Complete sequence of two potentially epidemic KPC-harbouring plasmids in Klebsiella pneumoniae ST11 strains in China

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Abstract

Background: Klebsiella pneumoniae carbapenemase (KPC) has spread across the world. The present study focuses on explore sequence and evolution pathway of two potentially epidemic KPC-harbouring plasmids in Klebsiella pneumonia isolated from Southern China.

Methods: Eighteen carbapenemases-producing Klebsiella pneumonia isolates were collected in a tertiary teaching hospital in 2014 in Southern China. The KPC production, antimicrobial susceptibility and MLST were tested for all isolates. Plasmids were purified for all isolates. Plasmids of six isolates were randomly selected to perform Single-molecule real-time sequencing, annotation and bioinformatics analysis. Conjugation and transformation experiment were also performed for the six selected plasmid samples. Furthermore, PCR screening based on the plasmid sequences information were performed for other twelve isolates, to identify the prevalence of the identified plasmids.

Results: Two new KPC plasmids, pF5 and pF77, were identified and detected to be highly epidemic in the collected isolates with the rate of 77.8% (14/18), and 92.9% of the plasmid-harbouring isolates were identified in sequence type (ST) 11 group. pF5 belongs to the incompatibility group IncR and IncFIIk, and pF77 belongs to IncFIIk. The two plasmids were non-conjugative. By sequencing and comparing the plasmids sequences in detail, we found these plasmids carrying bla KPC-2 , bla CTX-M-65 , bla SHV-12 , catA2 and fosA3 antimicrobial genes. Additionally, the two plasmids both showed a close relationship with the other two reported KPC-harbouring plasmids pKP1034 and pCT-KPC, which have highly similar backbones and have been considered to evolve from some recombination events of other closely related plasmids pHN7A8, pKPC-LK30 and pKPHS2. We noticed that these previous reported plasmids and our plasmids all have been detected in the period of 2013-2014 in Southern China, share similar backbone and carried by the clonal ST11 K. pneumonia strains.

Conclusions: The plasmids were identified and detected to be highly epidemic in the collected isolates. A potential evolutionary pathway from pHN7A8-, pKPC-LK30 and pKPHS2-derived plasmids in Klebsiella pneumoniae strains in China was hypothesised.

Background

Carbapenem-resistant *Klebsiella pneumoniae* has been a significant cause of infections worldwide. This pathogen seriously threats public health because of drug resistance against the last clinical resort of carbapenems, thus rendering almost all available treatment options ineffective [1]. One of the primary mechanisms of this pathogen causing resistance is producing the enzyme of *Klebsiella pneumoniae* carbapenemase (KPC), which is encoded by different variants of bla_{KPC} genes and has spread globally [2].

Plasmids are essential vehicles for the spread of drug-resistance genes such as bla_{KPC}. Among the 22 bla_{KPC} variants, bla_{KPC-2} is the most dominant one [3]. Different types of plasmids have been identified to carry bla_{KPC-2}, among which the IncFIIk plasmids are the most frequently detected and widely reported [4, 5]. Recently, two such plasmids pKP1034 and pCT-KPC (also known as pCY-KPC334) were identified from the south part of China [6, 7]. They both convey high levels of antibiotic resistance ability by carrying bla_{KPC-2}, $bla_{CTX-M-65}$, bla_{SHV-12} and many other drug-resistant genes[6, 7]. For the similarity of the sequences of the two plasmids, they were considered to relate to each other [7].

In this study, we identified two new KPC-harbouring IncFIIk plasmids pF77 and pF5, and they are closely relate to the reported plasmids pKP1034 and pCT-KPC, which are reported to be identified in Southern China. So we investigated characteristics of the plasmids and the isolates carrying them in detail. This information also provides us with deep insights into the diversity as well as the potential evolutionary pathway of the IncFIIk type KPC-harbouring plasmids in *K. pneumoniae*. In addition, we conducted a survey of this type of plasmid among a collection of KPC-positive *K.*

pneumoniae isolates and uncovered its significant prevalence in a tertiary teaching hospital in Southern China, and its association with KPC–2-harboring ST11 strains.

Materials And Methods

2.1 Clinical isolates collection

Eighteen KPC-harbouring *K. pneumoniae* isolates were collected from a tertiary teaching hospital from May to September 2014 in Fujian, China. The isolates were subcultured on blood agar and cultured at 37 °C for 18–24 h. A monoclonal colony was selected, inoculated in 2 mL of LB culture medium and cultured at 37 °C, 180 rpm overnight. The bacterial culture was stored at −80 °C in 50% glycerol. The species identification was confirmed by two methods as follows: by VITEK Gram-negative Plus Identification card (Biomerieux Vitek, Inc., France) and by PCR, sequencing, and Blast search of the 16S sequences.

2.2 Confirmation of KPC production and antimicrobial susceptibility testing

All presumptive KPC-producing isolates were subjected to the confirmation test for KPC production by the modified Hodge test (MTH) according to the Clinical and Laboratory Standards Institute (CLSI) criteria.

Antimicrobial susceptibility testing for the strains was determined using VITEK2 GNS cards (Biomerieux, France) in a VITEK–60 system (Biomerieux, France). The minimum inhibitory concentration (MIC) of 21 antimicrobial agents, namely, ampicillin, ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, cefazolin, cefuroxime, cefuroxime axetil, cefotetan, ceftazidime, ceftriaxone, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin and trimethoprim/sulfamethoxazole were determined.

2.3 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) analysis of the isolates was performed based on the MLST website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Chromosomal DNA was obtained by the alkaline lysis method using a commercial genomic DNA purification kit (Tiangen Biotech Co., Ltd) according to the manufacturer's instructions. Allele sequences and sequence types (STs) were verified at the website http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html.

2.4 Plasmids purification

Plasmid DNA of eighteen isolates was obtained and purified by using a commercial genomic DNA purification kit (Tiangen Biotech Co., Ltd) according to the manufacturer's instructions.

2.5 Plasmid sequencing, annotation, and bioinformatics analysis

The plasmids of six isolates were randomly selected from the eighteen isolates for sequencing. Complete sequencing of the plasmids was performed by Single-molecule real-time (SMRT) sequencing. SMRT cell library preparation was conducted by the recommendations provided by the manufacturer and performed using a Pacbio RS II instrument. Each sequence was assembled by the Hierarchical Genome Assembly Process [8, 9]. Glimmer and GeneMark were used to predict the coding sequences (CDSs) [10, 11]. The CDSs were annotated using BLAST against the NR database.

Antibiotic resistance genes were identified by the ARDB database (http://ardb.cbcb.umd.edu/) [12]. Plasmid replicon typing analysis was based on the plasmid MLST website (http://pubmlst.org/plasmid/) [13]. Whole-sequence comparative analysis among closely related plasmids was carried out by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6 Conjugation and transformation experiment of the plasmids

The six selected isolates were subjected to conjugation experiments. The conjugation experiments were conducted following a previous report [14]. Rifampin-resistant *Escherichia coli* EC600 ($LacZ^-$ Nal^r Rif^r) was used as the recipient strain. Briefly, the donor and recipient strains were cultured overnight and then mixed with fresh Luria–Bertani (LB) broth and incubated for 24 h at 35 °C. Then, the mixture was inoculated on LB agar plates containing rifampin (700 μg/ml) and imipenem (1.5 μg/ml) for 24 h at 35 °C. The colonies that grew on the plates were picked up for antimicrobial susceptibility testing. All the experiments were repeated three times.

Total plasmids were extracted from the six selected isolates for the transformation experiments via the method mentioned in 2.4. They were then transformed into *E. coli* DH5α competent cells (Tiangen, China) by the heat shock method, as reported before [15]. Transformants grew overnight on the LB agar containing ceftriaxone (64 mg/L) and then were selected. After testing the existence of the plasmid in the positive transformants via the method mentioned in 2.7, antimicrobial susceptibility of the transformants was determined via the method in 2.1.

2.7 PCR screening for the distribution of the pF77-like plasmids

For the similar parts shared between the identified KPC-harbouring plasmids in the selected isolates, we named this type of plasmid as pF77-like plasmid.

The distribution of the pF77-like plasmids in other twelve KPC-harbouring *K. pneumoniae* isolates was investigated by PCR. PCR primers were designed according to the sequences data of pF77 (Fig 1) [4, 16]. The PCR cycling conditions were as follows: an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min and a final extension step of 72°C for 5 min.

Results And Discussions

3.1. General features of the plasmids

Two new KPC-carrying plasmids, named as pF5 and pF77, were identified in the six selected isolates. pF5 was identified in two of the six isolates, and pF77 was identified in the other four isolates.

pF5 is a 105,125 bp plasmid with a GC content of 54.3% containing 176 open reading frames (ORFs). pF77 is a 57,009 bp plasmid with a GC content of 54.6% containing 96 ORFs. Among the ORFs of the two plasmids, about 50% exhibit known functions in GenBank including: plasmid replication (*repA1, repA2,* and *repA3; repB* only in pF5), stability (*stbAB* and *ssb,* both only in present pF5), partition (*parA* and *parB),*„ SOS inhibition (*psiA* and *psiB),*„ error-prone repair (*umuC* and *umuD),*„ antibiotic resistance (bla_{KPC-2}, $bla_{CTX-M-65}$, bla_{SHV-12} and *catA2; fosA3* only presents in pF5), antirestriction proteins

(*ardA* and *ardB)*,, post-segregation killing proteins (*sok-mok-hok)*,, the toxin-antitoxin system (*pemIK)*,, methyltransferases, transposase and integrase.

When blasting the backbone sequences of pF77 and pF5, they show both 99% identity to two reported plasmids, pKP1034 and pCT-KPC. However, the query coverage ranges from 48% to 79%, which means pF77 and pF5 are smaller than pKP1034 and pCT-KPC. In addition, although many genes pKP1034 and pCT-KPC were kept in pF77 and pF5, genes such as plasmids stability genes *stbA/B,* some antibiotic resistance genes (bla_{TEM-1} and *rmtB)* and the mercury-resistance clusters are lost in pF77 and/or pF5.

The differences in genetic elements indicate a highly versatile characteristic of this group of the plasmids. They can carry necessary genetic elements and help host cells improve the adaption ability under certain stress conditions, such as the presence of antibiotic and heavy metal toxin compounds [17, 18]. They can also help host cells maintain the cost-effectiveness by losing unnecessary elements, for example, the mercury-resistance clusters can be costly for the plasmid to successfully maintained in the strains living without the mercury pressure, which might be the reason for the loss of the clusters in the plasmids [19].

3.2. Plasmid sequences comparison and analysis

The backbone of pF77 can be divided into three modules, and all of them are flanked by IS*26.* The first module (from 26065 to 39750) contains $bla_{CTX-M-65}$ and the second module (from 39751 to 54956) contains $bla_{SHV-12}$and bla_{KPC-2}. These two modules are similar to the multi-resistant region (MRR) of pKP1034 or pCT-KPC, and they were considered to origin from the same sequence of plasmid pHN7A8 [6]. The third module contains (from 1 to 26064) *catA2,* which was only similar to the sequence of pKP1034 (Fig. 1). pF77 is identified to belong to the incompatibility group IncFIIk.

Despite containing the same three modules with pF77, pF5 extends with the other one module between the first and second modules. This extra module is also flanked by IS*26.* The resistant gene *fosA3* keeps in this module. By comparing the sequences in detail, this module is similar with the sequence of pCT-KPC via deleting the mercury-resistance clusters and a reversion event of part of the sequences (Fig. 1). This is a conserved region among most IncR plasmids such as pKPHS2 [6]. Thus pF5 is a multi-replicon plasmid and belongs to both IncR and IncFIIkgroup. Such a presence of multiple replicons can facilitate the plasmid to interact with the broader range of hosts [4].

Additionally, an unreported genetic element structure of the bla_{KPC-2} gene has been identified in plasmids pF77 and pF5. Unlike the classic non-Tn*4401* mobile element structure of bla_{KPC} in pKP1034 and pCT-KPC, which is a typical core element structure of IS*Kpn8*–bla_{KPC-2}–ΔIS*Kpn6* [20], the element structure upstream the bla_{KPC-2} in pF77 and pF5 consisted of *insB* and *insA (*Fig. 2), which together constitute IS*1* instead of IS*Kpn8 [*21]. As far as we know, this type of core element structure of IS*1*–bla_{KPC-2}–ΔIS*Kpn6* has not been reported yet.This structure derives from the IS*1*-mediated homologous recombination (HR) event of deletion of the sequences between bla_{KPC-2} and *insB* in pCT-KPC and pKP1034 (Fig. 1 and 2).

We also noticed that these previously reported plasmids and our plasmids were all detected in the period of 2013–2014 in Southern China. They share similar backbone and were carried by the clonal ST11 *K. pneumonia* strains. In addition to their sequence comparison, a hypothesised evolutionary pathway was considered for these plasmids. pF77 is supposed to derive from plasmids pHN7A8 and pKPC-LK30, and pF5 is supposed to derive from plasmids pHN7A8 and pKPHS2 (Fig 1). While pKP1034 also being hypothesised to evolve from plasmids pHN7A8 and pKPC-LK30 [6], and pCT-KPC has been hypothesised to evolve from plasmids pHN7A8 and pKPHS2 [7, 22, 23], it seems there is a highly diversity of the group of plasmids generated from pHN7A8, pKPC-LK30 and pKPHS2, via IS*26*-mediated HR events including deletion and reversion.

3.3. Microbiological characterisation of the isolates

Antimicrobial susceptibility tests showed that the isolates were all resistant to most of the antimicrobial agents. However, two isolates harbouring pF77 and one harbouring pF5 were susceptible to amikacin (≤ 2 μg/ml), gentamicin (4 μg/ml) and tobramycin (2 μg/ml), while the other two isolates harbouring pF77 were resistant to all of the three antimicrobilas. For the other one isolate harbouring pF5, it was susceptible to amikacin (≤ 2 μg/ml) and was resistant to gentamicin and tobramycin. The detailed results are presented in Table 3.

No *Escherichia coli* EC600 transconjugant recovered by conjugation of both two plasmids in all experiments, which is the same result with that of pKP1034 and pCT-KPC. But the *E. coli* DH5α transformants positive with plasmids pF5 and pF77 confer significantly higher resistance to β-lactams than their parental strain *E. coli* DH5α component cells (Table 3), which confirm the antimicrobial resistance ability of the plasmids.

MLST analysis revealed that the six isolates all belonged to the epidemic ST11 *K. pneumoniae* (Table 2). For other non-sequenced isolates, all of them belonged to ST11, except one isolate belonged to ST524.

3.4. Prevalence and dissemination of the pF77-like plasmids

By PCR the conserved locations of the sequences, eight out of twelve non-sequenced isolates were tested to carry the pF77-like plasmids. Therefore, this type of plasmids were epidemic in our collected isolates (14/18, 77.8%). Moreover, the majority (13/14, 92.9%) of the plasmid were harboured in ST11 isolates and one of the isolates belonged to ST524, which is very close to ST11 with only a single nucleotide mutation at 278 position of *infB* (C→T). Interestingly, pKP1034 and pCT-KPC were also identified in ST11 *K. pneumoniae* strains in Southern China (Table 2). Therefore, this type of plasmids seems to disseminate mainly in ST11 clonal strains in *K. pneumoniae.* Considering the three modules of pF77 discussed in 3.2 are highly conserved in the identified plasmids, the genes and structures in the modules may contribute to their successful dissemination in the strains [24].

Conclusions

In this study, we identified two newly reported KPC-harbouring plasmids pF5 and pF77. They were grouped as pF77-like plasmids and share three conservative modules in their sequences. The pF77-like plasmids were found to be highly prevalent in the collected KPC-harbouring *K. pneumoniae* isolates. Plasmid sequences comparison suggested a potential evolutionary pathway of pHN7A8-, pKPC-LK30- and pKPHS2-derived plasmids in *Klebsiella pneumoniae* strains in China. Through this pathway, a diversity of plasmids has emerged from frequent HR events between variable integration sites. This mechanism can facilitate them to rapidly adapt to a different environment and be able to confer a high level of antibiotic resistance. Furthermore, clonal dissemination and a regional epidemic of pF77-like plasmids in the ST11 *K. pneumoniae* isolates have been identified in our study, which highlights a broad concern of their further evolvement to be a growing clinical and public health threat. Further studies of the distributions of this type of plasmids in other regions and their prevalence in other species are required to determine the ongoing evolutional path of this type of plasmids, and this can be vital for us to understand their contributions to the KPC epidemic and virulence.

Abbreviations

MLST: multi-locus sequence typing (MLST) method; Inc: incompatibility; STs: sequences types, homologous recombination (HR).

Declarations

Acknowledgements

Not available.

Authors' contributions

ZG, SH and YK: designed the experiments. YZ, DL, YG and ZZ performed the experiments. YZ, PD and ZH: performed the plasmid sequencing and bioinformatics analysis. YC: collected and identified the isolates. YZ and DL: wrote and edited the manuscript.

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Availability of data and materials

The annotated sequences of pF5 and pF77 were submitted to GenBank under accession numbers CP016403 and CP016402, respectively.

Ethics approval and consent to participate

Present study approved form Peking University People's Hospital ethical committee 2015PHB037–01.

Consent for publication

The clinical isolate samples used in this research were part of the routine hospital laboratory procedure. Oral consent was taken from the patients. We do not use patients name or personal information so no need to take writing consent.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Primers used in this study

no.	Name	Sequence	Size (bp)	Target	Reference
1	KPC-F	TGTCACTGTATCGCCGTC	1011	*bla*$_{KPC}$ gene	[18]
2	KPC-R	CTCAGTGCTCTACAGAAAACC			
3	FII-F	CTGATCGTTTAAGGAATTTT	260	The *copA* region of the FII replicon	[4]
4	FII-R	CACACCATCCTGCACTTA			
5	RepA1-F	TAGGCTTCACCTCCCGTTT	474	The *repA* gene of the FII replicon	This study
6	RepA1-R	GCTTTCGCTATCAGTTCATCC			
7	IS-F	AGATGCCAAGGTCAATGC	436	Junction between *IS*Kpn6 and downstream gene	This study
8	IS-R	TGGATTACCAACCACTGTCA			
9	YGBK-F	TGATGACCAACAGCCAGGT	290	pF77-like plasmid gene of *ygbk*	This study
10	YGBK-R	CCACAATAAAGCGGGTAAATCC			

Table 2. Plasmids and isolation information

Plasmid	GenBank no.	Length	Replicon type	Isolation information					
				Strain	Strain ST	Year	Source	Country	Area
pF77	CP016402.1	57,009	IncFIIk	*K.pneumoniae*	ST11	2014	human	China	Fujian
pF5	CP016403.1	105,125	IncFIIk, IncR	*K.pneumoniae*	ST11	2014	human	China	Fujian
pKP1034	KP893385.1	136,848	IncFIIk, IncR	*K.pneumoniae*	ST11	2013	human	China	Zhejiang
pCT-KPC	KT185451.1	151,466	IncFIIk, IncR	*K.pneumoniae*	ST11	2013	human	China	Guangzhou

Table 3. Antimicrobial susceptibility profile of isolates and strains

MIC (μg/mL)[interpretation]	pF5-habouring isolates	pF77-habouring isolates	Transformant T-F5	Transformant T-F77
Ampicillin	≥32 [R]	≥32 [R]	≥32 [R]	≥32 [R]
Ampicillin/sulbactam	≥32 [R]	≥32 [R]	≥32 [R]	≥32 [R]
Piperacillin	≥128 [R]	≥128 [R]	64 [I]	64 [I]
Piperacillin/tazobactam	≥128 [R]	≥128 [R]	64 [I]	64 [I]
Cefazolin	≥64 [R]	≥64 [R]	≥64 [R]	≥64 [R]
Cefuroxime	≥64 [R]	≥64 [R]	32 [R]	16 [I]
Cefuroxime axetil	≥64 [R]	≥64 [R]	16 [I]	32 [R]
Cefotetan	≥64 [R]	≥64 [R]	≥64 [R]	≥64 [R]
Ceftazidime	≥64 [R]	≥64 [R]	≥64 [R]	≥64 [R]
Ceftriaxone	≥64 [R]	≥64 [R]	≥64 [R]	≥64 [R]
Cefepime	≥64 [R]	≥64 [R]	16 [I]	16 [I]
Aztreonam	≥64 [R]	≥64 [R]	≥64 [R]	≥64 [R]
Imipenem	≥16 [R]	≥16 [R]	2 [I]	2 [I]
Meropenem	≥16 [R]	≥16 [R]	2 [I]	2 [I]
Amikacin	≤2 [S]	≥64 [R] or ≤2 [S]	≤2 [S]	≤2 [S]
Gentamicin	≥16 [R] or 4 [S]	4 [S] or ≥16 [R]	≤1 [S]	≤1 [S]
Tobramycin	≥16 [R] or 4 [S]	2 [S] or ≥16 [R]	≤1 [S]	≤1 [S]
Ciprofloxacin	≥4 [R]	≥4 [R]	≤0.25 [S]	≤0.25 [S]
Levofloxacin	≥8 [R]	≥8 [R]	≤0.25 [S]	≤0.25 [S]
Nitrofurantoin	≥512 [R]	≥512 [R]	≤16 [S]	≤16 [S]
Trimethoprim/sulfamethoxazole	≥320 [R]	≥320 [R]	≤20 [S]	≤20 [S]

MIC, minimum inhibitory concentration; R, resistant; I, intermediate; S, susceptible

Figures

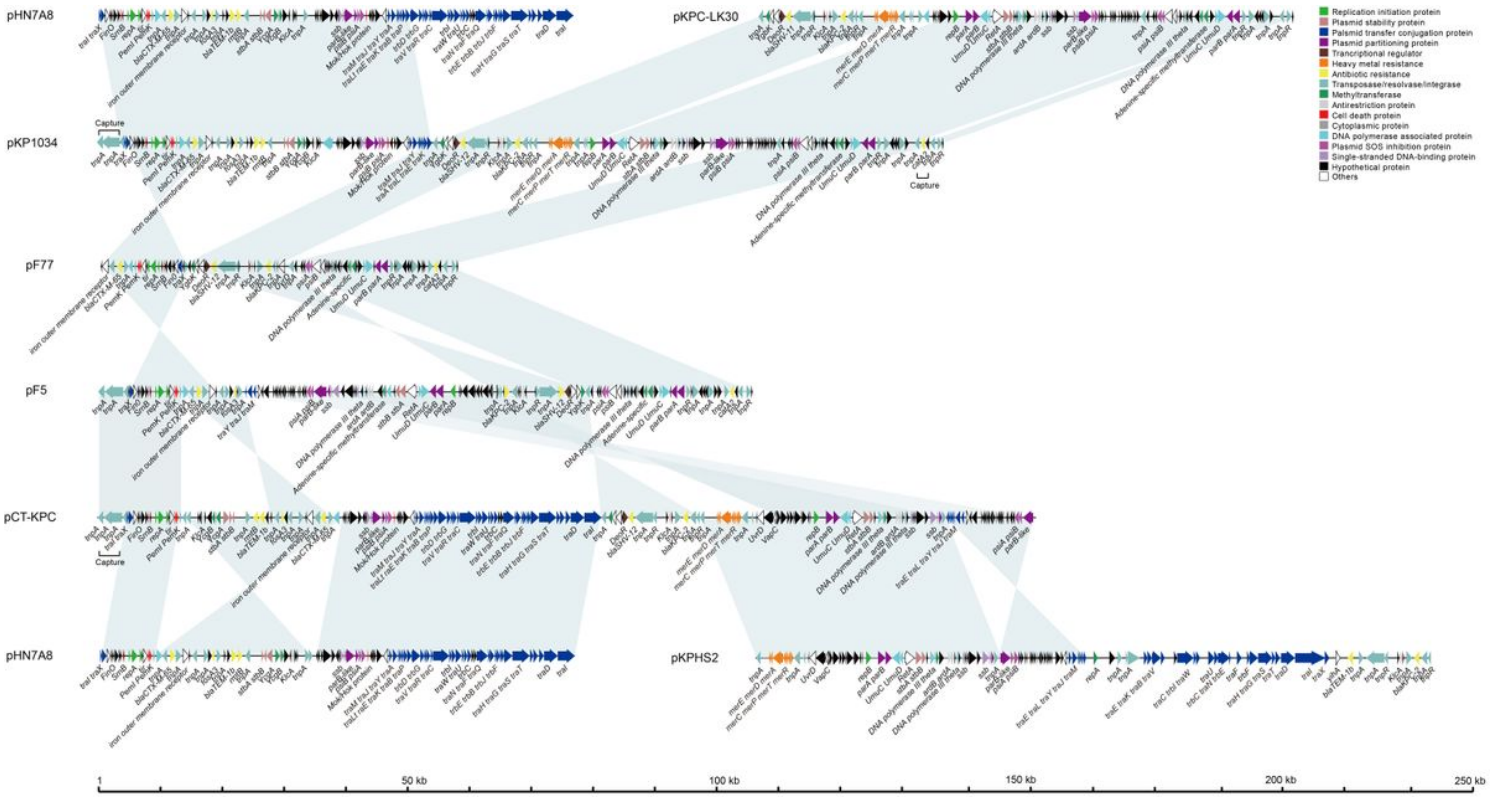

Figure 1

Comparative genomic analysis of the plasmids pF77 (CP016402), pF5 (CP016403), pKP1034 (KP893385), pCT-KPC (KT18545), pHN7A8 (JN232517), pKPC-LK30 (KC405622) and pKPHS2 (CP003224). Light blue shadings indicate regions of homology. The ORFs of genes are indicated by arrows and coloured by different predicted gene functions which are defined in the right panel.

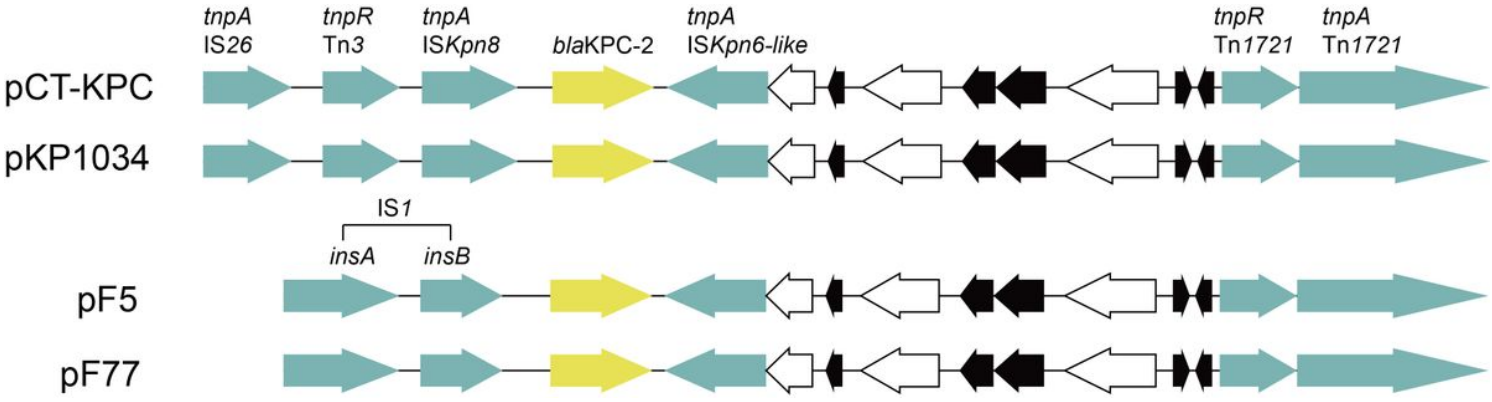

Figure 2

Compare the genetic element structure of the blaKPC-2 gene in plasmids pCT-KPC, pKP1034, pF5 and pF77. The black arrows are hypothetical proteins. The white arrows indicate other proteins of KorC, KlcA and putative replication protein.