

Characterization of In100, a New Integron Carrying a Metallo- β -Lactamase and a Carbenicillinase, from *Pseudomonas aeruginosa*

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In100, a new integron carrying a carbapenemase gene (*bla*_{VIM-2}) associated with a carbenicillinase (*bla*P1b) and aminoglycoside resistance genes (*aacA4* and *aadA2*), was detected in a *Pseudomonas aeruginosa* clinical isolate. The particular gene cassette organization of In100 seems to reflect the evolution of antibiotic usage in therapeutics.

Carbenicillin was introduced in therapeutics in 1967 and was especially useful, on its own or when associated with aminoglycosides, for the treatment of infections caused by *Pseudomonas aeruginosa* (23), a well-recognized nosocomial pathogen with intrinsic resistance to various antimicrobial agents and the ability to develop multidrug resistance (18). Nevertheless, an increasing frequency of *P. aeruginosa* isolates resistant to these antibiotics and to newer β -lactams has been observed during the last decades, thus determining the use of the most potent β -lactams, carbapenems (14). Intensive use of these antibiotics eased the emergence of carbapenem resistance in *P. aeruginosa* (7), among others, due to the production of metallo- β -lactamases, mainly IMP and VIM types (9, 10, 22). Initially reported in Japan (17), the new metallo- β -lactamases, have spread worldwide, nowadays being reported in gram-negative isolates from Europe (3, 7, 10, 20), Asia (9, 11), and, more recently, North America (26).

Most IMP- and VIM-like gene cassettes are found on class 1 integrons, with variable structures among isolates (11). In theory, gene cassettes can be incorporated at different positions in the integron (5); however, it seems that cassettes are preferentially integrated adjacent to the *attI1* site (5, 19), with the order of the gene cassettes possibly reflecting antibiotic pressure (4).

In this study, we report the genetic characterization of a novel class 1 integron carrying *bla*_{VIM-2} associated with carbenicillinase and aminoglycoside resistance genes, identified in a *P. aeruginosa* blood isolate (FF-PS2) from a patient located in the intensive care unit of a hospital in Coimbra, Portugal, in 2000. MICs of β -lactam antibiotics were determined by the Etest method (AB Biodisk, Solna, Sweden), and the susceptibility to aminoglycosides and ciprofloxacin was determined by the disk diffusion method (16). Analytical isoelectric focusing (IEF) was performed with crude extracts on a pH 3-to-9 polyacrylamide gel (Phast gels; Amersham Biosciences, Uppsala,

Sweden), and β -lactamases were visualized with nitrocefin (100 μ M) (Oxoid, Basingstoke, United Kingdom). A bioassay (3) was employed in order to detect metallo- β -lactamase production. *bla*_{VIM} and *bla*_{IMP} genes were detected by a multiplex PCR assay with VIM/IMP primers (3, 24), and class 1 integrons were detected with previously described specific primers (12, 22). Extraction of plasmid DNA was carried out with the QIAGEN plasmid kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Transfer of resistance genes was screened by mating with rifampin-resistant *P. aeruginosa* strain PAO or rifampin-resistant *Escherichia coli* strain K802N. Transconjugant selection was performed in Mueller-Hinton agar plates containing rifampin (100 μ g/ml) (Sigma, St. Louis, Mo.) and amoxicillin (30 μ g/ml) (Sigma) or imipenem (2 μ g/ml) (Merck Sharp & Dohme, Portugal) when *Escherichia coli* K802N and *P. aeruginosa* PAO were used as recipients, respectively. A possible chromosomal location of the *bla*_{VIM-2} gene was determined by hybridization of I-CeuI-digested genomic DNA with probes labeled with the ECL enhanced chemiluminescence kit (Amersham Biosciences Europe GmbH) for *bla*_{VIM-2} and the 16S and 23S rRNA genes, as previously described (8, 13). The PCR product obtained with the class 1 integron primers INT/5'CS (22) and 3'CS (12) was purified with the StrataPrep PCR purification kit (Stratagene, Amsterdam, The Netherlands), and the insert was ligated into the pPCR-Script Cam SK(+) cloning vector (Stratagene, Amsterdam, The Netherlands) and transformed into *Epicurian coli* XL10-Gold Kan ultracompetent cells (Stratagene, Amsterdam, The Netherlands). Luria-Bertani agar plates supplemented with ampicillin (50 μ g/ml), chloramphenicol (30 μ g/ml), X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), and IPTG (isopropyl- β -D-thiogalactopyranoside) were used as selection plates. The nucleotide sequence of the class 1 integron containing *bla*_{VIM-2} was determined in both directions through a primer-walking strategy using specific designed primers in both direct and cloned PCR products.

P. aeruginosa FF-PS2 was detected in the context of the regular screening for metallo- β -lactamase producers among carbapenem-resistant nonfermenters from clinical and environmental sources. The isolate was highly resistant to piper-

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FIG. 1. Schematic representation of the *P. aeruginosa* FF-PS2 class 1 integron, In100.

cillin (>256 $\mu\text{g/ml}$), piperacillin-tazobactam (>256 $\mu\text{g/ml}$), ceftazidime (32 $\mu\text{g/ml}$), cefepime (>32 $\mu\text{g/ml}$), imipenem (>32 $\mu\text{g/ml}$), and meropenem (>32 $\mu\text{g/ml}$) but remained susceptible to aztreonam (8 $\mu\text{g/ml}$). It was also resistant to tobramycin, gentamicin, amikacin, netilmicin, and ciprofloxacin. By IEF, three β -lactamase bands with pIs of 5.3, 5.7, and >8.2 were observed.

Confirming the positive bioassay, suggestive of a metallo- β -lactamase production, a multiplex PCR assay to amplify both *bla*_{VIM} and *bla*_{IMP} genes from FF-PS2 genomic DNA revealed an amplicon compatible with the presence of *bla*_{VIM}. No plasmid DNA was detectable by agarose gel electrophoresis, and conjugation experiments failed to demonstrate the occurrence of conjugative transfer of β -lactamase determinants. These results suggested the chromosomal location of *bla*_{VIM}, which was confirmed after the I-CeuI technique and hybridization with *bla*_{VIM-2} and rRNA probes.

Sequencing of a ca. 4,000-bp fragment revealed a class 1 integron containing an original array of four gene cassettes, named In100 (Fig. 1). Most integrons containing *bla*_{VIM} genes usually carry two or more gene cassettes (10, 21), but this is the first report of a class 1 integron containing a carbapenemase gene associated with another β -lactamase gene cassette. A closer look at the genetic structure of this integron revealed a gene cassette array that might reflect the evolution of antibiotic usage. The *bla*_{VIM-2} gene cassette in In100, identical to that reported for In56 (20), was found in the first position of the integron, which indicates it was the most recently acquired gene cassette (Fig. 1). The start codon (ATG) of *bla*_{VIM-2} was preceded by two putative promoter regions (with a weak promoter, Pc; -35 [TGGACA] and -10 [TAAGCT]) and a P2 promoter in its inactive form (6). An *aacA4* gene cassette encoding an aminoglycoside 6'-*N*-acetyltransferase was found immediately downstream of the *bla*_{VIM-2} cassette. The derived amino acid sequence of the *aacA4* allele demonstrated homology to AAC (6')-II enzyme, which modifies gentamicin, tobramycin, and netilmicin. The simultaneous presence of *bla*_{VIM-2} and *aacA4* is a common occurrence among integrons carrying metallo- β -lactamase genes (10, 11, 21). A higher number of nucleotides (24 bp) compared to those in the available GenBank *aacA4* sequences were observed in the upstream region of the start codon. Interestingly, the sequence immediately downstream of *aacA4* gene is identical to that of the *blaP1b* gene cassette described in In28 (GenBank accession no. AF313472), coding for PSE-1/CARB-2. Although PSE-1 has been reported as the most common acquired β -lactamase in *P. aeruginosa* isolates (1), its gene has never been associated with another β -lactamase gene in the same integron. The IEF analysis revealed a β -lactamase with pI \sim 5.7, compatible to the production of PSE-1, as well as a β -lactamase band of pIs 5.3 and >8.2, consistent with the presence of VIM-2 and chromosomal AmpC, respectively.

The fourth cassette present in In100 contained the *aadA2* gene coding for the aminoglycoside-3'-adenyltransferase and

confers resistance to streptomycin and spectinomycin (2). The *aadA2* gene is the most frequently integrated gene cassette in class 1 integrons (12), a fact that might be related to the early emergence of integrons carrying *aadA*, since streptomycin clinical availability and the resulting increase in selective pressure preceded that of other aminoglycosides and β -lactams.

The common human clinical use of β -lactams and aminoglycosides has been argued to contribute to the simultaneous presence of gene cassettes encoding β -lactamases and aminoglycoside-modifying enzymes in the same integron (21, 25). However, other ecological niches where antibiotics are used on a large scale, namely in animals for food production, may also contribute as sources and or reservoirs of integrons carrying resistance genes (15). The recent characterization of an integron carrying the same array of genes as In100, except *bla*_{VIM-2}, in a pig *P. aeruginosa* isolate might also support this possibility (S. Quinteira, unpublished data).

Novel gene cassettes are preferentially inserted at the 5'CS region of the integron, thus ensuring a stronger expression, forming a queue that ranges from the newest to the oldest inserted cassette (5, 19). *P. aeruginosa* In100 gene cassette organization can be viewed as a historical reflection of antibiotic usage in clinical therapeutics, thus further supporting the notion that the bacterial genome is an increasingly useful tool to unravel not only the basic phylogenetic patterns among microorganisms but also the specific direction of recent evolution within microbial species.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank sequence databases and assigned accession no. AY560837.

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