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ORIGINAL RESEARCH

Complete-Genome Sequencing and Comparative Genomic Characterization of an IMP-4 Producing *Citrobacter freundii* Isolate from Patient with Diarrhea

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Background: *Citrobacter freundii* is the most common class of pathogens in the genus *Citrobacter* and is an important pathogen associated with certain underlying diseases or immune dysfunction. The aim of this study was to elucidate the resistance mechanism of clinically derived carbapenem-resistant *C. freundii* isolate and to characterize the genetic environment and delivery pattern of the IncN1 plasmid carrying the bla_{IMP-4} gene from *C. freundii* isolate.

Materials and Methods: We identified a clinical isolate of *C. freundii* L91 carrying bla_{IMP-4} and performed phylogenetic analysis by whole-genome sequencing. The complete genomic sequence of L91 was obtained using the Illumina HiSeq 4000-PE150 and PacBio *RS* II platforms. Antimicrobial susceptibility testing was determined by the VITEK 2 system. Plasmid characteristics were presented by S1-pulsed-field gel electrophoresis (PFGE), Southern blotting and conjugation experiments.

Results: S1-PFGE, Southern blot and conjugation assay confirmed the presence of bla_{IMP-4} genes on a conjugative plasmid in this isolate. *C. freundii* L91 and transconjugant L91-*E. coli* 600 strains both showed resistance to carbapenems. In silico analysis further showed that pIMP-4-L91 is an IncN1 plasmid with a length of 51,042 bp. Furthermore, bla_{IMP-4} gene was found encoded in the bla_{IMP-4}-*qacG2-aacA4-catB3* cassette array within a class 1 integron. A conserved structure sequence (ΔIS*Kpn27*-bla_{IMP-4}-ΔIS*Sen2-hp-hp*-IS*6100*) was found in the upstream and downstream of the bla_{IMP-4}.

Conclusion: We performed a comprehensive phylogenetic analysis of carbapenemase-resistant *C. freundii* and elucidated the resistance mechanism of clinically derived *C. freundii* L91. Not only that, we also found that the bla_{IMP-4} gene is located on the IncN1 plasmid and has a horizontal transfer function and a certain ability to spread. To lower the risk of the dissemination of such *C. freundii* isolates in clinical settings, more surveillance is needed in the future.

Keywords: *Citrobacter freundii*, CPE, whole-genome sequencing, SNP, IncN, integron

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Background

The increasing prevalence of bacterial resistance has become a major problem affecting global public health.[1] Carbapenems are considered to be the last line of defense against multi-drug resistant Gram-negative strains, but with the advent of carbapenemase producing Enterobacteriaceae (CPE), it has made greater emphasis

on clinical care.[2] Carbapenemases, including *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and oxacillinase (OXA)-48 medicated antibiotic resistance.[3] IMP-type CPEs have been reported globally since the first report of IMP-1 from *Pseudomonas aeruginosa* in Japan.[4] The IMP-4 metalo-β-lactamase was first discovered in Hong Kong.[5] IMP-producing carbapenem-resistant bacteria have been found in many parts of the world, especially in Australia, causing severe outbreaks of various Gram-negative bacteria.[6]

Citrobacter belongs to Gram-negative bacilli and is widely found in water, food, soil, and intestines of animals and humans.[7] *Citrobacter* isolates are not only environmental pollutants with low virulence, but also can cause a wide range of infections, including the urinary tract, liver, biliary tract, peritoneum, intestine, bone, respiratory tract, endocardium, wound, soft tissue, and meninges.[8] *C. freundii* is the most common class of pathogens in the genus *Citrobacter* and is an important pathogen associated with certain underlying diseases or immune dysfunction.[9]

In fact, IMP-4 may be only a carbapenemase common in some areas, but on a global scale, the most common type is NDM-type metallo-β-lactamase.[10] The first metallo-β-lactamase reported in China is IMP-4, reported in 2001, which was detected on the plasmid from *Citrobacter youngae*.[11] Since then, only a few studies have analyzed the genomic background of the $bla_{\text{IMP-4}}$ gene among *C. freundii* isolates. Besides that, systematic analysis of the phylogeny and carbapenem resistance mechanism of *C. freundii* is still lacking. In this study, we identified a clinical *C. freundii* isolate L91 carrying $bla_{\text{IMP-4}}$, which was phylogenetically analyzed by whole-genome sequencing. In addition, we also elucidated the resistance mechanism of *C. freundii* isolate L91 and characterized the genetic environment and delivery pattern of the IncN1 plasmid carrying the $bla_{\text{IMP-4}}$ gene from this isolate.

Materials and Methods

Sample Collection

In the routine monitoring of carbapenem-resistant Enterobacteriaceae (CRE) isolates, we collected fecal samples from patients with diarrhea from the First Affiliated Hospital of Zhejiang University (FAHZU) since January 2016. We collected a fecal sample from a 91-year-old patient with advanced liver cancer on May 25, 2016, and isolated *C. freundii* L91 from it. The patient was initially diagnosed with cirrhosis and pulmonary infection at admission. The collected fecal samples were placed in 2–3 mL Brain Heart Infusion Broth (BD, Sparks, USA) was performed overnight before being applied on the screening plates. Then, Mac Conkey agar (OXOID, Hampshire, UK) plates were added to 2 mg/L meropenem (Meilunbio, Dalian, China) for preliminary screening of CRE isolates. The isolated *C. freundii* is numbered L91.

Verification of Carbapenemase-Producing *C. freundii*

Species confirmation of presumptive *C. freundii* L91 isolates were performed with matrix-assist laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker, Bremen, Germany). The carbapenemase gene was identified using PCR and DNA sequencing.

Plasmid Characterization and Conjugation Assay

The plasmid was characterized by S1-PFGE, and the location of bla_{IMP} was identified by Southern hybridization with digoxigenin-labelled bla_{IMP} probe using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). The plasmid conjugation experiment was carried out using *E. coli* 600 as a recipient strain. Next, the transconjugants were grown on agar (OXOID, Hampshire, UK) medium containing with 200 mg/L sodium azide and 2 mg/L meropenem. Finally, MALDI-TOFMS was identified for transconjugants, and bla_{IMP} was tested by PCR to ensure that the plasmid was successfully transferred to the recipient strain.

Antibiotic Susceptibility Testing of L91

L91 and L91-*E. coli* 600 strains were cultured on blood agar overnight at 37°C, while *E. coli* ATCC 25922 was used as a quality control. Minimum inhibitory concentrations (MICs) of piperacillin/tazobactam, ampicillin, cefazolin, cefoxitin, ceftriaxone, cefepime, ertapenem, imipenem, amikacin, aztreonam, gentamicin, tobramycin, ciprofloxacin, levofloxacin, tigecycline and nitrofurantoin antibiotics were determined by VITEK 2 system with AST-GN16 panel. The results of antimicrobial susceptibility testing were interpreted according to CLSI standards (https://clsi.org).

Whole-Genome Sequencing of L91

Genomic DNA was extracted using a DNA kit (Omega Bio-tek, Norcross, USA). The DNA was subsequently sequenced using Illumina-HiSeq 4000-PE150 (Illumina, San Diego, CA, USA) and PacBio RS II platform (Pacific Biosciences, California, USA). After sequencing, the complete genomic sequence of *C. freundii* L91 was generated using the Unicycler[12] by combining the sequencing results. The whole-genome sequence of the L91 was deposited in GenBank under the following accession numbers: SCVZ00000000. Additionally, the acquired antimicrobial resistance genes and replicon type of plasmid were identified using the online tools (http://www.genomicepidemiology.org/). The bacterial genome was annotated using the RAST server (http://rast.nmpdr.org/) and the transposon and IS elements were identified using the ISFinder database (https://www-is.biotoul.fr/). A circular image of multiple plasmid comparisons was generated using the BLAST Ring Image Generator (BRIG). The genetic environment surrounding the bla_{IMP-4} was annotated using Easyfig 2.2.3.

Phylogenetic Reconstruction and Analysis

Using the kSNP program to identify the core genomic single nucleotide polymorphism (SNP) on the WGS data of L91 using (GCA_901456285.1) as the reference genome.[13] kSNP is a program based on k-mer analysis. Kchooser is used to evaluate the optimal value of k-mer before kSNP is run. After the run of kSNP program, the output file was used for further analysis.[14] The maximum likelihood tree of the core SNP matrix output of kSNP was generated by using iTOL (https://itol.embl.de/).

Results

C. freundii L91 isolate was isolated from the feces of a 92-year-old male liver cancer patient. Single colony was selected from the plate of activated strains and identified as *C. freundii* by MALDI-TOF-MS. The isolate was recovered from the selected medium for PCR and sequencing, and the bla_{IMP-4} gene was confirmed.

Table 1 shows the results of the antibiotic susceptibility testing. The results show that *C. freundii* L91 is sensitive to amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, tigecycline, and nitrofurantoin. The isolate L91 showed resistance to imipenem, ertapenem, piperacillin/clavulanate, ampicillin, cefazolin, cefoxitin, ceftriaxone, cefepime, and aztreonam. The MIC values of imipenem, ertapenem are 16mg/L and 4mg/L, respectively. Moreover, the plasmid-transferred isolate L91-*E. coli* 600 showed the same drug resistance profile to L91. L91-*E. coli* 600 also showed resistance to imipenem and ertapenem, with the MIC values of 16 mg/L and 4 mg/L, respectively.

Table 1 MIC Values of Antimicrobials for *C. freundii* L91, Recipient Strain *E. coli* 600 and Transconjugant L91- *E. coli* 600

Antimicrobials	MIC Values (mg/L)		
	C. freundii L91	L91- *E. coli* 600	*E. coli* 600
Piperacillin/clavulanate	32/R	32/R	4/S
Ampicillin	32/R	32/R	1/S
Cefazolin	64/R	64R/	0.25/S
Cefoxitin	64/R	64/R	1/S
Ceftriaxone	64/R	64/R	0.25/S
Cefepime	8/R	64/R	0.25/S
Imipenem	16/R	16/R	0.5/S
Ertapenem	4/R	8/R	0.015/S
Amikacin	1/S	1/S	2/S
Aztreonam	16/R	1/R	1/S
Gentamicin	0.5/S	0.5/S	1/S
Tobramycin	0.5/S	0.5/S	0.25/S
Ciprofloxacin	0.25/S	0.5/S	0.5/S
Levofloxacin	1/S	1/S	0.5/S
Tigecycline	0.5/S	0.5/S	0.5/S
Nitrofurantoin	16/S	16/S	0.25/S

The genomic characteristics of *C. freundii* L91 were described in Table S1. The results showed that the L91 genome consisted of a 4,951,125 bp circular chromosome and two plasmids. The average G + C content of the circular chromosome was 51.1%, and the chromosome contained 4953 protein-coding genes and 83 tRNAs. In addition, the chromosome encodes the resistance genes *qnr-B38* and $bla_{CMY-109}$. The clinical isolate of *C. freundii* L91 contains two plasmids with size of 304, 128 bp and 51,042 bp, respectively.

S1-PFGE and Southern blot analysis demonstrated that the L91 isolate contained a ~50 kb plasmid, harbouring both bla_{IMP-4} and *qnrS1* genes (Figure S1). The whole-genome sequencing results showed that the plasmid pIMP-4-L91 was an IncN-type plasmid with a length of 51,042 bp and contained 80 protein-coding genes with a GC content of 50.7% (Table S1). The replication, partitioning, and transfer systems showed similarity to those of other sequenced plasmids deposited in GenBank. The plasmid pIMP-4-L91 in this study and the *C. freundii* plasmid pIMP-HK1500 (accession number KT989599.1), pIMP-FJ1503 (accession number

KU051710.1) from Hong Kong, *C. freundii* plasmid pP10159-2 from Chongqing (Accession number KU051710.1), *C. freundii* plasmid pIMP-ECL14-57 (accession number MH727565.1) from Zhengzhou showed extremely high similarity. The plasmid map of pIMP-4-L91 shows the genes and their locations (Figure 1A). By comparison, we found that another ~304, 128 bp plasmid contained 318 protein-coding genes with a GC content of 48.9% (Table S1). The comparison analysis of the plasmid found that the plasmid did not carry any drug-resistant genes, and there was no exact plasmid type.

The genome sequence of 48 strains of *C. freundii* carrying the carbapenemase encoding gene was downloaded from the Pathogen Detection (https://www.ncbi.nlm.nih.gov/pathogens/), and phylogenetic analysis was performed together with *C. freundii* L91. As shown in Figure 2, *C. freundii* L91 is closely related to (GCA_004004805.1) from France carrying $bla_{\text{OXA-48}}$. In addition, four isolates from China (GCA_002252125.1), (GCA_002252025.1), (GCA_001702455.1) and (GCA_002215385.1) showed closely phylogenetic relationships, three of which carried $bla_{\text{KPC-2}}$, and another isolate carries $bla_{\text{IMP-4}}$.

Discussion

In this study, we isolated a clinical isolate of *C. freundii* L91 carrying $bla_{\text{IMP-4}}$ and performed phylogenetic analysis by whole-genome sequencing. The plasmid carrying $bla_{\text{IMP-4}}$ in *C. freundii* L91 is of the IncN1 type. According to the study, the resistance of *Citrobacter* to carbapenems is increasing year by year.[15] In fact, the carbapenemase-resistant genes frequently reported in *C. freundii* are bla_{KPC},[16,17] bla_{NDM},[18,19] and bla_{VIM},[20,21] and only a few studies have reported the bla_{IMP} gene in *C. freundii*. Not only that, the research of the whole-genome sequencing of *C. freundii* isolates with $bla_{\text{IMP-4}}$ is even rarer.[22] Therefore, we performed phylogenetic analysis of *C. freundii* L91 by whole-genome sequencing. In addition, we also elucidated the resistance mechanism of L91 and characterized the genetic environment of the IncN1 plasmid carrying the $bla_{\text{IMP-4}}$.

Antibiotic susceptibility testing showed that L91 was resistant to imipenem and ertapenem, indicating that the resistant phenotype was consistent with the resistant genotype (Table 1). The plasmid-transferred isolate L91-*E. coli* 600 also showed resistance to imipenem and ertapenem, indicating that the plasmid carrying the $bla_{\text{IMP-4}}$ gene has horizontal metastatic ability and is capable of expressing drug resistance. This result indicates that the transferability of the plasmid increases the risk of drug-resistant bacteria and poses a great challenge to clinical treatment.

The IMP-type enzymes are among the clinically most important metallo-β-lactamase and can hydrolyze almost all β-lactams including carbapenems.[23] Up to now, IMP-type enzymes have been reported in *Enterobacteriaceae*,[24] *Acinetobacter*,[5] and *Pseudomonas*.[25] IMP-type metalloenzymes have been reported worldwide, with a higher prevalence in southern Europe and Asia.[23] The $bla_{\text{IMP-4}}$ gene in *C. freundii* has rarely been reported.[22] In China, KPC-type enzyme is the most common carbapenemase, followed by NDM-type enzyme, and the detection rate of IMP-type enzyme is relatively low.[26] In this study, $bla_{\text{IMP-4}}$ was found in the $bla_{\text{IMP-4}}$-*qacG2-aacA4-catB3* cassette array in a class 1 integron. This cassette array was described in *Acinetobacter baumannii* from a Hong Kong outbreak[5] and from Singapore,[27] *Enterobacter cloacae* from Australia,[28] *Klebsiella pneumoniae* pJIBE401,[29] and *Enterobacteriaceae* in silver gulls in Australia,[30] where the $bla_{\text{IMP-4}}$ cassette is in a *sul1*-type class 1 integron. Importantly, a conserved structure sequence (ΔIS*Kpn27*-$bla_{\text{IMP-4}}$-ΔIS*Sen2-hp-hp*-IS*6100*) was found in the upstream and downstream of the $bla_{\text{IMP-4}}$ (Figure 1B). Downstream of $bla_{\text{IMP-4}}$ also contained restriction modification systems; It contained an *ecoRIIR* gene. The *ecoRIIR* have 100 % nucleotide identity with the *Escherichia coli* modification methylase gene, *ecoRII*.[31] This restriction modification system not only assists in the defense against phage infection, but also contributes to the spread and maintenance of plasmids encoding these systems.[32] The results of phylogenetic analysis showed that *C. freundii* L91 is closely related to (GCA_004004805.1) from France carrying $bla_{\text{OXA-48}}$, and it is far from the other four isolates from China (GCA_002252125.1, GCA_002252025.1, GCA_001702455.1, GCA_002215385.1). Interestingly, *C. freundii* L91 has the same type of antibiotic resistance genes as (GCA_004004805.1), but *C. freundii* L91 is from the clinic (GCA_004004805.1) from the environment (Figure 2). This result indicates that the genomic sequences of L91 and (GCA_004004805.1) are similar, and the SNPs are small, and there may be a genetic relationship.

In this study, $bla_{\text{IMP-4}}$ was located on an IncN type plasmid (~50 kb), and plasmids belonging to the IncN incompatible group typically have a broad host range and high transmission efficiency, and they play an important role in the transmission of clinically important resistance determinants.[23,33,34] Location of the major carbapenem resistance genes such as bla_{IMP},[35,36]

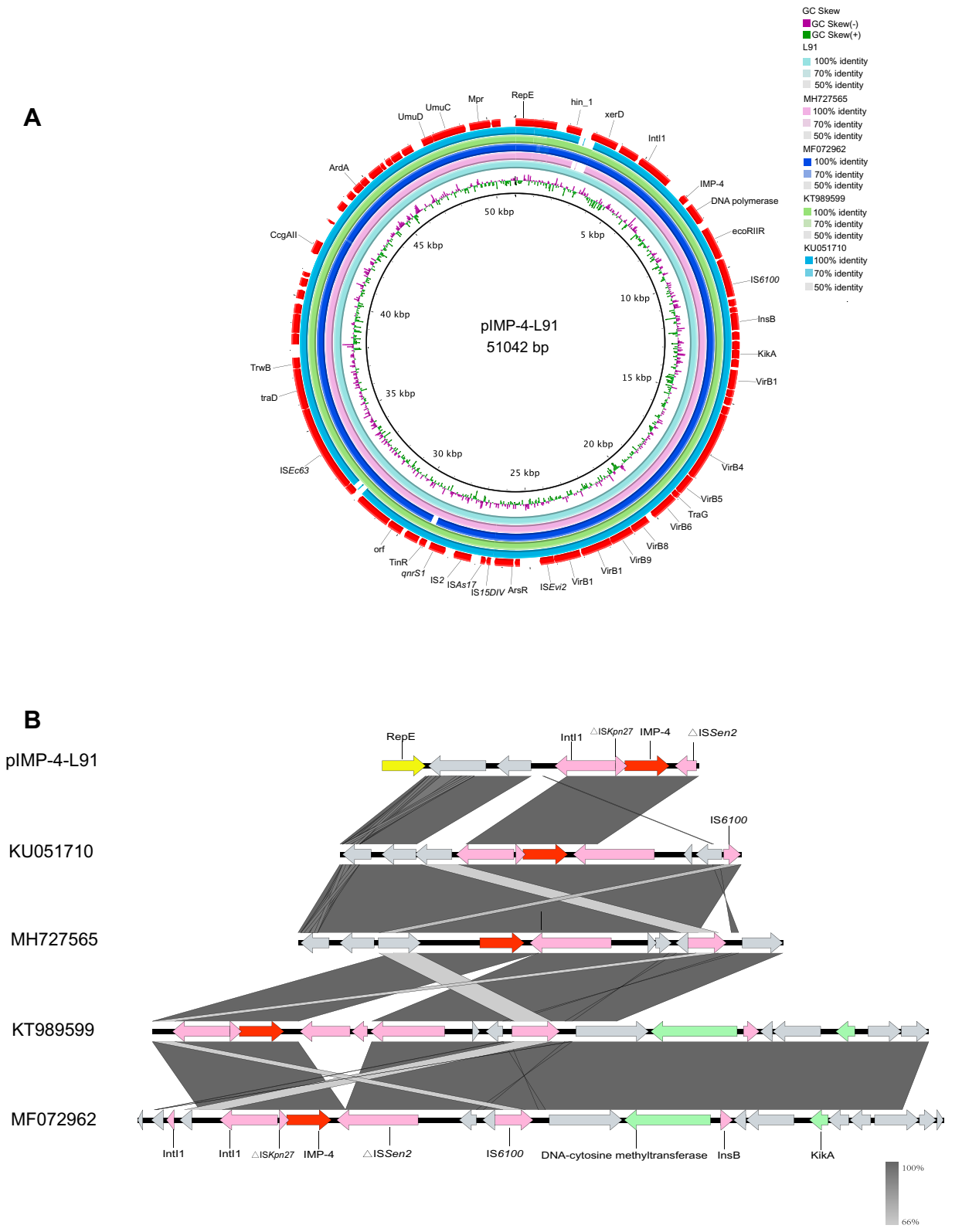

Figure 1 Genomic analyses of pIMP-4-L91 plasmid. (**A**) Comparison of the pIMP-4-L91plasmid sequence identified in isolate L91 with the bla_{IMP-4}. This figure was generated using BRIG. (**B**) Genetic context of bla_{IMP-4} on pIMP-4-L91 and related plasmids. The GenBank accession numbers are KU051710.1, MH727565.1, KT989599.1, MF072962.1. Open reading frames (ORFs) are shown as arrows, and indicated according to their putative functions. Shared regions with high degree of sequence similarity are indicated by gray. Conjugal transfer associated genes were colored as pink; Red arrows point antibiotic resistance genes, and yellow arrows indicate the replication initiator. Hypothetical protein encoded genes are colored by grey.

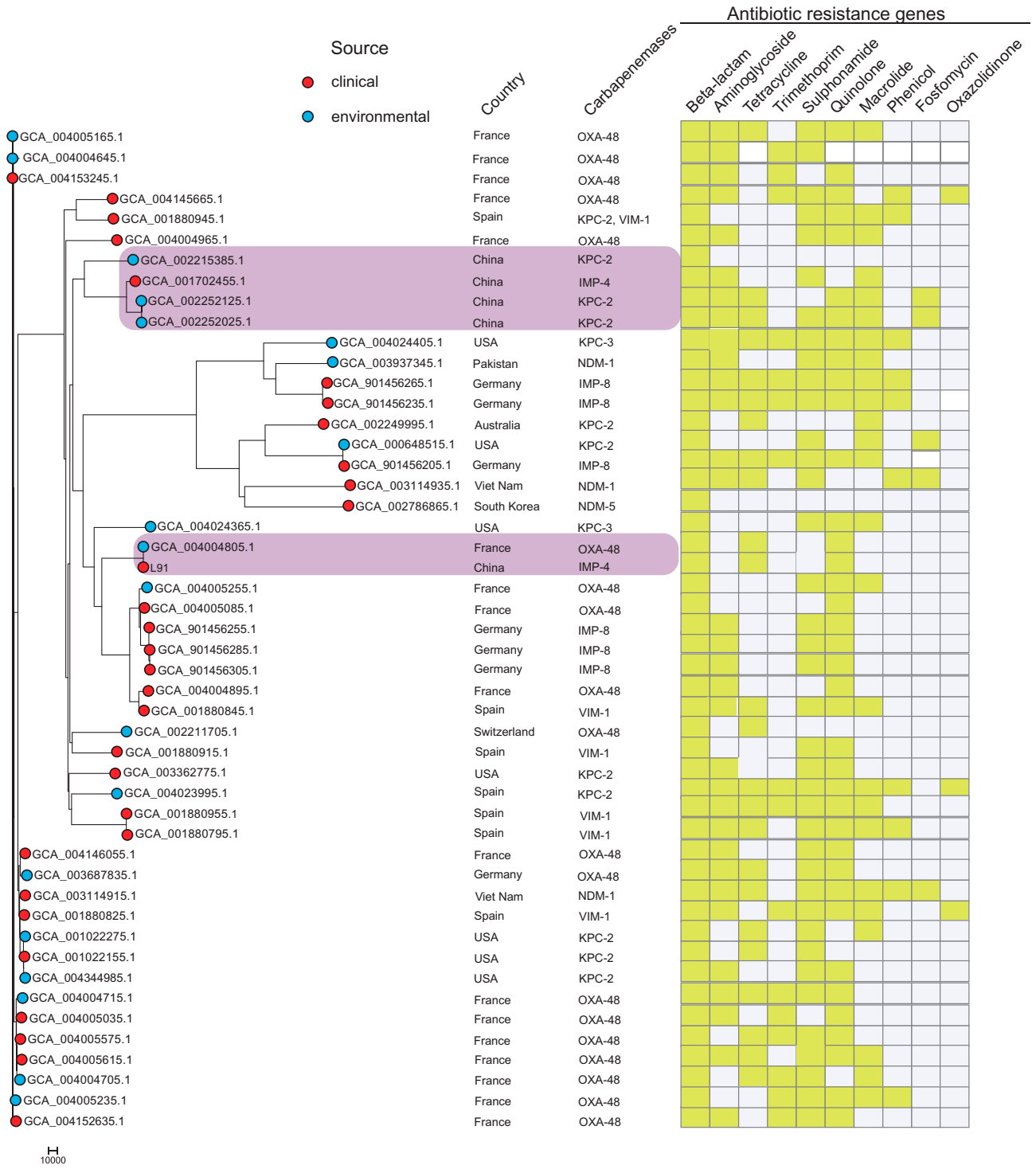

Figure 2 At the far left of the figure is the maximum likelihood core-gene phylogeny generated by kSNP. The red circle represents the isolate from the hospital and the blue circle represents the isolate from the environment. The figure also indicates which country the isolate is from and the carbapenemase gene carried. The gene sequence of the isolate was uploaded to Center of Genomic Epidemiology (http://www.genomicepidemiology.org/) to obtain antibiotic resistance gene type contained in the isolate. The heatmap is used to display the types of antibiotic resistance genes. Yellow indicates that the isolate carries such genes, and colorless means that the genes are not carried.

bla$_{KPC}$,[37,38] *bla*$_{NDM}$[39,40] and *bla*$_{VIM}$[34,41] has been found on different IncN-type plasmids. The IncN plasmids can be further divided into three subgroups, namely IncN1to IncN3, with their reference plasmids R46 (accession number AY046276), p271A[42] and pN-Cit,[43] respectively. In our study, the *C. freundii* L91 belongs to the IncN1 type plasmid, and we found many plasmids similar to p-IMP-4 through comparison with the database

(Figure 1A), indicating that this type of plasmids spread widely. There is no obvious difference between the five plasmids in Figure 1A. After comparison, all five plasmids carry bla_{IMP-4} and *qnrS1* resistance genes, and all plasmid types are IncN1. In addition, the plasmid carries multiple mobile transfer elements, which plays an important role in the propagation of the plasmid.[44] The backbone of IncN1 type plasmid includes regions of replication, maintenance, and conjugal transfer. Certain plasmid types have been implicated in the endemic spread of IMP-4 in Australia. These include the IncM2 type (pEl1573) in Sydney, the IncA/C type in Melbourne and the IncHI2 type in Queensland.[29,45,46] In Hong Kong, IMP-4 is more common in carbapenemase-producing Enterobacteriaceae (CPE) and is mostly located on the IncA/C- and IncN-type plasmids.[47–49] However, in China, little is known about the bacterial clones and plasmid types involved in bla_{IMP-4} transmission. Therefore, routine genomic monitoring of clinical strains resistant to carbapenem and plasmids carrying bla_{IMP} genes is urgently needed.

The emergence of carbapenem-resistant *C. freundii* highlights the importance of preventing the transmission of hospital-acquired pathogens. The increase in Enterobacteriaceae bacteria carrying the carbapenemase gene poses a challenge to clinical treatment. This situation requires us to carry out more monitoring to reduce the spread of such bacteria.

Conclusion

Collectively, we identified a bla_{IMP-4} positive *C. freundii* isolate and reported its complete genomic sequence by using PacBio sequencing reads including Illumina sequencing reads. We performed a comprehensive phylogenetic analysis of carbapenemase-resistant *C. freundii* and elucidated the resistance mechanism of clinically derived *C. freundii* L91. Not only that, we also found that the bla_{IMP-4} gene is located on the IncN1 plasmid and has a horizontal transfer function and a certain ability to spread. To lower the risk of the dissemination of such *C. freundii* isolates in clinical settings, more surveillance is needed in the future.

Data Sharing Statement

Full datasets analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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