A novel Tn1696-like composite transposon (Tn6404) harboring bla_{IMP-4} in a *Klebsiella pneumoniae* isolate carrying a rare ESBL gene \( \text{bla}_{SFO-1} \)

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Genetic determinants of a clinical *Klebsiella pneumoniae* isolate (KP1814) coproducing IMP-4 and a rare ESBL gene SFO-1 was investigated. KP1814 belongs to a novel sequence type (ST) assigned to ST2270. WGS identified four circular DNA sequences in KP1814, including two multidrug-resistance (MDR) plasmids, one virulence plasmid, and one circular form. The MDR plasmid pKP1814-1 (299.9 Kb) is untypeable, and carries two large mosaic multiresistance regions (MRRs). \( \text{bla}_{SFO-1} \) and \( \text{bla}_{IMP-4} \) co-exists on MRR1, and \( \text{bla}_{SFO-1} \) is associated with an IS/Tn-independent genetic context. \( \text{bla}_{IMP-4} \) is carried by a novel In804-like integron (intI1-\( \text{bla}_{IMP-4} \)-Kl.pn.I3-qacG2-aacA4-catB3A) associated with a novel Tn1696-like transposon (designed Tn6404) flanked by IS5075. The other MDR plasmid pKP1814-3 is a 95,701-bp IncFII plasmid, and is a hybrid of a *Shigella flexneri* plasmid pSF07201 and an *E. coli* plasmid pCA08. All resistance genes of pKP1814-3 were detected in a ~16-kb IS26-flanked composite transposon carried by a Tn5396 transposon. The circular form (18.3 Kb) was composed of two parts belonging to pKP1814-1 and pKP1814-3, respectively. The plasmid pKP1814-2, carrying multiple virulence factors, encodes IncFiB\(_K\) and IncFiI\(_K\) replicons with a size of 187,349 bp. The coexistence of MDR and virulence plasmids largely enhances the bacterial fitness in the host and environment.

SFO-1 is a rare class A ESBL first identified on a self-transferable plasmid in a clinical *Enterobacter cloacae* isolate from Japan in 1999\(^1\). By contrast to the known plasmid-borne \( \beta \)-lactamas which are produced constitutively, SFO-1 is induced by imipenem, and can hydrolyze most \( \beta \)-lactams except cephamycins and carbapenems\(^2\). The \( \text{bla}_{SFO-1} \) gene was later detected in an outbreak clone of multidrug-resistance (MDR) *E. cloacae* in Spain\(^3\), and in three *Klebsiella pneumoniae* and one *Escherichia coli* clinical isolates in China\(^4,5\). However, the complete structure of \( \text{bla}_{SFO-1} \)-harboring plasmids is still unknown.

IMP-4 was first detected in Hong Kong in 2001\(^6\), and later widely disseminated in Australia and the mainland of China\(^7\). The spread of IMP-4 is frequently associated with class 1 integrons carried by plasmids\(^8,9\). In China, plasmid-borne \( \text{bla}_{IMP-4} \) has been sporadically reported in different provinces/cities (Chongqing, Hubei, Fujian, Zhejiang, Guangdong, Shanghai and Tianjin\(^10-18\)). However, few studies have reported the full structure of \( \text{bla}_{IMP-4} \)-carrying plasmids detected in China\(^16-18\), largely limits our understanding on the dissemination mechanism of \( \text{bla}_{IMP-4} \). In this study, we report a carbapenem-resistant *K. pneumoniae* isolate that simultaneously carries \( \text{bla}_{IMP-4} \) and \( \text{bla}_{SFO-1} \) genes. The plasmid content and genetic determinants of the isolate were comprehensively analyzed to gain a better understanding of the spread mechanism of \( \text{bla}_{IMP-4} \) and \( \text{bla}_{SFO-1} \) genes in China.

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Materials and Methods

Strain collection. The community-associated *K. pneumoniae* strain KP1814 was collected in the frame of a national-wide survey for antibacterial resistance among outpatients with community-associated infections\(^{19}\). This strain was isolated from an outpatient (the type of clinical specimen was unknown) in a secondary hospital in mid-south of China (Hubei province) in 2011.

Antimicrobial Susceptibility Testing. Antimicrobial susceptibility was determined using Vitek-2 with card GN-13 (Biomerieux, Marcy l’Etoile, France) and was interpreted according to the clinical breakpoints defined by EUCAST-v7.0 (http://www.eucast.org/clinical_breakpoints/). ESBL phenotype was confirmed by the double-disc synergy test (DDST) using cefotaxime (30 \(\mu\)g) and ceftazidime (30 \(\mu\)g) with clavulanate (10 \(\mu\)g) discs.

Plasmid analysis. Azide-resistant *E. coli* J53 was used as the recipient for the conjugative transfer of plasmids. Transconjugants were selected on MH media with sodium azide (100 \(\mu\)g/\(\mu\)l) and gentamicin (10 \(\mu\)g/\(\mu\)l) or ceftazidime (8 \(\mu\)g/\(\mu\)l). The selected transconjugants were later confirmed by PCR and susceptibility testing. The plasmid size was estimated by S1 nuclease pulsed-field gel electrophoresis (PFGE).

Whole-genome sequencing (WGS) and data analysis. Genomic DNA was extracted using Gentra Puregene Yeast/Bacteria Kit (Qiagen, Hilden, Germany). The genome was sequenced by Illumina HiSeq2500 (Illumina, San Diego, CA, USA) using a 2 \(\times\) 125-bp pair-end libraries, and was further scaffolded by Pacbio RS II platform (Pacific Biosciences, California, US) using a 10-Kb library. *De novo* assembly for reads yielded by Hiseq2500 was done by CLC Genomics Workbench v8.0 (QIAGEN, Hilden, Germany) after quality trimming (Qs \(\geq\) 20), and the scaffolding was performed by SSPACE standard version 3.0 with default settings\(^{20}\). Further gaps within scaffolds were closed using GapFiller with default settings\(^{21}\). Annotation was performed using the RAST server (rast.nmpdr.org) followed by manual curation using BlastP and ISfinder (https://www-is.biotoul.fr); the CGE server (https://cge.cbs.dtu.dk).

Nucleotide Sequence Genbank Accession Numbers. The three plasmids and the circular form have been deposited at DDBJ/EMBL/GenBank under the accession of KX839207- KX839210.

Results and Discussion

The characterization of KP1814. KP1814 was resistant to numerous drugs, including ertapenem, and remained susceptible to amikacin, ciprofloxacin, and levofloxacin (Table S1). The genome of KP1814 comprised of 1,809,057 bp, with 35 genes encoding function/hypothetical proteins (Fig. 1). Notably, pKOX-R1 and pRpNDM1-1 were identified in KP1814, which was overlooked in previous reports\(^{19}\). The three plasmids and the circular form have been deposited at DDBJ/EMBL/GenBank under the accession of KX839207- KX839210.

**The characterization of pKP1814-1.** The *bla\(_{\text{IMP-4}}\)* and *bla\(_{\text{SHV-1}}\)* genes co-locate on pKP1814-1. To the best of our knowledge, this is the first report on the co-existence of *bla\(_{\text{IMP-4}}\)* and *bla\(_{\text{SHV-1}}\)* on a plasmid. This plasmid was transferred to the azide-resistant *E. coli* J53 via conjugation, and the conjugation frequency was estimated of \(5 \times 10^{-5}\) per donor cell.

**The multi-resistance regions (MRRs) of pKP1814-1.** All drug-resistant genes of pKP1814-1 were detected on two MRRs (Fig. 1). MRR1 is bracketed by an interrupted IR\(_{\text{inv}}\) of Tn21 (22 bp) and an 81-bp IR\(_{\text{inv}}\) and is composed of three subregions (Fig. 2A). The first subregion is a novel Tn1696-like mercury and multidrug resistance transposon (designed Tn6404 by Tn Number Registry). The two IRs of Tn6404 (IR\(_{\text{inv}}\) and IR\(_{\text{inv}}\)) are interrupted by the insertion of ISS075 into two pieces (16 bp + 22 bp), respectively. This is consistent with that ISS075 targets a specific position in the terminal repeats of Tn21 family\(^{24}\). A similar Tn1696-like transposon is
found in an IMP-4-encoding IncA/C2 plasmid pIMP-PH114 (KF250428) detected from a K. pneumoniae strain that was recovered from a patient who was hospitalized in the Philippines25 (Fig. 2B).

Tn6404 encodes a blaIMP-4-harboring In809-like integron, replacing the In4 detected in Tn1696. Notably, In809 with four cassettes (blaIMP-4-qacG2-aacA4-catB3) is widely disseminated in Acinetobacter spp and Enterobacteriaceae isolates in Asia-pacific region, e.g. Hong Kong, Singapore, Australia, and Philippines25. Here, the In809-like integron carries a novel cassette array (blaIMP-4-Kl.pn.I3-qacG2-aacA4-catB3∆), in which a group II intron Kl.pn.I3 is inserted into the attC site of the blaIMP-4 cassette (Fig. 2A). To date, various genetic context of blaIMP-4 has been identified, e.g. blaIMP-4-qacG-aacA4-aphA15, blaIMP-4-Kl.pn.I3-mobC and blaIMP-4-Kl.pn.13-qacEΔ-sul17–10,16–18,22,26. Of note, the structure blaIMP-4-Kl.pn.I3 seems unique in isolates from China revealed by blasting in GenBank, thus could be an epidemiological marker for blaIMP-4 detected in China.

The 5′-conserved segment (CS) of the In809-like integron is identical to that of In4 carried by Tn169627, encoding a complete intI1 gene bounded by a 25-bp IRi. However, the reported blaIMP-4-Kl.pn.13-harboring integrons carry a truncated intI1 gene, mostly due to IS26 insertions18. The 25-bp IRi of the In809-like integron is located between the resII and resIII sites of the tnpR-Tn1696, in the same configuration previously described in In416 (AJ704863) detected on a VIM-4- and CMY-4-encoding plasmid pCC416 in Samonella28. The In809-like integron lacks a typical sul1-associated 3′-CS. Instead, a Tn5563a-like transposon locates at the 3′ end of the In809-like integron, and the IRR is inserted into catB3 resulting in a truncated gene. IRL of the Tn5563a-like transposon is missing caused by that the tnpR of Tn5563a is replaced by a tniR probably via recombination.

The mer operon (merRTPEADE) of Tn6404 is highly homologous with that of Tn5053 (L40585) (>99.9% nucleotide identity with 4-nucleotide difference), but is different with that of Tn1696 (~84–87% nucleotide identity; a replacement of merC by merF) (Fig. 2B), suggesting that the mer operons of Tn6404 and Tn1696 have independent origin. Notably, the Tn5563a-like transposon together with the mer operon consists of a 9.4-kb module, which is almost identical to that found on pJR-1 (CP005961) from P. mandelii (2-nucleotide difference) and on pUM505 (HM560971) from P. aeruginosa (3-nucleotide difference). This indicates that the 9.4-kb module may originate from Pseudomonas spp. Taken together, our findings support that the ubiquity and variety of elements in the Tn6404 is the result of frequent recombination processes.

The second subregion of MMR1 carries a module encoding a rare ESBL gene blaSFO-1, which locates adjacent to its regulator ampR as previously reported (Fig. 2A). The dissemination of ampR-blaSFO-1 is previously suggested to be mediated via IS26-composite transposon in K. pneumoniae and E. cloacae15. However, IS26 and/or other IS/Tn sequences were not detected here, and the genetic context of ampR-blaSFO-1 is identical to the available

Figure 1. The genetic map of pKP1814-1. A circular representation of pKP1814-1 was generated by using DNA plotter. From the inside outward, the first, second, and third circle show GC skew, GC content, and the regions with over 50% nucleotide identity to pKOX-R1(CP003684), respectively. The fourth and fifth circle show the genetic features in counterclockwise and clockwise directions, respectively. Genes are classified by different colours as shown in the legend, and the gene names of plasmid backbone are shown. Two MRRs are marked by gray arches.
sequenced part (9568 bp) of an IncA/C plasmid pHD0149-2 (KJ721805) detected from an *E. coli* strain in China, suggesting a second dissemination mechanism harnessed by *bla*<sup>SFO-1</sup>.

The third subregion is a Tn<sup>5393c</sup> encoding *strA-strB* bracketed by two 81-bp IRs, and locates at downstream of the *bla*<sup>SFO-1</sup>-encoding module (Fig. 2A). The Tn<sup>5393c</sup> might originate from *Aeromonas salmonicida*, since it shared a high similarity with that found in pRAS2 (CP005961) from *A. salmonicida* (8-nucleotide difference).

MRR2 is flanked by a disrupted IR chrA (22 bp) and a 19-bp IR of IS<sup>CR1</sup> (Fig. 2C), and is consisted of two sub-regions. The first subregion (IS<sup>5075</sup>-chrA-IS6100-<sup>mphR(A)</sup>-mrx-mph(A)-IS<sup>26</sup>-aac(3<sup>′</sup>)-IId-IS<sup>Cfr1</sup>-*bla*<sup>TEM-1b</sup>) is bracketed by a 12-bp IRR of IS<sup>5075</sup> and a 38-bp IR TEM, almost identical (3-nucleotide difference) to that detected in a Tn2-derived MRR of a *bla*<sup>IMP-4</sup>-harboring IncL/M plasmid pEl1573 (JX101693) from Australia<sup>29</sup>. It is suggested that such module can disseminate among different Inc groups via homologous recombination<sup>25</sup>. The second subregion of MRR2 carries an incomplete Tn<sup>1722</sup> transposon only encoding a truncated *tnpA* and a *tnpR*.

An IS<sup>CR1</sup>-containing class 1 integron In37::IS<sup>CR1</sup> (<sup>intI1</sup>-<sup>aac(6</sup>′<sup>)Ib-catB3-arr-3-qacEA1-sul1-<sup>ISCR1</sup>) is inserted into the res site of Tn<sup>1722</sup>. Such insertion event has also been identified in an IncR plasmid pKPS30 (KF793937) and a *bla*<sup>VIM-13</sup>-encoding transposon (GQ396666), suggesting acquisitions of class 1 integron at a hotspot located within the res site of Tn<sup>1722</sup><sup>30</sup>. The Tn<sup>1722</sup>-derived MRR subregion is supposed to be obtained from pKPS30 via multiple recombination events, since they are only differed by 1 SNP and a *tnpM* deletion (Fig. 2C).

The IS<sup>26</sup>-flanked composite transposon of pKP1814-3. The other MDR plasmid pKP1814-3 is a 95,701-bp IncFII plasmid, and was able to be co-transferred with pKP1814-1 to the azide-resistant *E. coli* strain J53. This plasmid is a hybrid of a *Shigella flexneri* plasmid pSF07201 (KJ201887) and an *E. coli* plasmid pCA08 (CP009233) (Fig. S1). All resistance genes of pKP1814-3 were detected in a 16-kb IS<sup>26</sup>-flanked composite transposon (IS<sup>26</sup>-<sup>intI1</sup>-<sup>drfA17</sup>-<sup>aadA5</sup>-<sup>qacEA1-sul1-<sup>chrA-padr-R</sup>-IS6100-mphR(A)-mrx-mph(A)-IS26</sup>) carrying a class 1 integron In<sup>54</sup>, of which the 5′-CS was disrupted by the insertion of IS<sup>26</sup> resulting in a truncated *intI1* (Fig. 3). Such IS<sup>26</sup>-flanked transposons have been widely disseminated in plasmids, e.g. pEC958 (HG941719), pCA08 (CP009233), and pKF3-140 (FJ876827). They are in different combinations and arrangement highlighting the role of this module in the mosaic characteristics of MRRs. It is known that IS<sup>26</sup>-flanked transposon could switch to be a circular form as a translocatable unit via a replicative mechanism of IS<sup>26</sup>, and widely disseminate by incorporating at a new location either via replicative transposition, homologous recombination or a Tnp26-catalyzed

![Figure 2. Features of the two MRRs of pKP1814-1.](image-url)
conservative reaction\(^{31,32}\). The IS26-flanked composite transposon was further inserted into a Tn5396 transposon, thus is flanked upstream by a truncated \(\text{tpnA}\)-Tn5396 and downstream by a truncated \(\text{tpnR}\)-Tn5396, which was nearly identical to that found in \(\text{pECO-824}\) (CP009860) with only 1-nucleotide difference. The Tn5396 composite transposon was further inserted into an IS1-like resulting in a truncated \(\text{insB}\) (Fig. 3), indicating multiple events involved in the acquisition of these TnIs here.

**The circular form of KP1814.** KP1814 harbored an 18,238-bp circular form. Of note, ~58.6% of the circular form was almost identical to a region (from \(\text{chrA}\) to \(\text{orf39}\)) of pKP1814-1, and ~79% was nearly identical to the IS26-flanked transposon of pKP1814-3 (Fig. S2). The circular form shares a 6.78-kb module (\(\text{chrA-padR-IS 6100-mpfr(A)-mrxA-mpfA-IS26}\)) with pKP1814-1 and pKP1814-3. Intriguingly, a recent study detected various TnIS48-like circular forms in *Acinetobacter baumannii*\(^{32}\). Taken together, this suggests an emerging role of circular forms in the dissemination of antibiotic resistance genes.

**The characterization of the virulence plasmid pKP1814-2.** pKP1814-2 is a 187,349-bp circular plasmid encoding \(\text{IncFIB}_C\) and \(\text{IncFII}_E\) replicons (Fig. S3). The plasmid backbone of pKP1814-2 has a high synteny (43–46% coverage) to pKP-19 (CP012884) and pKPN-d90 (CP015132), mainly comprising genes encoding replication/maintenance/modification (\(\text{repA, para/B, umuC/D, relB/E, samA/B, sbr, psiA/B, higA-like}\)), F-like type IV conjugative transfer (\(\text{traM/J/Y/A/E/K/B/V/C/F/Q/I/G/T/D/P}\)) and other genes encoding function/hypothetical proteins (Fig. S3). The shared regions of the three plasmids were highly conserved with ~98.3% nucleotide identity (about 1500-bp difference). Numerous virulence-associated genes and one heavy-metal-associated resistance gene were detected on pKP1814-2. This plasmid carried a gene encoding klebicin B, a bacteriocin involved in competitive exclusion of other bacteria to form nutritionally restricted niches\(^{34}\). Of note, an mrk gene cluster (\(\text{mrkABCD}\)) encoding type 3 fimbria was detected. This cluster is commonly found on the chromosome of *K. pneumoniae*, and was occasionally identified on a few plasmids in Enterobacteriaceae. Such plasmid-borne mrk cluster is suggested to profoundly enhance the ability of biofilm formation and increase plasmid conjugation efficiency\(^{35,36}\). pKP1814-2 harbored a gene cluster \(\text{glgB-glgC-glgA-glgP-pgm}\) involved in the glycogen metabolic pathway, which has been linked to environmental survival, symbiotic performance and colonization, and virulence\(^{37}\). This cluster may originate from *Klebsiella* spp. as the two best matches with 100% coverage were found on pCAV1374-228 (CP011634) of a *K. pneumoniae* strain, and on p_IncFIB_DHQP1002001 (CP016810) of a *K. pneumoniae* strain.

Additionally, a thiomersistance cluster, encoding heat-shock proteins ClpC, Hsp20, and \(\Delta\text{ftsH}\), was identified on pKP1814-2. This cluster could be exchanged across species as it was also detected on two *E. cloacae* plasmids: pENT-22e (CP009855) and pENT-4bd (CP008907). An intact \(\text{fisH}\) gene was further found out of the cluster. These heat-shock proteins are known to provide protection against stressful conditions and to contribute to the bacterial pathogenesis\(^ {38-40}\). The thiomersistance cluster additionally carried a \(\text{kefB}\) gene encoding a potassium/proton antiporter, which is known to play a role in protecting *E. coli* from electrophile toxicity\(^ {41}\). It is assumed that those genes and gene clusters coding for carbon-source metabolism, stress response, fimbria clusters, efflux pump, and bacteriocin maybe involved in virulence properties of the host as well as in increased survival and fitness under the hospital environment, overall contributing to the successful dissemination and maintenance of these plasmids in hosts.

In conclusion, we report the complete structure of a novel plasmid carrying \(\text{bla}_{\text{OXA-1}}\) and \(\text{bla}_{\text{IMP-4}}\). Our study, along with others, raises the concern that co-existence of MDR and virulence plasmids enables to increase the fitness and viability of hosts largely challenging the clinical structures and treatments.

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Author Contributions
K.Z., W.Y., P.S., H.F.L., B.H.W. and Y.H.X. participated in the design and/or discussion of the study. K.Z. and W.Y. carried out the major experiments. K.Z. and W.Y. analyzed the data. K.Z. and Y.H.X. wrote the paper. J.W.A.R. revised it for important intellectual improvement. All authors read and approved the final version to be published.

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