



Novel Carbapenemases FLC-1 and IMI-2 Encoded by an *Enterobacter cloacae* Complex Isolated from Food Products

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ABSTRACT Food for human consumption is screened widely for the presence of antibiotic-resistant bacteria to assess the potential for transfer of resistant bacteria to the general population. Here, we describe an *Enterobacter cloacae* complex isolated from imported seafood that encodes two carbapenemases on two distinct plasmids. Both enzymes belong to Ambler class A β -lactamases, the previously described IMI-2 and a novel family designated FLC-1. The hydrolytic activity of the novel enzyme against aminopenicillins, cephalosporins, and carbapenems was determined.

KEYWORDS antimicrobial resistance, carbapenemase, *Enterobacter*, plasmid

To combat antimicrobial resistance (AMR) effectively, it is important to monitor reservoirs that may be sources of transmission to humans. Relevant reservoirs are those that may be attributed to the AMR genes found in the general population and patients. Seafood has been implicated as a potential source of AMR genes entering populations when several aquatic bacteria carrying carbapenemase genes were identified in seafood imported from Southeast Asia (1, 2). Often, these genes are chromosomally located in nonpathogenic aquatic bacterial species, limiting them as relevant threats for the general population (3). However, more recent studies screening seafood imported from Southeast Asia have found carbapenemases encoded in human pathogens or on conjugative plasmids (4–6). As such, seafood imported from countries with high carbapenemase prevalence may need to be included in monitoring programs.

Proteins with carbapenemase activity fall into the three major Ambler classes A, B, and D β -lactamases (7). Genes of these classes have been described on mobile genetic elements, such as plasmids and chromosomally integrated elements, which adds to the concerns regarding these genes because they facilitate the spread of these genes among both commensal and pathogenic bacteria (6, 8, 9). The family of *Enterobacteriaceae* consists of many commensal, opportunistic, and infectious species that can readily exchange genetic material. The organisms are collectively referred to as carbapenemase-producing *Enterobacteriaceae* when they have acquired and express one of these genes.

Recently, *Enterobacter cloacae* complex and *Vibrio cholerae* isolates have been described with a distinctive phenotype of hydrolyzing penicillins, aztreonam, and carbapenems but not extended-spectrum cephalosporins (10).

In March 2017, we isolated an *E. cloacae* complex isolate, designated 3442, on a ChromID Carba plate (bioMérieux Benelux BV) from a sample of frozen vannamei white shrimp (*Litopenaeus vannamei*) originating in India. Species identification was per-

Citation Brouwer MSM, Tehrani KHME, Rapallini M, Geurts Y, Kant A, Harders F, Mashayekhi V, Martin NI, Bossers A, Mevius DJ, Wit B, Veldman KT. 2019. Novel carbapenemases FLC-1 and IMI-2 encoded by an *Enterobacter cloacae* complex isolated from food products. *Antimicrob Agents Chemother* 63:e02338-18. <https://doi.org/10.1128/AAC.02338-18>.

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Received 5 November 2018

Returned for modification 16 December 2018

Accepted 17 March 2019

Accepted manuscript posted online 25 March 2019

Published 24 May 2019

TABLE 1 MICs of *E. cloacae* complex 3442, *E. coli* recipients, transformant, and transconjugant of pIMI2 and transformant of pBAD-FLC

Antibiotic	MIC ($\mu\text{g/ml}$) for:						
	<i>E. cloacae</i> 3442	<i>E. coli</i> DH10B pIMI2 (transformant)	<i>E. coli</i> DH10B	<i>E. coli</i> E3110 pIMI2 (transconjugant)	<i>E. coli</i> E3110	<i>E. coli</i> LMG194 pBAD-FLC	<i>E. coli</i> LMG194
Ampicillin	>64	>64	4	>64	4	>64	4
Cefotaxime	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	2	≤ 0.25
Cefotaxime/clavulanic acid	0.25/4	$\leq 0.06/4$	$\leq 0.06/4$	0.12/4	0.12/4	0.12/4	$\leq 0.06/4$
Ceftazidime	≤ 0.5	0.5	0.5	0.5	0.5	1	0.5
Ceftazidime/clavulanic acid	$\leq 0.12/4$	0.25/4	0.25/4	0.25/4	0.25/4	0.25/4	0.25/4
Cefepime	0.12	0.25	≤ 0.06	0.25	0.12	0.5	0.12
Cefoxitin	64	16	8	8	16	8	8
Ertapenem	>2	>2	≤ 0.015	>2	≤ 0.015	>2	≤ 0.015
Imipenem	>16	16	0.25	>16	0.5	16	0.25
Meropenem	>16	8	≤ 0.03	>16	0.06	4	≤ 0.03
Temocillin	4	16	32	16	16	16	8

formed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Microflex LT/SH, Bruker Daltonics, Billerica MA). Susceptibility testing was performed with broth microdilution in the Sensititre panels EUVSEC and EUVSEC2 (Thermo Fisher, Waltham MA) and interpreted using epidemiological cutoff values for cephalosporins and carbapenems as defined for *Escherichia coli* by EUCAST. The isolate exhibited an unusual phenotype, i.e., non-wild type susceptible to carbapenems (meropenem, ertapenem, and imipenem) and susceptible to extended-spectrum cephalosporins (cefotaxime, ceftazidime, and cefepime) (Table 1).

Complete genomic DNA was isolated using the Genra Puregene kit (Qiagen), and whole-genome sequencing was performed using Illumina MiSeq PE300 and ONT Minlon sequencing. Hybrid assemblies were created using a SPAdes reconstructed genome consisting of one chromosome and three plasmids. The chromosome data were used to determine the MLST type as ST813, a sequence type first described last year in companion animals in Japan (11).

Plasmid p3442-FLC-1 is 93 kb and carries a novel carbapenemase with close sequence similarity to *bla*_{FRI-1} (10). Plasmid p3442-IMI2 is 78 kb and carries the carbapenemase *bla*_{IMI-2}. Both plasmids are closely related to the IncFII plasmids first described in *Yersinia* spp. but have new FII-Y alleles (submitted to pubMLST and designated FII-Y-9 and FII-Y-10) (12). The closest relative to both plasmids is pIMI-6, described in an *E. cloacae* complex clinical isolate from Canada, which carries the carbapenemase *bla*_{IMI-6} (8). The two IncFII-Y plasmids contain much overlap in their sequences, but both plasmids appear to have lost significant amounts of genetic material, and we hypothesize that these plasmids developed from a pair of complete IncFII-Y plasmids. Currently, the two plasmids together contain most of the functions of IncFII-Y plasmids, as shown in Fig. S1 in the supplemental material in a BLAST ring image generator (13) comparison with the *E. cloacae* complex plasmid pIMI-6 (8), although several regions are absent in both plasmids.

To assess the mobility of these plasmids, transformation and conjugation experiments were performed as previously described (14). Carbapenem-resistant transconjugants and transformants were tested for *bla*_{IMI} and *bla*_{FRI}. Out of >200 colonies, only *bla*_{IMI}-positive transconjugants and transformants were detected. Antibiograms of transformants and transconjugants are included in Table 1. Whereas only p3442-IMI2 may be both transformed and conjugated into *E. coli* recipient cells, p3442-FLC-1 may be transferred at lower frequencies, below the level of detection used in the current experiments.

Although *bla*_{IMI-2} transformants and transconjugants were carbapenem resistant, we hypothesized that the *bla*_{FRI}-related gene may also have carbapenemase activity. Because the plasmid carrying the gene could not be transformed or conjugated into *E. coli* cells, the gene was cloned into an arabinose-inducible expression vector, pBAD-FLC (Vectorbuilder), and expressed in *E. coli* LMG194 (pBAD TOP TA expression kit, Invitrogen, Saint-Aubin, France) (15). The MIC of *E. coli* LMG194 pBAD-FLC was determined by

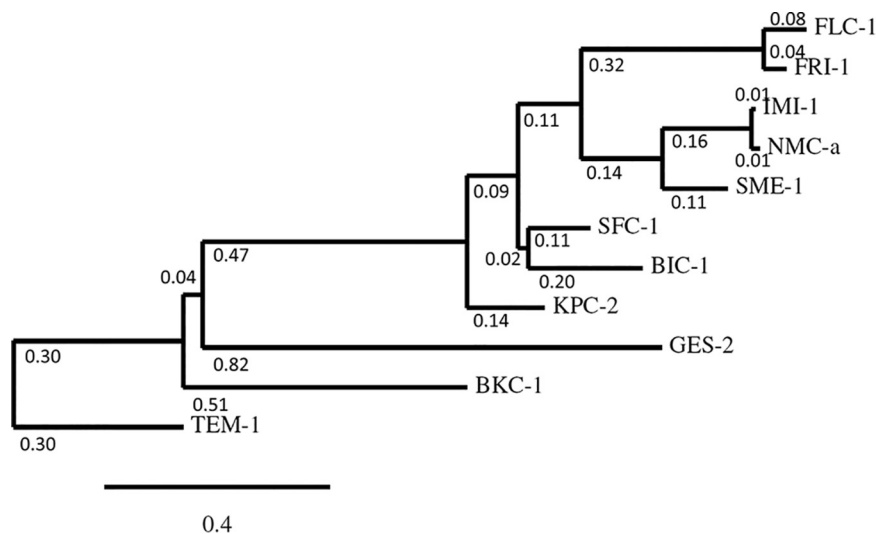


FIG 2 Phylogram of FLC-1 and 10 representative class A β -lactamases. Amino acid sequences were analyzed by Clustal Omega using the neighbor-joining method. Branch lengths are proportional to the number of amino acid changes.

broth microdilution after culture overnight in RPMI medium plus 0.2% glucose followed by dilution in Mueller-Hinton broth containing 0.2% arabinose and incubation at 37°C for 1 h to enable expression to start. Standard protocols were followed thereafter and *E. coli* LMG194 and ATCC 25922 were used as negative controls. *E. coli* LMG194 pBAD-FLC showed resistance against carbapenems and extended-spectrum cephalosporins (Table 1). The FRI variant was concluded to be a carbapenemase and is further referred to as FRI-like carbapenemase-1 (*bla*_{FLC-1}).

Multiple sequence alignments were made comparing FLC-1 with several members of plasmid-encoded Ambler class A carbapenemases (Fig. 1). All conserved residues among class A β -lactamases were present. The most related protein family was that of the French imipenemase (FRI), with 82% identity to FRI-1 and 87% to FRI-5 (10, 16, 17) (Fig. 2).

As previously described for other Ambler class A carbapenemases, *bla*_{FLC-1} is preceded by a lysR-type regulator, transcribed in the opposite direction, which is predicted to regulate the expression of the protein (18). This region was flanked by the remnants of two insertion elements, related to IS3 and ISEC25, which were likely responsible for the integration of the region onto an ancestor of the plasmid, which is also common for Ambler class A carbapenemases (8).

The soluble protein fractions of arabinose-induced *E. coli* LMG194 pBAD-FLC and *E. coli* LMG194 were prepared as described in the supplemental material, and their biochemical properties were evaluated. Analysis of the periplasmic protein fractions by SDS-PAGE showed induction of a protein between 25 and 35 kDa as expected (FLC-1 molecular weight, ~33 kDa; see Fig. S2 in the supplemental material). Hydrolysis of various β -lactam antibiotics was monitored with a Spark microplate reader (Tecan) at 23°C using 96-well UV-Star microplates. Phosphate-buffered saline (0.01% Triton X-100, pH 7.4) was used as the assay buffer. The extinction coefficients for the β -lactam antibiotics studied were $\Delta\epsilon_{235} = 900 \text{ M}^{-1} \text{ cm}^{-1}$ for ampicillin, $\Delta\epsilon_{297} = 10,940 \text{ M}^{-1} \text{ cm}^{-1}$ for meropenem, $\Delta\epsilon_{295} = 11,500 \text{ M}^{-1} \text{ cm}^{-1}$ for imipenem, $\Delta\epsilon_{300} = 6,920 \text{ M}^{-1} \text{ cm}^{-1}$ for ertapenem, and $\Delta\epsilon_{264} = 7,250 \text{ M}^{-1} \text{ cm}^{-1}$ for cefotaxime. To calculate kinetic parameters, including K_m and V_{max} , the measured initial velocities of the hydrolysis of the substrates were fit into the Michaelis-Menten equation using GraphPad Prism 7 software (see Fig. S3 in the supplemental material for Michaelis-Menten curves). Initially, cytoplasmic fractions of *E. coli* containing the plasmid showed hydrolysis of nitrocefin, while cytoplasmic fractions of *E. coli* lacking the plasmid did not (see Fig. S4a

TABLE 2 Kinetic parameters determined for the cytoplasmic fraction of *E. coli* LMG-194 producing FLC-1

Antibiotic	Protein concn ($\mu\text{g} \cdot \text{ml}$) ^{a,b}	K_m (μM)	$V_{\text{max}}/\mu\text{g protein}^c$	Relative k_{cat}/K_m
Ampicillin	5.53	1,649 \pm 174.2	(1,490 \pm 70) $\times 10^{-3}$	1.00
Meropenem	100	32.4 \pm 9.3	(2.05 \pm 0.14) $\times 10^{-3}$	0.07
Imipenem	17.68	177.2 \pm 12.5	(48.61 \pm 1.40) $\times 10^{-3}$	0.30
Ertapenem	44.21	29.6 \pm 11.7	(6.34 \pm 0.67) $\times 10^{-3}$	0.24
Cefotaxime	106.1	377.1 \pm 110.6	(7.85 \pm 1.25) $\times 10^{-3}$	0.02
Ceftazidime		ND ^d	<65 $\times 10^{-3e}$	
Cefepime		ND ^d	<34 $\times 10^{-3e}$	

^aProtein concentration of the cytoplasmic fraction.

^bThe *E. coli* strain producing FLC and the nontransformed strain were used to prepare cytoplasmic fractions. The highest tested concentration of both preparations was 176.83 $\mu\text{g}/\text{ml}$. None of the tested antibiotics were hydrolyzed by the nontransformed *E. coli* cytoplasmic fraction.

^cExpressed as $\mu\text{M}/\text{s}/\mu\text{g}$ of protein.

^dNot determinable.

^eBecause no substrate hydrolysis was detected, the V_{max} data for ceftazidime and cefepime have been reported as less than the limit of detection/ μg protein.

in the supplemental material). Expanding these measurements to several β -lactam antibiotics over time allowed for the determination of kinetic parameters of the protein-expressing cells (Table 2). The enzymatic activity of FLC-1 clearly showed greater efficiency of the enzyme toward carbapenems than toward cephalosporins (as evident by the relative k_{cat}/K_m values) (Table 2), with activity against ceftazidime and cefepime below the threshold of detection. Using nitrocefin as the substrate, the inhibition of FLC-1 enzymatic activity by clavulanic acid was tested, and the 50% inhibitory concentration was calculated (1.974 \pm 0.090 μM) (Fig. S4b).

Class A carbapenemases include members of GES, KPC, SME, and IMI/NMC-A enzymes plus SFC-1 and SHV-38 (19). With the exception of GES-1, most class A carbapenemases demonstrate higher carbapenemase activity of various degrees relative to extended-spectrum β -lactamases (19–22). FRI-1 is the closest member of the class A carbapenemases relative to FLC-1 and was found to be at least 15 times more efficient in degrading carbapenems than extended-spectrum cephalosporins (10). Here, we report a similar substrate preference for the FLC-1 enzyme, which hydrolyzes imipenem, ertapenem, and meropenem with greater efficiency than the cephalosporins tested (Table 2).

To control AMR and retain effective use of antimicrobials in human and veterinary medicine, a complete and correct overview of the impact that these human and animal reservoirs have on each other is essential. $bla_{\text{FLC-1}}$ was detected here in a sample of raw shrimp from India, but members of the FRI family, to which FLC is most closely related, and IMI, NMC-a, and SME have been described in a various global reservoirs (8, 10, 16, 17, 21, 23). Reliable databases of acquired resistance genes and point mutations leading to resistance are essential to determine the gene responsible for a particular resistant phenotype. The complete analysis presented here of the novel carbapenemase FLC-1 in its complete genetic carrier context will aid in the future for the recognition of its gene, $bla_{\text{FLC-1}}$, and related carbapenemases.

Accession numbers. The whole-genome sequence of isolate 3442 was submitted to GenBank, and the chromosome and individual plasmids are available under accession numbers CP033466 to CP033469. The protein sequence of $bla_{\text{FLC-1}}$ was submitted to GenBank under accession number ATX60370.1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02338-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

The work was funded by the Ministry of Agriculture, Nature and Food Quality (BO-43-013.03-002) and by the Netherlands Food and Consumer Product Safety Authority.

M.S.M.B., K.H.M.E.T., N.I.M., B.W., and K.T.V. conceived and designed the experiments.

M.S.M.B., K.H.M.E.T., M.R., Y.G., A.K., F.H., and V.M. performed the experiments. M.S.M.B., K.H.M.E.T., N.I.M., A.B., and K.T.V. analyzed the data. M.S.M.B., K.H.M.E.T., N.I.M., A.B., D.J.M., and K.T.V. wrote the manuscript.

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