Small-molecule inhibitors of bacterial metallo-β-lactamases

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Chapter 6

Biochemical evaluation of FLC-1, a novel carbapenemase encoded by an *Enterobacter cloacae* complex isolated from food products

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1. Introduction

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 the present chapter, the biochemical evaluations on a newly identified class A carbapenemase named FRI-like carbapenemase-1 (FLC-1) will be discussed.

2. Results and discussion

In March 2017, our collaborators from Wageningen Bioveterinary Research isolated an *E. cloacae* complex isolate, designated 3442, from a sample of frozen vannamei white shrimp (*Litopenaeus vannamei*) originating in India. The isolate exhibited an unusual phenotype, *i. e.*, resistant to carbapenems (meropenem, ertapenem, and imipenem) and susceptible to extended-spectrum cephalosporins (cefotaxime, ceftazidime, and cefepime) (table 1).

Sequencing analyses confirmed the presence of a 93 kb plasmid later named p3442-FLC-1 which carries a novel carbapenemase with close sequence similarity to bla_{FRI-1}.[10] It was hypothesized that the bla_{FRI}-related gene may 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, and expressed in *E. coli* LMG194.[11] The MIC of *E. coli* LMG194 pBAD-FLC was determined by broth microdilution after an overnight culture 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). This new FRI variant was concluded to be a carbapenemase and 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. 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.[10,12,13]

The soluble protein fractions of arabinose-induced *E. coli* LMG194 pBAD-FLC and *E. coli* LMG194 were prepared as described in the experimental section, 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 figure 1).

Table 1. Susceptibility data (MIC, μg/mL) of *E. cloacae* complex 3442, *E. coli* recipient, and transformant of pBAD-FLC.

Antibiotic	*E. cloacae* 3442	*E. coli* LMG194 pBAD-FLC	*E. coli* LMG194
Ampicillin	>64	>64	4
Cefepime	0.12	0.5	0.12
Cefotaxime	<=0.25	2	<=0.25
Cefotaxime/ clavulanic acid	0.25/4	0.12/4	<=0.06/4
Cefoxitin	64	8	8
Ceftazidime	<=0.5	1	0.5
Ceftazidime/ clavulanic acid	<=0.12/4	0.25/4	0.25/4
Ertapenem	>2	>2	<=0.015
Imipenem	>16	16	0.25
Meropenem	>16	4	<=0.03
Temocillin	4	16	8

The initial testing of the cytoplasmic fractions of *E. coli* containing the plasmid showed hydrolysis of nitrocefin, while cytoplasmic fractions of *E. coli* lacking the plasmid did not (figure 2a). 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 (IC_{50}) was calculated (1.97 ± 0.09 μM) (figure 2b).

Class A carbapenemases include members of GES, KPC, SME, and IMI/NMC-A enzymes plus SFC-1 and SHV-38.[14] With the exception of GES-1, most class A carbapenemases demonstrate higher carbapenemase activity of various degrees relative to extended-spectrum β-lactamases.[14–17] 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).

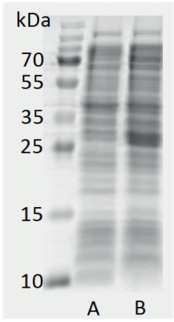

Figure 1. SDS-PAGE gel of *E. coli* LMG-194 pBAD-FLC. Periplasmic protein fractions were loaded before induction (lane A) and after 2 hr induction with 0.2% arabinose (lane B).

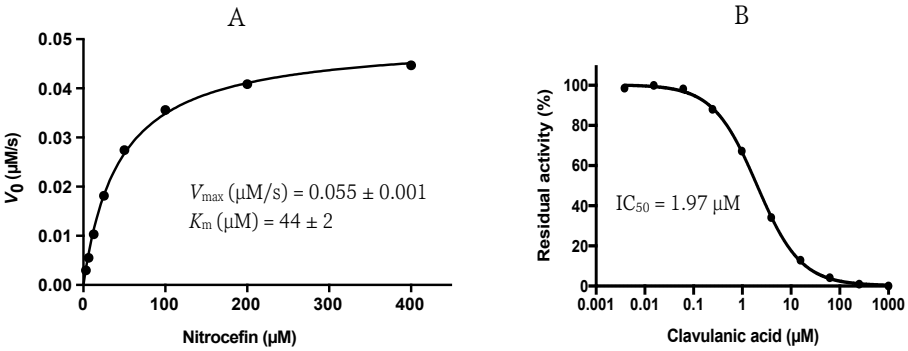

Figure 2. Inhibitory effect of clavulanic acid on FLC-1 enzymatic activity. (**A**) Michaelis-Menten plot of nitrocefin using 2 μg/mL of FLC-1 protein fraction. (**B**) Representative dose-response curve of FLC-1 inhibited by clavulanic acid. IC_{50} = 1.97 μM (± 0.09). Nitrocefin was used as chromogenic substrate.

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

Antibiotic	[Protein] (μg.mL)[a,b]	K_M (μM)	V_{max}/μg protein[c]	Relative k_{cat}/K_M
Ampicillin	5.53	1649 ± 174.2	(1490 ± 70) × 10^{-3}	1.00
Meropenem	100	32.4 ± 9.3	(2.05 ± 0.14) × 10^{-3}	0.07
Imipenem	17.68	177.2 ± 12.5	(48.61 ± 1.40) × 10^{-3}	0.30
Ertapenem	44.21	29.6 ± 11.7	(6.34 ± 0.67) × 10^{-3}	0.24
Cefotaxime	106.1	377.1 ± 110.6	(7.85 ± 1.25) × 10^{-3}	0.02
Ceftazidime		ND[d]	ND[d]	
Cefepime		ND[d]	ND[d]	

[a]Protein concentration of the cytoplasmic fraction.
[b]The *E. coli* strain producing FLC-1 and the non-transformed strain were used to prepare cytoplasmic fractions. The highest tested concentration of both preparations was 176.83 μg/mL. None of the tested antibiotics were hydrolyzed by the non-transformed *E. coli* cytoplasmic fraction.
[c]Expressed as μM/s/μg of protein.
[d]Not determinable.

3. Conclusion

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*$_{FLC-1}$ 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.[8,10,12,13,16,18] 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*$_{FLC-1}$, and related carbapenemases.

4. Experimental section

Preparation of bacterial cytoplasmic fractions

Cells were grown in YT2x (0.1% glucose, 50 μg/mL kanamycin). Upon reaching OD_{600} = 0.65, arabinose (0.2% final concentration) was added and after 2 h the cells were harvested by centrifugation at 6000 rpm (20 min, 4 °C). Pellets were resuspended in PBS (0.05% Triton X-100, 150 mM NaCl pH 7.4) and cells disrupted by two freeze-thaw cycles and three 30-second sonication cycles. Cell debris was removed by centrifugation at 12000 rpm (20 min, 4 °C). Protein content of the supernatant was determined by Pierce™ BCA protein assay kit following the manufacturer's protocol.

Kinetic experiments

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 $\Delta\varepsilon_{235}$ = 900 $M^{-1}cm^{-1}$ for ampicillin, $\Delta\varepsilon_{297}$ = 10940 $M^{-1}cm^{-1}$ for meropenem, $\Delta\varepsilon_{295}$ = 11500 $M^{-1}cm^{-1}$ for imipenem, $\Delta\varepsilon_{300}$ = 6920 $M^{-1}cm^{-1}$ for ertapenem, and $\Delta\varepsilon_{264}$ = 7250 $M^{-1}cm^{-1}$ 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 figure 3 for Michaelis-Menten curves).

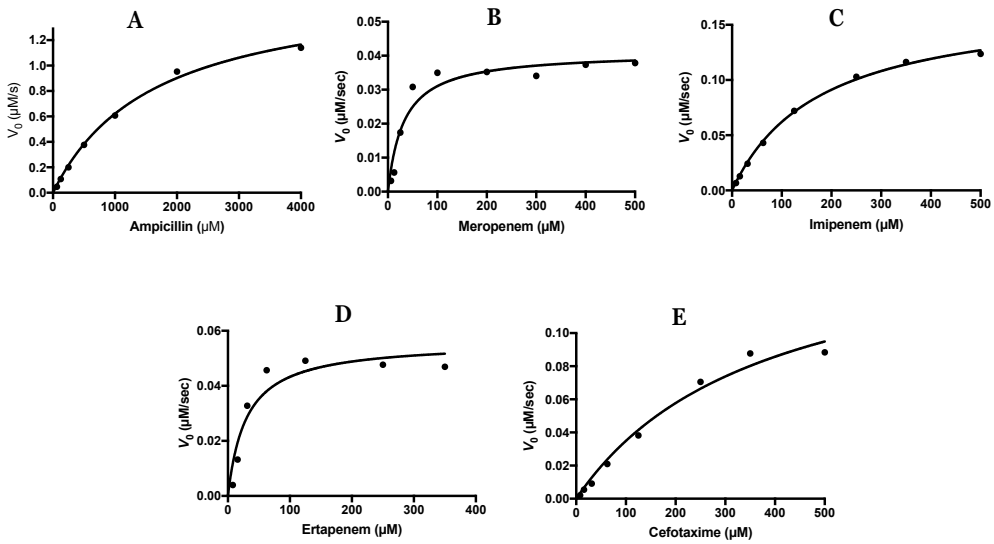

Figure 3. Michaelis-Menten plots of FLC-mediated hydrolysis of selected β-lactam antibiotics: (**A**) Ampicillin, (**B**) Meropenem, (**C**) Imipenem, (**D**) Ertapenem, (**E**) Cefotaxime.

IC_{50} determination

The inhibitory activity of clavulanic acid against FLC-1 fraction was assessed using nitrocefin as substrate. On a polystyrene 96-well plate and using the assay buffer described above, FLC-1 fraction (2 μg/mL) was incubated with clavulanic acid ranging from 1000 to 0.004 μM for 15 min at 25 °C. Nitrocefin with the concentration corresponding to K_M value (44 μM) was added to all the wells and absorption at 492 nm was monitored over 30 scan cycles. The initial velocity data was normalized using nitrocefin with enzyme in the absence of inhibitor as 100% activity and nitrocefin in the absence of enzyme as 0% activity. IC_{50} curve-fitting was performed on Log(concentration) vs. Activity (%) data using GraphPad Prism 7 software.

Acknowledgements

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