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ORIGINAL RESEARCH

# Characterization of the plasmid of incompatibility groups $IncFII_{pKF727591}$ and $Inc_{pKPHS1}$ from *Enterobacteriaceae* species

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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 $IncFII_{pKF727591}\beta$ plasmid p205880-qnrS and two $Inc_{pKPHS1}$ plasmids p11219-CTXM and p205880-NR1 were fully sequenced in this work. Detailed genomic comparison was applied to all available sequenced plasmids of $IncFII_{pKF727591}$ or $Inc_{pKPHS1}$ group.

**Results:** p205880-qnrS carried a novel transposon Tn*6396*, which was an IS*Kpn19*-compsite transposon and represented a prototype transposable element carrying a minimum core *qnrS1* module. p11219-CTXM harbored a novel transposon Tn*6559*, which was generated from integration of a truncated IS*903D*–$bla_{CTX\text{-}M\text{-}14}$–IS*Ecp1* unit into the Tn*3*-family cryptic unit transposon Tn*1722*. Two Inc groups, $IncFII_{pKF727591}$ and $Inc_{pKPHS1}$, of plasmids from *Enterobacteriaceae* species were proposed, and $IncFII_{pKF727591}$ was further grouped into two subgroups $IncFII_{pKF727591}\alpha$ and $IncFII_{pKF727591}\beta$. Each of the 11 $IncFII_{pKF727591}$ plasmids carried multiple accessory modules including at least one resistance module, and the relatively small $IncFII_{pKF727591}$ backbones could acquire a wealth of foreign genetic contents. The modular structures of plasmid backbones were conserved within each of $IncFII_{pKF727591}\alpha$ and $IncFII_{pKF727591}\beta$ subgroups but dramatically different, although with similar gene organizations, between these two subgroups. The $Inc_{pKPHS1}$ backbones were conserved with respect to modular structures, and only four of the 14 $Inc_{pKPHS1}$ plasmids carried accessory modules, two of which contained resistance genes.

**Conclusion:** A genomic comparison of sequenced $Inc_{pKPHS1}$ or $IncFII_{pKF727591}$ plasmids provides insights into modular differences and genetic diversification of these plasmids, some of which carries antimicrobial resistance genes.

**Keywords:** plasmids, $IncFII_{pKF727591}$, $Inc_{pKPHS1}$, Tn*6396*, Tn*6559*

## 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.[1] Nowadays, Inc classification is always based on replication initiation protein (Rep) sequences, and it is not necessarily confirmed by conventional conjugation-based incompatibility experiments.[1] At least 27 Inc groups have been identified in *Enterobacteriaceae* species,[1] 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*-compsite transposon Tn*6396*, p11219-CTXM harboring a novel Tn*1722*-derivated unit transposon Tn*6559*, and p205880-NR1 containing no resistance genes) and proposed two novel Inc groups ($IncFII_{pKF727591}$ and $Inc_{pKPHS1}$). p205880-qnrS belonged to $Inc_{pKPHS1}$, while p11219-CTXM and p205880-NR1 could be assigned to $IncFII_{pKF727591}$. Further detailed genomic comparison of all sequenced plasmids of $Inc_{pKPHS1}$ or $IncFII_{pKF727591}$ 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.[2] An unrooted neighbor-joining tree was generated from the aligned *repA* sequences of indicative plasmids.[2] 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,[2] with *Escherichia coli* EC600 as a recipient and the 205880 or 11219 isolates as a donor, for selecting an *E. coli* transconjugant that carried $bla_{CTX\text{-}M\text{-}14}$ (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,[2] to obtain an *E. coli* electroporant carrying $bla_{CTX\text{-}M\text{-}14}$ (p11219-CTXM). Double-disk synergy test was performed to detect the activity of extended-spectrum β-lactamase (ESBL) in indicative bacterial strains.[3] BioMérieux VITEK 2 was used to test bacterial antimicrobial susceptibility, which was interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.[4]

# Results and discussion

## Diversification of $IncFII_{pKF727591}$ 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 ≥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 $IncFII_{pKF727591}$ ($Inc_{reference\ plasmid}$), 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 $IncFII_{pKF727591}\alpha$ (n=8) and $IncFII_{pKF727591}\beta$ (n=3). As shown by pairwise comparison of *repA* nucleotide sequences, plasmids within each subgroup showed 100% identity, while those from different subgroups displayed ≥79% identity (Table S2A). Predicted RepA-binding iterons were located from 245 bp to 365 bp downstream of *repA* for $IncFII_{pKF727591}\alpha$ plasmids, but upstream from 366 bp to 460 bp for $IncFII_{pKF727591}\beta$ plasmids, and three copy numbers of iteron were found for all $IncFII_{pKF727591}$ 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 $IncFII_{pKF727591}$ plasmid) and pKp_Goe_414-4 (the first sequenced $IncFII_{pKF727591}\beta$ plasmid) were identified as the references for $IncFII_{pKF727591}\alpha$ and $IncFII_{pKF727591}\beta$, respectively. p205880-qnrS belonged to $IncFII_{pKF727591}\beta$.

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 $IncFII_{pKF727591}$ backbone regions (responsible for plasmid replication, maintenance and conjugal transfer). The eight $IncFII_{pKF727591}\alpha$ plasmids shared ≥88% of their backbone sequences with ≥99% nucleotide identity, and the three $IncFII_{pKF727591}\beta$ plasmids showed ≥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 |
|---|---|---|---|---|---|---|---|---|
| $IncFII_{pKF727591}\alpha$ | pKF727591[@] | KF727591 | 94,790 | 112 | 53.1 | 51,559 | $bla_{NDM-1}$ region[#], and ΔIS*Ecl6* | NA |
| | pKpn235-BG | KT852336 | 76,360 | 90 | 53.9 | 51,749 | $bla_{NDM-1}$ region[#], and ΔIS*Ecl6* | NA |
| | pKpn240-BG | KT852335 | 76,980 | 92 | 53.8 | 51,594 | $bla_{NDM-1}$ region[#], and ΔIS*Ecl6* | NA |
| | pB-3002cz | KJ958926 | 97,650 | 122 | 53.2 | 51,558 | $bla_{NDM-1}$ region[#], and ΔISEcl6 | 11,12 |
| | pEh1A | KR822246 | 96,120 | 105 | 53.1 | 51,559 | $bla_{NDM-1}$ region[#], and ΔIS*Ecl6* | 13 |
| | pCP020050 | CP020050 | 113,430 | 129 | 52.5 | 51,559 | *ars* region[#], and ΔIS*Ecl6* | NA |
| | pLN824135 | LN824135 | 118,320 | 142 | 52.5 | 46,102 | *dfrA14-qnrB1* region[#], IS*Ec10*, and ΔIS*Ecl6* | NA |
| | pCAV1217-71 | CP018674 | 70,610 | 95 | 52.4 | 46,100 | *glgC* region, IS*Ec27*, and ΔIS*Ecl6* | NA |
| $IncFII_{pKF727591}\beta$ | pKp_Goe_414-4[@] | CP018341 | 81,641 | 88 | 53.9 | 54,186 | MDR region[#] | NA |
| | p205880-qnrS[$] | MF190368 | 65,110 | 75 | 52.9 | 54,649 | IS*26*–ΔTn*1696*, and Tn*6396*[#] | This study |
| | p675920-2 | MF133496 | 79,370 | 83 | 54.1 | 54,726 | MDR region[#] | 7 |
| $Inc_{pKPHS1}$ | pKPHS1[@] | CP003223 | 122,800 | 131 | 49.5 | 113,828 | Tn*6558* region[#] | 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 |
| | p11219-CTXM | MF133442 | 122,080 | 128 | 50 | 110,993 | Tn*6559* region[#], and IS*Kpn24* | This study |
| | pCP020063 | CP020063 | 109,020 | 119 | 49.2 | 107,422 | IS*Kpn38* | NA |
| | pCP015755 | CP015755 | 109,350 | 125 | 49.3 | 109,350 | None | NA |
| | pCP016161 | CP016161 | 109,350 | 123 | 49.3 | 109,350 | None | NA |
| | pUCLAOXA232-4 | CP012565 | 112,060 | 117 | 49 | 112,060 | None | NA |
| | pUCLAOXA232-4.X | CP012570 | 111,240 | 115 | 49 | 111,240 | None | NA |
| | pIncFIB_DHQP1400954 | CP016924 | 111,540 | 116 | 49.3 | 111,540 | None | NA |
| | pKPN-04f | CP014756 | 121,030 | 118 | 49.3 | 109,640 | IS*3000*, and IS*Kpn25* | NA |
| | p205880-NR1 | MF144193 | 108,040 | 117 | 49.3 | 108,040 | None | This study |
| | pKP301b | KY354306 | 110,253 | 111 | 48.3 | 110,653 | None | NA |

**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). [@]Reference plasmids. [#]Carrying resistance gene.
**Abbreviation:** NA, not applicable.

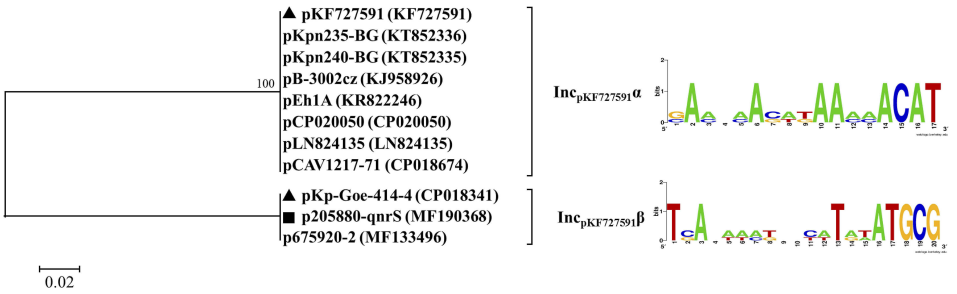


**Figure 1** A neighbor-joining phylogenetic tree for IncFII$_{pKF727591}$ plasmids. The degree of support (percentage) for both cluster of associated taxa, as determined by bootstrap analysis, is shown next to each branch. The bar corresponds to the scale of sequence divergence. The triangles indicate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

identity over ≥98% of their backbone sequences; by contrast, the backbones of IncFII$_{pKF727591}$α and IncFII$_{pKF727591}$β had ≤92% nucleotide identity across ≤70% of their backbone sequences (Table S2B). The modular structures of plasmid backbones were conserved within each of IncFII$_{pKF727591}$α and IncFII$_{pKF727591}$β subgroups but dramatically different between two subgroups.

Integration of accessory modules at various sites of IncFII$_{pKF727591}$ 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$_{pKF727591}$ 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$_{pKF727591}$ backbone. An 11-kb maintenance region carrying *parAB* (partition) and *ccdBA* (toxin-antitoxin) was found in all IncFII$_{pKF727591}$α plasmids, while another distinct 15-kb maintenance region containing *stbAB* (mediator of plasmid stability) and *resD* (resolvase) in all IncFII$_{pKF727591}$β plasmids.

All the three IncFII$_{pKF727591}$β plasmids contained a single IncFII$_{pKF727591}$β replicon, which six of the eight IncFII$_{pKF727591}$α plasmids contained a second Inc$_{pA1763\text{-}KPC}$ replicon beside the master IncFII$_{pKF727591}$α replicon (Figure 2). Notably, the Inc$_{pA1763\text{-}KPC}$ replicon was located within a 9.7- or 8.1-kb backbone region [carrying maintenance


**Figure 2** Linear comparison of complete sequences of IncFII$_{pKF727591}$ 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: ≥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 coexistent replicons $IncFII_{pKF727591}\alpha$ and $Inc_{pA1763\text{-}KPC}$, 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.[5]

## Accessory resistance modules of $IncFII_{pKF727591}$ plasmids

A large accessory module was integrated at a site between the two maintenance genes *mtsM* and *ccdA* in each of the eight $IncFII_{pKF727591}\alpha$ 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 $Inc_{pA1763\text{-}KPC}$ (see above) was found in six $IncFII_{pKF727591}\alpha$ plasmids except for pKpn235-BG and pKpn240-BG.The accessory modules from seven $IncFII_{pKF727591}\alpha$ plasmids, except for pCAV1217-71, carried resistance loci (Figure 3 and Table S3): i) a truncated Tn*125* transposon carrying $bla_{NDM-1}$[6] was harbored in pB-3002cz, pKpn235-BG and pKpn240-BG, while another truncated version of Tn*125* 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 $bla_{NDM-1}$ 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 Tn*6396* (Figure 4) were inserted at the same site within the *umuC* gene of the three $IncFII_{pKF727591}\beta$ 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 IS*26*–ΔTn*6346*–ΔGI*sul2*–IS*26* unit[7] into Tn*1721*,[8] which was further connected with a truncated IS*26*–$bla_{LAP-2}$–*qnrS1*–IS*26* unit.[7] The 26.9-kb MDR region was highly similar to the 24.7-kb MDR region but differed from it mainly by inversion of IS*26*–ΔTn*6346*–ΔGI*sul2*–IS*26* and further upstream insertion of an IS*26*–*pdk*–*catA2*–IS*26* unit. Tn*6396* 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. Tn*6396* represented a prototype transposable element carrying a minimum core *qnrS1* module. Different Tn*6396*

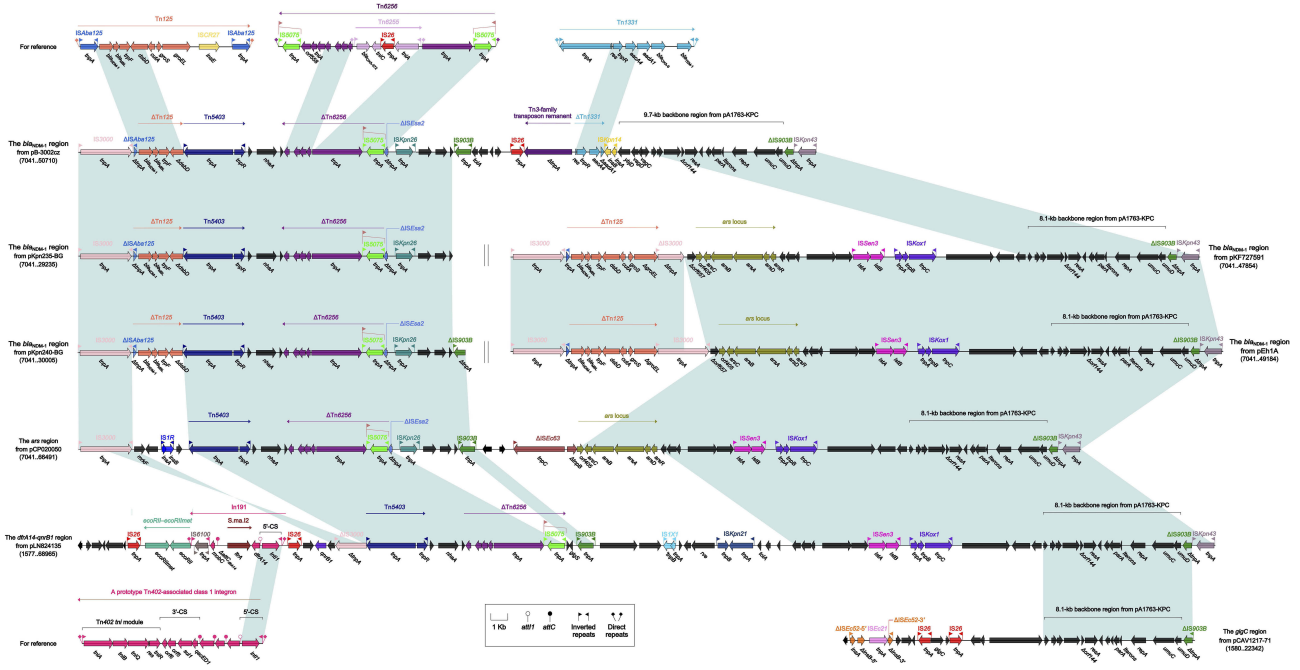

**Figure 3** Organization of selected accessory modules from $IncFII_{pKF727591}\alpha$ plasmids and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate e nucleotide positions within corresponding plasmids. The accession numbers of Tn*125*,[6] Tn*6256*[17] and Tn*1331*[18] for reference are JN872328, KP851978 and KC354802, respectively.

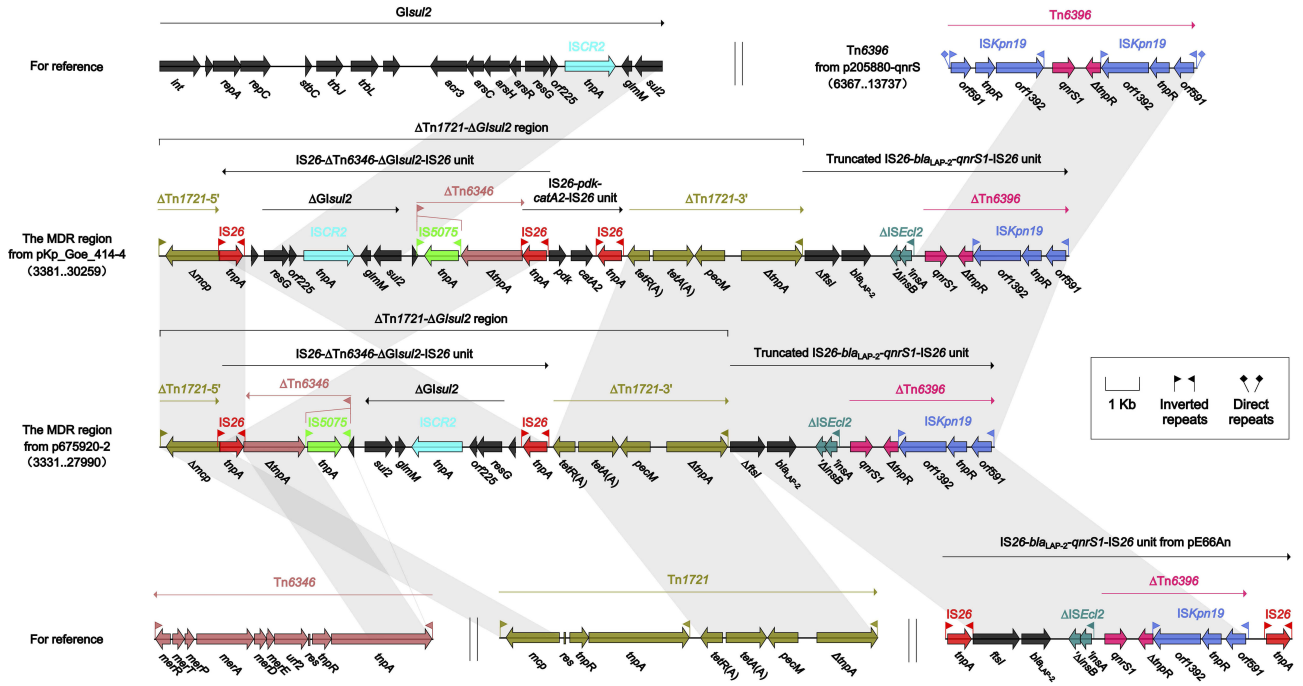


**Figure 4** Organization of accessory resistance modules from IncFII$_{pKF727591}$β plasmids and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate e nucleotide positions within corresponding plasmids. The accession numbers of GI*sul2*,[19] Tn*6346*,[20] Tn*172*[8] and IS*26*–*bla*$_{LAP-2}$–*qnrS1*–IS*26* unit[21] 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$_{pKPHS1}$ plasmids

Two additional new plasmids p11219-CTXM (carrying *bla*$_{CTX-M-14}$) 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*$_{CTX-M-14}$-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*$_{pKPHS1}$ (Table S4A) and had a backbone gene organization similar to pKPHS1 (Figure 5), were assigned into a novel Inc group named as Inc$_{pKPHS1}$ (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$_{pKPHS1}$ plasmids (Table S1). All plasmids carried a single iteron-regulated Inc$_{pKPHS1}$ replicon. pKPHS1, the first sequenced Inc$_{pKPHS1}$ plasmid, was identified as the Inc$_{pKPHS1}$ 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.[9]

Only four Inc$_{pKPHS1}$ plasmids had accessory modules, including the two resistance modules: Tn*6558* from pKPHS1 and Tn*6559* from p11219-CTXM (Table 1 and Figure 5). The highly similar Tn*6558* and Tn*6559* (Figure 6) were novel Tn*3*-family unit transposons generated from integration of truncated IS*903D*–*bla*$_{CTX-M-14}$–IS*Ecp1* units (representing the master prototype *bla*$_{CTX-M-14}$ genetic environments in China)[10] into the *mcp* gene of cryptic Tn*1722*,[8] and they slightly differed from one another by distinct truncations occurred within IS*903D*–*bla*$_{CTX-M-14}$–IS*Ecp1* unit or *mcp*.

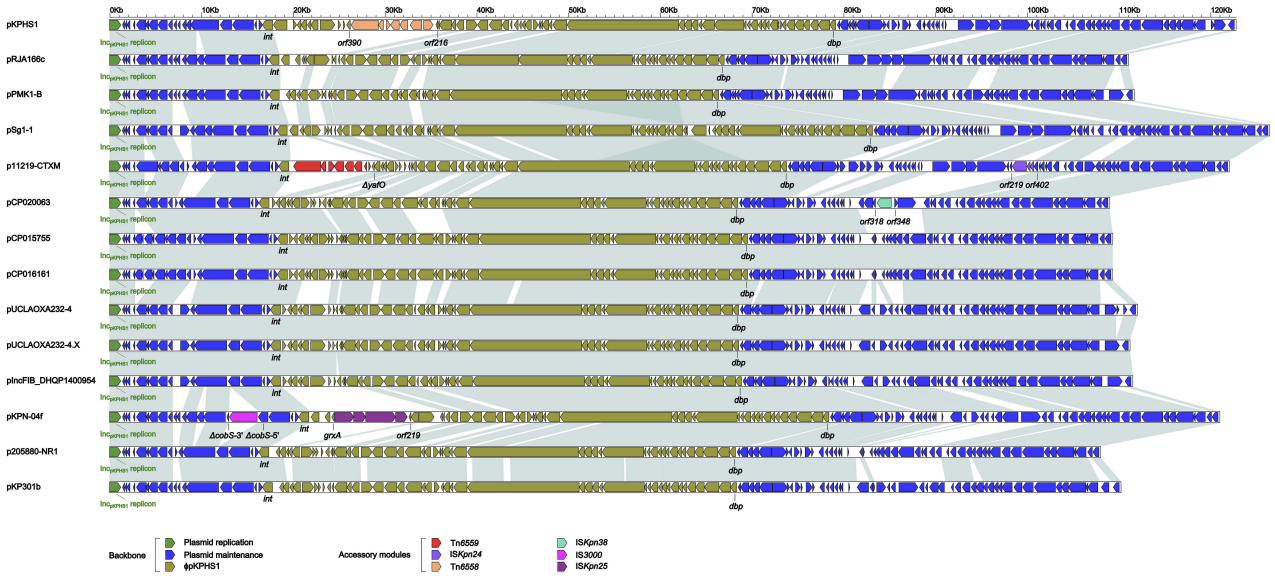


**Figure 5** Linear comparison of complete sequences of $Inc_{pKPHS1}$ plasmids. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading regions denote homology of plasmid backbone regions (≥90% nucleotide identity) but not accessory modules.

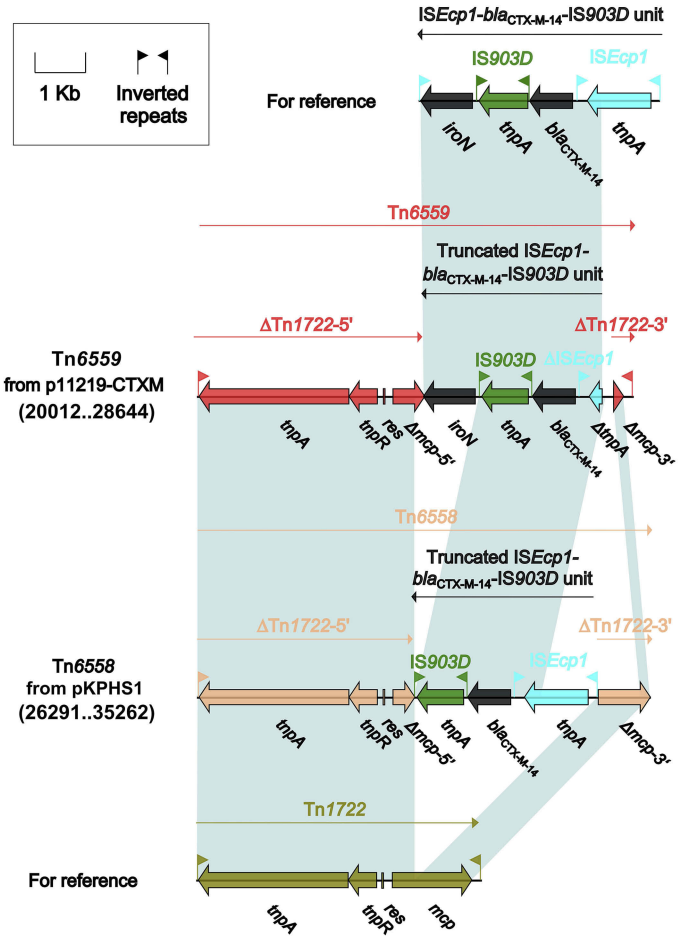


**Figure 6** Organization of Tn*6558* and Tn*6559* and comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids. The accession numbers of IS*Ecp1*–$bla_{CTX\text{-}M\text{-}14}$–IS*903D* unit[22] and Tn*1722*[8] 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 biohazards and all related procedures were approved by the Biosafety Committee of the Beijing Institute of Microbiology and Epidemiology.

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## 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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