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Short Communication

IncC *bla*$_{\text{KPC-2}}$-positive plasmid characterised from ST648 *Escherichia coli*

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ABSTRACT

Objectives: This study describes the characterisation of type 2 IncC plasmids pC-Ec20-KPC and pC-Ec2-KPC, carrying the*bla*$_{\text{KPC-2}}$ gene, from two multiresistant *Escherichia coli* recovered in University Hospital of Larissa (Greece) in 2018.

Methods: *E. coli* strains Ec-2Lar and Ec-20Lar were recovered from rectal swabs of two patients during monthly surveillance cultures. Transfer experiments by conjugation were carried out using rifampicin-resistant *E. coli* A15 laboratory strain as recipient. *bla*$_{\text{KPC}}$-carrying plasmids were characterised by *S1* profiling. Isolates were typed by MLST. Whole-genome sequencing was performed using the Sequel platform.

Results: Both *E. coli* isolates, belonging to ST648, transferred *bla*$_{\text{KPC-2}}$ to *E. coli* A15 by conjugation. Plasmid analysis revealed that the transconjugants harboured *bla*$_{\text{KPC}}$-positive plasmids of different sizes. Analysis of plasmid sequences showed that in both isolates the *bla*$_{\text{KPC-2}}$ gene was carried on a type 2 IncC plasmid (pC-Ec20-KPC and pC-Ec2-KPC, respectively). Both plasmids carried the ARI-B resistance island consisting of several resistance genes, intact and truncated copies of several mobile elements, and a 25 571-bp segment harbouring coding sequences for an iron transporter. The *bla*$_{\text{KPC-2}}$ gene was part of transposon Tn*4401a*, which was bounded by 5-bp direct repeats (TCCTT) suggesting its transposition into the IncC plasmids.

Conclusion: To our knowledge, this is the first report on complete nucleotide sequences of type 2 IncC plasmids. These findings, which hypothesise the acquisition of KPC-2-encoding transposon Tn*4401a* by an IncC replicon, indicate the ongoing need for molecular surveillance studies of multidrug-resistant pathogens. In addition, they underline the increasing clinical importance of the IncC plasmid family.

1. Introduction

The emergence and spread of carbapenemase-producing Enterobacterales is a major clinical and public-health concern. Plasmid-encoded class A carbapenemases of the KPC, IMI-2 and GES types effectively hydrolyse carbapenems and are partially inhibited by clavulanic acid [1]. However, KPCs are the most clinically common enzymes in this group. The first KPC-2-producing *Klebsiella pneumoniae* isolate was identified in 1996 in the eastern USA [2]. Within a few years, KPC-producing bacteria had spread globally. In Greece, KPC-2-producing *K. pneumoniae* have become prevalent since 2008 [3]. The worldwide spread of KPC-producers has been mainly contributed by the intercontinental dissemination of sequence type 258 (ST258) *K. pneumoniae* isolates [4]. Furthermore, sporadic isolation of Enterobacterales such as *Escherichia coli* and *Citrobacter freundii* strains with KPC has also been reported [5,6].

Between May 2010 and April 2011, an outbreak of KPC-2-producing *E. coli* ST410 was observed in University Hospital of Larissa (UHL) (Larissa, Greece) [5]. All isolates carried a KPC-2-encoding plasmid of the Inc group FIIK. To contain carbapenemase-producers, patients were isolated and enhanced control measures were applied in UHL. Since 2011, no further *E. coli* strains with KPC were isolated in UHL. The present study describes the characterisation of type 2 IncC plasmids pC-Ec20-KPC and pC-Ec2-KPC, carrying the *bla*$_{\text{KPC-2}}$ gene, from two multiresistant *E. coli* recovered in UHL in 2018.

2. Materials and methods

2.1. Bacterial isolates and confirmation of carbapenemase production

Two *E. coli* isolates (Ec-2Lar and Ec-20Lar) were recovered from rectal swabs of two patients in UHL during monthly surveillance cultures in May 2018. UHL is a 650-bed tertiary-care hospital in

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Thessaly (Central Greece) serving a population of 1 000 000 inhabitants. According to the policy of UHL, surveillance cultures for carbapenemase-producing Enterobacterales are performed upon patient admission to the intensive care unit and weekly until their discharge in order to isolate colonised patients. The routine protocol includes inoculation of rectal swabs on Brilliance^{TM} CRE chromogenic agar plates (Oxoid Ltd., Cambridge, UK), which are then incubated overnight at 37 °C. Colonies growing on chromogenic agar plates are identified and subjected to antimicrobial susceptibility testing using a VITEK® 2 automated system (bioMérieux, La Balmes-les-Grottes, France). Bacterial isolates non-susceptible to at least one carbapenem according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/) are first investigated for carbapenemase production by the modified Hodge test (MHT) using a meropenem disk (10 μg) as per Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. Bacteria that are positive by the MHT are screened for bla_{KPC}, $bla_{\text{VIM-like}}$, $bla_{\text{NDM-like}}$ and $bla_{\text{OXA-48-like}}$ genes by PCR [8]. Both strands of the PCR products are sequenced using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Typing

E. coli isolates underwent multilocus sequence typing (MLST) [9], and sequence types (STs) were assigned using the *E. coli* MSLT database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

2.3. Transfer of bla_{KPC} genes

Conjugal transfer of bla_{KPC} genes from clinical strains was carried out in mixed broth cultures using rifampicin-resistant *E. coli* A15 laboratory strain as recipient. Transconjugants were selected on MacConkey agar plates supplemented with rifampicin (150 mg/L) and ampicillin (50 mg/L).

2.4. Plasmid analysis

To define the genetic units of the bla_{KPC} gene, the plasmid content of all KPC-2-producing clinical and recombinant strains was analysed by pulsed-field gel electrophoresis (PFGE) of total DNA digested with *S1* nuclease (Promega, Madison, WI, USA). Following PFGE, DNA was transferred to a BrightStar-Plus Positively Charged Nylon Membrane (Applied Biosystems) and was hybridised with digoxigenin-labelled bla_{KPC} probes.

2.5. Whole-genome sequencing

Genomic DNA of *E. coli* was extracted using a NucleoSpin® Microbial DNA Kit (Macherey-Nagel, Düren, Germany) and was sequenced using long-read sequencing technology on the PacBio Sequel platform (Pacific Biosciences, Menlo Park, CA, USA). Library preparation was done following the manufacturer's recommendation for microbial multiplexing. DNA was sheared using g-tubes (Covaris, Woburn, MA, USA), and no size selection was performed during library preparation. HGAP4 De Novo Assembly Application (Pacific Biosciences) was used to perform genome assembly with minimum seed coverage of 30. Sequence gaps were filled by a PCR-based strategy using BLAST (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/) and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/). Comparative genome alignment was performed using Mauve v.2.3.1. Figures were generated from sequence data using BRIG v.0.95.

2.6. Nucleotide sequence accession numbers

The genomes and plasmids characterised during this study have been deposited in GenBank under accession nos. **CP035317**, **CP035318**, **MK388209**, **MK396099**, **MK410117**, **MK416183**, **MK439959**, **MK455767** and **MK455768**.

3. Results

E. coli isolates Ec-2Lar and Ec-20Lar exhibited resistance to penicillins, penicillin/β-lactamase inhibitor combinations, cephalosporins, aztreonam and carbapenems (Table 1). Both isolates were also resistant to various non-β-lactam antibiotics but remained susceptible to gentamicin and colistin. Ec-2Lar and Ec-20Lar, which were positive by the MHT, were found to carry the $bla_{\text{KPC-2}}$ carbapenemase gene.

MLST assigned both *E. coli* isolates to ST648 (*adk*-92, *fumC*-4, *gyrB*-87, *icd*-96, *mdh*-70, *purA*-58, *recA*-3). ST648 differs by six alleles from ST410 (*adk*-6, *fumC*-4, *gyrB*-12, *icd*-1, *mdh*-20, *purA*-18, *recA*-7), which included KPC-2-producing *E. coli* causing an outbreak in UHL during 2010–2011 [5]. ST648 has been associated with extended-spectrum β-lactamase (ESBL)-, NDM- and MCR-producing isolates in food animals and human beings worldwide [10,11].

The $bla_{\text{KPC-2}}$ genes from both clinical strains were transferred by conjugation (4×10^{-3} transconjugants/donor cell). All $bla_{\text{KPC-2}}$-positive recombinants exhibited similar resistance phenotypes, showing resistance to piperacillin, piperacillin/tazobactam, cephalosporins, imipenem, meropenem and aztreonam (Table 1), whilst they remained susceptible to ertapenem. In addition, all $bla_{\text{KPC-2}}$-positive recombinants were susceptible to non-β-lactam antibiotics, except for trimethoprim/sulfamethoxazole. Plasmid analysis revealed that the transconjugants harboured bla_{KPC}-positive plasmids of different sizes [ca. 190 kb (pC-Ec20-KPC) and ca. 220 kb (pC-Ec2-KPC)].

For isolate Ec-20Lar, assembly resulted in one genomic contig of 5 083 460 bp in size and three plasmidic contigs. After filling the gaps, plasmids pC-Ec20-KPC (190 688 bp), pB-Ec20 (106 616 bp) and pY-Ec20 (95 065 bp) were identified. On the other hand, for Ec-2Lar assembly resulted in one genomic contig of 5 027 415 bp in size and four plasmidic contigs. After filling the gaps, plasmids

Table 1

Antimicrobial susceptibility of *Escherichia coli* isolates Ec-2Lar and Ec-20Lar as well as the *E. coli* A15 transconjugants producing the KPC-2 carbapenemase.

Isolate	MIC (mg/L)														
	PIP	TZP	CTX	CAZ	FEP	ATM	IPM	MEM	ETP	GEN	AMK	CHL	SXT	CIP	COL
E. coli Ec-2Lar	>64	>64	>16	>16	>16	>16	4	4	4	2	8	16	>4	>2	≤1
E. coli A15_pC-Ec2-KPC	>64	>64	>16	>16	8	>16	8	8	1	1	2	8	>4	≤0.06	≤1
E. coli Ec-20Lar	>64	>64	>16	>16	>16	>16	4	4	8	2	8	16	>4	>2	≤1
E. coli A15_pC-Ec20-KPC	>64	>64	>16	>16	>16	16	8	4	1	1	2	8	>4	≤0.06	≤1
E. coli A15 (recipient)	≤0.5	1	≤0.06	≤0.25	≤0.12	≤0.25	≤0.12	≤0.12	≤0.5	0.12	0.5	≤1	1	≤0.06	≤1

MIC, minimum inhibitory concentration; PIP, piperacillin; TZP, piperacillin/tazobactam (inhibitor fixed at 4 mg/L); CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; COL, colistin.

pC-Ec2-KPC (227 878 bp), pB-Ec2 (95 745 bp), pY-Ec2 (95 047 bp) and pN-Ec2 (39 865 bp) were obtained. In addition, analysis of whole-genome sequencing data using VirulenceFinder 2.0 [12] showed that both isolates were positive for the presence of *air*, *eilA*, *gad* and *lpfA* virulence genes.

Analysis of plasmid sequences showed that in both isolates the *bla*$_{\text{KPC-2}}$ gene was carried on an IncC plasmid (pC-Ec20-KPC and pC-Ec2-KPC, respectively). In addition, analysis of sequenced plasmids by the core gene plasmid MLST (cgPMLST) scheme [13] indicated that both IncC plasmids carrying *bla*$_{\text{KPC-2}}$ belonged to cgST3.8. pC-Ec20-KPC and pC-Ec2-KPC were closely related to each other as well as to other previously described IncC plasmids [14], such as plasmid GR_12 recently characterised from a *K. pneumoniae* isolate from Greece (GenBank accession no. **CP027055**). The IncC backbones, which were highly syntenic with no genetic rearrangements [14], were composed of regions responsible for replication (*repA* gene), conjugative transfer (Tra1 and Tra2 regions) and plasmid maintenance (*higBA* and *parAB* operons and *xerD*- and *kfrA*-like genes) (Fig. 1). Plasmids pC-Ec20-KPC and pC-Ec2-KPC contained the i2 (462 bp between 38 820 and 38 821 in pRMH760) insertions [14], which are usually found only in type 2 IncC plasmids [14]. In addition, they contained the type 2 versions of the R1 (rhs2) and R2 (orf1847) regions [14]. Apart from the backbone, both plasmids carried the ARI-B resistance island. The ARI-B resistance island, which occurred upstream of Tra1, consisted of genes conferring resistance to β-lactams, aminoglycosides, tetracyclines, trimethoprim, streptomycin, rifampicin, sulfonamides and chloramphenicol (*bla*$_{\text{VEB-1}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{KPC-2}}$, *aadB*, *aadA1*, *tetA*, *dfrA23*, *dfrA14*, *strA*, *strB*, *sul1*, *sul2*, *arr-2*, *cmlA5* and *cmlA9* in pC-Ec20-KPC; Fig. 2), intact and truncated copies of several mobile elements, and a 25 571-bp segment (nt 19 922 to 45 492 in pC-Ec20-KPC) harbouring coding sequences for an iron transporter. The *bla*$_{\text{KPC-2}}$ gene was part of transposon Tn*4401a*, which is similar to that described in plasmid pNYC, lacking 100 bp upstream of *bla*$_{\text{KPC-2}}$. Direct repeats of 5 bp (TCCTT) were found at the boundaries of Tn*4401a*, suggesting its transposition into the IncC plasmids. The ARI-B of pC-Ec2-KPC carried an additional 39 492-bp sequence (nt 39 716 to 79 207) that

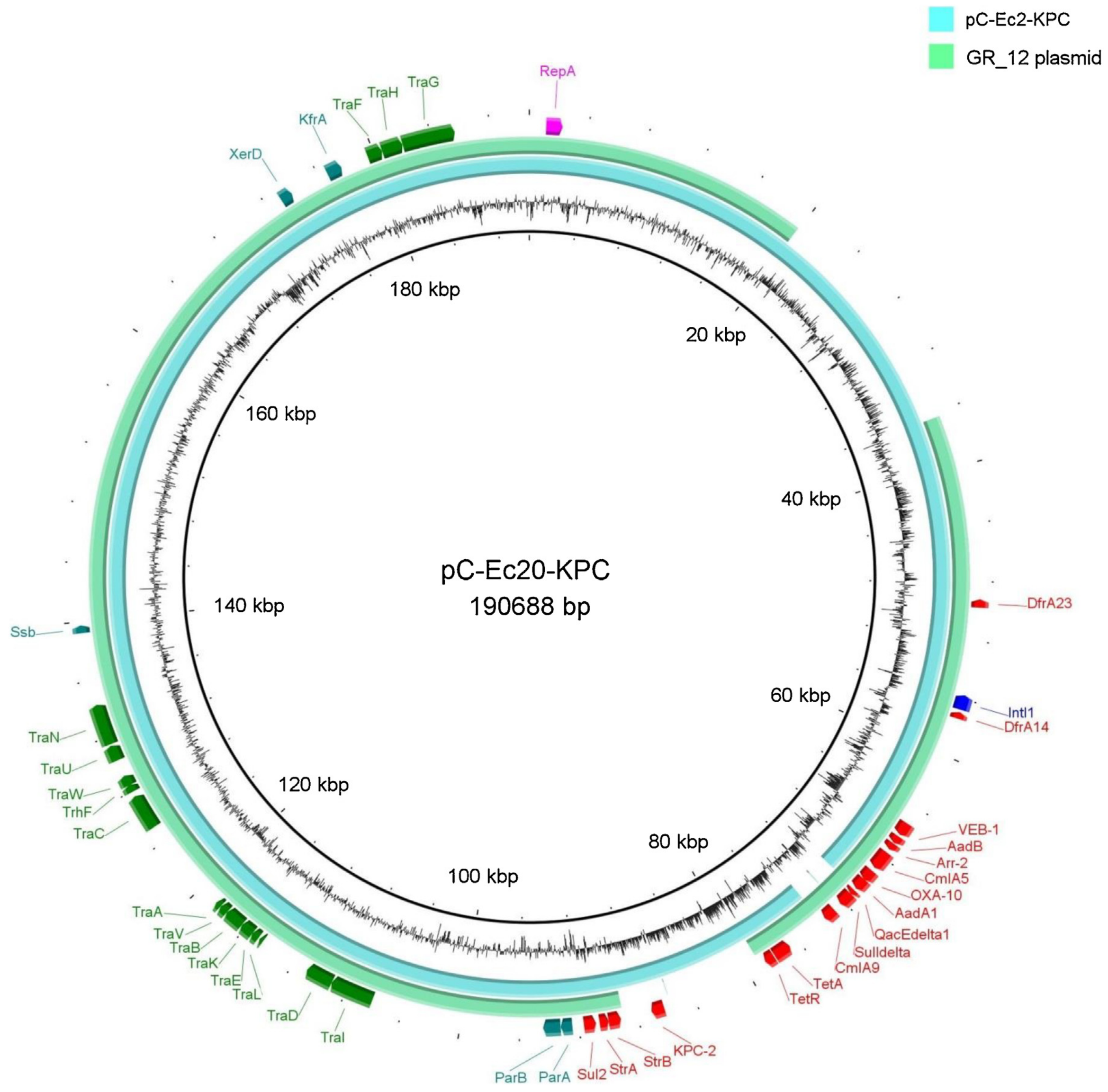

Fig. 1. Overview and alignment of KPC-2-encoding plasmids characterised from *Escherichia coli* isolates Ec-2Lar and Ec-20Lar. Indicative genes and the direction of transcription are shown by arrows in the outer circle. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and teal blue, respectively. Resistance genes are shown in red, whilst the *intI1* genes is shaded blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

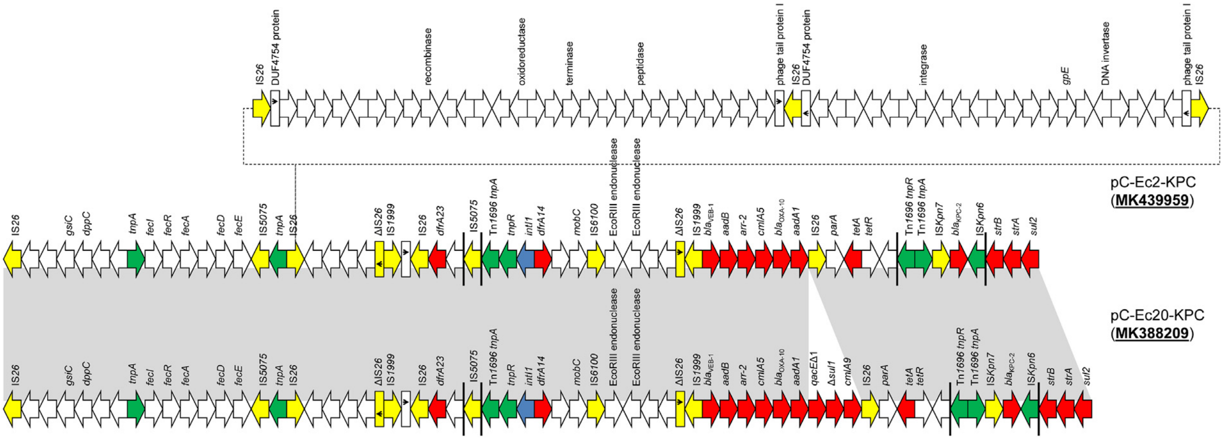

Fig. 2. Detailed comparison of ARI-B resistance island regions found in the IncC plasmids pC-Ec2-KPC and pC-Ec20-KPC. Arrows show the direction of transcription of open reading frames (ORFs), whilst truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes are shown in red. Insertion sequence (IS) elements and transposases are shown in yellow and green, respectively. *intI1* genes are shaded blue, whilst the remaining genes are shown in white. The grey shading corresponds to highly similar sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was bounded by two IS*26* elements in parallel orientation, harbouring coding sequences for proteins implicated in phage assembly as well as proteins with unknown function. However, a deletion of 10 984-bp was observed in the plasmid backbones of pC-Ec20-KPC and pC-Ec2-KPC (nt 19 083 to 30 066 in pRMH760) next to ARI-B. Like other type 2 IncC plasmids, pC-Ec20-KPC and pC-Ec2-KPC carried none of the *bla*$_{\text{CMY-2-like}}$ and ARI-A regions.

4. Discussion

Previous studies have shown that clonal dissemination of ST258 *K. pneumoniae* has contributed to the endemic spread of KPC-producers in Greek hospitals [3]. In addition, previous results have underlined that IncFIIK pKpQIL-like plasmids played a major role in Greece [15], since a highly conserved KPC-2-encoding pKpQIL-like plasmid was observed in a polyclonal population of KPC-producing *K. pneumoniae*, with ST258 as the predominant clonal lineage. A few studies have also reported the characterisation of KPC-2-producing Enterobacterales belonging to species other than *K. pneumoniae* [5,16]. However, in the current study, type 2 IncC plasmids pC-Ec20-KPC and pC-Ec2-KPC, carrying KPC-2-encoding transposon Tn*4401a*, have been characterised for the first time. Plasmids pC-Ec20-KPC and pC-Ec2-KPC, which were isolated from two ST648 *E. coli* recovered in a Greek hospital, also carried *bla*$_{\text{VEB-1}}$ and *bla*$_{\text{OXA-10}}$ β-lactamase-encoding genes. A previous study has also described the association of VEB-1 β-lactamase with IncC plasmids from Enterobacterales recovered in Greek hospitals [17]. These findings, which hypothesise the acquisition of KPC-2-encoding transposon Tn*4401a* by an IncC replicon, indicate the ongoing need for molecular surveillance studies of multidrug-resistant pathogens. In agreement with the results of previous studies [14,18–20], the current findings underline the increasing clinical importance of the IncC plasmid family as well as the rapid evolution of these molecules. IncC plasmids, being of broad host range and serving as reservoirs of various antimicrobial resistance genes including *bla*$_{\text{CMY}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$, will likely create challenges to human and animal health by limiting therapeutic options in cases of infection with a carrier organism.

Furthermore, BLASTN comparison revealed the presence of an additional 39 492-bp sequence in pC-Ec2-KPC. This finding reveals the ongoing evolution of genetic elements, even within the same clone. In conclusion, this study providing the complete genetic background of two ST648 KPC-2-producing *E. coli* contributes to the current knowledge of KPC-2-producing Enterobacterales.

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Competing interests

None declared.

Ethical approval

Not required.

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