Comparative analysis of KPC-2-encoding chimera plasmids with multi-replicon IncR:Inc_{pA1763-KPC}:IncN1 or IncFII_{pHN7A8}:Inc_{pA1763-KPC}:IncN1

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Background: IncR, IncFII, Inc_{pA1763-KPC}, and IncN1 plasmids have been increasingly found among *Enterobacteriaceae* species, but plasmids with hybrid structures derived from the above-mentioned incompatibility groups have not yet been described.

Methods: Plasmids p721005-KPC, p504051-KPC, and pA3295-KPC were fully sequenced and compared with previously sequenced related plasmids pHN84KPC (IncR), pKPHS2 (IncFII_K), pKOX_NDM1 (IncFII_V), pHN7A8 (IncFII_{pHN7A8}), and R46 (IncN1).

Results: The backbone of p721005-KPC/p504051-KPC was a hybrid of the entire 10-kb IncR-type backbone from pHN84KPC, the entire 64.3-kb IncFII_K-type maintenance, and conjugal transfer regions from pKPHS2, a 15.5-kb IncFII_V-type maintenance region from pKOX_NDM1 and a 5.6-kb Inc_{pA1763-KPC}-type backbone region from pA1763-KPC, and it contained a primary IncR replicon and two auxiliary Inc_{pA1763-KPC} and IncN1 replicons. The backbone of pA3295-KPC was a hybrid of a 7.2-kb IncFII_{pHN7A8}-type backbone region from pHN7A8, the almost entire 33.3-kb IncN1-type maintenance and conjugal transfer regions highly similar to R46, a 26.2-kb IncFII_K-type maintenance regions from pKPHS2, the above 15.5-kb IncFII_V-type maintenance region, and the above 5.6-kb Inc_{pA1763-KPC}-type backbone region, and it contained a primary IncFII_{pHN7A8} replicon and two auxiliary Inc_{pA1763-KPC} and IncN1 replicons. Each of p721005-KPC, p504051-KPC, and pA3295-KPC acquired a wealth of accessory modules, carrying a range of intact and residue mobile elements (such as insertion sequences, unit transposons, and integrons) and resistance markers (such as *bla*_{KPC}, *tetA*, *dfrA*, and *qnr*).

Conclusion: In each of p721005-KPC, p504051-KPC, and pA3295-KPC, multiple replicons in coordination with maintenance and conjugation regions of various origins would maintain a broad host range and a stable replication at a steady-state plasmid copy number.

Keywords: multi-replicon plasmids, multi-drug resistance, *bla*_{KPC-2}, mobile elements

Introduction

An IncR replicon alone is able to promote plasmid replication but often coexists with additional replicons such as IncC, IncFII, and IncH.¹ pEFER (GenBank accession number CU928144) is the first sequenced IncR single-replicon plasmid, but pHN84KPC (GenBank accession number KY296104) is more appropriate as the reference of IncR single-replicon plasmids because it contains the most complete IncR backbone, which is composed of *repB* (replication initiation) and *parAB*, *umuCD*, *vagCD*, *resD*, and *retA* (maintenance).¹ IncR single-replicon plasmids lack conjugal transfer genes, making it not self-transmissible.²

IncFII plasmids are usually low copy number plasmids with a narrow host range and circulated mainly among Enterobacteriaceae species.³ Due to significant variations at nucleotide and amino acid levels of backbone sequences, IncFII plasmids can be divided into multiple subgroups, namely IncFII_γ, IncFII_K, IncFII_{pHN7A8}, and IncFII_{p0716-KPC}, represented by pKOX_NDM1 (GenBank accession number JQ314407),⁴ pKPHS2 (GenBank accession number CP003224),⁵ pHN7A8 (GenBank accession number JN232517), and p0716-KPC (GenBank accession number KY270849),^{6,7} respectively. A single replicon IncFII_{pHN7A8}, three replicons IncFII_K, IncFIB, and IncR, and a single replicon IncFII_γ can be found in pHN7A8, pKPHS2, and pKOX_NDM1, respectively. p0716-KPC has a complex chimera backbone, which is composed of a primary replicon IncFII_{p0716-KPC}, the entire 64.3-kb IncFII_K-type maintenance, and conjugal transfer regions found in pKPHS2, a 15.5-kb IncFII_γ-type maintenance region found in pKOX_NDM1, a 5.6-kb backbone region from pA1763-KPC (GenBank accession number MH909340), and an unknown 3.2-kb conjugal transfer region. The pA1763-KPC backbone can be divided into two parts: 1) the above 5.6-kb Inc_{pA1763-KPC}-type backbone region composed of a novel replicon Inc_{pA1763-KPC} and several maintenance genes including *parA*, *ccdBA* and *resA*; and 2) the 64.3-kb IncFII_K-type maintenance and conjugal transfer regions found in pKPHS2.

IncN plasmids can be further divided into three subgroups IncN1, IncN2, and IncN3, in which backbones have conserved gene organization but with limited nucleotide sequence homology.⁸ The backbone of IncN1 reference plasmid R46 (GenBank accession number AY046276) includes regions of replication (*repA*_{IncN1}), maintenance (*mucAB*, *ardBR*, *ccgAE*, and *stbABC*), and conjugal transfer (*nuc*, *tivB*, *eex*, *dtr23*, and *rlx*).⁹

This study dealt with sequencing and genomic dissection of two IncR:Inc_{pA1763-KPC}:IncN1 multi-replicon plasmids p721005-KPC and p504051-KPC as well as an IncFII_{pHN7A8}:Inc_{pA1763-KPC}:IncN1 plasmid pA3295-KPC. The backbone of each plasmid displayed a very complex chimera structure, with integration of several accessory modules composed of mobile elements and associated resistance markers especially including *bla*_{KPC-2}.

Materials and methods

Bacterial strains

Klebsiella pneumoniae 721005 was isolated in 2013 from urine specimens of a 60-year-old male with paraplegia in a public hospital in Ningbo city of China. *K. pneumoniae* 504051 was isolated in 2013 from a blood specimen of a

68-year-old male with pancreatic neoplasm in the hospital mentioned previously. *K. pneumoniae* A3295 was recovered in 2016 from a sputum specimen of a pneumonia patient in a public hospital in Beijing city.

Conjugal transfer

Conjugal transfer experiments were carried out with rifampin-resistant *Escherichia coli* EC600 used as a recipient and each of the *bla*_{KPC}-positive 721005, 504051, and A3295 isolates as a donor. Three milliliters of overnight cultures of each of donor and recipient bacteria were mixed together, harvested, and resuspended in 80 μL of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm² hydrophilic nylon membrane filter with a 0.45 μm pore size (EMD Millipore) which was placed on BHI agar (BD Biosciences) plate and then incubated for mating at 37°C for 12–18 hours. Bacteria were washed from filter membrane and spotted on Muller-Hinton (MH) agar (BD Biosciences) plates containing 1,000 μg/mL rifampin together with 2 μg/mL meropenem, for selecting a *bla*_{KPC}-carrying *E. coli* transconjugant.

Electroporation

Electroporation experiments were carried out for the 721005 and 504051 isolates. To prepare competent cells for electroporation, 200 mL of overnight culture of *E. coli* TOP10 in Super Optimal Broth (SOB) at an optical density (OD₆₀₀) of 0.4–0.6 was washed three times with electroporation buffer (0.5 M mannitol and 10% glycerol) and concentrated into a final volume of 2 mL. One microgram of genomic DNA from the 721005 or 504051 isolate was mixed with 100 μL of competent cells for electroporation at 25 μF, 200 Ω, and 2.5 Kv. The resulting cells were suspended in 500 μL of SOB and an appropriate aliquot was spotted on SOB agar plates containing 1 μg/mL meropenem, for selecting a *bla*_{KPC}-carrying *E. coli* electroporant.

Sequencing and sequence assembly

Genomic DNA was isolated from each of the 721005, 504051, and A3295 isolates using a Blood & Cell Culture DNA Maxi Kit (Qiagen, Hilden, Germany). Genomic DNA of strain 504051 or A3295 was sequenced from a mate-pair library with average insert size of 5 kb (ranged from 2 kb to 10 kb) using a MiSeq sequencer (Illumina, San Diego, CA, USA). Quality control, removing adapters and low quality reads, were performed using *Trimmomatic* 0.36.¹⁰ The filtered clean reads were then assembled using *Newbler* 2.6,¹¹ followed by extraction of the consensus sequence with *CLC*

Genomics Workbench 3.0 (Qiagen Bioinformatics). Gapfiller V1.11 was used for gap closure.¹²

For the 721005 isolate, genome sequencing was performed with a sheared DNA library with average size of 15 kb (ranged from 10 kb to 20 kb) on a PacBio RSII sequencer (Pacific Biosciences, Menlo Park, CA, USA), as well as a paired-end library with an average insert size of 400 bp (ranged from 150 kb to 600 kb) on a HiSeq sequencer (Illumina). The paired-end short Illumina reads were used to correct long PacBio reads utilizing *proovread*,¹³ and then the corrected PacBio reads were assembled denovo utilizing *SMARTdenovo* (available from: <https://github.com/ruanjue/smartdenovo>).

Sequence annotation and comparison

Open reading frames and pseudogenes were predicted using RAST 2.0 with default parameters, combined with BLASTP/BLASTN searches against the UniProtKB/Swiss-Prot database and the RefSeq database.^{14–17} Annotation of resistance genes, mobile elements, and other features was carried out using the online databases including CARD,¹⁸ ResFinder,¹⁹ ISfinder,²⁰ INTEGRALL,²¹ and the Tn Number Registry.²² Gene organization diagrams were drawn in Inkscape 0.48.1 (<https://inkscape.org/en/>).

Phenotypic assays

Activity of Ambler class A/B/D carbapenemases in bacterial cell extracts was determined by a modified CarbaNP test.²³ Bacterial antimicrobial susceptibility was tested by BioMérieux Vitek 2 and interpreted according to the Clinical and Laboratory Standards Institute guidelines.

Nucleotide sequence accession numbers

The complete sequences of plasmids p721005-KPC, p504051-KPC, and pA3295-KPC were submitted to GenBank under accession numbers MG764550, MH477636, and MG764553, respectively.

Ethics statement

Ethics approval and informed consent were not required. All the bacterial isolates involved in this study were part of the routine hospital laboratory procedure.

Results and discussion

Overview of sequenced plasmids

High-throughput sequencing with genomic DNA of the 721005, 504051, and A3295 isolates generated the circular sequences of plasmids p721005-KPC, p504051-KPC, and pA3295-KPC, which were 64,198, 163,588, and 153,274 bp in length, with average G+C contents of 53.92%, 53.82%, and 54.52%, and contained 74, 59, and 60 predicted open reading frames in total, respectively (Table 1 and Figure S1). Each plasmid was composed of the backbone regions, and the accessory modules that were recognized as acquired DNA regions were associated with adjacent mobile elements and inserted at different sites of the backbone (Figure S1). pA3295-KPC, but not p721005-KPC and p504051-KPC, could be transferred from the A3295 isolate into *E. coli* EC600 through conjugation, generating the transconjugant A3295-KPC-EC600. p721005-KPC/p504051-KPC could be transferred from the 72005 or 504051 isolate into *E. coli* TOP10 through electroporation, yielding the electroporant 721005-KPC-TOP10 or 504051-KPC-TOP10, respectively. All these strains had class A carbapenemase activity (data not shown), and were resistant to all the cephalosporin and carbapenem drugs tested (Table 2), which resulted from presence of *bla*_{KPC-2} in all these plasmids.

Multi-replicon chimera structure

Each of p721005-KPC, p504051-KPC, and pA3295-KPC displayed a complex chimera structure. The sequences of p721005-KPC and p504051-KPC were almost identical (99% BLAST coverage and 99% nucleotide identity) to each other. The p721005-KPC/p504051-KPC backbone (Figure 1) was a hybrid of the entire 10-kb IncR-type back-

Table 1 Major features of plasmids analyzed

Category	Plasmids		
	p721005-KPC	p504051-KPC	pA3295-KPC
Total length (bp)	164,198	163,588	153,274
Total number of ORFs	210	211	222
Mean G+C content, %	53.92	53.82	54.52
Accessory modules	<i>IS1X3</i> -to- <i>IS26</i> region, <i>In207</i> - Δ <i>Tn2</i> region [#] , <i>catA2</i> region [#] , and <i>bla</i> _{KPC-2} region [#]	<i>IS1X3</i> -to- <i>IS26</i> region, Δ <i>ISEc15</i> -to- <i>IS26</i> region, <i>catA2</i> region [#] , and <i>bla</i> _{KPC-2} region [#]	<i>qnrS1</i> region [#] , <i>dfrA14</i> region [#] , <i>bla</i> _{KPC-2} region [#] , <i>IS26</i> , and <i>Tn6346</i> -related region

Notes: p721005-KPC, p504051-KPC, and pA3295-KPC were fully sequenced in this study, while pHN84KPC, pKOX_NDM1, pKPHS2, pHN7A8, and R46 were derived from GenBank. [#]Containing resistance genes.

Abbreviation: ORF, open reading frame.

Table 2 Antimicrobial drug susceptibility profiles

Antibiotics	MIC (mg/L)/antimicrobial susceptibility							
	721005	721005-KPC- -TOP10	504051	504051-KPC- -TOP10	A3295	A3295-KPC- -EC600	EC600	TOP10
Piperacillin	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≤4/S	≤4/S
Piperacillin/tazobactam	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≥128/R	≤4/S	≤4/S
Cefazolin	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤4/S	≤4/S
Ceftriaxone	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S	≤1/S
Cefepime	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S	≤1/S
Imipenem	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≤1/S	≤1/S
Meropenem	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≥16/R	≤0.25/S	≤0.25/S
Aztreonam	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≥64/R	≤1/S	≤1/S
Nitrofurantoin	128/R	≤16/S	128/R	≤16/S	≥512/R	32/S	≤16/S	≤16/S
Amikacin	≤2/S	≤2/S	≤2/S	≤2/S	≥64/R	≤2/S	≤2/S	≤2/S
Gentamicin	≤1/S	≤1/S	≤1/S	≤1/S	≥16/R	≤1/S	≤1/S	≤1/S
Ciprofloxacin	≥4/R	≤0.25/S	≥4/R	≤0.25/S	≥4/R	≥4/R	≤0.25/S	≤0.25/S
Levofloxacin	≥8/R	≤0.25/S	≥8/R	≤0.25/S	≥8/R	2/S	0.5/S	0.5/S
Trimethoprim/ sulfamethoxazole	≥320/R	≤20/S	≥320/R	≤20/S	≥320/R	≥320/R	≤20/S	≤20/S

Abbreviations: R, resistant; S, sensitive.

bone found in pHN84KPC, and an 85.4-kb p0716-KPC-derived backbone region that was composed of the almost whole p0716-KPC backbone except for the IncFII_{p0716-KPC} replicon and the 3.2-kb conjugal transfer region. p721005-KPC/p504051-KPC acquired four separate accessory modules, namely the cryptic IS_{IX3}-to-IS₂₆ region, the cryptic ΔISEc15-to-IS₂₆ region in p721005-KPC or the In207-ΔTn2 region in p504051-KPC, the *catA2* region, and the *bla*_{KPC-2} region (Table 1). A 3.6-kb IncN1-type backbone region, including the IncN1 replicon, was found in the *catA2* region. Taken together, p721005-KPC/p504051-KPC contained a primary IncR replicon, as well as two auxiliary replicons Inc_{pA1763-KPC} and IncN1.

The backbone (Figure 2) of pA3295-KPC was a hybrid of a 7.2-kb IncFII_{pHN7A8}-type backbone region found in pHN7A8, a 33.3-kb IncN1-type backbone region highly similar to R46, and a 47.3-kb backbone region found in p0716-KPC.⁷ The 7.2-kb region included an IncFII_{pHN7A8} replicon, a partial maintenance locus *pemIK*, and a partial conjugal transfer remnant region (*rlx*, *tivF16*, and *fnO*). The 33.3-kb region contained almost the entire IncN1-type maintenance and conjugal transfer regions. The 47.3-kb region could be further divided into a 26.2-kb IncFII_K-type maintenance region from pKPHS2, the 15.5-kb IncFII_V-type maintenance region from pKOX_NDM1, and the 5.6-kb backbone region from pA1763-KPC. These two 26.2-kb and 15.5-kb regions were also found in p721005-KPC/p504051-KPC. A total of four accessory modules, namely

the *qnrS1* region, the *dfrA14* region, the Tn6346-related region, and the *bla*_{KPC-2} region, were identified in pA3295-KPC. Remarkably, pA3295-KPC carried a primary IncFII_{pHN7A8} replicon, together with two auxiliary replicons Inc_{pA1763-KPC} and IncN1.

Accessory resistance regions

All of p721005-KPC, p504051-KPC, and pA3295-KPC contained the *bla*_{KPC-2} regions (Figure 3). The *bla*_{KPC-2} region from p721005-KPC could be divided into a *bla*_{TEM-1}-containing Tn2-related region and a *mer*-harboring Tn21-related region as observed in p0716-KPC,⁷ and a complex class 1 integron In207. In207 harbored two resistance regions, namely variable region 1 (VR1, containing a single gene cassette *dfrA25*) and VR2 (harboring *qnrB52* and a *bla*_{KPC-2}-carrying ΔTn6296).⁷ The *bla*_{KPC-2} region from p504051-KPC differed from p721005-KPC but by two major modular changes: 1) insertion of IS₂₆ into ISCR1 of In207, truncating In207 into VR1-carrying In207-5' and VR2-carrying In207-3'; and 2) translocation of In207-5' from the *bla*_{KPC-2} region to connect with the separate ΔISEc15-to-IS₂₆ region (also found as a cryptic accessory module in p721005-KPC), constituting the ΔIn207-Tn2 region. The *bla*_{KPC-2} region of pA3295-KPC was organized sequentially as ISK_{Pn19}, *tetA*(A)-containing ΔTn1721,²⁴ a truncated IS₂₆-*bla*_{SHV-12}-IS₂₆ unit,²⁵ a *bla*_{KPC-2}-carrying ΔTn6296 slightly differing from that from p721005-KPC/p504051-KPC, IS₂₆, a *tni* remnant, and the Tn21-related region.

Figure 2 Modular structure of pA3295-KPC.

Notes: Shown are deduced evolutionary history of plasmid backbone (**A**) and linear comparison of sequenced plasmids (**B**). Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on function classification. Shading denotes regions of homology (>95% nucleotide identity).

Abbreviation: MDR, multidrug resistance.

The Tn6346-related region (Figure 4A, also found in p675920-1) of pA3295-KPC was composed of a Tn3-family transposon remnant and the IS26- Δ Tn6346- Δ GIsl2-IS26 unit.²⁶ The *catA2* region (Figure 4A) of p721005-KPC/p504051-KPC differed from this Tn6346-related region by three major modular changes: 1) insertion of the IS26-*catA2*-IS26 unit; 2) inversion of the cryptic IS26- Δ Tn6346- Δ GIsl2-IS26 unit; and 3) integration of an IncN1-type backbone region containing *repA*_{IncN1} and its iterons, *ardK* and *mpr*. The *qnrS1* region (Figure 4B, also found in p675920-2) from pA3295-KPC was composed of a truncated IS26-*bla*_{lap-2}-*qnrS1*-IS26 unit and several unit transposon remnants and intact or residue ISs.²⁶ The *dfrA14* region (Figure 4C, as observed in pNDM-BTR) from pA3295-KPC harbored In191 containing a single gene cassette *dfrA14*, *ecoRII*-*ecoRIImet* (antirestriction system), and Δ IS1X2.²⁷

Conclusion

Multi-replicon plasmids are increasingly recognized, and existence of multiple replicons is one means by which plasmids with a narrow host range can be restructured to achieve broad host range.^{3,28} In this study, a detailed comparative genomics analysis was subjected to three *bla*_{KPC-2}-carrying chimera plasmids p721005-KPC, p504051-KPC, and pA3295-KPC, disclosing that their sequences were derived from different plasmids belonging to various incompatible groups. p721005-KPC/p504051-KPC might evolve from recombination of IncR plasmid pHN84KPC and IncFII plasmid p0716-KPC, displaying a IncR:Inc_{pA1763-KPC}:IncN1 multi-replicon structure. pA3295-KPC was a hybrid of IncFII_{pHN7A8} plasmid pHN7A8, IncN1 plasmid R46, and IncFII_{p0716-KPC} plasmid p0716-KPC, carrying three replicons IncFII_{pHN7A8}, Inc_{pA1763-KPC}, and IncN1. IncFII_{pHN7A8} replicon manifests as an antisense RNA-regulated replicon, for which an unstable antisense RNA prevents Rep translation at high concentrations by RNA interference, while IncR, IncN1, and Inc_{pA1763-KPC} replicons belong to iteron-regulated replicons, for which iterons (directly repeated sequences) are specifically bound by Rep monomers.²⁸ In each of these plasmids, replicons in coordination with maintenance and conjugation regions would maintain their stable replication at a steady-state plasmid copy number. Each of these plasmids integrated a wealth of accessory modules (Table 1), which were inserted at different sites across the backbone and carried a range of mobile elements (such as IS elements, unit transposons, and integrons) and associated resistance markers (such as *bla*_{KPC}, *tet*, *dfrA*, and *qnr*; Table 3), making host *K. pneumoniae* strains to be multidrug resistant.

Figure 4 Other accessory resistance regions from p7210005-KPC, p504051-KPC, and pA3295-KPC.

Notes: Shown are the *catA2* regions from p721005-KPC/p504051-KPC and the Tn6346-related region from pA3295-KPC (**A**), the *qnrS* region from pA3295-KPC (**B**), and the *dfpA* / 4 region from pA3295-KPC (**C**). Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate nucleotide positions within corresponding plasmids.

Table 3 Drug resistance genes in sequenced plasmids

Plasmid	Resistance marker	Resistance phenotype	Nucleotide position	Region located
p721005-KPC	catA2	Phenicol resistance	49298..49939	catA2 region
	bla _{TEM-1C}	β-lactam resistance	130135..130995	bla _{KPC-2} region
	dfrA25	Trimethoprim resistance	134543..135001	
	qacED1	Quaternary ammonium compound resistance	135185..135532	
	sulI	Sulfonamide resistance	135439..136365	
	qnrB	Quinolone resistance	140112..140687	
	bla _{KPC-2}	Carbapenem resistance	146267..147148	
	mer	Mercuric resistance	150777..154739	
P504051-KPC	sulI	Sulfonamide resistance	27342..28268	In207-ΔTn2 region
	qacED1	Quaternary ammonium compound resistance	28175..28522	
	dfrA25	Trimethoprim resistance	28706..29164	
	bla _{TEM-1C}	β-lactam resistance	32060..32920	
	catA2	Phenicol resistance	112233..112733	catA2 region
	qnrB	Quinolone resistance	139502..140077	bla _{KPC-2} region
	bla _{KPC-2}	Carbapenem resistance	145657..146538	
	mer	Mercuric resistance	150167..154129	
pA3295-KPC	qnrS1	Quinolone resistance	9398..10054	qnrS1 region
	dfrA14	Trimethoprim resistance	32034..32507	dfrA14 region
	tetA(A)	Tetracycline resistance	57453..58652	bla _{KPC-2} region
	bla _{SHV-12}	β-lactam resistance	62094..62954	
	bla _{KPC-2}	Carbapenem resistance	73465..74346	
	mer	Mercuric resistance	78958..82920	

Acknowledgment

This work was supported by the National Key R&D Program of China (2018YFC1200100) and the National Science and Technology Major Project of China (2018ZX10733402).

Author contributions

DZ and JH conceived the study and designed experimental procedures. DQ, YS, LH, XJ, and ZY performed the experiments. All the authors contributed to reagents and materials and data mining. DZ, DQ, YS, and JH wrote this manuscript. All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Figure 1 displays eight circular maps of plasmids, each showing the plasmid backbone and various genes. The plasmids are:

- Plasmid p721005-KPC (Total length 164,200b)
- Plasmid p504051-KPC (Total length 163,980b)
- Plasmid pA3295-KPC (Total length 153,270b)
- Plasmid pKOX_NDM1 (Total length 110,790b)
- Plasmid pKPHS2 (Total length 111,190b)
- Plasmid pH7A8 (Total length 76,890b)
- Plasmid pH8AKPC (Total length 40,630b)
- Plasmid R46 (Total length 58,870b)

The legend indicates the following color coding:

- Backbone:**
 - Plasmid replication (Blue)
 - Plasmid maintenance (Green)
 - Conjugal transfer (Red)
- Accessory modules:**
 - The *MCR*₁ region from pH8AKPC (Orange)
 - The *IS*₁ region from pH8AKPC (Yellow)
 - The *IS*₂ region from pH8AKPC (Light Green)
 - The *IS*₃ region from pH8AKPC (Light Blue)
 - The *IS*₄ region from pH8AKPC (Light Purple)
 - The *IS*₅ region from pH8AKPC (Light Orange)
 - The *IS*₆ region from pH8AKPC (Light Yellow)
 - The *IS*₇ region from pH8AKPC (Light Green)
 - The *IS*₈ region from pH8AKPC (Light Blue)
 - The *IS*₉ region from pH8AKPC (Light Purple)
 - The *IS*₁₀ region from pH8AKPC (Light Orange)
 - The *IS*₁₁ region from pH8AKPC (Light Yellow)
 - The *IS*₁₂ region from pH8AKPC (Light Green)
 - The *IS*₁₃ region from pH8AKPC (Light Blue)
 - The *IS*₁₄ region from pH8AKPC (Light Purple)
 - The *IS*₁₅ region from pH8AKPC (Light Orange)
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 - The *IS*₁₈ region from pH8AKPC (Light Blue)
 - The *IS*₁₉ region from pH8AKPC (Light Purple)
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 - The *IS*₉₉ region from pH8AKPC (Light Purple)
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Notes: Genes are denoted by arrows, and the backbone and accessory molecular regions are highlighted in black and grey, respectively. The innermost circle presents GC-skew $[(G-C)/(G+C)]$, with a window size of 500 bp and a step size of 20 bp. The next-to-innermost circle presents GC content.

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