


# Emergence of an Extensive Drug Resistant *Citrobacter portucalensis* Clinical Strain Harboring $bla_{SFO-1}$ , $bla_{KPC-2}$ , and $bla_{NDM-1}$

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**Background:** To explore the plasmid characteristics and transfer mechanisms of an extensive drug resistant (XDR) clinical isolate, *Citrobacter portucalensis* L2724hy, co-producing  $bla_{SFO-1}$ ,  $bla_{NDM-1}$ , and  $bla_{KPC-2}$ .

**Methods:** Species confirmation of L2724hy was achieved through 16S rRNA sequencing and Average Nucleotide Identity (ANI) analysis. Antimicrobial susceptibility testing (AST) employed the agar dilution and micro broth dilution methods. Identification of resistance genes was carried out by PCR and whole-genome sequencing (WGS). Essential resistance gene locations were verified by S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and southern hybridization experiments. Subsequent WGS data analysis delved into drug resistance genes and plasmids.

**Results:** The confirmation of the strain L2724hy as an extensive drug-resistant *Citrobacter portucalensis*, resistant to almost all antibiotics tested except polymyxin B and tigecycline, was achieved through 16S rRNA sequencing, ANI analysis and AST results. WGS and subsequent analysis revealed L2724hy carrying  $bla_{SFO-1}$ ,  $bla_{NDM-1}$ , and  $bla_{KPC-2}$  on plasmids of various sizes. The uncommon ESBL gene  $bla_{SFO-1}$  coexists with the *fosA3* gene on an IncFII plasmid, featuring the genetic environment IS26-*fosA3*-IS26-*ampR*- $bla_{SFO-1}$ -IS26. The  $bla_{NDM-1}$  was found on an IncX3 plasmid, coexisting with  $bla_{SHV-12}$ , displaying the sequence IS5-IS3000-IS3000-Tn2- $bla_{NDM-1}$ -*ble*-*trpF*-*dsbD*-*cutA*-*gros*-*groL*, lacking IS*Aa125*. The  $bla_{KPC-2}$  is located on an unclassified plasmid, exhibiting the sequence Tn2-*mpR*-ISK*pn27*- $bla_{KPC-2}$ -ISK*pn6*-*korC*. Conjugation assays confirmed the transferability of both  $bla_{NDM-1}$  and  $bla_{KPC-2}$ .

**Conclusion:** We discovered the coexistence of  $bla_{SFO-1}$ ,  $bla_{NDM-1}$ , and  $bla_{KPC-2}$  in *C. portucalensis* for the first time, delving into plasmid characteristics and transfer mechanisms. Our finding highlights the importance of vigilant monitoring of drug-resistance genes and insertion elements in uncommon strains.

**Keywords:** *Citrobacter spp.*, XDR,  $bla_{SFO}$ ,  $bla_{NDM}$ ,  $bla_{KPC}$ , IncFII

## Introduction

In the vast microbial landscape, the genus *Citrobacter* stands as a diverse group of bacteria known for its adaptability and ecological ubiquity. Within this genus, the relatively lesser-explored *C. portucalensis* has garnered attention for its potential contributions to various fields, especially in human health. Since its first report in 2017, diverse occurrences of *C. portucalensis* in wastewater, sputum, stool, sludge, poultry and, sea turtles across multiple countries have been discovered.<sup>1-6</sup> In 2021, Chinese researcher Cao et al isolated *C. portucalensis* carrying  $bla_{NDM-1}$  from clinical specimens for the first time.<sup>2</sup> Subsequently, in 2022, the coexistence of  $bla_{KPC-2}$  and  $bla_{NDM-1}$  was reported in an extensively drug-resistant *C. portucalensis* obtained from a hospital in China.<sup>7</sup> Additionally, in 2023,

researchers isolated *C. portucalensis* carrying *bla*<sub>NDM-1</sub> from an endangered marine animal that died from sepsis.<sup>8</sup> This growing body of evidence underscores the increasing clinical and extra-clinical dissemination of drug-resistant *C. portucalensis*, warranting heightened attention to address the associated public health concerns.

In 2017, the World Health Organization (WHO) designated carbapenem-resistant and extended spectrum beta-lactamases (ESBLs)-producing *Enterobacteriaceae* as “extremely important” on its list of antimicrobial resistant “priority pathogens”. Numerous antimicrobial resistance genes (ARGs) encoding carbapenemases and ESBLs were discovered. Among these, the *bla*<sub>SFO-1</sub>, an uncommon ESBLs gene exhibiting the ability to hydrolyze beta-lactams excluding cephamycins and carbapenems, was first identified on a self-transferring plasmid from *Enterobacter cloacae* isolated from Japan in 1999.<sup>9</sup> The *bla*<sub>SFO-1</sub> later manifested in an outbreak of *Enterobacter cloacae* in Spain.<sup>10</sup> Recently, its emergence has been documented in *Enterobacter hormaechei*, *Escherichia coli* and *Klebsiella pneumoniae* isolated from China.<sup>11–13</sup> Further investigations are crucial to the understanding of the epidemiology and clinical impact of *bla*<sub>SFO-1</sub>. Among the carbapenemase encoding genes, *bla*<sub>KPC</sub> stands out as the predominant in *Enterobacteriaceae* bacteria globally.<sup>14</sup> In China, the epidemic of *bla*<sub>KPC-2</sub>-carrying bacteria strains is of current concern.<sup>15</sup> Besides *bla*<sub>KPC</sub>, the rapid global spread of *bla*<sub>NDM</sub> since its discovery in 2008 has posed significant public health challenges as well.

In our study, we identified an extensively drug-resistant clinical isolate of *C. portucalensis* featuring a rare coexistence of *bla*<sub>SFO-1</sub>, *bla*<sub>KPC-2</sub>, and *bla*<sub>NDM-1</sub> genes. This report makes the first documented instance of *bla*<sub>SFO-1</sub> in *C. portucalensis*. Additionally, we investigated the plasmid characteristics and transfer mechanisms associated with drug-resistant genes in this particular strain.

## Materials and Methods

### Strain Isolation and Identification

In September 2020, we obtained a clinical isolate (L2724hy) from the faeces of a 56-year-old outpatient male patient with acute diarrhea at the First Affiliated Hospital of Zhejiang University. Initially, this isolate was mistakenly identified as *C. freundii* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).<sup>16</sup> To confirm its accurate species, we uploaded the 16s rRNA sequence of this strain to BioEZCloud (<https://www.ezbiocloud.net/>) and analyzed ANI, and the result indicated it to be *C. portucalensis*.<sup>17,18</sup>

### Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of L2724hy, transconjugant L2724hy-KPC-EC600, transconjugant L2724hy-NDM-EC600 and *E. coli* EC600 were determined by agar dilution method and micro-broth dilution method.<sup>19</sup> AST results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2023 (<https://clsi.org/>), while the breakpoints for tigecycline and colistin were interpreted based on EUCAST (<https://www.eucast.org/>).

### S1-PFGE-Southern Hybridization and Conjugation Assays

S1-PFGE was used to identify the size and number of plasmids. The location of the *bla*<sub>SFO-1</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub> were determined by S1-PFGE and hybridization experiments. Conjugation assays were conducted using *E. coli* EC600 as recipient and L2724hy as donor.<sup>20</sup> Subsequently, individual colonies of donor and recipient bacteria were carefully selected and suspended in LB liquid culture medium at 37 °C for 6 hours. Afterwards, the two suspensions were mixed in a 1:1 ratio (donor: recipient), introduced into 3 mL LB liquid medium, and incubated at 37 °C for 20 hours. An appropriate amount of bacterial solution was spread onto Mueller–Hinton agar (MHA) plates containing rifampicin (200 ug/mL) and meropenem (2 ug/mL). After the incubation at 37°C overnight, a single colony was selected for further cultivation. Moreover, the strain was identified through MALDI-TOF/MS and the presence of drug-resistant genes was confirmed through PCR.

## Whole-Genome Sequencing and Data Analysis

We utilized a Qiagen DNA purification kit in the extraction of the whole genomic DNA of strain L2724hy, which was then sequenced via Oxford Nanopore technology to generate raw data SRA. Then, we used Unicycler 0.4.9 to assemble the SRA, resulting in the acquisition of the whole-genome sequence.<sup>21</sup> The prokka v1.14.6 annotation tool was used to annotate the whole-genome sequence.<sup>22</sup> To predict drug-resistant genes, the data were submitted to ResFinder (<https://cge.food.dtu.dk/services/ResFinder/>), and the identification of insertion sequence and transposons was performed using ISFinder (<https://www-is.biotoul.fr/>). Plasmid types were confirmed through PlasmidFinder (<https://cge.food.dtu.dk/services/PlasmidFinder/>), and the MLST of the strains was determined via Pubmlst (<https://pubmlst.org/>). The discovery of transfer-related components was conducted using OriTfinder (<https://bioinfo-mml.sjtu.edu.cn/oriTfinder/>). The genetic environment surrounding *bla*<sub>SFO-1</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub> on the plasmid was presented using Easyfig 2.2.3.<sup>23</sup> Additionally, the comparison of multiple plasmids was carried out using the BLAST Ring Image Generator (BRIG) software.<sup>24</sup>

## Results

### Strain Isolation and Identification

L2724hy was isolated from the fecal sample of a patient with acute diarrhea and initially identified as *C. freundii* using MALDI-TOF/MS. Subsequent sequencing of the 16S rRNA lead to the reclassification of L2724hy as *C. portucalensis* (99.93% similarity for *C. portucalensis* and 99.86% similarity for *C. freundii*). Further ANI analysis confirmed L2724hy as *C. portucalensis*, showing a 98.73% ANI to *C. portucalensis* ATCC (CP044098) and a 94.52% ANI to *C. freundii* ATCC (CP033744).

### Antimicrobial Susceptibility Testing

As presented in Table 1, the results of antimicrobial susceptibility testing indicated that *C. portucalensis* exhibited resistance to almost all antibiotics, with the exception of tigecycline and polymyxin B. For both transconjugant L2724hy-

**Table 1** The MIC Values of L2724hy, L2724hy-KPC-EC600, L2724hy-NDM-EC600 and EC600

Antimicrobials	MIC Values (mg/L)			EC600
	L2724hy	L2724hy-KPC-EC600	L2724hy-NDM-EC600	
Aztreonam	>128 (R)	>128 (R)	>128 (R)	0.125 (S)
Imipenem	16 (R)	4 (R)	4 (R)	0.5 (S)
Meropenem	>32 (R)	4 (R)	4 (R)	0.06 (S)
Ceftriaxone	>128 (R)	64 (R)	128 (R)	0.06 (S)
Cefotaxime	>128 (R)	16 (R)	128 (R)	0.03 (S)
Ceftazidime	>128 (R)	64 (R)	>128 (R)	0.5 (S)
Levofloxacin	>64 (R)	0.5 (S)	0.25 (S)	1 (I)
Ciprofloxacin	>64 (R)	0.25 (S)	0.25 (S)	0.5 (S)
Amikacin	>128 (R)	1 (S)	2 (S)	2 (S)
Gentamicin	>128 (R)	1 (S)	1 (S)	2 (S)
Piperacillin	>128 (R)	>128 (R)	128 (R)	1 (S)
Fosfomicin	>512 (R)	4 (S)	2 (S)	8 (S)
Chloramphenicol	>128 (R)	8 (S)	8 (S)	8 (S)
Trimethoprim/Sulfamethoxazole	>8 (R)	≤0.125 (S)	≤0.125 (S)	≤2.375 (S)
Amoxicillin/Clavulanic acid	>128 (R)	128 (R)	128 (R)	4 (S)
Cefepime	128 (R)	16 (R)	16 (R)	<0.008 (S)
Ceftazidime-avibactam	64 (R)	1 (S)	64 (R)	0.5 (S)
Tigecycline	0.06 (S)	0.06 (S)	≤0.03 (S)	<0.05 (S)
Polymyxin B	2 (I)	2 (I)	2 (I)	0.5 (I)

**Note:** The quality control strains were *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.

**Abbreviations:** S, susceptible; R, resistant; I, intermediate.

KPC-EC600 and L2724hy-NDM-EC600, we observed a decrease in the MIC values of carbapenems (imipenem, meropenem), levofloxacin, ciprofloxacin, amikacin, gentamicin, fosfomycin, chloramphenicol, and trimethoprim/sulfamethoxazole (Table 1). Notably, the transconjugant L2724hy-NDM-EC600 was highly resistant to ceftazidime-avibactam (MIC=64 mg/l), as was L2724hy, emphasizing the hydrolysis of ceftazidime-avibactam by NDM-1.

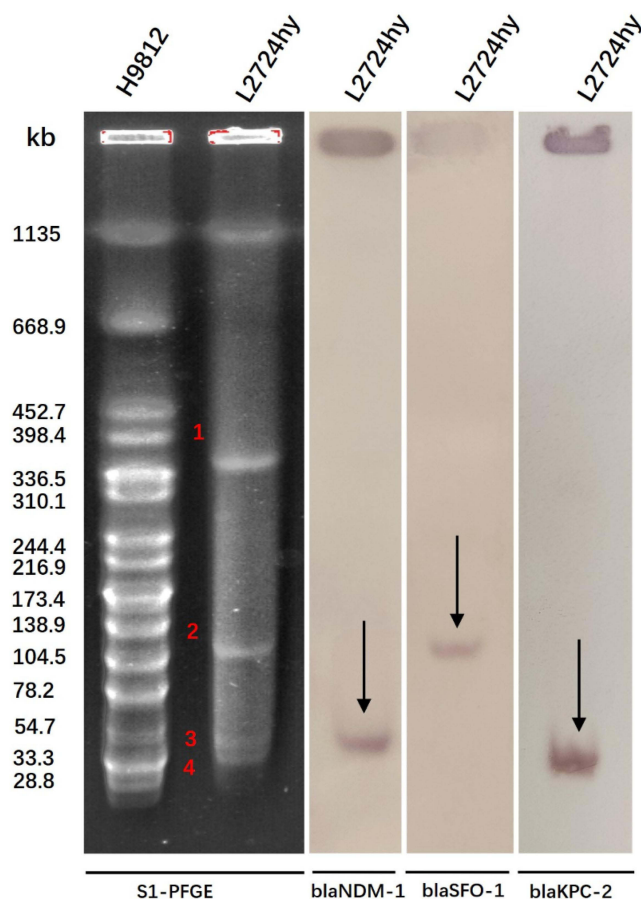
## S1-PFGE-Southern Hybridization and Conjugation Experiment

S1-PFGE profiles revealed that L2724hy harbored one chromosome and four plasmids (Figure 1). However, due to overlap or small size, not all plasmids were discernible on the electrophoresis profiles. The presence of *bla*<sub>SFO-1</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub> genes on plasmids of different sizes was verified through Southern blotting hybridization. Specifically, the *bla*<sub>SFO-1</sub> gene was identified on a ~130 kb plasmid, the *bla*<sub>NDM-1</sub> gene was found on a ~54 kb plasmid, and the *bla*<sub>KPC-2</sub> gene was located on a ~41 kb plasmid (Figure 1). These results are consistent with the data obtained from whole-genome sequencing.

The transconjugants were identified as *E. coli* by MALDI-TOF mass spectrometry. PCR confirmed that the transconjugants, namely L2724hy-KPC-EC600 and L2724hy-NDM-EC600, carried the *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> genes, respectively. This indicates the transferability of *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub> to *E. coli* EC600.

## Genomic Characteristics

The MLST analysis showed that strain L2724hy belongs to ST85. Strain L2724hy has a circular chromosome (5,031,620 bp) and eleven plasmids of various sizes (Table 2). The chromosome contains 5316 coding sequences and 121 RNAs, including 86 tRNAs, 25 rRNAs, and 10 ncRNAs. Its average G+C content is 51.9%. ResFinder identified ten ARGs in



**Figure 1** S1-PFGE profiles and southern blotting hybridization for L2724hy. S1-PFGE determines the number and size of plasmids in the strain. Southern blotting hybridization indicates the location of resistance genes *bla*<sub>SFO-1</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub>.

**Table 2** The Genomic Characteristics of L2724hy

L2724hy	Sizes	Type	G+C%	ARGs
Chromosome	5,031,620 bp	ST85	51.9	<i>bla</i> <sub>CMY-46</sub> , <i>qnrB18</i> ,
p1-L2724hy	365,162 bp	IncHI2/IncHI2A	46.5	
p2-L2724hy-SFO-1	131,226 bp	IncFII	53.7	<i>bla</i> <sub>SFO-1</sub> , <i>fosA3</i>
p3-L2724hy-NDM-1	54,000 bp	IncX3	49.2	<i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>NDM-1</sub>
p4-L2724hy	45,944 bp	IncR	51.9	<i>mph(A)</i>
p5-L2724hy-KPC-2	41,244 bp	Undefined	49.1	<i>bla</i> <sub>KPC-2</sub>
p6-L2724hy	5,933 bp	Undefined	51.1	<i>aac(6')-Ib-cr</i> , <i>aac(6')-Ib-Hangzhou</i>
p7-L2724hy	5,234 bp	Undefined	50.7	
p8-L2724hy	5,040 bp	Undefined	50.0	
p9-L2724hy	2,182 bp	Undefined	50.1	
p10-L2724hy	2,152 bp	Undefined	49.9	
p11-L2724hy	2,013 bp	Undefined	49.3	

strain L2724hy, with *bla*<sub>CMY-46</sub> and *qnrB18* located on the chromosome, while the remaining eight were situated on plasmids. The plasmid type of p2-L2724hy-SFO-1 is IncFII, carrying the *bla*<sub>SFO-1</sub> and *fosA3* genes. Meanwhile, p3-L2724hy-NDM-1 carries two ARGs, *bla*<sub>NDM-1</sub> and *bla*<sub>SHV-12</sub>, and is classified as IncX3. p4-L2724hy is an IncR plasmid with *mph(A)*, whereas *bla*<sub>KPC-2</sub> is on an unclassified p5-L2724hy-KPC-2. Another unclassified plasmid, p6-L2724hy, contains two resistance genes, including *aac(6')-Ib-cr* and *aac(6')-Ib-Hangzhou*.

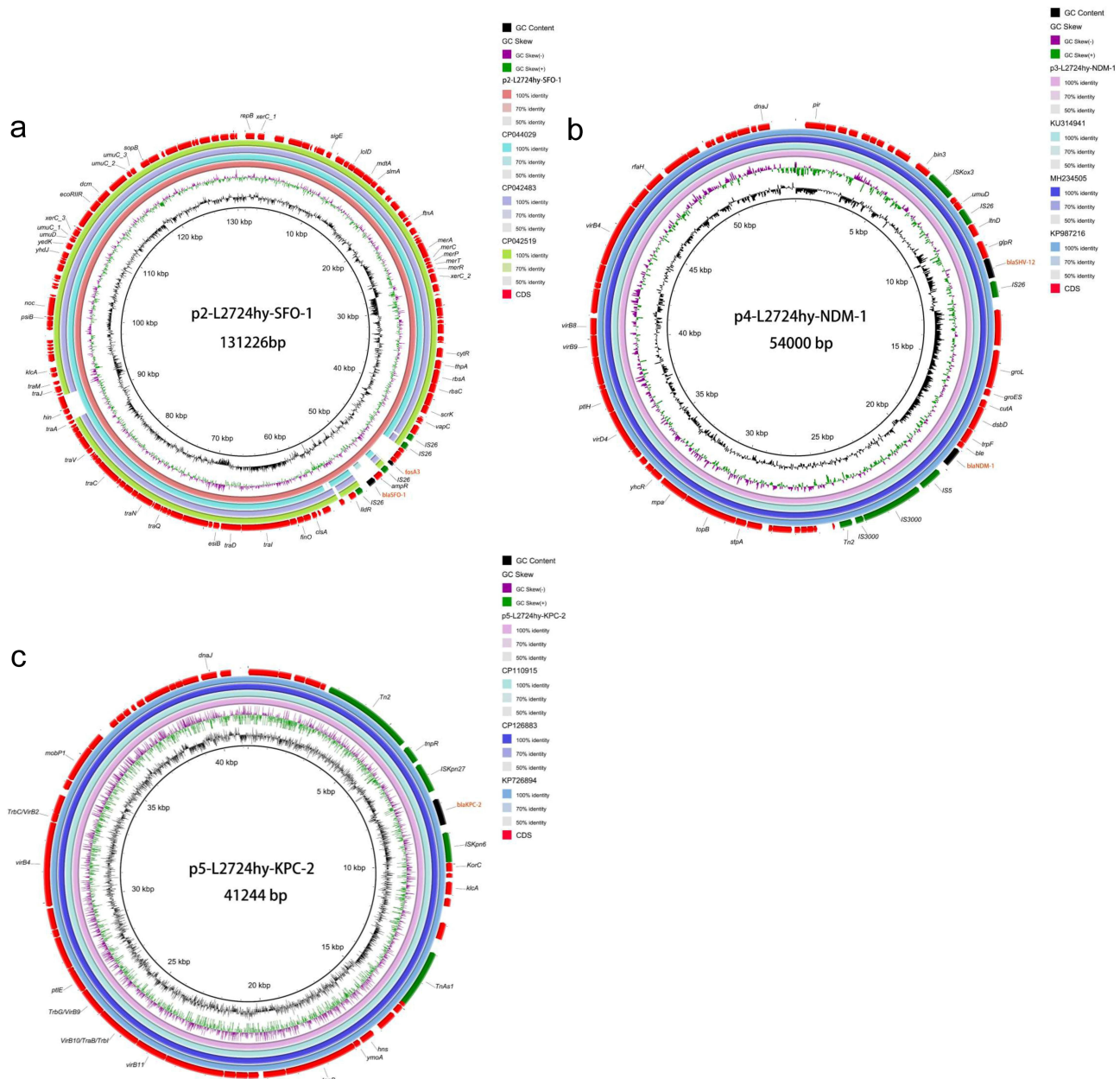
Our analysis revealed that these distinct drug-resistance genes are consistent with antibiotic phenotypes in L2724hy, which included resistance to carbapenems (*bla*<sub>NDM-1</sub>, *bla*<sub>KPC-2</sub>), cephalosporins (*bla*<sub>CMY-46</sub>, *bla*<sub>SFO-1</sub>, *bla*<sub>SHV-12</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>KPC-2</sub>), 4-quinolones (*qnrB18*), fosfomycin (*fosA3*), and aminoglycosides (*aac(6')-Ib-cr*, *aac(6')-Ib-Hangzhou*).

### Plasmid Characteristics of *bla*<sub>KPC-2</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>SFO-1</sub>

L2724hy carries plasmids of various types, including IncHI2/IncHI2A, IncFII, IncX3, IncR, and unclassified plasmids, as identified by PlasmidFinder. Among these, the IncFII plasmid, designated as p2-L2724hy-SFO-1, has a length of 131,226 bp and carries the resistance genes *bla*<sub>SFO-1</sub> and *fosA3*. Comparative genomics with three closely related plasmid genomes (CP044029 with 95% coverage and 100% identity, CP042483 with 93% coverage and 100% identity, and CP042519 with 93% coverage and 100% identity) revealed primary variations, most notably the absence of the resistance gene *bla*<sub>SFO-1</sub> and the transcriptional activator *ampR* (Figure 2a). Additionally, we also identified two strains of *K. pneumoniae* (Genebank: CP114855, and JQ724541) that closely resembled the genetic environment (IS26-*ampR*-*bla*<sub>SFO-1</sub>-IS26) surrounding *bla*<sub>SFO-1</sub> in L2724hy (Figure 3a). However, these two strains lack the *fosA3* gene, which is upstream of the IS26-*ampR*-*bla*<sub>SFO-1</sub>-IS26 sequence in L2724hy. In our study, IS26, which is a critical mobile element, is present on both sides of *fosA3*. Based on the OriTfinder results, p2-L2724hy-SFO-1 possesses mobile genetic elements associated with transfer, including an oriT region, type IV coupling protein (T4CP), and various type IV secretion system components (T4SS), which includes *traI*, *traD*, *traQ*, *traN*, *traC*, *traV*, *traA*, *traJ*, and *traM* (Figures 2a and 4).

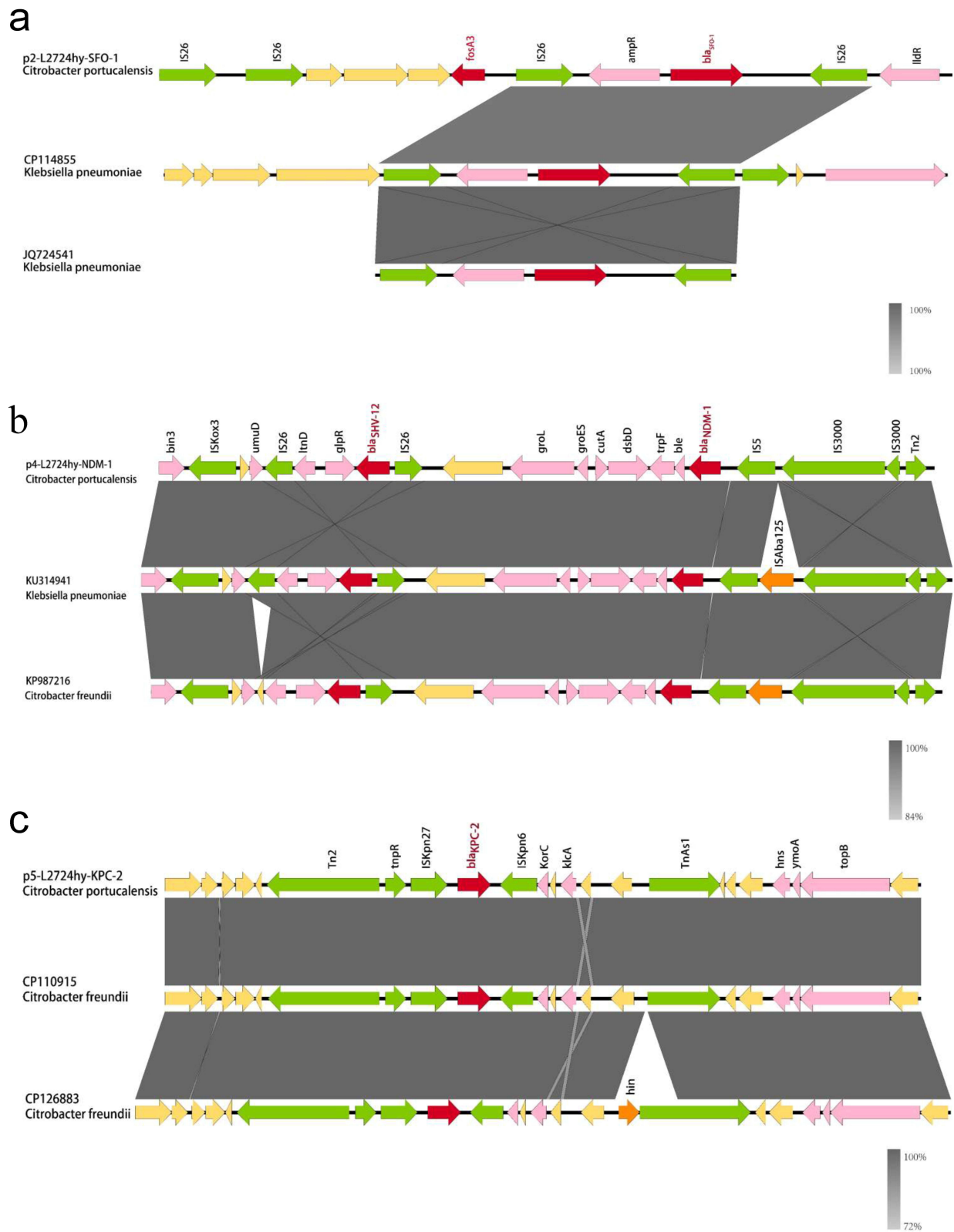
p4-L2724hy-NDM-1 was classified as an IncX3 plasmid, with a length of 54,000 bp and carried two resistance genes, *bla*<sub>NDM-1</sub> and *bla*<sub>SHV-12</sub> (Figure 2b). The NCBI BLAST search revealed 29 highly similar plasmids to p4-L2724hy-NDM-1, all exhibiting 100% coverage and 100% identity. Specifically, plasmids KU314941, MH234941, and KP987216, originating from distinct strains (*K. pneumoniae*, *E. coli*, and *C. freundii*) were selected for a comparative analysis of plasmid characteristics. The analysis revealed that these plasmids share similar backbones (Figure 2b), underscoring the widespread of *bla*<sub>NDM-1</sub> across different species. Upon analyzing the genetic environment of *bla*<sub>NDM-1</sub> in L2724hy (Figure 3b), a conserved sequence (*ble-trpF-dsbD-cutA-gros-groL*) downstream of *bla*<sub>NDM-1</sub> on the plasmid was observed. The upstream of *bla*<sub>NDM-1</sub> comprises an IS5-IS3000-IS3000-Tn2 sequence, with the notable deletion of IS*Aa125* deviating from the typical *bla*<sub>NDM-1</sub> structure. Additionally, an IS26 insertion element surrounding *bla*<sub>SHV-12</sub> was noted suggesting a potential role in facilitating the dissemination of *bla*<sub>SHV-12</sub>.



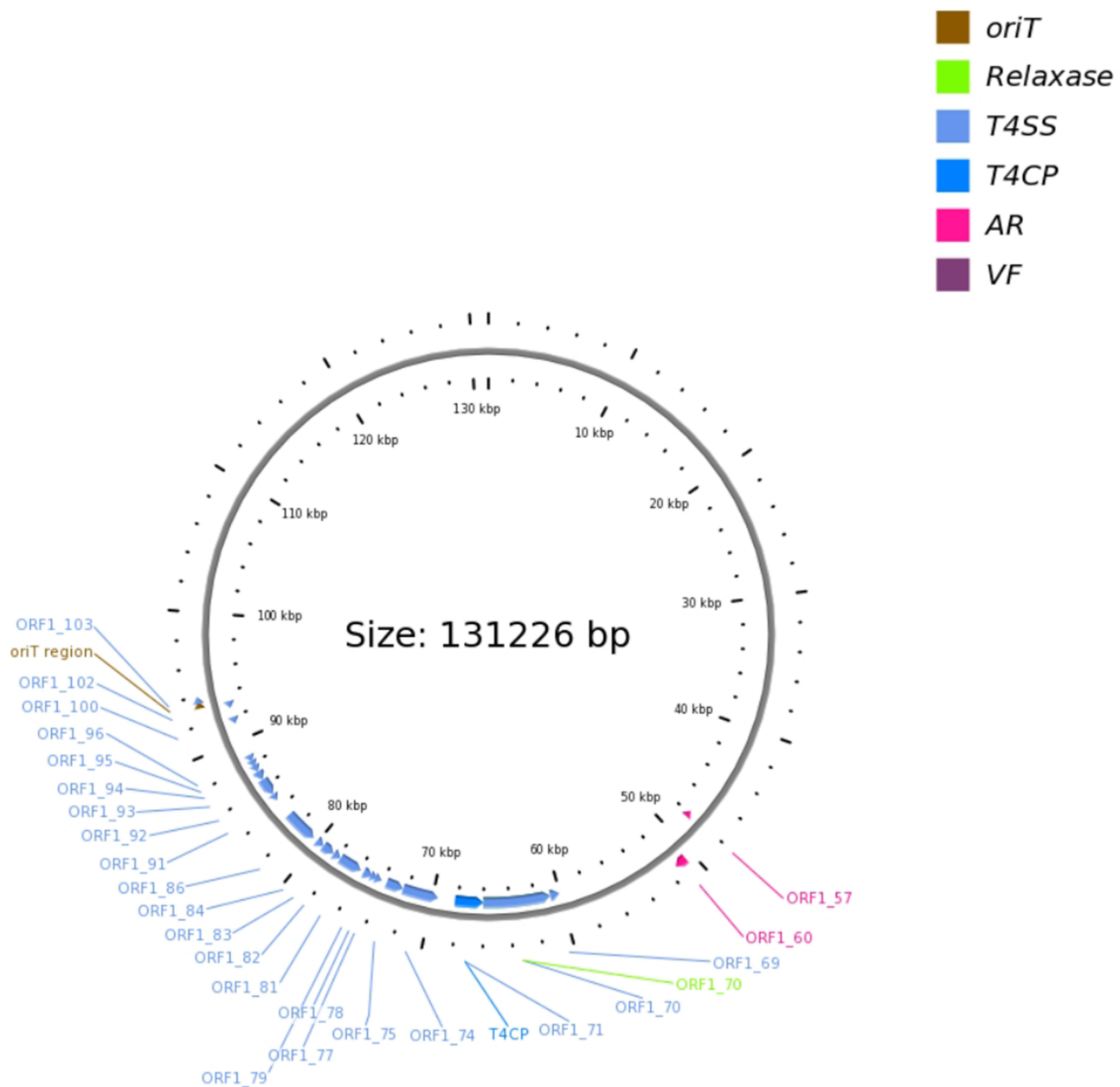


**Figure 2** Comparative analysis of plasmids p2-L2724hy-SFO-1, p4-L2724hy-NDM-1, and p5-L2724hy-KPC-2 in L2724hy. The BRIG circle maps show plasmid resistance genes and mobile genetic elements (MGEs). (a) plasmid p2-L2724hy-SFO-1 carrying *bla*<sub>SFO-1</sub> and *fosA3*. (b) plasmid p4-L2724hy-NDM-1 harboring *bla*<sub>NDM-1</sub> and *bla*<sub>SHV-12</sub>. (c) plasmid p5-L2724hy-KPC-2 bearing *bla*<sub>KPC-2</sub>.

The plasmid p5-L2724hy-KPC-2, carrying *bla*<sub>KPC-2</sub> gene, is currently unclassified and 41,244 bp in length. As shown in Figure 2c, the plasmid contains multiple-insertion elements (*ISKpn27*, *ISKpn6*), transposons (*Tn2*, *tnpR*, *TnAsI*), and transfer-related type IV secretion systems (*virB2*, *virB4*, *virB9*, *virB10*, and *virB11*). The plasmids CP110915, CP126883, and KP726894, are the most similar to p5-L2724hy-KPC-2, all displaying 100% coverage and 100% identity. Upon examination of these highly similar plasmids, it was observed that CP110915 and CP126883 originated from *C. freundii*. As illustrated in Figure 3c, a comparison of the genetic context of the two most similar *C. freundii* plasmids (CP110915, CP126883) with p5-L2724hy-KPC-2 revealed a similar genetic environment for *bla*<sub>KPC-2</sub> (*Tn2-tnpR-ISKpn27-bla*<sub>KPC-2</sub>-*ISKpn6-KorC*) which is entirely consistent with CP110915 from the same region but exhibits a small gap with CP126883 from a different region.



**Figure 3** Genetic environment of *bla*<sub>SFO-1</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub> in L2724hy. (a) *bla*<sub>SFO-1</sub> (b) *bla*<sub>NDM-1</sub> (c) *bla*<sub>KPC-2</sub>. Red arrows denote drug resistance genes, green arrows denote transposons, pink arrows denote other genes, and yellow arrows represent hypothetical proteins.



**Figure 4** A conjugative plasmid p2-L2724hy-SFO-I. ORF1-57, *fosA3*; ORF1-60, *bla<sub>SFO-1</sub>*.

**Abbreviation:** AR (ARGs), acquired antibiotic resistance determinant genes ;ORF, Open Reading Frame

## Discussion

In recent years, the coexistence of ESBLs and carbapenemase genes in *Enterobacteriaceae*, posing challenges to clinical antibiotic treatment.<sup>25</sup> To the best of our knowledge, our study is the first to report the coexistence of *bla<sub>SFO-1</sub>* with *bla<sub>NDM-1</sub>* and *bla<sub>KPC-2</sub>*. Furthermore, there are no reports of the ESBL gene *bla<sub>SFO-1</sub>* in *C. portucalensis*.

Since its discovery in 2017, *C. portucalensis* has been found in humans, animals, and environment. However, the misidentification of *C. portucalensis* as *C. freundii* has led to an underestimation of its prevalence in clinical settings.<sup>7,26</sup> In this study, L2724hy *C. portucalensis* was initially misidentified as *C. freundii* using MALDI-TOF/MS, and was later identified as *C. portucalensis* by a combination of 16s rRNA sequencing and ANI analysis. Thus, it is essential to enhance the whole-genome sequencing of less common strains.



Data analysis reveals that the plasmids carrying *bla*<sub>SFO-1</sub> include IncA/C, IncHI2, unclassified types.<sup>12,13,27,28</sup> In our study, plasmidFinder revealed that strain L2724hy carries the *bla*<sub>SFO-1</sub> gene on an IncFII plasmid that has not been reported before. According to the genetic environment of *bla*<sub>SFO-1</sub> (IS26-*ampR*-*bla*<sub>SFO-1</sub>-IS26), the presence of IS26 on both sides of *bla*<sub>SFO-1</sub> suggests a potential correlation with plasmid-mediated horizontal transmission of *bla*<sub>SFO-1</sub>, which is consistent with prior research findings.<sup>29</sup> Notably, we also observed that the *fosA3*, is located upstream of IS26-*ampR*-*bla*<sub>SFO-1</sub>-IS26 and shares the same IS26 with *ampR*-*bla*<sub>SFO-1</sub>. The *fosA3* has been reported to be closely associated with the transmission of ESBL gene *bla*<sub>CTX-M</sub> by the insertion element IS26.<sup>30</sup> Therefore, in our study, we believe that IS26 is a vital insertion element that gradually integrates with *fosA3* and *bla*<sub>SFO-1</sub> on the IncFII plasmid, forming a multidrug resistance region, which plays a crucial role in mediating the spread of drug resistance genes. Importantly, this study marks the first instance of *bla*<sub>SFO-1</sub> and *fosA3* coexisting on a plasmid.

NDM-1, a carbapenemase, can hydrolyze nearly all beta-lactam antibiotics, including carbapenemase.<sup>31</sup> The emergence of carbapenemase-producing strains of NDM-1 represents a significant risk to global public health, necessitating a high level of vigilance. In our study, we observed an absence of IS*Aba125* from IS5 to IS3000 when comparing it to classical bacteria. According to previous studies, IS*Aba125* is typically involved in the formation of *Tn125*, a complex transposon associated with the plasmid-mediated spread of *bla*<sub>NDM-1</sub>.<sup>32,33</sup> Our findings suggest that the lack of IS*Aba125* does not impede the horizontal transfer of *bla*<sub>NDM-1</sub>. In the conjugation experiment, we successfully transferred *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub> into EC600 for expression, affirming the transferability of the plasmids in our study. As mentioned above, the genetic environment of *bla*<sub>KPC-2</sub> in this study aligns entirely that of *C. freundii* (Genebank: CP110915) from the same region, strongly suggesting that *bla*<sub>KPC-2</sub> was transmitted from CP110915.

## Conclusion

In conclusion, our study reported for the first time the coexistence of *bla*<sub>SFO-1</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>KPC-2</sub> in a clinical isolated *C. portucalensis* strain, presenting challenges in multidrug resistance. IS26 is an essential insertion element that facilitates the propagation of drug-resistant genes on IncFII plasmids. These findings emphasize the urgency of continued monitoring and understanding evolving drug resistance dynamics in uncommon strains.

## Nucleotide Sequence Accession Numbers

The nucleotide sequences in L2724hy containing a circular chromosome and eleven plasmids have been submitted to GenBank and assigned accession numbers CP136601-136612, respectively.

## Ethical Approval

This study was approved by the clinical research ethics committee of the First Affiliated Hospital, Zhejiang University School of Medicine [number 2020-IIT-591], and was conducted according to the ethical principles of the Declaration of Helsinki. Parental written consent was obtained.

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## Disclosure

The authors report no conflicts of interest in this work.

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