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ORIGINAL RESEARCH

Characterization of Plasmid Co-Harboring NDM-1 and SHV-12 from a Multidrug-Resistant *Citrobacter freundii* Strain ZT01-0079 in China

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Background: The emergence of multidrug-resistant *Citrobacter freundii* poses daunting challenges to the treatment of clinical infections. The purpose of this study was to characterize the genome of a *C. freundii* strain with an IncX3 plasmid encoding both the $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ genes.

Methods: Strain ZT01-0079 was isolated from a clinical urine sample. The Vitek2 system was used for identification and antimicrobial susceptibility testing. The presence of $bla_{\text{NDM-1}}$ was detected by PCR and sequencing. Conjugation experiments and Southern blotting were performed to determine the transferability of the $bla_{\text{NDM-1}}$- carrying plasmid. Nanopore and Illumina sequencing were performed to better understand the genomic characteristics of the strain.

Results: Strain ZT01-0079 was identified as *C. freundii*, and the coexistence of $bla_{\text{NDM-1}}$ and multiple drug resistance genes was confirmed. Electrophoresis and Southern blotting showed that $bla_{\text{NDM-1}}$ was located on a ~53kb IncX3 plasmid. The NDM-1-encoding plasmid was successfully transferred at a frequency of 1.68×10^{-3}. Both the $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ genes were located on the self-transferable IncX3 plasmid.

Conclusion: The rapid spread of the IncX3 plasmid highlights the importance of continuous monitoring of the prevalence of NDM-1-encoding *Enterobacteriaceae*. Mutations of existing carbapenem resistance genes will bring formidable challenges to clinical treatment.

Keywords: *C. freundii*, whole-genome sequencing, genomics analysis

Background

New Delhi metallo-β-lactamase-1 (NDM-1) is an enzyme capable of hydrolyzing most β-lactam antibiotics. Since its first detection in 2009 in *Klebsiella pneumoniae*,[1] 21 NDM variants have been reported from various *Enterobacteriaceae* species.[2–10] The increasing prevalence of NDM-producing bacteria is due primarily to intra- and inter-species exchanges of a variety of self-transferring plasmids. The coexistence of NDM and other resistance genes on a single plasmid is being reported with increasing frequency and confers high-level carbapenem resistance, which poses daunting challenges to clinical management.[7,11]

Citrobacter freundii, a member of the *Enterobacteriaceae* family, is a constituent of the commensal intestinal microbiota of animal and humans.[12] However, it can cause diarrhea, sepsis,[13] meningitis,[14] respiratory[15] and urinary tract,[16] and can serve as a reservoir of antibiotic resistance genes. In recent years, due to the abuse of antibiotics, *C. freundii* has acquired increasing resistance to

common antibiotics.[17] In addition, *bla*$_{NDM-1}$-positive *C. freundii* has been reported in numerous countries that including but are not limited to China,[2–5] India,[6,7] Japan,[8] Denmark[9] and South Africa.[10] We report a carbapenem-resistant strain of *C. freundii* with coexistent *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$ genes on a transferrable IncX3 plasmid. Antimicrobial susceptibility tests, conjugation experiments, and whole-genome sequencing were performed to study the molecular characteristics of the multidrug-resistant strain.

Methods

Bacterial Identification and Isolation

Strain ZT01-0079 was isolated from a urine sample of a patient with dysuria in 2018, in Guangzhou, China. Species identification was conducted by using the VITEK2 compact system (BioMerieux, France) and confirmed by 16S rDNA sequencing. The *bla*$_{NDM}$ gene was detected by PCR amplification as previously described.[18] The isolate was obtained by conventional collection, and verbal consent was obtained as no personal information is included. All experiments were conducted in accordance with relevant regulations and approved by the Chinese PLA Center for Disease Control and Prevention.

Susceptibility Testing

The VITEK2 system (BioMérieux, France) with AST-GN09 card (bioMérieux) was used for minimum inhibitory concentrations (MICs) determinations of strain ZT01-0079 and transconjugants. The E-test method was used to determine the MIC value of meropenem. Results were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI)[19] guidelines. The following agents were tested: amikacin, aztreonam, nitrofurantoin, ciprofloxacin, piperacillin, gentamicin, cefepime, ceftriaxone, ceftazidime, tobramycin, imipenem, levofloxacin and sulfamethoxazole/trimethoprim.

S1-PFGE, Southern Blotting and Conjugation Experiment

The genomic DNA of strain ZT01-0079 was prepared in an agarose plug with S1 endonuclease (Takara, Dalian, China). PFGE separates DNA fragments by the CHEF-DR III system (Bio-Rad, Hercules, USA). PFGE runs for 15 h under the conditions of a gradient of 6.0 V/cm, a pulse time of 0.20–26.0 s and an angle of 120°. The plasmid DNA was transferred to a positively charged nylon membrane (Solabio, China) and hybridized with a digoxigenin-labeled *bla*$_{NDM-1}$ specific probe.

Strain ZT01-0079 and *Escherichia coli* strain J53 were used as donors and recipients, respectively. The ZT01-0079 and J53 strains were mixed in (LB) broth at a ratio of 1:3 and incubated at 37°C for 18 h. Transconjugants were selected on MacConkey agar plates containing meropenem (4 μg/mL) and sodium azide (150 μg/mL) for 12 h. A susceptibility test was performed to determine the horizontal transferability of drug resistance, and the corresponding transconjugants were confirmed by PCR and sequencing of the NDM gene and 16s rRNA gene, S1-PFGE and southern blotting. The frequency of conjugation was calculated as the ratio of transconjugants to recipient cells.

Whole Genome Sequencing and Comparative Genome Analysis

Genomic DNA was extracted from strain ZT01-0079 using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Whole-genome sequencing was performed using the Illumina HiSeq 2500 sequencer in Novogene (Beijing, China), with a coverage of 109X. The nanopore sequencing library was prepared using the rapid sequencing kit SQK-RAD004 (Oxford Nanotechnology, UK). The mixed assembly of Illumina and Nanopore (Oxford Nanotechnology, UK) reads was performed using Unicycler[20] (v0.4.7). PlasmidFinder[21] (https://cge.cbs.dtu.dk/services/PlasmidFinder/) was used to determine the plasmid replicon type. Genotyping was performed to query the seven domesticated genes (*aspC, clpX, fadD, mdh, arcA, dnaG and lysP*) via the multi-locus sequence typing MLST web service[22] (https://cge.cbs.dtu.dk/services/MLST/).

Nucleotide Sequence Accession Numbers23

The complete sequence of the ZT01-0079 strain and plasmids pZY-1, pZY-2 and pZY-NDM1 have been deposited in GenBank with accession numbers CP055247, CP055248, CP055249 and CP022050.

Results

Microbiological Features of Strain ZT01-0079

Strain ZT01-0079 was identified as *C. freundii*. Susceptibility testing showed that *C. freundii* ZT01-0079 exhibited resistance to aztreonam, nitrofurantoin, ciprofloxacin, piperacillin, cefepime, ceftriaxone, ceftazidime,

tobramycin, levofloxacin, and imipenem (Table 1). S1-PFGE revealed that *C. freundii* ZT01-0079 contained three plasmids (~143kb, ~89kb and ~53kb) (Figure 1). Conjugation and southern blotting showed that the bla_{NDM-1} gene was located on the ~53kb plasmid and only the NDM-1-carrying plasmid was successfully transferred to J53 at a frequency of 1.69×10^{-3} events per donor cell. Transconjugants acquired resistance to aztreonam, piperacillin, cefepime, ceftriaxone, ceftazidime, and imipenem (Table 1). The E-test showed that strain ZT01-0079 (MIC = 8) and transconjugants (MIC = 8) are both resistant to meropenem. In silico, MLST found that *C. freundii* ZT01-0079 belongs to sequence type 19 (ST19). In addition to bla_{NDM-1}, *C. freundii* ZT01-0079 carried multiple resistance genes including bla_{SHV-12}, $bla_{CTX-M-55}$, $bla_{CMY-152}$, *aph (3'')-Ib, aph (6)-Id,* bla_{TEM-1B}, *sul2, floR, tet(A)* and *aac(3)-IId*. Both the 89kb plasmid (named pZY-1) and the 143kb plasmid (named pZY-2) carried resistance genes including *aph(3'')-Ib, aph(6)-Id* (aminoglycoside resistance) and *sul2* (sulfonamide resistance). pZY-1 also carried the $bla_{CTX-M-55}$ (fluoroquinolone resistance), bla_{TEM-1B} (cephalosporin resistance), *floR* (florfenicol resistance), *tet(A)* (tigecycline resistance) genes. pZY-2 also carried the *aac(3)-IId* (aminoglycoside resistance) gene, while the 53kb plasmid (named pZY-NDM1) carried the bla_{NDM-1} and bla_{SHV-12} genes (carbapenem resistance).

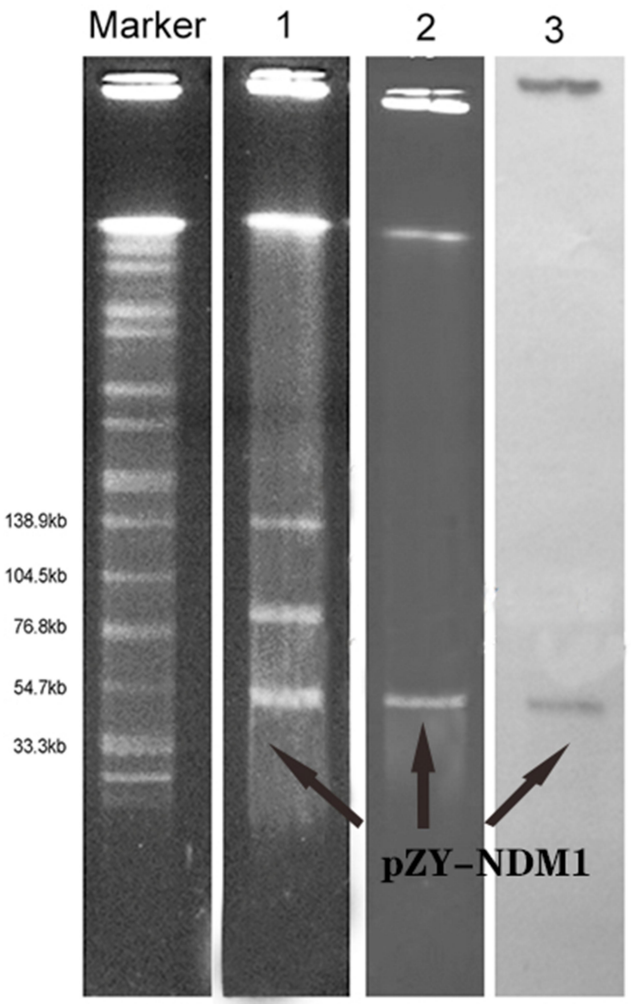

Figure 1 S1-PFGE pattern for strain ZT01-0079 and Southern blot analysis of bla_{NDM-1} genes. Lane marker, *Salmonella* serotype Braenderup strain H9812 as a reference size standard; Lane 1, PFGE result of S1-digested plasmid DNA of strain ZT01-0079; Lane 2, PFGE patterns for S1-digested plasmid DNA of the transconjugants J53.; Lane 3, Southern blotting of strain ZT01-0079 with the probes specific to the bla_{NDM-1} gene. The black arrows indicate the plasmid pZY-NDM1.

Table 1 Antibiotic Susceptibilities of *C. freundii* ZT01-0079, Transconjugant and *E. coli* J53 (Recipient)

Antimicrobial	MIC (μg/mL)		
	ZT01-0079	Transconjudant	Recipients (*E. coli* J53)
Amikacin	8	≤2	≤2
Aztreonam	≥64	16	≤1
Nitrofurantoin	128	≤16	≤16
Ciprofloxacin	≥4	≤0.25	≤0.25
Piperacillin	≥128	64	≤4
Gentamicin	≥16	≤1	≤1
Cefepime	≥64	8	≤4
Ceftriaxone	≥64	≥64	≤4
Ceftazidime	≥64	≥64	≤1
Tobramycin	≥16	≤1	≤1
Imipenem	≥16	≥16	≤1
Levofloxacin	≥8	≤0.25	≤0.25
Sulfamethoxazole-trimethoprim	≤20	≤20	≤20

Genetic Analysis of pZY-NDM1

Plasmid pZY-NDM1 is a 53573 bp circular plasmid with an average GC content of 49.24% and has 76 open reading frames. pZY-NDM1 belongs to type IncX3. BLAST comparison disclosed that the genome sequence of pZY-NDM1 shared >99% similarity with plasmids pNDM-HK3694 (Genbank accession JX104760.1) and p309074-NDM (Genbank accession MH909346.1). These three plasmids share a typical IncX plasmid backbone composed of replication (*repB*), plasmid stability *(parA, parB*), plasmid maintenance (*hns, top*) and type IV secretion system (taxA, *taxB, taxC, VirD1, VirD2, VirD3-4, VirD5* and *VirD6, VirD8, VirD9, VirD10, VirD11*) encoding regions (Figure 2A). The 19.66 kb

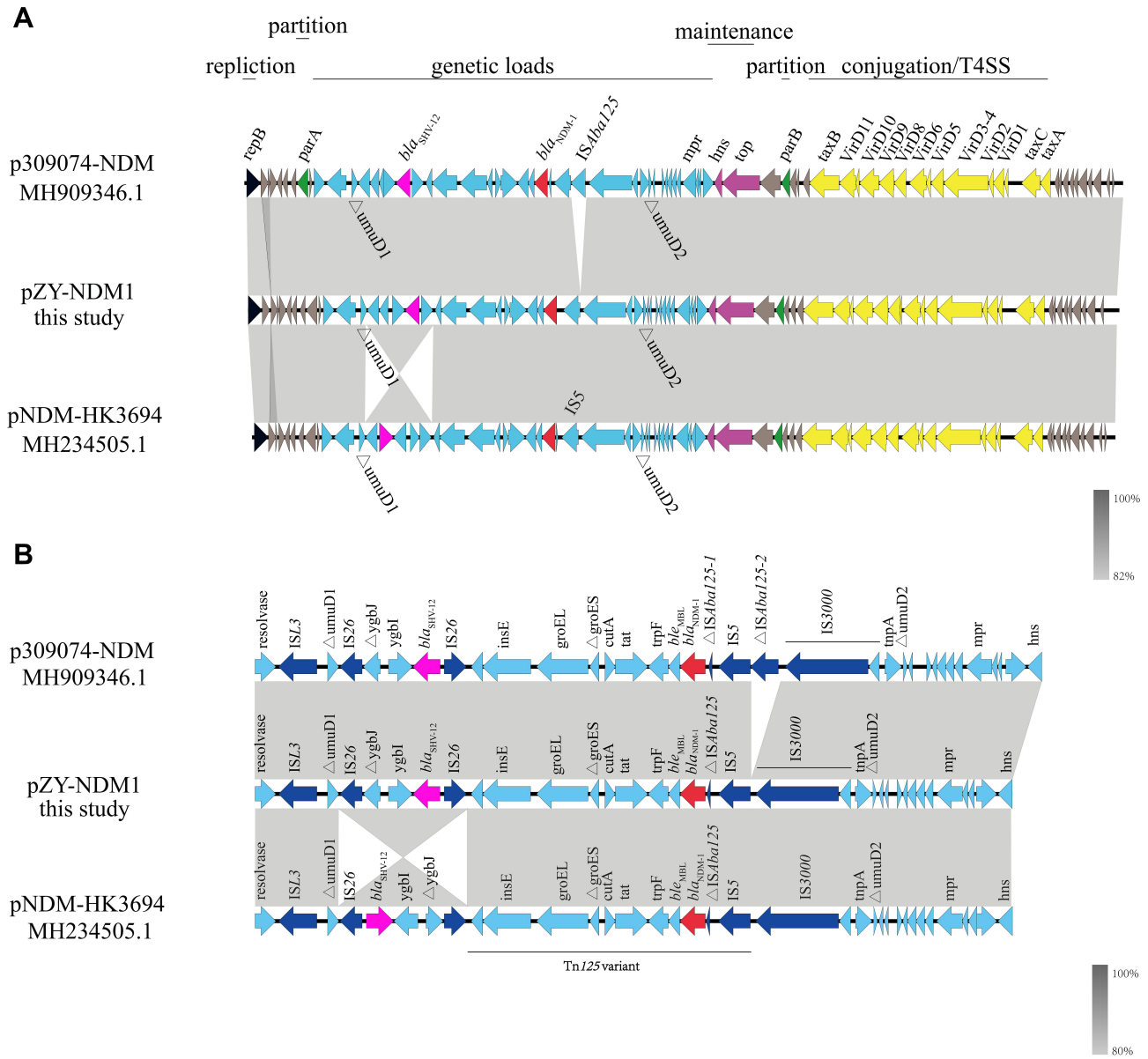

Figure 2 Schematic diagram of (**A**) plasmid comparison: plasmids p309074-NDM; pZY-NDM1 and pNDM-HK3694 and (**B**) Resistance area analysis: plasmids p309074-NDM; pZY-NDM1 and pNDM-HK3694. The open reading frames are indicated by arrows. The black, green, purple, and yellow arrows represent genes associated with replication, plasmid stability, plasmid maintenance and the type IV secretion system. The bla_{SHV-12} gene is shown in pink, while the bla_{NDM-1} gene is shown in red. The accessory modules are shown in blue. Other genes of the backbone are shown in dark gray. Homology regions among different plasmids are denoted by light gray.

genetic load region of pZY-NDM1 located between the *hns* and *resolvease* genes, contained Tn*3*, IS*3000*, the transposon Tn*125* variant encoding bla_{NDM-1} and a mobile element containing bla_{SHV-12} and IS*L3*. The transposon Tn*125* variant served as a mobile element for the transfer of bla_{NDM-1} and comprised IS*5*, ΔIS*Aba125, bla*$_{NDM-1}$, the bleomycin resistance gene *ble, trpF, tat, cutA, groES* and *groEL* as previously described.[23] Compared with p309074-NDM, the pZY-NDM1 plasmid has a deletion of an 891bp ΔIS*Aba125* fragment, which is located between IS*5* and IS*3000*. Compared with traditional Tn*125*, the upstream of the pZY-NDM1 plasmid lacks IS*Aba125*, and the downstream IS*Aba125* is replaced to ΔIS*Aba125* and IS*5* (Figure 2B).

The bla_{SHV-12}-carrying transposon was located in two opposite IS*26* elements and composed of Δ*ygbJ, ygbI* and bla_{SHV-12}. Compared with pNDM-HK3694, the bla_{SHV-12}-carrying transposon of pZY-NDM1 was reversed. In the transposon Tn*125* variant, the two ends of the bla_{SHV-12}-carrying

transposon are divided into two incomplete Δ*umuD1* and Δ*umuD2*, which may serve as insertion sites of regions containing the bla_{SHV-12}-carrying transposons, Tn*125* variant and *tnpA* (Figure 2B).

Discussion

The prevalence of NDM-1-producing bacteria is receiving increasing attention as a threat to global health. Most bla_{NDM-1}-positive isolates of diverse species of *Enterobacteriaceae* show high-level resistance to β-lactam antibiotics. Concurrently, the bla_{NDM-1} gene has also spread in many environmental and animal reservoirs, such as sewage, rivers, soil, and many mammals and poultry in Asia and the Middle East.[2,9,12,24] However, reports on *C. freundii* are still rare. Therefore, we investigated the genomic characteristics of multidrug-resistant *C. freundii* (strain ZT01-0079) isolated from a clinical urine specimen. This strain has the IncX3 plasmid pZY-NDM1 that co-harbors bla_{NDM-1} and bla_{SHV-12}. Plasmids belonging to the IncX3 group usually have a wide host range and self-conjugation.[12,25,26] Host-range plasmids usually appear with variable frequencies ranging from 10^{-3} to 10^{-6}.[27] pZY-NDM1 showed a relatively high transfer frequency, proving its great potential for cross-species transfer.

The coexistence of bla_{NDM-1} and other β-lactamase genes in IncX3 plasmids, which have a broad host range, mediates resistance to broad-spectrum antibiotics, such as carbapenems and cephalosporins, and can be transferred between *Enterobacteriaceae*.[28] Notably, there are few reports of such plasmids in *C. freundii*, and there is a potential risk of spreading this plasmid and monitoring should be strengthened.

Conclusions

In summary, we characterized the genomic basis of multidrug resistance in *C. freundii* ZT01-0079 strain. MLST revealed that strain ZT01-0079 belongs to ST19. ZT01-0079 carried multiple *bla* genes, which increased the level of carbapenem resistance. The transferable plasmid pZY-NDM1 carries bla_{NDM-1} and bla_{SHV-12} genes, and may thereby serve as a common vector for the rapid dissemination of carbapenemase-encoding genes. Our findings further underscore the threat of increased NDM-1 prevalence in *Enterobacteriaceae* and emphasize that increased resources and effort should be dedicated to monitoring the potentially rapid spread of NDM-1-encoding plasmids.

Data Sharing Statement

Genomic and plasmid sequences of strain ZT01-0079 were deposited in GenBank under accession CP055247, CP055248, CP055249 and CP022050.

Ethics Approval and Informed Consent

The authors state that all experimental protocols were approved by the institutional ethics committees of Academy of Military Medical Sciences.

Consent for Publication

The clinically separated samples used in this study are routine hospital procedures. We do not use patients' personal information, so written consent is not required.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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