Infection and Drug Resistance

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ORIGINAL RESEARCH

Replicon-Based Typing About IncG Plasmids and Molecular Characterization of Five IncG Plasmids Carrying Carbapenem Resistance Gene *bla*$_{KPC-2}$

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Purpose: To investigate the genetic diversity of IncG plasmids, we have proposed a typing scheme based on replicon *repA* and performed comparative genomic analysis of five IncG plasmids from China.

Methods: p30860-KPC, p116965-KPC, pA1705-KPC, pA1706-KPC and pNY5520-KPC total in five IncG plasmids from clinical isolates of *Pseudomonas* and *Enterobacteriaceae*, respectively, were fully sequenced and were compared with the previously collected reference plasmid p10265-KPC.

Results: Based on phylogeny, IncG-type plasmids are divided into IncG-I to IncG-VIII, the five plasmids belong to IncG-VIII. A detailed sequence comparison was then presented that the IncG plasmid involved accessory region I (Tn*5563a/b/c/d/e*), accessory region II (IS*pa19*), and accessory region III (*bla*$_{KPC-2}$-region). Expect for the pNY5520-KPC, the rest of the plasmids had the same backbone structure as the reference one. Within the plasmids, insertion sequences Tn*5563d* and Tn*5563e* were identified, a novel unknown insertion region was found in Tn*5563b/c/d/e*. In addition, Tn*6376b* and Tn*6376c* were newly designated in the study.

Conclusion: The data presented here including a typing scheme and detailed genetic comparison which provide an insight into the diversification and evolution history of IncG plasmids.

Keywords: IncG plasmids, *bla*$_{KPC-2}$, phylogenetic tree, multidrug resistance, mobile elements

Introduction

Infections with multidrug-resistant organisms are causing a global health crisis.[1,2] Acquired antimicrobial resistance genes in organisms are commonly captured and horizontally transferred by mobile genetic elements, such as plasmids. The IncG plasmid has become one of the major multidrug-resistant plasmids.

IncG plasmid was first isolated from clinical bacteria in Germany, in 1975.[3] Since then, IncG plasmids have been reported in the Americas, Europe and Asia, carried by *Pseudomonas* and *Enterobacteriaceae* in clinical and environment settings. Botts described four multidrug resistance plasmids on bacteria from the sediments of an urban coastal wetland in America, one of which was IncP-6 (IncG).[4] Pérez proposed that the plasmid types IncL, IncHI2, IncFIB, IncN, IncC, and IncP-6 (IncG) carrying carbapenem resistance genes might be responsible for the dissemination of carbapenemase genes in *K. oxytoca* isolates in Spain.[5] Other reports of plasmids from the literature are shown in Table 1. The scheme of replicon homology to identify Inc groups is widely recognized, and our previous study also proposed a novel group and three separately clustering subgroups of Inc$_{pSTY}$

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Table 1 IncG Plasmids Reported in the Literature

Plasmid	ARGs	Isolate	Source	Country	IncG subtype	Year
Rms149 (NCBI:AJ877225)	*sul1, qacEΔ1, aadA5, aac(3)-I,* bla_{A}	*Pseudomonas aeruginosa* Ps142	Clinical	Germany	IncG-V	1975
pRSB105 (NCBI:DQ839391)	*sul1, qacEΔ1, mel, mph, oxa10, dfrB2*	Uncultured bacterium	Activated sludge	Germany	IncG-V	2007
pRIO-5 (NCBI:JF785550)	bla_{BES-1}, bla_{TEM-1}	*Serratia marcescens* RIO-5	Clinical	Brazil	IncG-V	2012
pCOL-1 (NCBI:KC609323)	bla_{KPC-2}	*Pseudomonas aeruginosa* COL-1	Clinical	Colombia	IncG-V	2013
p10265-KPC (NCBI:KU578314)	bla_{KPC-2}	*Pseudomonas aeruginosa* strain 10265	Clinical	China	IncG-VIII	2016
pKOX3-P5-KPC (NCBI:KY913901)	bla_{KPC-2}	*Klebsiella oxytoca* KOX3	Clinical	China	IncG-VIII	2017
p121SC21	bla_{KPC-2}	*Citrobacter freundii* 121SC21	Wastewater	Japan	IncG-VIII	2017
pLNU-11 (NCBI:KX863568)	*tetAR, folP, qacEΔ1, dfrA10,* bla_{WDC-1}, *sulII, tetA/R, strA/B,* bla_{TEM-1}	*Citrobacter freundii* AtetA	The wetland sediments	America	IncG-V	2017
pCfr-33795cz (NCBI:MG558000)	*aacA4,* bla_{OXA-1}, *catB3, arr-3, sul1, qacEΔ1, mphA, mphP, mrx,* bla_{KPC-2}	*Citrobacter freundii* Cfr-33795	Clinical	Czech Republic	IncG-VI	2018
pMmo-37590cz (NCBI:MF497782)	*aacA4,* bla_{OXA-1}, *catB3, arr-3, sul1, qacEΔ1, mphA, mphP, mrx,* bla_{KPC-2}	*Morganella morganii* Mmo-37590			IncG-VI	
pKPC-1713 (NCBI:MH624132)	bla_{KPC-2}, *qnrS2*	*Aeromonas taiwanensis* 1713	River sediment	China	IncG-VIII	2019
-(ENA:PRJEB30102)	bla_{KPC-2}	*Klebsiella oxytoca* K9682	Clinical	Spain	IncG-VIII	2019
pCRE3-KPC (NCBI:MH919378)	bla_{KPC-2}, *aacC2, tmrB*	*Citrobacter braakii* CRE3	Clinical	China	IncG-VIII	2020
pWW19C-KPC2 (NCBI:PRJNA715927)	bla_{KPC-2}	*Enterobacter asburiae* WW19C	Raw sewage	Argentina	IncG-VIII	2021
pWW14A-KPC2 (NCBI:PRJNA715927)	bla_{KPC-2}	*Klebsiella quasipneumoniae subsp. quasipneumoniae* WW14A			IncG-VIII	
p5_CFTMDU	bla_{KPC-2}	*Citrobacter freundii* CFTMDU	Hospital Sewage	Japan	IncG-VIII	2022
p3_KVTMDU	bla_{KPC-2}	*Klebsiella variicola* KVTMDU			IncG-VIII	
pJBIWA001_5 (NCBI:SAMN18916998)	bla_{GES-5}, *aacA3, aadA16*	*Raoultella. planticola* JBIWA001	Lake water	Japan	IncG-VIII	2022

Note: The search deadline is April 26, 2022.

plasmids in *Pseudomonas*, which are formally genotyped via replicons.[6–8] However, to date, no research on the typing of IncG plasmids exists.[9]

In order to explore the drug resistance mechanism of bacteria, our laboratory has been collecting multi-drug resistant strains since 2016. So far, our laboratory has collected a total of five strains from *Pseudomonas* and *Enterobacteriaceae*

that carry IncG plasmid harboring bla_{KPC-2}. In this work, the first typing of the IncG plasmid replicon *repA* was performed. A collection of six fully sequenced bla_{KPC-2}-carrying IncG plasmids from different clinical strains were considered, including the reference p10265-KPC (as shown in our previous study and the first identified IncG-type plasmid in China);[10] Characteristics of the backbone region, accessory modules, and insertion sites were dissected by comprehensive genomic comparison of these six plasmids. Diversification and parallel evolution of IncG plasmids are revealed, and monitoring of bacterial resistance transmission by IncG plasmids is recommended.

Materials and Methods

Bacterial Strains and Identification

Except for the reference plasmid, the remaining five plasmids p30860-KPC, p116965-KPC, pA1705-KPC, pA1706-KPC, and pNY5520-KPC were all sequenced in this study and collected from hospitalized patients in four Chinese public hospitals (Table 2). All bacterial strains were subjected to species identification using 16S rDNA gene sequencing followed by genome sequence-based average nucleotide identity analysis (ANI) (http://www.ezbiocloud.net/tools/ani).[11]

Sequencing and Sequence Assembly

Bacterial strains 172116965, NY5520 and A1706 were subjected to draft-genome sequencing using a paired-end library with an average insert size of 400 bp (range: 150–600 bp) on a HiSeq sequencer (Illumina, CA, USA). In addition, bacterial strains 172116965, NY5520 and A1705 were subjected to complete-genome sequencing with a sheared DNA library with an average size of 15 kb (range: 10–20 kb) on a PacBio RSII sequencer (Pacific Biosciences, CA, USA). Bacterial strain 30860 was sequenced from mate-pair libraries with average insert size of 5 kb (range: 2–10 kb) using a MiSeq sequencer (Illumina, CA, USA). Sequence assembly were performed as previously described.[12–14]

Sequencing and Sequence Assembly

Open reading frames (ORFs) and pseudogenes were predicted using RAST 2.0 combined with BLASTP/BLASTN searches against the UniProtKB/Swiss-Prot database and the RefSeq database.[15–17] Annotation of resistance genes, mobile elements, and other features was carried out using online databases, including CARD,[18] ResFinder,[19] ISFinder,[20] INTEGRALL,[21] DANMEL,[22] and the Tn Number Registry. Multiple and pairwise sequence comparisons were

Table 2 Background Information of Strains Carrying IncG Plasmids Sequenced in This Study

plasmid	Accession number	Isolate	Specimen	Host	Total length(bp)	Mean G+C content(%)	Other plasmids in the isolate	Province	Year
p10265-KPC (referenec)	KU578314	*Pseudomonas aeruginosa* strain 10265	Urine	Homo sapiens	38939	58.20	-	Beijing	2013
p30860-KPC	MN477223	*Enterobacter cloacae* strain 30860	Blood	Homo sapiens	39013	58.19	p30860-NR (IncX8), p30860-tetA (IncR), p30860-HI2 (IncHI2)	Guangdong	2013
pA1706-KPC	MH909350	*Klebsiella pneumoniae* strain A1706	Sputum	Homo sapiens	39014	58.16	pA1706-NDM ($IncFII_{R100}$:IncR), pA1706-FIIK ($IncFII_{K:pKPHS2}$: $Inc_{pA1763-KPC}$)	Liaoning	2013
pA1705-KPC	MH909348	*Klebsiella pneumoniae* strain A1705	Urine	Homo sapiens	42055	58.50	pA1705-NDM ($IncFII_{R100}$:IncR), pA1705-FIIK ($IncFII_{K:pKPHS2}$: $Inc_{pA1763-KPC}$)	Liaoning	2013
p116965-KPC	MN539620	*Citrobacter sp.* strain 172116965	Abdominal drainage fluid	Homo sapiens	40013	58.09	-	Hunan	2017
pNY5520-KPC	CP096939	*Pseudomonas aeruginosa* NY5520	Urine	Homo sapiens	33003	58.17	pNY5520-IMP ($Inc_{p60512-IMP}$), pNY5520-NR (Unknow)	Henan	2019

performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and BLASTN, respectively. Gene organization diagrams were drawn using Inkscape 1.0 (https://inkscape.org/en/).

Phylogenetic Analysis

Using *repA*$_{p10265\text{-}KPC}$ as reference sequence, and using Coverage≥85% and Identity≥90% as screening conditions, 107 plasmids were obtained by blast in NCBI (the search deadline is August 18, 2022). All the plasmids in this study and one plasmid with the same Coverage and Identity values were randomly selected to be included in the phylogenetic analysis, with a total of 29 plasmids. The *repA*$_{IncG}$ sequences of plasmids were aligned using Clustal Omega and then maximum-likelihood phylogenetic trees were constructed from core sequences using MEGA 11 with a bootstrap iteration of 1000, and displayed using iTOL (https://itol.embl.de).[23]

Electroporation Transfer

Plasmid p116965-KPC as a representative IncG plasmid of arbitrary choice was transformed from its wild-type isolate into *E. coli* DH5α in *Enterobacteriaceae*, through electroporation experiments. To prepare competent cells for electroporation, 200 mL of overnight culture of *E. coli* DH5α in super optimal broth (SOB) at an optical density (OD600) of 0.4 to 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 DNA 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 brain heart infusion (BHI) agar plates containing 4 μg/mL meropenem to select for *E. coli* containing *bla*$_{KPC\text{-}2}$ plasmid. By PCR, transfected *E. coli* was further identified using *bla*$_{KPC\text{-}2}$ and *repA*$_{p116965\text{-}KPC}$ sequences (Table 3).

Antimicrobial Susceptibility Testing

Bacterial antimicrobial susceptibility was tested using BioMérieux VITEK 2 and interpreted as per the 2022 Clinical and Laboratory Standards Institute (CLSI) guidelines.[24]

Nucleotide Sequence Accession Numbers

The p30860-KPC, p116965-KPC, pA1705-KPC, pA1706-KPC and pNY5520-KPC sequences were submitted to GenBank under accession numbers MN477223, MN539620, MH909348, MH909350 and CP096939, respectively.

Results

Overview of Sequenced IncG Plasmids

Strains 30860, 172116965, and NY5520 came from *Enterobacter cloacae, Citrobacter* sp. and *Pseudomonas aeruginosa*, respectively, and strains A1706 and A1705 came from *Klebsiella pneumoniae* (Table 2). Screening for the resistance genes of the above strains was consistent with antimicrobial susceptibility testing (Figure 1, Tables 4 and S1), and indicated that the plasmids involved carried only the *bla*$_{KPC-2}$ gene. A phylogenetic tree (Figure 2A) constructed from the repA$_{IncG}$ core sequences of 29 arbitrarily selected representative plasmids (Table S2; last accessed August 18th 2022) clustered into eight major clades, which we designate as types IncG-I to IncG-VIII. Most branches in this tree had bootstrap values ≥70%,

Table 3 Primers of Genes

Gene	Primer sequence	Fragment length (bp)	Annealing temperature (°C)
bla$_{KPC\text{-}2}$	*bla*$_{KPC\text{-}2}$-F:CCATACACTCCGCAGGTTCC	432	58
	bla$_{KPC\text{-}2}$-R:CAAAAATGCGCTGGTTCCGT		
repA$_{p116965\text{-}KPC}$	*repA*$_{p116965\text{-}KPC}$-F: GAGCCGATCCGGTACAACTT	458	60
	repA$_{p116965\text{-}KPC}$-R: TCGTTCTTGGGCGTGTAGAG		

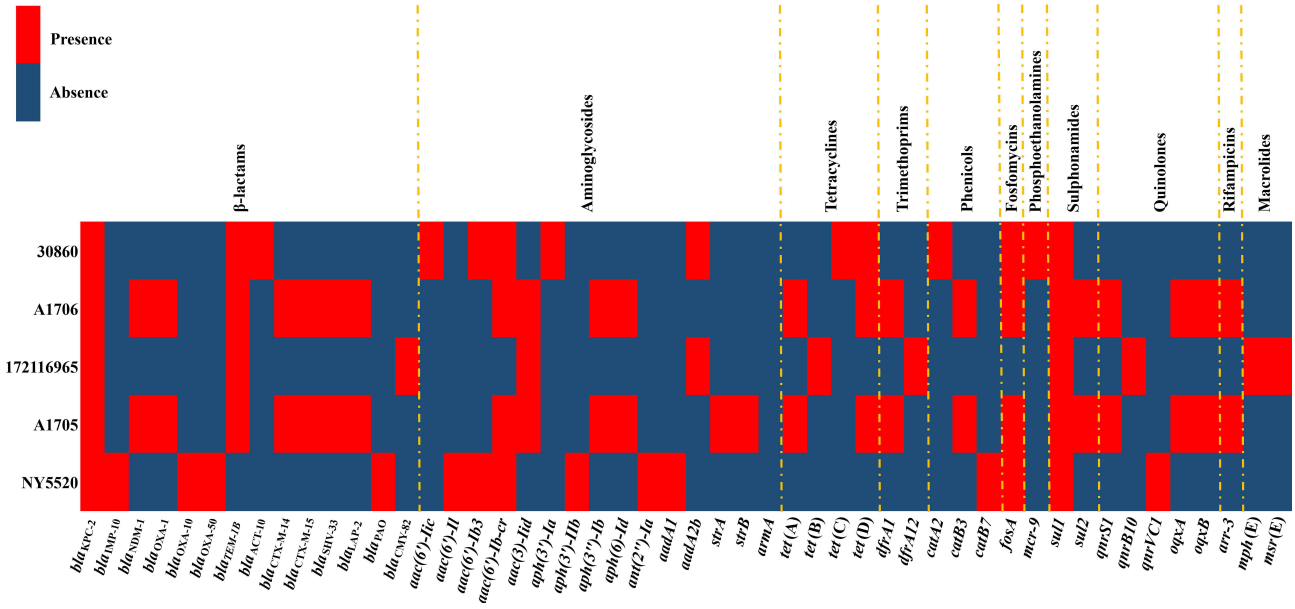

Figure 1 A heatmap of prevalence of resistance genes.
Notes: The original data are shown in Table S1.

suggesting that this maximum-likelihood phylogenetic tree robustly reflected the evolutionary relatedness of IncG plasmids. For genomic classification and phylogeny of IncG plasmids, the pairwise average nucleotide identity (ANI) values of the 29 sequenced plasmid *repA* sequences were calculated (Figure 2B and Table S3). A total of eight IncG types were identified, corresponding to the types in the phylogenetic tree, and thus both ANI-based plasmid typing and phylogenomic analysis yielded consistent results. These results confirm parallel diversification and evolution of IncG plasmids IncG-I to IncG-VIII. The phylogenetic tree suggests that plasmids p30860-KPC, pA1706-KPC, p116965-KPC, pA1705-KPC and pNY5520-KPC belong to type IncG-VIII. The evolutionary distances between p30860-KPC, pA1706-KPC, p116965-KPC and pA1705-KPC were relatively close, while the evolutionary distance between pNY5520-KPC and the other four plasmids was relatively large.

The five plasmids sequenced had complete and circular sequences and varied in size from approximately 33–42 kb (Figure 3). A pairwise sequence comparison using *BLASTN* showed that plasmids p30860-KPC, pA1706-KPC,

Table 4 Antimicrobial Drug Susceptibility Profile

Antibiotics	MIC (Minimum Inhibitory Concentration, μg/mL)/ Antimicrobial Susceptibility						
	30860	A1705	A1706	NY5520	172116965	172116965-KPC-2-DH5α	DH5α
Ceftazidime	32/R	≥ 64/R	≥ 64/R	≥ 64/R	≥ 64/R	32/R	<= 0.12/S
Imipenem	≥ 16/R	≥ 16/R	≥ 16/R	≥ 16/R	≥ 16/R	8/I	<= 0.25/S
Meropenem	8/I	≥ 16/R	≥ 16/R	≥ 16/R	≥ 16/R	≥ 16/R	<= 0.25/S
Ciprofloxacin	≥ 4/R	≥4/R	≥ 4/R	≥ 4/R	≥ 4/R	<= 0.25/S	<= 0.25/S
Levofloxacin	4/I	≥ 8/R	≥ 8/R	≥ 8/R	≥ 8/R	<= 0.12/S	<= 0.12/S
Tigecycline	2/S	≥ 8/R	≥ 8/R	≥ 8/R	<= 0.5/R	<= 0.5/S	<= 0.5/S
Amikacin	16/S	<= 2/S	<= 2/R	32/I	≥ 64/R	<= 2/S	<= 2/S
Tobramycin	≥ 16/R	8/I	≥ 16/R	≥ 16/R	≥ 16/R	<= 1/S	<= 1/S

Abbreviations: R, resistant; S, susceptible; I, susceptible-increased exposure.

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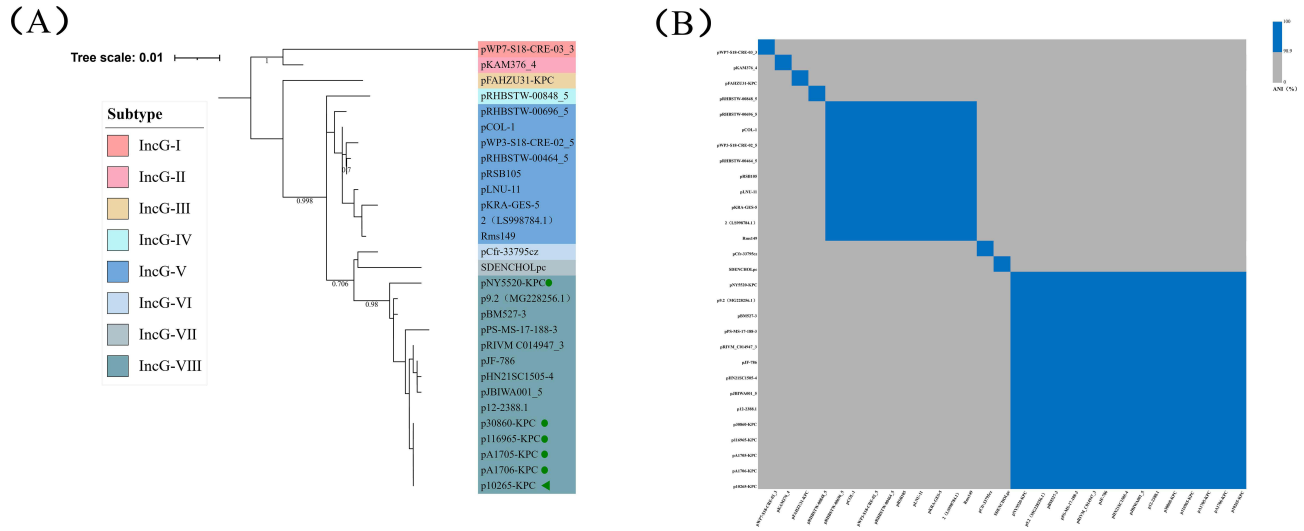

Figure 2 *repA*$_{IncG}$ typing.
Notes: (**A**) A maximum-likelihood phylogenetic tree of *repA*$_{IncG}$ sequences. The tree is constructed from core sequences. Bootstrap confidence values are shown next to each branch. The green colored dot represents plasmids from this study, and the green colored triangle represents the reference plasmid. (**B**) A heatmap of the pairwise average nucleotide identity (ANI) values of *repA*$_{IncG}$ sequences. The original data are shown in Table S3.

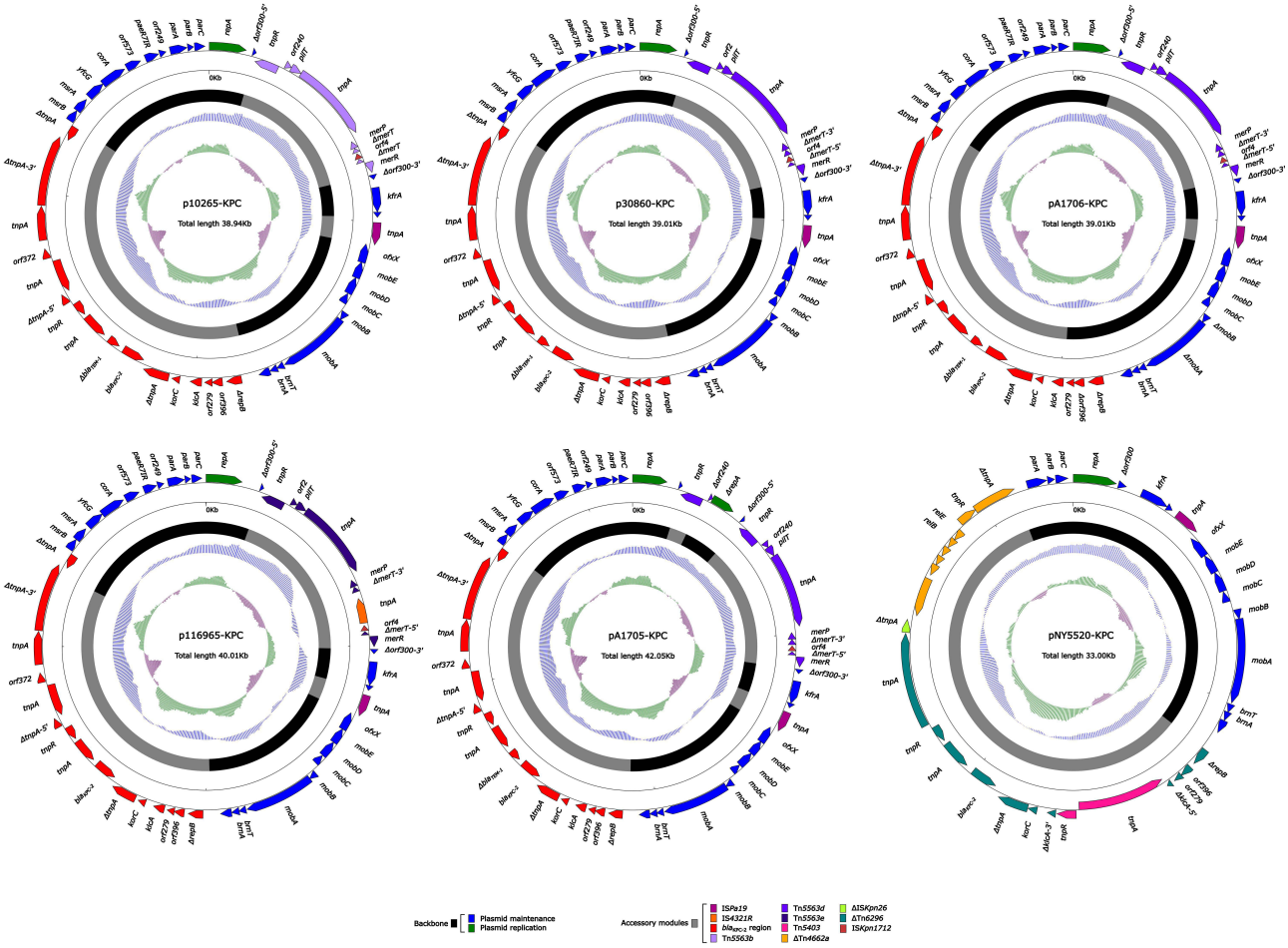

Figure 3 Schematic maps of the plasmids.
Notes: Genes are denoted by arrows and colored based on gene function classification. The innermost two circles indicate the GC-Skew [(**G-C**)/(G + (**C**)] and the GC content.

p116965-KPC, pA1705-KPC and the reference plasmid p10265-KPC[10] had >99.90% nucleotide identity across ≥96% of their complete sequences, while plasmid pNY5520-KPC and the other four plasmids showed ≥97.97% nucleotide identity across 56% to 69% of their complete sequences (Figure 4 and Table S4). The modular structure of each plasmid was divided into the backbone and the accessory modules that was represented by acquired DNA regions associated with mobile elements (Figures 5 and 6). A detailed sequence comparison applied to the collection of the five plasmids generated the following conclusions. i) Compared with p30860-KPC, pA1706-KPC, p116965-KPC and pA1705-KPC, pNY5520-KPC lacked some backbone genes downstream of accessory region III. ii) Compared with p30860-KPC, pA1706-KPC, p116965-KPC, and pA1705-KPC, pNY5520-KPC had only accessory regions II and III. iii) The composition of accessory region I of p30860-KPC (pA1706-KPC had the same sequence in the accessory region I), p116965-KPC and pA1705-KPC differed. iv) The composition of accessory region III of p116965-KPC and pNY5520-KPC differed from that of the other four plasmids.

Backbone Genes of Sequenced IncG Plasmids

The group of five plasmids in our study carry three partition genes *parA/B/C* and a downstream replicase gene *repA* with strong similarity (>97% identity and >96% identity, respectively) to that of reference plasmids p10265-KPC and Rms149 (the archetypical IncG-type plasmid). The *repA* gene encodes replication proteins, and the *parA* gene encodes an ATPase, whereas *parB* and *parC* encode auxiliary partition proteins, which constitute an IncG-type consecutive par-rep gene cluster. Therefore, these plasmids belong to the IncG incompatibility group. All five plasmids also contain the MOBP family mobilization module,[25–27] which is composed of five genes, *mobA* (relaxase/primase fusion protein), *mobB* (oriT recognition-like protein), *mobC* (relaxosome protein), and *mobD* and *mobE* (auxiliary proteins), and lack the conjugal

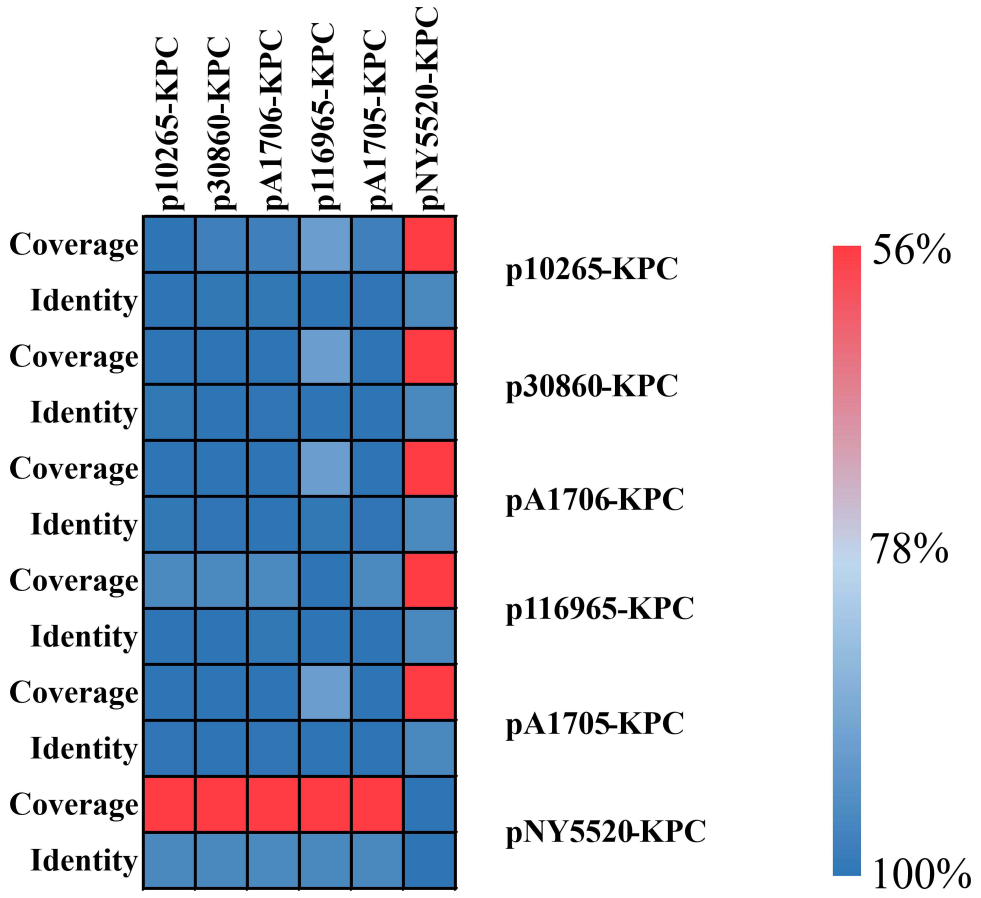

Figure 4 A heatmap of pairwise sequence comparison of the plasmids.
Notes: The original data are shown in Table S4.

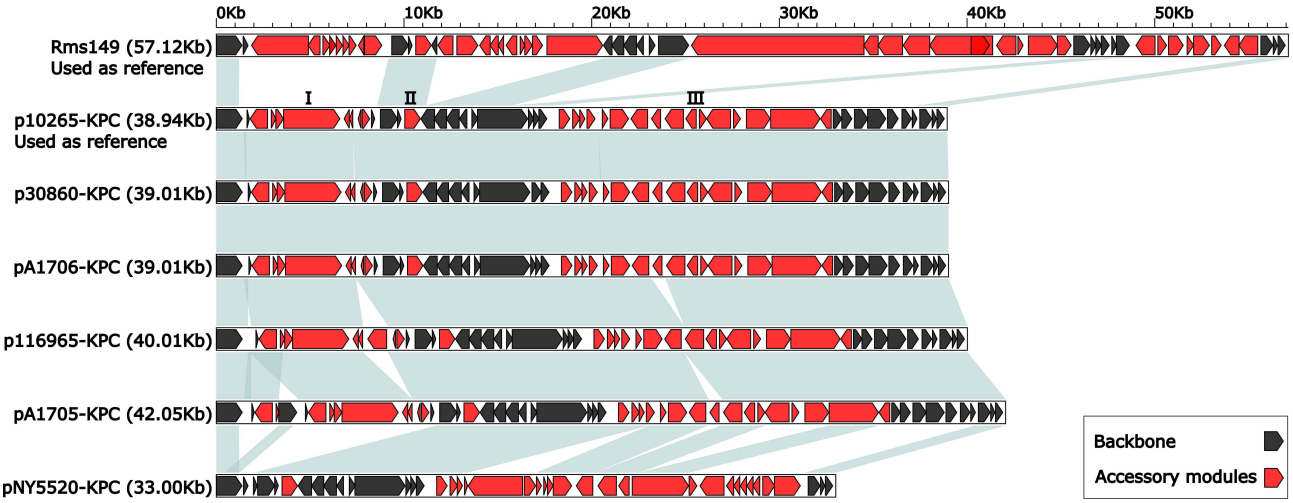

Figure 5 Linear comparison of plasmid genome sequences.
Notes: Genes are denoted by arrows and colored based on gene function classification. Shading regions denote shared regions of homology (>90% nucleotide identity).

transfer gene regions, consistent with p10265-KPC and Rms149. Additionally, the five plasmids and the reference plasmid share backbone genes *kfrA, ofxX, brnT*, and *brnN*. However, plasmids p10265-KPC, p30860-KPC, pA1706-KPC, p116965-KPC and pA1705-KPC had the backbone gene cluster *msrB-msrA-yfcG-corA-orf573*, while this backbone gene cluster was missing in plasmids pNY5520-KPC and Rms149 (Figures 3 and 5).

Accessory Modules of Sequenced IncG Plasmids

Accessory modules of the five sequenced IncG plasmids in this study were classified as Accessory regions I, II, and III. Plasmid pNY5520-KPC had only accessory regions II and III, but the other four plasmids had accessory regions I, II, and III. Accessory region II was insertion sequence IS*pa19*, which was inserted between the backbone genes *kfrA* and *ofxX*, corresponding to that of p10265-KPC. Accessory region I was mainly composed of Tn*5563d* or its variant Tn*5563e*. The transposon Tn*5563e* in p116965-KPC differed from the transposon Tn*5563d* in p30860-KPC, pA1706-KPC and pA1705-KPC, mainly by the insertion of IS*4321R*. However, an approximately 290 bp sequence was inserted into the *merT* gene of Tn*5563b/d/e*; the insertion sequence in Tn*5563b* differed by 7 bp from Tn*5563d/e*. Accessory region I of pA1705-KPC, which consisted of Tn*5563d, repA*$_{\text{IncG}}$, tnpR$_{\text{Tn}5563d}$, and Δ*orf240* (deletion of 102 bp compared with *orf240*), differed from that of the other plasmids (Figure 6A).

Accessory region III of plasmids p30860-KPC, pA1706-KPC and pA1705-KPC are inserted upstream of the backbone gene *msrB*, identical to reference plasmid p10265-KPC. Genomic comparison of p116965-KPC, pNY5520-KPC, and reference p10265-KPC for accessory region III revealed the following differences. i) Accessory region III of reference p10265-KPC is the Tn*6296* variant (the major gene environment of *bla*$_{\text{KPC}}$ in China.[28–30]). The *tnpA*$_{\text{Tn}6376}$ in plasmid p116965-KPC had an insertion of Tn*6583* (IS*Apu2-orf372*-IS*Apu1*); the novel variant Tn*6376* was designated as Tn*6376c*. ii) Compared with p116965-KPC, the Tn*6376c-3'* in plasmid p10265-KPC had an insertion of Δ*bla*$_{\text{TEM-1}}$; the variant structure was designated as Tn*6376b*. iii) Different from other plasmids, the structure of accessory region III in plasmid pNY5520-KPC was Δ*repB-orf396-orf279-klcA-5'*-Tn*5403-klcA-3'-korC*-ΔIS*Kpn6-bla*$_{\text{KPC-2}}$-Tn*6376*-Δ*tnpA*IS*Kpn26*-ΔTn*4662a*; Δ*tnpA*$_{\text{IS}Kpn26}$ was truncated at both ends, therefore, we hypothesize that IS*Kpn26* originally existed in pNY5520-KPC and the two ends of IS*Kpn26* were truncated due to the insertion of ΔTn*6296* and ΔTn*4662a*, and that Tn*5403* was inserted into the *klcA* gene after the insertion of ΔTn*6296*, causing a truncation of the *klcA* gene (Figure 6B).

Transferability and Bacterial Antimicrobial Susceptibility

The IncG plasmid p116965-KPC, representative of those used in this study, could be transferred from its wild-type isolate into DH5α through electroporation, generating *E. coli* transformants 172116965-KPC-2-DH5α. Sequences in *bla*$_{\text{KPC-2}}$

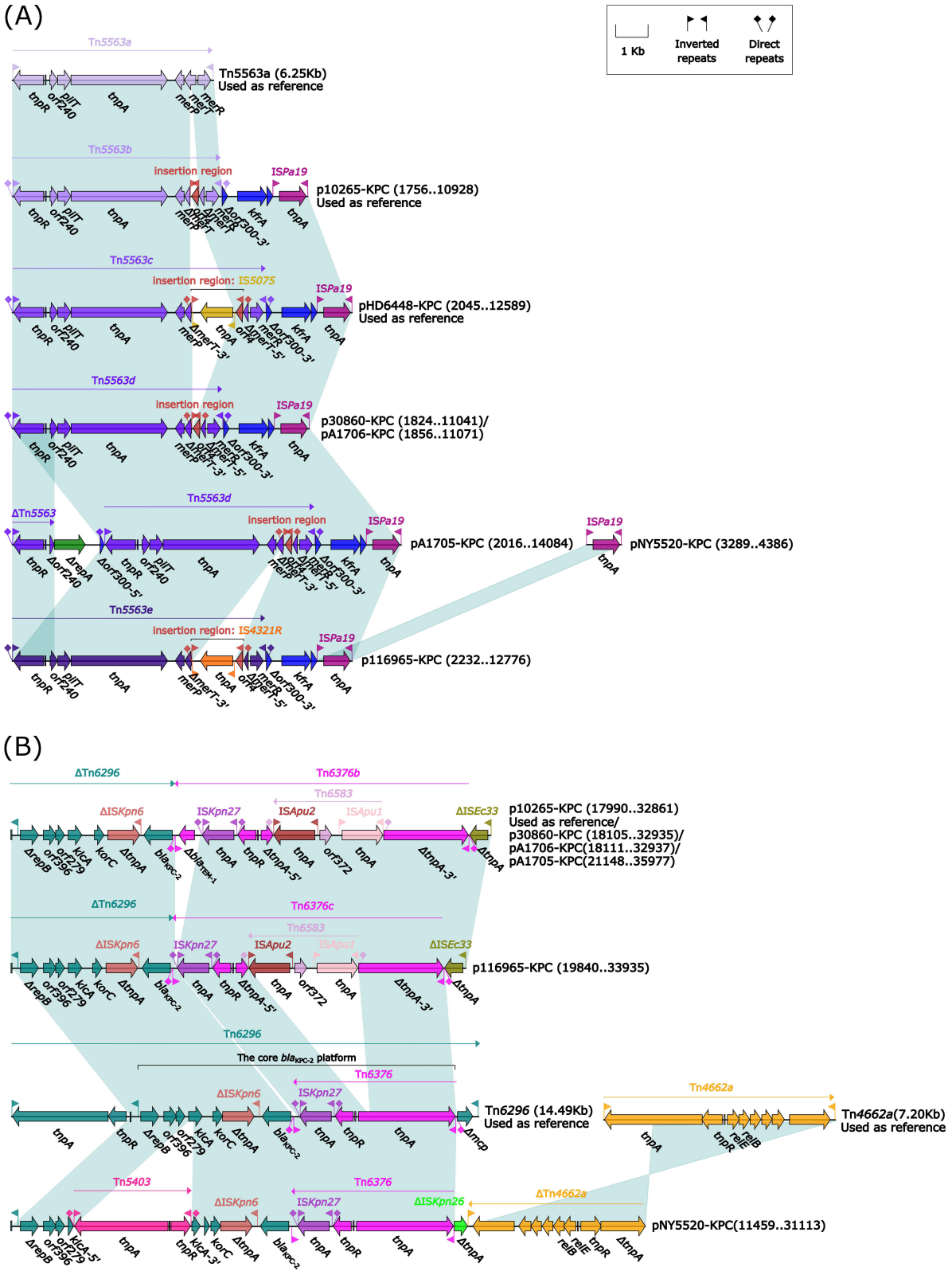

Figure 6 Accessory regions.

Notes: (**A**) Tn*5563a*/*b*/*c*/*d*/e and IS*pa19*. (**B**) bla_{KPC-2}-region. Genes are denoted by arrows and colored based on gene function classification. Shading regions denote shared regions of homology (>95% nucleotide identity).

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and *repA*$_{p116965\text{-}KPC}$ were used for verification and 172116965-KPC-2-DH5α was further confirmed by PCR detection and amplicon sequencing (Figure S1). As expected, all five bacterial strains and 172116965-KPC-2-DH5α were resistant to all the β-lactam. However, 172116965-KPC-2-DH5α remains susceptible to all other drugs tested (Table 4). In summary, transformant 172116965-KPC-2-DH5α harbored a non-conjugative plasmid p116965-KPC, which carries the *bla*$_{KPC\text{-}2}$ gene to mediate the resistance to β-lactams.

Discussion

Numerous IncG plasmids have now been reported. Despite this, a systematic summary of IncG plasmids has not previously been published. In 1975, the archetype IncG plasmid Rms149 was first found in clinical *Pseudomonas aeruginosa* strain Ps142 in Germany.[3] The nucleotide sequence analysis of Rms149 was published in 2005.[31] In 2007, IncG plasmid pRSB105, containing a novel macrolide resistance gene isolated from the environment, was reported in Germany.[32] Subsequently, in 2011 and 2013, IncG plasmids pRIO-5[33] and pCOL-1[34] were reported in Brazil and Colombia, respectively. From 2016 to 2022, IncG plasmids were reported four times in China, three times in Japan,[5,10,35–40] and once each in the United States, Spain, the Czech Republic and Argentina.[4,41,42] Given that all the strains harboring IncG plasmids were of clinical significance, so that it is possible that the real extent of the distribution of IncG plasmids is unknown.

To explore the genetic evolutionary relationship of IncG plasmids, we propose an ML typing scheme based on repA sequences that divides IncG type plasmids into eight subtypes: IncG-I to IncG-VIII. The archetypal IncG plasmid Rms149 belongs to the type IncG-V, the backbone and accessory elements of which are quite different from those of the plasmids in our study (Figure 5). We speculated that due to their distant evolutionary relationship, the plasmids in our study would exhibit large difference in their backbone structure and different characteristics in their accessible mobile elements. Although the plasmids in this study all belonged to IncG-VIII, according to the phylogenetic relationship of *repA*$_{IncG}$, the evolutionary distances among p30860-KPC, pA1706-KPC, p116965-KPC and pA1705-KPC were relatively close, while the evolutionary distance between pNY5520-KPC and the previous four plasmids was relatively distant. This result is consistent with the diversity of gene structure among these plasmids and further reveals the genetic evolution of IncG plasmids. Additionally, p30860-KPC was captured from *E. cloacae*, pA1706-KPC and pA1705-KPC were captured from *K. pneumoniae*, p116965-KPC was captured from *Citrobacter sp*, pNY5520-KPC was captured from *P. aeruginosa*, collected from samples taken at hospitals in Guangdong Province, Liaoning Province, Hunan Province, and Henan Province, respectively. At the same time, the IncG subtypes reported in the literature to date include IncG-V, IncG-VI and IncG-VIII, which have been found in continents such as Europe, Asia, North America, and South America. According to the literature, it is known that IncG-V plasmids are distributed in Europe, North America and South America, while IncG-VI plasmids are only reported in Europe. Compared to its distribution in Europe and South America, IncG-VIII plasmids are more widely distributed in Asia. The plasmids in this study came from different species and different regions yet have extremely high coverage and similarity (Figure 4), indicating that IncG plasmids similar to the reference p10265-KPC have strong horizontal transmission ability.

The accessory module of the IncG plasmid is relatively simple. Accessory modules of IncG plasmids in this study, mainly Tn*5563d/e*, IS*pa19* and Tn*6296* variants, showed high consistency. Among them, Tn*5563d* has a insertion of IS*4321R*, and the resulting sequence is named Tn*5563e*. Prototype Tn*5563a* was initially identified in plasmid pRA2 (accession number: U88088.2, an endogenous 33-kb plasmid from *Pseudomonas alcaligenes* NCIB 9867 (strain P25X).[43] Compared to the prototype Tn*5563a*, Tn*5563b* has a 286 bp insertion sequence, causing truncation of the *merT* gene,[10] whereas Tn*5563c* is inserted with IS*5057* compared with Tn*5563d*.[44] Remarkably, at the position of the 286 bp insertion sequence of Tn*5563b*, Tn*5563c/d/e* have a 293 bp insertion sequence, and 284 bp in the two sequences are identical. Therefore, we speculate that these two sequences belonged to the same unknown insertion region. The insertion region has an ORF size of 210 bp bounded by 20 bp inverted repeats (IRL: GGTCGCTTCAGAATTCGGAA; IRR: TTCCGTTTTCTGAGGTGACC) and further flanked by a 9 bp direct repeat (DR: CCATCATGG) at both ends. The 293 bp insertion sequence of Tn*5563d* was assigned as prototype of the unknown insertion region. Although the 293 bp insertion sequence of Tn*5563c/e* coincides with the structure of the unknown insert element, IS*5075* and IS*4321R* are inserted between the ORF and DR of Tn*5563c* and Tn*5563e*, respectively. The difference between the 286 bp insertion

sequence of Tn*5563b* and the unknown insertion region is the loss of a 7 bp DR. Additionally, compared with Tn*5563c/d/e*, Δ*merT*-3' in Tn*5563b* has lost 45 bp. Therefore, in terms of evolutionary relationship, it is possible that the unknown insertion region inserted into Tn*5563a* resulted in Tn*5563d*, followed by Tn*5563b/c/e* due to insertion, transposition and other events (Figure 6A).

The bla_{KPC-2}-region of IncG plasmids in this study all belonged to the Tn*6296* variant. This region of p30860-KPC, pA1705-KPC, and pA1706-KPC were consistent with reference p10265-KPC (coverage: 100%, identity: 100%), which is composed of ΔTn*6296* and Tn*6376b*. However, the bla_{KPC-2}-region of p116965-KPC differed from reference p10265-KPC only in that Tn*6376b* was replaced by Tn*6376c*. Compared with the prototype Tn*6376*, Tn*6376c* had a difference of 104 bp in IRR_Tn*6376* and IRR_IS*Kpn27*, and an insertion of Tn*6583*; compared with Tn*6376b*, Tn*6376c* had difference of 252 bp, and a deletion of Δbla_{TEM-1}. This suggests that Tn*6376c* experienced different insertion and transposition between IRR_Tn*6376* and IRR_IS*Kpn27*. In contrast, the bla_{KPC-2}-region of pNY5520-KPC had an insertion of Tn*5403* in truncated Tn*6296*, and due to insertion of ΔTn*4662a*, had ΔIS*Ec33* replaced by ΔIS*Kpn26*, as well as a deletion of DRs_Tn*6376* (Figure 6B).

Conclusion

In Conclusion, most IncG plasmids carrying the bla_{KPC-2} gene may be caused by artificial selection. However, the IncG plasmids are widely spreading in a variety of bacteria species by intercellular transfer, belonging to broad-spectrum plasmids. As the IncG plasmids have apparently become the repository for the bla_{KPC-2} gene, monitoring of bacterial resistance transmission via the IncG plasmids should be strengthened. In this work, we provided the first typing scheme against IncG plasmids. Based on the replicon sequences (IncG-I to IncG-VIII), IncG plasmids could be classified into eight subtypes. To further present the genetic structure of the IncG plasmids, we performed a detailed genomic comparison of five IncG plasmids carrying carbapenem resistance gene bla_{KPC-2} from clinical *Pseudomonas* and *Enterobacteriaceae* species. The insertion regions Tn*5563d*, Tn*5563e* and a novel unknown insertion region were identified, and novel insertion regions Tn6376b and Tn6376c named. We speculated that evolution may occur between Tn5563 and its variants due to translocation, insertion, or deletion. This study not only reveals the gene structure of the IncG plasmid carrying bla_{KPC-2}, but also provides deeper genetic insight into characteristics of the IncG plasmids.

Data Sharing Statement

The datasets generated for this study can be found in the complete nucleotide sequences of plasmids p30860-KPC, p116965-KPC, pA1705-KPC, pA1706-KPC and pNY5520-KPC were submitted to GenBank under accession numbers MN477223, MN539620, MH909348, MH909350 and CP096939 respectively.

Ethics Statement

This study used the clinical bacterial isolates obtained from the Chinese public hospitals as listed in Table 2. Since the bacterial isolation was part of the routine hospital laboratory procedures. According to relevant legislation/policy of China (http://www.nhc.gov.cn/fzs/s3576/201808/14ee8ab2388440c4a44ecce0f24e064c.shtml), this study was not subject to ethical review. 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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All experiments and data analyses were done in Dr. Dongsheng Zhou's laboratory.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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