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CASE REPORT

IncN1 ST7 Epidemic Plasmid Carrying blaIMP-4 in One ST85-Type *Klebsiella oxytoca* Clinical Isolate with Porin Deficiency

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Purpose: *Klebsiella oxytoca* is an opportunistic pathogen causing nosocomial infections. This study was designed to characterize the genomic features of a carbapenem-resistant *K. oxytoca* strain and analyze its molecular characteristics.

Materials and Methods: The strain wzx-IMP was isolated from the blood of a 2-year-old girl diagnosed with acute myeloid leukemia-M7. Species identification was performed, and the minimal inhibitory concentration of the strain was measured. Multilocus sequence typing was performed to identify the subtypes of *K. oxytoca*. The transfer capacity of the blaIMP-4-harboring plasmid was investigated by conjugation experiments, and the genome characteristics of the strain were examined using whole-genome sequencing.

Results: wzx-IMP belongs to the ST85 type and is resistant to imipenem and meropenem, which harbored the blaIMP-4 gene. The blaIMP-4 gene was located in an IS26-associated class 1 integron of pwzx_IMP, which contains conserved IncN1-type backbone regions with a replication gene and its accessory structure for plasmid replication. The blaIMP-4-carrying plasmid in wzx-IMP was successfully transferred to *Escherichia coli* EC600 by conjugation. Whole-genome sequencing showed that the wzx-IMP isolate included the blaOXY-1-1 gene, accompanied by OmpK36 absence.

Conclusion: We report an ST85-type carbapenem-resistant *K. oxytoca* strain, which produces blaIMP-4 located in an IncN1-type plasmid and accompanied by OmpK36 porin deficiency.

Keywords: *Klebsiella oxytoca*, blaIMP-4, ST85, IncN1, OmpK36

Introduction

Antimicrobial resistance is a global issue associated with an increased and often unrestricted antibiotic use in clinical settings, which leads to the dissemination of carbapenem-resistant Enterobacterales (CRE) in healthcare facilities (World Health Organization, 2017).[1] CRE constitutes a large group of bacteria with different mechanisms for drug resistance. Among them, carbapenem-resistant *Klebsiella pneumoniae* accounts for approximately 60%, followed by *Escherichia coli* and *Enterobacter cloacae*, but data on carbapenem-resistant *K. oxytoca* are limited.[1–5] Carbapenemases comprise three of the four Ambler classes as follows: Class A (eg, *K. pneumoniae* carbapenemases and some variants of Guiana extended-spectrum β-lactamases), class B (eg, metallo-β-lactamases (MBLs), including New Delhi MBLs (NDMs), Verona integron-encoded MBLs, and imipenemase (IMP)), and class D (eg, OXA-48-like carbapenemases).[1] Acquired MBLs first appeared in *Pseudomonas*

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aeruginosa in the 1980/1990s; soon after, these MBLs spread into *Enterobacteria*. Unlike NDMs, IMP-type β-lactamases are not often detected in CRE from China; one of the most commonly observed IMP variants is IMP-4, which was firstly detected in *Acinetobacter* spp. in Hong Kong in 2001. Since then, IMP-4-type carbapenemases have spread globally.[6–10] The *blaIMP-4* gene is often integrated into broad-host-range conjugative plasmids and carried on IncA/C- and IncN-type plasmids, which are transferred between different Gram-negative bacilli (eg, *Enterobacteriaceae, Acinetobacter* spp., and *Pseudomonas aeruginosa*).[11–13] The horizontal transfer of *blaIMP-4* in these plasmids is frequently associated with class 1 integrons. Plasmids belonging to the IncN incompatibility group are important mobile genetic platforms for disseminating clinically important resistance genes among enterobacterial species.[14–19] The IncN group can be further divided into three subgroups: IncN1, IncN2, and IncN3. These subgroups have similar backbone gene organization but with limited nucleotide sequence homology over the backbones. Plasmid-borne *blaIMP-4* has been sporadically reported in different Gram-negative bacilli in China. However, only a few studies have reported the complete sequence of *blaIMP-4*-harboring plasmids, limiting our understanding of the transmission mechanism of *blaIMP-4* between different Gram-negative bacilli.[13,20–22] In addition to producing carbapenemases, deficiency of outer membrane protein (OMP) combined with high-level AmpC cephalosporinase production also leads to *Enterobacteriaceae* resistance.[23] Still, not many reports on producing MBL in combination with the deficiency of OmpK36 porin in carbapenem-resistant Klebsiella oxytoca strain in China.[23]

In this study, we characterized the genomic features of an IMP-4-producing accompanied with deficiency of OmpK36 porin *K. oxytoca* wzx-IMP ST85 strain, a rare sequence type, and the *blaIMP-4* gene is carried with an IncN1-type plasmid, isolated from a girl with a bloodstream infection in China. To our knowledge, the *blaIMP-4*-carrying *K. oxytoca* ST85 strain identified in this study has not been reported previously.

Materials and Methods

Clinical Case, Bacterial Isolates, and Susceptibility Testing

The patient was a 2-year-old girl admitted to a cancer hospital in September 2019 who was diagnosed with acute myeloid leukemia-M7. The carbapenem-resistant *K. oxytoca* strain wzx-IMP was isolated from blood specimens on the next 4 days after hematopoietic stem cell transplantation. The patient received intravenous teicoplanin and meropenem, with voriconazole to prevent fungal infections empirically at first. When the child's condition was still getting worse, the antimicrobial drugs were switched to tigecycline and cefoperazone/sulbactam. The patient condition improved after antibiotic conversion. The antimicrobial susceptibility test results showed sensitivity to tetracycline, which was consistent with the improvement in the patient's symptoms. The patient was discharged 4 weeks after transplantation.

The species was identified using the Phoenix 100 Automated Microbiology System (Becton-Dickinson, New Jersey, USA), reidentified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with using a Microflex LT mass spectrometer (Bruker Daltonik), and analyzed using MALDI Biotyper (Bruker Corporation, Massachusetts, USA).

The minimal inhibitory concentrations (MICs) of the CRE strain were measured using the Phoenix 100 Automated Microbiology System and interpreted using the Clinical Laboratory Standards Institute criteria (CLSI, 2019), except for polymyxin, which was interpreted using the European Committee on Antimicrobial Susceptibility Testing criteria (EUCAST, 2019). Nineteen antibiotics belonging to 11 classes of antimicrobials were used for susceptibility tests in this study, including penicillins (ie, ampicillin), β-lactam/β-lactamase inhibitor complexes (ie, amoxicillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam), aminoglycosides (ie, gentamicin and amikacin), monocyclic β-lactams (ie, aztreonam), chloramphenicols (ie, chloramphenicol), cephalosporins (ie, cefazolin, cefotaxime, ceftazidime, and cefepime), carbapenems (ie, imipenem and meropenem), fluoroquinolones (ie, ciprofloxacin and levofloxacin), folate metabolic pathway inhibitors (ie, trimethoprim-sulfamethoxazole), tetracyclines (ie, tetracycline), and colistin.

Bacterial Genotyping

Multilocus sequence typing (MLST) for *K. oxytoca* was performed using previously described methods.[24] The polymerase chain reaction (PCR) products were purified and sequenced, and allelic profiles and sequence types were assigned using the *K. oxytoca* MLST website (http://pubmlst.org/koxytoca/).

Conjugation Experiment

The transfer capacity of the *blaIMP-4*-harboring plasmid was investigated by conjugation experiments, which were conducted using previously described methods.[25] Rifampin-resistant *E. coli* EC600 was used as the recipient, and the wzx-IMP strain was used as the donor. Transconjugants were selected on Mueller–Hinton (MH) agar supplemented with sodium rifampin (200 μg/mL) and meropenem (2 μg/mL) and identified by detecting antimicrobial susceptibility and resistance genes using PCR.

Whole-Genome Sequencing

The genomic DNA of the isolate was extracted using a QIAamp DNA Mini Kit (Qiagen, USA). The Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) and a long-read MinION sequencer (Nanopore, Oxford, UK) were used for whole-genome sequencing. The de novo hybrid assembly of both short Illumina reads and long MinION reads was performed using Unicycler. The whole-genome sequence was automatically annotated by the Prokaryotic Genome Annotation Pipeline server (NCBI, Maryland, USA).

Plasmid Analysis

We used the oriTfinder to quickly detect the origins of transfer (oriTs) and three other transfer-associated modules, such as relaxase, type IV coupling proteins (T4CP), and type IV secretion system (T4SS), in the *blaIMP-4*-carrying plasmid. The graphical circular map of the *blaIMP-4*-carrying plasmid was converted using the CGView Server.[26] Comparisons of the *blaIMP-4*-carrying plasmid with similar plasmids were performed using the BRIG and Easyfig tools.[27,28]

Nucleotide Sequence Accession Numbers

The sequences of the *blaIMP-4*-carrying plasmid were submitted to the GenBank database (NCBI, Maryland, USA) with the following accession number: pwzx_IMP (MW590809). All relevant data are available from the corresponding author upon reasonable request.

Results

Bacterial Identification and Susceptibility Testing

The isolate was identified as *K. oxytoca* using the Phoenix 100 system and MALDI-TOF-MS. Regarding antimicrobial susceptibility profiles, as shown in Table 1, the wzx-IMP strain was susceptible to amikacin, aztreonam, chloramphenicol, levofloxacin, trimethoprim-sulfamethoxazole, tetracycline, and colistin (MICs, ≤0.5μg/mL), intermediately susceptible to gentamicin and piperacillin-tazobactam, and resistant to ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, cefazolin, cefotaxime, ceftazidime, cefepime, imipenem, and meropenem.

MLST and Conjugation Experiments

MLST was performed for the wzx-IMP isolate. Based on the MLST results, the *K. oxytoca* isolate belongs to the ST85 type, which has not been reported previously in carbapenem-resistant *K. oxytoca*. We analyzed the genome data in the GenBank database (accessed on June 10, 2021) and found that no ST85-type *K. oxytoca* sequences are currently available in the database. The *blaIMP-4*-carrying plasmid in the wzx-IMP strain was successfully transferred to *E. coli* EC600 by conjugation. Transconjugants exhibited phenotypes resistant to imipenem and meropenem. PCR assays showed that the transconjugants under study were positive for the *blaIMP-4* gene.

Table 1 Antimicrobial Susceptibility Testing

Antimicrobial Agent	MIC (μg/mL)/ Antimicrobial Susceptibility	MIC Breakpoint (μg/mL)[a]		
		S	I	R
Amikacin	≤8/S	≤16	32	≥64
Aztreonam	≤2/S	≤4	8	≥16
Chloramphenicol	≤4/S	≤8	16	≥32
Levofloxacin	≤1/S	≤0.5	1	≥2
Trimethoprim-sulfamethoxazole	≤0.5/9.5/S	≤2/38	–	≥4/76
Tetracycline	≤2/S	≤4	8	≥16
Polymyxin	≤0.5/S	≤2[b]	–	>2[b]
Gentamicin	8/I	≤4	8	≥16
Piperacillin-tazobactam	64/4/I	≤16/4	32/4–64/4	≥128/4
Ampicillin	>16/R	≤8	16	≥32
Amoxicillin-clavulanate	>16/8/R	≤8/4	16/8	≥32/16
Ampicillin-sulbactam	>16/8/R	≤8/4	16/8	≥32/16
Cefazolin	>16/R	≤2	4	≥8
Cefotaxime	>32/R	≤1	2	≥4
Ceftazidime	>16/R	≤4	8	≥16
Cefepime	>16/R	≤2	–	≥16
Imipenem	8/R	≤1	2	≥4
Meropenem	>8/R	≤1	2	≥4

Notes: [a]CLSI guideline for MIC breakpoints of Enterobacteriaceae except polymyxin. [b]EUCAST guideline for MIC breakpoints of polymyxin.
Abbreviations: S, susceptible; I, intermediate; R, resistant; –, missing breakpoints.

WGS and Molecular Characterization

The wzx-IMP isolate had a chromosome and two plasmids (PlasmidA and pwzx_IMP), which were 6013415 bp, 140577 bp, and 62892 bp in length, had guanine–cytosine content of 55.91%, 51.90%, and 52.50%, respectively.

WGS showed that the isolate included *blaOXY-1-1*, a chromosomally encoded gene. Concerning the chromosomal outer membrane proteins OmpK35 and OmpK36, we did not find OmpK36 porins, and analysis of Ompk35 sequences did not reveal any nonsense point mutation insertion and/or deletion causing a reading frameshift with a premature stop codon or gross disruption by an insertion sequence.

Plasmid Analysis and Comparisons

pwzx_IMP was a 62,892-bp circular plasmid and was identified as an IncN1 group structure. It contains 45 predicted open reading frames. Through the oriTfinder server, we found that pwzx_IMP had a complete set of oriTs, relaxase, T4 cP, and T4SS (Figure 1), indicating that the plasmid has a strong self-transfer ability, which was consistent with the results of the conjugation experiments.

The *blaIMP-4*-carrying plasmid belonged to the IncN1 ST7 lineage. Four genes were involved in antimicrobial resistance, including the carbapenemase-encoding gene *blaIMP-4*, the *qnrS1* gene for quinolone resistance, the

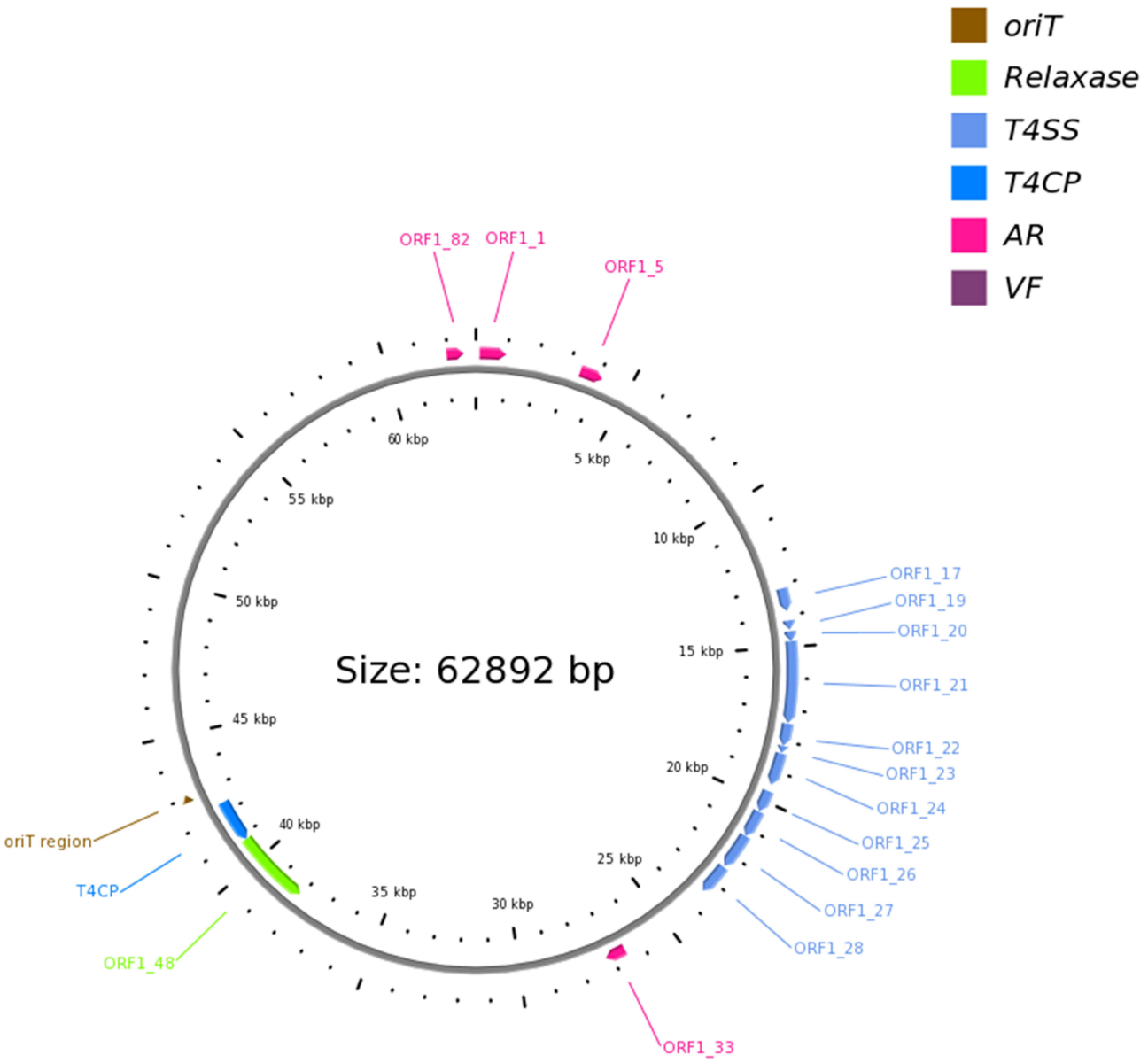

Figure 1 A scaled representation of the circular pwzx_IMP plasmid generated by the oriTfinder utility showing the locations and sizes of oriT (saddle brown), the relaxase gene (green), T4CP (dodger blue), genes coding for components of both T4SSs (blue) and AR (pink) within this replicon.

sulfonamide resistance gene *sul1*, and the *aac* gene (Figure 2).

The pwzx_IMP plasmid contained conserved IncN1-type backbone regions, containing a replication gene and its accessory structure for plasmid replication (Figure 2). The *blaIMP-4* gene mapped to pwzx_IMP was located at the proximal end of a truncated integrase gene, which included *Δintl1* and *blaIMP-4* and was designated In823,[13] preceded by IS26 in the upstream region, followed by another IS26 sequence in the downstream region.

A BLAST search of the pwzx_IMP plasmid sequence against the GenBank database showed that several similar previously published IncN1 plasmids were found (Figure 3): pIMP-HZ1 (accession no. KU886034) from the *K. pneumoniae* strain Kp1, pIMP-HK1500 (accession no. KT989599) from the *Citrobacter freundii* strain CRE1500, and pIMP-GZ1517 (accession no. KT982618) from the *E. coli* strain CRE1517, which are all found in China with an 84% query coverage and overall 100% nucleotide identity. However, the plasmid with the *C. freundii* strain ECL-14-57 (accession no. MH727565) exhibited an 84% query coverage and overall 99.89% nucleotide identity; the plasmid from the *K. oxytoca* strain pKOX3 (accession no. KY913900) exhibited an 81% query coverage and overall 99.90% nucleotide identity; the plasmid from the *K. pneumoniae* strain BKP19 (accession no. VWRO01000005) exhibited a 62% query coverage and an overall 99.95% nucleotide identity. The structural characteristics of pwzx_IMP compared to pIMP-HZ1, pIMP-HK1500, pIMP-GZ1517, and p24854-IMP, pECL-14-57, pKOX3 and pBKP19 are presented in Figure 4. KT982618, KT989599, and VWRO01000005 carry *blaIMP-4* only, whereas KU886034, KY913900, and MH727565 carry both *blaIMP-4* and *qnrS1*; however, pwzx_IMP harbors the *blaIMP-4, qnrS1, sul1*, and *aac* resistance genes. Both the *sul1* and *aac* genes are absent in similar plasmids.

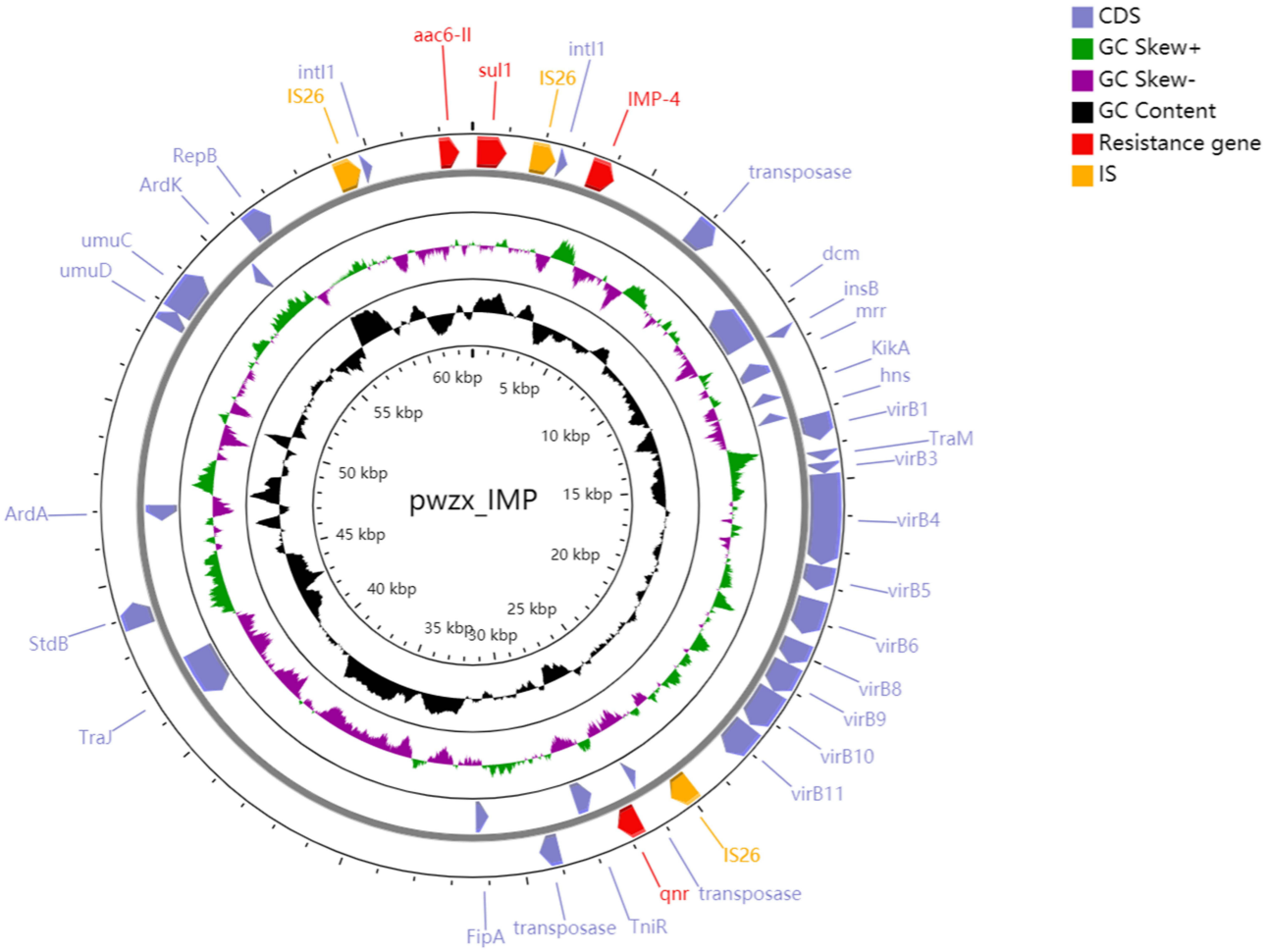

Figure 2 Backbone structure of the pwzx_IMP.

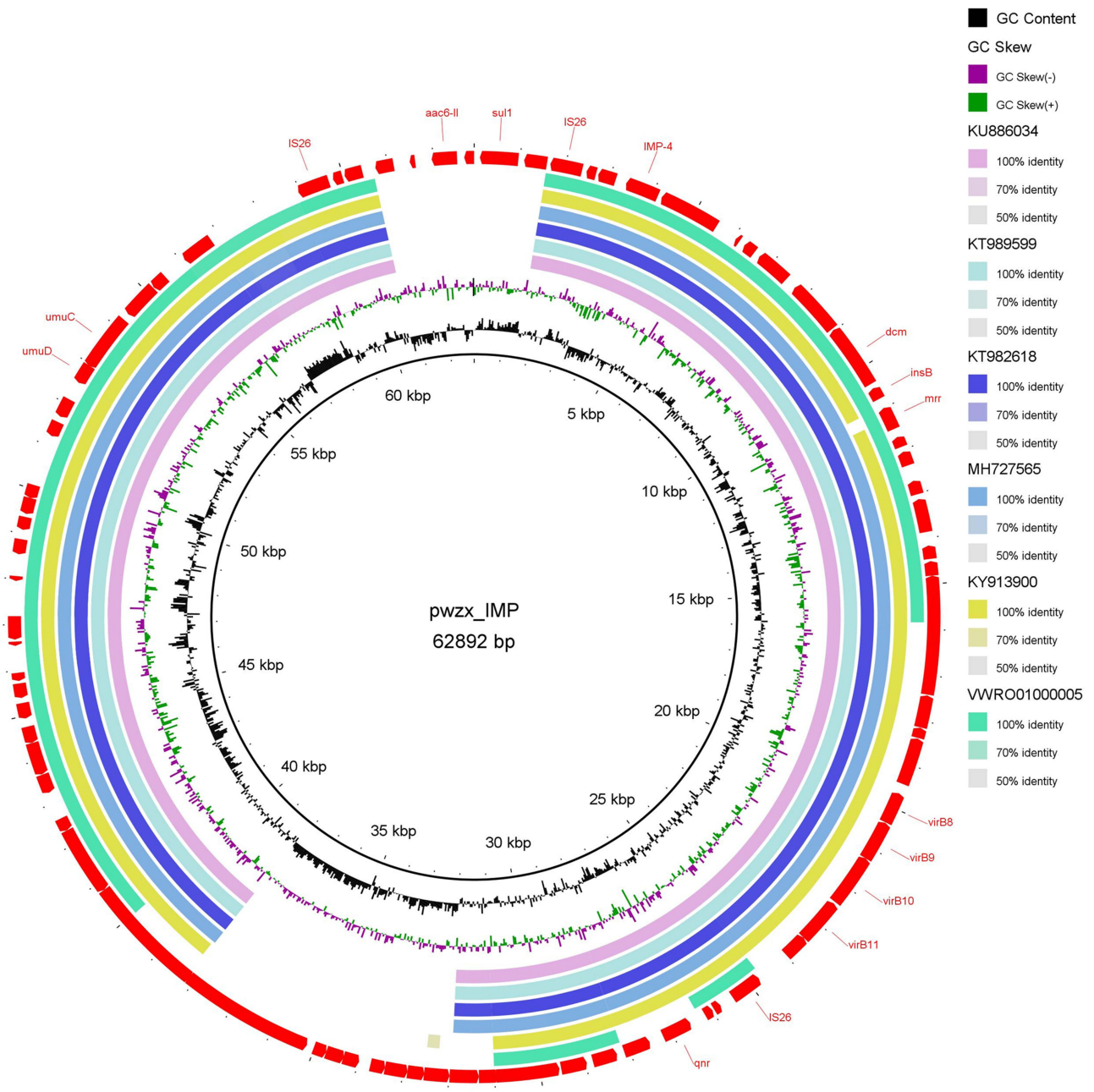

Figure 3 Sequence alignment of the pwzx_IMP plasmid in the NCBI GenBank database revealed several highly identical plasmids from different Gram-negative bacillus strains. KU886034 from the K. pneumoniae strain Kp1, KT989599 from the Citrobacter freundii strain CRE1500, and KT982618 from the E. coli strain CRE1517, MH727565 from the Citrobacter freundii strain ECL-14-57, KY913900 from the K. oxytoca strain pKOX3, VWRO01000005 from the K. pneumoniae strain BKP19.

Discussion

The chromosome of *K. oxytoca* encodes a class A β-lactamase-designated OXY (previously called K1 or KOXY).[29] The β-lactamase OXY group comprises the OXY-1, OXY-2, OXY-3, OXY-4, OXY-5, and OXY-6 subgroups.[30–32] Strains that overproduce the chromosomally encoded β-lactamase OXY are resistant to all β-lactamase inhibitors.[3,24,33,34] In this study, the isolate included *blaOXY-1-1*, a chromosomally encoded gene, which is the most common OXY gene type. IMP-4 carbapenemases, first identified in Hong Kong and China in the 1990s and initially restricted to Asia and the Pacific, have since become the predominant carbapenemase type worldwide;[35,36] however, unlike NDMs, IMP-type β-lactamases are not often detected in CRE from China.[37,38] The *blaIMP* genes are often found together

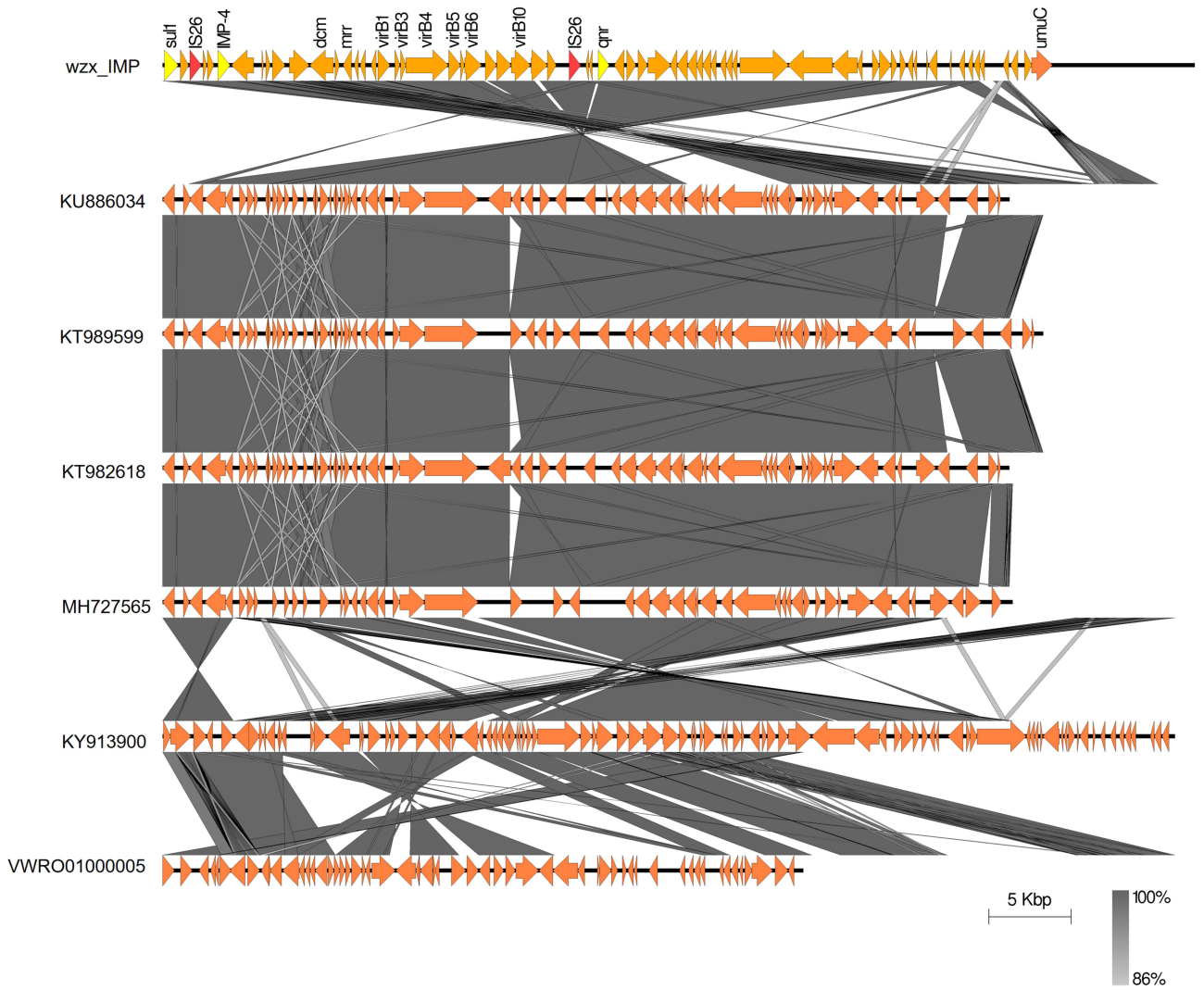

Figure 4 Comparative analysis of the homologous regions shared by seven plasmids. The figure was produced using EasyFig v. 2.2.3, and BLASTN was used to compare sequence homology with the following threshold parameters: minimum length of 100 bp accompanied by 90% identity. Antimicrobial resistance genes are indicated in yellow, IS elements are indicated in red, and all other genes are indicated in orange in pwzx_IMP.

with other resistance genes in the variable gene cassette arrays of class 1 integrons, and these integrons are further associated with mobile elements, such as transposons and plasmids, leading to the easily mobilization of cassette-borne resistance genes across various bacterial species.[39] IncN plasmids have been reported globally but are mainly prevalent in China and the USA. In the study by Hao et al, examining IncN1 plasmids in China (including the mainland and Taiwan, China), the most common carbapenemase type was *blaIMP* (63.9%; 23/36), followed by *blaNDM* (19.4%; 7/36) and *blaKPC* (19.4%; 7/36), and among the 25 IncN1 plasmids reported in the USA, *blaKPC* was the most common carbapenemase type, which accounted for 88% (22/25).[40] In China, Chen et al have reported a Chinese *Klebsiella oxytoca* strain ZC101 with IMP-4 and OmpK36 porin deficiency, the OmpK36 loss of strain was due to the IS5 insertion to OmpK36,[23] however, the strain wzx-IMP has a large number of points mutations and deletions. In short, we report for the first time an ST85-type *K. oxytoca* strain in China, which carried an IncN1-type plasmid, producing IMP-4–type MBLs along with OmpK36 porin deficiency.

Note that the backbone structures of pwzx_IMP identified in this study have been reported in other members of the *Enterobacteriaceae* family, including *E. coli, Klebsiella species, C. freundii*, and *Enterobacter cloacae*, along with *Pseudomonas* species. Based on the data reported in this study, it is reasonable to hypothesize that these resistance-encoding genes may have been recruited into a variable genetic locus flanked

by transposons and insertion sequence elements, while conserving the remaining plasmid scaffold. The successful transmission of these related episomes among various bacterial species challenges people with an interest in public health, which should be a cause of concern for clinicians, microbiologists, and administrators for infection control measures.

Conclusion

In summary, this study reports the first *K. oxytoca* ST85 strain harboring the class B β-lactamase *blaIMP-4* in an IncN1-type plasmid recovered from a child with a bloodstream infection in China. This work highlights the important role played by mobile plasmids identified in *K. oxytoca* and other bacteria as a modern threat to the successful treatment of infections.

Ethical Approval

This study obtained permissions from the Bioethics Committee of Affiliated Cancer Hospital of Zhengzhou University & Henan Cancer Hospital and participants (consent to participate was obtained from participants) to review patient records and use the data.

Consent Statement

All authors reporting this patient's details of the manuscript "IncN1 ST7 epidemic plasmid carrying blaIMP-4 in ST85-type Klebsiella oxytoca clinical isolate with porin deficiency" state that publication of their clinical details was obtained from the parent of the patient.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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