Complete sequence of two KPC-harbouring plasmids from *Pseudomonas aeruginosa*

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**Objectives:** KPC-producing *Pseudomonas aeruginosa* are increasingly isolated in the Americas and in the Caribbean islands. Here, we determined the whole-plasmid sequence of two plasmids carrying the \( \text{bla}_{\text{KPC-2}} \) gene from multidrug-resistant *P. aeruginosa* clinical isolates from Colombia.

**Methods:** The two plasmids, pCOL-1 and pPA-2, were transferred to *Escherichia coli* recipient strain TOP10 and completely sequenced using high-throughput pyrosequencing for pCOL-1 and classical Sanger sequencing for pPA-2.

**Results:** Both plasmids could be transferred to *E. coli* by transformation and displayed no other resistance marker besides KPC. Plasmid pCOL-1 was 31 529 bp in size, contained 31 open reading frames (ORFs) and belonged to the IncP-6 replicon group. It exhibited genes involved in replication, mobilization and partitioning, but none involved in conjugation. Plasmid pPA-2 was 7 995 bp in size and contained seven ORFs. It exhibited a replicase gene of IncU, but was lacking genes involved in mobilization, partitioning and conjugation. Only 2072 bp matched Tn4401, including the \( \text{bla}_{\text{KPC-2}} \) gene, part of ISKpn6 and a 73 bp segment located upstream of the \( \text{bla}_{\text{KPC-2}} \) gene, containing the P1 promoter. Sequence identity was interrupted by a Tn3 transposon, itself interrupted by an IS26 element inserted within the \( \beta \)-lactamase \( \text{bla}_{\text{TEM-1}} \) gene.

**Conclusions:** Here we present the genetic features of the very first plasmids carrying the \( \text{bla}_{\text{KPC-2}} \) gene from *P. aeruginosa*. The emergence of the \( \text{bla}_{\text{KPC-2}} \) gene on unrelated plasmids, differing in size and in incompatibility group, and harbouring different genetic structures containing the \( \text{bla}_{\text{KPC-2}} \) genes in *P. aeruginosa* isolates suggest that this resistance trait may follow a dissemination scheme in *P. aeruginosa* similar to that seen in Enterobacteriaceae.

**Keywords:** carbapenemases, antibiotic resistance, KPC, Gram-negative

**Introduction**

Carbapenem resistance in *Pseudomonas aeruginosa* is mainly related to acquired carbapenem-hydrolysing \( \beta \)-lactamases or OprD2 porin deficiency.\(^1\)\(^,\)\(^2\) Carbapenemases are able to hydrolyse most if not all \( \beta \)-lactams, including carbapenems.\(^3\) The carbapenemases currently found in *P. aeruginosa* belong to Ambler classes A (GES and KPC), B (IMP, VIM, NDM, SPM, AIM and GIM) and rarely D (OXA-198).\(^1\)\(^,\)\(^2\)\(^,\)\(^6\) Currently, the class A carbapenemases most frequently found in *P. aeruginosa* are the KPC enzymes.\(^1\)

KPC carbapenemases were initially described in a *Klebsiella pneumoniae* isolate in 2001 in North Carolina,\(^5\) and have since rapidly emerged and disseminated throughout the world, in particular in enterobacterial species.\(^1\) KPC-producing *P. aeruginosa* isolates, initially reported in 2006 from Colombia\(^6\) and subsequently in Puerto Rico, in Trinidad and Tobago, in the USA\(^7\) and in China,\(^8\) are increasingly being isolated in the Americas and in the Caribbean region.\(^1\)\(^,\)\(^9\) The rapid spread of \( \text{bla}_{\text{KPC}} \) genes has been linked to their location on a Tn4401, a Tn3-based transposon capable of high-frequency transposition,\(^10\) which itself is present on a wide variety of plasmids varying in size, nature and structure between enterobacterial isolates.\(^5\)\(^,\)\(^11\) Another explanation is likely to be linked to its presence in epidemic clones such as *K. pneumoniae* ST258.\(^3\) Several KPC-producing *P. aeruginosa* isolates from different Colombian hospitals have revealed the spread of different clones, harbouring either chromosome- or plasmid-encoded \( \text{bla}_{\text{KPC}} \) genes. The plasmids were different in size and contained \( \text{bla}_{\text{KPC}} \) genes associated...
with two different genetic structures. Most of the isolates contained the isoform Tn4401b located on an ~40 kb plasmid, and one isolate contained a novel genetic environment of a blaKPC-2 gene located on an ~10 kb plasmid. The aim of this work was to characterize the complete sequence of and to provide further insight into these two different plasmids isolated from KPC-producing P. aeruginosa isolates from Colombia.

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

P. aeruginosa COL-1 was chosen as a representative KPC-2-producing P. aeruginosa isolate responsible for an outbreak in Medellin and harbouring KPC-2 embedded in a Tn4401b structure, while P. aeruginosa PA-2 was chosen as a KPC-2-producing P. aeruginosa isolate possessing a KPC-2 gene in a different genetic environment compared with Tn4401b. Escherichia coli TOP10 (Invitrogen, Eragny, France) was used for electroporation experiments as previously described. Transformants were selected on Trypticase soy agar plates supplemented with 100 mg/L amoxicillin. Routine antibiograms were determined by the disc diffusion method on Mueller–Hinton agar (Bio-Rad, Marnes-La-Coquette, France), and the susceptibility breakpoints were as recommended by the CLSI.

Genetic and molecular analyses

Whole DNAs were extracted using a QiaAmp DNA Mini Kit (Qiagen, Les Ulis, France). Plasmid DNA was extracted using the Qiagen Maxi Kit (Qiagen) and by the Kieser extraction method.

Table 1. ORFs identified in pCOL-1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Position (strand)</th>
<th>Length of the corresponding protein (amino acids)</th>
<th>Function</th>
<th>Amino acid identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>repA IncP-6</td>
<td>1–1374 (+)</td>
<td>458</td>
<td>replicase</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>Δorf6(1)</td>
<td>1839–1873 (+)</td>
<td>547</td>
<td>unknown</td>
<td>—</td>
</tr>
<tr>
<td>tnpA Tn4401</td>
<td>1929–3569 (+)</td>
<td>547</td>
<td>resolvase</td>
<td>100% with Tn4401a</td>
</tr>
<tr>
<td>orf6</td>
<td>2909–3685 (+)</td>
<td>293</td>
<td>OrfB transposase</td>
<td>100% with Tn4401a</td>
</tr>
<tr>
<td>blaKPC-2</td>
<td>10075–10454 (+)</td>
<td>259</td>
<td>transposase</td>
<td>100% with Tn4401a</td>
</tr>
<tr>
<td>tnpA Tn501-like</td>
<td>10509–11522 (+)</td>
<td>341</td>
<td>OrfA transposase</td>
<td>100% with Tn501</td>
</tr>
<tr>
<td>kfrA</td>
<td>12452–13396 (+)</td>
<td>275</td>
<td>carbapenemase</td>
<td>100% with KPC-2</td>
</tr>
<tr>
<td>tnpA Tn501-like</td>
<td>13748–14572 (+)</td>
<td>128</td>
<td>putative transposase</td>
<td>100% with TnpA YP_245473</td>
</tr>
<tr>
<td>mobE</td>
<td>15328–16303 (+)</td>
<td>163</td>
<td>hypothetical protein</td>
<td>98% with hypothetical protein pRSB105</td>
</tr>
<tr>
<td>mobD</td>
<td>16301–17301 (+)</td>
<td>216</td>
<td>mobilization protein</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>mobC</td>
<td>17301–18301 (+)</td>
<td>227</td>
<td>mobilization protein</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>mobB</td>
<td>18301–19301 (+)</td>
<td>227</td>
<td>mobilization protein</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>mobA</td>
<td>19301–20301 (+)</td>
<td>227</td>
<td>mobilization protein</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>orf3</td>
<td>20412–21399 (+)</td>
<td>227</td>
<td>mobilization protein</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>tnpA Tn501-like</td>
<td>22565–23430 (+)</td>
<td>227</td>
<td>hypothetical protein</td>
<td>99% with Tn501 transposes family</td>
</tr>
<tr>
<td>mobR Tn501-like</td>
<td>22606–23208 (+)</td>
<td>227</td>
<td>truncated transposase Tn3 family</td>
<td>99% with Tn501 transposes family</td>
</tr>
<tr>
<td>orf6</td>
<td>23833–24195 (+)</td>
<td>227</td>
<td>hypothetical protein</td>
<td>100% with pFBAOT6 YP_067863</td>
</tr>
<tr>
<td>tnpA ISAp1</td>
<td>24522–25787 (+)</td>
<td>227</td>
<td>transposase</td>
<td>100% with pFBAOT6 YP_067863</td>
</tr>
<tr>
<td>orf9</td>
<td>26286–25885 (+)</td>
<td>227</td>
<td>hypothetical protein</td>
<td>100% with pFBAOT6 YP_067863</td>
</tr>
<tr>
<td>corA</td>
<td>26381–27348 (+)</td>
<td>227</td>
<td>deleted magnesium and cobalt transporter</td>
<td>99% with pFBAOT6 YP_067863</td>
</tr>
<tr>
<td>GST-family protein</td>
<td>28049–29340 (+)</td>
<td>227</td>
<td>GST-family protein</td>
<td>81% with pFBAOT6 YP_067863</td>
</tr>
<tr>
<td>msrA</td>
<td>28721–28728 (+)</td>
<td>227</td>
<td>putative methionine sulfoxide reductase</td>
<td>100% with pFBAOT6 YP_067863</td>
</tr>
<tr>
<td>ΔTn3-family tnpA</td>
<td>29345–29701 (+)</td>
<td>227</td>
<td>truncated transposase Tn3 family</td>
<td>95% with transposase EHV82687</td>
</tr>
<tr>
<td>parA</td>
<td>30063–30698 (+)</td>
<td>227</td>
<td>ATPase involved in partitioning</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>parB</td>
<td>30784–31041 (+)</td>
<td>227</td>
<td>auxiliary partitioning protein</td>
<td>100% with pRSB105</td>
</tr>
<tr>
<td>parC</td>
<td>30978–31361 (+)</td>
<td>227</td>
<td>auxiliary partitioning protein</td>
<td>100% with pRSB105</td>
</tr>
</tbody>
</table>
Sanger sequencing was used to sequence small plasmids, using an Applied Biosystems sequencer (ABI 3100), and analysed with software available over the Internet at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov). Plasmid assembly was performed using the program DNA Strider 3.0 (CEA, Saclay, France). High-density pyrosequencing was used for larger plasmids, using an Illumina Genome Analyzer IIx system (Illumina Inc., San Diego, CA, USA). The complete sequencing work flow was performed by the DNA Vision company (Gosselies, Belgium). Plasmid assembly was performed using CLC Genomics Workbench 5 software (CLC Genomics, Copenhagen, Denmark).

Nucleotide accession numbers
The annotated nucleotide sequences of plasmids pCOL-1 and pPA-2 were submitted to the GenBank database and are accessible under the accession numbers KC609323 and KC609322, respectively.

Results and discussion
The plasmidic location of the \textit{bla}\textsubscript{KPC-2} gene in \textit{P. aeruginosa} COL-1 isolated from a hospital in Medellin, Colombia, was initially suggested by Cuzon et al.\textsuperscript{9} Subsequently, plasmids of similar size to pCOL-1 were identified in several KPC-producing \textit{P. aeruginosa} isolates from different Colombian cities.\textsuperscript{9} A second plasmid, pPA-2, different in size from pCOL-1, was recovered from a clinical isolate of \textit{P. aeruginosa} isolated in Bogota, Colombia.\textsuperscript{9} The whole-cell DNAs of these two \textit{P. aeruginosa} isolates were electroporated into \textit{E. coli} TOP10. Transformants of \textit{E. coli} TOP10 could be obtained with both natural plasmids.

The two transformants were resistant to amoxicillin, ticarcillin, piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, aztreonam and cefepime, and remained susceptible or with an intermediate range of susceptibility to carbapenems (imipenem MICs 1.5 and 3 mg/L, meropenem MICs 0.38 and 1.5 mg/L, and ertapenem MICs 0.50 and 3 mg/L for pCOL-1 and pPA-2, respectively). No other antibiotic resistance marker was co-transferred, as evidenced by susceptibility testing. The MICs of non-\textit{β}-lactam antibiotics for \textit{E. coli} TOP10 (pCOL-1), \textit{E. coli} TOP10 (pPA-2) and the parental \textit{E. coli} TOP10 strain were identical and remained in the susceptible range (tetracycline 6 mg/L, chloramphenicol 4 mg/L, rifampicin 1 mg/L, amikacin 0.125 mg/L, gentamicin 0.125 mg/L, nalidixic acid 4 mg/L, aztreonam 0.5 mg/L, ceftazidime 4 mg/L, cefepime 4 mg/L, cefotaxime 4 mg/L, ticarcillin 2 mg/L, amoxicillin 4 mg/L, piperacillin 2 mg/L, piperacillin/tazobactam 1 mg/L).

Figure 1. Structural features of plasmid IncP-6 carrying the \textit{bla}\textsubscript{KPC-2} gene (pCOL-1) in \textit{P. aeruginosa} COL-1 in comparison with other IncP-6 plasmids pRSB105 (GenBank DQ839391) and pRIO-5 (GenBank JF785550).\textsuperscript{14,15} White boxes indicate plasmid scaffold regions that are held in common between plasmids or are of unknown function. Resistance genes are indicated by grey boxes, except for the \textit{β}-lactamase genes, which are indicated by black boxes. Transposon-related genes (\textit{tnpA}, \textit{tnpR} and \textit{tnpM}), insertion sequences and integrase genes are indicated by hatched boxes. Replicase genes are indicated by boxes with vertical lines. The transfer and replication origins are indicated by black circles. Genes encoding mobilization and partition systems are indicated by dotted boxes.
ciprofloxacin 0.012 mg/L, tigecycline 0.5 mg/L and colistin 0.38 mg/L), indicating that no other expressed resistance marker was transferred with the plasmids.

**General features of plasmid pCOL-1**

High-throughput sequencing revealed that the plasmid pCOL-1 was 31529 bp in size with an average GC content of 60% and contained 31 open reading frames (ORFs). The replication module present on this plasmid belonged to the IncP-6 group. This plasmid exhibited an array of genes involved in replication, mobilization and partition, but none involved in conjugation (Table 1 and Figure 1). This is in accordance with previous observations of a plasmid unable to transfer the carbapenem resistance marker via conjugation.9

The plasmid architecture observed in pCOL-1 was similar to that of other IncP-6 plasmids previously sequenced (Figure 1).

The plasmid backbone exhibited high gene synteny with plasmid pRSB105, which encodes several resistance determinants including the \( \text{bla}_{\text{OXA-10}} \) gene and macrolide resistance genes.14 This IncP-6 replicon module has also recently been identified in association with the extended-spectrum \( \beta \)-lactamase (ESBL) \( \text{bla}_{\text{BES-1}} \) gene in a *Serratia marcescens* clinical isolate from Brazil.15 In comparison, the plasmid pRIO-5 carrying the ESBL \( \text{bla}_{\text{BES-1}} \) gene exhibited only four genes of the IncP-6 backbone involved in partition and replication (\( \text{parA}, \text{parB}, \text{parC} \) and \( \text{repA} \)) (Figure 1).

**Plasmid pCOL-1 harboured the isoform Tn4401b**

Only a single antibiotic resistance gene was identified on pCOL-1, the \( \text{bla}_{\text{KPC-2}} \) gene, which is compatible with susceptibility testing results. The \( \text{bla}_{\text{KPC-2}} \) gene was a part of the Tn4401 isoform b transposon, which confirms the results obtained by Cuzon et al.17

![Figure 2. Major genetic features of plasmid pPA-2 carrying the \( \text{bla}_{\text{KPC-2}} \) gene in *P. aeruginosa* PA-2. Schematic representation of plasmid pPA-2 and a comparison with plasmid RA3 from *Aeromonas salmonicida*.18 The \( \text{tra/vir} \) locus is indicated within a large white box. White boxes indicate plasmid scaffold regions that are held in common between plasmids or are of unknown function. Resistance genes are indicated by grey boxes, except for the \( \beta \)-lactamase genes, which are indicated by black boxes. Transposon-related genes (\( \text{tnpA}, \text{tnpR} \) and \( \text{tnpM} \)), insertion sequences and integrase genes are indicated by hatched boxes. Replicase genes are indicated by boxes with vertical lines. The transfer and replication origins are indicated by black circles. Genes encoding mobilization and partition systems are indicated by dotted boxes.](https://academic.oup.com/jac/article-abstract/68/8/1757/866056)
et al., who used a PCR mapping approach to identify the Tn4401b isofrom. This isoform exhibited the complete form, i.e. without any deletion as observed in Tn4401a (−100 bp) and Tn4401c (−200 bp). The sequence upstream of the bla\textsubscript{KPC-2} gene was identical to previously sequenced Tn4401b, and\textsubscript{Kpn7}, which has recently been found to be involved in the expression of the bla\textsubscript{KPC-2} gene. Both promoters P1 and P2, known to be involved in bla\textsubscript{KPC-2} gene expression in \textit{E. coli}, were identified. The transposon Tn4401b was inserted into the IncP-6 backbone within orf6, which codes for a protein of unknown function (Figure 1). orf6 is close to the oriV and replicase region. In pRIO-5, this part of the IncP-6 backbone was missing, suggesting that the DNA sequences located next to the replicase gene might serve as hotspots of DNA integration, as observed for other plasmid types.

**General features of plasmid pPA-2**

Plasmid pPA-2 was 7995 bp in size with an average GC content of 56%, and contained seven ORFs. This plasmid exhibited a replicase gene, but was lacking the genes involved in mobilization, partition and conjugation (Figure 2 and Table 2). These results are in accordance with the fact that this plasmid could not be transferred through conjugation.\textsuperscript{9} The kIC gene, coding for an anti-restriction protein, and the korC gene, coding for a transcriptional regulator, were also identified on this plasmid. The korC gene is likely to be functional since the region upstream of the gene and the gene itself were intact. According to the replicase sequence, this plasmid belonged to the IncU incompatibility group. Resistance plasmids assigned to the IncU incompatibility group have been isolated from a number of \textit{Aeromonas} species and \textit{E. coli} strains from seawater fish hatcheries and diseased fish, as well as from clinical environments. Members of the IncU plasmid group are particularly implicated in the dissemination of antibiotic resistance in \textit{Aeromonas} strains associated with aquatic environments.\textsuperscript{17}

**Plasmid pPA-2 harboured a novel genetic environment containing the \textit{bla}_{KPC-2} gene**

The \textit{bla}_{KPC-2} gene in this plasmid was not associated with an entire transposon Tn4401. Only 2072 bp matched to Tn4401, including the \textit{bla}_{KPC-2} gene and part of IS\textsubscript{Kpn6} that was fused to another gene traN (Figure 3a). This IS\textsubscript{Kpn6}-like element shared a 1039 bp fragment with IS\textsubscript{Kpn6}. Target site duplication and the inverted repeat left described in Tn4401 are present, but the ORF encoding the putative transposase (439 amino acids in IS\textsubscript{Kpn6}) is truncated in its N-terminus by a 117 bp fragment encoding the C-terminal region of a protein sharing 100% amino acid identity with the TraN protein from plasmid pB479T described in \textit{Aeromonas punctata} (GenBank accession no. NC_006143). Upstream of the \textit{bla}_{KPC-2} gene, only a 73 bp segment is identical to Tn4401, which is interrupted by a Tn3 transposon, itself interrupted by an IS26 element inserted within the \textit{bla}_{TEM-1} gene. Similar structures have recently been described in \textit{K. pneumoniae} isolates from China and Argentina.\textsuperscript{18}

\textit{Tn3} insertion occurred immediately upstream of the −35 box of the P1 promoter responsible for the expression of the \textit{bla}_{KPC} gene. In this situation, the \textit{bla}_{KPC} gene is likely to still be expressed from its native P1 promoter,\textsuperscript{19} as well as by the outward-directed promoter of \textit{Tn3}. This p\textsubscript{OUT} promoter has previously been shown to be involved in the expression of a gentamicin resistance gene in \textit{Enterobacteriaceae} (Figure 3b).

**Conclusions**

Unlike the widespread occurrence of KPC-producing \textit{Enterobacteriaceae}, \textit{P. aeruginosa} isolates expressing KPC carbapenemase seem to be geographically limited. The presence of \textit{bla}_{KPC} in
P. aeruginosa isolates is worrying in a species that is known to be prone to becoming carbapenem resistant by multiple mechanisms. The association of Tn4401, a high-frequency transposable element lacking target site specificity, together with successful plasmid backgrounds is an additional source of concern since blaKPC genes possess all the required features for efficient dissemination in Enterobacteriaceae, as well as in non-enterobacterial species such as P. aeruginosa or Acinetobacter baumannii.

Two different plasmids carrying the same carbapenemase blaKPC-2 gene in P. aeruginosa have been described here. Therefore, in Colombia, at least two different plasmid backbones associated with blaKPC-2 are circulating: (i) the IncP-6-type plasmid, which possesses the ability to replicate both in Pseudomonas species and in Enterobacteriaceae and contains a full Tn4401 element;14 and (ii) the IncU-type plasmid, which is frequently associated with antibiotic resistance in Aeromonas species and contains a truncated form of the Tn4401 transposon.18 In Enterobacteriaceae the blaKPC gene was associated with IncFII, as well as with IncL/M- and IncN-type, plasmids.11,21 In a Pseudomonas putida isolate from Brazil, the blaKPC-2 gene was associated with a Tn4401C isoforom located on a 65 kb IncN plasmid, a plasmid backbone different from those described in this work and frequently encountered in Enterobacteriaceae.22 These findings show a high diversity of plasmids carrying the blaKPC-2 gene, which is, in most cases, related to the presence of Tn4401, a key genetic determinant for its interplasmid mobility.

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Transparency declarations
None to declare.

References