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Short Communication

Description of novel resistance islands harbouring $bla_{\text{CTX-M-2}}$ in IncC type 2 plasmids

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ABSTRACT

Objectives: We sequenced two IncA/C plasmids harbouring $bla_{\text{CTX-M-2}}$ in *Klebsiella pneumoniae* clinical isolates and compared their antibiotic resistance islands.

Methods: Transconjugants were obtained from two clinical *K. pneumoniae* isolates harbouring $bla_{\text{CTX-M-2}}$. Plasmid DNA from transconjugants underwent short-read whole-genome sequencing, reads were assembled, and gaps were closed by PCR and sequencing. Determination of plasmid replicons, antibiotic resistance genes, identification and characterisation of insertion sequence (IS) elements, and comparison with publicly available plasmid sequences were performed.

Results: $bla_{\text{CTX-M-2}}$ was located in a complex class 1 integron In*35*::IS*CR1*::$bla_{\text{CTX-M-2}}$, inserted in two different transposons designated Tn*7057* and Tn*7058*, that reside in the resistance islands of plasmids pUR-KP0923 and pUR-KP1025, respectively. The general modules of both transposons were In*35*::IS*CR1*::$bla_{\text{CTX-M-2}}$–Tn*1000*-like–Tn*2**–IS*Kpn11-12-13* variable module–ΔTn*21*. In Tn*7057* there was ΔIS*10R*–*catA2* associated with an additional IS*Kpn13*. Both plasmids belonged to IncC type 2 and ST3. pUR-KP0923 was 167 138 bp in length and had a 37 926-bp resistance island at position 4 (RI-4). Plasmid pUR-KP1025 was 168 128 bp with a RI-4 of 36 222 bp.

Conclusion: This report describes the molecular nature of two transposons (Tn*7057* and Tn*7058*) harbouring $bla_{\text{CTX-M-2}}$ that reside in IncC type 2 ST3 plasmids. These transposons mediate resistance to oxyimino-cephalosporins, gentamicin and, in the case of Tn*7057*, chloramphenicol. CTX-M-2 is an important extended-spectrum β-lactamase (ESBL) to South American epidemiology. It is remarkable that despite being only two plasmid sequences, the information revealed here could contribute to a better understanding of the resistance islands from IncC type 2 plasmids.

1. Introduction

CTX-M-2 was the first enzyme belonging to the CTX-M family of extended-spectrum β-lactamases (ESBLs) to be detected in South America and for many years was the most frequently isolated in this region [1–3]. Although in recent years there has been a replacement in favour of CTX-M-15- and CTX-M-9-derived ESBLs, CTX-M-2 has continued to circulate significantly among enterobacterial isolates of human and animal origin [2,4–6].

Regarding the localisation of $bla_{\text{CTX-M-2}}$, most of the reports are associated with conjugative incompatibility group A/C (IncA/C) plasmids, mainly identified by the classical PCR replicon typing scheme [4,5].

IncA/C were among the earliest plasmids to be associated with different β-lactamases in Gram-negative bacteria, and two groups have been described, namely IncA and IncC [7,8].

Plasmids from the IncC group are often found in multiple antibiotic-resistant Gram-negative bacteria belonging to several

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different species, indicating a broad host range. These plasmids have played a major role in the dissemination of genes encoding cephalosporinases and carbapenemases [9]. In this regard, most of the reports describing these plasmids refer to CMY and NDM enzymes [8] and there is scarce information about the presence of CTX-M enzymes in IncC plasmids in publicly available databases.

Regarding their characterisation, IncC plasmids have been classified into two groups, known as type 1 and type 2, primarily based on variations observed in the plasmid backbone and in the resistance islands (RIs), including the loss of areas adjacent to them [7,10]. Within the RIs, some differences can be observed, such as the presence of ARI-A and/or ARI-B and even the absence of both, as is the case with certain IncC [7].

In addition to ARI-A/B, other important RIs have been detected in both types, inserted near or in the *rhs* gene, and several RI insertion sites have been described within the *rhs* gene. The location of these RIs is numbered according to the position where they are inserted. For instance, most of the plasmids carrying resistance genes in RI position 4 (RI-4) do not include an ARI-B insertion. This implies that the backbone is complete and uninterrupted at this position [8].

In this study, we sequenced and compared two IncC plasmids harbouring $bla_{\text{CTX-M-2}}$ in *Klebsiella pneumoniae* clinical isolates and described their respective antibiotic resistance islands.

2. Materials and methods

2.1. Sample characteristics

Klebsiella pneumoniae strains KP0923 and KP1025 harbouring $bla_{\text{CTX-M-2}}$ were isolated from urine cultures from two paediatric patients admitted to Hospital del Niño, Centro Hospitalario Pereira Rossell (Montevideo, Uruguay) during 2010 and 2011, respectively. The two isolates were genetically unrelated [4]. Transconjugants were obtained from both strains and carried a single IncA/C plasmid of 140–160 kb in size harbouring $bla_{\text{CTX-M-2}}$ [4], hereinafter referred to as pUR-KP0923 and pUR-KP1025, respectively.

2.2. Conjugation frequency and characterisation of transconjugants

Conjugal transfer was assessed by mating experiments using *Escherichia coli* J53 (*pro met* Rif^{r} Nal^{r}) as recipient as previously described [4]. Transconjugants were selected on Luria–Bertani agar plates supplemented with rifampicin (150 μg/mL) and ceftriaxone (1 μg/mL). The presence of $bla_{\text{CTX-M-2}}$ in the transconjugants was assessed by PCR as described previously [4]. Conjugation frequencies were calculated as the number of transconjugants (Tc) per donor (d).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of clinical isolates and transconjugants was performed using the broth microdilution ARGNF Sensititre panel (Thermo Fisher, Waltham, MA, USA). The amikacin minimum inhibitory concentration (MIC) was determined by Etest (bioMérieux, Paris, France). All results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (www.eucast.org).

2.4. Plasmid sequencing and analysis

Plasmid DNA was obtained using a QIAGEN® Large-Construct Kit (QIAGEN) according to the manufacturer's instructions.

Whole-genome sequencing (WGS) of plasmid DNA from both transconjugants was performed using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) with a 2 × 250-bp or 2 × 300-bp paired-end approach. Raw reads were assembled using SPAdes 3.1 [11], and gaps were closed following a PCR and amplicon sequencing approach. Gene annotation was performed using the RAST platform [12] and were manually revised using plasmid pR55 (GenBank accession no. **JQ010984**) as reference [13]. Sequence alignments were performed using Mauve 2.4 (http://darlinglab.org/mauve/mauve.html), and physical maps were generated using Easyfig 2.2 (http://mjsull.github.io/Easyfig/). Plasmid multilocus sequence typing (pMLST) was performed *in silico* using the pMLST database (https://pubmlst.org/plasmid/). Comparisons with publicly available plasmid sequences were performed with BLAST (http://blast.ncbi.nlm.nih.gov/), and *in silico* determination of plasmid replicons and antibiotic resistance determinants were performed using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), respectively. Identification and characterisation of insertion sequence (IS) elements was performed using the ISfinder database (http://www-is.biotoul.fr) [14].

2.5. Nucleotide accession numbers

Plasmid sequences of KP0923 and KP1025 and their respective RIs are available in the GenBank database under accession numbers **MN207908** and **MW266061** (RI) for pUR-KP0923 and **MN065773** and **MW273770** (RI) for pUR-KP1025.

Transposons numbers were designated by The Transposon Registry (https://transposon.lstmed.ac.uk/) [15] as Tn*7057* and Tn*7058* located in pUR-KP0923 and pUR-KP1025, respectively.

3. Results and discussion

3.1. Conjugation frequency and whole-genome sequencing (WGS) results

Conjugal transfer to *E. coli* J53 was successful both for pUR-KP0923 (4.2×10^{-3} Tc\d) and pUR-KP1025 (5.5×10^{-3} Tc\d). According to Harmer and Hall, this constitutes a high conjugation frequency from IncC type 2 plasmids [7].

Antimicrobial susceptibility results for donors and transconjugants are shown in Table 1.

To further investigate the genetic features of these elements, a WGS approach was adopted and complete circular molecules of 167,138 bp and 168,128 bp in size were obtained for pUR-KP0923 (50 × coverage, 214 predicted ORFs) and pUR-KP1025 (30 × coverage, 219 predicted ORFs), respectively, with a mean G+C content of 52.5%.

3.2. Description of the plasmid backbone

In silico analysis of sequences revealed that both plasmids belonged to the IncC group type 2, carrying a *repA2* allele identical to that of the IncC reference plasmid pR55 (GenBank accession no. **JQ010984**) [13], and were classified within the ST3/ST3.3 lineage according to the pMLST/cgPMLST typing schemes [16].

Overall, the genetic organisation of both plasmids consisted of: (i) a backbone region of size 129,207 bp (pUR-KP0923) and 131,901 bp (pUR-KP1025), comprising core genes encoding replication, plasmid maintenance, partitioning and transfer functions; and (ii) a resistance island (RI-4) inserted in the *rhs2* gene (Fig. 1A).

The difference in size detected in the backbone region of pUR-KP1025 was basically explained by the presence of two insertion sequences belonging to the IS*4* family: IS*10R* located between nucleotides 34,586 and 35,794 and IS*186B* between nucleotides 100,292 and 101,404. IS*10R* was found interrupting a thymidylate kinase gene (*A058*) that is listed in the pMLST scheme [16], how-

Table 1
Minimum inhibitory concentrations (MICs) for *Klebsiella pneumoniae* strains KP0923 and KP1025, their respective transconjugants in *Escherichia coli* J53 (pUR-KP0923 and pUR-KP1025, respectively) and the recipient strain *E. coli* J53

Antimicrobial	MIC (μg/mL)[a]				
	KP0923	*E. coli* J53(pUR-KP0923)	KP1025	*E. coli* J53(pUR-KP1025)	*E. coli* J53
Ampicillin/sulbactam	>16	>16	>16	>16	≤8
Amoxicillin/clavulanic acid	>16	>16	>16	>16	≤8
Cefotaxime	>32	>32	>32	>32	≤1
Cefotaxime/clavulanic acid	≤0.25	≤0.25	0.5	≤0.25	≤0.25
Ceftazidime	16	8	16	8	≤2
Ceftazidime/clavulanic acid	0.5	0.5	1	0.5	0.5
Cefepime	16	8	16	8	≤2
Amikacin	16	2	16	2	0.25
Gentamicin	>8	>8	>8	>8	≤4
Ciprofloxacin	>2	≤0.06	0.25	≤0.06	≤0.06
Nitrofurantoin	>64	≤32	>64	≤32	≤32
Chloramphenicol	>16	>16	>16	≤8	≤8

[a] MIC determination was performed by Sensititre; amikacin results were obtained by Etest.

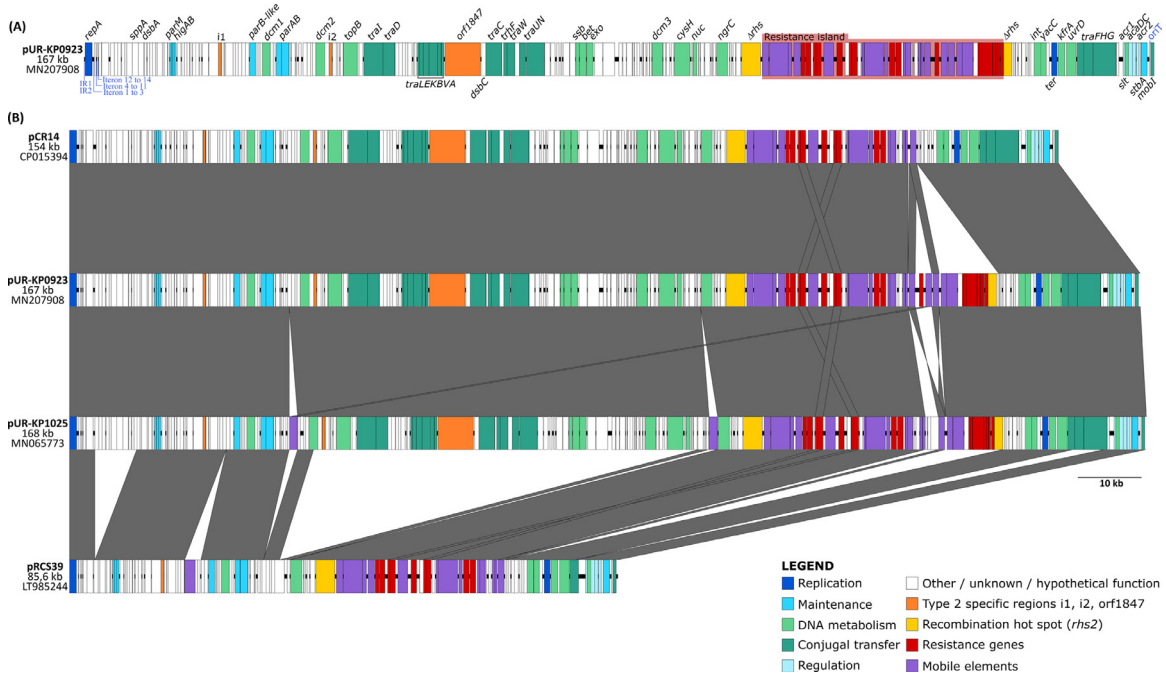

Fig. 1. (A) Linear map of plasmid pUR-KP0923 showing the genes belonging to the backbone and (B) BLASTn sequence comparison of pUR-KP0923 and pUR-KP1025 with two IncC type 2 plasmids harbouring $bla_{CTX\text{-}M\text{-}2}$ (pCR14 and pRCS39). Homologous segments (≥99% identity) are shown as grey blocks. Genes are represented by bars and coloured according to their function as shown in the legend.

ever we were able to identify the corresponding *A058* allele with the sequences on both sides of IS*10R*.

Several characteristics of IncC type 2 plasmids were also found. For instance, the occurrence of *orf1847* (5544 bp) between *traA* and *dsbC*, an *rhs2* gene (4263 bp) interrupted by a resistance island (RI-4) as mentioned before, and the presence of insertions *i1* and *i2* located upstream of *dcm1* and downstream of *dcm2*, respectively (Fig. 1A). Nevertheless, the ARI-A and ARI-B resistance islands, present in certain IncC plasmids, were absent [8].

Two IncC plasmids harbouring $bla_{CTX\text{-}M\text{-}2}$, pCR14 (GenBank accession no. **CP015394**) [17] and pRCS39 (GenBank accession no. **LT985244**) [8] were compared with both plasmids reported here (Fig. 1B). pCR14 was found to have a similar backbone region, but pRCS39 differs in this section. In this latter plasmid, a large region appears to be absent, including the conjugative machinery mediated by the *tra* genes and the type 2 specific region harbouring *i2* and *orf1847*.

3.3. Resistance island (RI) characterisation

According to Ambrose et al., the RI was located in position 4 (RI-4) as mentioned previously and was flanked by 5-bp direct repeats (5′-AATCT-3′) in both plasmids [8]. The RIs showed a slightly different length in pUR-KP0923 (37,926 bp) and pUR-KP1025 (36,222 bp) (Fig. 2).

The RI of each plasmid was composed by new transposons, designated Tn*7057* and Tn*7058* [15], that reside within pUR-KP0923 and pUR-KP1025, respectively.

Tn*7057* and Tn*7058* are hybrids of three Tn*3* family transposons: (i) Tn*1696* [18], with which they share the genes *tnpA* and *tnpR*, and both inverted repeats IRR and IRL, the former interrupted by an IS*4321R*; (ii) Tn*1000*/Tn*2**, carrying *tnpA, tnpR* and $bla_{TEM\text{-}1}$; and (iii) Tn*21* [18], with which they share the module comprising *tniB, tniA, urf2* and the mercury operon.

Tn*7057* and Tn*7058* also have three additional insertion sequences (IS*Kpn11*, IS*Kpn12* and IS*Kpn13*), the last one represented

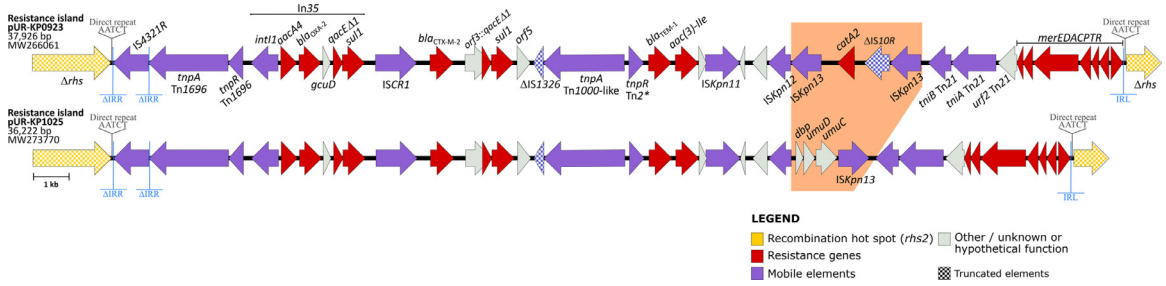

Fig. 2. Resistance islands (RIs) of plasmids pUR-KP0923 and pUR-KP1025 flanked by Δ*rhs* and comprising Tn*7057* and Tn*7058*, respectively. Genes are represented by arrows, directed according to their transcription direction and coloured by their function as shown in the legend. The orange block displays the differences between both RIs.

by two copies in Tn*7057*; and one complex class 1 integron comprised by In*35*::IS*CR1*::*bla*$_{CTX-M-2}$.

In addition, an *aac(3)-IIe* gene conferring resistance to gentamicin is located downstream of *bla*$_{TEM-1}$ in both transposons; and *catA2*, a chloramphenicol resistance determinant, is located downstream of the first copy of IS*Kpn13* in Tn*7057* followed by IS*10R*, which is truncated by the second copy of IS*Kpn13*. Unlike Tn*7057*, Tn*7058* has a module composed by IS*Kpn11*, IS*Kpn12, dbp* (a putative DNA-binding protein), the *umuD–umuC* genes (involved in SOS response) and a single copy of IS*Kpn13* (Fig. 2).

The class 1 integron found in the RIs is known as In*35* according to the composition of its variable region (*aacA4–bla*$_{OXA-2}$–*gcuD*). Downstream, it also presents the IS*CR1* element followed by the *bla*$_{CTX-M-2}$ ESBL gene, thus configuring a complex class 1 integron (Fig. 2). The antibiotic resistance genes embedded in such a platform usually confer resistance to amikacin, kanamycin and tobramycin (*aacA4*), broad-spectrum β-lactams and β-lactamase inhibitors (*bla*$_{OXA-2}$), sulfonamides (*sul1*) and oxyimino-cephalosporins (*bla*$_{CTX-M-2}$). The presence of *bla*$_{CTX-M-2}$ in this kind of platform has not only been frequently reported in our country but also has remained unchanged for at least 25 years [3,19,20].

The presence of IncC plasmids carrying *bla*$_{CTX-M-2}$ in RI-4 is scarce both in the literature and databases. We compared our plasmids with the RI-4 of pCR14 and pRCS39 and they had highly similar RIs comprising the region downstream of the first part of the *rhs* gene to IS*Kpn12* of pUR-KP1025 and to the first copy of IS*Kpn13* of pUR-KP0923. The structures found downstream of such insertion sequences are missing in pCR14 and pRCS39, including the Tn*21*::mercury operon module and the second part of *rhs* (Fig. 1B).

Other members of the CTX-M family have been found in IncC type 2 plasmids but in different positions to the one here reported. For instance, *bla*$_{CTX-M-15}$ was found to be inserted in ARI-B (GenBank accession no. **MT151380**) and *bla*$_{CTX-M-25}$ was found downstream of the *rhs* gene (GenBank accession no. **KF056330**).

4. Conclusions

To date, many of the reports and characterisations of IncC plasmids were focused on elements encoding NDM-type carbapenemases or CMY-type cephalosporinases, while very few studies investigated the role of such plasmids in spreading CTX-M-type ESBLs.

This report describes the molecular nature of two transposons (Tn*7057* and Tn*7058*) harbouring *bla*$_{CTX-M-2}$ that reside within pUR-KP0923 and pUR-KP1025, respectively. These transposons mediate resistance to oxyimino-cephalosporins, gentamicin and, in the case of Tn*7057*, chloramphenicol.

Although both transposons present evidence of multiple sequence insertion events, the presence of direct repeats (5′-AATCT-3′) adjacent to both corresponding inverted repeats (IRR/IRL) suggests that these transposons could be mobilised as a unit.

With this work, we contributing to providing complete plasmid sequences from type 2 IncC plasmids belonging to the ST3 clonal complex and harbouring *bla*$_{CTX-M-2}$, including the description of novel RI structures owing to the new transposons characterised.

It is remarkable that despite being only two plasmid sequences, the information revealed here could contribute to a better understanding of the platform of RIs from IncC type 2 plasmids.

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Competing interests

None declared.

Ethical approval

Not required.

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