Metallo-β-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones

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Objectives: To study the prevalence, nature, involved genetic elements and epidemiology of metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa* and *Pseudomonas putida* isolated in a Spanish hospital between 2005 and 2008.

Methods: Etests were used for susceptibility testing and screening for MBLs, confirmed through bla_{VIM} PCRs and sequencing. Clonal relatedness was evaluated by PFGE and multilocus sequence typing (MLST). MBL-carrying plasmids were characterized by restriction fragment length polymorphism, Southern blot and electroporation. MBL genetic elements were studied by cloning and sequencing.

Results: MBL-producing *P. putida* was detected in eight patients (one clone each; two harbouring bla_{VIM-1} and six harbouring bla_{VIM-2}), representing 14% of all the infections by the *P. putida/fluorescens* group. MBLs were detected in only 0.3% of *P. aeruginosa* infections (11 patients) during the same period. PFGE revealed four *P. aeruginosa* clones: one producing bla_{VIM-13} (two patients); and three producing bla_{VIM-2} (two patients, six patients and one patient, respectively). MLST indicated that the VIM-13 clone was the internationally spread sequence type (ST)235, while the major VIM-2 lineage corresponded to ST179, which is associated with chronic respiratory infections. The VIM-1 integron was shown to have both plasmid and chromosomal location, while the VIM-13 integron was only chromosomal. The VIM-2 integron was located in the same transposon (Tn402/Tn5053-like) in all *P. aeruginosa* and *P. putida* isolates, suggesting its crucial role in the dissemination of VIM-2.

Conclusions: The high diversity and proportion of MBL-positive *P. putida* suggests an environmental reservoir of these resistance determinants. Dissemination of these multidrug resistance elements to successful *P. aeruginosa* clones presents a major epidemiological and clinical threat.

Keywords: MBLs, bla_{VIM}, integrons, *Pseudomonas* spp.

Introduction

The growing threat of antimicrobial resistance in *Pseudomonas aeruginosa* is driven by its extraordinary capacity for developing resistance to almost any antibiotic by the selection of mutations in chromosomal genes, in conjunction with the emergence and dissemination of horizontally acquired resistance during the last decade.[1–3] This transferable resistance is frequently driven by integrons located in plasmids or transposons.[4,5] Class B carbapenemases [or metallo-β-lactamases (MBLs)] are certainly among the transferable determinants having a higher impact on antimicrobial therapy.[4–6] Horizontally acquired MBLs were first described in the early 1990s and currently include eight groups of enzymes (IMP, VIM, SPM, SIM, GIM, DIM, AIM and KHM).[5] Indeed, MBL-producing *P. aeruginosa* strains have been responsible for large outbreaks in several hospitals worldwide.[5]

Considerably less is known about chromosomal and transferable carbapenem resistance in other environmental *Pseudomonas* species, although MBLs have been reported particularly in *Pseudomonas putida*.[5] Moreover, their role as potential reservoirs of multidrug resistance determinants still remains poorly studied. In this study, we investigated the prevalence, nature, involved genetic elements and epidemiology of MBL-producing *Pseudomonas* spp. in a Spanish hospital during a 4 year period.

Materials and methods

Bacterial strains and susceptibility testing

All *Pseudomonas* spp. isolates (one per patient) resistant to imipenem and/ or meropenem, recovered from clinical samples during a 4 year period [2005 (first detection) to 2008] in a Spanish hospital [Hospital Son Dureta (HSD), a 900 bed public reference hospital of the Balearic Islands], were screened for class B carbapenemase production using MBL Etest strips (AB Biodisk, Solna, Sweden). The API 20 NE system (bioMér-ieux, Marcy l'Étoile, France) was used for the identification of *Pseudomonas* spp. isolates. MICs of piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin and colistin were determined by the Etest method. Breakpoints were applied following CLSI recommendations.

Molecular epidemiology

The clonal relatedness was investigated by PFGE, following conventional protocols.[6] The MBL-producing *P. aeruginosa* strains previously detected by our group in Majorcan hospitals were included in the analysis for comparative purposes.[7,8] Additionally, multilocus sequence typing (MLST) was performed, following previously published protocols,[9] in one isolate of each *P. aeruginosa* PFGE clone.

Characterization of MBL-encoding genes and their carrier genetic elements

The presence of MBL-encoding genes was explored by PCR and sequencing using previously described primers and conditions.[6] Their potential location in plasmids was evaluated by electroporation into PAO1, as previously described.[6] MICs for the antibiotics listed above were determined by Etest on the obtained transformants. Additionally, Southern blot hybridization experiments with bla_{VIM} PCR probes were performed with the genomic and plasmid DNA of each strain, using the ECL kit (GE-Healthcare, Little Chalfont, Buckinghamshire, UK). Lastly, MBL-harbouring plasmids were compared through the analysis of restriction fragment (BamHI and HindIII) length polymorphisms (RFLPs). The integrons harbouring the MBL-encoding genes were characterized by PCR followed by DNA sequencing, using previously described protocols.[6]

Cloning experiments

For the characterization of integrons too large to be amplified by PCR, cloning experiments were performed. Genomic DNA was digested with EcoRI and/or BamHI and ligated to the pUCP24 plasmid digested with the same enzymes. The ligation product was transformed into *Escherichia coli* XL1 Blue and plated on LB agar with 5 mg/L gentamicin and 30 mg/L ampicillin. The transformants were first checked by PCR of the corresponding bla_{VIM} gene and then the cloned fragments were fully sequenced using the walking primer approach.

Determination of mutational resistance mechanisms on P. aeruginosa strains

Previously described protocols were used, and included real-time RT–PCR to determine the expression of efflux pumps and *ampC*, and PCR and sequencing of the quinolone resistance-determining regions (QRDRs) and *oprD*.[2,6]

Nucleotide sequence accession numbers

GenBank EF577407 (VIM-13 integron), EF577408 (VIM-1 integron) and GQ227991 (VIM-2 integron/ transposon).

Results

Prevalence of MBL-producing Pseudomonas spp., resistance profile and epidemiological relatedness

Nineteen non-duplicated (one per patient) *Pseudomonas* spp. (11 *P. aeruginosa* and 8 *P. putida*) isolates showing a positive MBL Etest were detected in HSD during the study period. The percentage of infections by MBL-positive strains was 47-fold higher for the *P. putida/fluorescens* group (8/57, 14.03%) than for *P. aeruginosa* (11/3694, 0.3%). PFGE analysis revealed the presence of four *P. aeruginosa* clones, detected in one patient, two patients, two patients and six patients, respectively. On the other hand, each of the eight *P. putida* isolates belonged to a different clone. Their characteristics are shown in Table 1.

Two of the *P. putida* clones produced VIM-1, while the other six produced VIM-2. One of the *P. aeruginosa* clones produced the recently characterized VIM-13 and was found to be identical to the first isolate producing this enzyme, detected in another Majorcan hospital.[8] The other three *P. aeruginosa* clones produced VIM-2. Clone PAV2-2, detected in six patients, was found to have an identical PFGE profile to the mucoid VIM-2-producing strain found to be responsible for chronic respiratory infections in three patients in a Majorcan chronic care institution.[7] The medical records revealed that at least four of the six patients suffered chronic obstructive pulmonary disease and were chronically infected, since detection of the strain in respiratory samples for >6 months was documented.

Regarding MLST results (Table 1), the VIM-13 clone had the same allelic profile as the internationally widespread sequence type (ST)235,[10] while PAV2-2 belonged to ST179. Lastly, PAV2-1 (two patients) was shown to constitute a new ST, named ST811, whereas PAV2-3 (one patient) belonged to ST155.

Characterization of the genetic elements harbouring MBL genes

Figure 1 shows the integron structures. The *intI1* sequence revealed that they were all class 1 integrons, and contained the GGG between −10 and −35 boxes activating the P2 promoter, conserved in all our integrons. The integron from PAVIM-13 contained an AacA4 (with the reported polymorphism Ala108Thr) gene cassette downstream of bla_{VIM-13}.[8] The integron harbouring bla_{VIM-1} of *P. putida* strains PPV1-1 and PPV1-2 showed a similar structure, but containing an additional gene, *aadA1*. All VIM-2-producing *P. aeruginosa* and *P. putida* harboured the same integron, showing the AacA4 Ala108Thr variant upstream of bla_{VIM}. Cloning experiments and sequencing revealed that the VIM-2 integron was left-flanked by a transposase-encoding gene (99% nucleotide identity with Tn*5393 tnpA* of GenBank accession number AF313472). Moreover, the integron was right-flanked by the transposition module (*tniR*, *tniQ*, *tniB* and *tniA* genes) of the mercury resistance transposon Tn*5053* (GenBank accession number L40585). Furthermore, a 100% nucleotide identity was documented with the *tni* module of the Tn*402/5053*-like Tn*6017* transposon from *Citrobacter youngae* (GenBank accession number AF288045).[11] IRi and IRt were shown to be located 177 bp downstream of *intI1* and 119 bp upstream of *tniA*, respectively (Figure 1), suggesting that the VIM-2 integron could be mobilized using the *tni* machinery.

Table 1. PFGE and MLST clones, characteristics (sample, date and ward), MBL types and location, MICs and mutational resistance mechanisms of *P. aeruginosa* and *P. putida* strains, and the PAO1 obtained transformants harbouring the natural plasmids from *P. putida* strains

Clone (PFGE/MLST)	Sample/ward[a]	Date of isolation (dd/mm/yy)	MBL/location[b]	MICs[c] (mg/L)												Mutational mechanisms[d]					
				PIP	TZP	ATM	CAZ	FEP	IPM	MEM	GEN	TOB	AMK	CIP	CST	GyrA	ParC	OprD[e] (strain)	ampC	mexB	mexY
P. aeruginosa																					
PAV13	urine/HML	16/07/08	VIM-13/C	48	48	3	48	32	>32	>32	>256	32	12	>32	2	T83I	S87L	ABJ10119 (PA14)	NC[f]	1.3±0.3	17.8±8.4
PAV13/ST235	BAS/ICU and PNL	18/07/08	VIM-13/C	64	64	8	64	64	>32	>32	128	32	12	>32	2	T83I	S87L	ABJ10119	NC	1.1±0.5	32.6±9.9
PAV2-1/ST811	BAS/HML	04/05/05	VIM-2/C	48	32	4	24	48	>32	>32	>256	96	16	>32	2	T83I	S87L	EAZ62682 (PA2192)	NC	1.3±0.5	3.2±0.3
PAV2-1	sputum/HML	30/06/05	VIM-2/C	32	24	4	24	32	>32	>32	>256	64	16	>32	2	T83I	S87L	EAZ62682	NC	1.9±0.2	3.4±1.2
PAV2-2	sputum/PNL	11/06/07	VIM-2/C	64	48	4	48	32	>32	8	32	12	2	>32	1	T83I	S87L	EAZ62682	NC	1.8±0.9	12.3±4.3
PAV2-2	sputum/PNL	26/06/07	VIM-2/C	96	96	0.75	>256	128	>32	>32	>256	>256	32	>32	1.5	T83I	S87L	EAZ62682	NC	2.7±0.8	21.8±8.6
PAV2-2	urine/IM	04/07/07	VIM-2/C	24	24	6	16	24	>32	8	>256	>256	16	>32	2	T83I	S87L	EAZ62682	NC	7.5±2.9	13.7±4.3
PAV2-2	sputum/ICU, PNL and IM	11/12/07	VIM-2/C	4	3	0.25	12	8	>32	3	>256	64	12	>32	2	T83I	S87L	EAZ62682	NC	29.5±12.2	6.5±3.1
PAV2-2	sputum/PNL	16/03/08	VIM-2/C	16	12	3	16	24	>32	4	>256	128	16	>32	2	T83I	S87L	EAZ62682	NC	1.7±0.3	6.3±3.2
PAV2-2/ST179	sputum/IM	16/07/08	VIM-2/C	64	64	24	48	48	>32	>32	>256	192	32	>32	2	T83I	S87L	EAZ62682	84.17±11.73	4.6±1.1	42.3±15.3
PAV2-3/ST155	sputum/ICU	30/08/07	VIM-2/C and P	12	8	1.5	24	32	>32	>32	>256	128	12	0.25	2	—	—	EAZ62682 aa417X	NC	2.1±0.7	36.3±12.7
P. putida																					
PPV1-1	peritoneal fluid/ ICU	22/06/05	VIM-1/C and P	>256	>256	24	>256	>256	>32	>32	48	16	3	>32	2						
PPV1-2	urine/NRL	11/09/08	VIM-1/P	64	64	12	>256	>256	>32	>32	16	6	2	>32	2						
PPV2-1	blood/ surgery	19/07/05	VIM-2/C	8	8	12	12	6	>32	24	4	2	1	3	2						
PPV2-2	peritoneal fluid/ICU	30/11/05	VIM-2/P	64	128	12	48	24	>32	>32	12	6	3	0.125	2						
PPV2-3	blood/IM	03/07/06	VIM-2/P	48	32	8	24	8	>32	>32	1.5	1.5	0.25	0.032	0.75						
PPV2-4	catheter/ICU	23/10/06	VIM-2/P	96	96	16	48	16	>32	>32	2	2	0.38	0.047	0.75						
PPV2-5	sputum/PNL	02/03/07	VIM-2/P	24	24	3	12	3	>32	>32	0.5	1	0.047	0.064	0.5						
PPV2-6	blood/OCL	18/09/07	VIM-2/P	16	16	24	24	6	>32	>32	12	4	2	32	2						
Transformants																					
PAO1			—	3	2	1.5	1	1	2	0.25	2	1	4	0.125	1						
PAO1 (pPPV1-1)			VIM-1/P	256	96	4	>256	>256	>32	>32	96	24	4	0.125	1						
PAO1 (pPPV2-2)			VIM-2/P	64	96	1.5	48	24	>32	>32	48	16	4	0.125	1						
PAO1 (pPPV2-4)			VIM-2/P	48	32	1.5	32	32	>32	>32	128	24	6	0.125	1						

[a]HML, haematology; IM, internal medicine; PNL, pneumonology; OCL, oncology; BAS, bronchial aspirate; NRL, neurology; ICU, intensive care unit.
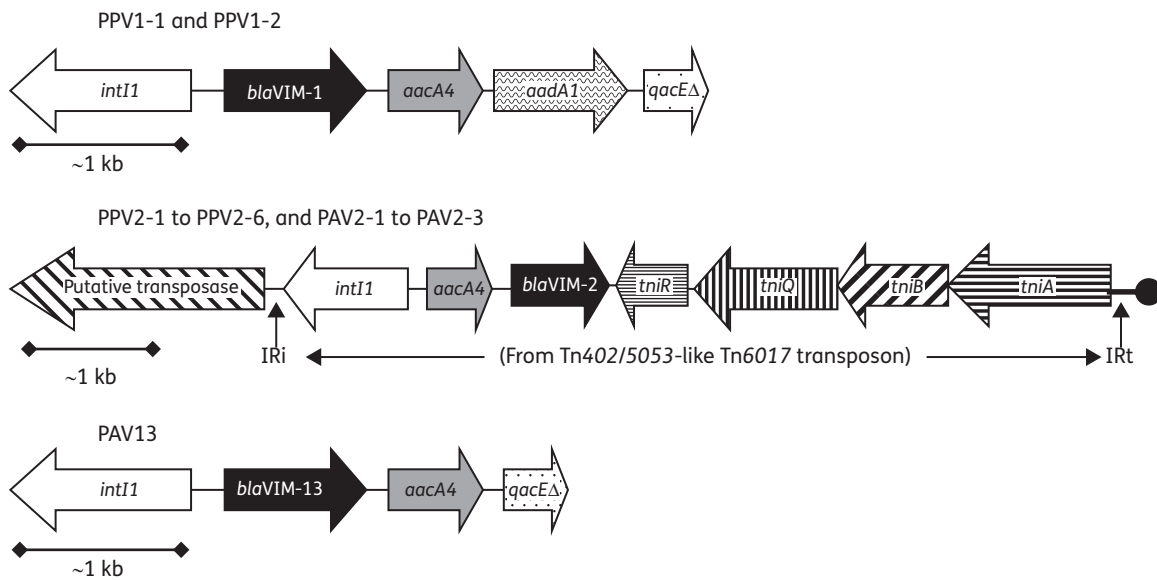
[b]C, chromosomal; P, plasmid.

[c]PIP, piperacillin; TZP, piperacillin/tazobactam; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin; CST, colistin.

[d]Only determined in *P. aeruginosa* strains. Mutations detected in GyrA, ParC or OprD, and relative expression of *ampC* and efflux pump genes *mexB* and *mexY* with respect to PAO1 basal expression.

[e]OprD variant of each isolate, with the corresponding GenBank accession number (*P. aeruginosa* strains where each OprD variant proceeds from are also shown). In PAV2-3, a premature stop codon was generated by mutation (amino acid 417).

[f]NC, no significant changes with regard to PAO1.

Figure 1. Structure of the integrons carrying the MBL genes of the *P. putida* and *P. aeruginosa* strains described in this work.

Chromosomal or plasmid location of the genetic elements harbouring MBL genes

According to Southern blot results, all the *P. putida* clones had their MBL genes located in plasmids, with the exception of the PPV2-1 clone, which showed a chromosomal location (Table 1). Furthermore, in clone PPV1-1, the bla_{VIM-1} probe hybridized with both plasmid and chromosomal DNA. The plasmids harbouring the bla_{VIM-1} integron in the PPV1-1 and PPV1-2 clones provided totally different RFLP patterns, and among the five *P. putida* clones producing plasmid-located VIM-2, an important variability in the RFLP patterns was detected (data not shown). Nevertheless, in all cases the bla_{VIM-2} probe hybridized with the same 3–4 kb BamHI–HindIII restriction band. The restriction map analysis showed a BamHI site 130 bp downstream of *intI1* and a HindIII site 474 bp downstream of bla_{VIM-2}, resulting in a restriction fragment of ~3300 bp. This was hence the band where the probe hybridized. MBL-harbouring plasmids from PPV1-1, PPV2-2 and PPV2-3 *P. putida* clones could be transferred by electroporation to *P. aeruginosa* PAO1. The transformants and the co-transferred resistance profile can be observed in Table 1. This resistance included all β-lactams (except aztreonam), and gentamicin and tobramycin, conferred by AacA4.

MBL genes had only a chromosomal location in all *P. aeruginosa* clones, with the exception of PAV2-3, apparently having an additional plasmidic bla_{VIM-2} copy. The bla_{VIM-2} probe hybridized with the 3–4 kb BamHI–HindIII restriction fragment of the plasmid DNA, as occurred in *P. putida* strains. The PAV2-3 RFLP was different to those of *P. putida*, although it shared some common bands (data not shown).

Mutational resistance in MBL-producing *P. aeruginosa* strains

Almost all *P. aeruginosa* clones showed diverse mutational resistance mechanisms, including QRDR mutations, efflux pump overexpression and AmpC hyperproduction or OprD inactivation (Table 1).

Discussion

The dissemination of multidrug-resistant (MDR) *P. aeruginosa* in the nosocomial environment is an epidemiological threat that compromises the effectiveness of the available therapeutic options. As genetic capitalism predicts,[12] the most successful clones are also more likely to acquire MDR determinants and be selected under antibiotic pressure, hence being spread. Indeed, recent research demonstrates the presence of internationally widespread (successful) *P. aeruginosa* clones, such as ST111 or ST235, linked to several resistance determinants located in diverse horizontally acquired elements (integrons, transposons and plasmids),[10,13] suggesting that the driver of this incipient MDR *P. aeruginosa* pandemic is a global spread of successful clones combined with the local acquisition of MDR determinants.

This work certainly illustrates this notion. Successful MDR MBL-producing *P. aeruginosa* clones were indeed detected in various Majorcan hospitals. The VIM-2-producing ST179 clone was not only detected in several patients from two different institutions, but also was found to cause chronic respiratory infections in an important proportion of the patients. Its long-term persistence (and maybe its frequent mucoid phenotype) might be among the keys to its success. It is noteworthy that the ST179 clone was detected in chronically infected cystic fibrosis patients from Canada two decades ago.[14] Meanwhile, ST235 is an internationally widespread clone that has been previously associated with PER, OXA and VIM enzymes.[10] ST235 has also been detected in Spain, linked to the production of GES extended-spectrum β-lactamases;[15] this work adds VIM-13 to its growing list of produced β-lactamases. Moreover, the here reported presence of diverse chromosomal resistance mechanisms in all these clones may also be a contributing factor explaining their success.

Certainly, the transferable resistance armamentarium of these global clones seems to be local. This is supported by the presence of a new VIM enzyme (VIM-13),[8] the presence of a

specific polymorphism in the AacA4 enzyme (Ala108Thr)[8] not detected in other areas or the detection in several clones of a defined VIM-2 transposon–integron structure. Hence, a crucial role of this transposon (through the *tni* machinery) in the horizontal dissemination of bla_{VIM-2} among the *P. putida* and *P. aeruginosa* clones is clearly suggested by our results.

Furthermore, the finding of diverse *P. putida* clones harbouring the same VIM-2 transposon in plasmids, together with the high proportion (14%) of MBL-producing strains compared with *P. aeruginosa* (0.3%), suggest that *P. putida* is a reservoir of these MDR transferable elements, which could be amplified by transfer to successful *P. aeruginosa* clones.

Our findings suggest that, in addition to preventing the dissemination of MBL-producing *P. aeruginosa* in hospitals, the detection and control of MBL-producing *P. putida* should be a priority, in spite of its lower clinical relevance.

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None to declare.

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