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Molecular characterization of an atypical IncX3 plasmid pKPC-NY79 carrying bla_{KPC-2} in a *Klebsiella pneumoniae*

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Abstract

The IncX family of plasmids has recently been expanded to include at least four subtypes, IncX1-IncX4. The revised classification provides an opportunity for improving our understanding of the sequence diversity of the IncX plasmids and the resistance genes they carried. We described the complete nucleotide sequence of a novel IncX3 plasmid, pKPC-NY79 (42,447 bp) from a sequence type 258 *Klebsiella pneumoniae* strain that was isolated from a patient who was hospitalized in New York, United States. In pKPC-NY79, the plasmid scaffold and genetic load region were highly similar to homologous regions in pIncX-SHV (IncX3, JN247852) and the bla_{KPC} carrying pKpQIL ($IncFII_k$, GU595196), respectively, indicating that it has possibly arisen through recombination of plasmids. The bla_{KPC-2} gene, as part of a transposon Tn*4401*a, was found within the genetic load region. The backbone of pKPC-NY79 differs from pIncX-SHV by a deletion involving the gene tandem *hns-topB* (encoding H-NS protein and topoisomerase III, respectively) and a putative ATPase gene. Unexpectedly, the impact of the *hns-topB* deletion on host fitness and plasmid stability was found to be small. In conclusion, the findings contribute to a better understanding of the plasmid platforms carrying bla_{KPC} and of variations in the backbone of the IncX3 plasmids.

(Word count 199)

Introduction

In *Enterobacteriaceae*, the most frequent class A carbapenmases are the KPC enzymes [26]. The first KPC-producing isolate was identified in a *Klebsiella pneumonia*e strain from North Carolina, USA in 1996. Since then, carbapenem-resistant, KPC-producing isolates have increased in frequency, species distribution and geographical distribution [3,19]. The spread of KPC involves clones, plasmids and transposons. Globally, KPC-producing *K. pneumoniae* isolates were often found to be sequence type (ST) 258 or its variant ST11 [22,26]. These KPC-producing isolates carry plasmids which encode the bla_{KPC} genes as part of a transposon Tn*4401* or its variants [18,22]. On the basis of sequence deletions upstream of bla_{KPC}, five isoforms of Tn*4401*a to Tn*4401*e have been identified which were associated with different levels of bla_{KPC} gene expression [18]. In China, a variant of Tn*4401*, harboring IS*Kpn8*, was reported to carry bla_{KPC} genes in most isolates [22]. While at least 13 different alleles have been reported, KPC-2 and KPC-3 occur most widely [8,26]. The bla_{KPC} genes have been reported on plasmids with narrow ($IncFII_k$, ColE) and broad (IncN, IncL/M and IncA/C) host range or on untypable plasmids. The first bla_{KPC}-carrying plasmids to be completely sequenced were p9 (IncN, FJ223607), p12 (IncN, FJ223605) and p15S (ColE, FJ223606) [9]. The other plasmids that have been completely sequenced were pKP048 ($IncFII_k$, FJ628167), pSLMT ($IncFII_k$, HQ589350), pKpQIL ($IncFII_k$, GU595196), pKpQIL-IT ($IncFII_k$, JN233705), pKPHS2 ($IncFII_k$, CP003224) and pKPN101-IT ($IncFII_k$, JX283456).

The IncX plasmids are narrow host range plasmids of *Enterobacteriaceae*. Such plasmids have most often been reported in *E. coli*, *Salmonella* spp. and *Klebseilla* spp [15,20]. They are known to encode type IV secretion system (T4SS), enabling their own conjugative transfer, and to carry genes encoding biofilm formation and antimicrobial resistance. PCR-based replicon typing (PBRT) procedures demonstrated that the IncX plasmids occur infrequently in 1% or less of the *Escherichia coli* populations [2,15]. Recently, comparative analysis of completely sequenced IncX plasmids demonstrated that the IncX plasmids could be subdivided into four subgroups, IncX1 to IncX4, and the prototype plasmid R6K belonged to the IncX2 subgroup [14]. The basic core structures of the IncX plasmids include sequences encoding replication, partitioning, T4SS, transcriptional activator and putative DNA transfer protein [14,20]. Based upon the *taxC* gene, a revised typing scheme was proposed and these plasmids were shown to be more prevalent than previously acknowledged [14]. In this study, we report the complete sequence of a novel IncX3 plasmid, designated pKPC-NY79 carrying bla_{KPC} originating from a patient with epidemiological link to the United States.

Material and methods

Bacterial strain

In 2011, we identified a *K. pneumoniae* strain CRE79 carrying bla_{KPC-2} from a urine sample of a 71 year-old man who was repatriated from New York, United States, where he had been hospitalized for intracranial hemorrhage. The organism was identified by the Vitek II (bioMérieux SA, Marcy l'Etoile, France). The disc diffusion method and Etest (AB Biodisk, Solna, Sweden) were used to determine the susceptibility to antibiotics [4]. Combined disc test was used to determine the carbapenemase phenotype using EDTA or boronic acid as inhibitors [10].

Molecular studies

The major carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM} and bla_{OXA-48}) were detected by PCR using previously described primers (Supplementary file, Table S1) [10,13]. The multilocus sequence type (MLST) of the strain was determined using the Pasteur Institute scheme [6]. In brief, internal portions of seven housekeeping genes were amplified and sequenced: *rpoB* (beta-subunit of RNA polymerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *infB* (translation initiation factor) and *tonB* (periplasmic energy transducer). A different allele number was given to each distinct sequence within a locus, and a distinct sequence type (ST) number was attributed to each distinct allelic profile. Allele sequences and STs are available on the web site at http:/www.pasteur.fr/mlst. The transferability of bla_{KPC} was tested by filter mating using *Escherichia coli* J53 (azide-resistant) as the recipient [13]. Transconjugants were selected on MacConkey medium containing sodium azide (100 µg/ml) and meropenem (0.5 µg/ml).

Plasmid DNA was extracted with QIAGEN Large Construct kit (Qiagen, Hilden, Germany). Subsequently, the plasmids were converted to the linear forms by incubation with *Aspergillus oryzae* S1 nuclease (Sigma Chemical Cp., St Louis, MO, USA) and were sized by pulsed-field gel electrophoresis [12]. The complete sequence of the plasmid pKPC-NY79 carrying bla_{KPC} in a J53 transconjugant (originating from *K. pneumoniae* strain CRE79) was obtained by using the 454 GS FLX system (Roche, USA) according to the manufacturer's instruction. The library yielded a total of 64,798 reads with average read length of 500 bp. The reads were assembled by the GS de novo Assembler (version 2.6) into two contigs. The gaps were closed by

PCR and Sanger sequencing. The plasmid was annotated by RAST Server and each predicted open reading frames (ORFs) was further blast against the NCBI non-redundant protein database using BLASTP [1,13]. Additional bioinformatics analyses were conducted as previously described [12,13].

Plasmid stability and fitness cost

Stability tests were conducted as described previously [23]. Three ul of an overnight growth of the bacteria in Luria-Beranti (LB) broth were inoculated into 3 ml of a fresh LB broth and incubated for 12 hours at 37 ^{o}C (time zero). The above process was repeated every 12-hourly (equivalent to 10 generations each). At time zero and after passage in the absence of antibiotic for 50, 100, 150 and 200 generations, a sample of the culture was diluted and spread onto LB plate. One hundred colonies were picked and replica plated onto a pair of plain and antibiotic-containing (0.5 µg/ml meropenem) LB plates. Plasmid stability was determined through the percentage of colonial growth on the antibiotic-containing plates. Testing was conducted on two separate occasions.

Two growth parameters including the lag phase and the doubling time in the exponential phase were used to assess the fitness cost associated with the introduction of pKPC-NY79. The test strains were *E. coli* J53 and a J53 transconjugant with the plasmid (J53/pKPC-NY79). Bacteria were cultured in LB broth at 37 ^{o}C with shaking. Growth was monitored every 15 minutes by the optical density until the readings reach a plateau. A growth curve of optical density versus time was plotted and the doubling time calculated as previously described [5]. The lag phase was obtained by extrapolating the tangent at the exponential part of the growth curve back to the inoculum level [24]. This experiment was carried out on three separate occasions.

Results and discussion

Phenotypic and genotypic characteristics of the bacterial strain

The *K. pneumoniae* strain CRE79 was resistant to all β-lactams, including imipenem (>32 µg/ml), ertapenem (>32 µg/ml), meropenem (>32 µg/ml), and multiple non-β-lactam antibiotics (amikacin, ciprofloxacin, chloramphenicol, cotrimoxazole, nitrofurantoin). It was susceptible only to gentamicin, colistin and fosfomycin (8 µg/ml). The extensively drug-resistant phenotype was consistent with those exhibited by KPC epidemic clones circulating in different countries [8,17,19]. Combined disc testing showed that carbapenem-resistance could be reversed by boronic acid but not EDTA. MLST identified CRE79 as ST 258, which is a major lineage

associated with dissemination of *bla*$_{KPC}$ in many countries [3,16]. PCR assays showed that it was positive for *bla*$_{KPC}$ but negative for the other major carbapenemase genes (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{OXA-48}$). The *bla*$_{KPC}$ carrying plasmid was conjugatively transferred to *Escherichia coli* J53 at frequency of 10^{-6} per donor cells. In the transconjugant, resistance to β-lactam antibiotics including carbapenems was the only resistance trait transferred. Pulsed-field gel electrophoresis and S1 nuclease digestion gave a single plasmid band of ~40 kb. The complete sequence of the plasmid is presented here.

Nucleotide sequence analysis of pKPC-NY79

The plasmid (designated as pKPC-NY79, GenBank accession JX104759) is a 42,447 bp circular plasmid with an average GC content of 48.5% and 38 putative open reading frames (ORFs) (Table 1). The plasmid backbone shares high sequence homology to IncX3 plasmids [8,14], exemplified by pIncX-SHV (JN247852) and pEC14_35 (JN935899) which were used as references for annotating pKPC-NY79. When this plasmid was tested by the initially described PBRT [2], the result was negative. Testing with the revised IncX typing scheme based upon the taxC genes [14] detected the plasmid as belonging to the IncX3 subgroup. Figure 1 shows the backbone regions shared by pIncX-SHV and pKPC-NY79 comprise sequences encoding replication (replication initiation protein, *pir*; replication accessory protein, *bis*), partitioning (*parA*), conjugation/type IV secretion system (T4SS, with 11 genes, *pilX1* to *pilX11*), transcriptional activator (*actX*) and putative DNA transfer proteins (*taxA* and *taxC*). However, pKPC-NY79 was modified by a deletion involving the putative plasmid stability tandem genes *hns–topB* (encoding a putative DNA-binding protein and a putative type III topoisomerase, respectively) and a gene encoding ATPase (*atpase*), as comparing with pIncX-SHV. In pKPC-NY79, the genetic load region between the resolvase, *res* gene and *parB* is 16.4 kb in length, including the *bla*$_{KPC-2}$ carrying Tn*4401*a transposon and an upstream 8.2 kb region with three transposases (IS*26*-*tnpA*, Tn*3*-*tnpA* and IS*As12*-*tnpA*), one resolvase (Tn*3*-*tnpR*) and a truncated *umuD* which putatively encodes for an ultraviolet repair protein. The *tnpR* gene in the right extremity of Tn*4401*a was truncated (Figure 1). Of note, the genetic load region from position 6441 to 21387 (JX104759) is almost identical (query coverage 100%, identity 99.9%) to the same gene array in the epidemic *bla*$_{KPC-3}$ carrying plasmid pKpQIL (GU595196, IncFII$_k$) which was associated with the spread of *bla*$_{KPC}$ in the United States, Israel and Italy [8,17,19]. The finding suggested that pKPC-NY79 might have evolved from recombination events involving IncX3/pIncX-SHV-like and IncFII$_k$/pKpQIL-like plasmid ancestors. In this regard, it is interesting that two such plasmids (IncX3/pIncX-SHV and IncFII$_k$-FIB$_k$/pKpQIL-IT) were found in the *K. pneumoniae* strain 55873 [8]. The two

156 *bla*$_{KPC}$ alleles that were found in the two highly homologous regions in pKPC-NY79 (*bla*$_{KPC-2}$, His271, codon
157 CAC) and pKpQIL (*bla*$_{KPC-3}$ Tyr271, codon TAC) could possibly arise through nucleotide substitution [27].
158 Similar phenomena have been reported for *bla*$_{CTX-M}$ alleles carried on highly similar plasmid IncFII
159 (*bla*$_{CTX-M-14/24}$), IncN (*bla*$_{CTX-M-1/32}$) and IncL/M (*bla*$_{CTX-M-3/15}$) platforms [12,21].
160
161 Plasmid stability of pKPC-NY79 and its effect on bacterial host fitness
162 Previous studies have demonstrated that plasmid-encoded H-NS proteins such as sfh have a "stealth" function
163 that allow the plasmids to be transferred to the new bacterial hosts with minimal effects on their fitness [7].
164 Given the deletion of the tandem genes *hns–topB* in pKPC-NY79, we assessed the impact of introducing the
165 plasmid into strain J53 and the stability of the plasmid in absence of antibiotics. The findings showed that
166 introduction of the plasmid significantly increased the lag phase and doubling time of the host strain but the
167 magnitude of the effects were small (Table 2). Stability of pKPC-NY79 with the *hns–topB* deletion could be
168 maintained at 100% over 50 generations of multiplication in the absence of antibiotics. However, a drastic loss
169 of pKPC-NY79 was observed after 100 generations of multiplication, with 75% and 28.5% retaining the
170 plasmid at 150 and 200 generations respectively. As reviewed recently, plasmid-encoded *hns*-like genes occur in
171 a wide range of different plasmids [25]. Unlike previous studies which demonstrated a major adverse effect on
172 fitness [7], our results showed that this IncX3 plasmid variant with *hns* deletion only has a modest effect on the
173 host and plasmid stability was only affected after prolonged propagation in the absence of antibiotics. The
174 discordant observations could possibly be related to host species (*Salmonella* spp. vs. *E. coli*), plasmid size (42
175 kb vs. >100 kb) and host adaptive mutations [23,25].
176
177 In conclusion, we hereby described the complete sequence of a *bla*$_{KPC-2}$-carrying IncX3 plasmid with some
178 unique features in the backbone sequences. The findings contribute to a better understanding of the replicon
179 types of plasmids involved in the dissemination of the *bla*$_{KPC}$ genes. The natural distribution of the IncX3 group
180 of plasmids and the roles that they play in the dissemination of emerging resistance genes remains to be
181 elucidated [11].
182
183 **Acknowledgement**

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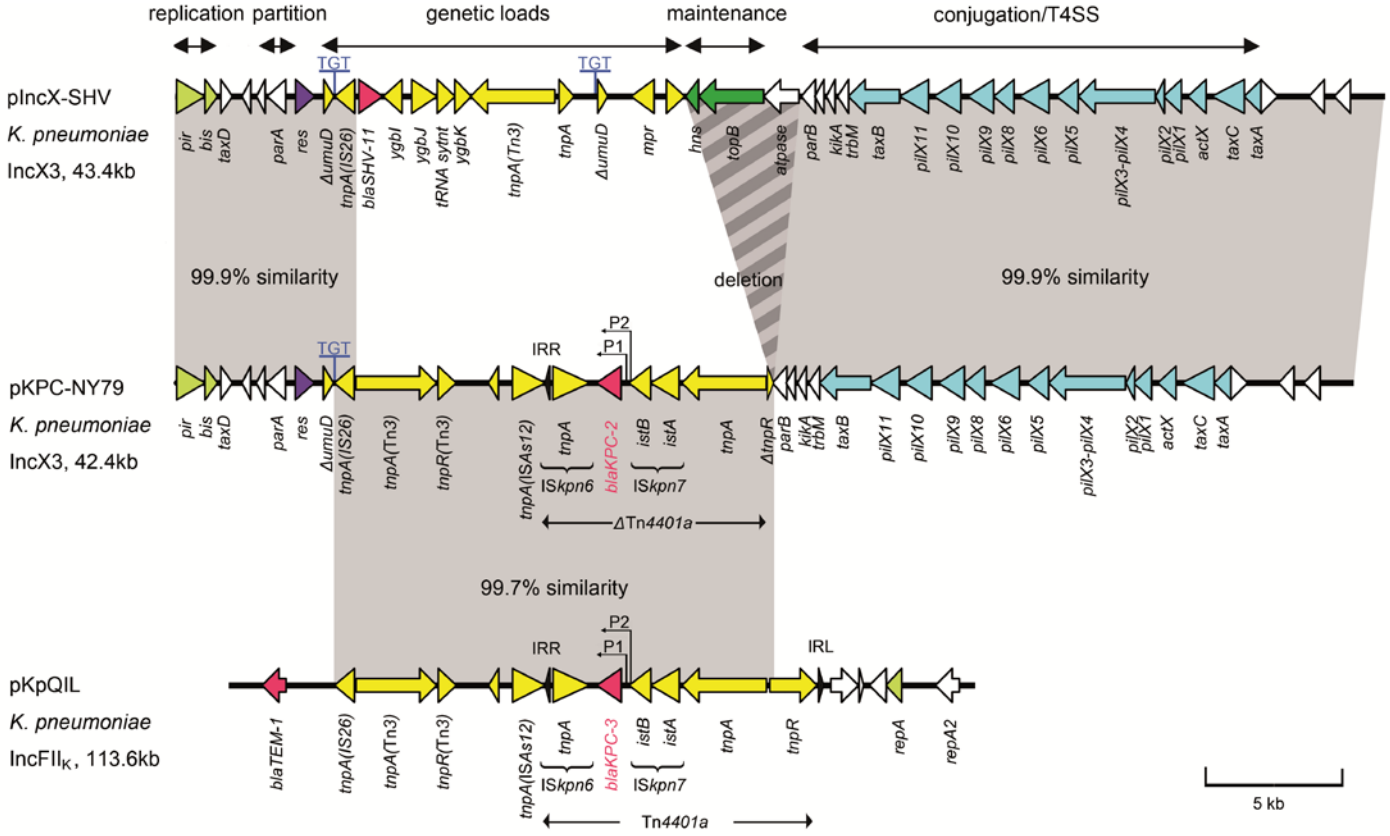
Fig. 1 Comparative analysis of linear plasmid maps for two plasmids, pIncX-SHV and pKPC-NY79 and a partial sequence in pKpQIL. The function blocks of the plasmids are indicated above the linear maps. The lengths of the ORFs are drawn in proportion to the size of the ORFs. Homologous ORFs in the plasmid maps are represented in the same colour. Mobile elements are labelled in yellow. The promoters, P1 and P2 of *bla*$_{KPC}$ gene are identified and annotated as described previously [18]. The accession numbers were: pIncX-SHV (JN247852); pKPC-NY79 (JX104759) and pKpQIL (GU595196).



193 **Table 1 Open reading frames identified in pKPC-NY79**

ORF number	Gene name (alternative name)	Position in JX104759	Strand	Size (bp)	Function
1.	*pir* (*repB*)	1-1014	+	1014	replication initiator protein
2.	*bis*	1023-1466	+	444	conserved hypothetical protein
3.	*taxD*	1585-2007	+	423	DNA distortion polypeptide
4.	*hyp*	2659-2348	–	312	hypothetical protein
5.	*hyp*	3176-2865	–	312	hypothetical protein
6.	*parA*	3911-3249	–	663	plasmid partition protein
7.	*res*	4274-4954	+	681	resolvase
8.	*ΔumuD*	5283-5639	+	357	truncated DNA polymerase
9.	*tnpA (*IS*26)*	6389-5673	–	717	transposase of IS26
10.	*tnpA (*Tn*3)*	6450-9347	+	2898	transposase of Tn*3*
11.	*tnpR (*Tn*3)*	9415-10056	+	642	resolvase of Tn*3*
12.	*hyp*	11581-11222	–	360	hypothetical protein
13.	*tnpA (*IS*As12)*	12085-13269	+	1185	transposase of IS*As12*
14.	*tnpA (*IS*kpn6)*	13546-14865	+	1320	transposase of IS*kpn6*
15.	*blaKPC-2*	15996-15115	–	882	Carbapenemase
16.	*istB (*IS*kpn7)*	17063-16284	–	780	transposase of IS*kpn7*
17.	*istA (*IS*kpn7)*	18085-17060	–	1026	transposase of IS*kpn7*
18.	*tnpA (*Tn*4401a)*	21221-18192	–	3030	transposase of Tn*4401a*
19.	*ΔtnpR (*Tn*4401a)*	21330-21388	+	59	truncated resolvase of Tn*4401a*
20.	*parB*	21966-21454	–	513	plasmid partition protein
21.	*hyp*	22271-21945	–	327	hypothetical protein
22.	*kikA*	22671-22357	–	315	killer family protein
23.	*trbM*	23151-22759	–	393	VirB/Tra/Trw family

					protein
24.	*taxB*	24983-23148	–	1836	conjugal transfer protein
25.	*pilX11*	26020-24986	–	1035	conjugal transfer protein
26.	*pilX10*	27181-26216	–	966	conjugal transfer protein
27.	*pilX9*	28356-27427	–	930	conjugal transfer protein
28.	*pilX8*	29090-28362	–	729	conjugal transfer protein
29.	*pilX6*	30340-29285	–	1056	conjugal transfer protein
30.	*pilX5*	31389-30619	–	771	conjugal transfer protein
31.	*pilX3-pilX4*	34152-31399	–	2754	conjugal transfer protein
32.	*pilX2*	34485-34177	–	309	conjugal transfer protein
33.	*pilX1*	35095-34451	–	645	conjugal transfer protein
34.	*actX*	35978-35328	–	651	Transcription anti-terminator
35.	*taxC*	37350-36190	–	1161	DNA transfer relaxase
36.	*taxA*	37967-37353	–	615	DNA transfer auxiliary protein
37.	*hyp*	37949-38554	+	606	hypothetical protein
38.	*hyp*	40225-39674	–	552	hypothetical protein
39.	*hyp*	41161-40580	–	582	hypothetical protein

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Table 2. Effect of pKPC-NY79 on bacterial growth parameters

	Mean time (minutes) ± standard deviation		*P* value
	J53	J53, pKPC-NY79	
Lag phase	134.6 ± 0.2	144.1 ± 0.6	<0.001
Doubling time	25.4 ± 0.1	26.4 ± 0.4	0.02

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