array included five gene cassettes: *aacA4*→bla_{IMP-9}→*aacA4*→*catB8*→bla_{OXA-10}. The bla_{IMP-9} gene was found in an identical genetic context in the other six bla_{IMP}-positive isolates.

Interestingly, 59be of the bla_{IMP-9} gene cassette is most closely related (only one nucleotide difference, position 776 G→C in AB074437) to that of bla_{IMP-11} (89% homologous to bla_{IMP-9}) found in *P. aeruginosa* from Japan (S. Iyobe et al., unpublished data), while it was more divergent (83% homology) from that of bla_{IMP-5}, despite a higher similarity (91%) in their β-lactamase genes.

Nucleotide sequence analysis of the amplicon obtained from isolate 96 showed a hybrid P_{ant} promoter identical to that of In1 in R46 (17) and also to that carried by the bla_{IMP-4} gene-containing integron described in a *Citrobacter youngae* from the same area (7).

The β-lactam MICs determined by the agar dilution method (19) and clinical data of the seven bla_{IMP}-carrying *P. aeruginosa* isolates are shown in Table 3. Interestingly, upon MIC testing some isolates appeared to be carbapenem intermediate or even susceptible and meropenem appeared to be consistently more active than imipenem against those isolates. The isolates were sensitive or borderline resistant to ciprofloxacin, amikacin, or gentamicin. All seven bla_{IMP}-positive isolates were also

TABLE 3. Susceptibility profiles and MBL production of bla_{IMP-9}-carrying *P. aeruginosa* isolates and their transconjugants

Organism	Hospital[a]	Ward	Isolation date (day/mo/yr)	Sex[b] age (yr)	Origin	MBL test result	MIC of β-lactam (μg/ml)[c]						
							IMP	MEM	ATM	CAZ	CTX	TZP	Car
96	H1	NICU[d]	03/04/00	M/65	Sputum	+	32	8	8	256	256	128	1,024
96T	Trans					+	32	8	4	256	256	64	1,024
121	H1	NICU	17/03/00	M/60	Sputum	+	16	8	32	256	256	256	ND[e]
101	H1	NICU	05/06/00	M/68	Sputum	+	16	2	16	32	256	128	ND
3584	H2	GM[h]	31/09/00	M/85	Sputum	+	8	4	32	256	256	128	1,024
3584T	Trans					+ (ph)[f]	4	4	8	256	256	128	1,024
3695	H2	GM	–/–/00	M/60	Sputum	+	8	1	16	256	256	128	ND
6104	H4	NICU	13/06/00	M/60	Sputum	+	8	4	4	128	64	32	ND
6104T	Trans					+ (ph)	1	2	4	1	32	1	ND
67	H7	ICU	10/10/00	M/68	Sputum	+	64	16	4	128	256	128	1,024
67T	Trans					+ (ph)	16	4	4	128	256	128	1,024
50814	Recipient				NCTC[g]	−	0.5	0.125	0.5	2	ND	0.5	<4

[a] H, hospital; numbers 1 to 11 were allocated to the 11 participating hospitals. Trans, transconjugant.
[b] M, male; F, female.
[c] IMP, Imipencm; MEM, meropenem; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; TZP, piperacillin-tazobactam; CAR, carbenicillin.
[d] NICU, neurology intensive care unit.
[e] ND, not determined.
[f] ph, phantom effect (a "keyhole" appears in the middle of the MBL test strip).
[g] NCTC, National Collection of Type Culture.
[h] GM, geriatric medicine.

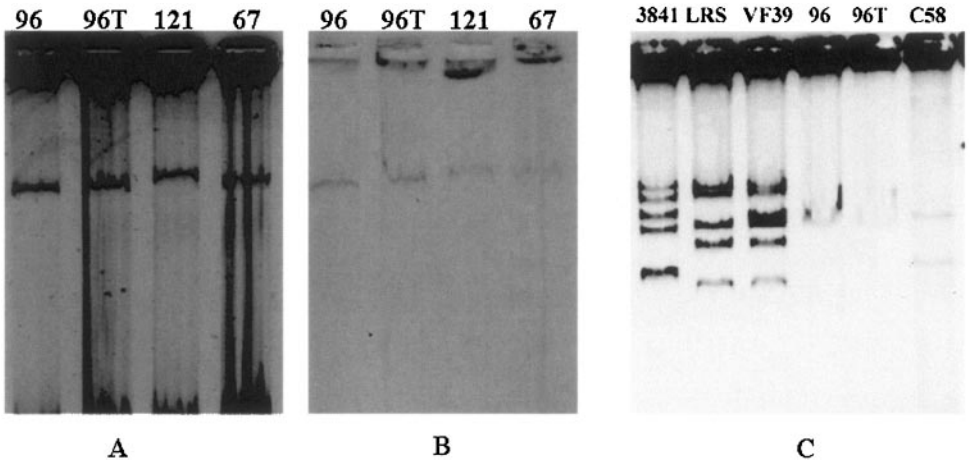

FIG. 1. Plasmids (A), hybridization profiles (B), and sizing of plasmids (C) of IMP-9-producing *P. aeruginosa* isolates and their transconjugants. The plasmids were prepared by a modification of the Eckhardt method. (A) Plasmid patterns of the isolates of 96 and its transconjugants, 121 and 67. (B) Hybridization profiles of plasmid preparations from panel A with digoxigenin-labeled specific probes. (C) Sizing of plasmid pOZ176 on the basis of the published sizes of the standard plasmids from *R. leguminosarum* strains 3841, LRS39401, and VF39 and *A. tumefaciens* C58.

resistant to potassium tellurite, with MICs of 10^{-3} M by a quantitative method (23).

Conjugal transfer of resistance (1) to carbapenems and other β-lactams was demonstrated with four of the $bla_{\text{IMP-9}}$-carrying *P. aeruginosa* isolates with *P. aeruginosa* NCTC 50814 as the recipient, but not with *Escherichia coli* UB1637/R (4). In all cases, the transfer of $bla_{\text{IMP-9}}$ was confirmed by Southern hybridization with a specific probe and PCR amplification with primers IMP-1 and -2 (Fig. 1A and B and data not shown). Resistance to tellurite was also transferred. Tellurite MICs for transconjugants were higher than that for recipient strain NCTC 50814 (10^{-4} M versus $\leq 10^{-5}$ M), although they were lower than those for donors (see above).

The plasmid content of *P. aeruginosa* 96 and its transconjugant was investigated by a modification of the Eckhardt method for isolation of large plasmids (8, 9). The size of the plasmids carried by *P. aeruginosa* 96 and by its transconjugant 96T was almost the same as that of the larger plasmid of *Agrobacterium tumefaciens* C58 (pAtC58 [543 kb]), the D plasmid (pRL10JI) of *Rhizobium leguminosarum* 3841 (488 kb), and the D plasmid (ca. 500 kb) of *R. leguminosarum* VF39 by visual estimation (Fig. 1C). From the plotted standard curve, the size of the plasmid was determined to be about 450 to 500 kb and the plasmid was designated pOZ176.

IncP group incompatibility tests (22) showed that pOZ176 and an IncP9-carrying plasmid (R2) could be transferred reciprocally and could replicate in the same cell. Their coreplication was confirmed by the phenotypic changes in resistance markers, plasmid profile, and PCR detection of the bla_{IMP} gene. In contrast, introduction of pOZ176 eliminated the IncP2 plasmid from *P. aeruginosa* PAO1(pBS31), *P. putida* ML4262(pBS228), and *P. putida* ML4262(R2) (Table 1), as confirmed by changes in the properties of the transconjugants. Furthermore, to test for the possibility of plasmid hybridization and incompatibility between IncP2 plasmids, *P. aeruginosa* 96 was crossed with PU21 (12) and PU21(pOZ176) was then crossed with PAO2003(CAM) (containing an IncP2 plasmid encoding functions for camphor degradation) (2), and PAO2003 was used as a control. Loss of the Cam^+ phenotype (14) was observed in 90% of the resulting transconjugants, indicating the incompatibility of the IncP2 plasmids from *P. aeruginosa* PAO2003 and 96. However, the phenotypes of both CAM and pOZ176 were also found in some of the transconjugants, which suggests the possibility of generation of CAM-pOZ176 recombinant plasmids; such IncP2 hybrid plasmid formation was first reported in *P. aeruginosa* in 1974 (12). The evidence for pOZ176 being an IncP2 plasmid is as follows: (i) pOZ176 mediates transferable tellurite resistance (13); (ii) pOZ176 was not stable with an IncP2 plasmid and was able to form a recombinant plasmid with a Cam^+ plasmid (IncP2), possibly via homologous DNA recombination or transposon-mediated cointegration (14); and (iii) the plasmid is large with a limited host range (13).

Randomly amplified polymorphic DNA typing (18) showed that the seven bla_{IMP}-positive isolates from four hospitals were nonclonal, except isolates 96 and 121, which were from the same hospital and ward. This finding, together with identification of the $bla_{\text{IMP-9}}$ gene on a transferable plasmid, suggests that spreading of carbapenem resistance mediated by IMP-9 MBL in *P. aeruginosa* in our area is largely due to horizontal transfer of the gene, most likely on a large R plasmid similar to pOZ176.

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REFERENCES

1. **Bennett, P. M., and M. H. Richmond.** 1976. Translocation of a discrete piece of deoxyribonucleic acid carrying an *amp* gene between replicons in *Escherichia coli*. J. Bacteriol. **126:**1–6.
2. **Chandler, P. M., and V. Krishnapillai.** 1974. Isolation and properties of

recombination-deficient mutants of *Pseudomonas aeruginosa*. Mutat. Res. **23:**15–23.
3. **Chu, Y. W., M. Afzal-Shah, E. T. Houang, M. I. Palepou, D. J. Lyon, N. Woodford, and D. M. 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. **De La Cruz, F., and J. Grinsted.** 1982. Genetic and molecular characterization of Tn*21*, a multiresistant transposon from R100.1. J. Bacteriol. **151:**222–228.
5. **Docquier, J. D., M. L. Riccio, C. Mugnaioli, F. Luzzaro, A. Endimiani, A. Toniolo, G. Amico Sante, and G. M. Rossolini.** 2003. IMP-12, a new plasmid-encoded metallo-β-lactamase from a *Pseudomonas putida* clinical isolate. Antimicrob. Agents Chemother. **47:**1522–1528.
6. **Goodner, B., G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Qurollo, B. S. Goldman, Y. W. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Lartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo, and S. Slater.** 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science **294:**2323–2328.
7. **Hawkey, P. M., J. Xiong, H. Ye, H. Li, and F. H. M'Zali.** 2001. Occurrence of a new metallo-β-lactamase IMP-4 carried on a conjugative plasmid in *Citrobacter youngae* from the People's Republic of China. FEMS Microbiol. Lett. **194:**53–57.
8. **Hynes, M. F., R. Simon, P. K. Müller, K. Niehaus, M. Labes, and A. Pühler.** 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202:**356–362.
9. **Hynes, M. F., and N. F. McGregor.** 1990. Two plasmids other than the nodulation plasmid are necessary for the formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol. Microbiol. **4:**567–574.
10. **Iyobe, S., H. Kusadokoro, A. Takahashi, S. Yomoda, T. Okubo, A. Nakamura, and K. O'Hara.** 2002. Detection of a variant metallo-β-lactamase, IMP-10, from two unrelated strains of *Pseudomonas aeruginosa* and an *Alcaligenes xylosoxidans* strain. Antimicrob. Agents Chemother. **46:**2014–2016.
11. **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.
12. **Jacoby, G. A.** 1974. Properties of R plasmids determining gentamicin resistance by acetylation in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **6:**239–252.
13. **Jacoby, G. A.** 1986. Resistance plasmids of *Pseudomonas aeruginosa*, p. 265–293. *In* I. C. Gunsalus, J. R. Sokatch, and L. N. Ornston (ed.), The bacteria, vol. X. Academic Press, Inc., Orlando, Fla.
14. **Jacoby, G. A., L. Sutton, L. Knobel, and P. Mammen.** 1983. Properties of IncP-2 plasmids of *Pseudomonas* spp. Antimicrob. Agents Chemother. **24:**168–175.
15. **Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J.-M. Frere, and G. M. Rossolini.** 1999. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. **43:**890–901.
16. **Lee, K., J. B. Lim, J. H. Yum, D. Yong, Y. Chong, J. M. Kim, and D. M. Livermore.** 2002. bla_{VIM-2} cassette-containing novel integrons in metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Pseudomonas putida* isolates disseminated in a Korean hospital. Antimicrob. Agents Chemother. **46:**1053–1058.
17. **Levesque, C., S. Brassard, J. Lapointe, and P. H. Roy.** 1994. Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. Gene **142:**49–54.
18. **Mahenthiralingam, E., M. E. Campbell, J. Foster, J. S. Lam, and D. P. Speert.** 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J. Clin. Microbiol. **34:**1129–1135.
19. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—fifth edition. NCCLS document M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
20. **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.
21. **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.
22. **Sagai, H., K. Hasuda, S. Iyobe, L. E. Bryan, B. W. Holloway, and S. Mitsuhashi.** 1976. Classification of R plasmids by incompatibility in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **10:**573–578.

22a. **Senda, K., Y. Arakawa, S. Ichiyama, K. Nakashima, H. Ito, S. Ohsuka, K. Shimokata, N. Kato, and M. Ohta.** 1996. PCR detection of metallo-β-lactamase gene (bla_{IMP}) in gram-negative rodo resistant to broad-spectrum β-lactams. J. Clin. Microbiol. **34:**2909–2913.

23. **Summers, A. O., and G. A. Jacoby.** 1977. Plasmid-determined resistance to tellurium compounds. J. Bacteriol. **129:**276–281.
24. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**147–151.
25. **Ye, H. F., H. Y. Li, D. M. Chen, Y. M. Yang, H. L. Chen, and J. H. Xiong.** 2002. Surveillance of the resistance of common pathogenic bacteria in some hospitals in Guangzhou area from 1998–2000. Chin. J. Infect. Dis. **20:**265–269. (In Chinese.)