

## Description of an original integron encompassing *bla*<sub>VIM-2</sub>, *qnrVC1* and genes encoding bacterial group II intron proteins in *Pseudomonas aeruginosa*

P. T. Belotti<sup>1</sup>, L. Thabet<sup>2</sup>, A. Laffargue<sup>1</sup>, C. André<sup>1</sup>, L. Coulange-Mayonnove<sup>1</sup>, C. Arpin<sup>1</sup>, A. Messadi<sup>2</sup>, F. M'Zali<sup>1</sup>, C. Quentin<sup>1</sup> and V. Dubois<sup>1\*</sup>

<sup>1</sup>UMR 5234 CNRS, University Bordeaux 2, 146 rue Leo Saignat, 33076 Bordeaux, France; <sup>2</sup>Laboratory of Biology, Aziza Othmana Hospital, Tunis, Tunisia

\*Corresponding author. Tel: +33-5-57-57-10-75; Fax: +33-5-57-57-95-14; E-mail: veronique.dubois@bacterio.u-bordeaux2.fr

Received 26 September 2014; returned 6 October 2014; revised 16 March 2015; accepted 28 March 2015

**Objectives:** A burn unit of a hospital in Tunis underwent an endemic situation caused by imipenem-resistant *Pseudomonas aeruginosa*. For nine non-repetitive isolates of a clonal VIM-2-producing strain, the *bla*<sub>VIM-2</sub> genetic background was characterized and the associated *qnrVC1* gene molecularly analysed.

**Methods:** The imipenem resistance mechanism was investigated by phenotypic and molecular tests, and resistance transfer was studied by conjugation and transformation experiments. The integron's structure was characterized by sequencing, and *qnrVC1* expression was explored after cloning experiments.

**Results:** The nine VIM-2-producing strains were collected from eight patients and one environmental sample. All transfer assays failed, suggesting a chromosomal location of *bla*<sub>VIM-2</sub>. This latter was found to be part of a class 1 integron of ~7500 bp, which also contains *bla*<sub>OXA-2</sub>, *aadA1* and two copies of the *aadB*, *arr-6* and *qnrVC1* genes. *qnrVC1* exhibited higher homology with the chromosomally encoded *qnr* genes of Vibrionaceae than with plasmid-mediated *qnr* genes of Enterobacteriaceae. The *qnrVC1* gene cassette possesses a promoter allowing its expression, and it conferred decreased fluoroquinolone susceptibility to *Escherichia coli*. Additionally, on the same integron, genes encoding an uncommon group IIC-attC intron were detected.

**Conclusions:** A VIM-2-producing *P. aeruginosa* outbreak led us to characterize an integron harbouring a *qnrVC1* cassette and a new group IIC-attC intron. This is the first known description of a *qnr* determinant in a *P. aeruginosa* strain. Its presence conferred a low level of resistance to quinolones in *E. coli*, which might favour the emergence of highly resistant mutants.

**Keywords:** *P. aeruginosa*, VIM-2 carbapenemase, burn unit, imipenem resistance

### Introduction

*Pseudomonas aeruginosa* is a saprophytic organism that is widespread in moist environments and has become a major nosocomial pathogen in patients with impaired defence systems, such as burn patients.<sup>1</sup> Currently, carbapenems are the first-line treatment for MDR *P. aeruginosa* infections. However, carbapenem resistance is increasing dramatically worldwide. The most reported mechanisms are the loss of the OprD porin, and more recently, the production of carbapenemases, especially the so-called metallo-β-lactamases (MBLs).<sup>2</sup> MBLs are mainly encoded by mobile gene cassettes inserted into integrons that often harbour other resistance determinants.

In this study, several clonal strains of imipenem-resistant *P. aeruginosa* were collected in a burn unit of a Tunisian hospital.<sup>3</sup>

One of them carried an uncommon integron encompassing *bla*<sub>VIM-2</sub>, associated with *qnrVC1* and genes encoding a bacterial group II intron. The expression of *qnrVC1* and the subsequent conferred resistance were investigated.

### Materials and methods

#### Bacterial strains

Between November 2007 and August 2008, nine non-repetitive imipenem-resistant isolates of *P. aeruginosa* belonging to a clonal strain were collected in the Burn Centre of the Aziza Othmana Hospital in Tunis. Identification to species level was confirmed by the API 20NE system (bioMérieux, Marcy-l'Étoile, France). An azide-resistant mutant of *Escherichia coli* C600, *E. coli* TOP10 and *P. aeruginosa* ATCC 27853 were

the recipient strains in conjugation and transformation experiments. All strains were grown at 37°C on Mueller–Hinton agar (Sanofi-Diagnostics Pasteur, Marnes la Coquette, France) or in Luria broth (GibcoBRL, Cergy Pontoise, France).

### Antibiotic susceptibility testing

Susceptibility to 23 antimicrobial agents was determined for the nine clinical strains by the disc diffusion method (<http://www.sfm-microbiologie.org>). The presence of MBLs was investigated by imipenem discs either supplemented or not with EDTA (750 µg). Imipenem MICs were determined alone and in combination with a fixed concentration of EDTA (320 mg/L) by the agar dilution method (<http://www.sfm-microbiologie.org>). Quinolone MICs were determined using Etest strips according to the manufacturer's recommendations (bioMérieux).

### Plasmid content analysis and conjugation and transformation experiments

Imipenem resistance transfer to an azide-resistant mutant of *E. coli* was attempted by a filter-mating technique. Putative transconjugants were selected on agar plates containing ampicillin (100 mg/L) and sodium azide (300 mg/L). Plasmid DNA was extracted using an alkaline-lysis method.<sup>4</sup> Putative plasmid DNA extract obtained from the representative clinical isolate Pa25 was electroporated into *E. coli* TOP10 and *P. aeruginosa* ATCC 27853 with selection on agar plates containing ampicillin (100 mg/L) or ticarcillin (100 mg/L).

### PCR experiments and DNA sequencing

Total DNA was extracted from the *P. aeruginosa* strains as previously described.<sup>4</sup> The detection of carbapenemase genes (*bla<sub>VIM</sub>*, *bla<sub>IMP</sub>*, *bla<sub>KPC</sub>* and *bla<sub>NDM-1</sub>*) and plasmid-mediated quinolone resistance determinants [*qnrA*, *qnrB*, *qnrS*, *qepA* and *aac(6')-Ib-cr*] and the characterization of the integron structure were performed by PCR in the nine clonally related strains under standard conditions<sup>4</sup> using published<sup>4–8</sup> or laboratory-designed primers. Mutations in the quinolone resistance-determining regions of the *gyrA*, *gyrB*, *parC* and *parE* genes were characterized by sequencing after PCR using published primers.<sup>9</sup> For sequencing, PCR products purified with the GeneJET PCR Purification kit (Fermentas, Villebon sur Yvette, France) and plasmids were used as templates in a cycle reaction using the AmpliTaq DNA polymerase FS Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Division, PerkinElmer) and an automatic ABI 3130xl sequencer (Applied Biosystems Division, PerkinElmer). Sequences were analysed using the 'BioEdit' software and the NCBI web site (<http://www.ncbi.nlm.nih.gov>).

### Cloning experiments

*qnrVC1* expression was explored by cloning the *qnrVC1* gene cassette using PCR products from the Pa25 strain and the pGEMT easy kit (Promega, Charbonnières, France) or the *Pseudomonas/E. coli* shuttle vector pCN60 (ATCC 77101). The DNA fragment amplified included the *qnrVC1* promoter region (primers *arr6-F* 5'-ATGTCGAGTGACTGGACTCC-3' and *aaddi* 5'-CAGGAACCGGATCAAGAGT-3' for cloning into pGEMT, and *qnrVF-Pst* 5'-CACACACTGCAGATGTCGAGTGACTGGACTCC-3' and *qnrVR-Hind* 5'-CACACAAAGCTTCAGGAACCGGATCAAGAGT-3' for cloning into pCN60, both restricted by HindIII and PstI). The recombinant plasmids were transformed by electroporation either into *E. coli* TOP10 and selected on ampicillin (100 mg/L) agar plates or into *P. aeruginosa* ATCC 27853 and selected on ticarcillin (100 mg/L) agar plates.

### Molecular typing

For the nine VIM-2-producing isolates, PFGE analysis was performed according to the manufacturer's instructions (Bio-Rad, Marnes la

Coquette, France) using whole-cell DNA digested with SpeI restriction enzyme.

### Nucleotide sequence accession number

The nucleotide sequence reported here is available in GenBank (accession number JX861889).

## Results and discussion

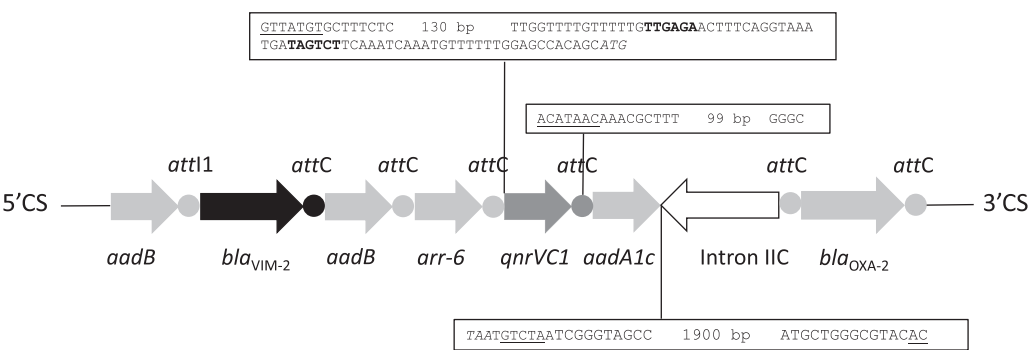
### Origin of the *P. aeruginosa* strains

Nine non-repetitive isolates collected from eight patients and one environmental sample (tap water) were demonstrated to be clonally related by PFGE (data not shown). These isolates, responsible for a limited outbreak from November 2007 to January 2008, were recovered from blood cultures (*n*=4), cutaneous wounds (*n*=3) and an invasive device (*n*=1). They exhibited high-level imipenem resistance (mode MIC >128 mg/L, range MIC 128 to >128 mg/L), and addition of EDTA restored the imipenem activity at least in part (mode MIC=32 mg/L, range MIC 8–64 mg/L), suggesting the presence of an MBL. PCR amplification and sequencing revealed the presence of *bla<sub>VIM-2</sub>*. No other carbapenemase was detected in these strains. MBL-producing *P. aeruginosa* have been described worldwide, and VIM-2 is the most prevalent MBL, reported from all continents,<sup>10</sup> particularly in southern European countries such as Italy, Greece and France.<sup>11</sup> Recent studies have reported the dissemination of VIM-2 *P. aeruginosa* in Tunisia, mainly due to the spread of clonal strains.<sup>12–14</sup>

### *bla<sub>VIM-2</sub>* genetic support and environment

Despite repeated attempts, imipenem resistance transfer to an azide-resistant mutant of *E. coli* yielded no transconjugants (<10<sup>−8</sup>). Plasmid analysis of Pa25 failed to detect a plasmid, and transformation by electroporation of putative plasmid DNA extract into *E. coli* TOP10 or *P. aeruginosa* ATCC 27853 was unsuccessful, suggesting a chromosomal location of the gene.

In the nine clinical isolates, characterization of the *bla<sub>VIM-2</sub>* flanking regions showed that this gene was located on the same large and original integron of ~7500 bp (Figure 1). It was found to contain *bla<sub>VIM-2</sub>* and *bla<sub>OXA-2</sub>* (β-lactam resistance), two copies of *aadB* (gentamicin and tobramycin resistance), *arr-6* (rifampicin resistance) and *aadA1a* (streptomycin and spectinomycin resistance). In addition, this integron contains a *qnrVC1* gene cassette encoding quinolone resistance that has been previously reported in a class 1 integron of a clinical Brazilian *Vibrio cholerae* strain.<sup>15</sup> The *qnrVC1* determinant showed higher homology with the chromosomally encoded *qnr* genes of Vibrionaceae, especially *Photobacterium profundum*, than with the plasmid-mediated *qnr* genes of Enterobacteriaceae.<sup>16</sup> In our strains, the *qnrVC1* gene cassette was composed of an *attC* site that is similar to the *Vibrio parahaemolyticus* repeat site and is characteristic of superintegrons. Moreover, this *attC* exhibits the core site GTTATGT and the inverse core site ACATAAC. Moreover, the gene cassette contains a 5'-untranscribed region including a functional promoter.<sup>17</sup> This is the first known description of *qnrVC* in *P. aeruginosa* located in a new integron. In addition, this integron possesses genes encoding a bacterial group II intron. This intron belongs to the group IIC-*attC* introns, and the feature of this group is to be inserted in the inverse core site of the *attC*



**Figure 1.** Schematic representation of the variable region of the integron characterized in *P. aeruginosa* Pa25. The arrows indicate the translation orientation. The solid lines represent the sequenced fragment from *P. aeruginosa* Pa25 with the ORFs in boxes. The sequences above correspond to the recombination site in the beginning of the *qnrVC1* gene cassette and to the *attC* site, respectively. The core site and the inverse core site are underlined, the *qnrVC1* promoter is in bold and the start codon (ATG) of *qnrVC1* is indicated in italics. The sequence below represents the integration site of the group II intron in the *attC* site of the *aadA1* gene cassette. The stop codon (TAA) of *aadA1* is indicated in italics, and the inverse core site of *aadA1* is underlined.

**Table 1.** Quinolone MICs for the clinical strain Pa25, the recipient *E. coli* TOP10 and *P. aeruginosa* ATCC 27853 strains, and the transformed strains containing the *qnrVC1* gene with its own promoter inserted into the vector pGEMT or pCN60

Strain	MIC (mg/L)					
	NAL	NOR	OFX	MXF	CIP	LVX
<i>E. coli</i> TOP10	1	0.023	0.016	0.004	0.003	0.008
<i>E. coli</i> TOP10 (pGEMT <i>qnrVC1</i> )	2	0.5	0.38	0.125	0.125	0.19
<i>E. coli</i> TOP10 (pCN60 <i>qnrVC1</i> )	2	0.75	0.5	0.19	0.25	0.25
<i>P. aeruginosa</i> Pa25	>256	>256	>32	>32	>32	>32
<i>P. aeruginosa</i> ATCC 27853	>256	1.5	2	1.5	0.19	0.75
<i>P. aeruginosa</i> (pCN60 <i>qnrVC1</i> )	>256	1.5	1.5	1.5	0.19	0.75

NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; MXF, moxifloxacin; CIP, ciprofloxacin; LVX, levofloxacin.

recombination site of the *aadA1* gene cassette (Figure 1) in the opposite orientation from the gene cassette. In this study, the sequence shows 78% homology with the most closely related sequence found in an integron from a South Korean strain of *P. aeruginosa* (GenBank number JF429900). Recently, a potential role for group IIC-*attC* introns in gene cassette formation has been suggested,<sup>18,19</sup> and its presence in our integron likely represents this role.

Expression of the qnrVC1 determinant

As for some rare gene cassettes, the one described here encompassing *qnrVC1* possesses a promoter. In the one previously described gene cassette that is similar, this promoter seemed functional by 5'RACE.<sup>17</sup> To explore the expression of *qnrVC1* and its role in quinolone resistance, the gene with its promoter were cloned into *E. coli* and into *P. aeruginosa*. Various transformants containing recombinant plasmids constructed using pGEMT in *E. coli* and pCN60 in *E. coli* and *P. aeruginosa* were obtained. By sequencing, the recombinant plasmids were chosen to be unaffected by the pGEMTplac promoter. Quinolone MIC determination showed that the *qnrVC1* gene with its own promoter conferred decreased susceptibility to fluoroquinolones, especially ciprofloxacin, in *E. coli* (MIC 0.125 versus 0.006 mg/L for the

recipient strain; Table 1). QnrVC1 protein affected nalidixic acid only slightly, in contrast to most plasmid-mediated Qnr in Enterobacteriaceae.<sup>16</sup> However, the level of decreased susceptibility to fluoroquinolones was close to that observed with the other *qnr* genes, i.e. a 42-fold increase in the ciprofloxacin MIC.<sup>20</sup> In contrast, *qnrVC1* seemed to have no effect in *P. aeruginosa*, possibly due to the inefficiency of the promoter or the protein in this species. However, in the clinical strain Pa25, fluoroquinolone MICs were very high (ciprofloxacin MIC >32 mg/L) and quinolone resistance-determining region sequencing revealed two common substitutions, Ile for Thr-83 in GyrA and Leu for Ser-87 in ParC, that explain the high-level quinolone resistance in this strain.<sup>9</sup> No other plasmid-mediated quinolone resistance was detected in the clinical strain.

Conclusions

In conclusion, this burn unit underwent a prolonged outbreak of VIM-2-producing *P. aeruginosa* belonging to different clones. Analysis of these epidemic strains led us to characterize an original integron containing the *qnrVC1* cassette and a new group IIC-*attC* intron. This study emphasizes the strategic role of integrons in the emergence of new resistance determinants, which may be linked to group IIC-*attC* intron presence, and the role of

*P. aeruginosa* in the dissemination of these determinants from environmental strains to clinical ones. This is the first known description of a *qnrVC* or even a *qnr* gene in *P. aeruginosa* from where these resistant genes could be transferred to enterobacterial species. We showed that *QnrVC1* confers low-level fluoroquinolone resistance to *E. coli*, and *Qnr* proteins are known to facilitate the emergence of high-level quinolone-resistant mutants by raising the level at which they can be selected.<sup>20</sup>

## Acknowledgements

Part of this study was presented at the Forty-ninth Interscience Conference of Antimicrobial Agents and Chemotherapy, San Francisco, CA, USA, 2009 (C2-365), at the Fifty-second Interscience Conference of Antimicrobial agents and Chemotherapy, San Francisco, CA, USA, 2012 (C2-719) and at the Twenty-fourth European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 2014 (number ECCMI-3724).

## Funding

This work was supported by grants from the Ministère de l'Éducation Nationale et de la Recherche and from the UMRCNRS 5234, Université de Bordeaux 2, Bordeaux, France.

Sequencing experiments were performed at the Genotyping and Sequencing Facility of Bordeaux [supported by grants from the Conseil Régional d'Aquitaine (20030304002FA) and from the European Union, FEDER (2003227)].

## Transparency declarations

None to declare.

## References

- Branski LK, Al-Mousawi A, Rivero H et al. Emerging infections in burns. *Surg Infect (Larchmt)* 2009; **10**: 389–97.
- Miriagou V, Cornaglia G, Edelstein M et al. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. *Clin Microb Infect* 2010; **16**: 112–22.
- Thabet L, Zoghalmi A, Boukadida J et al. Comparative study of antibiotic resistance in bacteria isolated from burned patients during two periods (2005–2008, 2008–2011) and in two hospitals (Hospital Aziza Othmana, Trauma and Burn Center). *Tunis Med* 2013; **91**: 134–8.
- Dubois V, Poirel L, Marie C et al. Molecular characterization of a novel class 1 integron containing *bla<sub>GES-1</sub>* and a fused product of *aac3-Ib/aac6'-Ib'* gene cassettes in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2002; **46**: 638–45.
- Carrer A, Poirel L, Eraksoy H et al. Spread of OXA-48-positive carbapenem-resistant *Klebsiella pneumoniae* isolates in Istanbul, Turkey. *Antimicrob Agents Chemother* 2008; **52**: 2950–4.
- Dubois V, Arpin C, Quentin C et al. Decreased susceptibility to cefepime in a clinical strain of *Escherichia coli* related to plasmid- and integron-encoded OXA-30  $\beta$ -lactamase. *Antimicrob Agents Chemother* 2003; **47**: 2380–1.
- Ploy MC, Denis F, Courvalin P et al. Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Antimicrob Agents Chemother* 2000; **44**: 2684–8.
- Poirel L, Schrenzel J, Cherkaoui A et al. Molecular analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland. *J Antimicrob Chemother* 2011; **66**: 1730–3.
- Akasaka T, Tanaka M, Yamaguchi A et al. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. *Antimicrob Agents Chemother* 2001; **45**: 2263–8.
- Hawkey PM, Jones AM. The changing epidemiology of resistance. *J Antimicrob Chemother* 2009; **64** Suppl 1: i3–10.
- Walsh TR. Emerging carbapenemases: a global perspective. *Int J Antimicrob Agents* 2010; **36** Suppl 3: S8–14.
- Hammami S, Boutiba-Ben Boubaker I, Ghazzi R et al. Nosocomial outbreak of imipenem-resistant *Pseudomonas aeruginosa* producing VIM-2 metallo- $\beta$ -lactamase in a kidney transplantation unit. *Diagn Pathol* 2011; **6**: 106.
- Ktari S, Mnif B, Znazen A et al. Diversity of  $\beta$ -lactamases in *Pseudomonas aeruginosa* isolates producing metallo- $\beta$ -lactamase in two Tunisian hospitals. *Microb Drug Resist* 2011; **17**: 25–30.
- Mansour W, Poirel L, Bettaieb D et al. Metallo- $\beta$ -lactamase-producing *Pseudomonas aeruginosa* isolates in Tunisia. *Diagn Microbiol Infect Dis* 2009; **64**: 458–61.
- da Fonseca EL, Dos Santos Freitas F, Vieira VV et al. New *qnr* gene cassettes associated with superintegron repeats in *Vibrio cholerae* O1. *Emerg Infect Dis* 2008; **14**: 1129–31.
- Cattoir V, Nordmann P. Plasmid-mediated quinolone resistance in Gram-negative bacterial species: an update. *Curr Med Chem* 2009; **16**: 1028–46.
- da Fonseca EL, Vicente AC. Functional characterization of a cassette-specific promoter in the class 1 integron-associated *qnrVC1* gene. *Antimicrob Agents Chemother* 2012; **56**: 3392–4.
- Leon G, Roy PH. Potential role of group IIC-*attC* introns in integron cassette formation. *J Bacteriol* 2009; **191**: 6040–51.
- Rodriguez-Martinez JM, Nordmann P, Poirel L. Group IIC intron with an unusual target of integration in *Enterobacter cloacae*. *J Bacteriol* 2012; **194**: 150–60.
- Rodriguez-Martinez JM, Cano ME, Velasco C et al. Plasmid-mediated quinolone resistance: an update. *J Infect Chemother* 2011; **17**: 149–82.