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Circulation of bla_{KPC-3}-Carrying IncX3 Plasmids among *Citrobacter freundii* Isolates in an Italian Hospital

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ABSTRACT Colonizations due to carbapenem-resistant *Enterobacteriaceae* (CRE) are a source of antimicrobial resistance transmission in health care settings. Eleven *Citrobacter freundii* strains producing KPC-3 carbapenemase were isolated from rectal swabs during a 3-year surveillance program. bla_{KPC-3}-carrying plasmids were found to belong to the IncX3 group in 9 of the 11 strains, and complete nucleotide sequences were obtained for 2 of them. Our results highlight the possible role of *C. freundii* as reservoir of resistance genes.

KEYWORDS KPC, IncX3, *Citrobacter freundii*, KPC-3, antibiotic resistance

Carbapenem-resistant *Enterobacteriaceae* (CRE) species are a common cause of health care-associated infection, causing high morbidity and mortality rates. The most prevalent CRE species associated with nosocomial infection in Italy was shown to be *Klebsiella pneumoniae*, and KPC was found to be the most common carbapenemase (1). The extraordinary spread of carbapenem resistance observed worldwide (2) can be explained by the presence of bla_{KPC} genes on mobile genetic elements that are horizontally transferred (3), not only among *K. pneumoniae* bacteria but also to other *Enterobacteriaceae*, including *Citrobacter freundii* (4, 5). In this study, we describe the emergence of carbapenem-resistant (CR) *Citrobacter freundii* during an active surveillance program designed to detect all CRE from rectal swabs of patients from the clinical and surgical wards of the L. Spallanzani National Institute for Infectious Diseases (INMI) in Rome, Italy.

Rectal swabs were taken from all patients upon admission to the hospital and then once weekly for the entire duration of their stay. Carbapenem-resistant strains, including 11 CR-*C. freundii*, were isolated using selective culture plates (chromID CARBA; bioMérieux, Marcy l'Etoile, France). Identification was performed by the use of a Vitek 2 system (bioMérieux, Marcy l'Etoile, France) and mass spectrometry (MS) (matrix-assisted laser desorption ionization–time of flight MS [MALDI-TOF MS]; Brucker Daltonics, Germany), and *C. freundii* isolates were confirmed using the specific *recN* biomarker (6, 7) (data not shown). Antimicrobial susceptibility was determined by reference broth microdilution (8), and MICs were interpreted according to the European Committee on Antimicrobial Susceptibility Testing recommendations (EUCAST breakpoint tables v6.0). Since the beginning of our surveillance program in 2013, the species of CRE most commonly isolated from rectal swabs was *K. pneumoniae*, as expected (data not shown). The first CR-*C. freundii* strain to be isolated was collected in March 2014, together with an *Escherichia coli* strain which was also carbapenem resistant and which was grown from the same swab from the same patient, who had been transferred from another hospital to our surgical unit. This *E. coli* strain was also included in this study and characterized.

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In the following months, our surveillance continued, with no CR-*C. freundii* strains detected until August 2015; between August 2015 and January 2017, a total of 10 nonduplicated CR-*C. freundii* strains were isolated in the surgery and postsurgery wards and in the intensive care unit (ICU) (Table 1).

All strains were analyzed by multilocus sequence typing (MLST) as described previously (9, 10), and the resulting sequence types (ST) were designated using the Pasteur database (http://pubmlst.org/cfreundii) and the scheme of the University of Warwick, United Kingdom (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) for *C. freundii* isolates and *E. coli* isolates, respectively. Bacterial genotyping revealed that the CR-*C. freundii* strains belonged to 5 different STs: ST91 (4/11), ST96 (2/11), ST22 (2/11), ST118 (2/11), and ST119 (1/11) (Table 1). None of the observed STs have been previously reported in association with a specific resistance mechanism. The *E. coli* strain that was coisolated with *C. freundii* belonged to ST5, which has been found in association with OXA, NDM, and IMP carbapenemases (11–13); to the best of our knowledge, this is the first report of a KPC-3-producing ST5 *E. coli* strain.

Beta-lactamase genes (bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM}, bla_{SHV}, bla_{TEM}, and $bla_{CTX-M-GROUP}$) were screened by PCR followed by sequencing for the identification of variants (14, 15).

Our results showed the presence of the bla_{KPC-3} gene in all CR-*C. freundii* isolates; the two ST96 strains also carried bla_{VIM-2} and extended-spectrum-beta-lactamase (ESBL) $bla_{CTX-M-9}$ genes. The bla_{SHV-11} gene was identified in 9/11 *C. freundii* strains, while bla_{TEM-1} was detected in 6/11 strains. The *E. coli* strain also carried bla_{KPC-3} and bla_{SHV-11} genes (Table 1).

Plasmid replicons were typed using a PCR-based replicon typing (PBRT) kit (Diatheva) (16): all isolates, with the exception of two ST118 isolates, carried IncX3 replicons (Table 1). To verify whether bla_{KPC-3} was located on IncX3, the bla_{KPC-3}-carrying plasmid DNAs from these strains were purified by the use of a PureYield Plasmid Midiprep kit (Promega, USA) and transformed into competent *E. coli* DH5α cells (Invitrogen, USA). Transformants were selected on Luria-Bertani agar plates containing ampicillin (Sigma; 50 μg/ml) and were screened for the presence of the bla_{KPC-3} gene. The PBRT kit was used to assign the replicons on the transferred plasmids. Our KPC-3 transformation experiments conducted on the bla_{KPC-3}-positive transformants obtained from all IncX3-carrying CR-*C. freundii* strains showed that the bla_{KPC-3} gene was located on IncX3 and that the bla_{VIM-2} gene was located on a different plasmid only in the two isolates belonging to ST96.

Complete plasmid sequences were obtained for the *C. freundii* Cfr-30 and Cfr-145 prototypic transformants using an Illumina MiSeq next-generation sequencer with 2x300PE (Illumina Inc., CA, USA) according to the manufacturer's instructions. *De novo* assembly was performed using Galaxy version 20150522 of the A5 pipeline through the ARIES public Galaxy server (https://w3.iss.it/site/aries/) (17), and open reading frames (ORFs) were annotated using the Sequin server (http://www.ncbi.nlm.nih.gov/Sequin/). GenBank files of the pCfr-30 and pCfr-145 plasmid sequences were deposited at the NCBI GenBank database (see below). As shown in Fig. 1, the pCfr-30 and pCfr-145 plasmids (both 53,292 bp in size) showed 100% nucleotide identity and a typical IncX3 scaffold compared with the DNA sequence of the pIncX-SHV plasmid (JN247852) (18), with which they showed 99% nucleotide identity. The insertion of the Tn*4401a* transposon (3) carrying bla_{KPC-3} occurred in the *umuD* gene for the pCfr-30 plasmid and in the *tnpA* transposase gene of the Tn*3* transposon for pCfr-145 (Fig. 1). The same interruption of the *umuD* gene found in pCfr-30 had been previously identified in pKPC-Ny79 (JX104759) (19). However, the pKPC-Ny79 plasmid carried a bla_{KPC-2} gene variant and showed a deletion involving the ATPase and *hns-topB* genes. Interestingly, both plasmids showed 100% identity with a bla_{KPC-3}-carrying IncX3 plasmid (KU934011) of a *Serratia marcescens* strain recently isolated in Italy from a kidney-liver-transplanted patient, in which the Tn*4401a* transposon was also inserted in the *tnpA* of Tn*3*-like pCfr-145, although in the opposite orientation. In both plasmids, the bla_{SHV-11} gene was found to be associated with an IS*26* element. These findings are further evidence of the

TABLE 1 Molecular typing and antibiotic susceptibility profiles of strains characterized in this study

Code	Date of first screening[a]	Date of isolation of CRE	Ward[b]	Species	MLST	β-Lactamase genes	Replicon(s)[c]	Antibiotic MIC value (mg/liter)[d]														
								AMK	AMC	FEP	CTX	CAZ	CIP	CST	ERT	FOF	GEN	IPM	MEM	TZP	TGC	SXT
30	15 March 2014	15 March 2014	S	*C. freundii*	ST91	KPC-3, SHV-11	X3^{A}, FIB	≤2	≥32	≥64	≥64	≥64	≥4	≤0.5	≥8	≥256	≥16	≥16	≥16	≥128	≤0.5	≥320
31	15 March 2014	15 March 2014	S	*E. coli*	ST5	KPC-3, SHV-11	X3^{A}, FIB, *colE*	2	≥32	2	≥64	≥64	≤0.25	≤0.5	2	≥16	≤1	8	≥16	≥128	≤0.5	≤20
365	26 July 2015	03 August 2015	S	*C. freundii*	ST91	KPC-3, SHV-11	X3^{A}	≤2	≥32	I–2	8	≥64	≥4	1	4	≥256	≥16	≥16	≥16	≥128	≤0.5	≥320
138	02 November 2015	11 November 2015	PS	*C. freundii*	ST96	KPC-3, VIM-2, SHV-11, TEM-1, CTX-M-9	X3^{A}, HI2	16	≥32	≥64	≥64	≥64	≥4	≤0.5	≥8	≤16	≥16	≥16	≥16	≥128	1	≥320
145	06 January 2016	13 January 2016	PS	*C. freundii*	ST119	KPC-3, SHV-11, TEM-1	X3^{B}	≤2	≥32	≥64	≥64	≥64	≥4	≤0.5	≥8	≤16	8	≥16	≥16	≥128	1	≥320
19	21 March 2016	28 March 2016	S	*C. freundii*	ST91	KPC-3, SHV-11	X3^{A}	≤2	≥32	8	8	≥64	≥4	≤0.5	≥8	≥256	≥16	≥16	≥16	≥128	≤0.5	≥320
167	21 March 2016	23 April 2016	PS	*C. freundii*	ST118	KPC-3, TEM-1	HI1, N	≤2	≥32	16	≥64	32	≥4	≤0.5	≥8	≤16	≥16	≥16	≥16	≥128	1.5	≥320
134	06 May 2016	13 May 2016	ICU	*C. freundii*	ST22	KPC-3, SHV-11	X3	≤2	≥32	16	≥64	32	≥4	≤0.5	≥8	≤16	≤1	≥16	≥16	≥128	1.5	≥320
124	22 June 2016	28 June 2016	PS	*C. freundii*	ST96	KPC-3, VIM-2, SHV-11, TEM-1, CTX-M-9	X3^{A}, HI2, N	16	≥32	≥64	≥64	≥64	≥4	≤0.5	≥8	≤16	≥16	≥16	≥16	≥128	≤0.5	≥320
80	30 June 2016	06 July 2016	PS	*C. freundii*	ST118	KPC-3, TEM-1	HI1	≤2	≥32	≥64	≥64	≥64	≥4	≤0.5	≥8	≤16	8	≥16	≥16	≥128	1.5	≥320
87	29 July 2016	06 September 2016	S	*C. freundii*	ST91	KPC-3, SHV-11	X3^{A}	≤2	≥32	8	8	≥64	≥4	≤0.5	≥8	≥256	≥16	≥16	≥16	≥128	≤0.5	≥320
162	27 December 2016	11 January 2017	PS	*C. freundii*	ST22	KPC-3, SHV-11, TEM-1	X3^{A}, A/C, FIIS	≤2	≥32	16	≥64	32	≥4	≤0.5	≥8	≤16	≤1	≥16	≥16	≥128	1.5	≥320

[a]Date of rectal swab performed upon admission to the ward.

[b]S, surgery; PS, postsurgery; ICU, intensive care unit.

[c]bla_{KPC-3} genes carrying replicon plasmids are underlined. X3^{A}, IncX3 plasmid presenting the insertion carrying bla_{KPC-3} in the *umuD* gene; X3^{B}, IncX3 plasmid presenting the insertion carrying bla_{KPC-3} in the *tnpA* Tn*3*.

[d]AMK, amikacin; AMC, amoxicillin-clavulanic acid; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; ERT, ertapenem; FOF, fosfomycin, GEN; gentamicin; IPM, imipenem; MEM, meropenem; TZP, piperacillin-tazobactam; TGC, tigecyclin, SXT, trimethoprim-sulfamethoxazole.

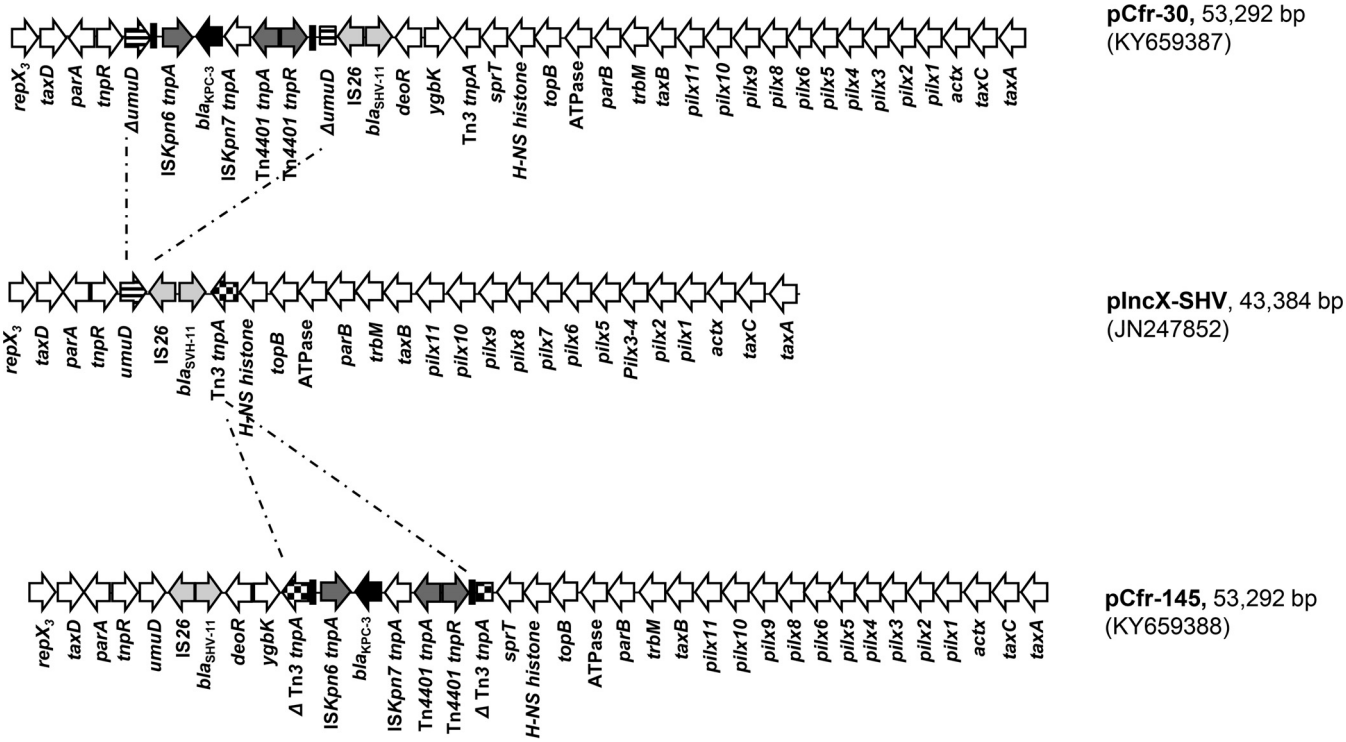

FIG 1 Linear maps of plasmids pCfr-30, pCfr-145, and pIncX-SHV. White arrows represent predicted open reading frames (ORFs); the functions of the plasmids are indicated below the arrows in the linear maps. The ORFs of pCfr-30 and pCfr-145 were identified in this study; ORFs of pIncX-SHV were deduced from the data available under GenBank accession no. JN247852. Transposase genes of the Tn*4401a* transposons are indicated by gray arrows; the bla_{KPC-3} genes inside Tn*4401a* are indicated by black arrows. The *umuD* and Tn*3 tnpA* genes targeted by the Tn*4401* transposition events are indicated by striped and dotted arrows, respectively.

circulation of these plasmids in hospital settings and in *Enterobacteriaceae* other than *K. pneumoniae*. Specific PCR assays were devised to detect the integration regions of Tn*4401a*, discerning the *umuD* or *tnpA* integration sites, respectively (KPC-Fw [5′-GCT ACACCTAGCTCCACCTTC-3′] [14]; *umuD* Rv [5′-TTTTCCATCGCCAGCAACC-3′]; and *tnpA* Rv [5′-GTCGAGTGCCCGGTATTTAG-3′]). The KPC-3-IncX3 plasmids were then classified on the basis of the integration sites as IncX3-type A and IncX3-type B, respectively (Table 1). PCR results showed that all the ST91 and ST96 strains and one of the two ST22 *C. freundii* strains isolated in 2017, as well as the *E. coli* strain, contained an IncX3-type A plasmid with the integration of bla_{KPC-3} in the *umuD* gene (Table 1). These results were confirmed by PstI restriction fragment length polymorphism (RFLP) analysis performed on plasmids from transformants Cfr-30, Cfr-138, and Cfr-145 obtained from *C. freundii* and *E. coli* 31 (Ec-31) from *E. coli* (data not shown). Plasmid DNAs of transformants Cfr-30, Ec-31, and Cfr-138 showed identical profiles (IncX3-type A), differing in only one band from the profile of Cfr-145 (IncX3-type B); this additional band was due to the integration of the bla_{KPC-3} gene in the Tn*3* transposase. It is noteworthy that all of the IncX3-carrying CR-*C. freundii* strains described above were from the surgical and postsurgical wards, except for one of the two ST22 strains, which was isolated in 2016 from the ICU (Table 1). This strain was also the only one that was negative in both IncX3-integration-site PCRs, suggesting a possible Tn*4401a* insertion in other regions of the plasmid scaffold.

Taken together, the data presented in our report confirm the finding of an IncX3-type A plasmid in different *C. freundii* clones circulating in surgical wards and in an *E. coli* strain coisolated from the index patient. The fact that this patient had been colonized prior to admission to the surgical ward of our hospital in 2014, while all our other subjects had negative swabs upon arrival, supports the hypothesis that an IncX3–KPC-3 plasmid was maintained within the ward.

Further, the presence of the same IncX3 plasmid in the *E. coli* and *C. freundii* strains in the index patient suggests that the bla_{KPC-3}-carrying IncX3 plasmid was probably transferred *in vivo* between the two species in the gut of the patient. We cannot exclude the possibility that *E. coli* may have been the original donor; however, IncX3 seems highly prevalent in *C. freundii*, having been previously identified not only in association with the bla_{KPC-3} gene but also in association with bla_{NDM} (20, 21).

In conclusion, the observation of several cases of CR-*C. freundii* isolates carrying IncX3–KPC-3 plasmids suggests their ability to persist in hospital wards; further, the fact that the same ST22 clone circulating in different wards acquired two different KPC-3–IncX3 plasmids in the course of several months (Table 1) suggests that these plasmids can be effectively transferred and maintained within *Enterobacteriaceae* strains of different origins and sources.

All our CR-*C. freundii* isolates were colonizers since all *C. freundii* strains obtained from clinically relevant sites of patients treated in the surgical wards were also analyzed by MLST, revealing a heterogeneity of STs and, more importantly, the absence of the IncX3 plasmid and resistance determinants (data not shown). No other CRE were isolated from these patients, and no CR-*C. freundii* strains were isolated in the entire hospital, except for those described in this study; our results therefore reinforce the importance of maintaining an active surveillance of all CR CRE, together with effective infection control practices.

Accession number(s). Sequence data have been submitted to GenBank under the following accession numbers: for pCfr-30, KY659387; for pCfr-145, KY659388.

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