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Characterization of a conjugative IncFII/IncR plasmid harboring bla_{KPC-2} in a clinical ST91 *Citrobacter freundii* isolate

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Abstract

Background Carbapenem-resistant *Citrobacter freundii* is increasingly reported and recognized as an opportunistic host of mobile carbapenemase-encoding elements in healthcare-associated settings. However, lineage-associated plasmid vehicles and their transfer-related features in this species remain incompletely characterized.

Objective This study aims to characterize the first IncFII(pBK30683)/IncR plasmid carrying bla_{KPC-2} in *C. freundii* ST91, focusing on its transferability, stability, and the role of plasmid lineages in carbapenem resistance dissemination.

Methods We investigated a urine-derived ST91 *C. freundii* isolate (MAS5905) through antimicrobial susceptibility testing, whole-genome sequencing, conjugation, 20-day plasmid stability, and growth curve assays. Comparative genomics included plasmid phylogeny of pMAS5905-KPC-like sequences and ST91 phylogenomics.

Results The 5.64 Mb genome comprised a chromosome and three plasmids. bla_{KPC-2} was located on an IncFII(pBK30683)/IncR plasmid pMAS5905-KPC carrying a complete conjugation module, the *mer* operon, and the anti-CRISPR determinant AcrIE9, with the bla_{KPC-2} embedded in an IS*26*-bracketed ΔTn*6296*-like module (IS*Kpn27*-bla_{KPC-2}-ΔIS*Kpn6*). The plasmid transferred to *E. coli* at 1.93×10^{-5} was retained at 100% over 20 days, and imposed a significant fitness cost. Phenotypically, MAS5905 was resistant to multiple β-lactams (including ertapenem and meropenem) and fluoroquinolones, while remaining susceptible to cefepime, ceftazidime, trimethoprim-sulfamethoxazole, aminoglycosides, tetracyclines, and nitrofurantoin. Plasmid comparisons resolved six IncFII/IncR clades with a conserved backbone and broad host range. ST91 phylogenomics revealed multiple clades and, in the analyzed public dataset, a high carbapenemase gene prevalence (75.76%), dominated by bla_{KPC} (31.82%) and $bla_{OXA-48-like}$ (30.30%).

Conclusion To our knowledge, this is the first characterization of an IncFII(pBK30683)/IncR plasmid carrying bla_{KPC-2} in a clinical *C. freundii* ST91 isolate. The findings highlight plasmid lineages as drivers of carbapenem resistance

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dissemination and underscore the need for plasmid-focused genomic surveillance, particularly for monitoring transferable resistance vehicles across clinically relevant hosts.

Keywords *Citrobacter freundii*, ST91, bla_{KPC-2}, IncFII(pBK30683)/IncR, Conjugative plasmid

Introduction

The rapid global dissemination of carbapenem-resistant Enterobacterales (CRE) poses an urgent public health threat. *Citrobacter freundii*, typically regarded as an opportunistic pathogen of secondary clinical importance, has been reported in healthcare-associated infections such as urinary tract infections, bloodstream infections, and wound infections [1]. Carbapenemase-producing *C. freundii*, which carry genes such as bla_{KPC} and bla_{NDM}, are of particular concern because the encoded enzymes hydrolyze carbapenems, compromising their efficacy and severely limiting treatment options [2, 3]. These carbapenemase genes are frequently located on large conjugative plasmids, promoting horizontal gene transfer across species and thereby accelerating the dissemination of antimicrobial resistance (AMR) [4, 5].

Among *C. freundii* lineages, sequence type 91 (ST91) has gained prominence owing to its association with carbapenemase production and evidence of regional expansion in surveillance datasets [6, 7]. In France, *C. freundii* ST91 has been linked to the spread of bla_{OXA-48}, bla_{NDM-1}, and $bla_{OXA-181}$ [7]. Moreover, bla_{KPC-2} has been detected in *C. freundii*, sometimes co-occurring with other resistance determinants and further constraining treatment options [8, 9]. Beyond clinical settings, *C. freundii* can persist in environmental reservoirs, including wastewater, and on medical devices, which may facilitate onward AMR transmission [10]. The acquisition and mobilization of these genes on mobile genetic elements (MGEs), particularly conjugative plasmids, are central drivers of their dissemination [5]. However, the lineage-specific genetic features and mechanisms governing plasmid transfer in *C. freundii* remain poorly understood. Importantly, the clinical relevance of *C. freundii* increasingly lies in its capacity to serve as a reservoir and conduit for mobile carbapenemase-encoding plasmids, rather than in its intrinsic virulence or prevalence.

Research on carbapenemase dissemination within Enterobacterales has focused predominantly on *Klebsiella pneumoniae* and *Escherichia coli*, whereas *C. freundii* has only recently received systematic attention [11, 12]. Plasmids carrying bla_{KPC} often encode complete conjugation systems and exhibit high transfer potential, enabling rapid interspecies and intraspecies spread [13, 14]. Consistent with this, plasmids bearing IncFII or IncR replicons are frequently linked to bla_{KPC} and commonly harbor MGEs that facilitate gene mobilization [15–17]. Despite these advances, the phylogenetic distribution of carbapenemase genes within *C. freundii* ST91 and the host range, conjugation modules, and genetic context of IncFII/IncR-type bla_{KPC} plasmids remain incompletely characterized.

In this study, we characterized the complete genome of *C. freundii* MAS5905 (ST91) recovered from a urine sample, with a focus on plasmid architecture, transferability, and resistance mechanisms. To our knowledge, this is the first report of an IncFII(pBK30683)/IncR-type plasmid carrying bla_{KPC-2} in *C. freundii.* We delineated the conjugation machinery and genetic context of pMAS5905-KPC, and demonstrated plasmid transfer, stable maintenance in the absence of selection, and an associated fitness cost. To contextualize these findings, we reconstructed a phylogeny of pMAS5905-KPC-like plasmids and a core-genome single nucleotide polymorphisms (SNPs) phylogeny of ST91 isolates, resolving lineage structure and the distribution of carbapenemase genes. Collectively, these results address key gaps in the host range and genetic architecture of IncFII(pBK30683)/IncR-type bla_{KPC} plasmids and inform surveillance and control strategies for carbapenem-resistant *C. freundii* (CRCF) and related pathogens.

Materials and methods

Bacterial strain and identification

C. freundii MAS5905 was isolated from a urine sample from a patient in Ma'anshan, Anhui, China, in 2023 during routine clinical diagnostics and public health surveillance. Identification was performed using the VITEK 2 (GN identification cards), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16S rRNA gene sequencing. PCR amplification and Sanger sequencing with previously reported primers confirmed the presence of the bla_{KPC} gene [18]. Sequence analysis was performed with the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/).

Antimicrobial susceptibility testing (AST)

Minimum inhibitory concentrations (MICs) were determined by broth microdilution using the Sensititre™ Gram Negative GN4F AST plate (Thermo Fisher Scientific, Waltham, MA, USA). AST was performed against twenty-four antimicrobial agents. Tigecycline MICs were interpreted according to the U.S. Food and Drug Administration (FDA) breakpoints [19]. MICs for all other agents were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2025, https://

clsi.org) performance standards. *E. coli* ATCC 25922 was used as a quality control strain.

Whole-genome sequencing (WGS) and bioinformatics analysis

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and sequenced on the Illumina NovaSeq 6000 platform with paired-end 150 bp reads, as well as on the PacBio Sequel IIe platform. PacBio long reads were quality filtered using NanoFilt v2.8.0 [20], followed by long-read assembly using Canu v2.2 [21]. The resulting assemblies were polished using Pilon v1.24 [22] with Illumina short reads. Assembly quality was assessed using BUSCO v5.5.2 [23]. Average nucleotide identity (ANI) values were calculated using FastANI v1.3.34 [24] to confirm species identity with a ≥ 95% cutoff [25]. Genome annotation was conducted using Prokka v1.14.6 [26] and Prodigal v2.6.3 [27], while RAST 2.0 (https://rast.nmpdr.org/, accessed on 27 August 2025) [28], Pfam v37.0 [29], and the NCBI non-redundant (NR) protein database were used for functional annotation. Insertion sequences (ISs) were identified through the ISfinder database [30]. Antimicrobial resistance genes (ARGs) were predicted using ResFinder v4.3.1 with default parameters [31, 32] and the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/, accessed on 9 September 2025) [33]. The Resistance Gene Identifier (RGI) v6.0.3 was run with an E-value cutoff of 1e-5, identity ≥ 80%, and coverage ≥ 80%. Plasmid replicons were identified using PlasmidFinder v2.1.6 [34]. Plasmid transferability was assessed in silico using oriTfinder v2.0 (https://bioinfo-mml.sjtu.edu.cn/oriTfinder/, accessed on 28 August 2025) [35], identifying *oriT*, relaxase, type IV coupling protein (T4CP), and type IV secretion system (T4SS) components, and recording the presence of ARGs, metal resistance genes, and anti-CRISPR loci. BLAST searches against the NCBI database were used to identify sequences with high plasmid similarity. Gene-cluster visualization and map generation were performed using ChiPlot (https://www.chiplot.online/, accessed on 25 October 2025) and Proksee (https://proksee.ca/, accessed on 4 October 2025) [36].

Plasmid conjugation assay and plasmid stability testing

Conjugation was performed by filter mating to assess transferability of the bla_{KPC-2}-carrying plasmid from *C. freundii* MAS5905 [37]. Streptomycin-resistant *E. coli* EC600 served as the recipient. Transconjugants were selected on MacConkey agar containing streptomycin (1500 μg/mL) and meropenem (2 μg/mL), and confirmed by MALDI-TOF MS and PCR. AST for the recipient and transconjugants was performed as described above. Conjugation frequency (CF) was calculated as follows: CF = (number of transconjugant bacterial colonies × $10x$)/(number of recipient bacterial colonies × $10y$), where x and y represent the dilution factors. Plasmid stability was evaluated as previously described [38], with slight modifications. Cultures were grown in Luria–Bertani (LB) broth at 37 °C with shaking (200 rpm) and passaged every 24 h into fresh LB broth at a 1:1000 dilution for 20 consecutive days. On even-numbered days (days 2–20), serial dilutions were plated on LB agar, and approximately 60 colonies per time point were randomly selected and PCR-screened for bla_{KPC-2} [18]. All experiments were performed in triplicate.

Fitness cost assessment

Fitness cost was evaluated by growth curve analysis. Growth kinetics were measured using the Bioscreen C Automated Growth Curve Analysis System (FP-1100-C, Oy Growth Curves Ab Ltd, Finland), and OD_{600} was recorded at 37°C. Each strain was assayed in triplicate. Growth differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

Phylogenetic analysis of pMAS5905-KPC-like plasmids

Plasmid sequences showing ≥ 80% nucleotide identity to pMAS5905-KPC were retrieved from NCBI ($n = 36$; Supplementary Table S1). Pairwise distances were computed with GGDC 3.0 (Genome-to-Genome Distance Calculator 3.0, https://ggdc.dsmz.de/ggdc.php, accessed on 14 September 2025). A phylogeny was inferred with FastME v2.1.6.1 [39] under the minimum-evolution framework with 1,000 bootstrap replicates, and the tree was visualized and annotated in iTOL v7 (https://itol.embl.de, accessed on 17 September 2025).

Phylogenomics of ST91 *C. freundii*

A total of 3,052 publicly available *C. freundii* genomes were downloaded from the NCBI Pathogen Detection database (https://www.ncbi.nlm.nih.gov/pathogens/isolates/, accessed on 19 July 2025), with detailed information provided in Supplementary Table S2. Sequence types (STs) were assigned with MLST v2.23.0 (https://github.com/tseemann/mlst) using the PubMLST database (https://pubmlst.org/), and the analysis then focused on ST91 genomes. In total, 66 ST91 *C. freundii* genomes, including MAS5905 and 65 publicly available assemblies, were included in this analysis (Supplementary Table S3). GFF3 files were generated with Prokka v1.14.6 [26]. Core genes were inferred with Roary v3.13.0 [40] at 95% identity. SNP-sites v2.5.1 [41] was used to extract SNPs from the core-gene alignment, after which recombination was removed with Gubbins v3.3.1 [42]. A maximum-likelihood tree was built from the recombination-filtered core-genome SNPs using IQ-TREE v2.3.4 under the GTR + G

model with 1,000 bootstrap replicates [43]. The final tree was visualized using iTOL v7 (https://itol.embl.de).

Statistical analyses

Data were analyzed using GraphPad Prism 9.5.0. One-way ANOVA was applied to assess significant differences, with $P < 0.05$ considered statistically significant.

Results

General features of *C. freundii* MAS5905 genome and mobility of its plasmids

The genome of *C. freundii* MAS5905 consisted of a circular chromosome (5,061,872 bp; 51.93% GC) and three plasmids, totaling approximately 5.64 Mb (Fig. 1A; Table 1). The plasmids were pMAS5905-1 (293,639 bp; 48.27% GC), pMAS5905-2 (163,225 bp; 53.77% GC), and pMAS5905-KPC (122,033 bp; 53.29% GC). Replicon typing assigned pMAS5905-2 to IncFII/IncFIB and pMAS5905-KPC to IncFII(pBK30683)/IncR, whereas pMAS5905-1 was non-typable. The $bla_{\text{KPC-2}}$ gene was located on pMAS5905-KPC. No additional acquired AMR genes were detected on pMAS5905-1 or pMAS5905-2. The chromosome also carried ARGs affecting multiple antimicrobial classes, including β-lactams ($bla_{\text{CMY-150}}$), fluoroquinolones (*qnrB38* with target substitutions in *gyrA* and *parC*), aminoglycosides (*aac(6′)-Ib*), fosfomycin (*uhpT*), as well as peptide antimicrobials (*bacA*, *pmrF*). In addition, multidrug efflux and envelope regulators were present (*acrD, acrAB-tolC*, *emrAB*, *mdfA*, *mdtB/C/G/M*, *msbA*, *kpnE/kpnF*, *marA*/*marR*, *soxS*, *H-NS*, *CRP*, *leuO*, *rsmA*, *cpxA*, *kdpE*), with potential impacts on other classes such as macrolides, tetracyclines, phenicols, rifamycins, and glycylcyclines (Table 1).

A complete conjugation module was identified on pMAS5905-KPC, including *oriT*, relaxase, T4CP, and T4SS components, consistent with a conjugative plasmid. In contrast, pMAS5905-1 and pMAS5905-2 encoded relaxase, T4CP, and T4SS components, as well as multiple metal resistance genes, but lacked a detectable *oriT* site. Additionally, pMAS5905-KPC carried a complete *mer* metal resistance operon (*merA, merD, merE, merR, merP,* and *merT*) and an anti-CRISPR determinant AcrIE9 (Fig. 1B). Comparative genomic analysis was performed between pMAS5905-KPC and eight IncFII(pBK30683)/IncR-type plasmids available in the NCBI database (accession numbers: CP046896.1, MN823984.1, CP021195.1, CP109830.1, MN823986.1, MN823985.1, CP160802.1, and CP100079.1) (Fig. 1B). All nine plasmids were recovered from isolates in China, with the eight reference plasmids originating from *K. pneumoniae* ($n = 4$), *Serratia marcescens* ($n = 3$), and *E. coli* ($n = 1$), collected between 2012 and 2023 (Supplementary Table S1). The IncFII(pBK30683)/IncR backbone was highly conserved across these plasmids, whereas sequence divergence was primarily concentrated in accessory resistance regions. Notably, pMAS5905-KPC represents the first report of an IncFII(pBK30683)/IncR-type KPC plasmid in *C. freundii*. On pMAS5905-KPC, $bla_{\text{KPC-2}}$ resided in a ΔTn*6296*-like module bracketed by IS*26*, with the core IS*Kpn27*-$bla_{\text{KPC-2}}$-ΔIS*Kpn6* arrangement (Fig. 1C), supporting IS*26*-mediated acquisition and dissemination.

Transferability, plasmid stability, and fitness cost of $bla_{\text{KPC-2}}$-harboring plasmid

Conjugation assays demonstrated that the $bla_{\text{KPC-2}}$-carrying plasmid was successfully transferred from *C. freundii* MAS5905 to *E. coli* EC600, with a conjugation frequency of 1.93×10^{-5} (Table 2). The presence of $bla_{\text{KPC-2}}$ in the transconjugant MAS5905T(KPC) was confirmed by PCR. Plasmid stability assays showed that the $bla_{\text{KPC-2}}$ plasmid was stably maintained in both the donor *C. freundii* MAS5905 and the *E. coli* transconjugant MAS5905T(KPC) over 20 consecutive days of serial passage, with 100% retention throughout the experiment (Fig. 2A). Significant growth differences ($P < 0.001$) were observed among the donor strain MAS5905, the recipient *E. coli* EC600, and the $bla_{\text{KPC-2}}$-positive transconjugant MAS5905T(KPC) (Fig. 2B). While the transconjugant exhibited a comparable lag phase and exponential phase to *E. coli* EC600, it reached a significantly lower stationary-phase OD ($P < 0.001$), indicating a measurable fitness cost associated with carriage of the $bla_{\text{KPC-2}}$ plasmid in the absence of antimicrobial pressure.

Phenotypic and genotypic characterization of resistance

AST results for *C. freundii* MAS5905, the recipient *E. coli* EC600, and the transconjugant MAS5905T(KPC) were summarized in Table 3. *C. freundii* MAS5905 exhibited resistance to multiple β-lactam agents, including penicillins (ampicillin and piperacillin), cephalosporins (cefazolin and ceftriaxone), monobactams (aztreonam), carbapenems (ertapenem and meropenem), and β-lactam/β-lactamase inhibitor combinations (ampicillin-sulbactam, ticarcillin-clavulanic acid, and piperacillin-tazobactam), as well as fluoroquinolones (ciprofloxacin and levofloxacin). The isolate remained susceptible to cefepime, ceftazidime, trimethoprim-sulfamethoxazole, aminoglycosides (amikacin, gentamicin, tobramycin), tetracyclines (tetracycline, minocycline, tigecycline), and nitrofurantoin. WGS confirmed the presence of $bla_{\text{KPC-2}}$ gene, consistent with the observed β-lactam and carbapenem resistance phenotypes (Table 1). The *E. coli* transconjugant MAS5905T(KPC) displayed a resistance pattern largely consistent with the donor strain, showing elevated MICs to β-lactams and carbapenems, confirming successful transfer of the $bla_{\text{KPC-2}}$-harboring plasmid. Both donor and transconjugant strains were susceptible

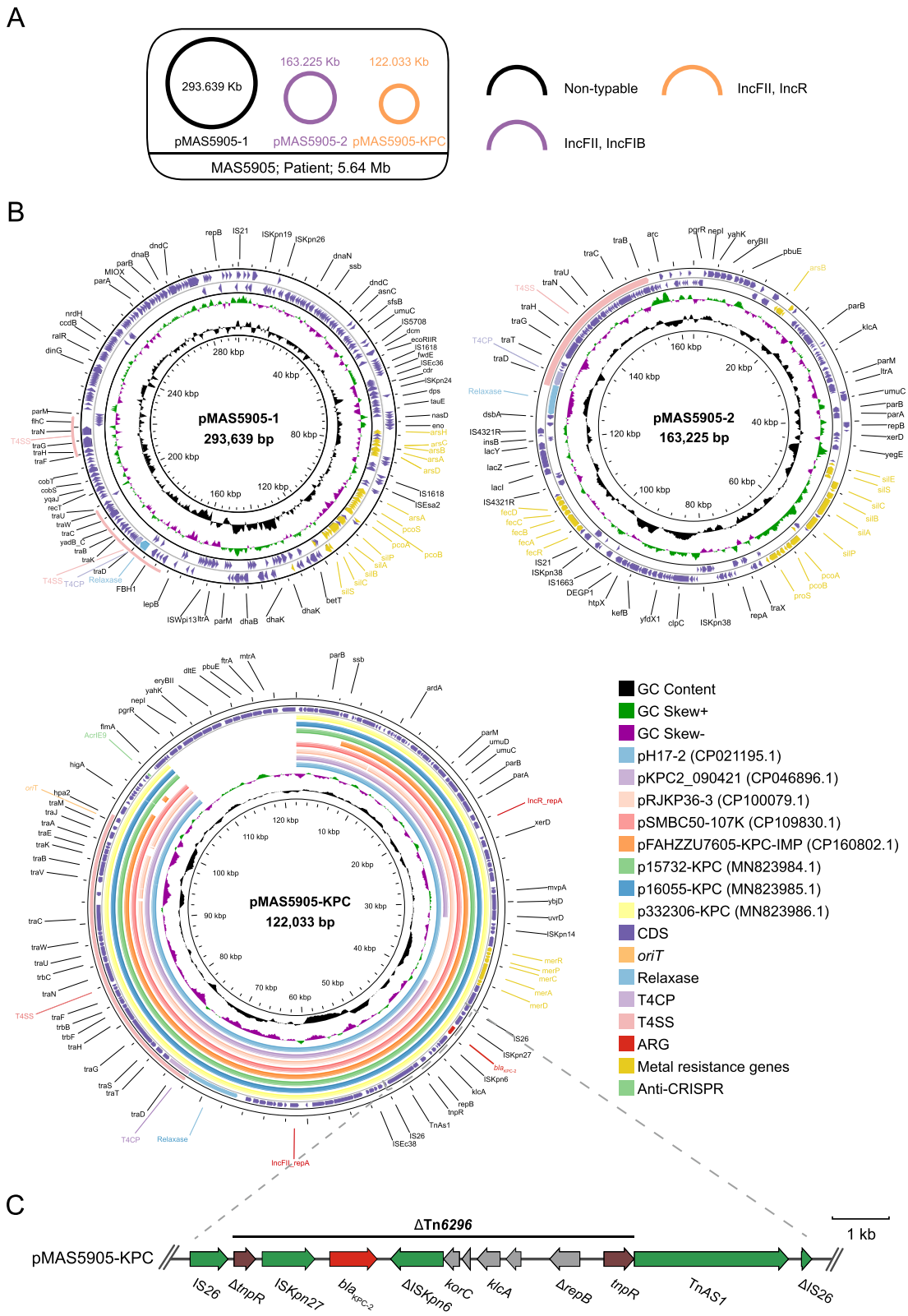

Fig. 1 Genomic architecture and comparative analysis of plasmids pMAS5905-1, pMAS5905-2, and pMAS5905-KPC. **A** Overview of the *C. freundii* MAS5905 genome. **B** Circular maps of plasmids pMAS5905-1, pMAS5905-2, and pMAS5905-KPC, showing different genomic features. Reference plasmids from *K. pneumoniae*, *S. marcescens*, and *E. coli* are shown for comparison, including pH17-2 (CP021195.1), pKPC2_090421 (CP046896.1), pRJKP36-3 (CP100079.1), pSMBC50-107K (CP109830.1), and others. **C** Genetic context of bla_{KPC-2} in *C. freundii* MAS5905

Table 1 Genetic characteristics of *C. freundii* MAS5905

Genetic characteristics	MAS5905 chromosome	pMAS5905-1	pMAS5905-2	pMAS5905-KPC
Size	5,061,872 bp	293,639 bp	163,225 bp	122,033 bp
GC Content (%)	51.93	48.27	53.77	53.29
CDSs	4,824	371	180	147
rRNA	25	-	-	-
sRNA	143	4	5	5
tRNA	86	-	1	-
Resistance genes	*aac(6')-If*, *acrA*, *acrB*, *acrD*, *bacA*, *baeR*, $bla_{CMY-150}$, *cpxA*, *CRP*, *emrA*, *emrB*, *emrR*, *EF-Tu*, *gyrA*, *H-NS*, *kdpE*, *kpnE*, *kpnF*, *leuO*, *marA*, *marR*, *mdfA*, *mdtB*, *mdtC*, *mdtG*, *mdtM*, *msbA*, *parC*, *pmrF*, *qnrB38*, *rsmA*, *soxS*, *uhpT*	-	-	bla_{KPC-2}
bla_{KPC-2} gene location	-	-	-	IncFII(pBK30683)/IncR plasmid

Note: -, none

Table 2 Conjugation frequency for MAS5905 and *E. coli* EC600

Donor	Recipient	Number of bacterial colonies (dilution factor)		CF
		MacConkey (SM)	MacConkey (MEM + SM)	
MAS5905	EC600	61 ($x=10^{-6}$)	118 ($y=10^{-1}$)	1.93×10^{-5}

SM streptomycin, *MEM* meropenem, *CF* conjugation frequency, *CF* = (number of transconjugant bacterial colonies × 10*x*)/(number of recipient bacterial colonies × 10*y*), where *x* and *y* represent the dilution factors

to non-β-lactam agents, including aminoglycosides, tetracyclines, sulfonamides, and nitrofurans, whereas the recipient *E. coli* EC600 remained fully susceptible to all tested antimicrobials.

Distribution of pMAS5905-KPC-like plasmids

We performed phylogenetic and comparative genomic analyses to investigate the distribution and genetic diversity of pMAS5905-KPC-like plasmids (Fig. 3). A total of 36 homologous plasmids sharing ≥ 80% nucleotide sequence identity with pMAS5905-KPC were retrieved from the NCBI database (Supplementary Table S1). These plasmids were predominantly identified in *K. pneumoniae*, *S. marcescens*, and *E. coli*, with sporadic occurrences in *K. aerogenes* and *C. freundii*. Most isolates originated from human clinical samples in China between 2012 and 2024, with additional records from Indonesia and the United States (USA).

Phylogenetic reconstruction divided these plasmids into six major clades (Clades 1–6), within which pMAS5905-KPC clustered most closely with plasmids from *K. pneumoniae*, *S. marcescens*, and *E. coli* carrying the same IncFII(pBK30683)/IncR replicon type (Fig. 3). All plasmids encoded a complete conjugation module (*oriT*, relaxase, T4CP, and T4SS), indicating a high potential for horizontal dissemination across Enterobacterales. Most pMAS5905-KPC-like plasmids shared a highly similar accessory gene composition, as evidenced by the

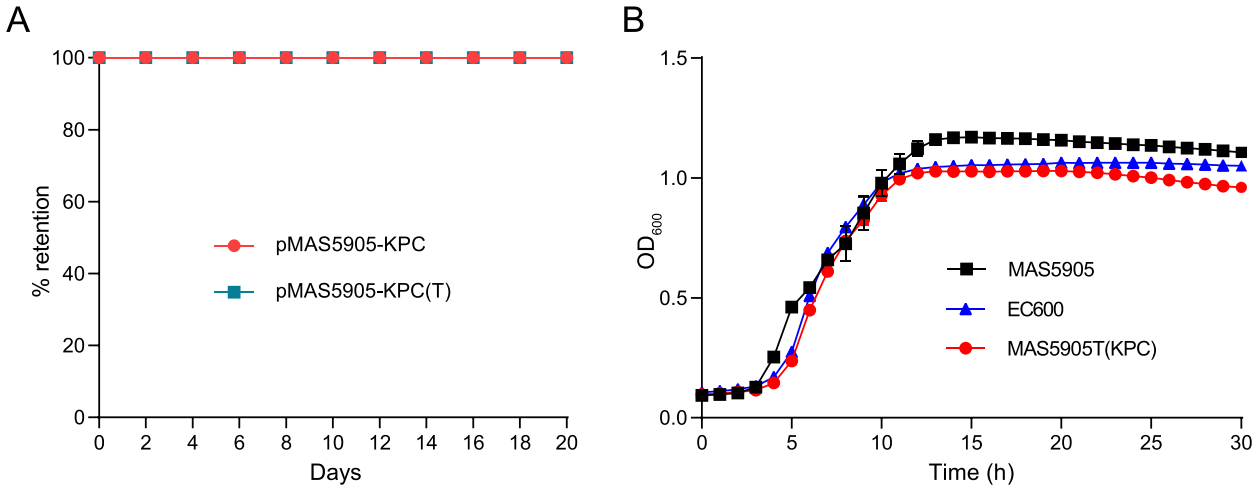

Fig. 2 Stability of the bla_{KPC-2} plasmid and growth curves of donor, recipient, and transconjugant strains. **A** Retention of the bla_{KPC-2}-carrying plasmids pMAS5905-KPC and pMAS5905-KPC(T) over 20 days of serial passage in the donor *C. freundii* MAS5905 and the *E. coli* transconjugant MAS5905T(KPC), respectively. **B** Growth curves of the donor strain MAS5905, the recipient strain *E. coli* EC600, and the transconjugant MAS5905T(KPC) within 30 h

Table 3 Minimum inhibitory concentrations (MICs) and antimicrobial susceptibility profiles of *C. freundii* MAS5905, its bla_{KPC-2}-positive transconjugant: MAS5905T(KPC), and *E. coli* EC600

Antimicrobial class/agent	MICs (μg/mL) [Antimicrobial susceptibility]		
	MAS5905	MAS5905T(KPC)	*E. coli* EC600
Aminoglycosides			
Amikacin	< 8[S]	< 8[S]	< 8[S]
Gentamicin	< 2[S]	< 2[S]	< 2[S]
Tobramycin	< 2[S]	< 2[S]	< 2[S]
Penicillins			
Ampicillin	> 16[R]	> 16[R]	< 8[S]
Piperacillin	> 64[R]	> 64[R]	< 16[S]
Monobactams			
Aztreonam	> 16[R]	> 16[R]	< 1[S]
Cephalosporins			
Cefazolin	> 16[R]	> 16[R]	4[S]
Cefepime	< 4[S]	< 4[S]	< 4[S]
Ceftazidime	4[S]	4[S]	< 1[S]
Ceftriaxone	16[R]	16[R]	< 0.5[S]
Carbapenems			
Doripenem	2[I]	2[I]	< 0.5[S]
Ertapenem	4[R]	2[R]	< 0.25[S]
Imipenem	2[I]	2[I]	< 0.5[S]
Meropenem	4[R]	4[R]	< 0.5[S]
β-lactam/β-lactamase inhibitors			
Ampicillin-Sulbactam	> 16/8[R]	> 16/8[R]	< 4/2[S]
Ticarcillin-Clavulanic acid	> 64/2[R]	> 64/2[R]	< 8/2[S]
Piperacillin-Tazobactam	128/4[R]	64/4[R]	< 8/4[S]
Fluoroquinolones			
Ciprofloxacin	2[R]	< 0.5[S]	< 0.5[S]
Levofloxacin	2[R]	< 1[S]	< 1[S]
Sulfonamides			
Trimethoprim-Sulfamethoxazole	< 2/38[S]	< 2/38[S]	< 2/38[S]
Tetracyclines			
Tetracycline	< 4[S]	< 4[S]	< 4[S]
Minocycline	2[S]	2[S]	< 1[S]
Tigecycline	< 1[S]	< 1[S]	< 1[S]
Nitrofurans			
Nitrofurantoin	< 32[S]	< 32[S]	< 32[S]

R resistant, *I* intermediate, *S* susceptible

conserved distribution of AMR, metal resistance, and anti-CRISPR determinants across the phylogeny (Fig. 3). All plasmids harbored bla_{KPC-2}, and several additionally carried bla_{IMP-4}, bla_{TEM-1B}, bla_{TEM-1C}, and *qnrS1*. In addition, most plasmids contained *mer* operon metal resistance genes (*merA, merD, merE, merR, merP,* and *merT*), and several possessed the anti-CRISPR determinant AcrIE9. These features are commonly associated with enhanced plasmid stability and adaptability under selective environmental and host pressures.

Phylogenomic structure and carbapenemase gene distribution of ST91 *C. freundii*

To elucidate the population structure of the ST91 *C. freundii* lineage, a maximum-likelihood phylogenetic tree was constructed using core-genome SNPs from MAS5905 and 65 publicly available ST91 genomes (Fig. 4; Supplementary Table S3). The analysis revealed substantial genomic diversity within the ST91 lineage, which was separated into several well-supported clades. MAS5905 was located on an independent branch, distinct from other Chinese isolates, indicating a separate evolutionary trajectory within this lineage. Among the 66 ST91 *C. freundii* isolates, 68.18% (45/66) were derived from clinical sources, 19.70% (13/66) from environmental sources, and 12.12% (8/66) from unknown sources. The isolates were geographically widespread, recovered predominantly from the USA, followed by the United Kingdom, Canada, Germany, Bulgaria, and China, with sporadic isolates reported from Fiji, the Netherlands, Portugal, Singapore, Japan, and other countries. These isolates were collected between 2012 and 2024, highlighting both the long-term persistence and global dissemination of the ST91 lineage.

Carbapenemase gene profiling showed that 75.76% (50/66) of the isolates carried at least one carbapenemase gene, while 16.67% (11/66) possessed combinations of two. The predominant genotype was bla_{KPC} (31.82%, 21/66), followed by $bla_{OXA-48-like}$ (30.30%, 20/66), bla_{IMP} (13.64%, 9/66), bla_{NDM} (10.61%, 7/66), and bla_{VIM} (6.06%, 4/66). MAS5905 carried only bla_{KPC-2}, consistent with its carbapenem-resistant phenotype and the absence of additional carbapenemase determinants.

Discussion

This study resolved the complete genome of *C. freundii* ST91 strain MAS5905 and revealed a conjugative IncFII(pBK30683)/IncR plasmid carrying bla_{KPC-2}. The plasmid exhibited efficient transfer, long-term stability, and a measurable fitness cost in *E. coli*, indicating a balance between persistence and burden. Comparative and phylogenomic analyses placed pMAS5905-KPC within a broader IncFII(pBK30683)/IncR plasmid lineage circulating across Enterobacterales and showed that, in the analyzed public dataset, ST91 is genetically diverse and commonly carries carbapenemase genes. These findings expand the known host range of IncFII(pBK30683)/IncR-type bla_{KPC} plasmids to *C. freundii* and suggest that this species can serve as an opportunistic host and conduit for transferable carbapenemase-encoding plasmids. Importantly, this study is not intended to infer the prevalence or clinical burden of carbapenem-resistant *C. freundii*, but rather to provide a case-based, plasmid-centric genomic characterization of a transferable bla_{KPC-2} vehicle.

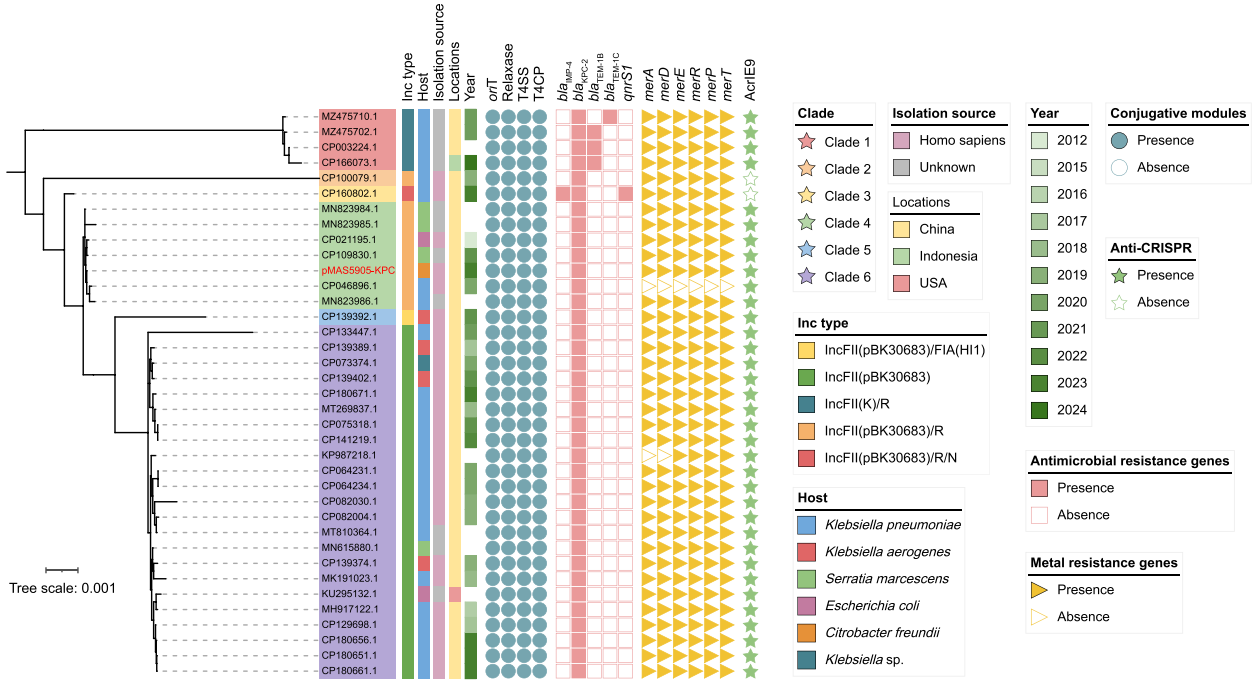

Fig. 3 Phylogeny of pMAS5905-KPC-like plasmids and distribution of conjugation, carbapenemase, metal resistance, and anti-CRISPR determinants. A distance-based phylogeny was built for pMAS5905-KPC and homologous plasmids with ≥ 80% nucleotide sequence identity. pMAS5905-KPC is highlighted in red. The right panel shows the presence (filled) or absence (open) of conjugation modules (circles), antimicrobial resistance genes (squares), *mer* operon metal resistance genes (triangles), and the anti-CRISPR determinant AcrIE9 (star). Metadata bars denote Inc replicon type, host species, isolation source, country of origin, collection year, and clade assignment (1–6). T4CP, type IV coupling protein. T4SS, type IV secretion system

MGEs, such as plasmids, integrons, transposons, and ISs, play a crucial role in the evolution of bacteria and the horizontal dissemination of adaptive cargo, notably ARGs [44]. Previous studies have shown that IS*26* and Tn*6296* have been recognized as important vehicles for the transmission of the $bla_{\text{KPC-2}}$ gene across plasmids and bacterial hosts [45, 46]. In pMAS5905-KPC, the $bla_{\text{KPC-2}}$ locus resides within an IS*26*-bracketed ΔTn*6296*-like module, consistent with pervasive IS*26*-mediated mobilization among Chinese Enterobacterales [45, 46]. IS*26* captures resistance cassettes and drives structural diversification via targeted deletion and recombination [47], accounting for the recurrent appearance of near-identical $bla_{\text{KPC-2}}$ modules on distinct plasmid backbones. These patterns indicate that $bla_{\text{KPC-2}}$ is not confined to a single plasmid backbone or bacterial host, underscoring the potential for interspecies spread and supporting plasmid-focused genomic surveillance.

Conjugative plasmids remain central drivers of clinical AMR evolution and its dissemination within and between species [48]. The IncFII(pBK30683)/IncR scaffold of pMAS5905-KPC aligns with epidemic bla_{KPC} vectors characterized in *K. pneumoniae* and *E. coli*. Its occurrence in *C. freundii* provides evidence for cross-genus permeability of bla_{KPC} plasmid pools and suggests that vehicles optimized in *K. pneumoniae* can be repeatedly redeployed into secondary hosts under healthcare-associated selection pressures. These observations extend dissemination models centered on *K. pneumoniae* ST11 and *E. coli* ST131 by demonstrating intergeneric spread likely enabled by modular conjugation systems and adaptive cargo [49, 50]. Accordingly, surveillance should track plasmid lineages alongside bacterial hosts and incorporate plasmid-backbone typing into routine resistance monitoring to enable earlier detection of emerging cross-species vectors such as pMAS5905-KPC.

Beyond classical resistance determinants, pMAS5905-KPC carried two adaptive modules with ecological and epidemiological implications. The *mer* operon may promote heavy metal-driven co-selection, a well-documented mechanism sustaining AMR at the clinical-environmental interface [51, 52]. In parallel, the *acrIE9* gene, encoding an anti-CRISPR protein that antagonizes type I-E CRISPR-Cas systems, may facilitate the establishment and maintenance of foreign plasmids in CRISPR-positive hosts [53, 54]. The co-occurrence of metal resistance, which may enable co-selection, and anti-defense mechanisms provides a plausible explanation for the remarkable persistence and broad host range of IncFII/IncR plasmids beyond a single clonal background [54, 55]. Although correlative in our dataset, these associations suggest synergistic drivers of plasmid dissemination under multifactorial environmental pressures and warrant targeted

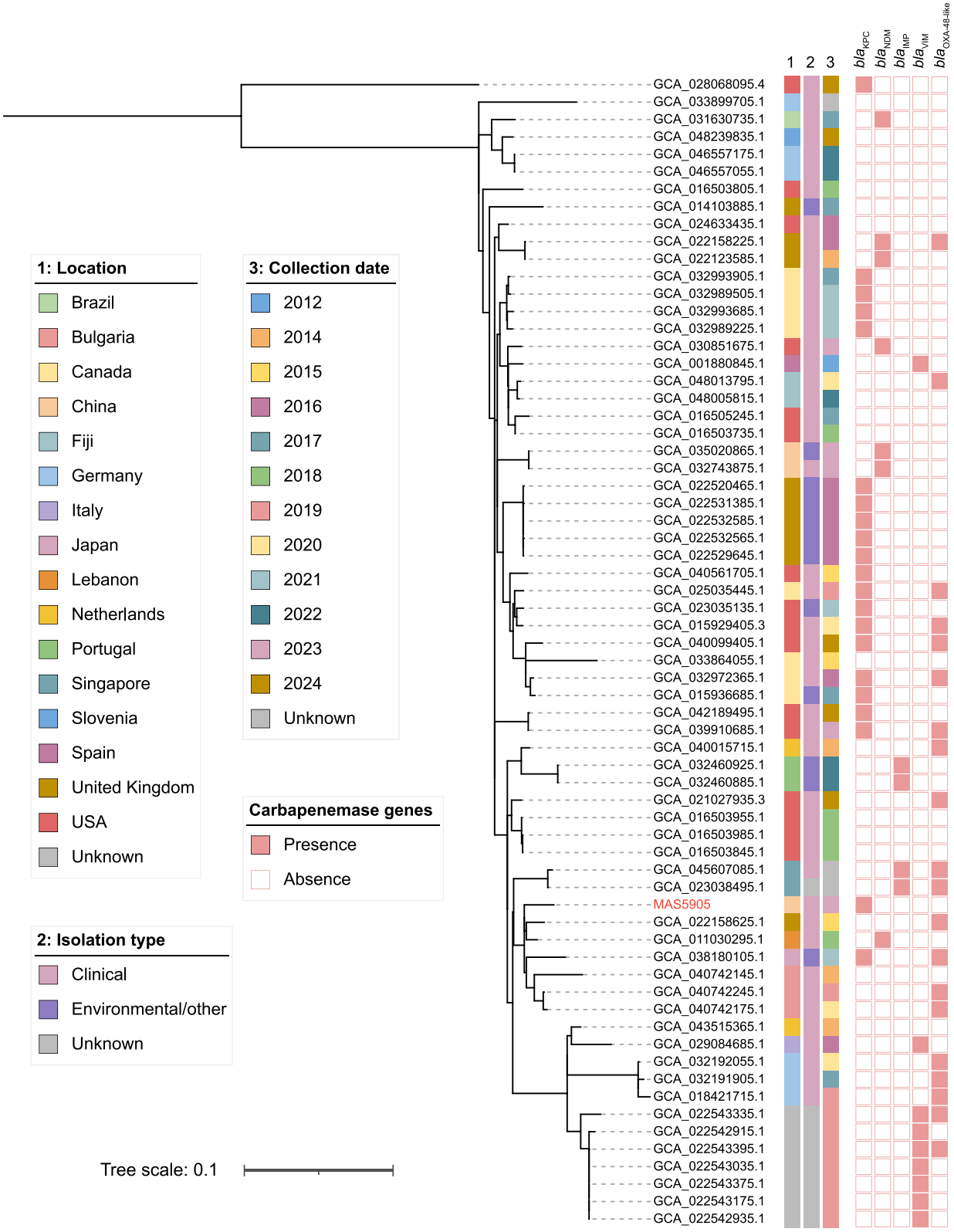

Fig. 4 Phylogenomics of 66 ST91 *C. freundii* isolates and distribution of carbapenemase genes. The maximum-likelihood phylogenetic tree was constructed based on core-genome SNPs from MAS5905 and 65 publicly available ST91 genomes. MAS5905 is highlighted in red. The heatmap on the right shows the presence (red) or absence (white) of carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and $bla_{OXA-48-like}$) in each strain. Metadata annotations include location of origin (1), isolation type (2), and collection year (3)

validation, including tests of *acrIE9* activity and metal-dependent selection.

Previous studies have reported *C. freundii* ST8, ST22, and ST91 in France as high-risk clones [7]. In this study, we focused on *C. freundii* ST91. In the analyzed public dataset, ST91 appears globally distributed and is associated with a relatively high proportion of carbapenemase genes, although these observations may be influenced by sampling bias. Within this lineage, MAS5905 occupies an independent branch, supporting multiple introductions rather than single-source diffusion. In our dataset, ST91 frequently harbors bla_{KPC} and $bla_{\text{OXA-48-like}}$, in line with French surveillance that designates ST91 as high risk and documents circulation of multiple carbapenemase genes in *C. freundii* [7]. In Germany, $bla_{\text{OXA-48-like}}$ predominates at the species level, whereas ST-specific analyses link ST91 more often to bla_{NDM}, underscoring the need to monitor both clonal expansion and plasmid traffic [56]. These observations suggest that *C. freundii* may serve as an opportunistic host facilitating the persistence and onward transfer of multidrug resistance plasmids in clinical settings.

This study has inherent limitations, most notably its focus on a single clinical isolate. Functional assays were performed on a single clinical isolate and one laboratory recipient, constraining generalization across hosts and environments. The comparative plasmid set is skewed toward publicly deposited clinical genomes and may underrepresent environmental reservoirs. Furthermore, temporal and geographic sampling is uneven, limiting precise reconstruction of dissemination routes.

Conclusions

In conclusion, this study reports, to our knowledge, the first characterization of an IncFII(pBK30683)/IncR plasmid carrying $bla_{\text{KPC-2}}$ in a clinical *C. freundii* ST91 isolate, demonstrating conjugative transfer and stable maintenance with a measurable fitness cost. Our findings underscore plasmid lineages, rather than bacterial clones alone, as primary drivers of carbapenem resistance dissemination across Enterobacterales. Furthermore, this study advocates integrating plasmid-backbone typing into routine surveillance and strengthening infection control and genomic monitoring at the healthcare-environment interface, particularly as a complementary measure in under-monitored settings.

Abbreviations

CRE	Carbapenem-resistant Enterobacterales
AMR	Antimicrobial resistance
ST91	Sequence type 91
MGEs	Mobile genetic elements
SNPs	Single nucleotide polymorphisms
CRCF	Carbapenem-resistant *C. freundii*
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
BLAST	Basic Local Alignment Search Tool
AST	Antimicrobial susceptibility testing
MICs	Minimum inhibitory concentrations
FDA	Food and Drug Administration
CLSI	Clinical and Laboratory Standards Institute
WGS	Whole-genome sequencing
ANI	Average nucleotide identity
NCBI	National Center for Biotechnology Information
NR	Non-redundant
ISs	Insertion sequences
ARGs	Antimicrobial resistance genes
CARD	Comprehensive Antibiotic Resistance Database
RGI	Resistance Gene Identifier
T4CP	Type IV coupling protein
T4SS	Type IV secretion system
CF	Conjugation frequency
LB	Luria-Bertani
ANOVA	Analysis of variance
GGDC	Genome-to-Genome Distance Calculator
STs	Sequence types

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-026-04795-1.

Supplementary Material 1.

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Not applicable.

Authors' contributions

XL, ZL, ZS, BZ, and DW designed the research project. XL, ZL, ZS, GY, HG, and XB performed the experiments. XL ZL, ZS, BZ, and DW analyzed and discussed the data. XL wrote the manuscript. XL, ZL, ZS, GY, HG, XB, BZ, and DW reviewed and discussed the manuscript.

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Data availability

The complete genome sequence of strain MAS5905 sequenced in this study has been deposited at GenBank under BioProject ID no. PRJNA1354676.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. The Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China (protocol code ICDC-LSM-2025009) reviewed the study and waived the requirement for informed consent because it was a retrospective analysis involving only anonymized bacterial isolates and did not include any direct human participation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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