

# Genetic and Biochemical Characterization of FRI-1, a Carbapenem-Hydrolyzing Class A $\beta$ -Lactamase from *Enterobacter cloacae*

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**An *Enterobacter cloacae* isolate was recovered from a rectal swab from a patient hospitalized in France with previous travel to Switzerland. It was resistant to penicillins, narrow- and broad-spectrum cephalosporins, aztreonam, and carbapenems but remained susceptible to expanded-spectrum cephalosporins. Whereas PCR-based identification of the most common carbapenemase genes failed, the biochemical Carba NP test II identified an Ambler class A carbapenemase. Cloning experiments followed by sequencing identified a gene encoding a totally novel class A carbapenemase, FRI-1, sharing 51 to 55% amino acid sequence identity with the closest carbapenemase sequences. However, it shared conserved residues as a source of carbapenemase activity. Purified  $\beta$ -lactamase FRI-1 hydrolyzed penicillins, aztreonam, and carbapenems but spared expanded-spectrum cephalosporins. The 50% inhibitory concentrations (IC<sub>50</sub>s) of clavulanic acid and tazobactam were 10-fold higher than those found for *Klebsiella pneumoniae* carbapenemase (KPC), IMI, and SME, leading to lower sensitivity of FRI-1 activity to  $\beta$ -lactamase inhibitors. The *bla*<sub>FRI-1</sub> gene was located on a ca. 110-kb untypeable, transferable, and non-self-conjugative plasmid. A putative LysR family regulator-encoding gene at the 5' end of the  $\beta$ -lactamase gene was identified, leading to inducible expression of the *bla*<sub>FRI-1</sub> gene.**

Carbapenem resistance in *Enterobacteriaceae* may be related to two mechanisms: (i) overexpression of a  $\beta$ -lactamase possessing no (or weak) activity against carbapenems (e.g., extended-spectrum beta-lactamase [ESBL] and cephalosporinases) combined with decreased outer membrane permeability and (ii) expression of enzymes able to hydrolyze carbapenems, namely, the carbapenemases (1). The most clinically relevant carbapenemases are classified into three groups according to protein sequence identity: (i) the *Klebsiella pneumoniae* carbapenemase (KPC)-type enzymes (Ambler class A), first described in the United States but now found worldwide (2, 3); (ii) the VIM, IMP, and NDM metallo- $\beta$ -lactamases (Ambler class B) (1, 4); and (iii) the OXA-48-type enzymes (Ambler class D), widespread among Mediterranean countries and progressively disseminating to other geographical areas (5).

Ambler class A carbapenemases hydrolyze a large variety of  $\beta$ -lactams, including penicillins, cephalosporins, carbapenems, and aztreonam (6). Their hydrolytic activity is *in vitro* inhibited by clavulanic acid and tazobactam. Four main types of class A carbapenemases are known: NmcA/IMI, SME, KPC, and several variants of the GES type (GES-2, -4, -5, -6, and -11) (7).

The SME family includes three variants (SME-1 to -3). These enzymes have all been chromosome encoded in *S. marcescens* isolates (8–11). The chromosome-encoded NmcA and IMI enzymes have been detected in rare isolates of *Enterobacter* spp. (12, 13). The gene encoding the IMI-2 variant has been identified sporadically as plasmid located in environmental strains of *Enterobacter* spp. (14). The GES-type family includes 27 variants, only a few of which possess carbapenemase activity (7). All the GES variants possess the ability to hydrolyze broad-spectrum cephalosporins, but only some variants (mainly GES-2, GES-4, and GES-5 in *Enterobacteriaceae*) possess amino acid substitutions within their active sites (positions 104 and 170 according to the Ambler classification) that enlarge their spectra of activity against carbapenems (7, 15). Although still rare, GES enzymes have been identified

worldwide. The most prevalent Ambler class A carbapenemase is KPC. This plasmid-encoded enzyme was first identified in 2001 in the United States (16). Since then, KPC-producing *Enterobacteriaceae* have spread worldwide, mostly due to the clonal dissemination of KPC-producing *K. pneumoniae* isolates of sequence type 258 (ST258) (2, 3). While this study was being completed, another class A carbapenemase, BKC-1, was identified from *K. pneumoniae* in Brazil (17).

The aim of this study was to characterize at the genetic and biochemical levels the molecular mechanisms of resistance to carbapenems from an *Enterobacter cloacae* isolate.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *E. cloacae* isolate DUB was recovered from a urine sample from a patient, hospitalized in a suburb of Paris, with a previous history of travel (without hospitalization) in Switzerland. Identification of the clinical isolate was done by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper CA system; Bruker Daltonics, Billerica, MA, USA). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning experiments and azide-resistant *E. coli* J53 for conjugation assays. The kanamycin-resistant pBK-CMV (Invitrogen, Saint-Aubin, France) was used as the cloning vector. Bacterial cultures were grown in Trypticase soy (TS) broth at 37°C for 18 h unless otherwise indicated.

TABLE 1 MICs of  $\beta$ -lactams for *E. cloacae* DUB, *E. coli* TOP10 transformed with the natural *bla*<sub>FRI-1</sub>-bearing plasmid (pDUB), *E. coli* TOP10 harboring a recombinant plasmid (pFRI), and the *E. coli* TOP10 reference strain

$\beta$ -Lactam(s)	MIC ( $\mu$ g/ml)			
	<i>E. cloacae</i> DUB	<i>E. coli</i> TOP10(pDUB) <sup>a</sup>	<i>E. coli</i> TOP10(pFRI) <sup>b</sup>	<i>E. coli</i> TOP10
Amoxicillin	>256	128	>256	2
Amoxicillin + CLA <sup>c</sup>	>256	64	96	2
Ticarcillin	>256	>256	>256	2
Ticarcillin + CLA	256	96	96	2
Piperacillin	128	12	24	1
Piperacillin + TZB <sup>d</sup>	96	8	12	1
Cefalotin	>256	256	>256	4
Cefoxitin	>256	2	2	2
Ceftazidime	4	2	2	0.12
Ceftazidime + CLA	0.78	0.5	0.75	0.12
Ceftazidime + TZB	4	1.5	1.5	0.12
Cefotaxime	1	0.38	0.5	0.06
Cefotaxime + CLA	0.5	0.12	0.19	0.06
Cefotaxime + TZB	1	0.09	0.5	0.06
Cefepime	0.5	0.19	0.19	0.02
Cefepime + CLA	0.06	0.06	0.06	0.02
Cefepime + TZB	0.25	0.06	0.06	0.02
Cefpirome	1.5	0.25	0.38	0.02
Cefpirome + CLA	1	0.06	0.09	0.02
Cefpirome + TZB	0.75	0.06	0.38	0.02
Aztreonam	256	256	256	0.09
Aztreonam + CLA	256	8	32	0.09
Aztreonam + TZB	256	64	256	0.09
Imipenem	8	0.75	4	0.06
Imipenem + CLA	4	0.38	2	0.06
Imipenem + TZB	4	0.5	1.5	0.06
Meropenem	3	0.12	0.38	0.02
Meropenem + CLA	2	0.03	0.12	0.02
Meropenem + TZB	4	0.09	0.25	0.02
Ertapenem	24	0.12	0.75	0.06
Ertapenem + CLA	8	0.03	0.12	0.06
Ertapenem + TZB	4	0.06	0.38	0.06

<sup>a</sup> pDUB, natural plasmid carrying the *bla*<sub>FRI-1</sub> gene.

<sup>b</sup> pFRI, the *bla*<sub>FRI-1</sub> gene cloned in the pBK-CMV plasmid.

<sup>c</sup> CLA, clavulanic acid at a fixed concentration of 4  $\mu$ g/ml.

<sup>d</sup> TZB, tazobactam at a fixed concentration of 4  $\mu$ g/ml.

**Susceptibility testing.** Antimicrobial susceptibilities were determined by the disc diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints as updated in 2015 (<http://www.eucast.org>). MICs were determined using the Etest technique (bioMérieux, La Balme-Les-Grottes, France).

**Detection of carbapenemase activity.** Carbapenemase activity was analyzed by using two techniques, namely, the biochemical Carba NP test (18) and UV spectrophotometry, as previously described (19). Discrimination between Ambler class A, B, and non-A non-B carbapenemases was assessed using the Carba NP test II results, as previously described (20).

**Molecular detection of carbapenemase-encoding genes.** PCR screening for the most common class A carbapenemase genes (*bla*<sub>KPC</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>GES</sub>, *bla*<sub>SFC-1</sub>, and *bla*<sub>IMI/NMC-A</sub>) was done as previously described (21).

**Plasmid extraction and conjugation assays.** Plasmid DNA of *E. cloacae* DUB was extracted and analyzed using the Kieser method, as described previously (22). Recombinant plasmid DNA was prepared using Qiagen maxi columns (Qiagen, Courtaboeuf, France). Transfer of the imipenem resistance marker into *E. coli* TOP10 was attempted by electroporation. Transformants were selected on ticarcillin (100  $\mu$ g/ml)-con-

taining TS agar plates (Oxoid, Dardilly, France). Transfer of the  $\beta$ -lactam resistance marker into azide-resistant *E. coli* J53 was also attempted by liquid mating-out assays at 37°C. Transformants were selected on azide (100  $\mu$ g/ml)- and ticarcillin (100  $\mu$ g/ml)-containing TS agar plates. Plasmid typing was performed on electrotransformant strains by using the PCR-based replicon-typing (PBRT) method, as described previously (23).

**Cloning experiments, recombinant plasmid analysis, and DNA sequencing.** PCR amplification of the identified *bla*<sub>FRI-1</sub> gene was performed by using the internal primers FRI-1A (5'-TGAACTCATTCGCC TCTCAG-3') and FRI-1B (5'-CTGCTTCGTCATGTTTGTCG-3'). Whole-cell DNA of the *E. cloacae* isolate was extracted using a QIAamp DNA minikit (Qiagen, Courtaboeuf, France). Partially Sau3AI-restricted DNA was ligated into the BamHI-restricted pBK-CMV plasmid and introduced into *E. coli* TOP10 by electroporation. Recombinant plasmids were selected on ticarcillin (100  $\mu$ g/ml)- and kanamycin (50  $\mu$ g/ml)-containing Trypticase soy agar plates. The recombinant plasmid possessing the shortest insert, namely, pFRI, was retained for further analysis. Both strands of the cloned DNA inserts of recombinant plasmids were sequenced by using an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced protein sequences were analyzed with software avail-



ceftazidime and carbapenems (Table 1). The positivity of the Carba NP test identified the expression of carbapenemase. PCR experiments carried out on purified DNA of whole-cell *E. cloacae* DUB with primers specific for the most common carbapenemase genes (*bla*<sub>KPC</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub>) remained negative. The Carba NP test II was therefore performed to identify the type of carbapenemase. The inhibition of carbapenemase activity by tazobactam suggested that this carbapenemase belonged to the Ambler class A group. Accordingly, additional PCR experiments were performed using primers specific for rarer Ambler class A carbapenemase genes (*bla*<sub>GES</sub>, *bla*<sub>SFC-1</sub>, and *bla*<sub>IMI/NMC-A</sub>), but they also remained negative. IEF analysis revealed that strain DUB produced an acquired  $\beta$ -lactamase with a pI value of ca. 8.4.

**Cloning, conjugation, and transformation of the  $\beta$ -lactamase gene.** Shotgun cloning resulted in the selection of an *E. coli* TOP10(pFRI) recombinant strain that expressed a clavulanic acid-inhibited carbapenemase phenotype with resistance or reduced susceptibility to penicillins, ceftazidime, aztreonam, and carbapenems. The addition of clavulanic acid partially restored the activities of the  $\beta$ -lactams (Table 1). IEF analysis showed that *E. coli* TOP10(pFRI) produced a  $\beta$ -lactamase with a pI value of 8.4, identical to that identified in *E. cloacae* DUB (data not shown). Analysis of the plasmid DNA extract of the *E. cloacae* DUB isolate revealed a ca. 110-kb plasmid. Transfer of the imipenem resistance marker into *E. coli* TOP10 by electroporation was successful. In contrast, mating-out assays failed to give any transconjugant. PBRT analysis performed using a DNA extract of an *E. coli* TOP10 transformant (pDUB) (23) failed to identify a plasmid of any known incompatibility group.

**Identification of  $\beta$ -lactamase FRI-1.** DNA sequence analysis of the 2,863-bp insert of pFRI revealed an open reading frame (ORF) of 885 bp encoding a 295-amino-acid preprotein, FRI-1 (French imipenemase), with a relative molecular mass of 32.5 kDa. The G+C content of this ORF was 39%. The signal peptide cleavage site was identified between the alanine and serine residues at positions 23 and 24 (AS-QV) of FRI-1 (Fig. 1). The  $\beta$ -lactamase FRI-1 contained four conserved motifs of class A serine  $\beta$ -lactamases, namely, <sup>70</sup>SSFK<sup>73</sup>, <sup>130</sup>SDN<sup>132</sup>, <sup>166</sup>EXXXN<sup>170</sup>, and <sup>234</sup>KTG<sup>236</sup> (25) (Fig. 1). Interestingly, FRI-1 contains the amino acid residues that, associated, have been identified as a source of carbapenemase activity in class A  $\beta$ -lactamases; <sup>69</sup>C, <sup>105</sup>H, <sup>167</sup>L, <sup>237</sup>S, <sup>238</sup>C, and <sup>241</sup>Y (25–27). The  $\beta$ -lactamase FRI-1 shares 55%, 54%, 53%, 53%, and 51% amino acid identity with the class A carbapenemases NMC-A, IMI-1, SME-1, SFC-1, and KPC-2, respectively (Fig. 1). It shares 42% amino acid identity with the recently identified  $\beta$ -lactamase BKC-1 (data not shown). A dendrogram was generated from the amino acid sequence alignment of FRI-1 with main class A  $\beta$ -lactamases. It showed that FRI-1 is more closely related to the subgroup that includes SME-1, IMI-1, and NMC-A than to that of KPC-2, SFC-1, and BIC-1 (Fig. 2).

**Biochemical features of  $\beta$ -lactamase FRI-1.** The purification state of FRI-1 was estimated to be >95% by SDS-PAGE analysis (data not shown). Kinetic parameters of the purified  $\beta$ -lactamase FRI-1 showed that it possessed quite significant carbapenemase activity (Table 2). The highest  $k_{cat}$  value for carbapenems was obtained with imipenem and was approximately 39- and 12-fold higher than those for meropenem and ertapenem, respectively (Table 2). The  $k_{cat}/K_m$  values obtained for FRI-1 were close to

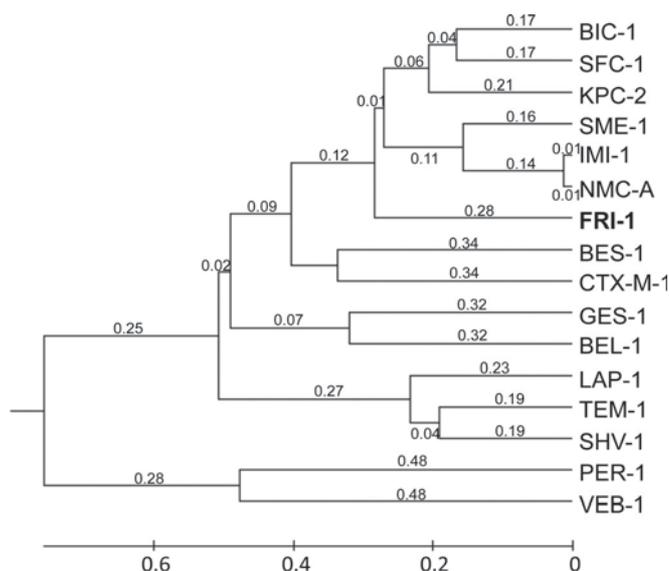


FIG 2 Dendrogram obtained for 16 representative class A  $\beta$ -lactamases by neighbor-joining analysis. The alignment used for the tree calculation was performed with the ClustalW program. Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The distance along the vertical axis has no significance. The  $\beta$ -lactamases (GenBank accession numbers) are BIC-1 (GQ260093), SFC-1 (AY354402), KPC-2 (AY034847), SME-1 (Z28968), IMI-1 (U50278), NMC-A (Z21956), BES-1 (AF234999), CTX-M-1 (X92506), GES-1 (AF156486), BEL-1 (DQ089809), LAP-1 (EF026092), TEM-1 (AY458016), SHV-1 (AF148850), PER-1 (Z21957), and VEB-1 (AF010416).

those obtained for NMC-A and IMI-1 (Table 2). Notably, the  $k_{cat}$  value for aztreonam was very high (Table 2), which correlates with the high MIC values obtained with the *E. coli* TOP10 transformant and the *E. coli* TOP10(pFRI) recombinant strain (Table 1). Inhibition studies, as measured by IC<sub>50</sub>, showed that the activity of FRI was weakly inhibited by clavulanic acid (IC<sub>50</sub>, 90  $\mu$ M) and tazobactam (IC<sub>50</sub>, 15  $\mu$ M). These values are in the same range as those found for SFC-1, 72.8 and 6.9  $\mu$ M for clavulanic acid and tazobactam, respectively (28, 29). In addition, we observed that *bla*<sub>FRI-1</sub> expression was inducible (~8- to 10-fold) by imipenem (5  $\mu$ g/ml) or by cefoxitin (50  $\mu$ g/ml and 200  $\mu$ g/ml).

**Genetic environment of the *bla*<sub>FRI-1</sub> gene.** Part of the 5,750-bp insert of the recombinant plasmid pFRI was sequenced to identify the flanking sequences of the *bla*<sub>FRI-1</sub> gene (Fig. 3). The *bla*<sub>FRI-1</sub> gene was bracketed by two insertion sequences (IS). The closest IS shared 93% identity with IS*Raq1* (GenBank accession no. AY528232), identified in *Rahnella aquatilis*. This IS*Raq1*-like IS was truncated by the insertion of another IS belonging to the IS66 family, sharing 87% identity with IS*Kpn24* (GenBank accession no. NC\_014312). Upstream of *bla*<sub>FRI-1</sub>, a gene encoding FRI-R, a LysR transcriptional regulator sharing 63% amino acid identity with SmeR, the regulator of SME-1, was identified. The *friR* gene possessed a G+C content similar to that of the *bla*<sub>FRI-1</sub> gene (39%).

## DISCUSSION

Our study identified a novel plasmid-encoded class A carbapenemase from the urine of a French patient who had traveled in Switzerland. The  $\beta$ -lactamase FRI-1 shares the highest amino acid identity with the chromosome-encoded Ambler class A carbapen-

TABLE 2 Steady-state kinetic parameters of the  $\beta$ -lactamase FRI-1 and comparison of parameters obtained for the  $\beta$ -lactamases NMC-A (24), IMI-1 (13), SME-1 (11), KPC-2 (16), SFC-1 (28), and GES-4 (15)

Parameter	$\beta$ -Lactam	Value <sup>a</sup>						
		FRI-1	NMC-A	IMI-1	SME-1	KPC-2	SFC-1	GES-4
$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	Benzylpenicillin	1,060	260	36	19.3	63	NA	130
	Amoxicillin	>17,000	816	190	181	NA	NA	19
	Ticarcillin	120	81	NA	NA	NA	NA	NA
	Piperacillin	>2,600	NA	6.1	NA	NA	NA	NA
	Cefotaxime	>220	286	3.4	<0.98	17	8.3	17
	Cefepime	28	NA	NA	NA	12	NA	NA
	Ceftazidime	–	–	<0.01	NA	0.5	2.1	2.5
	Aztreonam	>8,300	707	51	108	66	162	NA
	Imipenem	1,790	1,040	89	104	31	54	7.7
	Ertapenem	150	NA	NA	NA	NA	NA	NA
	Meropenem	46	12	10	8.9	3.6	6.5	NA
$K_m$ ( $\mu\text{M}$ )	Benzylpenicillin	567	28	64	16.7	30	NA	160
	Amoxicillin	>5,000	90	780	488	NA	NA	62
	Ticarcillin	393	152	NA	NA	NA	NA	NA
	Piperacillin	>3,000	NA	13	NA	NA	NA	NA
	Cefotaxime	>5,000	956	190	–	100	89	700
	Cefepime	3,400	NA	NA	NA	540	NA	NA
	Ceftazidime	–	–	270	–	230	52	1,500
	Aztreonam	>5,000	125	93	259	420	484	–
	Imipenem	1,614	92	170	202	90	82	4.7
	Ertapenem	98	NA	NA	NA	NA	NA	NA
	Meropenem	70	4.35	26	13.4	13	26	NA
$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1}/\text{s}$ )	Benzylpenicillin	1,870	9,300	560	1,160	2,100	NA	780
	Amoxicillin	3,400	9,060	240	370	NA	NA	310
	Ticarcillin	305	530	NA	NA	NA	NA	NA
	Piperacillin	867	NA	470	NA	NA	NA	NA
	Cefotaxime	44	300	18	–	170	93	24
	Cefepime	8	NA	NA	NA	22	NA	NA
	Ceftazidime	–	52	0.02	–	2.1	40	1.7
	Aztreonam	1,660	5,600	550	420	160	3.5	–
	Imipenem	1,109	11,000	520	520	340	660	81
	Ertapenem	1,531	NA	NA	NA	280	250	NA
	Meropenem	657	2,700	380	660	NA	NA	NA

<sup>a</sup> –, no detectable hydrolysis; NA, no data available.

emases NMC-A from *E. cloacae* (12) and IMI-1 (13). Biochemical characterization of FRI-1 showed significant hydrolysis of carbapenemase, and its protein structure analysis identified conserved amino acid residues as a source of its carbapenemase activity. As observed for other class A  $\beta$ -lactamases, such as NMC-A (12), IMI-1 (13), and SFC-1 (30), FRI-1 confers a high level of resistance to aztreonam but does not confer significant resistance to broad-spectrum cephalosporins, such as ceftazidime, cefotaxime, and cefepime (Table 1). Therefore, its resistance profile differs

from that of KPCs that hydrolyze all extended-spectrum  $\beta$ -lactams.

Analysis of the immediate upstream genetic environment of the  $bla_{\text{FRI-1}}$  gene identified a LysR-type transcriptional regulator, FriR, as previously observed for other class A carbapenemases, such as NMC-A, IMI-1, and SME (13, 31, 32). Notably, the G+C content of the  $bla_{\text{FRI-1}}$  gene and its regulator  $friR$  (39%) differed from that of *E. cloacae* genes (ca. 55%), suggesting the acquisition of the  $friR$ - $bla_{\text{FRI-1}}$  locus through a horizontal gene transfer process. These results further emphasize that acquisition of carbapenemase genes by a group of *Enterobacteriaceae* results from acquisition “in block” of the  $\beta$ -lactamase gene and its regulator from a nonenterobacterial species acting as the reservoir. The presence of IS upstream and downstream of the DNA fragment  $friR$ - $bla_{\text{FRI-1}}$  suggested that those elements might have been involved in the mobilization process. The plasmid location of the  $bla_{\text{FRI-1}}$  gene identified in an enterobacterial species adds to the list of carbapenemase genes able to disseminate worldwide.

Finally, this work highlights the need to use, not only molecular-based techniques, but also biochemical methods for the screening



FIG 3 Schematic map of the structures surrounding  $bla_{\text{FRI-1}}$  identified in the *E. cloacae* DUB isolate. The genes and their corresponding transcriptional orientations are indicated by horizontal arrows. Sau3AI restriction sites that allowed cloning in the pBK-CMV plasmid (pFRI) are indicated.

of carbapenemase-producing strains due to the growing diversity of carbapenemases.

## ACKNOWLEDGMENTS

This work was financed by INSERM U914, Paris, France, and the University of Fribourg, Fribourg, Switzerland.

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