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ORIGINAL RESEARCH

Emergence of OXA-484-Producing *Klebsiella variicola* in China

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Purpose: The frequent and inappropriate use of antibiotics has caused a dramatic rise in the number, species, and degree of multi-drug resistant bacteria, making them more prevalent and difficult to treat. In this context, the aim of the present study was to characterize the OXA-484-producing strains isolated from a perianal swab of a patient by using whole-genome analysis.

Patients and Methods: In this study, carbapenemase-producing *Klebsiella variicola* was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), average nucleotide identity (ANI) and PCR. S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern blotting were utilized to characterize the plasmid profiles of *K. variicola* 4717. In particular, WGS was performed to obtain genomic information on this clinical isolate, and assemble all the plasmids of the $bla_{\text{OXA-484}}$-harboring strain.

Results: The antimicrobial susceptibility pattern of *K. variicola* 4717 revealed that it was resistant to a range of antibiotics, including aztreonam, imipenem, meropenem, ceftriaxone, cefotaxime, ceftazidime, levofloxacin, ciprofloxacin, piperacillin-tazobactam, methylene-sulfamer oxazole, amoxicillin-clavulanic acid, cefepime, and tigecycline. Its susceptibility to chloromycin was intermediate, while it was still susceptible to amikacin, gentamicin, fosfomycin, and polymyxin B. The presence of two companion plasmids, p4717_1 and p4717_2, together with a plasmid carrying the $bla_{\text{OXA-484}}$ gene was observed. An in-depth investigation of p4717-OXA-484 uncovered that it is an IncX3-type plasmid and shares a similar segment encoded by IS26. Given the similar genetic background, it was conceivable that $bla_{\text{OXA-484}}$ could have developed from $bla_{\text{OXA-181}}$ through a series of mutations.

Conclusion: Herein, we described the first genome sequence of *K. variicola* strain harbouring the class D β-actamase $bla_{\text{OXA-484}}$ in an Inc-X3-type plasmid. Our work also uncovered the genetic characterization of *K. variicola* 4717 and the importance of initiating antimicrobial detection promptly.

Keywords: *Klebsiella variicola*, OXA-484, IncX3, mutation

Introduction

Klebsiella variicola, a species of the *Klebsiella* genus, exhibits genetic and biochemical distinctions in comparison to *Klebsiella pneumoniae*.[1] *K. variicola* was first reported in 2004 and mainly isolated from natural environments.[2] Additionally, they have an ability to fix nitrogen and promote the plant growth.[3] As regarding its characteristic of antimicrobial resistance in recent years, *K. variicola* was considered as an emerging human pathogen which should be monitored.[4] A hypermucoviscous multidrug-resistant *K. variicola* coproducing IMP-4 and NDM-1 was obtained from a pediatric patient.[5] Multidrug-resistant *K. variicola* has caused outbreaks in a Bangladeshi neonatal unit with high mortality. Given all that, more attention should be paid to *K. variicola*, which may be a reservoir of antimicrobial resistance genes. However, in most clinical laboratories, *K. variicola* was often misidentified as *K. pneumoniae*, until the

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PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used for species identification.[6] In addition, *K. variicola* can also be recognized on the basis of average nucleotide identity (ANI). Thus, resistance mechanism and genetic characteristic of *K. variicola* remain largely unknown until now.

OXA-48 is a class of carbapenemases that mainly appear in European countries, Middle East and Mediterranean countries.[7] To the best of our knowledge, at least 35 variants of OXA-48 have been reported, the difference between them being a few amino acid substitutions.[8] Among them, OXA-484, which differs from OXA-48 in five amino acid substitutions (Thr104Ala, Asn110Asp, Glu168Gln, Ser171Ala, Arg214Gly), was collected from *K. pneumoniae* strains in the UK.[9] It has also been documented that *Escherichia coli* is hosting the $bla_{\text{OXA-484}}$ gene.[10] In this study, we were the first to document the infection caused by OXA-484-producing *K. variicola* in China.

Materials and Methods

Species Identification and Antimicrobial Susceptibility Testing

Isolates were collected from a tertiary hospital in Zhengzhou, Henan province, China, during our routine surveillance of multi-drug resistance bacteria. The species was identified using both MALDI-TOF/MS (Bruker Daltonik GmbH, Bremen, Germany) and average nucleotide identity (ANI) analysis.[11] The carbapenemase-encoding genes were detected using PCR and sanger sequencing. Antimicrobial susceptibility testing (AST) was conducted using the agar dilution method and broth microdilution method.[12] The Clinical Laboratory Standards Institute (CLSI) guidelines (https://clsi.org/standards/) were used for the interpretation of susceptibility results. The resistance breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org) were used for polymyxin B and tigecycline. *K. pneumoniae* ATCC700603 and *E. coli* ATCC 25922 were used as quality controls strains.[13]

Localization and Transferability of $bla_{\text{OXA-484}}$

The size and number of plasmids carried by *K. variicola* 4717 was visualized by S1-PFGE and southern blotting. The conjugation assay aimed to determine the transfer capacity of the $bla_{\text{OXA-484}}$-harbouring plasmid. The sodium azide-resistant strain *E. coli* J53 used as the recipient and the $bla_{\text{OXA-484}}$-positive *K. variicola* 4717 was selected as the donor. MH agar plates supplemented with NaN_3 (200 mg/L) and imipenem (2 mg/L) as the screen plate for the $bla_{\text{OXA-484}}$-positive transconjugants. Then, MALDI-TOF MS and PCR were used to confirm the presumptive conjugant.[14]

Whole Genome Sequencing and Bioinformatics Analysis

Genomic DNA of *K. variicola* 4717 was obtained through a Bacterial DNA Kit (QIAGEN, Hilden, Germany) according to the instruction.[15] The extracted genome of *K. variicola* 4717 was sequencing on the Illumina NovaSeq 6000 (Illumina, San Diego, CA, United States) for short-read data and Oxford Nanopore platform (Oxford Nanopore Technologies, Oxford, United Kingdom) for long-read data at different depths.[16] Then, the Illumina short reads and Nanopore long reads were assembled using Unicycler for whole genome sequence. Prokka (https://www.psc.edu/resources/software/prokka/) was used for genome annotation. Genomic data were analyzed using various online tools. ResFinder (https://cge.food.dtu.dk/services/ResFinder/) and the BacWGSTdb (http://www.bacdb.cn/BacWGSTdb/) were employed to detect acquired antimicrobial resistance genes and virulence genes, respectively.[17] Multilocus sequence typing (MLST) of *K. variicola* isolates by analyzing the seven housekeeping genes (*leuS, pgi, pgk*, phoE, *pyrG, rpoB*, and *fusA*) using an online database (http://mlstkv.insp.mx/).[18] Plasmid finder was used to identify the replicon type of plasmids (https://cge.food.dtu.dk/services/PlasmidFinder/). The origin of transfers in DNA sequences of bacterial mobile genetic elements was identified on oriTfinder (https://tool-mml.sjtu.edu.cn/oriTfinder/oriTfinder.html).[19] The NCBI blast was used to align the plasmids which were similar to the $bla_{\text{OXA-484}}$-harbouring plasmid. The comparisons images of multiple plasmids were conducted by using the BLAST Ring Image Generator (BRIG) in concentric rings and the figures of genetic context surrounding the $bla_{\text{OXA-484}}$-harbouring plasmid and other related plasmids were generated by Easyfig 2.0 software in linear graph.

Biofilm Formation Assays

Biofilm formation was measured in accordance with the assays outlined in previous researches.[20] In a word, the bacterial overnight culture was diluted in LB and the 200 μL of the mixture was dispensed into the each well of 96-well plate. After static culture at 37°C for 24 hours, PBS was utilized to clean the microwells three times in order to eliminate all non-adherent bacteria. Methanol was used for fixation, and 0.1% crystal violet solution were added to each well for subsequent stain. After washing the plate with PBS three times and discarding the washing solution, 100 μL of DMSO were added to dissolve the crystal violet attached to the biofilm, and the plate should be incubated for 5 minutes. Finally, the OD (optical density) was measured at 590 nm. LB broth was used for the negative control.

Results

Species Identification

The isolate FAHZZU 4717 was obtained from a stored strain which was collected from a perianal swab of a patient admitted to ICU in the First Affiliated Hospital of Zhengzhou University in 2021. FAHZZU 4717 was identified as *K. variicola* based on the ANI analysis. Moreover, the genomic sequences of FAHZZU 4717 share 99.24% similarity and 87% coverage with the genome of the variicola reference strain F2R9, which has been archived as ATCC BAA-830 (no. CP072130).

Antimicrobial Susceptibility Profiles of *K. variicola* 4717

K. variicola 4717 showed intermediate to chloromycin, displayed susceptibility to amikacin, gentamicin, fosfomycin, tigecycline, polymyxin B, and showed resistance to aztreonam, imipenem, meropenem, ceftriaxone, cefotaxime, ceftazidime, levofloxacin, ciprofloxacin, piperacillin-tazobactam, methylene-sulfamer oxazole, amoxicillin-clavulanic acid, and cefepime (Table 1).

Table 1 Susceptibility of *K. variicola* 4717

Antibiotics	MIC Values (μg/mL)	Antimicrobial Susceptibility
Penicillins		
Piperacillin/tazobactam[a]	>128	R
Beta-lactam		
Ceftazidime	16	R
Ceftriaxone	16	R
Cefepime	1	S
Cefotaxime	16	R
Imipenem	8	R
Meropenem	16	R
Aztreonam	16	R
Fluoroquinolones		
Ciprofloxacin	2	R
Aminoglycosides		
Amikacin	2	R
Gentamicin	2	S
Tetracyclines		
Tigecycline	0.25	S
Phenicols		
Chloramphenicol	16	I
Polymyxin		
Colistin	2	S
Sulfonamide		
Trimethoprim/ sulfamethoxazole	>8	R

Note: [a]Tazobactam at a fixed concentration of 4mg/L.
Abbreviations: R, resistant; S, susceptible; I, intermediate.

Characterization of the Genome of *K. variicola* 4717

The genome of *K. variicola* 4717 consists of six contigs. Among them, contig 1 (5,672,190 bp) belong to the chromosome, while the contig 2 (168,009 bp), contig 3 (115,032 bp) and contig 4 (51,479) belong to three different plasmids (Table S1). However, only two plasmid replicons were identified, contig 2 belongs to the IncFII and IncFIB(K), and contig 4 belongs to the IncX3 which designated p4717-OXA-484. Moreover, MLST analysis showed that the allelic profile (*leuS, pgi, pgk, phoE, pyrG, rpoB*, and *fusA*) of *K. variicola* 4717 was 25:24:6:1:34:1:4, which indicates an unknown ST.

The ARGs existing in the genome of *K. variicola* 4717 were presented in Table S1. We identified the quinolone resistance genes *oqxB, oqxA*, and *qnrS1*; beta-lactam resistance genes bla_{LEN16}, $bla_{\text{DHA-1}}$, and $bla_{\text{OXA-484}}$; fosfomycin resistance gene *fosA*; sulphonamide resistance gene *sul1*; trimethoprim resistance gene *dfrA1*, and tetracycline resistance gene *tet(A)*.

K. variicola 4717 was found to possess approximately 60 virulence factors (Table S2), including those related to adhesion (*fimH, fimF*, and *fimC*), type VI secretion systems (*vipA/tssB, impA/tssA, dotU/tssL, vasE/tssK, hcp/tssD*, and *sciN/tssJ*), iron acquisition (*entA, entD, entE, fepA, fepB*, and *fes*), and biofilm formation (*ompA, mrkC, mrkD*, and *mrkH*). In addition, experiments on biofilm formation demonstrated that *K. variicola* 4717 has a moderate capacity for biofilm production (Figure 1).

Characterization of $bla_{\text{OXA-484}}$ Bearing Plasmid

The p4717-OXA-484 is 51,457 bp long, with an average GC content of 46.4% (Figure S1). Its replicon was identified as the IncX3 incompatibility group. Combined with the result of BLASTn search, linear alignment of the plasmid genomes suggests that p4717-OXA-484 is similar to three different plasmids from *E. coli, K. pneumoniae*, and *K. variicola*, namely pLB_OXA-181_PT109 (no. CP041033), pSECR18-2374D (no. CP041931), and pKS22 (no. KT005457), respectively (Figure 2A). Additionally, like the three plasmids mentioned above, the $bla_{\text{OXA-484}}$ was consistently associated with a composite transposon which was flanked by two copies of IS*26*. The similar region contains transposons (Tn*2*), insertion sequences (IS*Kpn19*, IS*3000*) and other genes (*tnpR, umuD*, DNAase and *qnrS1*) (Figure 2B). There were no transconjugants obtained after three conjugation assays. Simultaneously, the outcome of oriTfinder analysis revealed that no origin site of DNA transfer (oriT) was predicted (Figure S2).

Existence of Two Accompanying Plasmids p4717_1 and p4717_2

In addition to the p4717-OXA-484, two accompanying plasmids were also found in the clinical strain *K. variicola* 4717 and designated as p4717_1 and p4717_2. The p4717_1 was classified as IncFII/IncFIB$_{\text{K}}$-type hybrid plasmid which

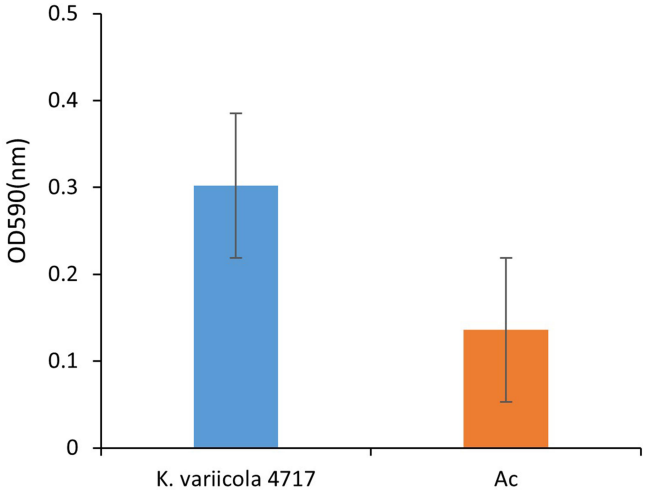

Figure 1 Biofilm formation analysis of *K. variicola* 4717. Ac, the negative control.

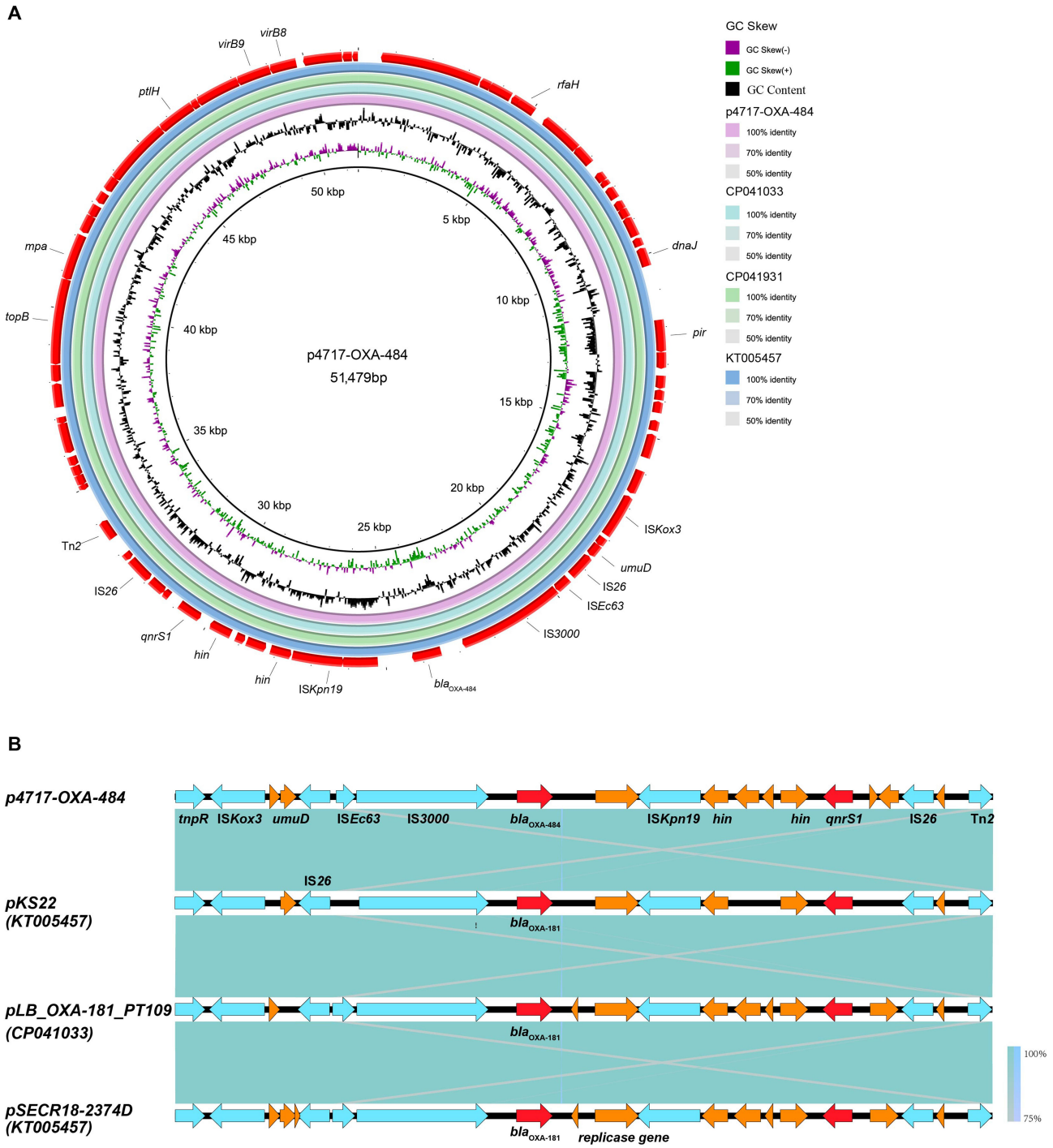

Figure 2 Genomic analyses of plasmid p4717-OXA-484. (**A**) Comparative analysis of plasmids p4717-OXA-484 with pLB_OXA-181_PT109 (no. CP041033), pSECR18-2374D (no. CP041931) and pKS22 (no. KT005457). (**B**) Comparison of genes surrounding $bla_{OXA-484}$ on p4717-OXA-484, pLB_OXA-181_PT109 (no. CP041033), pSECR18-2374D (no. CP041931) and pKS22 (no. KT005457). Open reading frames (ORFs) are shown as arrows and indicated according to their putative functions. Red indicates antimicrobial resistance genes, light blue indicates genes related to mobile elements, and the Orange represents other functional genes. Regions with a high degree of homology are indicated by blue shading.

showed high similarity with three known plasmids restricted to *K. variicola*, namely pKP91 (no. CP000966), p15WZ-82_res (no. CP032357) and pM142-3 (no. CP063868). Surprisingly, no acquired resistance genes were carried by p4717_1 (Figure 3A).

The PlasmidFinder analysis revealed that p4717_2 had no replicons, indicating the plasmid was untypable. The backbone and structure of p4717_2 was similar to that of pBSI138_P3 (no. MT269850), plasmid p4_1_2.1 (no.

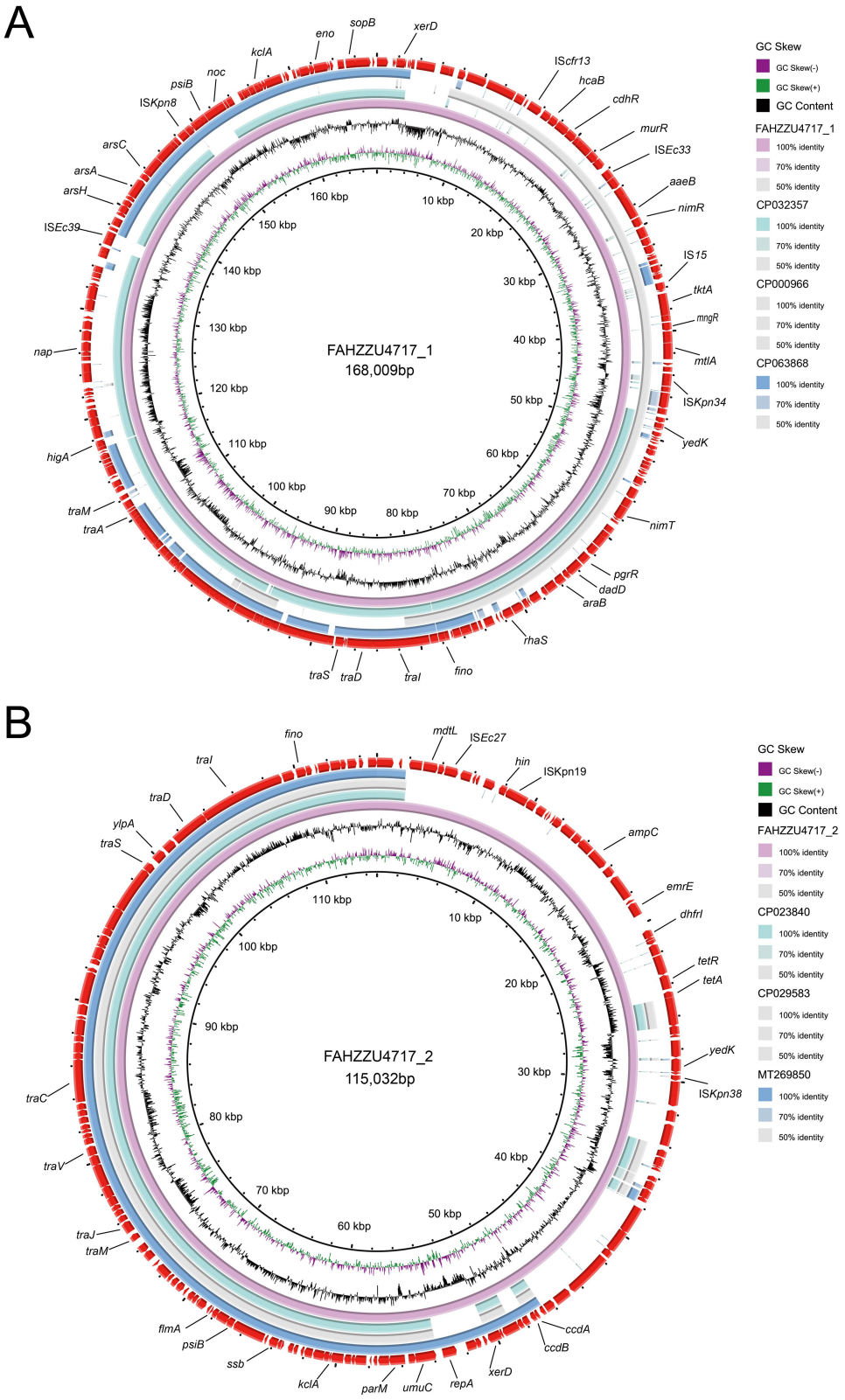

Figure 3 Alignment of two companion plasmids p4717_1 and p4717_2. (**A**) The p4717_1 showed high similarity with three known plasmids, namely pKP91 (no. CP000966), p15WZ-82_res (no. CP032357) and pM142-3 (no. CP063868). (**B**) The p4717_2 was similar with pBS1138_P3 (no. MT269850), plasmid p4_1_2.1 (no. CP023840) and plasmid pDA33140-112 (no. CP029583).

CP023840) and plasmid pDA33140-112 (no. CP029583) (Figure 3B). In comparison to p4717-OXA-484, p4717_2 was found to contain a greater variety of antibiotic resistance genes, including *dfrA1* (trimethoprim), *sul1* (sulphonamide), *qnrS1* (quinolone), *tet(A)* (tetracycline), *qacE* (disinfectant) and $bla_{\text{DHA-1}}$ (β-lactam). Obviously, the existence of ARGs in p4717_2 partly explains the phenotypic resistance of the clinical isolate *K. variicola* 4717.

Discussion

In comparison to *K. pneumoniae, K. variola* strains containing carbapenemases resistance genes have not been given as much attention. In our study, the identification of *K. variicola* carrying $bla_{\text{OXA-484}}$ suggested the wider dissemination than previously thought. Initially, the resistance profile of *K. variicola* in the environment was extensively researched. In 2017, a *K. variicola* isolate producing NDM-9 was identified from an urban river in South Korea. Later the presence of beta-lactamase $bla_{\text{TEM-116}}$ in *K. variicola* was reported from an urban riverine environment in India.[21] Other studies also have demonstrated that *K. variicola* is a pathogenic organism in animals. In Brazil, a polymyxin-resistant *K. variicola* was collected from birds.[22] A previous study described the isolation of *K. variicola* in a horse with respiratory disease for the first time.[23] *K. variicola* has been identified as a cause of clinical mastitis in cows, with evidence of its presence in milk from affected animals.[24] Shen et al suggested that even low infection levels of *K. variicola* AHKv-S01 can caused a significant reduction in chicken embryo hatchability.[25] As well, the circulation of multidrug-resistant *K. variicola* in healthy livestock animals has also been reported.[26]

Contrary to the existing studies that investigate the role of *K. variicola* in environmental resistance and animal disease, there is growing evidence of its clinical impact. Previous research has demonstrated that *K. variicola* is associated with a range of infections, including bloodstream infections, urinary infections, and respiratory tract infections. Akine et al has reported a case of meningitis caused by *K. variicola* after neurosurgery, and the effect of *K. variicola* has been underestimated in terms of causing urinary tract infection disease.[3,27] Studies have demonstrated that bloodstream infections caused by *K. variicola* are more fatal than *K. pneumoniae*, and this pathogen is mainly isolated from blood samples in clinical settings.[28] Moreover, *K. variicola* and *K. pneumoniae* isolated from the same patient had the different antimicrobial susceptibility profiles for *K. variicola* was imipenem-susceptible, but *K. pneumoniae* was resistant.[29,30] However, *K. variicola* 4717 isolated in this work showed resistance to both imipenem and meropenem. The biofilm formation assay showed *K. variicola* 4717 was a moderate biofilm producer due to the expression of fimbriae factors, granting it the ability to adhere and colonize. Understanding the relevant pathogenic and resistant characteristics of *K. variicola* was indispensable, which contributes to the identification and clinical treatment of associated infections.

The bla_{KPC} gene is the most commonly reported gene conferring carbapenem resistance in *K. variicola*, while the OXA-48-like carbapenemase is relatively uncommon. In a retrospective study, only one strain carrying $bla_{\text{OXA-48}}$ was detected in 145 *K. variicola* strains.[6] It also has been suggested that the $bla_{\text{OXA-484}}$-bearing strains had a lower resistance to temocillin and carbapenems than the $bla_{\text{OXA-48}}$-carrying strains.[9] While, there were few reports on carbapenem resistance genes-harboring *K. variicola* in China. In contrast to previous reports about $bla_{\text{OXA-484}}$, the discovery of *K. variicola* 4717 complements a novel example of $bla_{\text{OXA-484}}$-positive strain. Utilizing the genome sequence of *K. variicola* 4717 as a reference point for comparative genomic analysis can provide further insight into the transmission mechanism of *K. variicola*.

Plasmids are often seen as a crucial genetic factor in the transfer of antibiotic resistance genes between different species.[31,32] The p4717-OXA-484 belonged to the IncX3 plasmids which were particularly renowned for their broad host range and high transmissibility.[33,34] But the results of the oriTfinder analysis indicated that p4717-OXA-484 may not be capable of conjugation and transfer due to the lack of oriT modules, which was in line with the unsuccessful results of the conjugation transfer experiment. It was likely that the $bla_{\text{OXA-484}}$ gene, which encodes for carbapenemase in *K. variicola* 4717 is a result of mutation from the $bla_{\text{OXA-181}}$ gene that is present in *E. coli* and *K. pneumoniae* isolates, given that the genetic structures are analogous.

Conclusion

Our initial discovery of a *K. variicola* strain harboring the $bla_{\text{OXA-484}}$ gene encouraged us to explore its resistance mechanism and genetic features. Our research shed light on the current prevalence of ARGs and the importance of bacterial identification. Paying close attention to *K. variicola* and monitor its carriage of ARGs is critical to prevent it from developing into a novel pathogen.

Ethics Approval

The ethical protocol was approved by the Ethics Committee of First Affiliated Hospital of Zhejiang University (no. 2021-631).

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Disclosure

The authors report no conflicts of interest in this work.

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