High-level carbapenem resistance in a *Citrobacter freundii* clinical isolate is due to a combination of KPC-2 production and decreased porin expression

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An imipenem-resistant isolate of *Citrobacter freundii* ZJ163 (MIC 256 µg ml⁻¹) isolated from a Chinese hospital was investigated. The *C. freundii* ZJ163 isolate exhibited high-level resistance to carbapenems, penicillins, cephalosporins, cefoxitin, aztreonam, quinolones and aminoglycosides. Isoelectric focusing (IEF) demonstrated three β-lactamases with pIs of 5.4 (TEM-1), 6.7 (KPC-2) and 7.9 (CTX-M-14). Two different transconjugants (types A and B) were obtained by conjugation studies. The type A transconjugant exhibited reduced susceptibility or resistance to penicillins, cephalosporins and aztreonam, but was susceptible to carbapenems, quinolones and aminoglycosides. The antimicrobial susceptibility patterns of the type B transconjugant were similar to that of type A, except for its significantly reduced carbapenem susceptibility (imipenem MIC 2 µg ml⁻¹). IEF, specific PCRs and DNA sequence analysis indicated that the type A transconjugant produced CTX-M-14 β-lactamase with a pI of 7.9, that the type B transconjugant produced KPC-2 β-lactamase with a pI of 6.7 and that the β-lactamase with a pI of 5.4 was TEM-1. PCR analysis and sequencing confirmed the presence of the *ampC* gene in the chromosomal DNA from *C. freundii* ZJ163, although no activity of AmpC β-lactamase was detected by IEF. Urea/SDS-PAGE analysis of outer-membrane proteins revealed that the levels of the 41 and 38 kDa porins were decreased in *C. freundii* ZJ163. It was concluded that production of KPC-2 combined with decreased expression of porins contributes to high-level resistance to carbapenems in *C. freundii* ZJ163.

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INTRODUCTION

Citrobacter freundii is an opportunistic pathogen that can cause diarrhoea, septicaemia, meningitis, and urinary tract and respiratory system infection, especially in high-risk groups. *C. freundii* has now become one of the most important nosocomial infection pathogens in China. According to monitoring data during 1994–2001 for the Chinese Net for the Nosocomial Pathogens Resistance Surveillance, *C. freundii* was ranked tenth among Gram-negative organisms.

Increased emergence of members of the *Enterobacteriaceae* possessing extended-spectrum β-lactamases (ESBLs) has been accompanied by the widespread use of cephalosporins. Carbapenems are considered to be one of the few

remaining therapies for infections caused by multidrug-resistant isolates, especially strains producing high-level AmpC or ESBLs. Over the past two decades, whilst carbapenem resistance has become a serious problem for non-lactose-fermenting bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Flavobacterium meningosepticum*, it has remained uncommon in the *Enterobacteriaceae*. However, recently identification of carbapenem-resistant members of the *Enterobacteriaceae* has increased. The production of carbapenemase is a common mechanism of carbapenem resistance.

Carbapenem-hydrolysing KPC β-lactamases are a group of recently identified carbapenemases that belong to Bush group 2f, molecular class A. KPCs are capable of hydrolysing carbapenems, penicillins, cephalosporins and aztreonam, and are inhibited by clavulanic acid and tazobactam. The initial report of one of these β-lactamases, KPC-1, was from carbapenem-resistant *Klebsiella pneumoniae* isolated in North Carolina, USA (Yigit *et al.*, 2001).

Abbreviations: ESBL, extended-spectrum β-lactamase; pI, isoelectric point; OMP, outer-membrane protein.

The GenBank/EMBL/DDBJ accession numbers for the *bla*_{KPC-2} and *ampC* gene sequences of *Citrobacter freundii* ZJ163 are EF062508 and EF426097.

KPC-2 was then found in isolates of *K. pneumoniae* (Moland *et al.*, 2003), *Salmonella enterica* (Miriagou *et al.*, 2003), *Klebsiella oxytoca* (Yigit *et al.*, 2003) and *Enterobacter* sp. (Hossain *et al.*, 2004). Soon after, KPC-3 was found in *K. pneumoniae* (Woodford *et al.*, 2004) and *Enterobacter cloacae* (Bratu *et al.*, 2005) from New York, USA. Recently, KPC-2 was found outside the USA in France (Naas *et al.*, 2005), Israel (Navon-Venezia *et al.*, 2006) and South America (Villegas *et al.*, 2006), and KPC-2 was first identified in *P. aeruginosa* isolates, outside the family *Enterobacteriaceae* (Villegas *et al.*, 2007). KPC-2 has emerged in China. A *K. pneumoniae* isolate from Hangzhou, China, producing KPC-2 was reported (Wei *et al.*, 2007). Almost simultaneously, we identified KPC-2 in three isolates of *Serratia marcescens* from the same city but a different hospital (Zhang *et al.*, 2007).

However, there has been no report of KPC in *C. freundii* so far. In this report, we describe a clinical isolate of carbapenem-resistant *C. freundii* that produces carbapenem-hydrolysing KPC-2 and expresses a reduced level of porins. This is the first report of detection of the plasmid-mediated carbapenem-hydrolysing KPC-2 in *C. freundii*.

METHODS

Bacterial strains. A clinical isolate of carbapenem-resistant *C. freundii* ZJ163 was collected from a urine sample of a patient in the rehabilitation ward in the Second Affiliated Hospital of Zhejiang University in June 2006. The patient was initially diagnosed with an aneurysm at the end of May 2006. The patient received surgery in another hospital in Shanghai and was then transferred back to our hospital for rehabilitation at the end of June. Levofloxacin was provided immediately after hospitalization and *C. freundii* ZJ163 was then isolated from the patient on the second day. The identity of the isolate was confirmed using a VITEK system (bioMérieux). *Escherichia coli* EC600 (LacZ⁻ Nal^R Rif^R) was used as a recipient in conjugal transfer experiments. *E. coli* ATCC 25922 was used for quality control in antimicrobial susceptibility tests.

Antimicrobial susceptibility testing. MICs were determined using an agar dilution method according to the recommendations of the

Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (NCCLS, 2006).

Conjugal transfer experiments. Conjugation experiments were carried out in mixed broth cultures. Rifampicin-resistant *E. coli* EC600 was used as the recipient. Overnight cultures of donor strain (200 µl) and recipient strain (100 µl) were mixed with 600 µl fresh Luria–Bertani broth and incubated for 4 h at 35 °C. The mixture was then inoculated on Mueller–Hinton agar plates containing rifampicin (700 µg ml⁻¹) plus cefotaxime (2 µg ml⁻¹), or rifampicin (700 µg ml⁻¹) plus imipenem (0.5 µg ml⁻¹) for 24 h at 35 °C. Colonies that grew on the selective medium were identified by a VITEK system. Plasmid DNA preparations were obtained by an alkaline lysis technique using an AxyPrep Plasmid Miniprep kit (Axygen Scientific). Plasmids were separated by electrophoresis in a 0.8% agarose gel containing ethidium bromide in 0.5 × TBE buffer at a constant voltage for 1 h.

Isoelectric focusing (IEF) of β-lactamases. Crude β-lactamase extracts were prepared by ultrasonic treatment of bacterial cells. IEF was performed on a PhastGel polyacrylamide gel (Amersham Biosciences) using the PhastSystem (Pharmacia Biotech) following the method of Mathew *et al.* (1975). β-Lactamase activity was visualized by staining the gel with nitrocefin (Oxoid). The isoelectric points (pIs) were determined after comparison with the known β-lactamases TEM-1 (pI 5.4), SHV-1 (pI 7.6) and SHV-5 (pI 8.2).

PCR amplification and DNA sequence analysis of bla genes.

The primers used to amplify *bla* genes, class I integrase and class I integron are listed in Table 1. PCR was carried out using approximately 0.3 µg template DNA, 0.5 µM each primer, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTPs and 2.5 U *Taq* DNA polymerase (Promega) in a total volume of 50 µl. Amplification was conducted in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems). The PCR products were cloned into a pGEMT-Easy vector (Promega) and recombinant plasmids were transformed into *E. coli* DH5α as described previously (Chen *et al.*, 2006). Inserts were sequenced using an ABI 3730 Sequencer (Applied Biosystems). The DNA sequences were identified using the BLAST program (www.ncbi.nlm.nih.gov/blast/).

Analysis of outer-membrane proteins (OMPs). Protein concentration was determined with a Coomassie Brilliant Blue protein assay kit (Bio-Rad). The OMPs of *C. freundii* ATCC 8090 and *C. freundii* ZJ163 were isolated as described by Hernández-Allés *et al.* (1999). Electrophoretic analysis of OMPs by urea/SDS-PAGE was performed

Table 1. Primers used in this study

Gene	Primers	Sequence (5'→3')	Product size (bp)	Reference
<i>bla</i> _{KPC}	KPC-F	GCTACACCTAGCTCCACCTTC	1050	Moland <i>et al.</i> (2003)
	KPC-R	TCAGTGCTCTACAGAAAACC		
<i>bla</i> _{TEM}	TEM-F	TCGGGGAAATGTGCG	972	Yu <i>et al.</i> (2007)
	TEM-R	TGCTTAATCAGTGAGGCACC		
<i>bla</i> _{CTX-M-9} group	CTX-M-F	GCGCATGGTGACAAAAGAGAGTGCAA	881	Yu <i>et al.</i> (2007)
	CTX-M-R	GTTACAGCCCTTCGGCGATGATTC		
Class I integrase	Int1 F	CAGTGGACATAAGCCTGTTC	160	Koeleman <i>et al.</i> (2001)
	Int1-R	CCCGAGGCATAGACTGTA		
Class I integron	5'-CS	GGCATCCAAGCAGCAAG	Uncertain	Lévesque <i>et al.</i> (1995)
	3'-CS	AAGCAGACTTGACCTGA		
<i>ampC</i>	AmpC-F	TTGCTTTTAATTACGGAAGTATTT	1241	Barlow & Hall (2002)
	AmpC-R	TAAGTGTAGATGACAGCAGGAAA		

in 11.6 % acrylamide/0.4 % bisacrylamide/0.1 % SDS gels containing 20 % urea. Samples were boiled for 5 min in sample buffer before electrophoresis. The 0.75 mm thickness mini gel was run at a constant current of 20 mA for 85 min using a Mini-PROTEAN 3 slab electrophoresis cell (Bio-Rad). The gel was visualized by staining with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Antimicrobial susceptibility

The results of susceptibility testing are shown in Table 2. *C. freundii* ZJ163 exhibited high-level resistance to imipenem and meropenem, with MICs for both drugs of 256 µg ml⁻¹. The isolate was also resistant to penicillins, cephalosporins, cefoxitin, aztreonam, quinolones and aminoglycosides.

Plasmids and transfer of imipenem resistance

Transfer of β -lactam resistance from the *C. freundii* ZJ163 isolate to *E. coli* EC600 by conjugation was successful. Several single colonies that grew on the plate containing rifampicin plus cefotaxime were reinoculated simultaneously onto a plate containing rifampicin plus cefotaxime and a plate containing rifampicin plus imipenem. Bacteria from the same colony that grew on the plate containing rifampicin plus cefotaxime but not on the plate containing rifampicin plus imipenem were named transconjugant A, whilst those that grew on both plates were named transconjugant B. Both transconjugants were identified as *E. coli* by the VITEK system. Transconjugant A was resistant or had intermediate resistance to penicillins, cephalosporins and aztreonam, but was susceptible to carbapenems, cefoxitin, quinolones and aminoglycosides.

The antimicrobial susceptibility patterns of transconjugant B were similar to those of transconjugant A, except for its significantly reduced carbapenem susceptibility, with MICs of 2 µg ml⁻¹ (Table 2). This suggested that the plasmid that existed in transconjugant B may play an important role in resistance to carbapenems in *C. freundii* ZJ163.

Plasmid profile results indicated that the *C. freundii* ZJ163 isolate harboured several plasmids. Transconjugants A and B both had a plasmid with a size of approximately 60 kb (Fig. 1).

IEF analysis

IEF analysis demonstrated that the *C. freundii* ZJ163 isolate produced three β -lactamases with pIs of 5.4, 6.7 and 7.9, respectively. As shown in Fig. 2, transconjugant A produced a pI 7.9 β -lactamase and transconjugant B produced a pI 6.7 β -lactamase.

PCR and DNA sequence analysis

The IEF results suggested the presence of *bla*_{TEM} (pI 5.4), *bla*_{KPC} (pI 6.7) and *bla*_{CTX-M} (pI 7.9). These three *bla* genes were detected in total plasmid DNA from the *C. freundii* ZJ163 isolate by PCR amplification. Furthermore, *bla*_{CTX-M} was detected in plasmid DNA from transconjugant A and *bla*_{KPC} was detected in transconjugant B. These three genes were identified as *bla*_{TEM-1} (pI 5.4), *bla*_{KPC-2} (pI 6.7) and *bla*_{CTX-M-14} (pI 7.9) by comparing their DNA sequences with known genes in GenBank. These results indicated that KPC-2 and CTX-M-14 were encoded on different conjugative plasmids. A transconjugant that produced TEM-1 was not obtained by conjugation experiments using

Table 2. Antimicrobial susceptibility results for *C. freundii* ZJ163, *Escherichia coli* EC600 and transconjugants A and B

CTX, cefotaxime; RIF, rifampicin; IPM, imipenem.

Antimicrobial agent(s)	MIC (µg ml ⁻¹)			
	<i>C. freundii</i> ZJ163	<i>E. coli</i> EC600	Transconjugant A (CTX + RIF)	Transconjugant B (IPM + RIF)
Imipenem	256	≤0.125	≤0.125	2
Meropenem	256	≤0.125	≤0.125	2
Aztreonam	>256	0.25	32	256
Ceftazidime	256	0.25	4	32
Ceftriaxone	>256	≤0.125	128	32
Cefotaxime	>256	≤0.125	32	16
Cefepime	>256	≤0.125	16	8
Cefoperazone/sulbactam	>256	0.25	32	64
Ampicillin	>256	8	>256	>256
Piperacillin	>256	1	256	256
Piperacillin/tazobactam	>256	1	16	128
Cefoxitin	>256	8	8	16
Ciprofloxacin	>32	≤0.125	≤0.125	≤0.125
Gentamicin	>256	0.5	0.5	0.25
Amikacin	>256	1	1	1

Fig. 1. Plasmid profiles of *C. freundii* ZJ163 and transconjugants. Plasmids prepared from *E. coli* V517 (54.2, 7.3, 5.6, 5.2, 3.9, 3.0, 2.7 and 2.1 kb) (lane 1), *C. freundii* ZJ163 (lane 2), transconjugant A (lane 3) and transconjugant B (lane 4).

selective medium containing rifampicin ($700 \mu\text{g ml}^{-1}$) plus ampicillin ($50 \mu\text{g ml}^{-1}$). *bla*_{TEM-1} was detected in total plasmid DNA but not in chromosomal DNA from *C. freundii* ZJ163, implying that *bla*_{TEM-1} was encoded on a third plasmid.

The presence of a chromosomal *ampC* gene was confirmed by PCR analysis and sequencing. The sequence showed 98.95 % nucleotide similarity (1134/1146) to the chromosomal *ampC* gene of *C. freundii* ATCC 6879. There was only one amino acid difference between them: Val-368 in *C. freundii* strain ATCC 6879 was replaced with Ala in *C. freundii* ZJ163. However, no activity of AmpC β -lactamase was detected by IEF. This was probably due to low enzyme activity.

Neither class I integrase nor class I integron was detected in the *bla*_{KPC}-encoding plasmid. Most *bla*_{KPC}-encoding

Fig. 2. IEF patterns of crude β -lactamase extracts from *C. freundii* ZJ163 and transconjugants. Lanes: 1, *C. freundii* ZJ163; 2, transconjugant A; 3, transconjugant B; 4, strain producing TEM-1 (pI 5.4), SHV-1 (pI 7.6) and SHV-5 (pI 8.2).

plasmids can be conjugated into *E. coli*, except for the *bla*_{KPC-1}-encoding plasmid described by Yigit *et al.* (2001). Some also encode *bla*_{TEM-1} (Hossain *et al.*, 2004; Miriagou *et al.*, 2003) and various ESBLs (Yigit *et al.*, 2003). However, only a *bla*_{KPC} gene was identified from the plasmid in transconjugant B in the current study. It is thus evident that *bla*_{KPC} spreads among different types of plasmid through mobile elements, and that the spread of transmissible plasmids among different clinical members of the *Enterobacteriaceae* results in the spread of drug resistance. To identify the existence of the integrons, we conducted careful PCR analyses of the plasmid encoding KPC and the total plasmids. We found that class I integrase and the variable regions of integrons were amplified in the total plasmid DNA from *C. freundii* but not in the plasmid DNA from transconjugant A or B. Fig. 3 shows PCR amplification of class I integrase and the variable regions of integrons from *C. freundii* and transconjugants A and B.

Fig. 3. PCR amplification of class I integrase (a) and the variable regions of integrons (b) from *C. freundii* and transconjugants of type A and B. Lanes: 1, positive amplification control (plasmid containing an integron, confirmed by DNA sequencing); 2, total plasmid DNA from *C. freundii* ZJ163; 3, plasmid DNA from transconjugant A; 4, plasmid DNA from transconjugant B; 5, negative amplification control; M, DNA molecular mass standard (Takara).

Therefore, we concluded that class I integrons, which may be related to aminoglycoside resistance, were not present on the KPC-encoding plasmid but were present on the TEM-encoding plasmid or other plasmids.

Analysis of OMPs

Transconjugant B, which only produced KPC-2, exhibited significantly reduced susceptibility to imipenem and meropenem (MICs $2 \mu\text{g ml}^{-1}$). However, it was not sufficient to explain the high-level carbapenem resistance in *C. freundii* ZJ163 (MICs $256 \mu\text{g ml}^{-1}$), as transconjugant B remained susceptible to carbapenems at NCCLS break-points. Therefore, OMPs were examined. Urea/SDS-PAGE analysis of OMPs showed that *C. freundii* ZJ163 and ATCC 8090 both expressed four major OMPs, with molecular masses of 47, 41, 38 and 32 kDa. The 41 and 38 kDa proteins were considered to be porins (Aoyama *et al.*, 1988). The four OMPs were analogous to *E. coli* LamB, OmpC, OmpF and OmpA, respectively. We found that the 41 and 38 kDa porins were decreased in *C. freundii* ZJ163. To confirm whether these two proteins were completely abolished or had decreased expression, we overloaded the sample from *C. freundii* ZJ163 in electrophoresis. As shown in Fig. 4, even with approximately fourfold overloading of total protein from *C. freundii* ZJ163, only very faint bands of 41 and 38 kDa were observed compared with the bands from ATCC 8090 (Fig. 4). Therefore, the levels of the 41

and 38 kDa porins were confirmed to be significantly decreased in *C. freundii* ZJ163 compared with ATCC 8090. We inferred from these results that production of KPC-2 combined with decreased expression of porins confers high-level carbapenem resistance in *C. freundii* ZJ163.

Carbapenems are important antibiotics for treatment of nosocomial infections. The emergence and spread of β -lactamases capable of hydrolysing carbapenems has caused problems regarding therapy and control. Carbapenem resistance in *C. freundii* has rarely been reported. In this study, we identified the carbapenem-hydrolysing enzyme KPC-2 and decreased levels of porins in *C. freundii*. KPCs have been detected in a variety of members of the *Enterobacteriaceae* (*K. pneumoniae*, *Salmonella enterica*, *K. oxytoca*, *Enterobacter* sp. and *E. coli*) and *P. aeruginosa* in different regions and countries (North Carolina, Maryland, New York and Boston, USA; Paris, France; Israel; South America; and Hangzhou, China). This indicates that the spread of *bla*_{KPC} genes is no longer a regional problem. Attention should be paid to preventing potential outbreaks of nosocomial infections by pathogens producing KPC.

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Fig. 4. Urea/SDS-PAGE analysis of OMPs extracted from *C. freundii* ATCC 8090 and *C. freundii* ZJ163. Lanes: 1, OMPs (2 μg) from *C. freundii* ATCC 8090; 2, OMPs (5 μg) from *C. freundii* ZJ163; 3, protein molecular mass standard (MBI Fermentas).

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