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Identification and characterization of the *Vibrio anguillarum prtV* gene encoding a new metalloprotease *

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Abstract We cloned and sequenced a *prtV*-like gene from *Vibrio anguillarum* M3 strain. This *prtV* gene encodes a putative protein of 918 amino acids, and is highly homologous to the *V. cholerae prtV* gene. We found that a *prtV* insertion mutant strain displayed lower gelatinase activity on gelatin agar, lower protease activity against azocasein, and lower activity for four glycosidases. This *prtV* mutant strain also had increased activity for two esterases in its extracellular products, as analyzed by the API ZYM system. In addition, the *prtV* mutant strain exhibited decreased growth in turbot intestinal mucus and reduced hemolytic activity on turbot erythrocytes. Infection experiments showed that the LD_{50} of the *prtV* mutant strain increased by at least 1 log compared to the wild-type in turbot fish. We propose that *prtV* plays an important role in the pathogenesis of *V. anguillarum*.

Keyword: *Vibrio anguillarum*; *prtV*; proteolysis; hemolysis; mucus; LD_{50}

1 INTRODUCTION

Vibrio anguillarum is the etiological agent of vibriosis, resulting in great economic loss in many marine and freshwater fish (Austin et al., 1993). Several bacterial activities have been implicated in the pathogenesis of *V. anguillarum*, including the ability to scavenge iron from the host (Crosa, 1980; Stock et al., 2002), resistance to serum killing (Welch et al., 2005; Boesen et al., 1999), colonization of the fish skin (Croxatto et al., 2007), and invasion into the fish from seawater (McGee et al., 1996; Milton et al., 1996). The bacteria also produce different enzymes and toxins, such as proteases and hemolysins (Milton et al., 1992; Hirono et al., 1996; Denkin et al., 1999; Mo et al., 2002; Rock et al., 2006). In our preliminary study, we obtained the sequence of a DNA fragment from a plasmid library of *V. anguillarum* by random sequencing that was highly homologous to the *prtV* gene in *V. cholerae*. Our bioinformatics analyses indicated that there were *prtV* homologous genes in the published genomes of many bacterial species, including *Vibrio* sp., *Moritella* sp., *Aeromonas* sp., *Bacillus* sp., and *Shewanella* sp. However, among these homologous sequences, only the *prtV* gene of *V. cholerae* and *inhA* and *inhA1* of *Bacillus thuringiensis* have been partially identified. Previous studies have shown that *V. cholera prtV* is required for killing *Caenorhabditis elegans* (Vaitkevicius et al., 2006). Purified PrtV protein has a cytotoxic effect leading to cell death, and it can degrade the extracellular matrix components fibronectin, fibrinogen, and plasminogen (Vaitkevicius et al., 2008). *B. thuringiensis* InhA can block the humoral defense system in the pupae of the silk moth *Hyalophora cecropia* against *Escherichia coli* (Edlund et al., 1976). Injection of purified InhA caused lethality of *Callosa miapromethea* pupae and *Drosophila melanogaster* (Siden et al., 1979). In addition, InhA1, a homolog of InhA, has been shown to be necessary for pathogenicity in insects infected via the oral route (Fedhila et al., 2002).

Extracellular proteases are considered to be putative virulence factors in several microbial diseases. *V. anguillarum* secretes a metalloprotease, EmpA, which is specifically expressed when grown

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in fish gastrointestinal tract mucus (Denkin et al., 1999). Injection of purified EmpA is lethal to flounder (*Paralichthys olivaceus)* (Mo et al., 2002). EmpA and its homologs, such as hemagglutinin/protease of *V. cholerae* and elastase, LasB, of *Pseudomonas aeruginosa*, which belong to the M4 family of peptidases, are different from the PrtV homolog, which belong to the M6 family of peptidases. In this report, we identified and characterized the *prtV* gene from the *V. anguillarum* M3 strain. This study provided insight into the *prtV* gene of *V. anguillarum* by demonstrating its role in bacterial pathogenesis.

2 MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

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

2.2 Cloning and identification of the *prtV* gene of *V. anguillarum*

In the preliminary study, an insertion DNA sequence from a pUC18-plasmid library of the M3 genome was found to be highly homologous to the *prtV* gene in *V. cholera*e. To obtain the full length *prtV*-like gene from *V. anguillarum*, a fosmid library of the M3 genome was constructed using the CopyControl™ Fosmid Library Production Kit (Epicentre, USA), followed by a two-step PCR assay (Asakawa et al., 1997) using primers complementary to the previously obtained M3 *prtV*-like fragment. The fosmid clone containing the predicted DNA band of the *prtV* fragment was selected and used for genome-walking according the method described by Sambrook et al. (2001). The DNA fragments amplified were cloned into the pGEM-T easy vector (Promega) and the DNA was sequenced. The full length of *prtV* gene was deposited in GenBank under accession no. EU984502.

2.3 Generation of the *prtV* insertion mutant strain

The *prtV* insertion mutant strain was generated following the method described by Milton et al. (1992). Briefly, a 691-bp internal fragment of *prtV* was generated by PCR and cloned into the *Sac*I/*Sal*I digested sites of the suicide plasmid pNQ705. The resulting recombinant plasmid was then transformed into *E. coli* SY17-1. The chloramphenicol-resistant transformants containing the recombinant plasmid were selected. Plasmid transfers from *E. coli* SY17-1 to *V. anguillarum* were done by bacterial mating. Briefly, all strains were grown to mid logarithmic growth phase. With an approximately 5:1 ratio, the recipient strain *V. anguillarum* and the donor strain *E. coli* were mixed, pelleted together, and then spotted on a trypticase soy agar plate. The matings were incubated at 28°C for 24 h. Transconjugants resistant to both chloramphenicol and ampicillin were selected and confirmed by PCR to contain the correct integration of the recombinant pNQ705 plasmid into the chromosomal *prtV* gene.

To construct a complementation strain, the complete *prtV* gene was obtained by PCR and introduced into the *Sac*I/*Eco*RI digested sites in the pACYC184 plasmid (Fermentas). The resulting recombinant pACYC184 plasmid was introduced into the *prtV* mutant strain by electroporation to generate the complemented strain, *prtV*-com.

2.4 Enzymatic activity

Extracellular products (ECPs) of *V. anguillaru*m strains were prepared using the cellophane plate technique (Inamura et al., 1985). Briefly, 200 μl of overnight culture were spread onto each of the cellophane plates and cultured at 28°C for 24 h. Bacterial cells were washed off with 3 ml of phosphate-buffered saline (PBS, 0.05 mol/L, pH 7.6). The bacterial suspensions were collected and adjusted to A_{540}=2.0, then centrifuged at 5 000 *g* for 30 min at 4°C. The supernatant was collected and filtered through a 0.22 μm pore-size filter to obtain the cell-free ECPs. The total protein concentration of ECPs was measured using the Bradford Protein Assay Kit (Beyotime, China). The API ZYM system (BioMerieux, France) was used to evaluate enzymatic activities of ECPs. Proteolytic activity in ECPs was assayed against azocasein (Sigma) (Inarmura et al., 1985). One unit of protease activity was defined as an increase of 0.01 unit of absorbance at 440 nm. Gelatinase activity of *V. anguillarum* strains was observed on TSS agar plates containing 1% gelatin.

2.5 Growth in fish intestinal mucus

Raw turbot intestinal mucus was prepared by the technique described by O'Toole et al. (1996). In brief, the gastrointestinal tract of turbot fish was cut open, and the mucus was rubbed from the tract surface and

homogenized in PBS. Mucus preparations were centrifuged twice at 20 000 *g* for 30 min at 4°C to remove the particulate and cellular material. The mucus supernatants were adjusted to 1 mg ml^{-1} with PBS and filtered through 0.45- and 0.2-μm-pore-size filters. The final mucus suspensions were stored at -20°C until use.

The growth of the *V. anguillarum* in the mucus was evaluated in TSS agar plates. In brief, overnight bacterial cultures were precipitated by centrifugation at 5 000 *g* for 10 min at 4ºC. The bacterial pellets were resuspended in PBS and adjusted to A_{540}=0.1. Equal volumes (1.5 ml) of bacterial suspension and mucus suspension were mixed and incubated at 28ºC. Samples of 100 μl mixture were removed at 0, 6, 12, 18 and 24 h, diluted, and spread on TSS plates.

2.6 Hemolytic activity

Two different assays were used to determine the hemolytic activity in turbot red blood cells. For the plate assay, the *V. anguillarum* was spotted on a TSS plate containing 5% turbot erythrocytes and incubated at 28°C. The hemolytic zone was recorded after 24 h. For the cell-contact hemolysis assay, the *V. anguillarum* suspension was adjusted to A_{540}=1.0 with PBS, mixed with 1% turbot erythrocytes resuspended in PBS, and then incubated for 4 h at 28°C. The percentage of total hemolysis (P_h) was calculated using the equation, $P_h=[(X-B)/(T-B)]\times 100$ (Blocker et al., 1999), where X was the A_{405} value obtained when erythrocytes were incubated with sample tested, B was the baseline value obtained when erythrocytes were incubated with PBS, and T was the control value obtained when erythrocytes were incubated with PBS containing 0.1% SDS.

2.7 Virulence determination

The 50% lethal doses (LD_{50}) of *V. anguillarum* strains were determined in healthy turbot *Scophthalmus maximus* (L.) obtained from a commercial fish farm. Fish with body weights of 11–13 g were used. Fish mortality was observed for 14 days and the LD_{50} was calculated by the method of Reed et al. (1938).

2.8 Statistical analyses

Data obtained were expressed as mean ±S.D. and analyzed for statistical significance (P<0.05) by a paired t-test.

3 RESULTS AND DISCUSSION

3.1 Identification of the *prtV* gene in *V. anguillarum*

Previously, we obtained a DNA fragment from a pUC18-plasmid genome library of *V. anguillarum* that was highly homologous to the *prtV* gene in *V. cholerae* (data not shown). Using the cloning strategy described in Materials and Methods, we obtained an approximately 3.2 kb DNA sequence containing an ORF of 2 778 bp, which encoded a putative protein of 918 amino acids, 77% identical to the PrtV protease (GenBank accession no. VCA0223) present in the genome of *V. cholerae* O1 biovar eltor str. N16961. The putative *prtV* gene of *V. anguillarum* also showed homology to a putative M6 secreted metalloprotease precursor (EU349011) of *V. splendidus*, a protease (ZP_01897859) of *Moritella* sp., an immune inhibitor A protein (YP_857947) of *Aeromonas hydrophila*, a M6 family metalloendopeptidase (YP_001488784) of *B. pumilus*, and two secreted metal-dependent proteases (NP_347382 and YP_001125830) of *Clostridium acetobutylicum* and *Geobacillus thermodenitrificans*.

Using interPro (http://www.ebi.ac.uk/Tools/Inter ProScan/), an M6 peptidase domain was observed at amino acids 93–747 (numbers refer to positions in the putative full-length PrtV protein), which contains a conserved zinc-binding motif (HEYGH) (Jongeneel et al. 1989) located at amino acids 330–334. Two polycystic kidney disease (PKD) domains were observed (at amino acids 758–835 and at 842–918). A series of phosphorylation sites that are signatures of cAMP- and cGMP-dependent protein kinases and casein kinase II were also predicted. The conserved zinc-binding metallopeptidases in pathogenic bacteria have been shown to display a variety of pathological actions including digestion of host connective tissue, cytotoxicity to host cells, and maturation of other enzymes (Miyoshi et al., 2000). The PKD domains are supposed to be involved in protein-protein or protein-carbohydrate interactions (Finn et al., 2006). These findings suggest that *V. anguillarum* PrtV, similarly to other M6 peptidases such as InhA of *B. thuringiensis* (Edlund et al., 1976; Siden et al., 1979) and PrtV of *V. cholerae* (Vaitkevicius et al., 2006), might be important for the virulence of *V. anguillarum*. The potential phosphorylation sites suggest a possible role in modulating other proteases activities (Kalderon et al., 1989).

3.2 Effect of *prtV* mutation on enzymatic activity of *V. anguillarum*

To investigate the function of *prtV*, the *prtV*:Km^r chromosomal mutant was constructed in *V. anguillarum* M3 and confirmed by PCR analyses (data not shown). The *prtV* mutant strain and the parental M3 wild-type strain showed comparable growth rates on TSS agar and in TSS broth, indicating that the mutation in the *prtV* gene did not affect bacterial growth in normal laboratory conditions.

The *prtV* mutant strain was then tested for gelatinase activity on gelatin agar. The activity of the *prtV* mutant strain was less than that of the wild-type strain. The clearance zone of the *prtV* mutant strain (zone B) is smaller than that of the wild-type strain (zone A) (Fig.1). Complementation of the *prtV* mutant strain (*prtV*-com) with the recombinant pACYC184 plasmid containing the *prtV* gene, restored the gelatinase activity to the wild-type level (zones C and D) (Fig.1). The result of the complementation test ruled out polar effects caused indirectly by the *prtV* insertion mutation. However, the clear zone was not completely lost in the *prtV* mutant strain, suggesting that other, unrelated, extracellular proteases might exist in the mutant strain. In fact, EmpA has been shown to be an extracellular protease that exhibits gelatinase activity (Milton et al., 1992). Thus, mutation of *prtV* partially affected the gelatin hydrolysis activity of *V. anguillarum*.

The protein concentration and the proteolytic activity against azocasein were determined simultaneously in ECPs. Lower protein concentration (2.31±0.13 mg ml^{-1}) (P<0.05) and

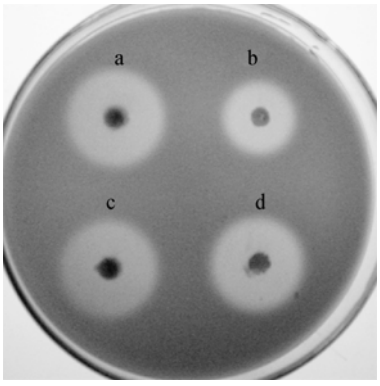

Fig.1 Proteolytic activity of *V. anguillarum* strains on 1% gelatin agar

A sample of an overnight culture was spotted onto tryptic soy agar containing 1% gelatin at 28°C for 24 h. a, wild-type strain; b, *prtV* mutant strain; c, *prtV*-com (complemented strain); d, *prtV*-com strain isolated from fish in the virulence test.

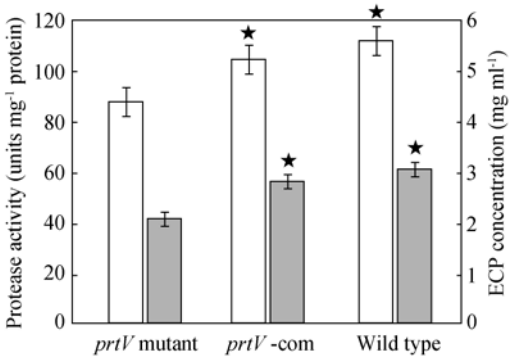

Fig.2 Protein concentration and proteolytic activity against azocasein in ECPs of *V. anguillarum*

Bacterial strains were cultured on a TSS agar plate at 28ºC for 24 h and ECPs were extracted using the cellophane plate technique (Inamura et al., 1985). Bar limits represent standard deviations. The asterisks denote significant differences (P<0.05) between the values of wild-type or *prtV*-com strain, and the *prtV* mutant strain. The white bricks represent the proteolytic activity and the grey bricks represent the protein concentration.

proteolytic activity (87.8±5.6 units mg^{-1} protein) (P<0.05) were seen in the *prtV* mutant strain, compared with those of the wild-type (3.02±0.11 mg ml^{-1}, 112.1±4.7 units) (Fig.2). As expected, the *prtV*-com strain restored the protein concentration (2.82±1.13 mg ml^{-1}) (P>0.05) and proteolytic activity (104.8±3.05 units) (P>0.05) to close to the wild-type levels. The *prtV* mutation reduced the proteolytic activity of ECPs by about 21.7% of the total proteolytic activity, which is consistent with the observation in *V. cholerae*, where *prtV* mutation abolished 10%–20% of total proteolytic activity (Vaitkevicius et al., 2006).

In addition, the activities of 19 hydrolytic enzymes were measured using the API ZYM strips (Table 1). In the wild-type strain, the highest enzymatic activity (>40 nmol of hydrolyzed substrate) was found for alkaline phosphatase and leucine arylamidase, relatively high activity for trypsin and *N*-acetyl-*β*-glucosaminidase (20–30 nmol), and low activity for esterase/lipase (5–10 nmol). In the *prtV* mutant strain, decreased activity (0–10 nmol) was found for the four glycosidases (alkaline phosphatase, leucine arylamidase, trypsin and *N*-acetyl-*β*-glucosaminidase), but increased activity was found for esterase (10–20 nmol) and esterase/lipase (20–30 nmol). The *prtV*-com strain showed activities of the above-mentioned enzymes close to those of the wild-type. This result indicated that PrtV might affect the activities of other enzymes, supporting the hypothesis that *V. anguillarum* PrtV might have a role in modulating other proteases activities at the phosphorylation level.

Table 1 Enzymatic activities of ECPs determined with the API-ZYM system*

Enzymes	Range of values		
	prtV mutant	*prtV*-com	wild-type
Alkaline phosphatase	0–1	4–5	>5
Leucine arylamidase	1–2	4–5	>5
Trypsin	0–1	3–4	3–4
N-acetyl-*β*-glucosaminidase	0–1	3–4	3–4
Esterase	2–3	2–3	0
Esterase/lipase	3–4	3–4	1–2

*: ECPs were prepared from bacterial suspensions adjusted to A_{540}=2.0, and 60 μl of the ECP preparation was added to each microcapsule on the test trays. Values 0–5 correspond to 0–≥40 nmol of hydrolyzed colorimetric substrate assigned to each reaction (0 for 0 nmol, 1 for 5 nmol, 2 for10 nmol, 3 for 20 nmol, 4 for 30 nmol, and 5 for 40 nmol, respectively). Assays were run in duplicate.

3.3 Hemolytic activity of *prt*V mutant strain

Two assays were used to evaluate the hemolytic activity of *V. anguillarum* strains. In the agar plate assay, no hemolytic zone was generated by the wild-type, *prtV* mutant, or *prtV*-com strains, but activities were detected in the cell-contact hemolysis assay. As shown in Fig.3, the wild-type strain had the highest hemolysis value (P_h=90.6%±1.5%), the *prtV*-com strain had a relatively high value (P_h =85.9%±7.0%), and the *prtV* mutant strain had the lowest hemolysis value (P_h=75.6%±3.9%). A significant difference (P<0.05) in hemolysis value was observed between the wild-type and the mutant strain. These results suggest that *V. anguillarum* PrtV might be capable of disrupting the fish erythrocytes. Many metalloproteases have been reported to be cytotoxic. For example, *V. cholerae* PrtV is able to induce cell rounding and detachment in human intestinal cell line HCT8 (Vaitkevicius et al., 2008). However, there are limited documented data on metalloproteases hemolytic activity. A tissue-destructive metalloprotease from *Legionella pneumophila* has been reported to display both hemolytic and cytotoxic activities (Keen et al., 1989). *V. vulnificus* metalloprotease was found to proteolyze basement membrane components, causing breakdown of capillary vessels and leakage of blood components into surrounding tissues (Miyoshi et al., 1998). However, the *V. cholerae* hemagglutinin/protease was found to process and activate the recombinant pro-HlyA *in vitro* (Nagamune et al., 1996). Although the detailed action of PrtV is not clear, its proteolytic activity might be essential to the hemolysis or the potential cytotoxicity of this protein.

3.4 Growth in turbot intestinal mucus

The assays for growth in turbot intestinal mucus were performed in the wild-type, *prtV* mutant, and *prtV*-com strains. All the strains assayed were able to grow and increase their cell number (Fig.4). The highest cell numbers were seen in the wild-type strain (3.3 log increase from $(5.44\pm1.14)\times10^5$ to $(9.59\pm1.43)\times10^8$ CFU ml^{-1}), relatively high cell numbers were seen in the *prtV*-com strain (2.8 log increase from $(3.31\pm1.06)\times10^5$ to $(2.28\pm1.08)\times10^8$ CFU ml^{-1}), and the lowest cell numbers were found in the *prtV* mutant strain (1.2 log increase from $(4.55\pm0.99)\times10^5$ to $(8.10\pm1.91)\times10^6$ CFU ml^{-1}). A significant difference (P<0.05) in cell number was observed between the wild-type and the *prtV* mutant strain from the 12 h samples. These experiments indicate that *V. anguillarum* PrtV might facilitate the growth of the bacterium in the fish intestine. Bordas et al. (1996) showed that inhibitory substances in the mucus layer are involved in the prevention of bacterial colonization and invasion. Thus, the role of PrtV might be in resistance to the mucous inhibition during the use of the mucus as a carbon source supporting bacterial multiplication (Ichinose et al., 1994).

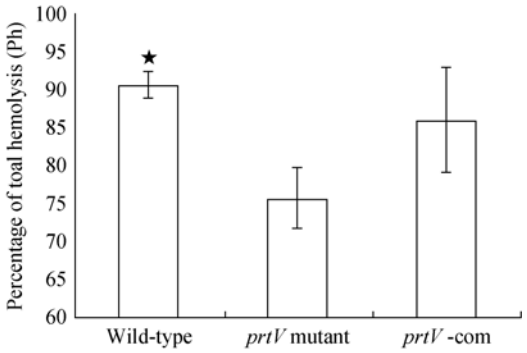

Fig.3 Percentage of hemolysis of turbot erythrocytes by *V. anguillarum* strains

Overnight cultures were resuspended in PBS (A_{540}=1.0) and incubated with 1% turbot erythrocytes at 28ºC for 4 hours. Bar limits represent standard deviations. The asterisks denote significant differences (P<0.05) between the values of the wild-type and *prtV* mutant strain.

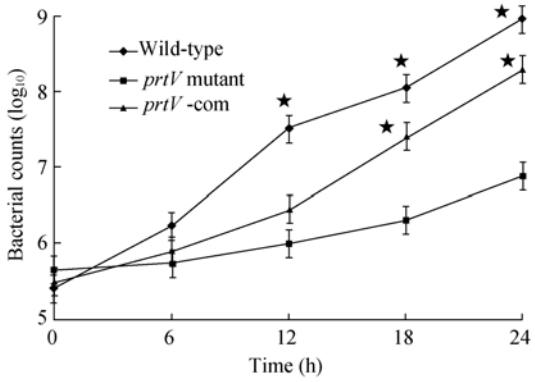

Fig.4 Bacterial counts of *V. anguillarum* strains in turbot intestinal mucus

Overnight cultures were resuspended in PBS (A_{540}=0.1) and incubated with an equal volume of turbot intestine mucus (1 mg ml^{-1}) at 28ºC with shaking. Bar limits represent standard deviations. The asterisks denote significant differences (P<0.05) between the values of the wild-type or *prtV*-com strain, and the *prtV* mutant strain.

3.5 Virulence of *prtV* mutant

Healthy turbot fish were infected with the wild-type, the *prtV* mutant and *prtV*-com strains by intraperitoneal injection (Table 2). Fish inoculated with 1.5×10^7 CFU/fish of wide-type strain suffered 100% mortality by two days, and the LD_{50} was $10^{6.0}$ CFU/fish. Fish inoculated with 1.3×10^7 CFU/fish of *prtV* mutant strain suffered only 10% mortality, and the LD_{50} of *prtV* mutant strain was $>10^7$ CFU/fish. Fish inoculated with the *prtV*-com strain restored the killing ability to almost the same level of wide-type with a LD_{50} of $10^{6.29}$ CFU/fish. These results indicate PrtV might play a role in the virulence of *V. anguillarum*, at least when infection is by the route of i.p. injection.

Table 2 Virulence of *V. anguillarum* strains in turbot, *Scophthalmus maximus* (L.)

Strains	Dose/fish（CFU）	Total mortality [a] (%)	Time of death in days (no. of deaths/total fish)	LD_{50} [c]
M3	1.5×10^7	100	1(8/10), 2(10/10)	$10^{6.0}$
	1.5×10^6	60	1(4/10), 2(6/10)	
	1.5×10^5	10	3(1/10)	
prtV mutant	1.3×10^7	10	4(1/10)	$>10^{7.0}$
	1.3×10^6	0	NA [b]	
	1.3×10^5	0	NA [b]	
prtV-com	1.1×10^7	80	1(4/10), 2(6/10), 3(8/10)	$10^{6.29}$
	1.1×10^6	40	1(2/10), 2(3/10), 4(4/10)	
	1.1×10^5	0	NA [b]	
PBS control		0	NA [b]	

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

In conclusion, our data demonstrated that *prtV* of *V. anguillarum* encoded a putative 918 amino acid protein belonging to the M6 peptidase family. Mutation of *prtV* resulted in decreased activity of proteolysis and hemolysis of *V. anguillarum*. The *prtV* mutant strain also showed reduced multiplication in fish intestinal mucus, suggesting that wild-type *prtV* confers resistance to the inhibitory effects present in the mucus. Infection experiment of fish showed that the virulence of the *prtV* mutant strain increased the LD_{50} by at least 1 log. Finally, complementation experiments restored the above-mentioned traits of *prtV* mutant strain to close to those of the wild-type strain. Our data suggest that PrtV plays an important role in the pathogenesis of *V. anguillarum* via its proteolytic action. Given its hemolysis role on fish erythrocytes, we hypothesize PrtV might interfere with components of erythrocyte cells by modulating the activities of hemolysin.

References

Asakawa S, Abe I, Kudoh Y, Kishi N, Wang Y, Kubota R, Kudoh J, Kawasaki K, Minoshima S, Shimizu N. 1997. Human BAC library: construction and rapid screening. *Gene*, **191**: 69-79.

Austin B, Austin D A. 1993. Vibrionaceae representatives. *In*: Bacterial fish pathogens: diseases in farmed and wild fish, 2nd ed. by Horwood E, Ltd. Chichester: United Kingdom, p. 265-307.

Blocker A, Gounon P, Larquet E, Niebuhr K, Cabiaux V, Parsot C, Sansonetti P. 1999. The tripartite type III secretion of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J. Cell Biolog.*, **147**: 683-693.

Bordas M A, Balebona M C, Zorrilla I, Borrego J J, Morinigo M A. 1996. Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream (*Sparus aurata* L.). *Appl. Environ. Microbiol.*, **62**: 3 650-3 654.

Boesen H T, Pedersen K, Larsen J L, Koch C, Ellis A E. 1999. *Vibrio anguillarum* resistance to rainbow trout

(*Oncorhynchus mykiss*) serum: role of O-antigen structure of lipopolysaccharide. *Infect. Immun.*, **67**: 294-301.

Crosa J H. 1980. A plasmid associated with virulence in the marine fish pathogen specifies an iron-sequestering system. *Nature* (London), **284**: 566-568.

Croxatto A, Lauritz J, Chen C, Milton D L. 2007. *Vibrio anguillarum* colonization of rainbow trout integument requires a DNA locus involved in exopolysaccharide transport and biosynthesis. *Environ. Microbiol*., **9**: 370-382.

Denkin S M, Nelson D R. 1999. Induction of protease activity in *Vibrio anguillarum* by gastrointestinal mucus. *Appl. Environ. Microbiol.*, **65**: 3 555-3 560.

Edlund T, Siden I, Boman H G. 1976. Evidence for two immune inhibitors from *Bacillus thuringiensis* interfering with the humoral defense system of saturniid pupae. *Infect. Immun*., **14**: 934-941.

Fedhila S, Nel P, Lereclus D. 2002. The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. *J. Bacteriol*., **184**: 3 296-3 304.

Finn R D, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Sean R E, Sonnhammer E L L, Bateman A. 2006. Pfam: clans, web tools and services. *Nucleic. Acids. Res*., **34**: D247-D251.

Hirono I, Masuda T, Aoki T. 1996. Cloning and detection of the hemolysin gene of *Vibrio anguillarum*. *Microb. Pathog.*, **21**: 173-182.

Ichinose Y, Ehara M, Honda T, Miwatani T. 1994. The effect on enterotoxicity of protease purified from *Vibrio cholerae* O1. *FEMS Microbiol. Lett.*, **115**: 265-271.

Inamura H, Nakai T, Muroga K. 1985. An extracellular protease produced by *Vibrio anguillarum*. *Bull. Jap. Soc. Sci. Fisheri.*, **51**: 1 915-1 920.

Jongeneel C V, Bouvier J, Bairoch A. 1989. A unique signature identifies a family of zinc-dependent metallopeptidases. *FEBS Lett*., **242**: 211-214.

Kalderon D, Rubin R M. 1989. cGMP-dependent protein kinase genes in Drosophila. *J. Biol. Chem.*, **264**: 10 738-10 748.

Keen M G, Hoffman P S. 1989. Characterization of a *Legionella pneumophila* extracellular protease exhibiting hemolytic and cytotoxic activities. *Infect. Immun.*, **57**: 732-738.

McGee K, Hörstedt P, Milton D L. 1996. Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. *J. Bacteriol.*, **178**: 5 188-5 198.

Milton D L, Norqvist A, Wolf-Wat H. 1992. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. *J. Bacterio.*, **174**: 7 235-7 244.

Milton D L, O'Toole R, Hörstedt P, Wolf-Watz H. 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J. Bacteriol.*, **178**: 1 310-1 319.

Miyoshi S, Nakazawa H, Tomochika K, Shinoda S. 1998. Characterization of the hemorrhagic reaction caused by *Vibrio vulnificus* metalloprotease, a member of the thermolysin family. *Infect. Imun.*, **66**: 4 851-4 855.

Miyoshi S, Shino S. 2000. Microbial metalloproteases and pathogenesis. *Microbes. Infect.*, **2**: 91-98.

Mo Z L, Chen S Y, Zhang P J. 2002. Properties of proteolytic toxin of *Vibrio anguillarum* from diseased flounder. *Chin. J. Oceanol.*, **20**: 316-322.

Mo Z L, Tan X G, Xu Y L, Zhang P J. 2001. A *Vibrio anguillarum* strain associated with skin ulcer on cultured flounder, *Paralichthys olivaceus*. *Chin. J. Oceanol.*, **19**: 319-326.

Nagamune K, Yamamoto K, Naka A, Matsuyama J, Miwatani T, Honda T. 1996. *In vitro* proteolytic processing and activation of the recombinant precursor of El Tor cytolysin/hemolysin (pro-HlyA) of *Vibrio cholerae* by soluble hemagglutinin/protease of *V. cholerae*, trypsin, and other proteases. *Infect. Immun*., **64**: 4 655-4 658.

O'Toole R, Milton D L, Wolf-Watz H. 1996. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. *Mol. Microbiol.*, **19**: 625-637.

Reed L J, Muench H. 1938. A simple method of estimating fifty percent end points. *Am. J. Hyg.*, **27**: 493-497.

Rock J L, Nelson D R. 2006. Identification and characterization of a hemolysin gene cluster in *Vibrio anguillarum*. *Infect. Immun.*, **74**: 2 777-2 786.

Sambrook J, Russell DW. 2001. Molecular cloning: A laboratory manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York, p. 474-489.

Siden I, Dalhammar G, Telander B, Boman H G, Somerville H. 1979. Virulence factors in *Bacillus thuringiensis*: purification and properties of a protein inhibitor of immunity in insects. *J. Gen. Microbiol.*, **114**: 45-52.

Stork M, Di Lorenzo M, Welch T J, Crosa L M, Crosa J H. 2002. Plasmid-mediated iron uptake and virulence in *Vibrio anguillarum*. *Plasmid*, **48**: 222-228.

Vaitkevicius K, Lindmark B, Ou G W, Song TY, Toma C, Iwanaga M, Zhu J, Andersson A, Hammarström M-L, Tuck S, Wai S N. 2006. A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *PNAS*, **103**: 9 280-9 285.

Vaitkevicius K, Rompikuntal P K, Lindmark B, Vaitkevicius R, Song T Y, Wai S N. 2008. The metalloprotease PrtV from *Vibrio cholerae*. *FEBS J.*, **275**: 3 167-3 177.

Welch T J, Crosa J H. 2005. Novel role of the lipopolysaccharide O1 side chain in ferric siderophore transport and virulence of *Vibrio anguillarum*. *Infect. Immun.*, **73**: 5 864-5 872.