

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

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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*_{NDM-1} and pP10159-2 harboring *bla*_{IMP-4} plus *qnrS1* were almost identical to IncX3 plasmid pNDM-HN380 and IncN1 plasmid pP378-IMP, respectively. The *bla*_{KPC-2}-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 IncHI4 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

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.

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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 mid-stream 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 μ L of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm² hydrophilic nylon membrane filter with a 0.45 μ m 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 μ g/mL rifampin together with indicated additional antibiotics for selecting an *E. coli* transconjugant carrying one of the following resistance markers: 4 μ g/mL meropenem for *bla*_{NDM-1} (pP10159-1), *bla*_{IMP-4} (pP10159-2) and *bla*_{KPC-2} (pP10159-3); 10 μ g/mL chloramphenicol for *catB3* (pP10159-4); and 10 μ g/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 (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 plasmid DNA, which was isolated from the P10159 strain using a Qiagen Plasmid Midi Kit, was mixed with 100 μ L of competent cells for electroporation at 25 μ F, 200 Ω and 2.5 Kv. The resulting cells were suspended in 500 μ L 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*_{NDM-1}, *bla*_{IMP-4}, *bla*_{KPC-2}, *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 OD₆₀₀ of 1.0 to 1.4. If required, ampicillin was used at 200 μ g/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 μ L of 20 mM Tris-HCl (pH 7.8) and lysed by sonication, followed by centrifugation at 10,000 \times g at 4°C for 5 minutes. Fifty microliters of the supernatant (the enzymatic bacterial suspension) were mixed with 50 μ L 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/ μ L imipenem. Substrate III: 0.054% phenol red plus 0.1 mM

ZnSO₄ (pH7.8), 0.6 mg/μL mg imipenem and 0.8 mg/μL tazobactam. Substrate IV: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH7.8), 0.6 mg/μL mg imipenem and 3 mM EDTA (pH7.8). Substrate V: 0.054% phenol red plus 0.1 mM ZnSO₄ (pH7.8), 0.6 mg/μL mg imipenem, 0.8 mg/μL tazobactam and 3 mM EDTA (pH7.8).

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

Nucleotide sequence accession numbers

The pP10159-1, pP10159-2, pP10159-3, pP10159-4 and pP10159-5 sequences were submitted to GenBank under accession numbers MF072961 to MF072965, respectively.

Results

C. freundii co-harboring five resistance plasmids

P10159 belonged to a novel ST252, with an allelic profile 108–62–71–7–1–1–57 corresponding to the seven house-keeping genes *aspC*, *clpX*, *fadD*, *mdh*, *arcA*, *dnaG*, and *lysP*. High-throughput sequencing with the genomic DNA of the P10159 strain revealed the circularly closed sequences of the five plasmids pP10159-1, pP10159-2, pP10159-3, pP10159-4 and pP10159-5, 42.8 kb to 228.7 kb in length with 53 to 270 predicted open reading frames (ORFs) (Figure S1 and Table 1). The modular structure of each plasmid was divided into the backbone regions and separate accessory modules, which were defined as acquired DNA regions associated with and bordered by mobile elements (Figure S1 and Table 1). Some of these accessory modules harbored drug resistance genes (Table 2).

pP10159-1, 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.

P10159 and all the transconjugants and transformants were resistant to ampicillin, ceftazidime, meropenem and ceftoxitin (Table 3). P10159 had class A + B carbapenemase activity, while the transconjugants and transformants harboring pP10159-1 or pP10159-2 had B activity and those harboring pP10159-3 had A activity (Table S1). These resistance phenotypes were consistent with the production

Table 1 Major features of plasmids analyzed

| Category | Plasmid | | | | | | | | | | |
|-----------------------------|--|------------|---|-----------|----------------------|-------------|--|--|----------|---|---|
| | pP10159-1 | pNDM-HN380 | pP10159-2 | pP378-IMP | pP10159-3 | pHS062105-3 | pECN49-KPC | pP10159-4 | pNDM-CIT | pP10159-5 | pR55 |
| Incompatibility group | IncX3 | | IncN1 | | Unknown | | IncH14 | | | Type 2 IncC | |
| Total length (bp) | 54,034 | 54,035 | 51,104 | 51,207 | 42,763 | 42,848 | 41,317 | 228,709 | 288,920 | 152,132 | 170,810 |
| Total number of ORFs | 62 | 62 | 62 | 64 | 53 | 53 | 52 | 270 | 314 | 190 | 203 |
| Mean G + C content, % | 49.0 | 49.0 | 50.4 | 50.5 | 49.8 | 49.7 | 49.1 | 47.0 | 47.7 | 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 | 208,301 | 89,438 | 129,510 |
| Accessory modules | The <i>bla</i> _{NDM-1} region [#] and ISKox3 | | Inc823b [#] , ΔTn6292 [#] and the IS1 remnant | | ΔTn6296 [#] | | The MDR region [#] , the <i>bla</i> _{CTX-M-3} region [#] , ISLad2, ISKpn26-IS1R, ISCFr4-ΔISCFr9, IS903B and ISCFr6:ISEc21 | The MDR region [#] , ΦCP4-6157 [#] , ISKpn26, ΔTn6292-3' and two separate copies of ISEc52 | | The MDR region [#] and Tn6187 [#] | The <i>bla</i> _{R-1SCR2} region [#] and Tn6187 [#] |

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 | <i>bla</i> _{SHV-12} | Beta-lactam resistance | 9,324–10,184 | The <i>bla</i> _{NDM-1} region |
| | <i>ble</i> | Bleomycin resistance | 17,458–17,823 | |
| pPI0159-2 | <i>bla</i> _{NDM-1} | Beta-lactam resistance | 17,827–18,639 | In823b ΔTn6292 |
| | <i>bla</i> _{IMP-4} | Beta-lactam resistance | 4,295–5,035 | |
| | <i>qnrS1</i> | Quinolone resistance | 27,662–28,318 | |
| pPI0159-3 | <i>bla</i> _{KPC-2} | Beta-lactam resistance | 7,737–8,618 | ΔTn6296 |
| pPI0159-4 | <i>aacA4cr</i> | Quinolone resistance | 85,056–85,655 | The MDR region |
| | <i>bla</i> _{OXA-1} | Beta-lactam resistance | 85,786–86,616 | |
| | <i>catB3</i> | Phenicol resistance | 86,754–87,386 | |
| | <i>arr3</i> | Rifampicin resistance | 87,471–87,923 | |
| | <i>qacEΔ1</i> | Quaternary ammonium compound resistance | 88,146–88,493 | |
| | <i>sulI</i> | Sulfonamide resistance | 88,487–89,326 | |
| | <i>bla</i> _{CTX-M-3} | Beta-lactam resistance | 114,790–115,665 | |
| | The <i>ter</i> locus | Tellurium resistance | 116,056–136,305 | |
| | <i>bla</i> _{SFO-1} | Beta-lactam resistance | 87,807–88,694 | |
| | <i>chrA</i> | Chromate resistance | 98,473–99,678 | |
| pPI0159-5 | <i>mph(A)</i> | Macrolide resistance | 103,297–104,202 | The plasmid backbone The MDR region |
| | <i>aacC2</i> | Aminoglycoside resistance | 105,261–106,121 | |
| | <i>tmrB</i> | Tunicamycin resistance | 106,134–106,676 | |
| | <i>bla</i> _{TEM-1} | Beta-lactam resistance | 118,230–119,090 | |
| | The <i>mer</i> locus | Mercuric resistance | 120,698–124,660 | |
| | <i>qacEΔ1</i> | Quaternary ammonium compound resistance | 128,336–128,683 | |
| | <i>sulI</i> | Sulfonamide resistance | 128,677–129,516 | |
| | <i>armA</i> | Aminoglycoside resistance | 132,848–133,621 | |
| | <i>msr(E)</i> | Macrolide resistance | 135,920–137,395 | |
| | <i>mph(E)</i> | Macrolide resistance | 137,451–138,335 | |

Abbreviation: MDR, multi-drug resistant.

Table 3 Antimicrobial drug susceptibility profiles

| Antibiotics | MIC (mg/L)/antimicrobial susceptibility | | | | | | | | |
|------------------|---|-----------|-----------|-----------|-----------|-----------|-----------|---------|---------|
| | PI0159 | NDM-EC600 | NDM-TOPI0 | IMP-EC600 | IMP-TOPI0 | KPC-EC600 | KPC-TOPI0 | 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 |
| Amikacin | >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 | 8/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 | 19/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 | 4/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 |

Abbreviations: MIC, minimum inhibitory concentration; S, sensitive; R, resistant; I, intermediately 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 *bla*_{NDM-1}-carrying IncX3 plasmids, including the first fully sequenced pNDM-HN380¹⁴ (Figure 1A). pP10159-1/pNDM-HN380 contained three resistance genes *bla*_{NDM-1}, *ble* and *bla*_{SHV-12}, which were all located in the *bla*_{NDM-1} regions (Figure 1B). The *bla*_{NDM-1} regions of pP10159-1/pNDM-HN380 were organized in order of a 4.1-kb truncated version of the composite transposon-like IS26-*bla*_{SHV-12}-IS26 unit,¹⁵ a *bla*_{NDM-1}-containing Δ Tn125 element derived from the IS*Aba125*-flanked composite transposon Tn125,¹⁶ IS3000 and a 583-bp Tn3 remnant (Figure 1B). An inversion of the truncated IS26-*bla*_{SHV-12}-IS26 unit in the *bla*_{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*_{IMP}-carrying IncN1 plasmids, including pIMP-HZ1¹⁷ and pP378-IMP¹⁸ (Figure 2A). Although pIMP-HZ1 was the first fully sequenced *bla*_{IMP}-carrying IncN1 plasmid, pP378-IMP¹⁸ was more appropriate as the reference for genomic comparison because it contained two relatively complete mobile elements, namely a class 1 integron In823 and a truncated Tn3-family unit transposon Δ Tn6292,¹⁹ harboring *bla*_{IMP-4} and *qnrS1*, respectively. pP10159-2 and pP378-IMP shared the same In823 and also two resistance genes *bla*_{IMP-4} and *qnrS1*.

There were two major modular differences between pP10159-2 and pP378-IMP. First, the CUP (conserved upstream repeat)-controlled regulon¹⁸ was composed of seven sequentially arranged operons CUPA, CUPB, CUP5, CUP4, CUP3, CUP2 and CUP1 in the backbone of pP10159-2, while the translocation of the CUP2 operon occurred in pP378-IMP (Figure 2B).

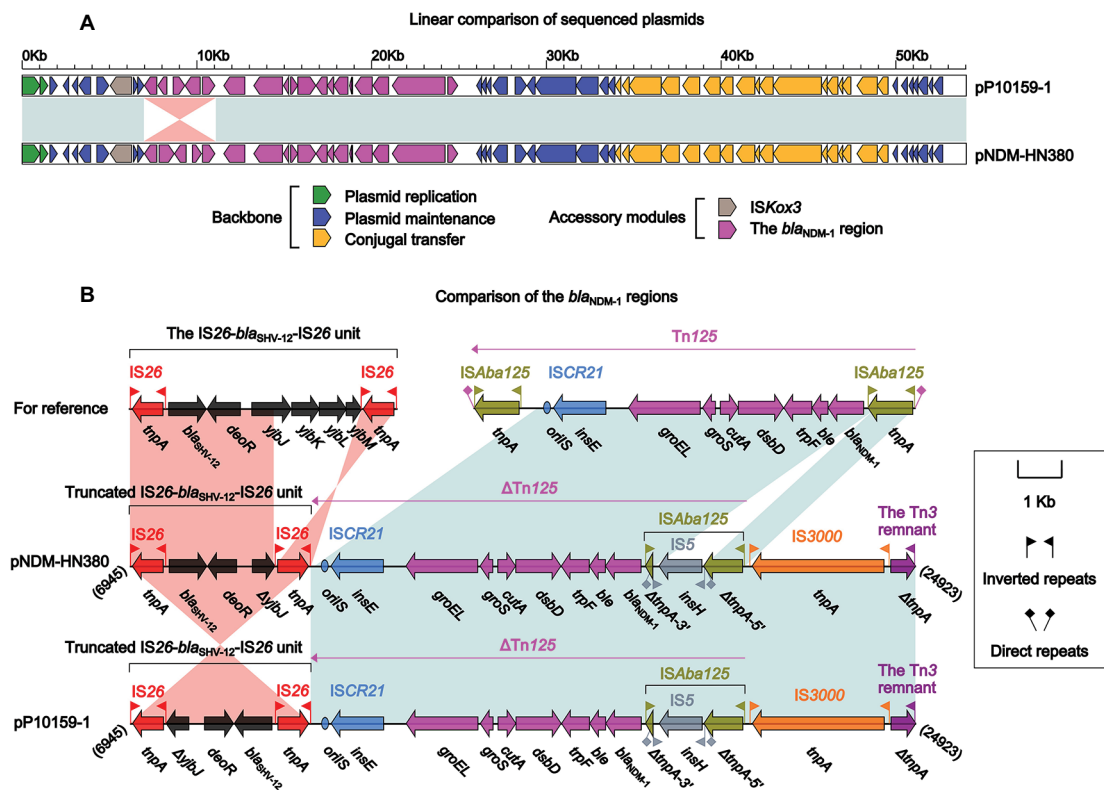


Figure 1 Comparison of pP10159-1 with pNDM-HN380.

Notes: Shown are linear comparison of the two sequenced plasmids pP10159-1 and pNDM-HN380 (A), and that of the *bla*_{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.

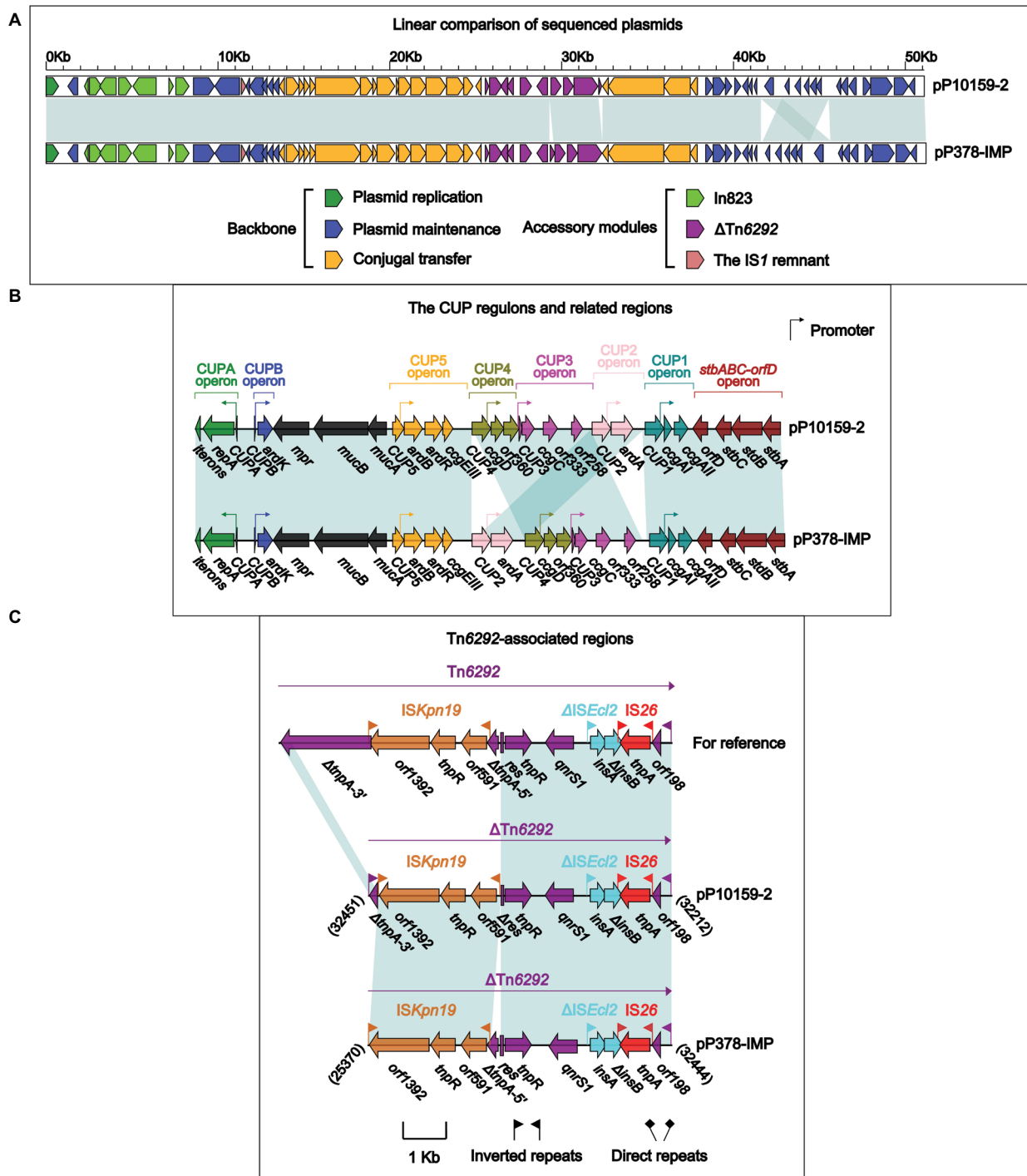


Figure 2 Comparison of pP10159-2 with pP378-IMP. **Notes:** Shown are linear comparison of the two sequenced plasmids pP10159-2 and pP378-IMP (A), that of the CUP regions of these two plasmids (B), and that of the Tn6292-associated regions of these two plasmids (C). Genes are denoted by arrows. Genes, 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.

Second, the core transposition module *tnpA* (transposase)-*res* (resolution site)-*tnpR* (resolvase) of Tn6292 was interrupted by *ISKpn19*, breaking *tnpA* into separate Δ *tnpA*-3' and Δ *tnpA*-5',¹⁸ whereas distinct additional deletion events occurred within the *tnpA*-*res*-*tnpR* modules of the Δ Tn6292 elements from P10159-2 and pP378-IMP (Figure 2C).

Comparison of pP10159-3 with pHS062105-3 and pECN49-KPC

pP10159-3, 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

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 *bla*_{KPC} platform (ie, the Tn6376 to *ΔrepB* region) into the cryptic Tn3-family transposon Tn1722, truncating and splitting it into Δ Tn1722-5' and Δ Tn1722-3', generated Tn6296 (Figure 3B) as initially observed in pKP048.²⁰ Each of pP10159-3, pHS062105-3 and pECN49-KPC carried a single accessory region Δ Tn6296, which carried the *bla*_{KPC-2} gene serving as the sole resistance determinant of these plasmids (Figure 3B). The three Δ Tn6296 elements of pP10159-3, pHS062105-3 and pECN49-KPC had undergone deletions and insertions relative to the prototype Tn6296: 1) Δ Tn1722-3' was lost from all the three Δ Tn6296 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 Δ Tn1722-5' 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 Δ Tn6296 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.^{19,21} 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 *bla*_{CTX-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, Δ IS1R, *ISKpn19*, Δ ISPa38, *orf402* (hypothetical protein), *orf291* (DNA polymerase), Δ Tn6308 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 *tnpAR-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 *tmi* module, leading to truncation of 3'-CS and *tmi*. 8KR included the chromate-resistance unit IRL_{chrA}-*chrA-orf98* and the macrolide-resistance unit IS26-*mph(A)-mrx-mphR(A)-IS6100*;²³ IRL_{chrA} was further disrupted by IS5075 in 8KR. Tn6309, a 217-bp region containing *attI1*, and a 15.5-kb region (8KR- Δ *tmiA-mer:IS5075-IRR*_{Tn6308}) were not found in Δ Tn6308 of pP10159-4 relative to Tn6308.

ISEcp1 was able to capture and arrange *bla*_{CTX-M-3}- Δ *orf477* at its downstream end while it moved,²⁴ while the *bla*_{CTX-M-3} region of pP10159-4 was 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 Tn6292¹⁸ remnant (designated Δ Tn6292; harboring no resistance genes) and eight con-

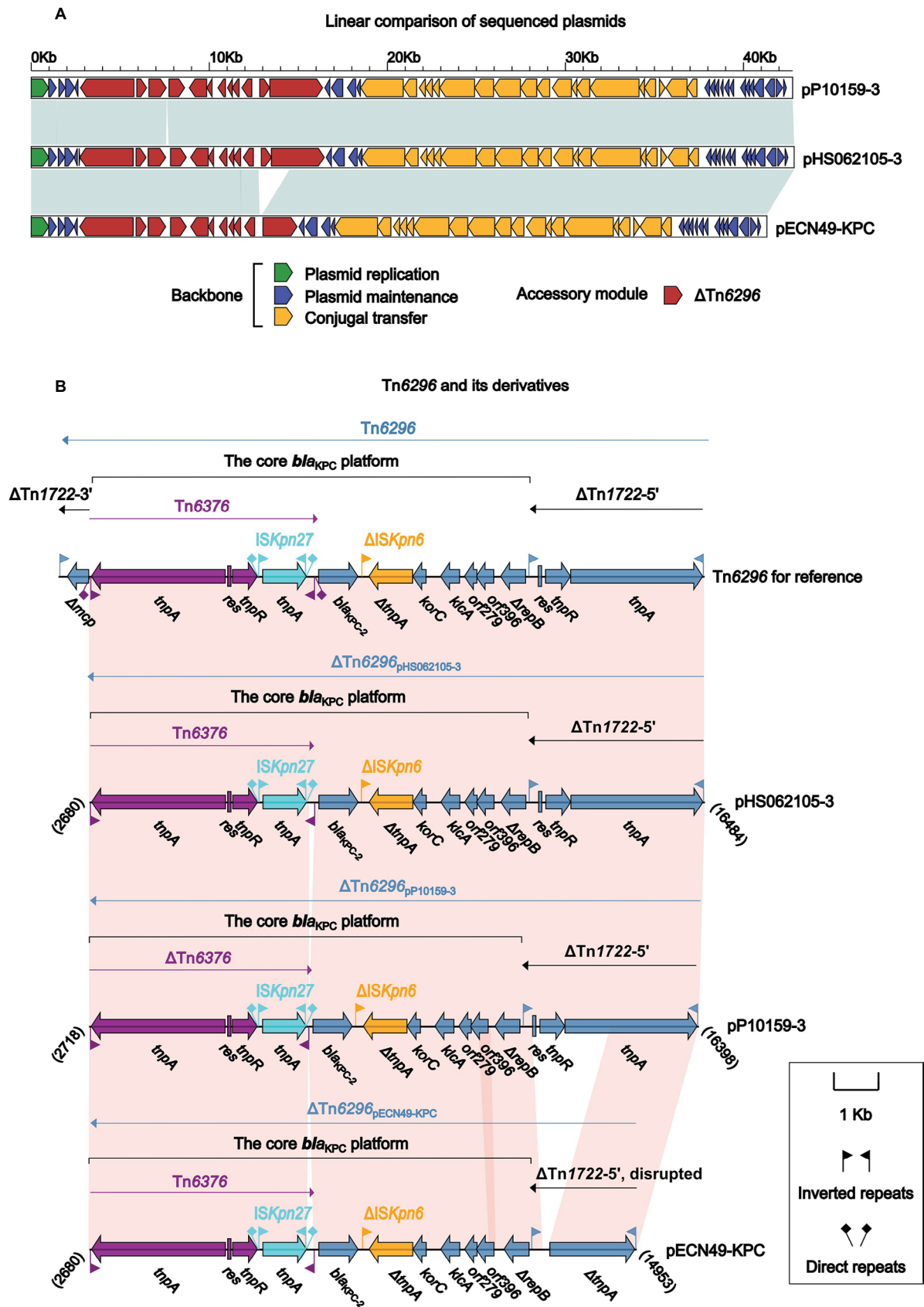


Figure 3 Comparison of pP10159-3 with pHS062105-3 and pECN49-KPC. **Notes:** Shown are linear comparison of the three sequenced plasmids pP10159-3, pHS062105-3 and pECN49-KPC (A), and that of Tn6296 and its three derivatives from these three plasmids (B). Genes are denoted by arrows. Genes, mobile elements 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.

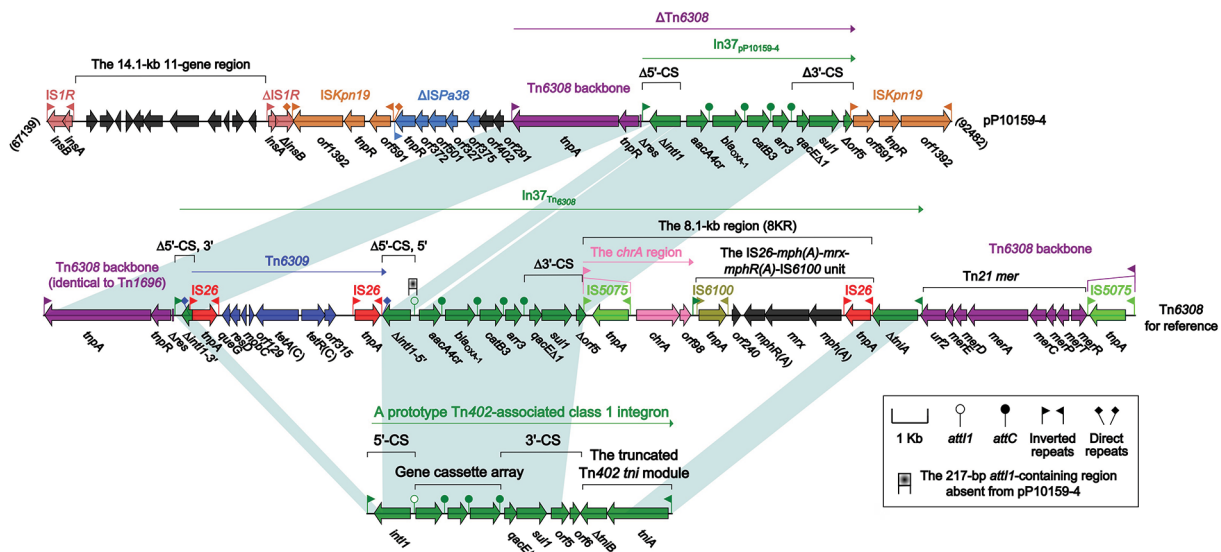


Figure 4 The MDR region from pP10159-4 and comparison with related regions.

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). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids.

Abbreviation: MDR, multi-drug resistant.

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 *mobI* 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 *bla*_{SFO-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 *ISCFr1* and *bla*_{TEM-1} (β -lactamase resistance) of the original *aacC2*-*tmrB* region as identified in pE11573,²⁹ a 7.4-kb Tn21 remnant that was the 3'-terminal IRR to Δ *tmiA* region (containing the *mer* locus) of Tn21³⁰ with the disruption of IRR by *IS4321R* and a Tn1548-associated region.

Tn1548 was an IS26-flanked composite transposon lacking DRs at its ends, and had an IS26-In27-*ISCR1*- Δ *ISEc28*-*armaA* (aminoglycoside resistance)-*ISEc29*-*msr(E)*-*mph(E)* (macrolide resistance)-*orf543*-*repAciN*-IS26 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.

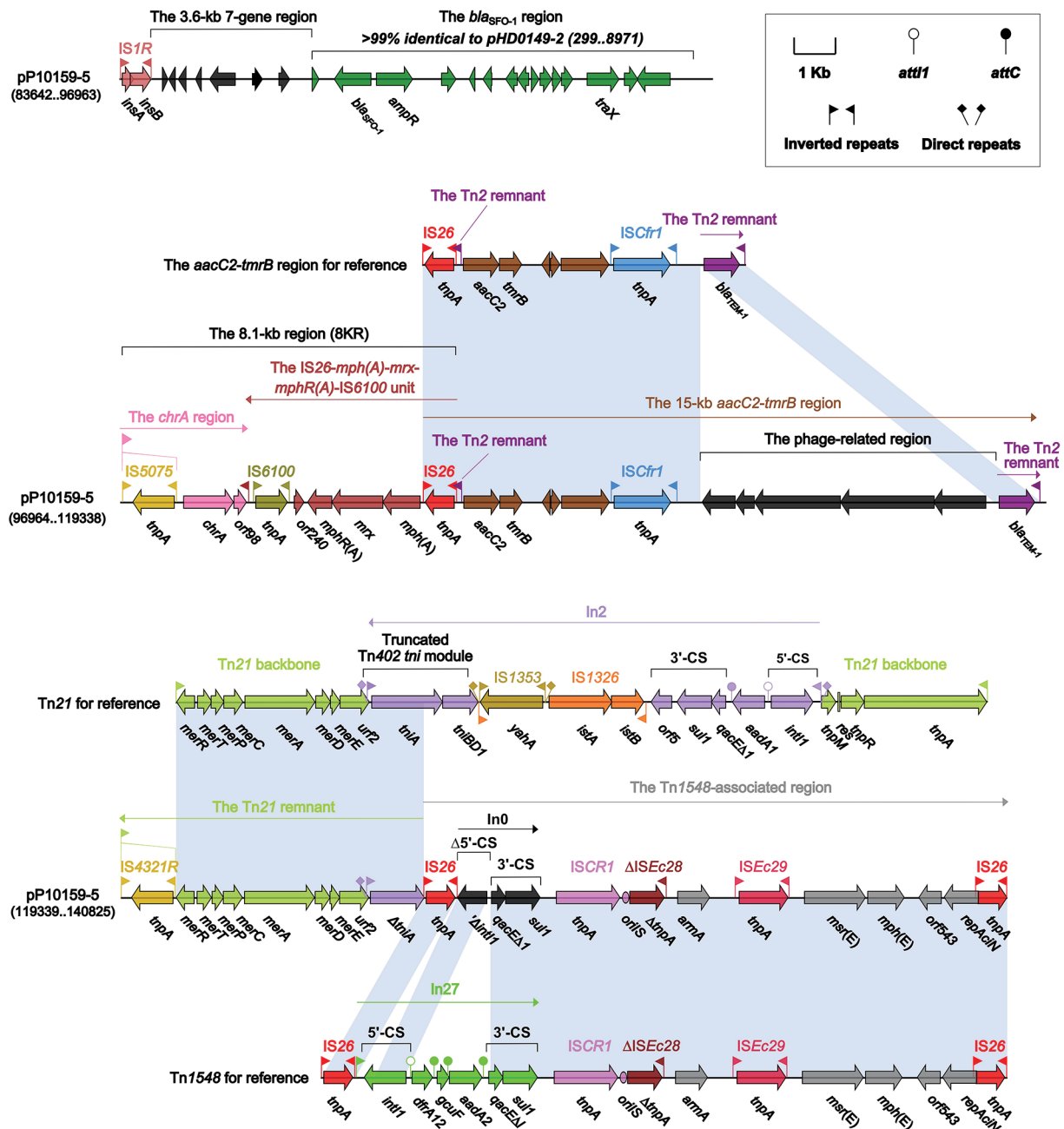


Figure 5 The MDR region from pP10159-5 and comparison with related regions. **Notes:** Genes are denoted by arrows. Genes, mobile elements and other features are colored based on function classification. Shading denotes regions of homology (>95% nucleotide identity). Numbers in brackets indicate the nucleotide positions within the corresponding plasmids. **Abbreviation:** MDR, multi-drug resistant.

Discussion

C. freundii isolates are resistant to cephalosporins due to inducible expression of chromosomally encoded AmpC β-lactamase,³² 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,³³ NDM,³⁴ and VIM,³⁵ have emerged during the past decade and, moreover, the coproduction of two or more carbapenemases, eg, KPC-2 + NDM-1³⁶ and NDM-1 + VIM-4 + OXA-181,³⁷ 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*.^{36,38,39} We recently determined the complete nucleotide sequences of four plasmids p112298-KPC (belonging to an unknown incompatibility group; accession number KP987215),³⁶ p112298-NDM (IncX3 type; KP987216),³⁶ p112298-catA (IncHI2; KY270851) and p112298-tetA (type 1 IncC)⁴⁰ 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.^{36,38,39} 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 *aacC2*), quinolones (*qnrS1* and *aacA4cr*), macrolides [*mph*(A) and *mph*(E)] and sulfonamides (two copies of *sulI*).

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.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

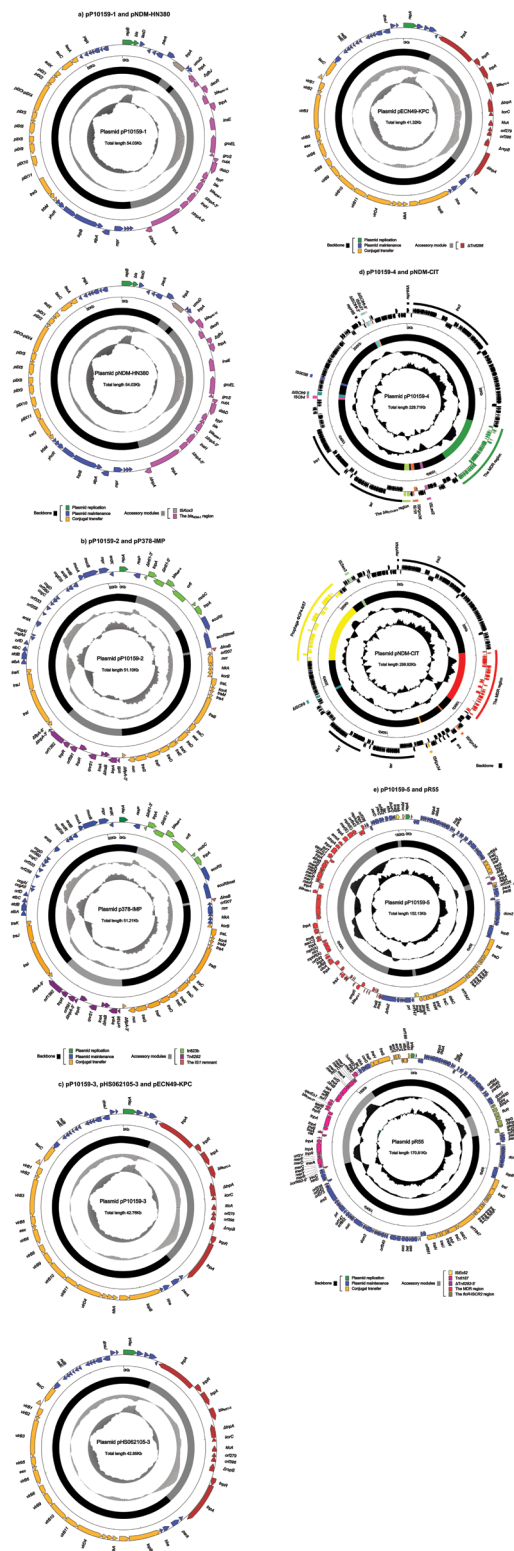


Figure S1 Plasmid schematic maps.

Notes: The nine plasmids pP10159-1 and pNDM-HN380 (A), pP10159-2 and pP378-IMP (B), pP10159-3, pHS062105-3 and pECN49-KPC (C), pP10159-4 and pNDM-CIT (D), and pP10159-5 and pR55 (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.

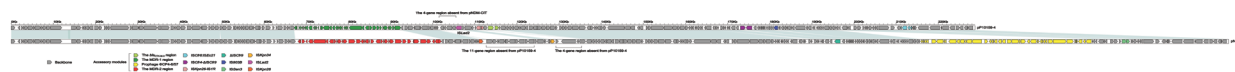


Figure S2 Linear comparison of pPI0159-4 and pNDM-CIT sequences.

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).

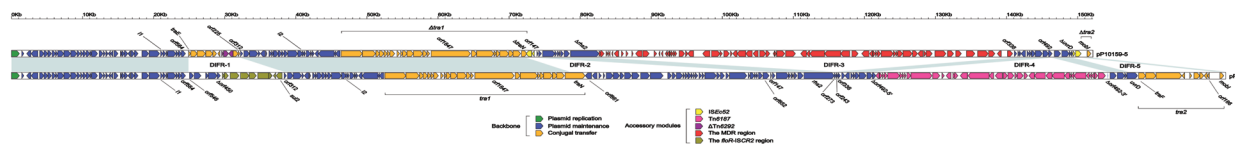


Figure S3 Linear comparison of pPI0159-5 and pR55 sequences.

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).

Table S1 Results of modified CARBA-NP test

| Bacterial strain | Substrate | | | | | Detecting carbapenemase activity (Ambler class) |
|------------------|-----------|--------|--------|--------|-----|---|
| | I | II | III | IV | V | |
| PI0159 | 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 | – |

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