**IncFIB-4.1 and IncFIB-4.2 Single-Replicon Plasmids: Small Backbones with Large Accessory Regions**

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**Purpose:** To establish a typing scheme for IncFIB replicon and to dissect genomic features of IncFIB-4.1/4.2 single-replicon plasmids.

**Methods:** A total of 146 representative fully sequenced IncFIB-replicon-containing plasmids were selected to construct a phylogenetic tree of repB IncFIB sequences. A collection of nine IncFIB-4.1/4.2 single-replicon plasmids from China were fully sequenced here and compared with the first sequenced IncFIB-4.1/4.2 single-replicon plasmids from GenBank to dissect their genomic diversity.

**Results:** In this study, a repB sequence-based scheme was proposed for grouping IncFIB replicon into seven primary types and further into 70 subtypes. A collection of nine IncFIB-4.1/4.2 single-replicon plasmids were fully sequenced here and compared with the first sequenced IncFIB-4.1/4.2 single-replicon plasmids from GenBank. These 11 plasmids had small backbones and shared only three key backbone markers repB together with its iterons, parABC, and stbD. Each plasmid contained one large accessory region (LAR) inserted into the backbone, and these 11 LARs had significantly distinct profiles of mobile genetic elements (MGEs) and resistance/metabolism gene loci. Antibiotic resistance regions (ARRs; the antibiotic resistance gene-containing genetic elements) were found in seven of these 11 LARs. Besides resistance genes, ARRs carried unit or composite transposons, integrons, and putative resistance units. IncFIB-4.1/4.2 single-replicon plasmids were important vectors of drug resistance genes. This was the first report of three novel MGEs: In1776, Tn6755, and Tn6857.

**Conclusion:** Data presented here provided a deeper insight into diversity and evolution of IncFIB replicon and IncFIB-4.1/4.2 single-replicon plasmids.

**Keywords:** IncFIB, IncFIB-4 single-replicon plasmids, large accessory regions, antibiotic resistance regions, In1776, Tn6755, Tn6857

**Introduction**

IncF plasmids, initially found in *Escherichia coli* K-12 in 1946,1 are widely spreading among *Escherichia coli*,2 *Klebsiella pneumonia*,3 *Salmonella enterica*,4 et al. They acted as vectors of diverse mobile genetic elements (MGEs) carrying various resistance,5–7 virulence8 or metabolism-related genes.9,10 By the intercellular transfer of IncF plasmids, these genes were accumulated and disseminated in different bacterial isolates, and thus enhances the adaption and survival of these IncF plasmid-containing isolates.

Based on replication initiation protein Rep sequences, different F-type replicons have been identified and can be further categorized into IncFII, IncFIA, and IncFIB.11 IncFII are regulated by antisense RNAs, while IncFIA and IncFIB function as iteron-regulated replicons.12,13 IncFIA and IncFIB often as auxiliary replicons coexist with the major IncFII replicon to constitute multi-replicon plasmids, as represented by the IncFII:IncFIB plasmid p28078-NDM (GenBank accession number MF156713), the IncFII:IncFIB plasmid pO86A1 (GenBank accession number AB255435), and the...
IncFII:IncFIA:IncFIB plasmid pCA08. Additionally, other replicons, such as IncR and IncN, can also cointegrate with IncFII, as represented by the IncFII:IncFIB:IncR plasmid pKPHS2 and the IncFII:IncN plasmid pA3295-KPC. This multi-replicon status will overcome the IncF incompatibility barrier and accomplish the broad host range, thereby facilitating dissemination of IncF plasmids. IncFIB as a sole replicon or an auxiliary replicon in a plasmid is also capable of guaranteeing the stable maintenance for inheritance, as presented by the plasmid pSE11-3 or Plasmid F.

There are few reports on characterization of IncFIB replicon and IncFIB single-replicon plasmids. In this work, a repB sequence-based scheme was proposed for grouping IncFIB replicon into seven primary types and further into 70 subtypes. Furthermore, a comprehensive comparative genomics analysis of 11 IncFIB-4.1/4.2 single-replicon plasmids (including nine ones sequenced in this work) provided a deeper insight into diversification and evolution of IncFIB-4.1/4.2 single-replicon plasmids.

**Materials and Methods**

**Bacterial Strains and Identification**

Nine clinical isolates, including *Klebsiella pneumoniae* BJ20, 71221, W08291, 10057 and A2359, *K. quasipneumoniae* A2508, 13294 and A1876, and *Leclercia adcarboxylata* L21, were collected from eight different Chinese public hospitals (Table S1). Each of them carried an IncFIB single-replicon plasmid that designated as pBJ20-tetA, p71221-mphA, pW08291-tetA, p10057-catA, pA2359-IMP, pA2508-emrE, p13294-1NR, pA1876-NR, and pL21-1NR, respectively (Table S2). These nine plasmids had complex molecular structures and contained multiple loci related to antibiotics, heavy metal, or metabolism resistance. To provide a comprehensive comparative genomics analysis of IncFIB-4.1/4.2 plasmids, these nine plasmids were involved into a comprehensive comparative genomics analysis.

**Genomic DNA Extraction, Sequencing, and Sequence Assembly**

Bacterial genomic DNA was isolated using the UltraClean Microbial Kit (Qiagen, NW, Germany), and sequenced from a sheared DNA library with average size of 15 kb (ranged from 10 kb to 20 kb) on a PacBio RSII sequencer (Pacific Biosciences, CA, USA), as well as a paired-end library with an average insert size of 350 bp (ranged from 150 bp to 600 bp) on a HiSeq sequencer (Illumina, CA, USA). The paired-end short Illumina reads were used to correct the long PacBio reads utilizing proovread, and then the corrected PacBio reads were assembled de novo utilizing SMARdenovo (https://github.com/ruanjue/smartdenovo).

**Sequence Annotation and Comparison**

Genome sequences were annotated by the Rapid Annotation using Subsystem Technology (RAST) combined with BLASTP/BLASTX/BLASTN, Domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and RefSeq database. Annotation of replication genes, resistance genes, MGEs, and other features were carried out using the online databases including PlasmidFinder, CARD, ResFinder, ISfinder, INTEGRALL, and Tn Number Registry. Multiple and pairwise sequence comparisons were performed using BLASTN. Gene organization diagrams were drawn using Inkscape 1.0 (https://inkscape.org/en/).

**Phylogenomic Analysis**

The repB sequences of indicated plasmids were aligned using Clustal Omega 1.2.2, and then maximum-likelihood phylogenetic trees were constructed from aligned sequences using MEGA X 10.1.8 with a bootstrap iteration of 1000.

**Conjugal Transfer and Electroporation Transfer**

Each indicated plasmid was transformed from its wild-type isolate into *E. coli* EC600 or TOP10 in Enterobacteriaceae, through conjugal transfer or electroporation experiments, respectively. Three milliliters of overnight cultures of each donor and recipient bacteria were mixed together, harvested, and resuspended in 80 mL of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm² hydrophilic nylon membrane filter with a 0.45 µm pore size
(Millipore) that was placed on BHI agar (BD Biosciences) plate and then incubated for mating at 26°C or 37°C for 12 h to 18 h. Bacteria were washed from filter membrane and spotted on BHI plates, for selecting mphA-carrying transconjugant and catA-carrying transconjugant. 1500 μg/mL rifampin (for EC600), together with 40 μg/mL azithromycin [for mph(A)] or 25 μg/mL chloramphenicol (for catA) was used as transconjugant selection, respectively.

To prepare competent cells for electroporation, 200 mL of overnight culture of E. coli TOP10 in Super Optimal Broth (SOB) at an optical density (OD600) of 0.4 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 SOB agar plates containing 40 μg/mL azithromycin [for mph(A)] or 25 μg/mL chloramphenicol (for catA), for selecting of E. coli electroporant, respectively.

**Bacterial Antimicrobial Susceptibility Testing**

Bacterial antimicrobial susceptibility was tested by E-test, and bacterial antimicrobial susceptibility was interpreted as per the 2020 Clinical and Laboratory Standards Institute (CLSI) guidelines.33

**Nucleotide Sequence Accession Numbers**

Complete sequences of plasmids pBJ20-tetA (K. pneumoniae BJ20), p71221-mphA (K. pneumoniae 71221), pW08291-tetA (K. pneumoniae W08291), pA2508-emrE (K. quasipneumoniae A2508), pL21-1NR (L. adcarboxglata L21), p10057-catA (K. pneumoniae 10057), p13294-1NR (K. quasipneumoniae 13294), pA1876-NR (K. quasipneumoniae A1876), and pA2359-IMP (K. pneumoniae A2359) were submitted to GenBank under accession numbers MN310373, MN310374, MN310376, MN310379, MN423365, MN423364, MT570100, MT549899, and MN423363, respectively.

**Results and Discussion**

**A Typing Scheme for repB_{IncFIB} Genes**

A phylogenetic tree (Figure 1) was constructed from the repB_{IncFIB} sequences of 146 arbitrarily selected representative plasmids (Table S3; last accessed November 2nd 2020) with IncFIB as the sole or auxiliary replicon. repB_{IncFIB} could be divided into seven major separately clustering clades (Figures 1 and S1), being designated as primary IncFIB types IncFIB-1 to IncFIB-7. Each primary type was further divided into various subtypes (eg IncFIB-1.1 to IncFIB-1.23), which were based on the criterion that repB_{IncFIB} sequences within each subtype displayed ≥95% nucleotide identity while <95% sequence identity was observed between different subtypes. Accordingly, a total of 70 IncFIB subtypes were identified.

**A Group of 11 IncFIB-4 Single-Replicon Plasmids Analyzed**

Five IncFIB-4.1 single-replicon plasmids and four IncFIB-4.2 single-replicon plasmids were fully sequenced here. A genomic comparison was applied to a total 11 plasmids (Table S2), which comprised the above nine ones and also two GenBank plasmids pSC138 and pFB2.3, which were the first sequenced IncFIB-4.1/4.2 single-replicon plasmids and had the minimum IncFIB-4.1/4.2 backbones, respectively.

The modular structure of each plasmid was divided into the backbone, and the accessory modules that manifested as acquired DNA regions associated/bordered with MGEs (Figure S2 and Table S4). These 11 plasmids had small backbones (6.9 kb to 24.3 kb in length) and contained no conjugal transfer genes. They shared only three key backbone markers repB together with its iterons (replication), parABC (partition), and stbD (mediator of plasmid stability), but displaying remarkable sequence divergence between IncFIB-4.1 and IncFIB-4.2. Each plasmid carried one large accessory region (LAR): the resulting 11 LARs varied in size from about 98 kb to nearly 236 kb and had significantly different profiles of MGEs and resistance/metabolism genes. At least 46 resistance genes, involved in resistance to 17 categories of antibiotics and heavy metals, were identified in these 11 plasmids (Table S5).
Figure 1. A maximum-likelihood phylogenetic tree of repB\textsubscript{IncFIB} sequences.

Notes: Bootstrap values are shown next to each branch. Bar denote scale of sequence divergence. Triangles indicate the first sequenced IncFIB-4.1/4.2 single-replicon plasmids while squares denote nine plasmids fully sequenced in this study.

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The six IncFIB-4.1 plasmids displayed >96% nucleotide identity across 25% to 100% of their backbone sequences, while the five IncFIB-4.2 plasmids showed ≥99% nucleotide identity over ≥84% of their backbone sequences; by contrast, IncFIB-4.1 and IncFIB-4.1 plasmids had ≤90% nucleotide identity across ≤90% of their backbone sequences (Table S6).

Modular Differences in IncFIB-4.1 Single-Replicon Plasmids

Based on a presumed prototype IncFIB-4.1 backbone, the six IncFIB-4.1 plasmids exhibited at least eight major modular differences (Figure S2): i) integration of two copies of IS903B led to a 5.1-kb backbone deletion and a 5.7-kb backbone inversion in pBJ20-tetA, respectively; ii) ISKpn26 was inserted at two different sites of the backbone in pBJ20-tetA and pW08291-tetA; iii) insertion of ISA – ISSen4 led to absence of the backbone gene orf306 in pBJ20-tetA, p71221-mphA, and pW08291-tetA; iv) insertion of IS903B caused a 3.8-kb backbone deletion in p71221-mphA and pW08291-tetA; v) a group II Kl.pn.15-like intron was integrated into p71221-mphA, pW08291-tetA, and pBJ20-tetA; vi) LAR integration resulted in 14.7-kb and 2.6-kb backbone deletions upstream and downstream, respectively, of LAR in both pL21-1NR and pSC138, and led to a 3.8-kb upstream-of-LAR deletion in pA2508-emrE; vii) there was the deletion of a 4.2-kb orf1953–to–orf426 backbone region from pA2508-emrE, pL21-1NR, and pSC138; viii) the copy numbers of 43-bp tandem repeat within parC (ParB-binding sites) varied as 8, 8, 8, 19, 11, and 11 in pBJ20-tetA, p71221-mphA, pW08291-tetA, pA2508-emrE, pL21-1NR, and pSC138, respectively. Together, IncFIB-4.1 backbones underwent massive deletion events, mostly resulting from accessory module integration.

The six LARs (Figure S2) had at least six major genetic differences. Firstly, antibiotic resistance regions (ARRs; the antibiotic resistance gene-containing genetic elements in LARS) were identified in pBJ20-tetA, p71221-mphA, pW08291-tetA, and pSC138. Secondly, ars loci (arsenic resistance) were found in pBJ20-tetA, p71221-mphA, pW08291-tetA, pA2508-emrE, and pL21-1NR. Thirdly, sil–cop regions, conferring resistance to silver (sil) and copper (cop), were present in the five plasmids except for pSC138. Fourthly, pA2508-emrE had five metabolism gene loci: phn (phosphonate uptake), urt (urea uptake), fec (iron uptake), lac (galactoside uptake), and gsi (glutathione uptake). Fifthly, a K-antigen gene cluster was present only in pL21-1NR. Sixthly, a 13.5-kb backbone region matching IncFII plasmid pKp_Goe_414-34 was found in pBJ20-tetA, p71221-mphA, and pW08291-tetA; a 8.3-kb one and a 25.7-kb one and a 7.4-kb one, and a 43.6-kb one matching IncFII plasmids p1220-CTXM35 and pKPN1705-2 (CP022825), p16005813A,36 and IncI plasmid p628-CTXM37 in pW08291-tetA and pA2508-emrE, pL21-1NR, and pSC138, respectively.

Modular Differences in IncFIB-4.2 Single-Replicon Plasmids

The five IncFIB-4.2 plasmids had very similar backbones with at least two major modular differences: orf537 in pFB2.3 and pA2359-IMP was deleted due to LAR integration (Figure S2); the 43-bp tandem repeats within parC varied in copy numbers, namely 23, 21, 17, 19, and 8 for p10057-catA, p13294-1NR, pA1876-NR, pA2359-IMP, and pFB2.3, respectively.

The five LARs (Figure S2) displayed at least six major genetic differences. Firstly, ARRs were found in p10057-catA, p13294-1NR, pA1876-NR, pA2359-IMP, and pFB2.3. Secondly, ars loci were found in p10057-catA, p13294-1NR, and pFB2.3. Thirdly, sil–cop regions were present in p13294-1NR, pA1876-NR, and pFB2.3. Fourthly, the IS15DI (a minor variant of IS26)-composite transposon Tn6755 was located in p13294-1NR. Fifthly, different IncFIB-4.2 plasmids had distinct profiles of metabolism gene loci: hut (histidine uptake) and ∆lac in p10057-catA; ehu (ectoine uptake), gsi and phn in p13294-1NR; ∆ehr and gsi in pA1876-NR; fec, lac and hut in pA2359-IMP; and ∆ehr in pFB2.3. Sixthly, backbone regions of IncHI1 plasmid pKP21HI138 were contained in the four plasmids except for pA1876-NR.

Diversified ARRs

The eight ARRs elements ARR_{PA2359-IMP} bla^IMP-38-carrying Tn6382 (Tn21 subfamily unit transposon) as described previously, ARR_{p71221-mphA}, ARR_{pW08291-tetA}, ARR_{pBJ20-tetA}, ARR_{p10057-catA}, ARR-1_{pSC138}, ARR-2_{pSC138}, and ARR_{pA2508-emrE} varied in size from 4.1 kb to 98.7 kb.
ARR\textsubscript{pW08291-tetA}, ARR\textsubscript{p71221-mphA} and ARR\textsubscript{pBJ20-tetA} (Figure 2) displayed similar modular structures. ARR\textsubscript{pW08291-tetA} was derived from Tn\textsubscript{3}-family tet\textsubscript{A}(A)-carrying unit transposon Tn1721: a \textit{tnpA} (transposase)–\textit{tnpR} (resolvase)–\textit{res}\textsubscript{-containing region within Tn1721 was replaced by In\textsubscript{363} together with IS\textsubscript{26}–\textit{mph}(A)–IS\textsubscript{6100} unit; and this modified Tn1721 further connected with a truncated IS\textsubscript{26}–\textit{bla}\textsubscript{SHV-12}–IS\textsubscript{26} unit. ARR\textsubscript{p71221-mphA} differed from ARR\textsubscript{pW08291-tetA} by additional insertion of \textit{aphA1}-carrying IS\textsubscript{26}-composite transposon Tn\textsubscript{4352} upstream of IS\textsubscript{26}–\textit{mph}(A)–IS\textsubscript{6100} unit.

Figure 2 Organization of ARR\textsubscript{p71221-mphA}, ARR\textsubscript{pW08291-tetA} and ARR\textsubscript{pBJ20-tetA}, and comparison to related regions.

Notes: Genes are denoted by arrows. Genes, MGEs and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within corresponding plasmids. Accession numbers of Tn1721, IS\textsubscript{26}–\textit{bla}\textsubscript{SHV-12}–IS\textsubscript{26} unit, IS\textsubscript{26}–\textit{bla}\textsubscript{LAP-2}–\textit{qnrS1}–IS\textsubscript{26} unit, and \textit{aacC2}–\textit{tmrB} region used as reference are X61367, CP003684, HF545433, and JX101693, respectively.

ARR\textsubscript{pW08291-tetA}, ARR\textsubscript{p71221-mphA} and ARR\textsubscript{pBJ20-tetA} (Figure 2) displayed similar modular structures. ARR\textsubscript{pW08291-tetA} was derived from Tn3-family tetA(A)-carrying unit transposon Tn1721: a \textit{tnpA} (transposase)–\textit{tnpR} (resolvase)–\textit{res}\textsubscript{-containing region within Tn1721 was replaced by In\textsubscript{363} together with IS\textsubscript{26}–\textit{mph}(A)–IS\textsubscript{6100} unit; and this modified Tn1721 further connected with a truncated IS\textsubscript{26}–\textit{bla}\textsubscript{SHV-12}–IS\textsubscript{26} unit. ARR\textsubscript{p71221-mphA} differed from ARR\textsubscript{pW08291-tetA} by additional insertion of \textit{aphA1}-carrying IS\textsubscript{26}-composite transposon Tn\textsubscript{4352} upstream of IS\textsubscript{26}–\textit{mph}(A)–IS\textsubscript{6100} unit.
ARR_pJ20-tetA contained the whole ARR_p71221-mphA, with addition of a truncated IS26-\textit{bla}\subtext{LAP-2}-\textit{qnrS}-IS26 unit\textsuperscript{45} and a truncated \textit{aacC2}–\textit{imrB} region.\textsuperscript{46}

ARR_p10057-catA (Figure 3) contained at least 12 antibiotic resistance loci: \textit{In}1776, an interrupted \textit{chrA}–\textit{orf98} unit, IS26–\textit{mph}(A)–IS6100 unit, IS26–\textit{bla}\subtext{SHV-12}–IS26 unit, a \textit{Tn}1548-related element, a truncated IS1\textit{R}-composite transposon \textit{Tn}9 carrying \textit{catA}\textsubscript{1},\textsuperscript{47} \textit{In}37,\textsuperscript{48} a truncated \textit{Tn}21-subfamily unit transposon \textit{Tn}6535,\textsuperscript{39} IS26–\textit{tetA}(D)–IS26 unit,\textsuperscript{42} a truncated IS26-composite transposon \textit{Tn}6322 carrying \textit{catA}\textsubscript{1},\textsuperscript{50} a truncated \textit{Tn}163-subfamily unit transposon \textit{Tn}5393c carrying \textit{strAB},\textsuperscript{51} a truncated integrative and mobilizable element \textit{Tn}6591 carrying \textit{su}l\textsubscript{2}.\textsuperscript{52} The concise class 1 integron \textit{In}1776 carried the gene cassette array (GCA) \textit{df}\textsubscript{rA12}–\textit{aadA2}:IS1\textit{R}–\textit{gcuF}, which highly resembled that of \textit{In}27.\textsuperscript{53} \textit{Tn}1548,\textsuperscript{54} an IS26-composite transposon lack of direct repeats (DRs; target site duplication signals for transposition), contained three resistance loci: \textit{ISEc29}–\textit{mph}(E)–IS26 unit, \textit{ISCR1}–\textit{armA} unit, and \textit{In}27. The \textit{Tn}1548-related element had two major modular differences relative to \textit{Tn}1548: \textit{ISEc29}–\textit{mph}(E)–IS26 unit was interrupted by insertion of \textit{ISKpn}21; and a \textit{qnrB4}–\textit{bla}\subtext{IDH}A\subscript{1} region was present instead of \textit{In}27.

ARR-1_pSC138 (Figure 4) harbored at least seven antibiotic resistance loci: \textit{ΔTn}1721, IS26–\textit{catA}2–IS26 unit,\textsuperscript{50} a truncated \textit{Tn}3-subfamily unit transposon \textit{Tn}2 carrying \textit{bla}_{\text{TEM-1}},\textsuperscript{55} \textit{Tn}4352, \textit{In}27, a truncated IS26–\textit{met}(B)–\textit{su}3–IS440 unit,\textsuperscript{56} and \textit{In}641.\textsuperscript{57} ARR-2_pSC138 (Figure S3) and ARR_pA2508-emrE (Figure S4) manifested as \textit{Tn}6092\textsuperscript{57} (a \textit{bla}_{\text{CMY-2}}-carrying \textit{ISEcp1}-based transposition unit that was another derivative of \textit{Tn}6538\textsubscript{a/b/c}),\textsuperscript{58} and a truncated \textit{Tn}21-subfamily unit transposon \textit{Tn}6535 carrying \textit{emrE},\textsuperscript{39} respectively.

**Other Key Accessory Regions**

Eight plasmids harbored five types of \textit{ars} loci (type A to E; Figure S4): incomplete type A in \textit{ΔTn}6736 of pBJ20-tetA, p71221-mphA and pW08291-tetA, type B in pl21-1NR, type C in p13294-1NR and pFB2.3, type D in pA2508-emrE and pFB2.3, and type E in two distinct \textit{ΔTn}6535 of pA2508-emrE and p10057-catA, respectively. \textit{ΔTn}6736 and \textit{ΔTn}6535 were the truncated versions of \textit{Tn}3-family prototype unit transposons \textit{Tn}6736 and \textit{Tn}6535,\textsuperscript{39} respectively. Different types of \textit{ars} loci exhibited considerable diversity in gene organization and nucleotide and amino acid sequences.

The prototype \textit{sil}–\textit{cop} region as initially characterized in plasmid R478\textsuperscript{59} was present in three IncFIB-4 plasmids, while those in other five IncFIB-4 plasmids underwent insertion, deletion, duplication, and inversion (Figure 5), especially including insertion of LAR\textsubscript{p71221-mphA}, \textit{Tn}6831\textsubscript{pFB2.3} (an \textit{IS}1\textit{X2}-composite transposon related to cytosome metabolism), and \textit{Tn}6732\subscript{pL21-1NR} (a cryptic \textit{IS}1\textit{F}-composite transposon genetically closed related to \textit{Tn}6732\textsubscript{a}).

\textit{Tn}6735\subscript{p13294-1NR} (Figure S5) was a 138.5-kb \textit{IS}1\textit{DI}-composite transposon and contained mainly \textit{phn}, \textit{ars}, \textit{sil}–\textit{cop} and two novel \textit{Tn}3-family unit transposons \textit{Tn}6736\textsubscript{b} (cryptic) and \textit{Tn}6857 (glucose metabolism).

**Transferability and Bacterial Antimicrobial Susceptibility**

p71221-mphA or p10057-catA as the IncFIB-4.1 or IncFIB-4.1 representative plasmid could be transferred from its wild-type isolate into TOP10 through electroporation, generating \textit{E. coli} electroporant TOP10/p71221-mphA or TOP10/p10057-catA, respectively. As expected, these two electroporants were resistant to azithromycin and chloramphenicol with very high minimum inhibitory concentration values ≥256 and ≥32, resp ectively. Repeated attempts to plasmid conjugal transfer failed, being consistent with the fact that these plasmids lacked conjugal transfer genes.

**Novel MGEs Identified**

There were totally three newly identified MGEs: integron \textit{In}1776, composite transposon \textit{Tn}6755, and unit transposon \textit{Tn}6857. Additional seven MGEs (composite transposons \textit{Tn}6732\textsubscript{b} and \textit{Tn}6831, unit transposons \textit{Tn}6736 and \textit{Tn}6756\textsubscript{b}, and \textit{IS} elements \textit{ISK}qu3, \textit{IS}Lead2 and \textit{IS}Lead3) were newly designated on the basis of standard MGE nomenclatures, but they had previously determined sequences.

**Concluding Remarks**

In summary, a \textit{repB} sequence-based typing and nomenclature scheme is proposed to identify different types or subtypes of IncFIB replicon, and a detailed comparison of the modular structures of IncFIB-4.1/4.2 single-replicon
Figure 3. Organization of ARR<sub>10057-catA</sub> and comparison to related regions.

**Notes:** Genes are denoted by arrows. Genes, MGEs and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within corresponding plasmids. Accession numbers of Tn<sub>4548</sub>, Tn<sub>4747</sub>, Tn<sub>6535</sub>, Tn<sub>6384</sub>, Tn<sub>6322</sub>, Tn<sub>5393c</sub> and Tn<sub>6591</sub> used as reference are AF550415, CP042858, V00622, CP009706, MF344563, BX664015, AF262622, and AE014073, respectively.
Figure 4 Organization of ARR-1<sub>psc138</sub> and comparison to related regions.

Notes: Genes are denoted by arrows. Genes, MGEs and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within corresponding plasmids. Accession numbers of Tn2<sup>55</sup>, Tn2<sup>60</sup> and IS26–mef<sup>(B)</sup>–sul3–IS440 <sup>unit</sup> used as reference are HM749967, AF071413, and FJ196385, respectively.
Plasmids is performed to understand the roles of gene loss/acquisition in diversification of IncFIB plasmids. The IncFIB-4.1/4.2 single-replicon plasmids have rather small backbones, which undergo massive deletion events and thus display considerable modular diversification. The IncFIB-4.1/4.2 minimum backbone includes only three key backbone markers $repB$ together with its iterons, $parABC$, and $stbD$, related to plasmid replication and maintenance. Each plasmid contains one LAR inserted into the backbone, and these LARs have significantly distinct profiles of MGEs and resistance/metabolism gene loci, thus displaying separate assembly and evolution histories. Seven of these 11 LARs contain one or two ARRs, and thus IncFIB-4.1/4.2 plasmids are important vectors of resistance genes, contributing to resistance to not only antibiotics (β-lactams including carbapenems, quinolones, aminoglycosides, tetracyclines, and phenicols) but also heavy metals (mercuric ions, copper, silver ions, and arsenic). Acquisition and accumulation of accessory regions containing resistance/metabolism markers would increase adaptability of IncFIB-4.1/4.2 plasmids-carrying bacteria under complex environments. Ten of these 11 LARs acquire one or two regions responsible for plasmid maintenance, which would facilitate stable replication of these IncFIB single-replicon plasmids at steady-state copy numbers. Data presented here provides a deeper insight into diversity and evolution of IncFIB replicon and IncFIB-4.1/4.2 single-replicon plasmids.

![Figure 5 Organization of sil–cop regions, and comparison to related regions. Notes: Genes are denoted by arrows. Genes, MGEs and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within corresponding plasmids. Accession numbers of prototype sil–cop region$^{15}$ and Tn$^{6732a}$ used as reference are BX664015 and CP035380, respectively.](https://doi.org/10.2147/IDR.S332949)
Data Sharing Statement
The datasets generated for this study can be found in the complete nucleotide sequences of plasmids pBJ20-tetA, p71221-mphA, pW08291-tetA, pA2508-emrE, pL21-1NR, p10057-catA, pA1876-NR, pL21-1NR, p13294-1NR, pA1876-NR, and pA2359-IMP were submitted to GenBank under accession numbers MN310373, MN310374, MN310376, MN310379, MN423365, MN423364, MT570100, MT549899, and MN423363, respectively.

Ethics Statement
This study uses the clinical bacterial isolates obtained from the Chinese public hospitals as listed in Table S1. The local legislation did not require the study to be reviewed or approved by an ethics committee, because the bacterial isolates involved in this study was part of the routine hospital Laboratory procedures. 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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