

# Characterization of the plasmid of incompatibility groups IncFII<sub>pKF727591</sub> and Inc<sub>pKPHS1</sub> from *Enterobacteriaceae* species

This article was published in the following Dove Press journal:  
*Infection and Drug Resistance*

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**Background:** Multiple incompatibility (Inc) groups of plasmids have been identified in *Enterobacteriaceae* species, but there are still quite a few sequenced plasmids that could not be assigned to any known Inc groups.

**Methods:** One IncFII<sub>pKF727591</sub>β plasmid p205880-qnrS and two Inc<sub>pKPHS1</sub> plasmids p11219-CTXM and p205880-NR1 were fully sequenced in this work. Detailed genomic comparison was applied to all available sequenced plasmids of IncFII<sub>pKF727591</sub> or Inc<sub>pKPHS1</sub> group.

**Results:** p205880-qnrS carried a novel transposon Tn6396, which was an ISKpn19-composite transposon and represented a prototype transposable element carrying a minimum core *qnrS1* module. p11219-CTXM harbored a novel transposon Tn6559, which was generated from integration of a truncated IS903D-bla<sub>CTX-M-14</sub>-ISEcp1 unit into the Tn3-family cryptic unit transposon Tn1722. Two Inc groups, IncFII<sub>pKF727591</sub> and Inc<sub>pKPHS1</sub>, of plasmids from *Enterobacteriaceae* species were proposed, and IncFII<sub>pKF727591</sub> was further grouped into two subgroups IncFII<sub>pKF727591</sub>α and IncFII<sub>pKF727591</sub>β. Each of the 11 IncFII<sub>pKF727591</sub> plasmids carried multiple accessory modules including at least one resistance module, and the relatively small IncFII<sub>pKF727591</sub> backbones could acquire a wealth of foreign genetic contents. The modular structures of plasmid backbones were conserved within each of IncFII<sub>pKF727591</sub>α and IncFII<sub>pKF727591</sub>β subgroups but dramatically different, although with similar gene organizations, between these two subgroups. The Inc<sub>pKPHS1</sub> backbones were conserved with respect to modular structures, and only four of the 14 Inc<sub>pKPHS1</sub> plasmids carried accessory modules, two of which contained resistance genes.

**Conclusion:** A genomic comparison of sequenced Inc<sub>pKPHS1</sub> or IncFII<sub>pKF727591</sub> plasmids provides insights into modular differences and genetic diversification of these plasmids, some of which carries antimicrobial resistance genes.

**Keywords:** plasmids, IncFII<sub>pKF727591</sub>, Inc<sub>pKPHS1</sub>, Tn6396, Tn6559

## Introduction

Plasmid is a small DNA molecule within a bacterial cell and capable of replicating independently from the host's chromosomal DNA. Plasmids are mobile genetic elements that commonly carry antimicrobial resistance genes and other genetic factors such as virulence genes. Plasmid-mediated transmission of antimicrobial resistance genes among *Enterobacteriaceae* and other bacteria imposes a major public health concern.

The original replicon-based scheme to classify plasmids into different incompatibility (Inc) groups was developed in 1970s, which is based on the experimental observations that plasmids with similar replication machinery are often unable to

stably co-exist within the same host cell and thus the plasmid shows incompatibility with the same Inc group plasmid.<sup>1</sup> Nowadays, Inc classification is always based on replication initiation protein (Rep) sequences, and it is not necessarily confirmed by conventional conjugation-based incompatibility experiments.<sup>1</sup> At least 27 Inc groups have been identified in *Enterobacteriaceae* species,<sup>1</sup> but there are quite a few sequenced plasmids that could not be assigned to any known Inc groups.

This study presented three sequenced plasmids (p205880-qnrS carrying a novel IS*Kpn19*-composite transposon Tn6396, p11219-CTXM harboring a novel Tn1722-derived unit transposon Tn6559, and p205880-NR1 containing no resistance genes) and proposed two novel Inc groups (IncFII<sub>pKF727591</sub> and Inc<sub>pKPHS1</sub>). p205880-qnrS belonged to Inc<sub>pKPHS1</sub>, while p11219-CTXM and p205880-NR1 could be assigned to IncFII<sub>pKF727591</sub>. Further detailed genomic comparison of all sequenced plasmids of Inc<sub>pKPHS1</sub> or IncFII<sub>pKF727591</sub> indicated considerable modular differences and genetic diversification of each group of plasmids.

## Materials and methods

### Bacterial strains and genome sequencing

*Klebsiella pneumoniae* 205880 and 11219 were recovered from the sputum specimens of two different patients with pneumonia in two different Chinese hospitals in 2012 and 2013, respectively. For each strain, genomic DNA isolation, genome sequencing, and sequence assembly and annotation were carried out as described previously.<sup>2</sup> An unrooted neighbor-joining tree was generated from the aligned *repA* sequences of indicative plasmids.<sup>2</sup> Plasmids p205880-qnrS, p11219-CTXM and p205880-NR1 had GenBank accession numbers MF190368, MF133442 and MF144193, respectively.

### Phenotypic assays

Plasmid conjugal transfer was carried out, as described previously,<sup>2</sup> with *Escherichia coli* EC600 as a recipient and the 205880 or 11219 isolates as a donor, for selecting an *E. coli* transconjugant that carried *bla*<sub>CTX-M-14</sub> (p11219-CTXM) or *qnrS1* (p205880-qnrS), respectively. Electroporation of plasmid p11219-CTXM from the 11219 isolate into *E. coli* TOP10 was performed, as described previously,<sup>2</sup> to obtain an *E. coli* electroporant carrying *bla*<sub>CTX-M-14</sub> (p11219-CTXM). Double-disk synergy test was performed to detect the activity of extended-spectrum  $\beta$ -lactamase (ESBL) in indicative bacterial strains.<sup>3</sup> BioMérieux VITEK 2 was used to test

bacterial antimicrobial susceptibility, which was interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>4</sup>

## Results and discussion

### Diversification of IncFII<sub>pKF727591</sub> plasmids

One new plasmid p205880-qnrS was fully sequenced (Table 1) and could be transferred from the wild-type 205880 isolate into EC600, through conjugation, giving a *qnrS*-positive transconjugant p205880-qnrS-EC600. As expected, these two strains were resistant to ciprofloxacin and levofloxacin with minimum inhibitory concentration (MIC) values  $\geq 4$ .

A collection of 11 plasmids including p205880-qnrS (Table S1), which had homologous *repA* (replication initiation) genes and similar backbone gene organizations, were assigned into a novel Inc group designated IncFII<sub>pKF727591</sub> (Inc<sub>reference</sub> plasmid), because all these RepA proteins had an IncFII super-family domain. The phylogenetic tree (Figure 1) based on *repA* sequences indicated that these 11 plasmids could be divided into two separately clustering subgroups IncFII<sub>pKF727591</sub> $\alpha$  (n=8) and IncFII<sub>pKF727591</sub> $\beta$  (n=3). As shown by pairwise comparison of *repA* nucleotide sequences, plasmids within each subgroup showed 100% identity, while those from different subgroups displayed  $\geq 79\%$  identity (Table S2A). Predicted RepA-binding iterons were located from 245 bp to 365 bp downstream of *repA* for IncFII<sub>pKF727591</sub> $\alpha$  plasmids, but upstream from 366 bp to 460 bp for IncFII<sub>pKF727591</sub> $\beta$  plasmids, and three copy numbers of iteron were found for all IncFII<sub>pKF727591</sub> plasmids (Table S1). Plasmids within each subgroup shared a conserved iteron motif, but those from different subgroups had dramatically different iteron motifs (Figure 1).

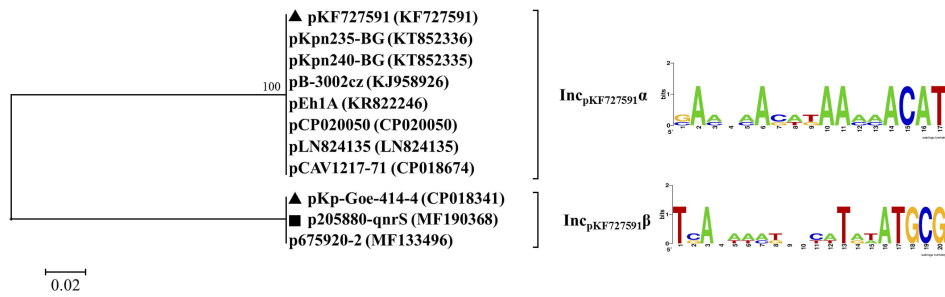
pKF727591 (the first sequenced IncFII<sub>pKF727591</sub> plasmid) and pKp\_Goe\_414-4 (the first sequenced IncFII<sub>pKF727591</sub> $\beta$  plasmid) were identified as the references for IncFII<sub>pKF727591</sub> $\alpha$  and IncFII<sub>pKF727591</sub> $\beta$ , respectively. p205880-qnrS belonged to IncFII<sub>pKF727591</sub> $\beta$ .

The modular structure (Table 1 and Figure S1) of each plasmid could be divided into one or more accessory modules (defined as acquired DNA regions associated or bordered with mobile elements) and the remaining IncFII<sub>pKF727591</sub> backbone regions (responsible for plasmid replication, maintenance and conjugal transfer). The eight IncFII<sub>pKF727591</sub> $\alpha$  plasmids shared  $\geq 88\%$  of their backbone sequences with  $\geq 99\%$  nucleotide identity, and the three IncFII<sub>pKF727591</sub> $\beta$  plasmids showed  $\geq 99\%$  nucleotide

**Table 1** Major features of plasmids analyzed

Inc group	Plasmid	Accession number	Total length (bp)	Total number of open reading frames	Mean G+C content, %	Length of the backbone (bp)	Accessory module (s)	Reference	
IncFII <sub>pKF727591α</sub>	pKF727591@	KF727591	94,790	112	53.1	51,559	<i>bla</i> <sub>NDM-1</sub> region <sup>#</sup> , and $\Delta$ ISEc16	NA	
	pKpn235-BG	KT852336	76,360	90	53.9	51,749	<i>bla</i> <sub>NDM-1</sub> region <sup>#</sup> , and $\Delta$ ISEc16	NA	
	pKpn240-BG	KT852335	76,980	92	53.8	51,594	<i>bla</i> <sub>NDM-1</sub> region <sup>#</sup> , and $\Delta$ ISEc16	NA	
	pB-3002cz	KJ958926	97,650	122	53.2	51,558	<i>bla</i> <sub>NDM-1</sub> region <sup>#</sup> , and $\Delta$ ISEc16	11,12	
	pEh1A	KR822246	96,120	105	53.1	51,559	<i>bla</i> <sub>NDM-1</sub> region <sup>#</sup> , and $\Delta$ ISEc16	13	
	pCP020050	CP020050	113,430	129	52.5	51,559	<i>ars</i> region <sup>#</sup> , and $\Delta$ ISEc16	NA	
	pLN824135	LN824135	118,320	142	52.5	46,102	<i>dfpA14-qnrB1</i> region <sup>#</sup> , ISEc10, and $\Delta$ ISEc16	NA	
	pCAV1217-71	CP018674	70,610	95	52.4	46,100	<i>gigC</i> region, ISEc27, and $\Delta$ ISEc16	NA	
	IncFII <sub>pKF727591β</sub>	pKp_Goe_414-4@	CP018341	81,641	88	53.9	54,186	MDR region <sup>#</sup>	NA
		p205880-qnrS <sup>\$</sup>	MFI90368	65,110	75	52.9	54,649	IS26- $\Delta$ Tn1696, and Tn6396 <sup>#</sup>	This study
p675920-2		MFI133496	79,370	83	54.1	54,726	MDR region <sup>#</sup>	7	
Inc <sub>pKPHS1</sub>	pKPHS1@	CP003223	122,800	131	49.5	113,828	Tn6558 region <sup>#</sup>	14	
	pRJA166c	CP019050	111,080	117	49	111,080	None	NA	
	pPMK1-B	CP008931	111,690	117	49.2	111,690	None	15	
	pSg1-1	CP012427	126,470	134	49.2	126,470	None	16	
	pI1219-CTXM	MFI133442	122,080	128	50	110,993	Tn6559 region <sup>#</sup> , and ISKpn24	This study	
	pCP020063	CP020063	109,020	119	49.2	107,422	ISKpn38	NA	
	pCP015755	CP015755	109,350	125	49.3	109,350	None	NA	
	pCP016161	CP016161	109,350	123	49.3	109,350	None	NA	
	pJCLA0XA232-4	CP012565	112,060	117	49	112,060	None	NA	
	pJCLA0XA232-4.X	CP012570	111,240	115	49	111,240	None	NA	
	plncFIB_DHQP1400954	CP016924	111,540	116	49.3	111,540	None	NA	
	pKPN-04f	CP014756	121,030	118	49.3	109,640	IS3000, and ISKpn25	NA	
	p205880-NR1	MFI44193	108,040	117	49.3	108,040	None	NA	
	pKP301b	KY354306	110,253	111	48.3	110,653	None	This study	

**Notes:** p205880-qnrS, p11219-CTXM and p205880-NR1 were sequenced in this study, while all the other plasmids were derived from GenBank (last accessed May 28, 2017). <sup>#</sup>Reference plasmids. <sup>\$</sup>Carrying resistance gene. **Abbreviation:** NA, not applicable.



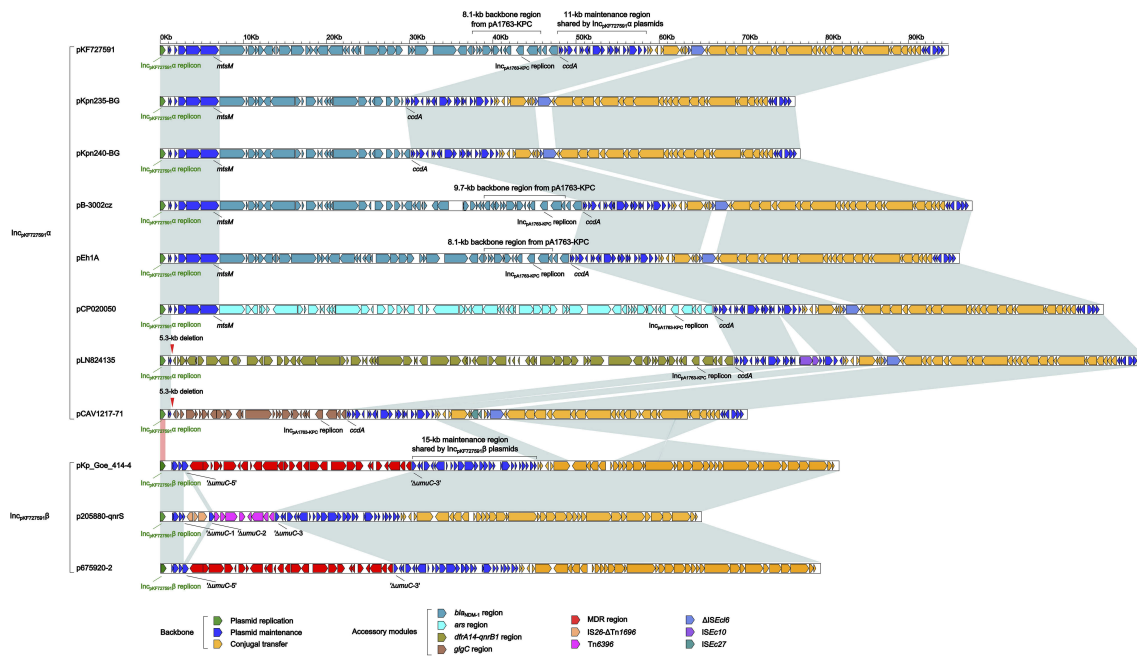
**Figure 1** A neighbor-joining phylogenetic tree for IncFII<sub>pKF727591</sub> plasmids. The degree of support (percentage) for both cluster of associated taxa, as determined by bootstrap analysis, is shown next to each branch. The bar corresponds to the scale of sequence divergence. The triangles indicate the reference plasmids, while the square denotes the plasmid sequenced in this study.

identity over  $\geq 98\%$  of their backbone sequences; by contrast, the backbones of IncFII<sub>pKF727591α</sub> and IncFII<sub>pKF727591β</sub> had  $\leq 92\%$  nucleotide identity across  $\leq 70\%$  of their backbone sequences (Table S2B). The modular structures of plasmid backbones were conserved within each of IncFII<sub>pKF727591α</sub> and IncFII<sub>pKF727591β</sub> subgroups but dramatically different between two subgroups.

Integration of accessory modules at various sites of IncFII<sub>pKF727591</sub> backbones led to the interruption of relevant backbone genes (eg, *umuC*), the disruption of the maintenance or conjugal transfer regions, or the deletion of surrounding backbone regions (eg, 5.3-kb deletion containing *mtsM*) (Figure 2). The IncFII<sub>pKF727591</sub> replicons and the conjugal transfer regions (encoding an F-type

type IV secretion system) were found in all 11 plasmids and thus represented the core IncFII<sub>pKF727591</sub> backbone. An 11-kb maintenance region carrying *parAB* (partition) and *ccdBA* (toxin-antitoxin) was found in all IncFII<sub>pKF727591α</sub> plasmids, while another distinct 15-kb maintenance region containing *stbAB* (mediator of plasmid stability) and *resD* (resolvase) in all IncFII<sub>pKF727591β</sub> plasmids.

All the three IncFII<sub>pKF727591β</sub> plasmids contained a single IncFII<sub>pKF727591β</sub> replicon, which six of the eight IncFII<sub>pKF727591α</sub> plasmids contained a second Inc<sub>pA1763-KPC</sub> replicon beside the master IncFII<sub>pKF727591α</sub> replicon (Figure 2). Notably, the Inc<sub>pA1763-KPC</sub> replicon was located within a 9.7- or 8.1-kb backbone region [carrying maintenance



**Figure 2** Linear comparison of complete sequences of IncFII<sub>pKF727591</sub> plasmids. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading regions denote homology of plasmid backbone regions (light blue:  $\geq 90\%$  nucleotide identity; light red:  $< 90\%$  nucleotide identity) but not accessory modules.

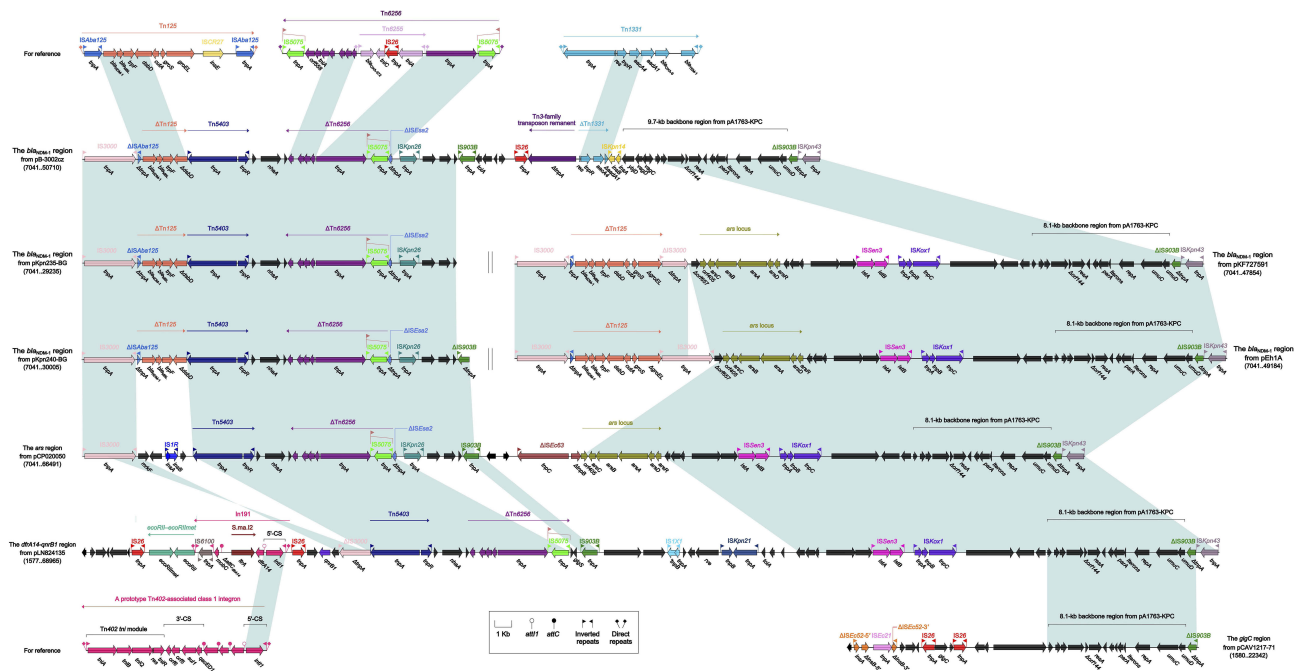
genes such as *parA* and *resA*; as observed in pA1763-KPC (GenBank accession number MH909340], which was a part of relevant accessory modules (see below; Figure 3). Two co-existent replicons IncFII<sub>pKF727591α</sub> and Inc<sub>pA1763-KPC</sub>, together with their supporting maintenance genes, will promote relevant plasmids to overcome incompatibility barrier with incoming plasmids. All the above replicons belonged to the iteron-regulated replicon, for which Rep monomers specifically bound to iterons.<sup>5</sup>

### Accessory resistance modules of IncFII<sub>pKF727591</sub> plasmids

A large accessory module was integrated at a site between the two maintenance genes *mtsM* and *ccdA* in each of the eight IncFII<sub>pKF727591α</sub> plasmids (Figure 3). These eight modules had some common regions but showed considerable modular differences across the whole modules, indicating their sole evolutionary origin followed by parallel mosaic diversification. The 9.7- or 8.1-kb backbone region from Inc<sub>pA1763-KPC</sub> (see above) was found in six IncFII<sub>pKF727591α</sub> plasmids except for pKpn235-BG and pKpn240-BG. The accessory modules from seven IncFII<sub>pKF727591α</sub> plasmids, except for pCAV1217-71, carried resistance loci (Figure 3 and Table S3): i) a truncated Tn125 transposon carrying *bla*<sub>NDM-1</sub><sup>6</sup> was harbored in pB-

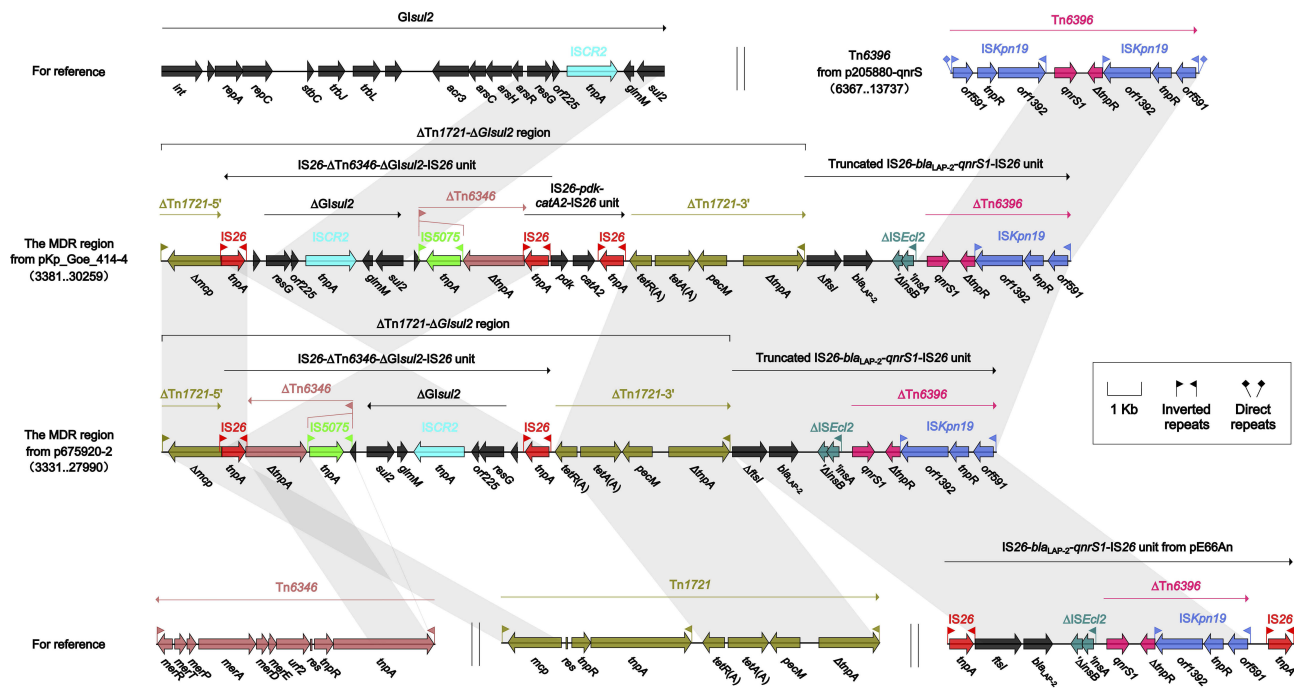
3002cz, pKpn235-BG and pKpn240-BG, while another truncated version of Tn125 in pKF727591 and pEh1A; ii) the *ars* (arsenical resistance) locus was found in pKF727591, pEh1A and pCP020050 and, notably, the first two plasmids showed coexistence of *bla*<sub>NDM-1</sub> and *ars*; and iii) *qnrB1* and *dfrA14*-carrying In191 were identified in pLN824135.

A 24.7-kb MDR region, another 26.9-kb MDR region and Tn6396 (Figure 4) were inserted at the same site within the *umuC* gene of the three IncFII<sub>pKF727591β</sub> plasmids p675920-2, pKp\_Goe\_414-4 and p205880-qnrS, respectively. The 24.7-kb MDR region, carrying multiple resistance genes (Table S3), was generated from integration of an IS26-ΔTn6346-ΔGI*sul2*-IS26 unit<sup>7</sup> into Tn1721,<sup>8</sup> which was further connected with a truncated IS26-*bla*<sub>LAP-2</sub>-*qnrS1*-IS26 unit.<sup>7</sup> The 26.9-kb MDR region was highly similar to the 24.7-kb MDR region but differed from it mainly by inversion of IS26-ΔTn6346-ΔGI*sul2*-IS26 and further upstream insertion of an IS26-*pdk-cata2*-IS26 unit. Tn6396 was a novel IS*Kpn19*-compsite transposon, which carried the *qnrS1*-*ΔtnpR* region and bracketed by 7-bp direct repeats (DRs: target site duplication signals for transposition) at both ends. Tn6396 represented a prototype transposable element carrying a minimum core *qnrS1* module. Different Tn6396



**Figure 3** Organization of selected accessory modules from IncFII<sub>pKF727591α</sub> plasmids and comparison with related regions. 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. The accession numbers of Tn125,<sup>6</sup> Tn6256<sup>17</sup> and Tn1331<sup>18</sup> for reference are JN872328, KP851978 and KC354802, respectively.





**Figure 4** Organization of accessory resistance modules from IncFII<sub>pKF727591</sub> β plasmids and comparison with related regions. 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. The accession numbers of Glsul2,<sup>19</sup> Tn6346,<sup>20</sup> Tn1721<sup>8</sup> and IS26-bla<sub>LAP-2</sub>-qnrS1-IS26 unit<sup>21</sup> for reference are CP001918, EU696790, X61367 and HF545433, respectively.

derivatives with distinct terminal truncations were found in various plasmids including pKp\_Goe\_414-4 and p675920-2 (Figure S2).

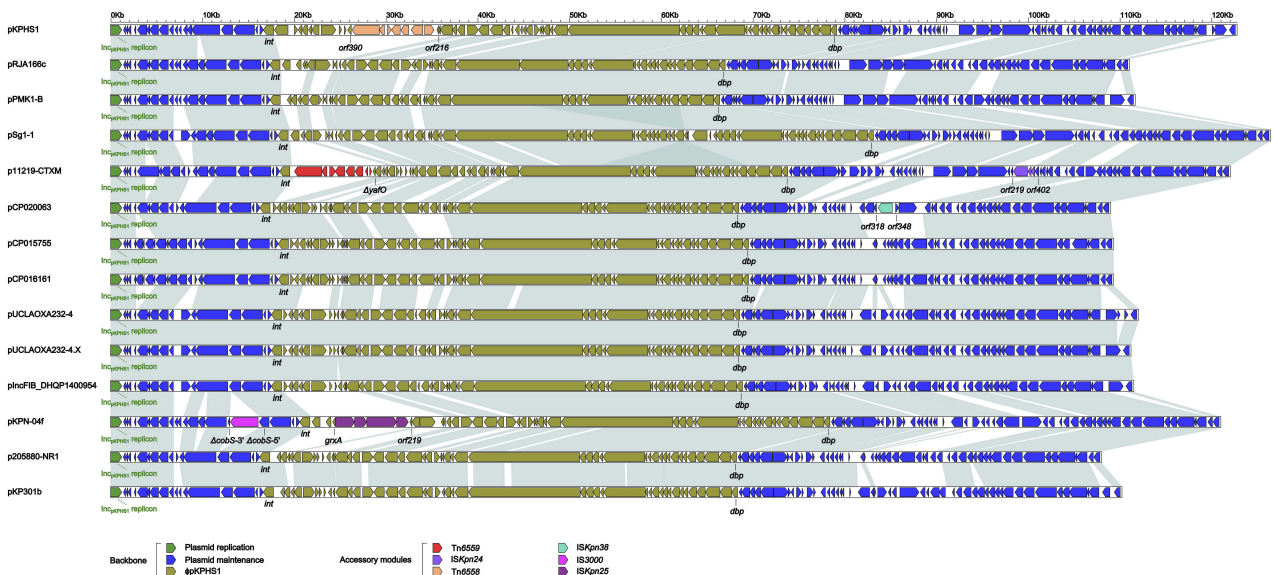
## Characterization of Inc<sub>pKPHS1</sub> plasmids

Two additional new plasmids p11219-CTXM (carrying bla<sub>CTX-M-14</sub>) and p205880-NR1 (containing no resistance genes) were fully sequenced (Table 1 and Figure S1). p11219-CTXM could not be transferred from the wild-type 11219 isolate into EC600 through conjugation, but could be transferred into TOP10 through electroporation, generating a bla<sub>CTX-M-14</sub>-positive electroporant 11219-CTXM-TOP10. These two wild-type and electroporant strains had ESBL activity (data not shown) and were resistant to cefazolin, cefuroxime and ceftazidime with MIC values ≥64.

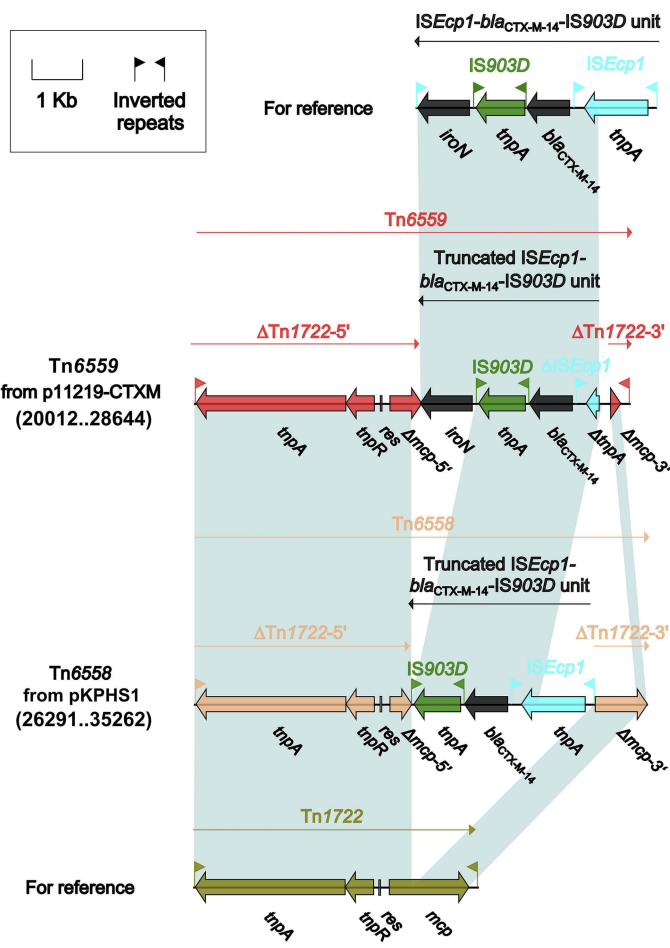
A total of 14 plasmids including p11219-CTXM and p205880-NR1 (Table S1), each of which carried a single repA gene with >96% nucleotide identity to repA<sub>pKPHS1</sub> (Table S4A) and had a backbone gene organization similar to pKPHS1 (Figure 5), were assigned into a novel Inc group named as Inc<sub>pKPHS1</sub> (Figure S3). These 14 RepA proteins did not any of known domain super-families. Four

copy numbers of a conserved iteron motif (Figure S3) were found 48 bp to 218 bp downstream of repA for all Inc<sub>pKPHS1</sub> plasmids (Table S1). All plasmids carried a single iteron-regulated Inc<sub>pKPHS1</sub> replicon. pKPHS1, the first sequenced Inc<sub>pKPHS1</sub> plasmid, was identified as the Inc<sub>pKPHS1</sub> reference. These 14 plasmids had >96% nucleotide identity over >75% coverage of their backbone sequences (Table S4B). Modular differences were found at multiple sites of the maintenance regions (Figure 5). None of conjugal transfer genes was found in all plasmids, which was consistent to the non-conjugative nature of p11219-CTXM. Remarkably, all these plasmids carried φpKPHS1 regions resembling SSU5 phage.<sup>9</sup>

Only four Inc<sub>pKPHS1</sub> plasmids had accessory modules, including the two resistance modules: Tn6558 from pKPHS1 and Tn6559 from p11219-CTXM (Table 1 and Figure 5). The highly similar Tn6558 and Tn6559 (Figure 6) were novel Tn3-family unit transposons generated from integration of truncated IS903D-bla<sub>CTX-M-14</sub>-ISEcp1 units (representing the master prototype bla<sub>CTX-M-14</sub> genetic environments in China)<sup>10</sup> into the mcp gene of cryptic Tn1722,<sup>8</sup> and they slightly differed from one another by distinct truncations occurred within IS903D-bla<sub>CTX-M-14</sub>-ISEcp1 unit or mcp.



**Figure 5** Linear comparison of complete sequences of *Inc*<sub>PKPHS1</sub> plasmids. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading regions denote homology of plasmid backbone regions ( $\geq 90\%$  nucleotide identity) but not accessory modules.



**Figure 6** Organization of Tn6558 and Tn6559 and comparison with related regions. 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 the nucleotide positions within the corresponding plasmids. The accession numbers of *ISecp1-bla*<sub>CTX-M-14</sub>-*IS903D* unit<sup>22</sup> and Tn1722<sup>9</sup> are KX646543 and X61367, respectively.

## Ethics approval and informed consent

This study needs not to be reviewed or approved by the ethics committee of the hospitals, because the bacterial isolate involved in this study was part of the routine hospital laboratory procedure. The research involving bio-hazards and all related procedures were approved by the Biosafety Committee of the Beijing Institute of Microbiology and Epidemiology.

## Acknowledgment

This work was supported by the National Key R&D Program (2018YFC1200100) of China and the Local Social Science Project (2018QD0031) of Heilongjiang Province.

## Author contributions

All authors contributed toward data analysis, drafting and revising the paper, 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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