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Complete Nucleotide Sequence of Two Multidrug-Resistant IncR Plasmids from *Klebsiella pneumoniae*

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We report here the complete nucleotide sequence of two IncR replicons encoding multidrug resistance determinants, including β-lactam (*bla*$_{DHA-1}$, *bla*$_{SHV-12}$), aminoglycoside (*aphA1*, *strA*, *strB*), and fluoroquinolone (*qnrB4*, *aac6′-1b-cr*) resistance genes. The plasmids have backbones that are similar to each other, including the replication and stability systems, and contain a wide variety of transposable elements carrying known antibiotic resistance genes. This study confirms the increasing clinical importance of IncR replicons as resistance gene carriers.

Since the first description of the novel IncR complex replicons in 2009 (1), multidrug resistance (MDR) plasmids containing IncR characteristic sequences and carrying multiple resistance genes, such as *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *bla*$_{DHA-1}$, *bla*$_{TEM-52}$, *qnrS1*, or *armA*, have been increasingly reported in *Enterobacteriaceae* isolates from various geographical regions (1–7). Moreover, IncR replicons have been found either as single replicons (7) or as part of multireplicon plasmids, i.e., plasmids harboring multiple replication initiation proteins (6, 8–10). However, the possible role of this emerging IncR complex in the spread of multidrug resistance is difficult to evaluate, since the plasmids belonging to this complex are known to be nontransferable. In order to further explore the role of these resistance plasmids, we analyzed the full nucleotide sequence of two IncR plasmids, pKPS30 and pKPS77, from *Klebsiella pneumoniae* strains isolated in France.

K. pneumoniae KPS30 (sequence type 11 [ST11]) was isolated from urine in 2008 at Tenon Hospital (Paris, France). KPS77 (ST15) was responsible for a urinary tract infection in a pregnant woman in 2008 at Saint Antoine Hospital (Paris, France). KPS30 exhibited an acquired *ampC* phenotype and was resistant to tobramycin, amikacin, fluoroquinolones, and cotrimoxazole. KPS77 exhibited an extended-spectrum β-lactamase (ESBL) phenotype and was susceptible to aminoglycosides but resistant to fluoroquinolones and cotrimoxazole. KPS30 carries the broad-spectrum β-lactamase genes *bla*$_{SHV-11}$ and *bla*$_{OXA-30}$ and the acquired *ampC*-type gene *bla*$_{DHA-1}$, whereas KPS77 carries *bla*$_{TEM-1}$ and the ESBL gene *bla*$_{SHV-12}$. Resistance to β-lactams and cotrimoxazole was transferred by electroporation for the two parental strains, whereas resistance to aminoglycosides was transferred from only KPS30 to the E_KPS30 (electroporant KPS30) transformant (Table 1).

TABLE 1 Susceptibility of parental and recipient strains

Antibiotic	MICs (mg/liter) for[a]:			
	KPS30	E_KPS30	KPS77	E_KPS77
Piperacillin/tazobactam	8	2	4	1
Cefotaxime	>128	32	16	4
Ceftazidime	>128	32	>128	32
Cefoxitin	>32	8	4	2
Cefepime	0.5	0.125	4	2
Gentamicin	S	S	S	S
Tobramycin	R	I	S	S
Amikacin	R	I	S	S
Ofloxacin	R	I	R	S
Ciprofloxacin	R	S	R	S
Cotrimoxazole	R	R	R	R

[a] Resistance according to the Société Française de Microbiologie (see http://www.sfm-microbiologie.org/). S, susceptible; I, intermediate; R, resistant.

Plasmid-based replicon typing (PBRT) (1, 11) allowed the identification of an IncR replicon in the two parental strains, KPS30 and KPS77. An additional IncFIIk replicon was detected in KPS30. The plasmid contents of the parental strains and transformants were determined by the Kado method (12) and confirmed the presence of two distinct plasmids in KPS30, whereas only one plasmid was present in the transformant E_KPS30 (data not shown). IncR plasmids harboring *bla*$_{DHA-1}$ and *bla*$_{SHV-12}$ could be transferred into *Escherichia coli* DH10B (Invitrogen, Cergy-Pontoise, France) only by electroporation. In order to analyze the complete sequence of the IncR replicons of KPS30 and KPS77, plasmid DNA was extracted from transformants using the Qiagen (Courtaboeuf, France) large construct kit. Sequencing was performed using shotgun and 3-kb paired-end sequencing on a 454/Roche GS FLX analyzer (Roche, Basel, Switzerland). The sequences obtained were assembled to a unique scaffold, and the predicted gaps were closed using PCR followed by sequencing with a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) in an ABI Prism 310 DNA sequencer (Applied Biosystems). Gene prediction and annotation were performed using the CAAT-Box tool (13).

pKPS30 is a 61,228-bp plasmid with an average G+C content of 54.4%. It includes a 12,391-bp backbone (nucleotides [nt] 1 to 7,690 and 56,528 to 61,228), which showed maximum identity

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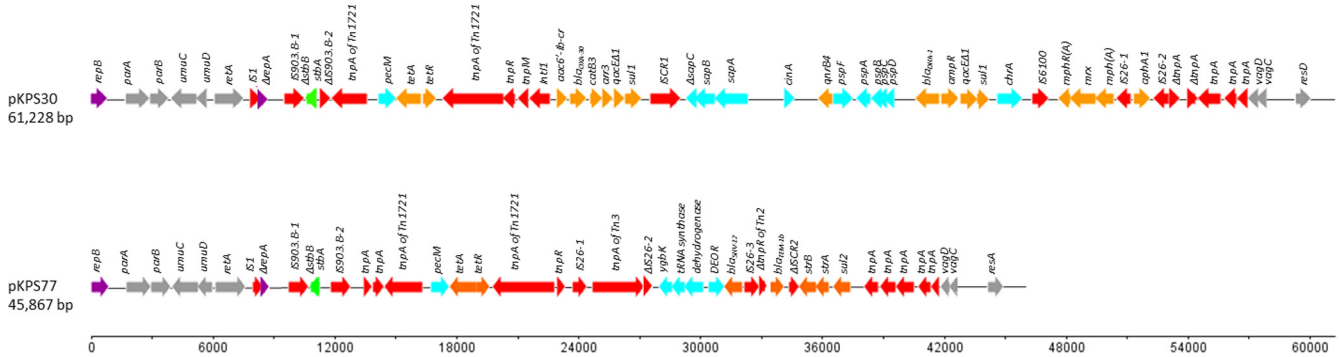

FIG 1 Linear maps of pKPS77 and pKPS30. Open reading frames (ORFs) are shown as arrows indicating the direction of transcription. Purple, replication initiation genes; gray, IncR backbone common to the two plasmids; red, transposon-related genes; orange, resistance genes; green, *stbA* and *stbB* genes; pale blue, other genes. ORFs encoding hypothetical proteins are not represented. The scale shows the number of base pairs.

with regions of IncR replicons. Using the GenBank BLAST tool, the highest similarity scores (100% coverage, 99% identity) were obtained with the corresponding regions of the recently described plasmid pKP1780 (GenBank accession number JX424614) harboring the bla_{VIM-1} metallo-β-lactamase gene of a *K. pneumoniae* strain isolated in Greece (7). pKPS77 is a 45,867-bp plasmid with an average G+C content of 54%. Its IncR backbone (nt 1 to 7,690 and 41,711 to 45,867) is similar to that of pKPS30. It showed the highest similarity with the respective regions of plasmid p1 from *K. pneumoniae* JM45 (GenBank accession number CP006657) encoding the ESBLs VEB-3 and CTX-M-24. The linear map of the plasmids pKPS30 and pKPS77 and the direction of gene transcription are shown in Fig. 1.

A comparative search of GenBank revealed 11 similar IncR plasmid backbones (98% to 99% identity) in *Enterobacteriaceae* (Table 2). Their backbone regions include a replication initiation gene, *repB*, 100% identical to *repB* of pKP1780, with a set of iterons suggested to be implicated in replication regulation. The upstream region specifies maintenance systems, including the *vagCD* operon encoding a toxin-antitoxin system and a resolvase probably contributing to multimer resolution (14). The downstream region encodes the ParA and ParB partition proteins. The scaffold also includes the *umuDC* operon, which is probably involved in SOS mutagenesis (15). As observed with all other fully sequenced IncR single replicon plasmids such as pKP1780 (GenBank accession number JX424614) (7), pKPC-LK30 (KC405622) (16), pKPN5 (CP000650), and pEFER (CU928144), no transfer-encoding region was identified. The remaining regions surrounding the IncR backbone in all 11 IncR plasmids whose sequences are stored in public databases contain various combinations of transposable elements and resistance genes. In pKPS30 and pKPS77, an IS*1*-like sequence and a truncated *repA* gene similar to the replication region of IncN plasmids were found at the boundary of the IncR region. It has an adjacent composite transposon containing *stbA* and truncated *stbB* genes (probably involved in plasmid stabilization [17]) flanked by two IS*903.B* insertion sequences (IS*903.B*-1 and IS*903.B*-2) (18, 19). A similar structure can be observed in pKP1780 (JX424614) (where Tn*5393* had transposed within IS*903.B*-1 [7]), pEFER (CU928144), and p1 (CP006657). Apart from these related structures, pKPS30 and pKPS77 include various MDR determinants.

In pKPS30, the MDR region consists of a 44,944-bp sequence in which we identified a mosaic structure, including 17 resistance genes, three complete and two truncated insertion sequences, a complete class 1 integron, and two composite transposons. The class 1 integron was part of an IS*CR1*-containing integron encoding bla_{DHA-1} and the *sap* and *psp* operons. The *sap* operon confers resistance to small cationic peptides (20), while the *psp* operon

TABLE 2 Completely sequenced plasmids in GenBank containing IncR characteristic regions

Plasmid name	Species	GenBank accession no.	No. of replication initiation proteins (Inc group)[a]	Transfer region	Reference or source (yr of submission)
pCROD1	*Citrobacter rodentium*	FN543503.1	1 (IncR)		27 (2010)
pEFER	*Escherichia fergusonii*	CU928144	1 (IncR)		Direct submission[b] (2012)
pKP1780	*Klebsiella pneumoniae*	JX424614	1 (IncR)		7 (2013)
pKPC-LK30	*K. pneumoniae*	KC405622	1 (IncR)		16 (2014)
pKPN101-IT	*K. pneumoniae*	JX283456	2 (IncR, IncFIIk)	FIIk	9 (2012)
pK245	*K. pneumoniae*	DQ449578	3 (IncR, NT, NT)		10 (2006)
pKP048	*K. pneumoniae*	FJ628167	2 (IncR, IncFIIk)	FIIk	28 (2010)
pKPN5	*K. pneumoniae*	CP000650	2 (IncR, NT)		Direct submission (2009)
pKPHS2	*K. pneumoniae*	CP003224	2 (IncR, IncFIIk)	FIIk	Direct submission (2011)
pTC2	*Providencia stuartii*	JQ824049	2 (IncR, IncA/C)	A/C	6 (2013)
p1	*K. pneumoniae*	CP006657	2 (IncR, IncFIIk)	FIIk	Direct submission (2013)
pKPS30	*K. pneumoniae*	KF793937	1 (IncR)		This study
pKPS77	*K. pneumoniae*	KF954150	1 (IncR)		This study

[a] Truncated replication initiation proteins were excluded.
[b] Direct submission, plasmid was submitted directly to GenBank (no publications are associated with the submission).

may play a role in maintaining cytoplasmic membrane integrity in response to various environmental stresses (21). These structures were previously described for the pRBDHA (GenBank accession number AJ971343) and pPMDHA (GenBank accession number AJ971344) plasmids isolated from *K. pneumoniae* in Paris, France (22). It has at its 3′ extremity (which includes IRt-IS*6100*) a macrolide resistance region [comprising the *mph*(R), *mrx*, and *mph*(A) genes], as is often observed with class 1 introns/transposons (In/Tn) (18). In addition to the IS*903.B*-containing element mentioned above, two other composite transposons were identified in pKPS30, (i) an *aph*(*3′*)-*I* gene as part of a Tn*4352*-like transposon (18) inserted downstream of the *mph*(A) region and (ii) a Tn*1721*-like transposon (18) carrying the tetracycline resistance *tetA* gene. This transposon was found not to be complete, as the class 1 In/Tn was inserted in its *res* site (TCAAG).

The remaining 28,051-bp sequence of pKPS77 includes genes conferring resistance to β-lactams (bla_{SHV-12}, bla_{TEM-1}), aminoglycosides (*strA*, *strB*), tetracycline (*tetA*), and sulfonamides (*sul2*). The bla_{SHV-12} gene is part of an IS*26*-flanked composite transposon, as previously described on other plasmids (18, 23), and *tetA* is part of a truncated Tn*1721* transposon (18). The structure, including a truncated Tn*2* element carrying bla_{TEM-1b} followed by the ΔCR2-*strB-strA-sul2* configuration (24), was similar to the one observed in the IncFIIk plasmid pKD01 (GenBank accession number JX424423) (25).

IncR plasmids that are able to carry various resistance genes are reported with increasing frequency in clinical strains (1–7). However, as they are nontransferable and nonmobilizable because they lack a transfer system and a relaxase (26), one might question their role in the spread of MDR elements in *Enterobacteriaceae*. In addition, IncR replicons are increasingly described as part of multireplicon plasmids (including associations with IncA/C, IncF, IncFIIk, or nontypeable backbones) (6, 8–10). These associations raise the issue of replication coordination and of the incompatibility phenomenon in multireplicon plasmids.

The present study included two IncR plasmids displaying a wide variety of transposable elements carrying resistance genes. It confirms the increasing clinical importance of this plasmid group. The pool of resistance genes carried by IncR replicons may spread to transmissible plasmids through transposition events or plasmid recombination leading to multireplicons, thus contributing to the high plasticity observed in bacterial plasmids.

Nucleotide sequence accession numbers. The sequences of pKPS30 and pKPS77 have been submitted to GenBank under accession numbers KF793937 and KF954150, respectively.

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