Comparative genomics of five different resistance plasmids coexisting in a clinical multi-drug resistant *Citrobacter freundii* isolate

**Background:** Plasmid-mediated multi-drug resistance (MDR) has been widely found in *Citrobacter freundii*. *C. freundii* P10159 was isolated from a human case of postoperative urinary tract infection in a Chinese teaching hospital.

**Methods:** The complete nucleotide sequences of five resistance plasmids pP10159-1, pP10159-2, pP10159-3, pP10159-4 and pP10159-5 from *C. freundii* P10159 were determined through high-throughput genome sequencing, and then compared with related plasmids sequences. Plasmid transfer, CarbaNP test of carbapenemase activity, and bacterial antimicrobial susceptibility test were performed to characterize resistance phenotypes mediated by these plasmids.

**Results:** pP10159-1 carrying *bla*<sup>NDM-1</sup> and pP10159-2 harboring *bla*<sup>IMP-4</sup> plus *qnrS1* were almost identical to IncX3 plasmid pNDM-HN380 and IncN1 plasmid p378-IMP, respectively. The *bla*<sup>KPC-2</sup>-carrying plasmids pP10159-3, pHS062105-3 and pECN49-KPC were highly similar to each other, and constituted a novel group of plasmids belonging to an unknown incomparability group. The MDR plasmids pP10159-4 and pP10159-5 had the backbones highly similar to IncH4 plasmid pNDM-CIT and type 2 IncC plasmid pR55, respectively, but their accessory resistance regions differed from pNDM-CIT and pR55, respectively. The five plasmids from the P10159 isolate contained a total of 24 different genes or gene loci, which contributed to resistance to 13 distinct antibiotic molecules or toxic compounds.

**Conclusion:** This is the first report of co-occurrence of five different resistance plasmids, with determination of their complete sequences. Data presented here provide a deeper insight into co-selection and maintenance of multiple plasmids and an extremely large number of resistance genes in a single bacterial isolate.

**Keywords:** *Citrobacter freundii*, multi-drug resistance, plasmids, mobile elements

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**Introduction**

*Citrobacter freundii*, a member of the family *Enterobacteriaceae*, is widely found in the environment as well as in the intestinal tract of humans and animals. *C. freundii* is generally considered a low-grade opportunistic pathogen that rarely causes infections, but it has been associated with a wide spectrum of infections of the central nervous system, the respiratory, gastrointestinal, urinary and respiratory tracts, the blood, and many other normally sterile sites in neonates and immunocompromised patients.¹

Plasmid-mediated multi-drug resistance (MDR) has been widely found in *C. freundii*. This study disclosed the co-occurrence of five resistance plasmids pP10159-1, pP10159-2, pP10159-3, pP10159-4 and pP10159-5, containing a total of 24 different resistance markers, in a single clinical *C. freundii* isolate.
Materials and methods

Bacterial strain

The use of human specimens and all related experimental protocols were approved by the Committee on Human Research of Southwest Hospital, and carried out in accordance with the approved guidelines. The indicated patient signed a written informed consent.

*C. freundii* P10159 was isolated in 2013 from a midstream urine specimen from an esophageal cancer patient with hospital-acquired postoperative urinary tract infection from a teaching hospital in Chongqing City, China. Bacterial species identification was performed using Bruker MALDI Biotyper (Bruker Daltonics) and 16S rRNA gene sequencing.² The sequence type (ST) of P10159 was determined based on the *C. freundii* multilocus sequence typing (MLST) scheme (https://pubmlst.org/cfreundii/). All PCR amplicons were sequenced on an ABI 3730 Sequencer.

Genomic DNA sequencing and plasmid sequence assembly

Bacterial genomic DNA was isolated using a Qiagen large construct kit and sequenced from a paired-end library with a mate-pair library with average insert size of 5 kb (ranging from 2 to 10 kb) at a mean coverage 108, using a MiSeq sequencer (Illumina, CA, USA). Reads were trimmed to remove poor quality sequences. In order to get the complete plasmid sequences, DNA contigs were assembled based on their contig coverage using Newbler 2.8.³ Gaps between contigs were filled using a combination of PCR and Sanger sequencing using an ABI 3730 Sequencer.

Sequence annotation and genome comparison

Open reading frames and pseudogenes were predicted using RAST 2.0⁴ combined with BLASTP/BLASTN searches against the UniProtKB/Swiss-Prot⁵ and RefSeq⁶ databases. Annotation of resistance genes, mobile elements and other features was carried out using CARD,⁷ ResFinder,⁸ ISfinder⁹ and INTEGRALL.¹⁰ Multiple and pairwise sequence comparisons were performed using MUSCLE 3.8.31¹¹ and BLASTN, respectively. Gene organization diagrams were drawn in Inkscape 0.48.1 (https://inkscape.org/en/).

Plasmid conjugal transfer

Plasmid conjugal transfer experiments were carried out with the rifampin-resistant *Escherichia coli* EC600 being used as recipient and the P10159 strain as donor. Three milliliters of overnight cultures of each of donor and recipient bacteria were mixed together, harvested and resuspended in 80 µL 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 a BHI agar (BD Biosciences) plate and then incubated for mating at 37°C for 12–18 h. Bacteria were washed from the filter membrane and spotted on Muller-Hinton (MH) agar (BD Biosciences) plates containing 1,000 µg/mL rifampin together with indicated additional antibiotics for selecting an *E. coli* transconjugant carrying one of the following resistance markers: 4 µg/mL meropenem for *bla*<sub>NDM-1</sub> (pP10159-1), *bla*<sub>IMP-4</sub> (pP10159-2) and *bla*<sub>KPC-2</sub> (pP10159-3); 10 µg/mL chloramphenicol for *catB3* (pP10159-4); and 10 µg/mL azithromycin for *mph(A)* (pP10159-5).

Plasmid electroporation

To prepare competent cells for plasmid electroporation, 200 mL of overnight culture of *E. coli* TOP10 in Super Optimal Broth (SOB) at an optical density (OD<sub>600</sub>) 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 plasmid DNA, which was isolated from the P10159 strain using a Qiagen Plasmid Midi Kit, was mixed with 100 µL of competent cells for electroporation at 25 µL, 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 the above indicated antibiotics for selecting of an *E. coli* electroporant carrying one of *bla*<sub>NDM-1</sub>, *bla*<sub>IMP-4</sub>, *bla*<sub>KPC-2</sub>, *catB3* and *mph(A)*.

Detection of carbapenemase activity

Activity of class A/B/D carbapenemases in bacterial cell extracts was determined via a modified CarbaNP test.¹² Overnight bacterial cell culture in MH broth was diluted 1:100 into 3 mL of fresh MH broth and bacteria were allowed to grow at 37°C with shaking at 200 rpm to reach an OD<sub>600</sub> of 1.0 to 1.4. If required, ampicillin was used at 200 µg/mL. Bacterial cells were harvested from 2 mL of the above culture and washed twice with 20 mM Tris-HCl (pH 7.8). Cell pellets were resuspended in 500 µL of 20 mM Tris-HCl (pH 7.8) and lysed by sonication, followed by centrifugation at 10,000 × g at 4°C for 5 minutes. Fifty microliters of the supernatant (the enzymatic bacterial suspension) were mixed with 50 µL of substrate I to V , respectively, followed by incubation at 37°C for 2 hours. Substrate I: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH7.8). Substrate II: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH7.8) and 0.6 mg/µL imipenem. Substrate III: 0.054% phenol red plus 0.1 mM imipenem. The result was read at OD<sub>500</sub>.
Five resistance plasmids coexisting in Citrobacter freundii

ZnSO₄ (pH 7.8), 0.6 mg/µL imipenem and 0.8 mg/µL tazobactam. Substrate IV: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 0.6 mg/µL imipenem and 3 mM EDTA (pH 7.8). Substrate V: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH 7.8), 0.6 mg/µL imipenem, 0.8 mg/µL tazobactam and 3 mM EDTA (pH 7.8).

Bacterial antimicrobial susceptibility test

Bacterial antimicrobial susceptibility was tested by the broth dilution method and interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines.¹³

P10159 and all the transconjugants and transformants were resistant to ampicillin, ceftazidime, meropenem and cefoxitin (Table 3). P10159 had class A β-lactamase activity, while the transconjugants and transformants harboring pP10159-1 or pP10159-2 had B activity, and those harboring pP10159-3 had A activity (Table 3). These resistance phenotypes were consistent with the production of resistance plasmids.

P10159-1, pP10159-2 and pP10159-3 could be transferred into E. coli through conjugation or electroporation, which generated the corresponding transconjugants NDM-EC600, IMP-EC600 and KPC-EC600 plus the respective electroporants NDM-TOP10, IMP-TOP10 and KPC-TOP10 (Table 3). This assay indicated that these three plasmids were conjugative. Repeated attempts failed to transfer pP10159-4 or pP10159-5 into E. coli through conjugation and electroporation.

Table 1 Major features of plasmids analyzed

| Category       | Plasmid               | pP10159-1 | pNDM-HN380-1 | pP10159-2 | pP378-IMP | pP10159-3 | pHS062105-3 | pECN49-KPC | pP10159-4 | pNDM-CIT | pP10159-5 | pR55    |
|----------------|-----------------------|-----------|--------------|-----------|-----------|-----------|--------------|-------------|-----------|-----------|-----------|---------|
| Incompatibility group | IncX3               | IncN1         | Unknown      | IncH14   |           |           |               |             |           |           |           |         |
| Total length (bp)    | 54,034               | 54,035      | 51,104       | 42,763    | 42,848    | 41,317    | 228,709      | 288,920     | 208,301   | 190,385   | 152,132  | 107,810 |
| Total number of ORFs | 62                   | 62          | 62           | 64        | 53        | 52         | 53           | 52          | 53        | 52        | 53       | 203     |
| Mean G + C content, % | 49.0                | 49.0        | 50.4         | 49.8      | 49.7      | 49.1       | 47.0         | 47.7        | 47.1      | 47.0      | 51.1     | 53.0    |
| Length of the backbone (bp) | 34,731              | 34,732      | 37,474       | 37,345    | 29,082    | 29,043     | 29,043       | 190,385     | 190,385   | 190,385   | 190,385  | 190,385 |
| Accessory modules    | The blaNDM-1 region* and ISK263 | Isb239* and ΔTn6292* and the IS/ remnant | ∆Tn6296* | The MDR region*, the blaNDM-1 region*, ISLod2, ISKpn26-1-1R, ISCR4-1-1SC9, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, the blaNDM-1 region*, ISLod2, ISKpn26-1-1R, ISCR4-1-1SC9, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 | The MDR region*, ISKpn26, ISCf6r-1-16SC21 and ISCf6r-1-16SC22 |

Notes: pP10159-1, pP10159-2, pP10159-3, pP10159-4 and pP10159-5 from C. freundii P10159 were sequenced in this work, while the other plasmids were derived from GenBank and used as references. Sequence comparison of each of these five sequenced plasmids with their reference plasmid(s) was interpreted in the text. These accessory modules contained resistance genes as listed in Table 2.

Abbreviation: MDR, multi-drug resistant.
**Table 2** Drug resistance genes in plasmids analyzed

| Plasmid | Resistance gene | Resistance phenotype | Nucleotide position in plasmid | Accessory or backbone region located |
|---------|----------------|----------------------|---------------------------------|-------------------------------------|
| pPI0159-1 | bla<sub>SHV-12</sub> | Beta-lactam resistance | 9,324–10,184 | The bla<sub>NDM-1</sub> region |
|         | ble             | Beta-lactam resistance | 17,458–17,823 |                                     |
|         | blao<sub>PO</sub> | Beta-lactam resistance | 17,827–18,639 |                                     |
| pPI0159-2 | blao<sub>SHV</sub> | Beta-lactam resistance | 4,295–5,035 | In823b |
|         | qnrS1           | Quinolone resistance  | 27,662–28,318 | ATn6292 |
| pPI0159-3 | blao<sub>PSC-2</sub> | Beta-lactam resistance | 7,737–8,618 | ATn6296 |
| pPI0159-4 | aoxAc4r         | Quinolone resistance  | 85,056–85,655 | The MDR region |
|         | blao<sub>DAE-1</sub> | Beta-lactam resistance | 85,786–86,616 |                                     |
|         | catB3           | Phenicol resistance  | 86,754–87,386 |                                     |
|         | arr3            | Rifampicin resistance | 87,471–87,923 |                                     |
|         | qacEΔI          | Quaternary ammonium compound resistance | 88,146–88,493 |                                     |
|         | sulI            | Sulfonamide resistance | 88,487–89,326 |                                     |
|         | blao<sub>CTX-M-1</sub> | Beta-lactam resistance | 114,790–115,665 | The bla<sub>CTX-M-1</sub> region |
| pPI0159-5 | blao<sub>PO-1</sub> | Beta-lactam resistance | 87,807–88,694 | The MDR region |
|         | chrA            | Chromosome resistance | 98,473–99,678 |                                     |
|         | mph(A)          | Macrolide resistance | 103,297–104,202 |                                     |
|         | aoxC2           | Aminoglycoside resistance | 105,261–106,121 |                                     |
|         | tnrB            | Tunicamycin resistance | 106,134–106,676 |                                     |
|         | blao<sub>SHV-1</sub> | Beta-lactam resistance | 118,230–119,090 |                                     |
|         | The ter locus   | Tellurium resistance | 116,056–136,305 | The plasmid backbone |
|         | sulI            | Sulfonamide resistance | 125,487–126,326 |                                     |
|         | aoxA            | Aminoglycoside resistance | 132,848–133,621 |                                     |
|         | mra(E)          | Macrolide resistance | 135,920–137,395 |                                     |
|         | mph(E)          | Macrolide resistance | 137,451–138,335 |                                     |

**Table 3** Antimicrobial drug susceptibility profiles

| Antibiotics | MIC (mg/L)/antimicrobial susceptibility |
|-------------|----------------------------------------|
|             | P10159 | NDM-EC600 | NDM-TOP10 | IMP-EC600 | IMP-TOP10 | KPC-EC600 | KPC-TOP10 | TOP10 | EC600 |
| Ampicillin   | >1,024/R | >1,024/R | >1,024/R | 512/R | 256/R | >1,024/R | >1,024/R | <4/S | <4/S |
| Ceftazidime  | >512/R | >512/R | >512/R | >512/R | 512/R | 64/R | 16/R | <4/S | <4/S |
| Imipenem     | 64/R | 64/R | 64/R | 64/R | 32/R | 64/R | 64/R | <1/S | <1/S |
| Meropenem    | 64/R | 16/R | 8/R | 4/R | 4/R | 32/R | 4/R | <1/S | <1/S |
| Cefoxitin    | >1,024/R | 1,024/R | 512/R | >1,024/R | 256/R | 128/R | 32/R | <8/S | <8/S |
| Ciprofloxacin| 64/R | <1/S | <1/S | 2/I | 2/I | <1/S | <1/S | <1/S | <1/S |
| Aminoglycosides | >1,024/R | <8/S | <8/S | <8/S | <8/S | <8/S | <8/S | <8/S | <8/S |
| Azithromycin | >512/R | <4/S | <4/S | <4/S | <4/S | <4/S | <4/S | <4/S | <4/S |
| Trimethoprim | >32/R | <0.25/S | <0.25/S | <0.25/S | <0.25/S | <1/S | <0.25/S | <0.25/S | <0.25/S |
| Sulfamethoxazole | >608/R | <4.75/S | <4.75/S | <4.75/S | <4.75/S | <4.75/S | <4.75/S | <4.75/S | <4.75/S |
| Chloramphenicol | 32/R | <8/S | <8/S | <8/S | <8/S | <8/S | <8/S | <8/S | <8/S |
| Nitrofurantoin | 16/S | 16/S | <4/S | 16/S | <4/S | 32/S | <4/S | <4/S | 8/S |
| Minocycline  | 2/S | <1/S | 4/S | <1/S | 4/S | <1/S | 4/S | <1/S | <1/S |
| Fosfomycin   | <64/S | <64/S | <64/S | <64/S | <64/S | <64/S | <64/S | <64/S | <64/S |
| Tigecycline  | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S |
| Colistin     | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S | <1/S |

**Abbreviation:** MDR, multi-drug resistant.
of one or all of NDM, IMP and KPC enzymes in the corresponding strains.

Comparison of pP10159-1 with pNDM-HN380

pP10159-1 showed 99% nucleotide identity (with 100% query coverage) to nine blaNDM-carrying IncX3 plasmids, including the first fully sequenced pNDM-HN380 \(^{14}\) (Figure 1A). pP10159-1/pNDM-HN380 contained three resistance genes bla\(_{\text{NDM-1}}\), ble and bla\(_{\text{SHV-12}}\), which were all located in the bla\(_{\text{NDM-1}}\) regions (Figure 1B). The bla\(_{\text{NDM-1}}\) regions of pP10159-1/pNDM-HN380 were organized in order of a 4.1-kb truncated version of the composite transposon-like IS\(_{\text{26}}\)-bla\(_{\text{SHV-12}}\)-IS\(_{\text{26}}\) unit,\(^{15}\) a bla\(_{\text{NDM-1}}\)-containing\(\Delta\)Tn\(_{125}\) element derived from the IS\(_{\text{Aba125}}\)-flanked composite transposon Tn\(_{125}\),\(^{16}\) IS\(_{3000}\) and a 583-bp Tn\(_{3}\) remnant (Figure 1B). An inversion of the truncated IS\(_{\text{26}}\)-bla\(_{\text{SHV-12}}\)-IS\(_{\text{26}}\) unit in the bla\(_{\text{NDM-1}}\) regions represented the only modular difference between pP10159-1 and pNDM-HN380 (Figure 1B).

Comparison of pP10159-2 with pP378-IMP

pP10159-2 displayed >99% nucleotide identity (with >97% query coverage) to 16 bla\(_{\text{IMP}}\)-carrying IncN1 plasmids, including pIMP-HZ1\(^{17}\) and pP378-IMP\(^{18}\) (Figure 2A). Although pIMP-HZ1 was the first fully sequenced bla\(_{\text{IMP}}\)-carrying IncN1 plasmid, pP378-IMP\(^{18}\) was more appropriate as the reference for genomic comparison because it contained two relatively complete mobile elements, namely a class 1 integron In\(_{823}\) and a truncated Tn\(_{3}\)-family unit transposon \(\Delta\)Tn\(_{6292}\),\(^{19}\) harboring bla\(_{\text{IMP-4}}\) and qnr\(_{\text{S1}}\), respectively. pP10159-2 and pP378-IMP shared the same In\(_{823}\) and also two resistance genes bla\(_{\text{IMP-4}}\) and qnr\(_{\text{S1}}\).

There were two major modular differences between pP10159-2 and pP378-IMP. First, the CUP (conserved upstream repeat)-controlled regulon\(^{18}\) was composed of seven sequentially arranged operons CUP\(_{\text{A}}\), CUP\(_{\text{B}}\), CUP\(_{\text{5}}\), CUP\(_{\text{4}}\), CUP\(_{\text{3}}\), CUP\(_{\text{2}}\) and CUP\(_{\text{1}}\) in the backbone of pP10159-2, while the translocation of the CUP\(_{\text{2}}\) operon occurred in pP378-IMP (Figure 2B).

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**Figure 1** Comparison of pP10159-1 with pNDM-HN380.

**Notes:** Shown are linear comparison of the two sequenced plasmids pP10159-1 and pNDM-HN380 (A), and that of the bla\(_{\text{NDM-1}}\) regions of these two plasmids (B). 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.
Second, the core transposition module \( \text{tnpA (transposase)-res (resolution site)-tnpR (resolvase)} \) of Tn6292 was interrupted by IS\( \text{Kpn19} \), breaking \( \text{tnpA} \) into separate \( \Delta \text{tnpA-3}' \) and \( \Delta \text{tnpA-5}' \), whereas distinct additional deletion events occurred within the \( \text{tnpA-res-tnpR} \) modules of the \( \Delta \text{Tn6292} \) elements from P10159-2 and P378-IMP (Figure 2C).

**Comparison of pP10159-3 with pHs062105-3 and pECN49-KPC**
p10159-3, pHs062105-3 and pECN49-KPC constituted a novel group of plasmids with almost identical backbones (>99% query coverage and >99% nucleotide identity). Their key backbone gene loci included repA for plasmid replication.

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**Figure 2** Comparison of pP10159-2 with pP378-IMP.

**Notes:** Shown are linear comparison of the two sequenced plasmids pP10159-2 and pP378-IMP (A), that of the CUP regions of these two plasmids (B), and that of the Tn6292-associated regions of these two plasmids (C). 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.
initiation, parA for plasmid partition, a toxin-antitoxin system relEB for post-segregational killing and a P-type IV secretion system for plasmid conjugal transfer (Figures S1C and 3A). Their RepA proteins belonging to the Rep_3 superfamily (pfam10134) and matched the RepA proteins from Klebsiella pneumoniae and Pantoea stewartii with >96% query coverage and >99% amino acid identity; all these RepA proteins could not be assigned into any known incompatibility groups.

The insertion of the core blaKPC platform (ie, the Tn6376 to ΔrepB region) into the cryptic Tn3 family- plasmid Tn1722, truncating and splitting it into ΔTn1722-5′ and ΔTn1722-3′, generated Tn6296 (Figure 3B) as initially observed in pKP048.20 Each of pP10159-3, pHS062105-3 and pECN49-KPC carried a single accessory region ΔTn6296, which carried the blaKPC gene serving as the sole resistance determinant of these plasmids (Figure 3B). The three ΔTn6296 elements of pP10159-3, pHS062105-3, and pECN49-KPC had undergone deletions and insertions relative to the prototype Tn6296: 1) ΔTn1722-3′ was lost from all the three ΔTn6296 elements; 2) a 123-bp deletion at the 3′-terminal region of Tn6376 was found in pP10159-3; and 3) a 1,754-bp deletion within ΔTn1722-5′ as well as a 73-bp insertion within the variable number tandem repeat (VNTR) region of orf396 was identified in pECN49-KPC. Notably, the above variations within ΔTn6296 accounted for the only modular difference between pHS062105-3, pP10159-3, and pECN49-KPC.

Comparison of pP10159-4 with pNDM-CIT

The pP10159-4 backbone was highly similar (98% query coverage and 98% nucleotide identity) to the reference IncHI4 plasmid pNDM-CIT.19,22 pP10159-4 and pNDM-CIT shared the core IncHI4 backbone gene loci,19 including repHI4A and repHI4B for replication initiation, parAB and parMR for partition, and the tra1 and tra2 regions for conjugal transfer. There were two major modular differences between these two backbones (Figure S2): 1) the deletion of an 11-gene region downstream of ISKpn26; containing the arsenic resistance (ars) locus and a 4-gene region (downstream of ISKpn34) from pP10159-4 relative to pNDM-CIT, and 2) the deletion of a distinct 4-gene region (downstream of ISLad2) from pNDM-CIT compared with pP10159-4. These large-fragment deletions might result from the insertion of the corresponding IS elements ISKpn26, ISKpn34 and ISLad2. Of the accessory regions of pP10159-4, only the 25.3-kb MDR region and the 2.9-kb blaCTX-M-3 region contained the resistance genes (Tables 1 and 2).

The MDR region (Figure 4) of pP10159-4 was organized as IS1R, a 14.1-kb 11-gene region encoding unknown functions, ΔIS1R, ISKpn19, ΔISPa38, orf402 (hypothetical protein), orf291 (DNA polymerase), ΔTn6308 and ISKpn19. The unit transposon Tn6308 belonged to the Tn21 subgroup of the Tn3 family and was initially identified in plasmid pP10164-3.22 Tn6308 (Figure 4) had a hybrid backbone, which was composed of the core transposition module trpAR-res of Tn1696 and the mercury resistance (mer) region of Tn21 and bordered by an intact 38-bp IRL and an IS5075-disrupted IRR (inverted repeat right); the class 1 integron In37 was inserted into the res site of Tn6308, truncating it from an original 120-bp fragment into an 83-bp remnant. Compared with the prototype class 1 integron, In37 in Tn6308 had a very complex mosaic structure and had undergone two major events: 1) an IS26-flanked composite transposon Tn6309, carrying the class C tetracycline resistance module tetA(C)-tetR(C), was inserted into intI1; and 2) an 8.1-kb region (8KR) was inserted at a site between 3′-conserved segment (3′-CS), and the Tn402 mini module, leading to truncation of 3′-CS and mini. 8KR included the chromate-resistance unit IRL₁r, chrA-orf98 and the macrolide-resistance unit IS26-mph(A)-mph(B)-IS6100;21 IRL₁r was further disrupted by IS5075 in 8KR. Tn6309, a 217-bp region containing attI₁, and a 15.5-kb region (8KR-Δn1- Δn1-mer-IS5075-IRR₃₆₃₈₉₆) were not found in ΔTn6308 of pP10159-4 relative to Tn6308.

ISEcp1 was able to capture and arrange blaCTX-M-3, Δorf477 at its downstream end while it moved,24 while the blaCTX-M-3 region of pP10159-4 is a close derivative of this ISEcp1-base unit with truncation at its 3′-end region.

Comparison of pP10159-5 with pR55

pP10159-5 shared 94% of its backbone with the reference type 2 IncC plasmid pR55,25 with 99% nucleotide identity. All the type 2 IncC signature sequences, including two small insertions i₁ and i₂ as well as two genes orf1847 and rhs2,26 were found in pR55 and pP10159-5, with the only exception that a 246-bp deletion occurred at the 3′-terminus of rhs2 in pP10159-5 (Figure S1E). pP10159-5 and pR55 possessed the core IncC backbone gene loci including repA for replication initiation, parAB and parM for partition, and the tra1 and tra2 regions for conjugal transfer (Figure S1E).

Linear sequence comparison of pP10159-5 and pR55 revealed five different regions (DIERs), namely DIER-1 to DIER-5 (Figure S3). DIER-1 was located between orf564 and orf312, and manifested as the traE and orf225 region composed of a 3′-terminal Tn6292Δ remnant (designated ΔTn6292; harboring no resistance genes) and eight con-
Figure 3  Comparison of pP10159-3 with pH5062105-3 and pECN49-KPC.

Notes: Shown are linear comparison of the three sequenced plasmids pP10159-3, pH5062105-3 and pECN49-KPC (A), and that of Tn6296 and its three derivatives from these three plasmids (B). 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.
Five resistance plasmids coexisting in Citrobacter freundii

jugal transfer genes [found in another type 2 IncC plasmid pSRC119-A/C27 but not in pR55] in pP10159-5; however, DIER-1 was manifested as the orf546 to sul2 region carrying the floR (florfenicol/chloramphenicol resistance)-ISCR2 region and five putative plasmid maintenance genes in pR55.

DIER-2 existed as the orf861 to orf852 region (consisting of 34 putative plasmid maintenance genes) in pR55 and was deleted due to the insertion of ISEc52 at a site between traN and orf147 in pP10159-5; the ISEc52 insertion also resulted in the truncation of traN in pP10159-5. The loss of the orf861 to orf852 region, a part of the plasmid maintenance region as observed in pR55, did not destroy the plasmid maintenance of pP10159-5. A 58.2-kb MDR region (DIER-3) was inserted into rhs2 in pP10159-5 relative to pR55, leading to the truncation of rhs2 as well as the deletion of the downstream orf273-orf243 region. The Tn3-family unit transposon Tn6187 (DIER-4; 32.6 kb in length) was inserted into orf492 in pR55 relative to pP10159-5, splitting orf492 into two separate parts and meanwhile leaving 5-bp direct repeats (DRs; target site duplication signals of transposition) at both ends of Tn6187. The traF to orf186 region (DIER-5; containing not only conjugal transfer but plasmid maintenance genes) was located between uvrD and mobl in pR55, but replaced by a second copy of ISEc52 in pP10159-5; the ISEc52 insertion further led to the truncation of uvrD in pP10159-5. These five DIERs were associated with not only accessory modules but backbone regions, and the insertion and deletion events occurred within the two conjugal transfer regions tra1 and tra2 of pP10159-5 would cause this plasmid to be non-conjugative.

All the resistance genes of pP10159-5 were harbored in the MDR region (Figure 5), which was sequentially organized as IS1R, a 3.6-kb 7-gene region of unknown functions, a 9.6-kb blaSFO-1 (β-lactam resistance) region as observed in pHD0149-2,28 the chrA- and mph(A)-carrying 8KR fragment as found in In37 of Tn6308,22 a 15-kb aacC2 (aminoglycoside resistance)-tmrB (tunicamycin resistance) region that was generated from the insertion of a 7-kb phage-related region at a site between ISGf1 and blaTEM-1 (β-lactamase resistance) of the original aacC2-tmrB region as identified in pEl1573,29 a 7.4-kb Tn21 remnant that was the 3′-terminal IRR to ΔmiA region (containing the mer locus) of Tn2130 with the disruption of IRR by IS4321R and a Tn1548-associated region.

Tn1548 was a IS26-flanked composite transposon lacking DRs at its ends, and had an IS26-In27-ISCRI-IASEc28-armA (aminoglycoside resistance)-ISEc29-msr(E)-mph(E) (macrolide resistance)-orf543-repAcIn-IIS26 structure. There were a number of Tn1548-associated elements with the replacement of In27 by different class 1 integrons,31 eg, In0 carrying no gene cassette array in pP10159-5 (Figure 5).

Remarkably, pP10159-5/pR55 belonged to the same incompatibility group and were genetically very closely related with respect to their plasmid backbones, but they carried totally different profiles of accessory modules that were inserted at different sites of the plasmid backbones; a similar observation was also found for pP10159-4/pNDM-CIT.
C. freundii isolates are resistant to cephalosporins due to inducible expression of chromosomally encoded AmpC β-lactamase, but in general still remain susceptible to carbapenems. C. freundii can persist in the hosts for long periods, which likely facilitates the acquisition and accumulation of various resistance determinants under high selective pressure within the hospital environments. Carbapenem-resistant C. freundii isolates expressing plasmid-encoding carbapenemases, such as KPC, NDM, and VIM, have emerged during the past decade and, moreover, the coproduction of two or more carbapenemases, eg, KPC-2 + NDM-1 and NDM-1 + VIM-4 + OXA-181, have also been identified in C. freundii, making the

Discussion

C. freundii isolates are resistant to cephalosporins due to inducible expression of chromosomally encoded AmpC β-lactamase, but in general still remain susceptible to carbapenems. C. freundii can persist in the hosts for long periods, which likely facilitates the acquisition and accumulation of various resistance determinants under high selective pressure within the hospital environments. Carbapenem-resistant C. freundii isolates expressing plasmid-encoding carbapenemases, such as KPC, NDM, and VIM, have emerged during the past decade and, moreover, the coproduction of two or more carbapenemases, eg, KPC-2 + NDM-1 and NDM-1 + VIM-4 + OXA-181, have also been identified in C. freundii, making the
corresponding isolates highly resistant to β-lactams including carbapenems.

Coexistence of two to four different resistance plasmids has been observed in *C. freundii*. We recently determined the complete nucleotide sequences of four plasmids p112298-KPC (belonging to an unknown incompatibility group; accession number KP987215), p112298-NDM (IncX3 type; KP987216), p112298-catA (IncHI2; KY270851) and p112298-tetA (type 1 IncC) coexisting in a clinical *C. freundii* isolate 112,298. In this follow-up study, high-throughput genomic sequencing disclosed the co-occurrence of five resistance plasmids pP10159-1, pP10159-2, pP10159-3, pP10159-4 and pP10159-5 in a clinical *C. freundii* isolate P10159. This is the first report of co-occurrence of five resistance plasmids of different incompatibility groups, with determination of their complete sequences, coexisting in a bacterial isolate.

There is the increasing prevalence of MDR *C. freundii* strains, which resulted from co-selection of genes encoding resistance to multiple antimicrobial classes, thereby leaving few or no options of antimicrobial treatment. In this study, the five plasmids from *C. freundii* P10159 contain a total of 24 different genes or gene loci involved in the resistance to β-lactams including carbapenems, aminoglycosides, quinolones, macrolides, phenicols, rifampicin, sulfonamides, tunicamycin, bleomycin, quaternary ammonium compounds, chromate, mercury and tellurium, which are associated with several mobile elements including insertion sequences, integrons and transposons. Notably, the existence of redundant resistance genes made the P10159 strain extremely highly resistant to the corresponding classes of antibiotics, including carbapenems (*bla*NDM-1, *bla*IMP-4 and *bla*KPC-2), aminoglycosides (*armA* and *aacA4cr*), quinolones (*qnrS1* and *aac4acr*), macrolides [*mph(A)* and *mph(E)*] and sulfonamides (two copies of *sul1*).

The co-selection and maintenance of multiple plasmids and an extremely large number of resistance genes in a single bacterial isolate reflect active and complex horizontal genetic transfer events that have taken place under selective pressures associated with many kinds of antibiotic molecules or toxic compounds.

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**Disclosure**

The authors report no conflicts of interest in this work.

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Supplementary material

Figure S1 Plasmid schematic maps.

Notes: The nine plasmids p10159-1 and pNDM-HN380 (A), p10159-2 and p378-IMP (B), p10159-3, pH5062105-3 and pECN49-KPC (C), p10159-4 and pNDM-CIT (D), and p10159-5 and pASS (E) are included in the comparative analysis. Genes are denoted by arrows, and the backbone and accessory module regions are highlighted in black and color, respectively. The innermost circle presents GC-skew ([G-C]/[G + C]), with a window size of 500 bp and a step size of 20 bp. The next-to-innermost circle presents GC content.
Table S1 Results of modified CARBA-NP test

| Bacterial strain | Substrate | I  | II  | III | IV  | V  | Detecting carbapenemase activity (Ambler class) |
|------------------|-----------|----|-----|-----|-----|----|-----------------------------------------------|
| P10159           |           | Red| Yellow| Yellow| Yellow| Red | A + B                                        |
| NDM-EC600        |           | Red| Yellow| Yellow| Red  | Red | B                                            |
| NDM-TOP10        |           | Red| Yellow| Yellow| Red  | Red | B                                            |
| IMP-EC600        |           | Red| Yellow| Yellow| Red  | Red | B                                            |
| IMP-TOP10        |           | Red| Yellow| Yellow| Red  | Red | B                                            |
| KPC-EC600        |           | Red| Yellow| Red   | Yellow| Red | A                                            |
| KPC-TOP10        |           | Red| Yellow| Red   | Yellow| Red | A                                            |
| TOP10            |           | Red| Red   | Red   | Red  | Red | –                                            |
| EC600            |           | Red| Red   | Red   | Red  | Red | –                                            |

Notes: 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).