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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 bla_{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 7995 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 Tn*4401*, including the bla_{KPC-2} gene, part of IS*Kpn6* and a 73 bp segment located upstream of the bla_{KPC-2} gene, containing the P1 promoter. Sequence identity was interrupted by a Tn*3* transposon, itself interrupted by an IS*26* element inserted within the β-lactamase bla_{TEM-1} gene.

Conclusions: Here we present the genetic features of the very first plasmids carrying the bla_{KPC-2} gene from *P. aeruginosa*. The emergence of the bla_{KPC-2} gene on unrelated plasmids, differing in size and in incompatibility group, and harbouring different genetic structures containing the bla_{KPC-2} genes in *P. aeruginosa* isolates suggests 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 β-lactamases or OprD2 porin deficiency.[1,2] Carbapenemases are able to hydrolyse most if not all β-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).[1,3,4] 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 bla_{KPC} genes has been linked to their location on a Tn*4401*, a Tn*3*-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.[1,11] Another explanation is likely to be linked to its presence in epidemic clones such as *K. pneumoniae* ST258.[1] Several KPC-producing *P. aeruginosa* isolates from different Colombian hospitals have revealed the spread of different clones, harbouring either chromosome- or plasmid-encoded bla_{KPC-2} genes. The plasmids were different in size and contained bla_{KPC-2} genes associated

with two different genetic structures. Most of the isolates contained the isoform Tn*4401b* located on an ~40 kb plasmid, and one isolate contained a novel genetic environment of a bla_{KPC-2} gene located on an ~10 kb plasmid.[9] 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 Tn*4401b* 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 Tn*4401b*.[9] *Escherichia coli* TOP10 (Invitrogen, Eragny, France) was used for electroporation experiments as previously described.[9] 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.[12]

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.[13]

Table 1. ORFs identified in pCOL-1

Gene name	Position (strand)	Length of the corresponding protein (amino acids)	Function	Amino acid identity
repA IncP-6	1–1374 (+)	458	replicase	100% with pRSB105
Δ*orf6*(1)	1839–1873 (+)	—	unknown	—
tnpR Tn*4401*	1929–3569 (−)	547	resolvase	100% with Tn*4401a*
tnpA Tn*4401*	3751–6777 (+)	1009	transposase	100% with Tn*4401a*
orfA IS*Kpn7*	6887–7909 (+)	341	OrfA transposase	100% with Tn*4401a*
orfB IS*Kpn7*	7909–8685 (+)	259	OrfB transposase	100% with Tn*4401a*
bla_{KPC-2}	9075–9954 (+)	293	carbapenemase	100% with KPC-2
tnpA IS*Kpn6*	10209–11522 (−)	438	transposase	100% with Tn*4401a*
Δ*orf6*(2)	11877–12143 (+)	—	unknown	—
kfrA	12452–13396 (+)	315	antirestriction protein	97% with KfrA YP_245474
tnpA	13748–14572 (+)	275	putative transposase	100% with TnpA YP_245473
ofxX	15328–14603 (−)	242	unknown	98% with hypothetical protein pRSB105
mobE	15975–15328(−)	216	mobilization protein	100% with pRSB105
mobD	16651–15971 (−)	227	mobilization protein	100% with pRSB105
mobC	17020–16667 (−)	118	mobilization protein	100% with pRSB105
mobB	17330–17647 (+)	106	mobilization protein	100% with pRSB105
mobA	17802–20300 (+)	833	mobilization protein	100% with pRSB105
orf1	20412–20900 (+)	163	hypothetical protein	100% with pRMS149 YP_245446.1
orf2	20900–21319 (+)	140	hypothetical protein	100% with pRSB105
tnpA Tn*501*-like	22565–21708 (−)	285	truncated transposase Tn*3* family	99% with Tn*501* transposase YP_003829287.1
tnpR Tn*501*-like	22606–23208 (+)	201	resolvase	99% with Tn*501* resolvase YP_003829288
orf3	23833–24195 (+)	121	hypothetical protein	100% with pFBAOT6 YP_067863
tnpA IS*Apu1*	24522–25787 (+)	422	transposase	100% with pFBAOT6 YP_067863
orf4	26286–25885 (−)	134	hypothetical protein	100% with pFBAOT6 YP_067863
corA	26381–27348 (−)	322	deleted magnesium and cobalt transporter	99% with pFBAOT6 YP_067863
GST-family protein	28049–27348 (−)	234	GST-family protein	81% with pFBAOT6 YP_067863
msrA	28721–28185 (−)	179	putative methionine sulfoxide reductase	100% with pFBAOT6 YP_067863
ΔTn*3*-family *tnpA*	29345–29701 (+)	119	truncated transposase Tn*3* family	95% with transposase EHV82687
parA	30063–30698 (+)	212	ATPase involved in partitioning	100% with pRSB105
parB	30744–30941 (+)	73	auxiliary partitioning protein	100% with pRSB105
parC	30978–31361 (+)	128	auxiliary partitioning protein	100% with pRSB105

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 DNAVision 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 bla_{KPC-2} gene in *P. aeruginosa* COL-1 isolated from a hospital in Medellin, Colombia, was initially suggested by Cuzon *et al.*[9] Subsequently, plasmids of similar size to pCOL-1 were identified in several KPC-producing *P. aeruginosa* isolates from different Colombian cities.[9] A second plasmid, pPA-2, different in size from pCOL-1, was recovered from a clinical isolate of *P. aeruginosa* isolated in Bogota, Colombia.[9] The whole-cell DNAs of these two *P. aeruginosa* isolates were electroporated into *E. coli* TOP10. Transformants of *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-β-lactam antibiotics for *E. coli* TOP10 (pCOL-1), *E. coli* TOP10 (pPA-2) and the parental *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,

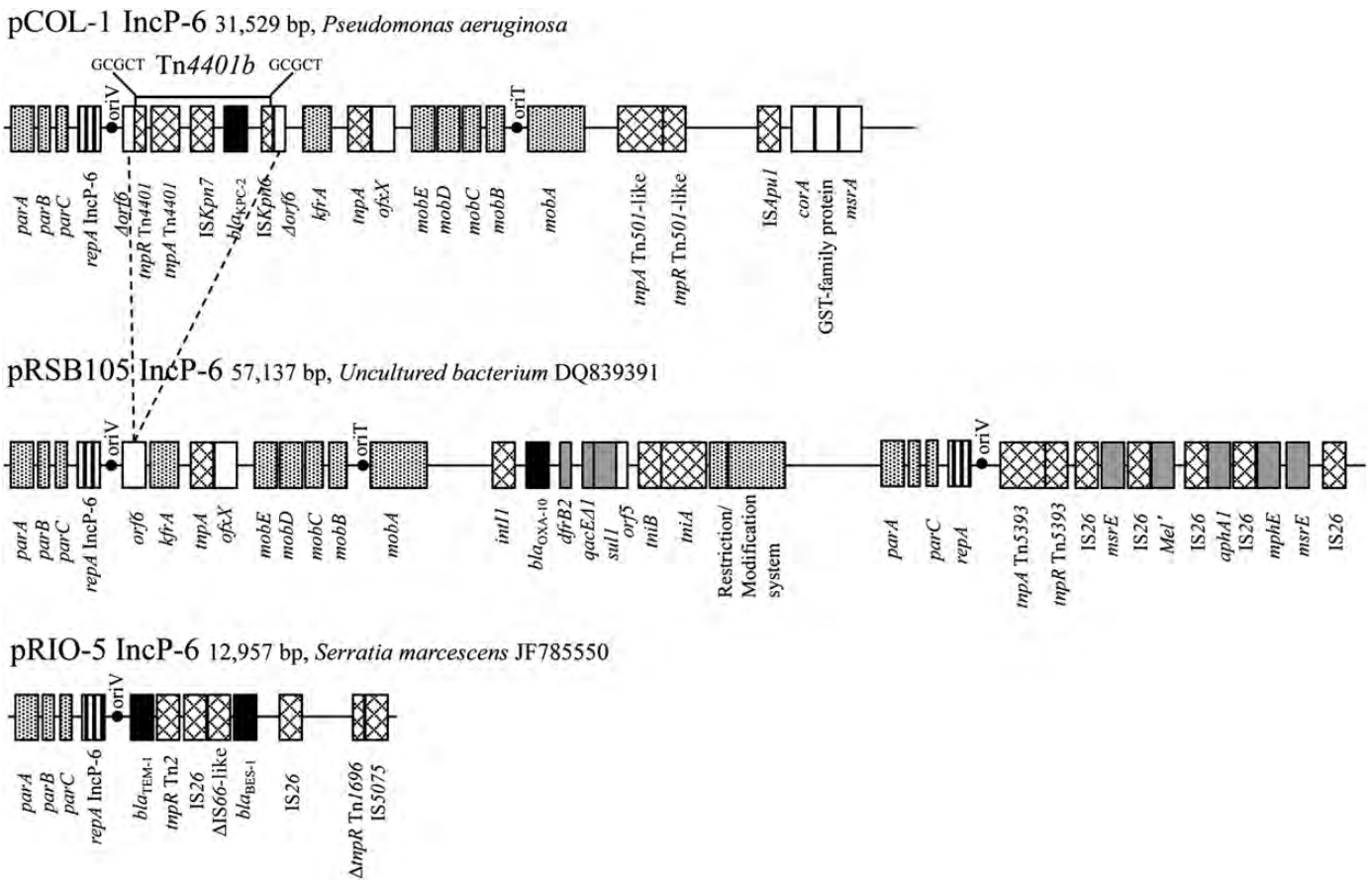

Figure 1. Structural features of plasmid IncP-6 carrying the bla_{KPC-2} gene (pCOL-1) in *P. aeruginosa* COL-1 in comparison with other IncP-6 plasmids pRSB105 (GenBank DQ839391) and pRIO-5 (GenBank JF785550).[14,15] White boxes indicate plasmid scaffold regions that are held in common between plasmids or are of unknown function. Resistance genes are indicated by grey boxes, except for the β-lactamase genes, which are indicated by black boxes. Transposon-related genes (*tnpA*, *tnpR* and *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.

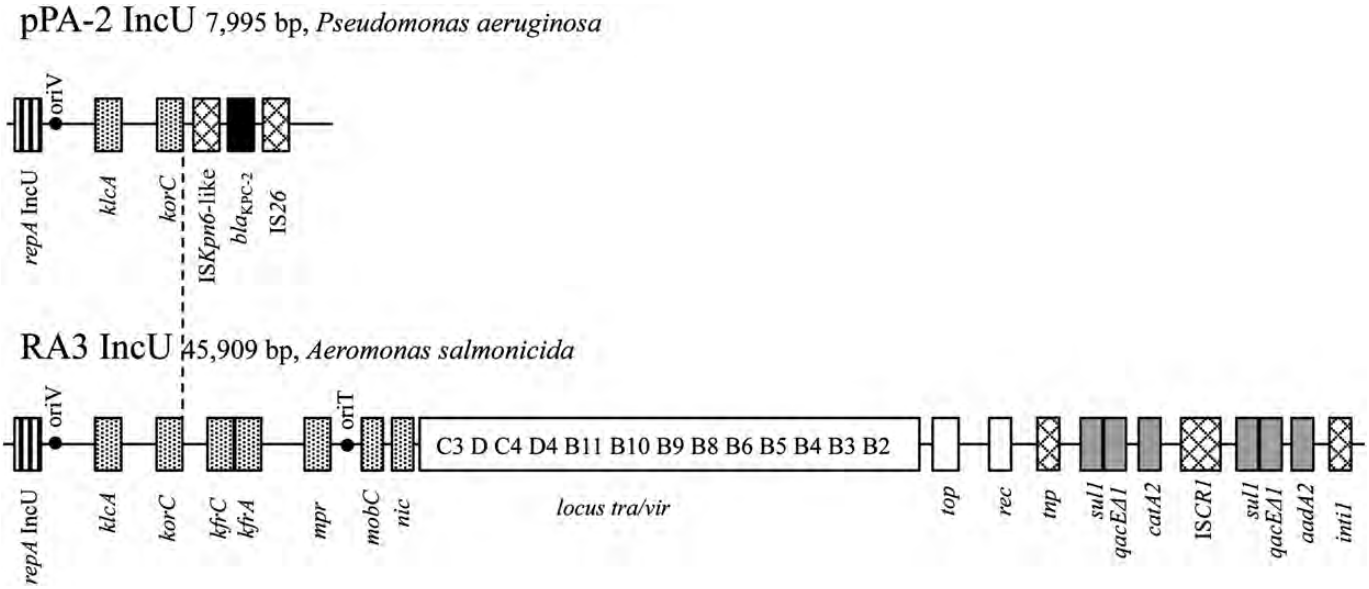

Figure 2. Major genetic features of plasmid pPA-2 carrying the bla_{KPC-2} gene in *P. aeruginosa* PA-2. Schematic representation of plasmid pPA-2 and a comparison with plasmid RA3 from *Aeromonas salmonicida*.[18] The *tra/vir* locus is indicated within a large white box. White boxes indicate plasmid scaffold regions that are held in common between plasmids or are of unknown function. Resistance genes are indicated by grey boxes, except for the β-lactamase genes, which are indicated by black boxes. Transposon-related genes (*tnpA*, *tnpR* and *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.

Table 2. ORFs identified in pPA-2

Gene name	Position (strand)	Length of the corresponding protein (amino acids)	Function	Amino acid identity
repA	1–843 (+)	276	replicase	100% with RA3
orf1	2250–2525 (+)	92	unknown	100% with pKP048_p014 YP_003754007
klcA	2639–3061 (+)	141	antirestriction protein	100% with pKP048_p014 YP_003754007
korC	3393–3686 (+)	98	transcriptional repressor protein	100% with pKP048_p014 YP_003754007
tnpA IS*Kpn6*-like	3694–4671 (+)	326	transposase	—
bla_{KPC-2}	5805–4927 (−)	293	carbapenemase	100% with KPC-2
tnpA IS*26*	6274–6987 (+)	238	transposase	100% with TnpA IS*26*

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 31 529 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 bla_{OXA-10} gene and macrolide resistance genes.[14] This IncP-6 replicon module has also recently been identified in association with the extended-spectrum β-lactamase (ESBL) bla_{BES-1} gene in a *Serratia marcescens* clinical isolate from Brazil.[15] In comparison, the plasmid pRIO-5 carrying the ESBL bla_{BES-1} gene exhibited only four genes of the IncP-6 backbone involved in partition and replication (*parA*, *parB*, *parC* and *repA*) (Figure 1).

Plasmid pCOL-1 harboured the isoform Tn4401b

Only a single antibiotic resistance gene was identified on pCOL-1, the bla_{KPC-2} gene, which is compatible with susceptibility testing results. The bla_{KPC-2} gene was a part of the Tn*4401* isoform b transposon, which confirms the results obtained by Cuzon

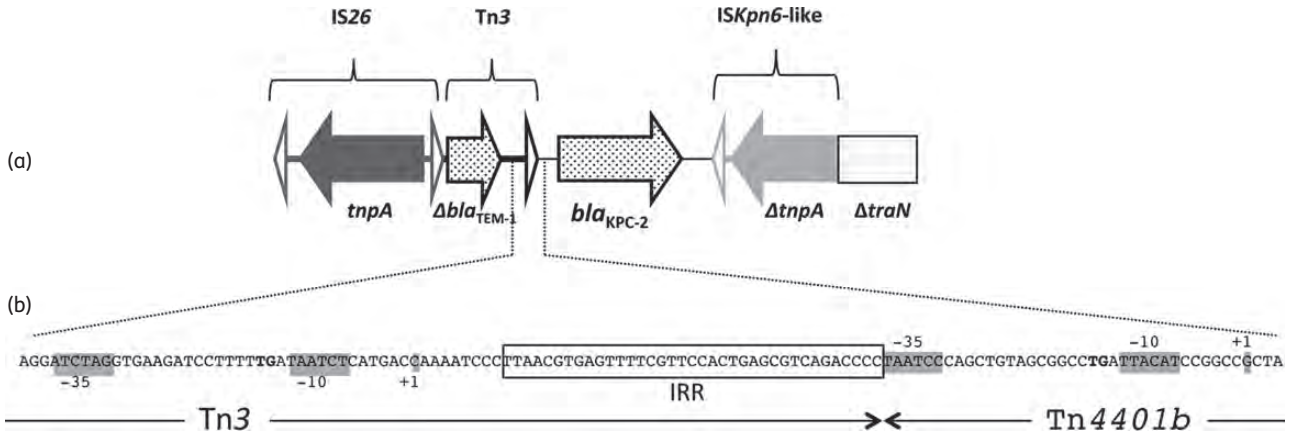

Figure 3. The genetic environment of the bla_{KPC-2} gene in plasmid pPA-2. (a) Schematic representation of the genetic environment of the bla_{KPC-2} gene. Inverted repeats of insertion sequences and of Tn*3* are indicated by large arrow heads. Brackets indicate the boundaries of mobile elements. Dotted lines indicate the part of the sequence that is detailed in part (b). Genes are indicated by large arrows with their names below. Truncated genes are indicated by the sign Δ. (b) Detailed analysis of the upstream sequence of the bla_{KPC-2} gene. Sequence identities with Tn*3* or Tn*4401b* sequences are indicated by arrows below the nucleotide sequences. The inverted repeat right (IRR) of Tn*3* is boxed. Sequences highlighted in grey represent the −35, −10 and +1 promoter sequences. Bold nucleotides **TGA** correspond to the extended −10 boxes.[16,20] With Tn*3* being inserted immediately upstream of the −35 box of the bla_{KPC} gene, it is likely that the bla_{KPC-2} gene is still expressed from its native P1 promoter, as well as from the outward promoter of Tn*3*, as previously described for gentamicin resistance gene expression.[20]

et al.,[9] who used a PCR mapping approach to identify the Tn*4401b* isoform. This isoform exhibited the complete form, i.e. without any deletion as observed in Tn*4401a* (−100 bp) and Tn*4401c* (−200 bp).[7] The sequence upstream of the bla_{KPC-2} gene was identical to previously sequenced Tn*4401b*,[7] and IS*Kpn7*, which has recently been found to be involved in the expression of the bla_{KPC-2} gene.[16] Both promoters P1 and P2, known to be involved in bla_{KPC-2} gene expression in *E. coli*, were identified. The transposon Tn*4401b* 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.[17]

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.[9] The *klcA* gene, coding for an antirestriction 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 *Aeromonas* species and *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 *Aeromonas* strains associated with aquatic environments.[18]

Plasmid pPA-2 harboured a novel genetic environment containing the bla_{KPC-2} gene

The bla_{KPC-2} gene in this plasmid was not associated with an entire transposon Tn*4401*. Only 2072 bp matched to Tn*4401*, including the bla_{KPC-2} gene and part of IS*Kpn6* that was fused to another gene *traN* (Figure 3a). This IS*Kpn6*-like element shared a 1039 bp fragment with IS*Kpn6*. Target site duplication and the inverted repeat left described in Tn*4401* are present, but the ORF encoding the putative transposase (439 amino acids in IS*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 pFBAOT6 described in *Aeromonas punctata* (GenBank accession no. NC_006143). Upstream of the bla_{KPC-2} gene, only a 73 bp segment is identical to Tn*4401*, which is interrupted by a Tn*3* transposon, itself interrupted by an IS*26* element inserted within the bla_{TEM-1} gene. Similar structures have recently been described in *K. pneumoniae* isolates from China and Argentina.[19]

Tn*3* insertion occurred immediately upstream of the −35 box of the P1 promoter responsible for the expression of the bla_{KPC} gene. In this situation, the bla_{KPC} gene is likely to still be expressed from its native P1 promoter,[16] as well as by the outward-directed promoter of Tn*3*. This pOUT promoter has previously been shown to be involved in the expression of a gentamicin resistance gene in Enterobacteriaceae (Figure 3b).[20]

Conclusions

Unlike the widespread occurrence of KPC-producing Enterobacteriaceae, *P. aeruginosa* isolates expressing KPC carbapenemase seem to be geographically limited. The presence of 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 Tn*4401*, a high-frequency transposable element lacking target site specificity, together with successful plasmid backgrounds is an additional source of concern since bla_{KPC} 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 bla_{KPC-2} gene in *P. aeruginosa* have been described here. Therefore, in Colombia, at least two different plasmid backbones associated with bla_{KPC-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 Tn*4401* 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 Tn*4401* transposon.[18] In Enterobacteriaceae the bla_{KPC} 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 bla_{KPC-2} gene was associated with a Tn*4401c* isoform located on a 65 kb IncFI 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 bla_{KPC-2} gene, which is, in most cases, related to the presence of Tn*4401*, a key genetic determinant for its inter-plasmid mobility.

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Transparency declarations

None to declare.

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