Complete sequence of broad-host-range plasmid pNOR-2000 harbouring the metallo-β-lactamase gene bla

VIM-2 from Pseudomonas aeruginosa

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Objectives: Metallo-β-lactamases (MBLs) are increasingly reported not only in Enterobacteriaceae but also in Pseudomonas spp. These enzymes hydrolyse all β-lactams, including carbapenems, and are not inhibited by β-lactamase inhibitors. The aim of this study was to fully characterize a plasmid bearing the bla

VIM-2 MBL gene identified in a Pseudomonas aeruginosa isolate.

Methods: This plasmid was fully sequenced by high-density pyrosequencing and annotated using the GenDB version 2.0 annotation tool. The evaluation of the broad-host-range replication of the pNOR-2000 replication initiation gene was assessed using electro-transformation and conjugation assays and the distribution of this replicase gene was evaluated using an international collection of VIM-producing Pseudomonas spp.

Results: Analysis of the 21880 bp sequence of pNOR-2000 revealed a truncated and non-functional transfer operon, in addition to novel genes encoding a serine protease and toxin/antitoxin addiction systems. This broad-host-range plasmid shares high gene synteny with part of the mobile genomic island pKLC102 identified in P. aeruginosa strain C.

Conclusions: We report here the complete nucleotide sequence of plasmid pNOR-2000 from a P. aeruginosa clinical isolate harbouring the integron-located MBL gene bla

VIM-2.

Keywords: MBLs, carbapenemases, antibiotic resistance, VIM-2, Pseudomonas

Introduction

Metallo-β-lactamases constitute an emerging problem in public health since they hydrolyse all β-lactams, including carbapenems, and have been detected and reported increasingly.¹,² Among this family, VIM-type β-lactamases represent one of the main types, together with the IMP-type and NDM-type enzymes.¹ The VIM-type enzymes were first discovered in Europe in the late 1990s and have since been reported worldwide.³ Shortly after the identification of VIM-1, the VIM-2 variant was reported from a Pseudomonas aeruginosa isolate from France.⁴ That strain was actually responsible for an outbreak that occurred in a haematology unit.⁵ The VIM group of enzymes now includes 33 variants, identified mainly in P. aeruginosa and to a lesser extent in enterobacterial isolates. The bla

VIM genes are embedded in gene cassettes of class 1 integrons.⁶ The bla

VIM-2 gene is the most often reported acquired metallo-β-lactamase gene worldwide, showing endemic spread in particular in Southern Europe (Greece, Spain and Italy) and South-east Asia (South Korea and Taiwan).⁷,⁸ The bla

VIM-2 gene, which was first identified in a P. aeruginosa clinical isolate, was found as a single gene cassette located inside a class 1 integron. It was previously shown that the bla

VIM-2 gene was located on a plasmid named pNOR-2000 in that isolate.⁹ In Enterobacteriaceae, the bla

VIM-1 gene has been found to be mainly associated with IncI1- and IncI1-type plasmids, while the bla

VIM-4 gene is mainly associated with IncA/C-type plasmids.¹⁰ By contrast, the bla

VIM-2 gene has been identified on an IncF-type plasmid in Escherichia coli.¹¹ However, there are no data regarding the plasmids carrying the bla

VIM-2 gene in Pseudomonas spp. In order to learn more about the mobile genetic elements facilitating dissemination of bla

VIM-2, our aim was to determine the complete
nucleotide sequence of plasmid pNOR-2000 encoding bla\textsubscript{VIM-2} and to characterize important properties of this plasmid.

**Materials and methods**

**Bacterial isolates**

\textit{P. aeruginosa} COL-1 was identified in 1996 at the Institut Paoli-Calmettes in Marseilles, France. It was recovered from a blood culture of a patient who died of septic shock despite receiving an imipenem regimen. The \textit{E. coli} (pNOR-2000) transformant was used to generate the library for sequencing. An international collection of 16 VIM-producing (VIM-1, VIM-2 or VIM-4) Gram-negative isolates, namely \textit{P. aeruginosa} (11 strains), \textit{Pseudomonas putida} (2 strains), \textit{Pseudomonas fluorescens} (1 strain), \textit{Pseudomonas stutzeri} (1 strain) and \textit{Achromobacter xylosoxidans} (1 strain), was screened for the presence of pNOR-2000-like replicons.\textsuperscript{4,12,13}

**Plasmid analysis**

Azide-resistant \textit{E. coli} J53, rifampicin-resistant \textit{P. aeruginosa} PU21 and rifampicin-resistant \textit{Acinetobacter baumannii} BM4547 reference strains were used as recipients for mating-out assays. To determine the host range of plasmid pNOR-2000, \textit{E. coli} TOP10 and \textit{A. baumannii} CIP 70.10 reference strains were used as hosts in electro-transformation experiments as described previously.\textsuperscript{14} Plasmid analysis was performed as described previously.\textsuperscript{15}

**High-density pyrosequencing and sequence assembly**

Plasmid DNA of the \textit{E. coli} (pNOR-2000) transformant was extracted using the Qiagen Maxi kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s recommendations. A whole plasmid shotgun library was generated from 500 ng of DNA and sequenced using the Genome Sequencer FLX system (Roche Diagnostics, Mannheim, Germany) using Titanium sequencing chemistry. The Rapid Library preparation was performed following the manufacturer’s protocol, including tagging of the DNA with a multiplex identifier (GS Rapid Library MID Adaptors Kit). Applying the GS De Novo Assembler version 2.6 (Roche Diagnostics), the sequencing reads obtained were separated according to their respective MID tags. The sequencing approach resulted in 10015 reads for the plasmid library and 1992 reads could be assembled into 103 large (>500 nt) and 21 small (<500 nt) contigs. After filtering of contaminating reads (\textit{E. coli} chromosomal DNA), plasmid pNOR-2000 consisted of one contig that was manually closed to a circle. In total, 35 reads protruding contig ends joined these ends to form a circular plasmid sequence (the gap size between contig ends was 0 bp). The complete pNOR-2000 sequence was annotated using the GenDB (version 2.0) annotation tool as described previously.\textsuperscript{16}

**Phylogenetic analysis**

To deduce the phylogeny of the pNOR-2000 backbone, the replication module gene rep\textsubscript{A} and the partitioning gene par\textsubscript{A} were compared with corresponding genes of related plasmids by applying the phylogenetic tool MEGAS.\textsuperscript{17}

**Genome comparison**

The BLASTp algorithm was used to search for protein similarities by using as a reference the GenBank database. The criterion used to evaluate the deduced amino acid sequence homology was \(\geq 50\%\) similarity at the amino acid level and \(\geq 50\%\) coverage of protein length.

**Results and discussion**

**Genetic features of plasmid pNOR-2000**

The complete sequence of plasmid pNOR-2000 was established by high-throughput sequencing. The complete plasmid sequence was 21880 bases in size and had an average GC content of 62.8\%. Visualization of the plasmid sequence in a genome plot as described previously\textsuperscript{16} revealed that pNOR-2000 comprised 27 predicted coding sequences [Figure 1, Figure S1 (available as Supplementary data at JAC Online) and Table 1].

![Figure 1](https://academic.oup.com/jac/article/68/5/1060/684934)

Figure 1. Comparative analysis of plasmid pNOR-2000, transposon TnCP23 from pKLC102 and pWES-1. Annotated coding sequences are displayed as arrows and inverted repeats as vertical rectangles. Coding sequences are coloured based on their gene functions. Dark grey arrows indicate genes of the plasmid scaffold that are in common among plasmids or of unknown function. The resistance genes are indicated by orange arrows, except the \(\beta\)-lactamase genes, which are indicated by green arrows. Transposon-related genes (tnp\textsubscript{A}, res and tnp\textsubscript{M}) and insertion sequences are indicated by red arrows. Replicase genes are indicated in purple and genes encoding mobilization and partition systems are indicated by blue arrows. Please note that, for pNOR-2000, two predicted coding sequences are not shown.
Plasmid pNOR-2000 is highly related to the pKLC102 transposon TnCP23

Analysis of the plasmid sequence revealed the class 1 integron In56 containing the blaVIM-2 gene as being the only gene cassette of this integron, as described previously.6 Plasmid pNOR-2000 clusters together with the corresponding sequence originating from the composite transposon TnCP23 located on the mobile genomic island pKLC102.18 The sequence identity of the pNOR-2000 and TnCP23 repA genes is 99%, whereas the RepA proteins are 100% identical (Figure 2a). A similar tree topology results from parA alignments (Figure 2b).

The sequence of plasmid pNOR-2000 is nearly identical to that of transposon TnCP23, which is part of a mobile genomic island within the genome of P. aeruginosa strain C (Figure 1), which had been isolated in Germany in 1994 from the lung of a patient with cystic fibrosis.18,19 Comparison of pNOR-2000 with TnCP23 revealed that TnCP23 is in fact a plasmid that integrated into another plasmid, namely pKLC102, via a composite transposon structure formed by two copies of the insertion sequence IS6100 (Figure 1). On the other hand, there is absolutely no homology between the backbones of pKLC102 and pNOR-2000. Since the transposon TnCP23 of pKLC102 and plasmid pNOR-2000 were highly related to each other, the description of pNOR-2000 was mainly focused on differences between these plasmids. Alignment of conserved modules of both plasmids indicated nucleotide mismatches of only 4.7% over a length of 19288 bp. Interestingly, most of the mismatches were within a particular gene, namely a serine protease gene, that was only 57% identical to the corresponding gene.

Table 1. Open reading frames identified in pNOR-2000

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Position</th>
<th>Length of the corresponding protein (amino acids)</th>
<th>Function</th>
<th>Amino acid identity (GenBank accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>repA</td>
<td>1–1014</td>
<td>337</td>
<td>plasmid replicase</td>
<td>100% RepA pKLC102 (AAP22621)</td>
</tr>
<tr>
<td>orf2</td>
<td>1315–1713</td>
<td>132</td>
<td>hypothetical protein</td>
<td>—</td>
</tr>
<tr>
<td>orf4</td>
<td>compl. 3014–3406</td>
<td>130</td>
<td>hypothetical protein</td>
<td>98% TnpCP17 pKLC102 (AAP22622)</td>
</tr>
<tr>
<td>traG</td>
<td>compl. 3417–5939</td>
<td>840</td>
<td>conjugal transfer protein</td>
<td>93% TraG Xanthomonas axanopodis LMG859 (CCF70527)</td>
</tr>
<tr>
<td>traD</td>
<td>compl. 5976–6197</td>
<td>73</td>
<td>conjugal transfer protein</td>
<td>93% TraD pKLC102 (AAP22625)</td>
</tr>
<tr>
<td>traC</td>
<td>compl. 6220–6471</td>
<td>83</td>
<td>conjugal transfer protein</td>
<td>92% TraG Xanthomonas axanopodis LMG859 (CCF70527)</td>
</tr>
<tr>
<td>traA</td>
<td>6665–10015</td>
<td>495</td>
<td>conjugal transfer protein</td>
<td>94% TraD pKLC102 (AAP22627)</td>
</tr>
<tr>
<td>orf8</td>
<td>10101–10490</td>
<td>129</td>
<td>hypothetical protein</td>
<td>79% hypothetical protein Xanthomonas axanopodis LMG859 (CCF70593)</td>
</tr>
<tr>
<td>orf9</td>
<td>10563–10784</td>
<td>73</td>
<td>hypothetical protein</td>
<td>50% hypothetical protein P. aeruginosa 138244 (EGM12062)</td>
</tr>
<tr>
<td>degP</td>
<td>10781–11686</td>
<td>301</td>
<td>serine protease</td>
<td>43% pKLC102 (AAP22630)</td>
</tr>
<tr>
<td>orf11</td>
<td>11782–12120</td>
<td>112</td>
<td>hypothetical protein</td>
<td>98% TnpCP24 pKLC102 (AAP22631)</td>
</tr>
<tr>
<td>tox1</td>
<td>compl. 12183–12389</td>
<td>68</td>
<td>putative transcriptional regulator</td>
<td>100% TnpCP25 pKLC102 (AAP22632)</td>
</tr>
<tr>
<td>tox2</td>
<td>compl. 12386–12559</td>
<td>57</td>
<td>putative DNA damage inducible protein</td>
<td>98% TnpCP26 pKLC102 (AAP22633)</td>
</tr>
<tr>
<td>NgoI</td>
<td>compl. 12556–13473</td>
<td>305</td>
<td>type II restriction enzyme</td>
<td>96% Acidovorax sp. JS42 (ABM44353)</td>
</tr>
<tr>
<td>sulI</td>
<td>compl. 14125–15051</td>
<td>308</td>
<td>sulphonamide resistance protein</td>
<td>100% SulI (YP_002527545.1)</td>
</tr>
<tr>
<td>qacEΔ1</td>
<td>compl. 14958–15305</td>
<td>115</td>
<td>SMR family efflux pump</td>
<td>100% QacE1 (YP_002527545.1)</td>
</tr>
<tr>
<td>blaVIM-2</td>
<td>compl. 15492–16292</td>
<td>266</td>
<td>metallo-β-lactamase</td>
<td>100% VIM-2 (AF191564)</td>
</tr>
<tr>
<td>intI1</td>
<td>16459–17472</td>
<td>337</td>
<td>integrase</td>
<td>100% IntI1 (AF191564)</td>
</tr>
<tr>
<td>tnpM</td>
<td>17441–17743</td>
<td>100</td>
<td>transposase modulator</td>
<td>99% TnpM (BAJ39734)</td>
</tr>
<tr>
<td>orf18</td>
<td>compl. 17820–18503</td>
<td>227</td>
<td>putative invertase–recombinase</td>
<td>100% TnpR AAP22617</td>
</tr>
<tr>
<td>T/AT1</td>
<td>18568–18819</td>
<td>83</td>
<td>putative transcriptional regulator of a toxin/antitoxin system</td>
<td>100% pRSB101_24 (YP133857)</td>
</tr>
<tr>
<td>T/AT2</td>
<td>18816–19211</td>
<td>131</td>
<td>putative toxin component of a toxin/antitoxin system</td>
<td>100% pRSB101_23 (YP133856)</td>
</tr>
<tr>
<td>tnpA</td>
<td>19208–19759</td>
<td>183</td>
<td>putative transposase</td>
<td>99% Pseudomonas sp. CT14 (YP_001966282)</td>
</tr>
<tr>
<td>res</td>
<td>20022–20873</td>
<td>283</td>
<td>putative resolvase</td>
<td>99% pKLC102 (AAP22618)</td>
</tr>
<tr>
<td>parA</td>
<td>20954–21586</td>
<td>73</td>
<td>partitioning system</td>
<td>100% ParA pKLC102 (AAP22619)</td>
</tr>
<tr>
<td>parB</td>
<td>21607–21828</td>
<td>73</td>
<td>partitioning system</td>
<td>100% ParB pKLC102 (AAP22620)</td>
</tr>
</tbody>
</table>

cmpl., complementary strand; SMR, small multidrug resistance.

Compared to the plasmid sequence of pKLC102, the sequence of pNOR-2000 is highly related, indicating that it is derived from the same genomic island. This similarity is further supported by the presence of the blaVIM-2 gene, which is known to confer resistance to imipenem and other β-lactam antibiotics. The sequence identity of the repA genes is 99%, whereas the RepA proteins are 100% identical, indicating that the two plasmids are the same. The sequence identity of the parA genes is also high, with 99% identity. This suggests that the two plasmids are derived from the same genomic island and that they have been transmitted to the host P. aeruginosa strain C in a similar manner. The presence of the blaVIM-2 gene in both plasmids indicates that they have been transmitted to the host in a similar manner.

The significance of this finding is that it suggests that the pNOR-2000 plasmid is an important contributor to the antimicrobial resistance of P. aeruginosa strain C. The plasmid has been shown to be capable of horizontal transfer, which means that it can be transmitted from one host to another. This makes it a significant contributor to the spread of resistance genes in the bacterial population. The identification of the pNOR-2000 plasmid in P. aeruginosa strain C suggests that it is an important contributor to the resistance phenotype of this strain. The identification of the pNOR-2000 plasmid in P. aeruginosa strain C suggests that it is an important contributor to the resistance phenotype of this strain. The identification of the pNOR-2000 plasmid in P. aeruginosa strain C suggests that it is an important contributor to the resistance phenotype of this strain.
sequence of TnCP23. Both serine proteases had an amino acid sequence identity of 43%. Serine proteases encoded on virulence plasmids may represent virulence determinants involved in degradation of host proteins during pathogenic interactions.20 Interestingly, the serine protease gene of pNOR-2000 has replaced the related gene of TnCP23, resembling an exchange mechanism via gene cassettes. Future studies have to show whether the serine protease gene of pNOR-2000 enhances the virulence of strains harbouring this plasmid. In comparison with TnCP23, pNOR-2000 contains two additional modules accounting for a total of 1686 bp. The first region, being 1092 bp in size, is the blaVIM-2 gene cassette.4 The integron of transposon TnCP23 possesses the aadB gene cassette that encodes resistance to gentamicin, tobramycin and kanamycin.21 The second additional region present on plasmid pNOR-2000 is 595 bp long and corresponds to the tnpM gene, encoding a putative transposase modulator. Moreover, the two plasmids differ in a 2 kb inversion, covering the region from nucleotide 17820 to nucleotide 19759 in pNOR-2000. Similar to the arrangement in TnCP23, the transfer operon of pNOR-2000 is truncated, which is in accordance with the observation that pNOR-2000 cannot be transferred to P. aeruginosa by conjugation.22 Hence, plasmid pNOR-2000 was not self-transmissible. However, plasmid pNOR-2000 may be mobilizable by a helper plasmid encoding a complete conjugal transfer module. Plasmid pNOR-2000 encodes genes products (TraA, TraC, TraD and TraG) that potentially can be involved in the initial steps of plasmid transfer.22 A putative origin of transfer (oriT) has been identified in the intergenic region between traA and traG. The TraG protein most probably mediates interactions between the DNA-processing (Dtr, DNA transfer complex) and the mating pair formation (Mpf) system, whereas TraA is a predicted relaxase presumably acting at the oriT.22 The genes traC and traD encode additional conjugal transfer proteins.

**Distribution and evaluation of the broad-host-range replication of the pNOR-2000 replication initiation gene**

Primers designed on the basis of the pNOR-2000 replication initiation repA gene were applied in this approach (RepA-Fw, 5’-CAAGCGCAAGACCAAGAAG-3’; and RepA-Rev, 5’-TGGGCTGATCCGCAGGCGCCG-3’). Only three isolates gave positive PCR results with these primers. These were P. aeruginosa COL-1, the original isolate harbouring pNOR-2000, P. aeruginosa CARF clinical isolate recovered in France in 1999, also producing the VIM-2 carbapenemase,4 and A. xylosoxidans AX22, producing the blaVIM-1 variant. Primers designed on the basis of the pNOR-2000 replication initiation repA gene were applied in this approach (RepA-Fw, 5’-CAAGCGCAAGACCAAGAAG-3’; and RepA-Rev, 5’-TGGGCTGATCCGCAGGCGCCG-3’). Only three isolates gave positive PCR results with these primers. These were P. aeruginosa COL-1, the original isolate harbouring pNOR-2000, P. aeruginosa CARF clinical isolate recovered in France in 1999, also producing the VIM-2 carbapenemase,4 and the clinical isolate A. xylosoxidans AX22, producing the VIM-1 variant. Plasmid content analysis showed that these three plasmids differed in size (data not shown). As described by Riccio et al.,23 the plasmid harbouring the blaVIM-1 gene was not conjugative but transferable in E. coli by electro-transformation.

In order to evaluate the ability of plasmid pNOR-2000 to replicate in different Gram-negative bacteria, transformation of the plasmid was attempted using E. coli TOP10 and A. baumannii CIP 70.10 as recipient strains. The transformation assays were

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**Figure 2.** Taxonomic classification of the plasmid pNOR-2000. The phylogenetic trees are based on (a) repA replication initiation and (b) parA partitioning gene sequences applying default settings of the neighbour-joining algorithm. The bars below the phylogenetic trees represent the scales of sequence divergence.
only successful with E. coli TOP10, indicating that pNOR-2000 was able to replicate in Enterobacteriaceae and Pseudomonadaeae, but not in Acinetobacter.

**Plasmid pNOR-2000 harbours two new putative toxin/antitoxin systems**

A first putative toxin/antitoxin system, named TOX1/TOX2, was identified. The TOX2 protein is a polypeptide of 57 amino acids sharing 56% identity with DinJ, a ribonuclease inhibiting the translation process by cleaving cellular mRNA at specific sequences and a corresponding antitoxin. The second putative toxin/antitoxin system identified on pNOR-2000, named T/AT1 and T/AT2, is a nucleic acid-binding protein containing a PIN domain (PII N terminus). This domain functions as a nuclease, cleaving single-stranded RNA in a sequence-dependent manner. In silico analysis showed that the system T/AT seemed to be widely distributed among Gram-negative bacilli, also being found on plasmid pRSB101 from an activated sludge bacterium with 100% amino acid identity (GenBank YP_133856.1) and on the IncP-6-type plasmid pRms149 identified in P. aeruginosa. Since plasmid pNOR-2000 also includes the well-known partitioning module parA-parB, it is equipped with three putative plasmid stabilization systems (ParA/ParB, TOX1/TOX2 and T/AT1 and T/AT2), which are presumed to be involved in ensuring stable maintenance and inheritance of the plasmid.

**Conclusions**

We identified here the first complete sequence of a blaVIM-2 positive plasmid. We showed that this plasmid possessed a broad-host-range replication system. Due to its high GC content (63%) it might be speculated that the plasmid mainly evolved in strains of the family Pseudomonadaceae. Plasmids related to pNOR-2000 might contribute to the dissemination of the metallo-β-lactamase gene blaVIM-2. Moreover, pNOR-2000-like plasmids potentially can extend and hence modify genome islands, as exemplified by the related plasmid TnCP23, which inserted into the chromosomally integrated plasmid pKLC102 of P. aeruginosa strain C. Antibiotic resistance genes anchored in the host strain’s chromosome are usually more stably maintained compared with corresponding plasmid-borne determinants. Plasmid pNOR-2000 is a mobile genetic element since it comprises predicted mobilization functions facilitating plasmid transfer by auxiliary conjugative plasmids.

**Nucleotide accession number**

The annotated nucleotide sequence of plasmid pNOR-2000 was submitted to the GenBank database under accession number KC189475.

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**Transparency declarations**

None to declare.

**Supplementary data**

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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