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Doctor Forum

Characterization of EmpA protease in *Vibrio anguillarum* M3

HAN Yifan[1)], MO Zhaolan[2),*], XIAO Peng[2)], HAO Bin[2)], LI Jie[2)], and YANG Guanpin[1),*]

1) *College of Marine Life Sciences*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*
2) *Institute of Oceanology*, *Chinese Academy of Sciences*, 7 *Nanhai Road*, *Qingdao* 266071, *P. R. China*

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Abstract EmpA is an extracellular metalloprotease secreted by *Vibrio anguillarum*. For better understanding its role in the pathogenicity of *V. anguillarum* strain M3, *empA* insertion mutant was constructed. In the mutant it decreased in extracellular proteolytic activity, swarming motility, hemolytic activity and virulence on turbot (*Scophthalmus maximus*). Significant decline (by 5-fold) of extracellular proteolytic activity and similar growth curve between mutant and wild type strains indicated that EmpA was the major extracellular protease of M3. LD_{50} of mutant increased by 38-fold compared with wild type. No pro-EmpA was detected in the supernatant of culture, indicating that EmpA autolyzed to mature protein after 24 h. Secretion of EmpA in M3 was similar to that in NB10 strain. Attenuated virulence of mutant was similar to that of M93Sm strain. It was demonstrated that specific operation of EmpA was different from that in previous studies and EmpA contributed to the swarming motility and hemolytic activity in *V. anguillarum* strain M3. The results provides insight into understanding the function of EmpA and its potential application in vaccine development.

Key words *Vibrio anguillarum*; pathogenicity; insertion mutation; EmpA

1 Introduction

Vibrio anguillarum is a rod-shaped gram-negative bacterium as a member of aquatic microflora in the intestine of marine fish (Olsson *et al*., 1998; Larsen *et al*., 1988). It is the causative agent of vibriosis, a systemic disease of both wild and cultured marine fishes (Austin *et al*., 2007). Outbreak of vibriosis results in high mortalities of infected fish. The disease is still a major obstacle for aquaculture industry at present. A few of virulence determinants of *V. anguillarum* have been reported, including iron-acquiring system (Crosa, 1980; Stork *et al*., 2002; Welch *et al*., 2005), serum resistance (Boesen *et al*., 1998), colonization and invasion of the host (Croxatto *et al*., 2007), and production of hemolysins and protease (Hirono *et al*., 1996; Denkin *et al*., 2004; Rock *et al*., 2006).

Extracellular proteases are considered putative virulence factors in several pathogens, including *V. cholera* (Silva *et al*., 2006), *V. vulnificus* (Wang *et al*., 2008), *V. harveyi* (Lee *et al*., 1999), *V. fisheri* (Stevens *et al*., 1997), *V. anguillarum* (Norqvist *et al*., 1990; Milton *et al*., 1992) and *Pseudomonas aeruginosa* (Zhang *et al*., 2007). For example, HapA, an extracellular metalloprotease secreted by *V. cholera*, can proteolytically degrade several physiologically important host proteins including mucin, fibronectin and lactoferrin when released into host body (Finkelstein *et al*., 1983). It also can proteolytically activate cholera toxin (CT) A subunit (Booth *et al*., 1984), El Tor cytolysin and hemolysin (Nagamune *et al*., 1996). EmpA is an extracellular metalloprotease of *V. anguillarum*. It shares significant sequence homology with other known proteases that can induce tissue destruction of host, such as LasB of *P. aeruginosa* (Nicas *et al*., 1985), VvP of *V. vulnificus* (Miyoshi *et al*., 1998) and HapA of *V. cholera* (Hase *et al*., 1990). EmpA has been identified as a virulence factor for *V. anguillarum* (Norqvist *et al*., 1990; Milton *et al*., 1992; Denkin *et al*., 2004). Induction of this protease activity varies between different *V. anguillarum* strains (Staroscik *et al*., 2005). Despite of the reports about obvious contribution of EmpA to virulence of *V. anguillarum*, the relationship between production of EmpA and pathogenesis is still controversial (Denkin *et al*., 2004).

In our previous study, we isolated and identified a pathogenic *V. anguillarum* strain M3 from diseased flounder *Paralichthys olivaceus* (Mo *et al*., 2001). M3 could secrete EmpA into the supernatant of the laboratory medium, and injection of the purified protein was lethal to flounder (Mo *et al*., 2002). In an attempt to confirm the relationship between EmpA and pathogenesis of *V. anguillarum* strain, the growth rate, motility, extracellular protease activity, hemolytic activity and LD_{50} were investigated in M3 after gene *empA* was mutated.

* Corresponding author.
MO Zhaolan, E-mail: zhlmo@ms.qdio.ac.cn;
YANG Guanpin, E-mail: yguanpin@ouc.edu.cn

2 Materials and Methods

2.1 Bacterial Strains and Growth Conditions

M3 strain of *V. anguillarum* was isolated from a diseased flounder (*Paralichthys olivaceus*) and used in this study (Mo *et al*., 2001). M3 grew in tryptic soy medium containing 1.5% NaCl (TSS) at 28℃. *E. coli* strains grew in LB medium at 37℃. When required, the media were supplemented with ampicillin (50 μg mL^{-1}), chloramphenicol (34 μgmL^{-1}) or tetracycline (100 μg mL^{-1}).

2.2 Generation of the Empa Insertion Mutant Strain

The *empA* insertion mutant was generated following the method of Milton *et al*. (1992). In brief, a 536-bp internal fragment of *empA* was generated by PCR with primers of empA-F1 (5'-GC*GAGCTC*AGATAACGAA AACCAAATGGCTCAA-3') and empA-R1 (5'-GC*G TCGAC*GATTTAAATGTATTTTGGCCATCACCA-3'), which were introduced into a *Sac*I restriction site and a *Sal*I restriction site, respectively. In addition, a stop codon (underlined letters) was included in each primer to ensure the inactivation of the metalloprotease gene once the plasmid had recombined into the chromosome. The resultant PCR product was cloned into the *Sac*I/*Sal*I digested sites of the suicide plasmid pNQ705. The recombinant plasmid was then transformed into *E. coli* SY17-1. The chloramphenicol-resistant transformants containing the recombinant plasmid were selected. Transfer of plasmid from *E. coli* SY17-1 to *V. anguillarum* was done by bacterial mating according to the previous method (Mo *et al*., 2007). The transconjugants resistant to both chloramphenicol and ampicillin were selected. Primers of empA-F2 (5'-GCGTAACGGCAAAAGCACCGCCGG ACATCA-3') and empA-R2 (5'-AAGATTTGAAAA TGTCGCTC-3'), complementary to the pNQ705 vector outside the linker region and to the downstream region of empA, were used to detect the integration of the recombinant pNQ705 plasmid into the chromosomal *empA* gene. The size of PCR product was 820 bp. The mutant was named strain MC-1.

2.3 Protease Assay

Two assays were used to detect protease activity of the *V. anguillarum* strains. Overnight culture was spotted on 2216E agar plates containing 1% gelatin and incubated at 28℃. After 24 h of incubation, 10% $HgCl_2$ (W/V) solution was flooded onto the plate and clearance zone was recorded. In another assay, the extracellular products (ECP) of the test strain were prepared and the proteolytic activity was examined. Briefly, supernatant of the bacterial culture was collected by centrifugation at 10000*g* for 5 min at 4℃, and then filtered through a 0.22μm pore-size filter. The resultant supernatant was used as ECP. A reaction mixture containing 0.5 mL of azocasein (Sigma) at 5 mgmL^{-1}, 0.4 mL of distilled water, and 0.1 mL of an ECP sample solution was incubated at 25℃ for 20 min. The reaction was stopped by addition of 3.5 mL of 5% trichloroacetic acid, the precipitate was removed by centrifugation at 5000 g for 5 min, and 4.5 mL of 0.5 molL^{-1} NaOH was added to the supernatant. The protease activity of each sample was measured at 440 nm. One unit of protease activity was defined as an increasing of 0.01 unit of absorbance.

2.4 Motility Assay

Bacterial strains were grown overnight in 2216E broth and subsequently adjusted to an OD_{540} value of 0.5. Aliquots of 3uL were spotted onto the surface of dishes with 2216E medium containing 0.3% agar. Movement of bacterial cells through the agar was monitored after 24 h at 25℃. Experiment was replicated three times.

2.5 Hemolytic Activity

Overnight bacterial culture was spotted on a 2216E agar containing 5% flounder erythrocytes and incubated at 28℃. The hemolytic zone was recorded after 24 h.

2.6 SDS-PAGE and Western Blot Analysis of ECP

ECP of the test strains were precipitated with 10% (w/v) trichloroacetic acid (TCA) and then dissolved in 50μL ReadyPrep reagent 3 (Bio-Rad), and equal volume (10μL per lane) of each sample was loaded on an SDS-12% polyacrylamide gel. Electrophoresis was done for 45 min at 200 V. The gels were fixed and stained with 0.1% Coomassie brilliant blue in 40% methanol-10% acetic acid and then destained in 40% methanol-10% acetic acid. For western blot analysis, ECP resolved in 12% SDS-PAGE gel was electrotransferred onto nitrocellulose membranes. The membrane was incubated with anti-EmpA rabbit polyclonal antibody overnight at 4 ℃, followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). Antibody complexes were detected by development in 3,3-diamino- benzidine tetrahydrochloride (DAB).

2.7 Virulence Determination of Empa Mutant

Healthy flounder with weight of 10±2.4 g were challenged with test strain grown on 2216E agar at 25℃ for 24 h. Ten fish in each test group were infected by intramuscular injection of 0.1 mL sterile seawater resuspended with 10^5-10^9 cells. Fish in control group were injected with sterile seawater without bacterial cells. After treatment fish were kept in aerated seawater at 20±2℃. Mortality was recorded daily for 14 d. Kidneys of dead fish were subjected to bacteria isolation. The isolated bacterium was confirmed to be the test strain by the agglutination test with anti-*V. anguillarum* M3 rabbit antiserum. LD_{50} was calculated by the method of Reed *et al*. (1938).

3 Results

3.1 Construction of an Insertion Empa Mutant of *V. anguillarum* M3

To construct an insertion mutant of *empA*, a 536-bp in-

ternal fragment of *empA* generated by primers pair of empA-F1/empA-R1 (Fig.1, lane 2) was cloned into the suicide vector pNQ705 (Fig.1, lane 3). Then the resultant recombinant plasmid was mobilized into *V. anguillarum* wild-type strain M3 by conjugal mating. The transconjugants were selected on TCBS containing chloramphenicol. Integration of the plasmid into *empA* gene was confirmed by PCR analysis using a primer (empA-F2) complementary to the plasmid just outside the linker region of pNQ705 and another primer (empA-R2) complementary to the *empA* gene just outside the 536-bp internal region (Fig.1, lane 1).

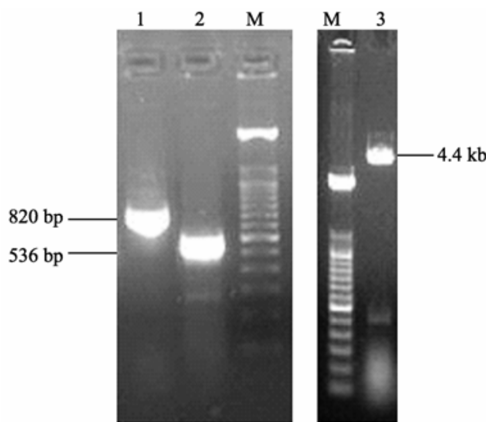
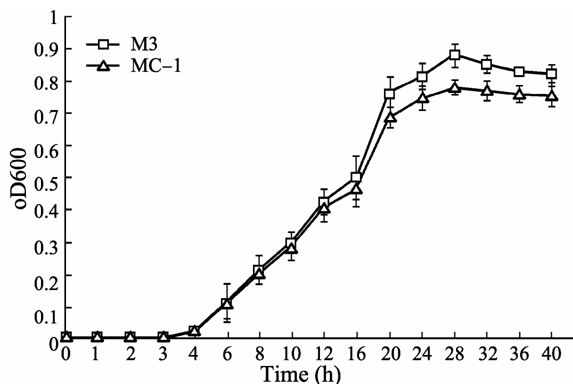


Fig.1 Confirmation of the *empA* mutant. Lane 1, PCR analysis of chromosome DNA of *empA* mutant by primers pair of empA-F2/empA-R2. An 820-bp DNA band was generated. Land 2, PCR analysis of chromosome DNA of wild-type M3 by primers pair of empA-F1/empA-R1. A 536-bp DNA band was generated. Lane 3, *Sac*I/*Sal*I digest of recombinant vector pNQ705 containing the 536-bp internal fragment of *empA* gene. A 4.4kb DNA band indicated the lined vector pNQ705 DNA, and a 536-bp DNA band indicated the internal DNA of *empA* gene. Lane M, marker DNA (100 bp DNA ladder).

3.2 EmpA is a Major Protease Secreted by the *V. anguillarum* Strain M3

When cultured in 2216E marine broth, no significant difference of growth ($p>0.05$) was observed between the wild-type strain M3 and the *empA* mutant strain MC-1 during 40 h (Fig.2A). For strain M3, protease activity was detected in the supernatant at optical densities higher than 0.4. The maximum protease activity was detected when the bacteria growth reached the stationary phase. However, little protease activity was detected in the supernatant of strain MC-1(Fig.2B). When supernatants of the cultures were analyzed by SDS-PAGE, an evident 36kD band was observed in M3 (Fig.3A, lane 1), while this band was not observed in MC-1(Fig.3A, lane 2). To further identify whether this band was EmpA, identical samples were loaded in the second gel and analyzed by western blot using a rabbit anti-EmpA serum. A reactive protein band with a size of 36kD was observed in supernatant of M3 (Fig.3B, lane 2), while no band in MC-1 (Fig.3B, lane 1). The result confirmed that the 36kD protein band was EmpA and it was a major protease secreted by the *V. anguillarum* strain M3. Based on this observation, we used an azocasein assay to quantitate production of EmpA from M3 cultured at different conditions.



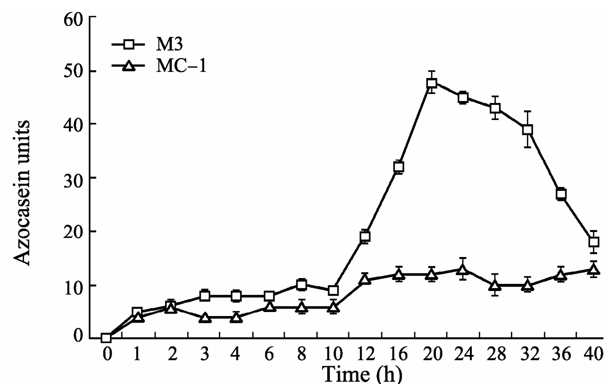


Fig.2 Growth and protease activity of *V. anguillarum* strains. A, Growth curve of wild-type and *empA* mutant; B, Azocasein activity of ECP of wild-type and *empA* mutant. M3=wild type of *V. anguillarum*, MC-1=EmpA insertion mutant of *V. anguillarum*.

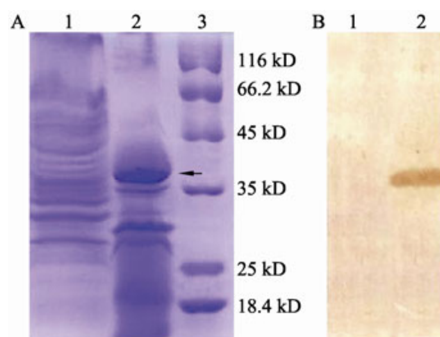


Fig.3 Detection of EmpA protein in the extracellular product (ECP) of *V. anguillarum* strains. A, SDS-PAGE analysis of ECP of *V. anguillarum* strains. Lane 1, *empA* mutant; Lane 2, wild-type M3, arrow indicating EmpA; Lane 3, protein molecular marker. B, Western blot analysis of ECP of *V. anguillarum* strains. Lane 1, *empA* mutant; Lane 2, wild-type M3.

3.3 Effects of the *empA* Mutation on the Virulence-Associated Phenotypes

To examine whether the disruption of *empA* gene had any effects on the virulence-related functions, hemolytic, proteolytic and motility activities were examined in the

empA mutant. The hemolytic activities of the wild-type and *empA* mutant strains were examined on agar plate with flounder blood. The *empA* mutant (Fig.4B) produced smaller hemolysis zone than wild-type stain (Fig.4A),

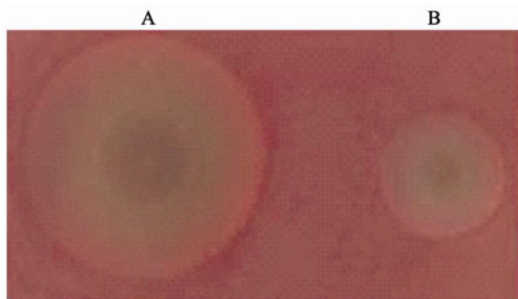


Fig.4 Hemolytic activity of *V. anguillarum* strains on flounder blood agar. A, wild-type strain; B, *empA* mutant.

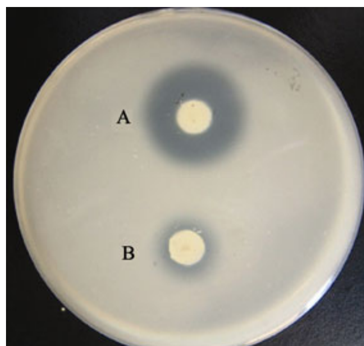


Fig.5 Proteolytic activity of *V. anguillarum* strains on gelatin agar. A, wild-type strain; B, *empA* mutant.

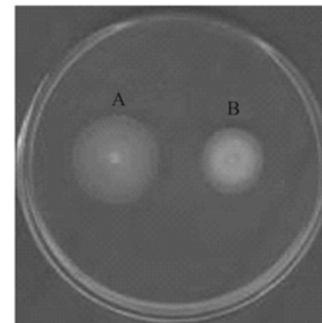


Fig.6 Swarming activity of *V. anguillarum* strains on 0.3% soft agar. A, wild-type strain; B, *empA* mutant.

indicating that EmpA contributed to the hemolytic activity of the *V. anguillarum* M3. Then the *empA* mutant strain was tested for proteolytic activity on gelatin agar. The *empA* mutant strain (Fig.5A) produced much smaller clearance zone than wild-type strain (Fig.5B), indicating that EmpA contributed to the major protease activity of the *V. anguillarum* M3 and was in agreement with the results indicated in Fig.2. Swarming activities of both wild-type and *empA* mutant were tested on 0.3% soft agar. As indicated in Fig.6, the *empA* mutant (Fig.6B) showed smaller swarming zone than the wild-type strain (Fig.6A), indicating that EmpA contributed to the swarming motility of *V. anguillarum* M3.

3.4 Virulence of the *empA* Mutant

Healthy flounder were challenged with wild-type or *empA* mutant strains by intraperitoneal injection (Table 1). LD_{50} of the wild-type strain was 1.3×10^6 CFU/fish, while LD_{50} of the *empA* mutant was 5.0×10^7 CFU/fish. Therefore, the virulence of the *empA* mutant was attenuated by 38-fold compared to wild-type strain. This result indicated that EmpA contributed to the virulence of the *V. anguillarum* M3 strain.

Table 1 Virulence of *V. anguillarum* strains in flounder *Paralichthys olivaceus*

Strains	Dose/fish (CFU)	Total % mortality [a]	LD_{50} [c]
M3	2.7×10^7	100	1.3×10^6
	2.7×10^6	80	
	2.7×10^5	60	
	2.7×10^4	40	
empA mutant	3.9×10^7	100	5.0×10^7
	3.9×10^6	70	
	3.9×10^5	30	
	3.9×10^4	0	
Control		0	N/A [b]

Note: a. The dead fish showed clinical symptoms of vibriosis, and *V. anguillarum* cells could be isolated from the fish; b. N/A, not applicable; no fish death occurred during the 14-day experiment; c. LD_{50} was calculated by the method of Reed *et al.* (1938)

4 Discussion

Previous studies revealed different induction and virulent display of EmpA in two strains of *V. anguillarum*, M93Sm and NB10. Strain M93Sm expressed EmpA only in the presence of fish gastrointestinal tract mucus, while NB10 expressed EmpA in either the presence or absence of the mucus (Denkin *et al*., 2004). The results of Denkin and Nelson (2004) showed that Atlantic salmon challenged with *empA* mutant of NB10 (NB12) was avirulent by both intraperitoneal injection and anal intubation. Differently, the *empA* mutant of M93Sm (M99) was fully virulent by intraperitoneal injection, but was attenuated when administered by anal intubation. In our study, M3 strain secreted EmpA into the supernatant of marine broth from optical densities higher than 0.4, and achieved the maximum expression at the stationary phase. It indicated that strain M3 expressed EmpA in the absence of mucus. A previous study also showed that M3 had high protease production by adding the extract of fish muscle tissue (Chen *et al*., 2004). These results indicated that M3 expressing EmpA was similar to strain NB10 but different

with M93Sm strain.

More experimental data indicated that EmpA was a major protease secreted by M3. Interruption of *empA* gene resulted in significant decrease of protease activity in the supernatant without any other alteration of growth conditions, implying that EmpA played an important role in the physiological life of *V. anguillarum*. Up till now, almost all documented data indicated that the EmpA was involved in the pathogenesis of *V. anguillarum*, but little is known about the specific role of EmpA in virulence. In this study, we investigated some phenotypes related with bacterial pathogenesis by mutating *empA* gene on chromosome M3 strain. Results indicated that mutation of *empA* resulted in decline of swarming motility, hemolytic motility and proteolytic activity in M3. It has been suggested that motility contributes to bacterial virulence by promoting penetration of the protective mucus barrier (Mekalanos *et al*., 1995), and hemolytic activity is an important bacterial virulence factor by exerting its role in disruption of erythrocytes and other cell types (Rowe *et al*., 1994). The results in this study support the previous conclusion that EmpA is a virulence factor of *V. anguillarum*.

It is interesting that introduction of EmpA in strain M3 was identical to NB10, and the virulence of *empA* mutant of M3 was identical to M93Sm strain. In the assay of western blot, no pro-EmpA was detected in supernatant of M3 culture media. Supernatant of culture media was collected after 16 h incubation in the previous study but after 24 h in this study. Pro-EmpA might autolyze to mature EmpA within 8 hours or was modified in periplasmic. These phenomena indicated that specific operation of EmpA in M3 was different to that in the other two strains and further research is required. At present, there are at least 23 serotypes of *V. anguillarum* and only O1, O2 and O3 are considered pathogenic to fish (Pedersen *et al*. 1999). Recently, different *V. anguillarum* strains were isolated from different samples with the presence of *empA* gene in our lab, showing that many of the non-pathogenic isolates encoded *empA* genes (data not shown). Thus the exact role of EmpA in non- pathogenic *V. anguillarum* isolates needs further investigation.

Acknowledgments

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