

Co-Production of NDM-1 and OXA-10 β -Lactamase in *Citrobacter braakii* Strain Causing Urinary Tract Infection

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Abstract: In this study, we describe, for the first time, the co-existence of bla_{NDM-1} and bla_{OXA-10} in a carbapenem-resistant *Citrobacter braakii* strain DY2019 isolated from a patient with urinary tract infection in China. We aimed to investigate the genomic context of two β -lactamase-producing plasmids and characterize the transmission mechanism of the carbapenemase-encoding gene. Whole-genome sequencing of strain DY2019 was performed with Nanopore and Illumina platforms, which revealed a chromosome sequence with the length of 4,830,928 bp, an IncC group plasmid pDY2019-OXA (size of 178,134 bp), and a novel IncHI2 group plasmid pDY2019-NDM (length 348,495 bp). A total of 16 antimicrobial resistance genes (ARGs) that confer resistance to nine different antibiotic groups were identified in strain DY2019, and 11 of them were carried by plasmid pDY2019-OXA. These data and analyses suggest that the carbapenem-resistant *C. braakii* strains may serve as potential reservoir of carbapenemase and highlight the need for further close surveillance of this species in clinical settings.

Keywords: *Citrobacter braakii*, carbapenem-resistant, NDM-1, OXA-10, whole-genome sequencing, urinary tract infection

Introduction

Genus *Citrobacter* is a Gram-negative, facultative aerobic, long rod-shaped Enterobacteriaceae.^{1,2} *Citrobacter* isolates are most commonly isolated from environmental habitats, including soil, water, sewage, and intestines of animals and humans.³⁻⁶ As an opportunistic pathogen, *Citrobacter freundii* complex (CFC) isolates are usually associated with a variety of nosocomial infections of the respiratory tract,⁷ urinary tract,⁸ blood,⁹ and even central nervous system.¹⁰ *Citrobacter braakii*, as a member of CFC, has rarely been recognized as a human pathogen.¹¹

Infections caused by *C. braakii* are usually treated with cephalosporins, fluoroquinolones, and carbapenems. Due to the irrational use of broad-spectrum antibiotics, *C. braakii* developed resistance to a certain number of antibiotics. New Delhi Metallo- β -lactamase 1 (NDM-1) is an Ambler class B β -lactamase that spreads across different Enterobacteriaceae species, including *C. braakii*.¹²⁻¹⁷

This work reports a carbapenem-resistant *C. braakii* strain causing urinary tract infection, which showed extensive resistance to most tested antibiotics. Nanopore and Illumina sequencing revealed the co-existence of bla_{NDM-1} -carrying and bla_{OXA-10} -carrying plasmids in the isolate. Our study aimed to (i) describe the complete sequence of novel IncHI2 bla_{NDM-1} -carrying and bla_{OXA-10} -carrying plasmids and (ii) evaluate the phenotypic characteristics of the IncHI2 bla_{NDM-1} -carrying plasmid.

Materials and Methods

Isolate DY2019 was recovered from a urine sample of a 71-years old female patient with urethritis in March 2019 in Dongyang, China. The isolate was found to harbor the *bla*_{NDM-1} gene by real-time PCR as part of ongoing routine work for surveillance of carbapenemase-producing bacteria. The carbapenemase-encoding genes were identified by PCR amplification as described previously.¹⁸ Bacterial identification was performed by both VITEK2 compact system (BioMérieux, France) and Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as described previously.^{6,19} Using ANI analysis, species were determined if the genome in question had >95% ANI value with the type genome using pyani with default settings (<https://github.com/widdowquinn/pyani>).

The VITEK 2 system with panel AST-GN-16 (bioMérieux, Marcy-l'Étoile, France) was employed for antimicrobial susceptibility testing (AST) of isolate DY2019, with *Escherichia coli* ATCC 25922 as control. AST results were interpreted following the standards of the Clinical and Laboratory Standards Institute.²⁰ Minimum inhibitory concentration (MIC) of strain DY2019 showed that DY2019 resistant to a various type of antibiotics, including cefotaxime (MIC =64 µg/mL), ceftazidime (MIC >128 µg/mL), cefpirome (MIC =16 µg/mL), piperacillin/tazobactam (MIC =128 µg/mL), ciprofloxacin (MIC =16 µg/mL), imipenem (MIC =4 µg/mL), and only susceptible to tigecycline (MIC =0.06 µg/mL) and colistin (MIC =1 µg/mL).

According to the manufacturer's instructions, total DNA (chromosomal and extrachromosomal) was extracted and purified using the QIAamp DNA Mini Kit (Qiagen, Germany). One microgram of the genomic DNA extract was subjected to whole-genome sequencing (WGS) using the Nanopore platform (Oxford Nanopore, Oxford, UK) and Illumina Novaseq-600 sequencer (Illumina, San Diego, United States) as described before.^{18,21} Protein-coding genes were initially identified and annotated using RAST (<https://rast.nmpdr.org/>), while insertion elements (IS) were determined using ISFinder (<https://www-is.biotoul.fr/>). Queries were generated using the ResFinder 3.1 database²² to identify acquired antibiotic resistance genes. PlasmidFinder and pMLST were used to identify plasmid incompatibility types.²³

S1-PFGE characterized the number and size of the plasmid of the DY2019. The location of *bla*_{NDM-1} gene was confirmed by Southern blot and hybridization with a digoxigenin-labeled *bla*_{NDM-1} probe using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). Conjugation transfer experiments were conducted to explore the transferability of plasmids with rifampicin-resistant *E. coli* 600 as the recipient strain as recipients, as described previously.²⁴ After that, Mueller-Hinton agar (OXOID, Hampshire, United Kingdom) plates contained both 200 µg/mL

Table 1 Characteristics of ARGs Carried by DY2019

Resistance Genes	Phenotype	Number of ARGs
Chromosome		1
<i>bla</i> _{CMY-93}	β-lactam resistance	1
pDY2019-NDM		4
<i>bla</i> _{NDM-1}	Carbapenem resistance	1
<i>aac</i> (6)- <i>Ib-cr</i>	Aminoglycoside resistance	1
<i>aac</i> (6)- <i>Ib3</i>	Aminoglycoside resistance	1
<i>tet</i> (D)	Tetracycline resistance	1
pDY2019-OXA		11
<i>bla</i> _{OXA-10}	β-lactam resistance	1
<i>bla</i> _{DHA-1}	β-lactam resistance	1
<i>qnrB4</i>	Fluoroquinolone resistance	1
<i>aadA1</i>	Aminoglycoside resistance	1
ARR-2	Rifamycin resistance	1
<i>sul1</i>	Sulfamethoxazole resistance	1
<i>sul2</i>	Sulfamethoxazole resistance	1
<i>dfrA14</i>	Trimethoprim resistance	1
<i>tet</i> (D)	Tetracycline resistance	1
<i>mph</i> (A)	Macrolide resistance	1
<i>cmIA1</i>	Chloramphenicol resistance	1

rifampicin (Meilunbio, Dalian, China) and two $\mu\text{g/mL}$ meropenem were used to select $bla_{\text{NDM-1}}$ carrying transconjugants. Plasmid typing was performed by using PlasmidFinder.²⁵

Results and Discussion

Strain DY2019 was finally identified as *C. braakii* by ANIb analysis. A chromosome of 4,830,928 bp in length with a GC content of 52.2%, a plasmid (pDY2019-NDM) of 348,495 bp in length with a GC content of 47.9%, and a plasmid (pDY2019-OXA) of 178,134 bp in length with a GC content of 52.5%, were assembled from the Illumina and Nanopore sequencing reads with a hybrid strategy utilizing Unicycler program.²⁶ Whole-genome sequencing showed that strain DY2019 harboured $bla_{\text{NDM-1}}$, $bla_{\text{OXA-10}}$, $aac(6')\text{-Ib-cr}$, $aac(6')\text{-Ib3}$, $qnrB4$, $aadA1$, $sul1$, $sul2$, $dfrA14$, $tet(D)$, $ARR-2$, $mph(A)$, $cmlA1$, $floR$, $bla_{\text{DHA-1}}$, and $bla_{\text{CMY-93}}$ (Table 1), which is consistent with the phenotypic results. It is worthy to note that one AmpC-type beta-lactamase encoding gene, $bla_{\text{CMY-93}}$, was found in the chromosome of strain DY2019 using ResFinder 3.1. This result further supported the previous hypothesis that bla_{CMY} genes were ubiquitous in CFC isolates.¹⁹

S1-PFGE and Southern blot demonstrated that DY2019 encoded two plasmids (Figure 1), and $bla_{\text{NDM-1}}$ was located on a ~ 348 kb novel IncHI2 plasmid (Figure 2). Interestingly, the principal NDM variant found in CFC was $bla_{\text{NDM-1}}$, which is usually located on the self-transferable IncX3 plasmid.^{1,27} However, despite repeated efforts, no transconjugants

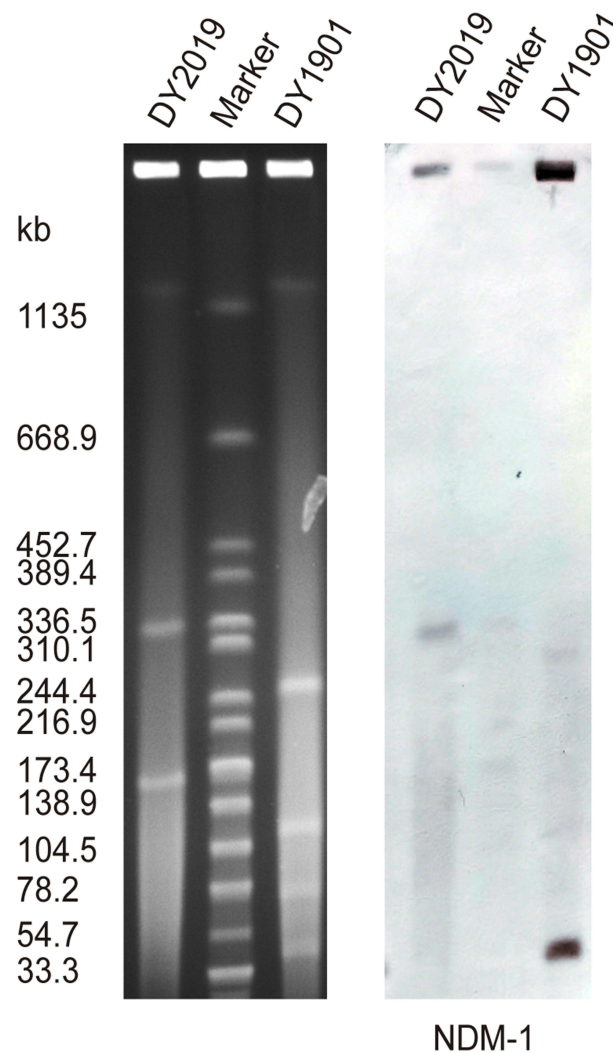


Figure 1 Plasmid profiles and Southern blot-hybridization of *C. braakii* DY2019. Southern blot-hybridization of S1-nuclease digested DNA using a specific probe (bla_{NDM}). M: XbaI digested total DNA of *Salmonella enterica* serotype Braenderup H9812 as a size marker and *Enterobacter cloacae* DY1901 as the control.

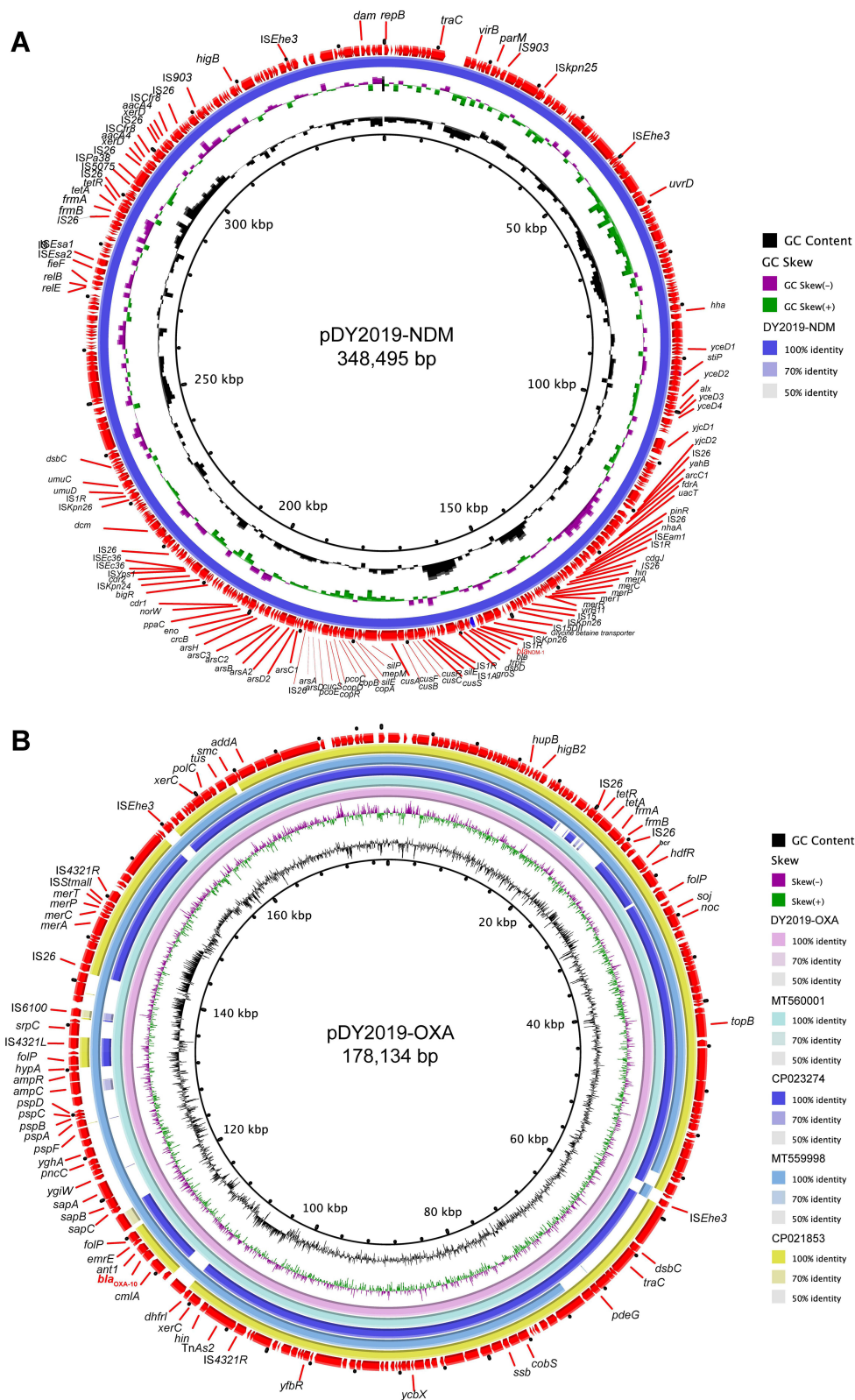


Figure 2 Major structural features and comparison of beta-lactamase-encoding plasmids. **(A)** Schematic illustration showing the structural features of pDY2019-NDM. **(B)** Linear maps of five NDM-I encoding plasmids, pDY2019-NDM, pCCI-1/2b (MT559998), pKC3-1/2b (MT560001), pPmi70746_1 (CP023274), and AR_0156 plasmid unitig_1 (CP021853). ORFs are portrayed by arrows and colored according to their putative functions. The alignment of the plasmids was performed and visualized by BLAST ring image generator (BRIG) software.

carrying *bla*_{NDM-1} were obtained. This could explain by the fact that pDY2019-NDM lacks a set of transfer (*tra*) genes (Figure 2). Resfinder Identified four antimicrobial resistance genes (ARGs) in pDY2019-NDM. These genes were associated with β -lactams resistance (*bla*_{NDM-1}), aminoglycoside resistance (*aac(6')-Ib-cr* and *aac(6')-Ib3*) and tetracycline resistance (*tet(D)*) phenotypes of strain DY2019. Interestingly, plasmid comparison based on full plasmid BLAST query revealed that pDY2019-NDM exhibited very low similarity to other plasmids (Figure 2A), which suggested that it is a novel *bla*_{NDM-1}-carrying plasmid. Genetic context characterization revealed that *bla*_{NDM-1} was located in an ISCR1 complex class 1 integron with a conserved structure of IS*Aba125*-*bla*_{NDM-1}-*ble*_{MBL}-*trpF*-*dsbD*. This conserved structure is normal among elements carrying *bla*_{NDM-1} and is commonly found in various *bla*_{NDM-1}-carrying plasmids in *Enterobacteriaceae*. It is noteworthy that *bla*_{NDM-1}-carrying IncHI2 plasmids were mainly Identified in the *Enterobacter cloacae* complex in China.^{28,29} This study further suggests that *bla*_{NDM-1}-positive IncHI2 plasmids might circulate in *Citrobacter* species.

Plasmidfinder also indicated that pDY2019-OXA was an IncC type plasmid. A total of 11 ARGs were Identified in pDY2019-OXA by ResFinder. These ARGs enabled pDY2019-OXA to exhibit resistance to different types of antimicrobial agents, including β -lactams (*bla*_{DHA-1} and *bla*_{OXA-10}), fluoroquinolone (*qnrB4*), sulphonamide (*sul* and *sul2*), chloramphenicol (*cmlA1*), aminoglycoside (*aadA1*), macrolide (*mph(A)*), tetracycline (*tet(D)*), rifampin (ARR-2), and trimethoprim (*dfiA14*). We further compared the sequence of pDY2019-OXA against the NCBI database by Blastn. The result showed that pDY2019-OXA shares the 99.95% identity (92% coverage) with plasmid pCC1-1/2b (MT559998), and 99.84% identity (99% coverage) with plasmid pKC3-1/2b (MT560001). It is worth noting that plasmids pKC3-1/2b and pCC1-1/2b were classified as type 1/2b IncC plasmid. The plasmid pCC1-1/2b was carried by a *C. freundii* isolate. In contrast, pKC3-1/2b was carried by a *Klebsiella pneumoniae* strain, and both of them were identified in food-producing animals originating from China. These data indicated that *bla*_{OXA-10}-carrying plasmid Identified in this study might disseminate from food-producing animals to humans, and the proliferation of IncC plasmids represents a potential public health risk.

It is reported that CFC represents approximately 29% of all opportunistic nosocomial infections.³⁰ However, there is little information about carbapenemase-producing CFC. In this work, we report an adult case of urinary tract infection caused by multi-antibiotic resistant *C. braakii*, although a single case limits this work. Furthermore, we describe the structure of pDY2019-NDM, a 348-kb IncHI2 plasmid carrying the *bla*_{NDM-1} gene, and pDY2019-OXA, a 178-kb IncC plasmid carrying the *bla*_{OXA-10} gene.

Conclusion

To the best of our knowledge, co-production of NDM-1 and OXA-10 in the same isolate has never been reported. The results of this study improved our understanding of the genetic context of *C. braakii* strains and their antibiotic resistance phenotypes and genotypes. In general, we highlighted the emergence of NDM-1-producing *C. braakii* as worrisome and emphasized the need for close surveys for controlling potential dissemination.

Nucleotide sequence accession numbers. The nucleotide sequences of the chromosome and the plasmids of *C. freundii* strain DY2019 have been deposited into DDBJ/EMBL/GenBank under accession numbers CP080539, CP080540, and CP080541, respectively.

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Disclosure

The authors report no conflicts of interest in this work.

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