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Virulence of metalloproteases produced by *Vibrio* species on Pacific oyster *Crassostrea gigas* larvae

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ABSTRACT: *Vibrio tubiashii*, a pathogen of shellfish larvae and juveniles, produces several extracellular products. Here, we document that culture supernatants of several marine *Vibrio* species showed toxicity to oyster larvae. Treatment of these supernatants with EDTA not only severely diminished proteolytic activities, but also dramatically reduced toxicity to the larvae. Culture supernatants of metalloprotease-deficient mutants of *V. tubiashii*, *V. cholerae*, and *V. splendidus* were impaired in their ability to cause larval death compared to the wild type strains. Culture supernatants of *Pseudomonas aeruginosa*, known to contain several secreted proteases, showed virtually no toxicity to oyster larvae. Purified *V. tubiashii* protease A (VtpA), but not the prototype metalloprotease, thermolysin from *Bacillus thermoproteolyticus*, was highly toxic to the larvae. In addition, toxicity of purified VtpA was much greater for 6 d old oyster larvae than for 16 d old larvae. Together, these results indicated that culture supernatants of a variety of *Vibrio* species are highly toxic to oyster larvae and that the production of a metalloprotease is required for this effect. We propose that there are, as yet uncharacterized, specific substrates contained in larval tissue that are degraded by VtpA as well as certain homologous metalloproteases produced by other marine *Vibrio* species which, in turn, may contribute to vibriosis.

KEY WORDS: *Vibrio tubiashii* · VtpA · Vibriosis · Extracellular products · Shellfish hatchery

INTRODUCTION

Bacterial diseases can be a major cause of mortality in rearing facilities of molluscan bivalve larvae, with such outbreaks resulting in substantial economic loss for shellfish growers (Brown 1981, Elston et al. 1981, 2008, Elston 1999). In addition, losses of hatchery- and nursery-reared shellfish larvae and juveniles have, in some cases, been accompanied by reports of failures of natural shellfish larval populations (Elston 1984, Perkins 1993, Prado et al. 2005). Although bacterial strains from other genera, such as *Pseudomonas*, have been demonstrated to be pathogens of larval bivalves (Brown 1973), members of the genus *Vibrio* are the most frequent cause of larval mortalities. Overall, vibriosis is considered to be the most serious disease of hatchery-reared oyster larvae (Tubiash et al. 1970, Hada et al. 1984, Elston et al. 2008).

The genus *Vibrio* is composed of gram-negative curved rods that are motile by means of a single polar flagellum. The genus is widespread in the aquatic environment throughout the world and includes more than 30 species, many of which are pathogenic not only for humans but also for various aquatic animals, including fish and shellfish. Bacterial infections of adult mollusk bivalves only rarely lead to disease while larval and juvenile bivalves are highly susceptible to infection by some species of the genus *Vibrio* (Elston & Leibovitz 1980, Nottage & Birkbeck 1987, Elston et al. 2008).

Vibrio tubiashii recently re-emerged as a pathogen of several species of bivalve larvae, causing substantial economic loss, especially in Pacific oyster-rearing facilities (Estes et al. 2004, Elston et al. 2008). Vibriosis was initially termed bacillary necrosis (Tubiash et al. 1965) and the causative bacterium later designated as *V. tubiashii* by Hada et al. (1984). Vibriosis is characterized by a rapid onset of reduced larval motility, detached vela,

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and necrotic soft tissue, accompanied by high mortality rates, often exceeding 90% (Sindermann 1988). Although first identified as a cause of such mortalities in 1965 (Tubiash et al. 1965), *V. tubiashii* re-emerged in 2006 and 2007 as a causative agent for mass larval mortalities of Pacific oysters and other species in some hatcheries in the Pacific Northwest (Elston et al. 2008).

Extracellular metalloproteases produced by a variety of marine *Vibrio* species have been established as virulence factors for their fish or shellfish hosts. For instance, metalloprotease Vvp from a strain of *V. vulnificus* pathogenic for eels is essential for colonization of mucosal surfaces in aquatic animals (Valiente et al. 2008). In *V. anguillarum*, a metalloprotease has been shown to contribute to its ability to invade host fish tissues and cause systemic disease in a number of fish species (Norqvist et al. 1990). In addition, an extracellular metalloprotease of *V. splendidus* is toxic when injected into adult oysters (Le Roux et al. 2007, Binesse et al. 2008). Moreover, it has been reported that extracellular products produced by *V. aestuarianus* are responsible for its virulence for *Crassostrea gigas* (Labreuche et al. 2006b). A subsequent study (Labreuche et al. 2006a) showed that protease concentration from *V. aestuarianus* injected into oysters is correlated with a decrease in phagocytic activities in the host. Another study reported that toxicity of culture supernatants of *V. proteolyticus* to larvae of European flat oysters *Ostrea edulis* was due to an extracellular metalloprotease and that the enzyme also rapidly broke down gill tissue of blue mussels *Mytilus edulis* (Nottage & Birkbeck 1987).

In a previous study, we reported that culture supernatants of *Vibrio tubiashii* were highly toxic to Pacific oyster larvae (Hasegawa et al. 2008). This toxicity was attributed to the activity of the secreted metalloprotease because (1) metalloprotease inhibitors, including EDTA, severely impaired the toxicity of *V. tubiashii* culture supernatants to Pacific oyster larvae, (2) culture supernatants of non-proteolytic *V. cholerae* carrying the *V. tubiashii* metalloprotease gene (*vtpA*) were highly toxic to Pacific oyster larvae, and (3) a VtpA$^-$ mutant of *V. tubiashii* showed much reduced larval mortality. Thus, we concluded that the VtpA is a major virulence factor for Pacific oyster larvae.

In this study, we purified the VtpA protein and analyzed its enzymatic activity and toxicity to oyster larvae in more detail. We also examined larval toxicity of culture supernatants of various *Vibrio* species and their correlation with protease production, using both inhibitors and their relevant mutant strains. In addition, we analyzed non-*Vibrio* related metalloproteases, including culture supernatants of *Pseudomonas aeruginosa* and purified thermolysin from *Bacillus thermoproteolyticus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. *Vibrio tubiashii*, *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, *V. splendidus*, *V. vulnificus*, and *V. parahaemolyticus* were grown in Luria-Bertani (LB) media supplemented with 3% sodium chloride, whereas *V. cholerae* and *Pseudomonas aeruginosa* were grown in pure LB media. All *Vibrio* strains were grown at 30°C and *P. aeruginosa* was grown at 37°C overnight. *Escherichia coli* Top 10 cells (Invitrogen) were used for routine cloning as well as His-tagged protein purification and grown in LB broth or on agar supplemented with appropriate antibiotics. When required, antibiotics were supplemented as follows: ampicillin (Ap), 100 µg ml^{-1}; kanamycin (Km), 50 µg ml^{-1}; chloramphenicol (Cm), 20 µg ml^{-1} and tetracycline (Tc), 10 µg ml^{-1}.

Construction of the *Vibrio tubiashii* VtpA$^-$ mutant. To obtain a cosmid library of *V. tubiashii*, genomic DNA of *V. tubiashii* strain RE22 was partially digested with *Sau3*AI. DNA fragments (approximately 20 kb) were ligated into pLAFR5 cosmid arms, which were produced by digesting pLAFR5 with *Sca*I and *Bam*HI to produce 2 DNA fragments (Keen et al. 1988). The ligation mix was packaged into lambda phageheads *in vitro* (Packagene Lambda DNA Packaging System, Promega) and transduced into *Escherichia coli* strain LE392, selecting for Tc-resistant colonies. The cosmid DNA containing the *vtpA* gene was obtained from the library by screening pools of colonies by standard PCR using *vtpA*–specific primers. A *vtpA* containing cosmid, pLAFR5-vtpA, was then mutagenized via the lambda red recombinase system (Datsenko & Wanner 2000), using hybrid lambda red *vtpA* primers and pKD3 (Datsenko & Wanner 2000) as the template. Competent cells of *E. coli* BW25113 carrying pKD46 (Datsenko & Wanner 2000) and pLAFR5-vtpA were electroporated with a linear PCR product containing the Cm resistance cassette flanked by *vtpA* sequences. The resulting Cm and Tc resistance clones were confirmed for the correct insertion by PCR, creating pLAFR5-vtpA$^-$. *E. coli* strain Top10 carrying pLAFR5-vtpA$^-$ was then conjugated into *V. tubiashii* RE22 by triparental matings with the helper *E. coli* strain HB101 carrying pRK2013 (Figurski & Helinski 1979). *V. tubiashii* carrying the mutagenized cosmid were selected for the marker (Cm) inserted into the *vtpA* gene and then tested for the loss of vector marker (Tc). The allelic exchange following double homologous recombination was confirmed by PCR. All PCR and cloning reactions were conducted using standard procedures (Ausubel et al. 1991, Saltikov & Newman 2003).

Purification of VtpA-His protein. A DNA fragment containing the entire coding region of *vtpA* was PCR amplified from *Vibrio tubiashii* RE22 chromosomal DNA by using primers 5′-GAG GAA TAA TAA ATG AAA CAA CGT CAA ATG CTT TG-3′ and 5′-GTC TAA TCT TAG TGT CAC GC-3′, which do not encode the native stop codon. The fragment was then cloned into pBAD-His vector (Invitrogen), thus fusing a 6×His tag to the open reading frame (ORF) to yield pBADvtpA-His. The insert was sequenced to verify the construct. To purify VtpA-His from bacterial cells, *Escherichia coli* strain Top10 carrying pBADvtpA-His was grown in LB medium supplemented with arabinose (0.2%, wt vol^{-1}) and ampicillin at 37°C and induced for several h. Cells from 250 ml culture were typically harvested by centrifugation when the culture reached an absorbance of light (A_{600}) value of 1.8. VtpA-His protein from the lysed cell pellet was purified using Ni-NTA column (Qiagen), according to the manufacturer's instructions. Protein concentrations were determined by using Bradford Assay kit (Pierce). Thermolysin was purchased from Calbiochem.

Larvae toxicity assay. Stationary-phase bacterial cultures were centrifuged and the filter-sterilized supernatants, as well as purified proteins, were used as the samples. We aliquoted 6, 10, and 16 d old oyster larvae (Coast Seafoods Company) in sterile seawater to a 96-well flat-bottom plate (Nunc) at a density of approximately 20 larvae in 100 µl per well. Toxicity to larvae was assessed by adding various amounts of undiluted or diluted supernatants or purified proteins (0.5 to 2 µl) to the wells in triplicate and varying incubation times (every 30 min up to 4 h) at room temperature to determine the threshold for toxicity. The same amount of growth medium or protein elution buffer was used as a control. Toxicity to larvae was determined by visualization on an inverted microscope. We considered oyster larvae dead when the larvae stop moving, the velum was grossly damaged, and the larvae appeared to be darkened, similar to phenotypes described in Garland et al. (1983). The concentration of EDTA used in these experiments was 10 mM.

Enzyme assays. *Vibrio* species supernatants and purified proteins were assayed for proteolytic activities using 1% azocasein as previously described by Hasegawa et al. (2008). The proteolytic units were calculated as the ratio between the absorbances from azocasein assays (at 420 nm) and the normalized bacterial growth (600 nm). For semiquantative assays for gelatinase, elastase, and mucinase activities, wells were

Table 1. *Vibrio* spp., *Pseudomonas* spp., *Escherichia* spp. Bacterial strains and plasmids used in this study. Ap^r: ampicillin resistant; Tc^r: tetracycline resistant; Cm^r: chloramphenicol resistant; Mob$^+$: can be mobilized; Tra$^+$: capable of self transfer; Km^r: Kanamycin resistant

Strains/Plasmids	Relevant characteristics	Source/ Reference
Strains		
V. tubiashii RE22	Wild-type	Estes et al. (2004)
V. tubiashii VtpA17	VtpA$^-$ derivative of RE22	Present study
V. cholerae C7258	Wild-type	Robert et al. (1996)
V. cholerae 638	HapA$^-$ derivative of C7258	Robert et al. (1996)
V. splendidus LGP32	Wild-type	Le Roux et al. (2007)
V. splendidus δ2989	Vsm$^-$ derivative of LGP32	Le Roux et al. (2007)
V. alginolyticus ATCC17749	Wild-type	Sakazaki (1968)
V. fluvialis 1959-82	Wild-type	Hickman-Brenner et al. (1984)
V. mimicus ATCC33653	Wild-type	Davis et al. (1981)
V. parahaemolyticus ATCC17802	Wild-type	Fujino et al. (1974)
V. vulnifucus ATCC27562	Wild-type	Amaro et al. (1992)
P. aeruginosa PA14	Wild-type	Rahme et al. (1995)
P. aeruginosa ΔLasB	LasB$^-$ derivative of PA14	M. Schuster (pers. comm.)
P. aeruginosa ΔLasR	LasR$^-$ derivative of PA14	M. Schuster (pers. comm.)
E. coli Top10	Cloning host	Invitrogen
E. coli BW25113	Conjugation donor	Datsenko & Wanner (2000)
Plasmids		
pBAD-His	Ap^r, protein expression vector	Invitrogen
pBADvtpA-His	Ap^r, *vtpA* gene in pBAD-His	Present study
pLARF5	Tc^r, cosmid cloning vector	Keen et al. (1988)
pLAFR5-vtpA	Tc^r, *vtpA* gene of RE22 in pLARF5	Present study
pLAFR5-ΔvtpA	Tc^r, Cm^r, *vtpA*$^-$ derivative of pLAFR5-vtpA	Present study
pKD3	Cm^r, template for PCR amplification for red recombinase-mediated recombination	Datsenko & Wanner (2000)
pKD46	Ap^r, plasmid encoding red recombinase	Datsenko & Wanner (2000)
pRK2013	Mob$^+$ Tra$^+$ Km^r	Figurski & Helinski (1979)

made in 1% substrate-containing agarose media and the bottoms of the wells were sealed with agarose. Supernatant samples were applied to the wells and the plates were incubated at 30°C. After 24 h, halos around the wells due to protease activity became visible in the assay plates. A reaction was scored as positive with at least 1 mm of visible halo. For the collagenase assay, 100 µl of supernatant was incubated with 400 µl of 1% Hide-Remazol Brilliant Blue R (Sigma) for 30 min at 37°C. The reactions were centrifuged and the absorbances at 595 nm were measured. When added, the concentration of EDTA was 10 mM. Proteolytic activity was also assessed using Zymogram gel electrophoresis as described previously (Hasegawa et al. 2008).

RESULTS

Toxicity of culture supernatants

Previously, we documented that culture supernatants of *Vibrio tubiashii* are highly toxic to Pacific oyster larvae due to the production of VtpA (Hasegawa et al. 2008). In addition, sequence analyses revealed that VtpA homologs are widespread amongst other *Vibrio* species (Hasegawa et al. 2008). To determine if culture supernatants of other *Vibrio* species show toxicity to oyster larvae that can be attributed to metalloprotease activities, we tested sterile culture supernatants from various marine *Vibrio* species in azocasein as well as larval toxicity assays. Also included in this study was a well-studied metalloprotease, elastase from *Pseudomonas aeruginosa*, known to be enzymatically and structurally related to this group of *Vibrio* metalloproteases (Häse & Finkelstein 1990, Miyoshi & Shinoda 2000, Cahan et al. 2001). As expected, the azocasein assay showed that culture supernatants of *V. fluvialis*, *V. mimicus*, *V. vulnificus, V. tubiashii*, *V. cholerae*, *V. splendidus*, and *P. aerugionosa* all contained considerable levels of extracellular proteases (Fig. 1A). When assayed for toxicity to 10 d old oyster larvae, all protease-containing culture supernatants of the tested *Vibrio* species caused substantial levels of mortality (Fig. 1B). Appropriate media were used as the negative control in this assay, which showed no toxicity to oyster larvae (data not shown). There was no dramatic difference between *V. tubiashii* and these *Vibrio* species in their proteolytic activities or their resulting toxicities. On the contrary, culture supernatants of *V. alginolyticus* and *V. parahaemolyticus* under our growth conditions did not contain significant azocasein activities and did not show considerable toxicity to oyster larvae (Fig. 1).

It has been known that elastase, a metalloprotease produced by *Pseudomonas aeruginosa*, is similar to *Vibrio* metalloproteases (Miyoshi & Shinoda 2000), showing 64% similarity to the VtpA (data not shown), which is much lower than similarities between *Vibrio* metalloproteases (81 to 87%, Hasegawa et al. 2008). Although *P. aeruginosa* produced high levels of azocasein activity, only minimal larvae mortality (ca. 13%) was observed (Fig. 1B); however, an unusual effect was observed, resulting in loss of overall larval movement, which the cilia were still actively moving (data not shown). A mutation in *lasR* (quorum sensing regulator), but not *lasB* (elastase), eliminated this effect

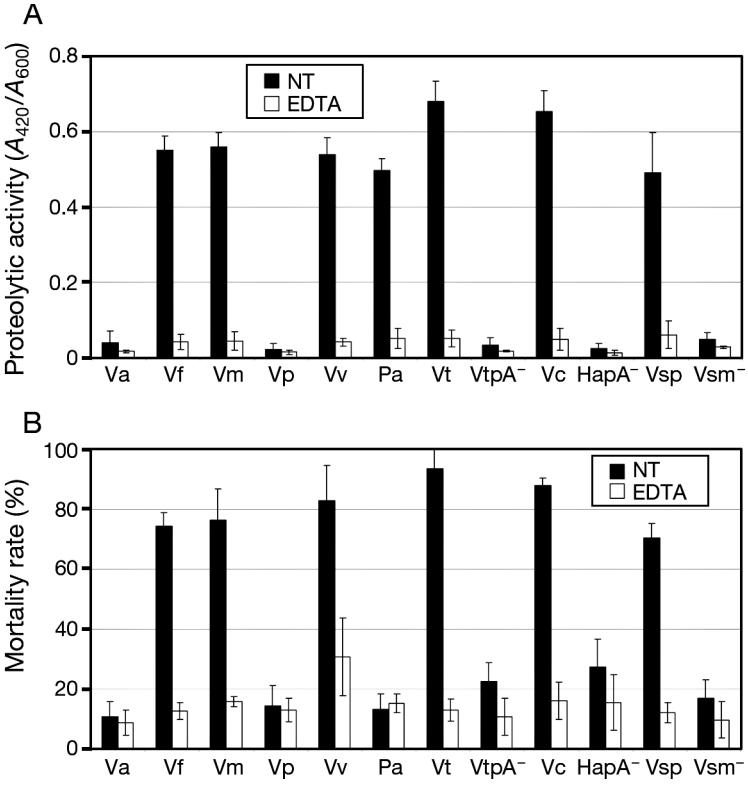

Fig. 1. *Vibrio* spp. and *Pseudomonas aeruginosa*. Analyses of supernatants. (A) Protease activity and (B) toxicity were assayed in absence or presence of 10 mM EDTA. All cells were harvested at late stationary phase. Proteolytic and toxic activities were determined using azocasein and 10 d old oyster larvae, respectively, as described in 'Materials and methods.' Va: *V. alginolyticus*; Vf: *V. fluvialis*;: Vm: *V. mimicus*;: Vp: *V. parahaemolyticus*; Vv: *V. vulnificus*; Pa: *P. aeruginosa*; Vt: *V. tubiashii*; VtpA⁻: the VtpA⁻ mutant of Vt; Vc: *V. cholerae*; HapA⁻: the HapA⁻ mutant of Vc; Vsp: *V. splendidus*; Vsm⁻: the Vsm⁻ mutant of Vsp. A_{420}, A_{600}: absorbance of light at 420 and 600 nm respectively. NT: non-treated. For the toxicity assay, filter-sterilized supernatants were added to final concentrations of 1%. Error bars indicate SD (N = 3)

(data not shown). Our previous study demonstrated that the metalloprotease inhibitor, EDTA, strongly reduced the proteolytic activity and thus the toxicity level of culture supernatants of *V. tubiashii* (Hasegawa et al. 2008). Treatment with EDTA not only significantly reduced the proteolytic activities of all tested supernatants (Fig. 1A), but also severely diminished toxicity levels of all the *Vibrio* species tested (Fig. 1B). By contrast, EDTA treatment did not affect the unusual toxicity observed for *P. aeruginosa* supernatants (data not shown).

Table 2. *Vibrio* spp. and *Pseudomonas aeruginosa.* Enzymatic activities found in culture supernatants from different bacterial species in the presence of various protease substrates. Toxicity was regarded as '+' when the mortality rate was more than 70% and '–' when the rate was less than 15%

Bacterial species	Gelatin	Elastin	Mucin	Casein	Collagen	Toxicity
V. tubiashii	+	–	+	+	+	+
V. cholerae	+	+	+	+	+	+
V. fluvialis	+	–	–	+	+	+
V. mimicus	+	–	–	+	+	+
V. splendidus	+	–	–	+	+	+
V. vulnificus	+	–	–	+	+	+
P. aeruginosa	+	+	–	+	+	–

Substrate specificity of culture supernatants

In order to correlate culture supernatant toxicity with digestion of various potentially physiologically relevant substrates, culture supernatants from various marine *Vibrio* species and *Pseudomonas aeruginosa* were examined for activity toward gelatin, elastin, mucin, casein, and collagen. Except for casein, all of the potential substrates are thought to be found in oysters (Galtsoff 1964). Table 2 shows the various substrate specificities of the tested supernatants. For *Vibrio* species tested, supernatants exhibiting larval toxicity also exhibited gelatinase, caseinase, and collagenase, but not elastase or mucinase, activities. Moreover, the *P. aeruginosa* supernatants that showed very limited toxicity to oyster larvae also degraded gelatin, elastin, casein, and collagen.

Effects of loss of the metalloprotease on pathogenicity

To further validate the role of metalloproteases in the observed toxicity of *Vibrio* supernatants, we analyzed isogenic *V. cholerae* and *V. splendidus* strains carrying mutations in their metalloprotease genes (*hapA* and *vsm*, respectively) with high homology to VtpA. In addition, we generated a novel, stable mutant of *V. tubiashii* by deleting the entire *vtpA* gene. Proteolytic activity of this mutant strain was fully restored by providing the *vtpA* gene *in trans* (data not shown). Fig. 1A shows that compared to the wild type, these metalloprotease deficient mutants produced only minimal levels of extracellular protease, which were comparable to those of the parent strains treated with EDTA. Zymographic analysis using gelatin as the substrate showed that proteolytic band profiles varied among these species (Fig. 2). In *V. cholerae* and *V. splendidus*, virtually all proteolytic bands disappeared in their mutant samples, whereas the *V. tubiashii* mutant still retained some observable proteolytic bands (Fig. 2). Similar to our previous results (Hasegawa et al. 2008), the *V. tubiashii* mutant showed dramatically reduced toxicity to oyster larvae (Fig. 1B). Likewise, the *V. cholerae* and *V. splendidus* mutants showed much-reduced larval toxicity compared to the parent strains (Fig. 1B).

Proteolytic activity and toxicity of purified His-tagged VtpA

Although we had evidence that VtpA was directly responsible for larval mortality observed in culture supernatants of *Vibrio tubiashii* (Hasegawa et al. 2008), we wanted to independently confirm the toxicity of this enzyme. To determine if VtpA is a structural

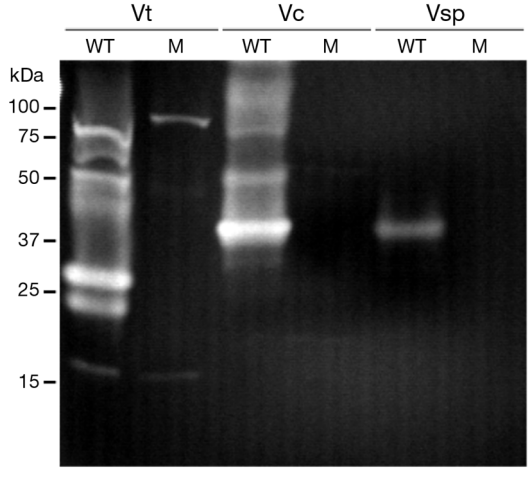

Fig. 2. *Vibrio* spp. and *Pseudomonas aeruginosa.* Zymography analysis of supernatants using a 10% gelatin polyacrylamide gel. WT: wild type strain, M: the metalloprotease mutant. Bands of proteolytic activity in the zymogram gel are shown as clear protein bands in a dark background. The molecular masses (kDa) on the left indicate the positions of molecular weight markers

toxin for oyster larvae, a His-tagged VtpA was purified and tested for toxicity. To test toxicity of another similar metalloprotease (41% similarity to the VtpA, data not shown) produced by a gram-positive bacterial species, we used thermolysin, a prototype metalloprotease from *Bacillus thermoproteolyticus*. Fig. 3A shows that both the thermolysin and purified VtpA presented high azocasein activities. On the other hand, VtpA was highly toxic to oyster larvae, whereas thermolysin was found to be much less toxic to oyster larvae in spite of high azocasein activities (Fig. 3B).

Relationship between proteolytic activities and toxicity levels in the purified VtpA

Clearly, certain metalloproteases from various marine *Vibrio* species are toxic to oyster larvae. To determine the relative potency of these metalloproteases, serial dilutions of protease containing samples, including culture supernatants and purified protease preparations, were tested for their toxicity. Azocasein units required to produce 50 or 20% of mortality in Pacific oyster larvae are summarized in Table 3. The levels of proteolytic activities required to kill 50% of the larvae were very similar for the *V. tubiashii* and *V. cholerae* supernatants, as well as the VtpA purified protein. By contrast, thermolysin required an approximately 10-fold higher concentration of protease units than VtpA to achieve 50% larval mortality. Similarly, *V. tubiashii*, *V. cholerae*, and VtpA produced 20% mortality at virtually equivalent protease units and were approximately 16-fold more lethal than thermolysin.

Effects of larval ages on susceptibility to VtpA

Several reports indicated that larval and juvenile stages of oysters are more susceptible to vibriosis than the adult stages (Nottage & Birkbeck 1987, Elston & Leibovitz 1980, Elston et al. 2008, Gómez-León et al. 2008). To examine if VtpA toxicity varies amongst different ages of oyster larvae, mortality rates of 6 d old and 16 d old larvae were analyzed using various dilutions of VtpA. At higher VtpA concentrations, no major difference was observed in mortality rate of both age groups, with over 90% of larvae being killed in either age group (Table 4). Even at the highest concentration used, a certain number of larvae appeared to be unaffected (data not shown), suggesting natural resistance or absence of the, as yet unidentified, VtpA substrates in these oysters. At lower concentrations of VtpA (50 and 25 ng ml^{-1}), a significant difference in survival rates was observed between the different ages. Overall, 6 d old oyster larvae were much more susceptible to the effects of VtpA compared to 16 d old oyster larvae (Table 4). Moreover, sensitivity of 24 h old larvae was comparable to that of 6 d old larvae (data not shown).

DISCUSSION

Bacterial diseases are a major cause of mortality of bivalve mollusk larvae and may result in major losses and great expense for shellfish growers. Members of the genus *Vibrio* are the most frequent cause of these mortalities. In 2006 and 2007, some of the commercial oyster hatcheries on the west coast of the United States

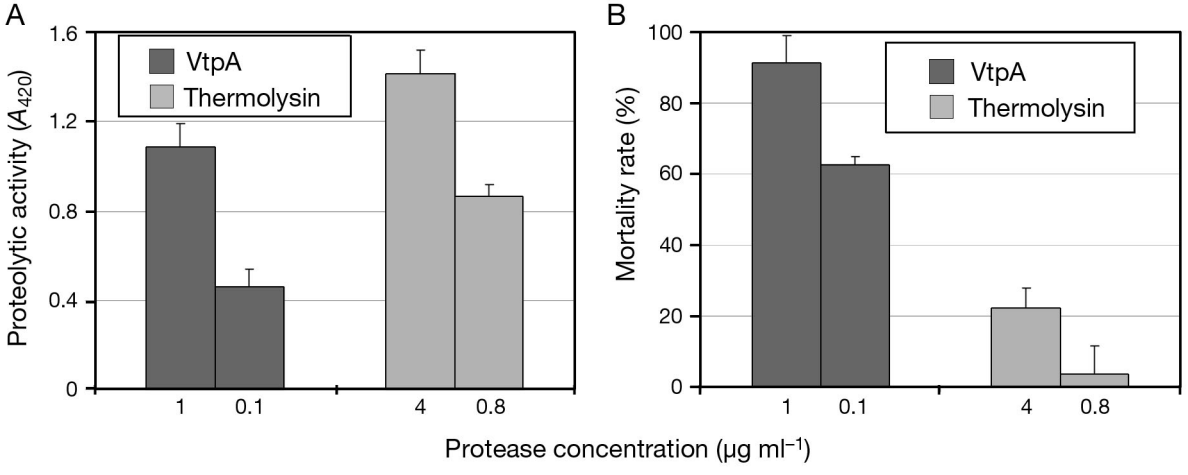

Fig. 3. *Vibrio* spp. and *Crassostrea gigas*. Analyses of purified *V. tubiashii* protease A (VtpA) and thermolysin. Quantitative assays for (A) proteolytic activities and (B) oyster toxicity of VtpA, and thermolysin at 2 different concentrations. Proteolytic and toxic activities were determined using azocasein and 10 d old oyster larvae, respectively, as described in 'Materials and methods.' A_{420}: light absorbance at 420 nm. Error bars indicate SD (N = 3)

Table 3. *Vibrio* spp. and *Crassostrea gigas.* Proteolytic units (absorbance at 420 nm, A_{420}) to kill 50 and 20% of Pacific oyster larvae. Data are means ± SD based on at least 3 independent experiments. sup: supernatants

Culture sup or purified proteins	Azocasein unit (A_{420}) 50% mortality	20% mortality
V. tubiashii sup	0.351 ± 0.08	0.129 ± 0.06
V. cholerae sup	0.364 ± 0.05	0.148 ± 0.08
VtpA	0.368 ± 0.01	0.152 ± 0.09
Thermolysin	3.28 ± 0.10	2.146 ± 0.32

were affected by outbreaks of *V. tubiashii* caused vibriosis, and resulted in a markedly reduced seed supply of Pacific oysters resulted (Elston et al. 2008). In spite of the economic importance of *Vibrio* species in the cultivation of larval and juvenile bivalves in shellfish hatcheries, very little is known about the virulence mechanisms employed by these pathogens, nor their presence at shellfish hatcheries. We have recently shown that the metalloprotease, but not the cytolysin, acts as one of the critical factors for the toxicity of *V. tubiashii* supernatants on Pacific oyster larvae (Hasegawa et al. 2008). However, we could not rule out the possibility that VtpA actually interacts with other still unknown factors, which, in turn, might cause toxicity to the larvae. Therefore, it was essential to prove that the VtpA is a structural toxin. A recent study shows that a purified metalloprotease from *V. splendidus* is toxic when injected into abductor muscles of adult oysters (Binesse et al. 2008). Here, we have also reported, for the first time, that simple exposure to metalloproteases from a variety of marine *Vibrio* species causes larval mortality. Previous studies have shown that the metalloproteases are potentially lethal to adult oysters when injected (Le Roux et al. 2007, Binesse et al. 2008). Moreover, we extended our previous observations by analyzing proteolytic profiles and larval toxicity of culture supernatants from other marine *Vibrio* species as well as similar metalloproteases from *Bacillus* and *Pseudomonas*.

Our data indicated that culture supernatants of multiple *Vibrio* species were highly toxic to Pacific oyster larvae, which was attributed to the production of metalloproteases. Our previous analyses showed not only that a non-pathogenic bacterial isolate lacked the ability to produce high levels of extracellular protease, but that a number of marine *Vibrio* species, including *V. cholerae*, *V. fluvialis*, *V. splendidus*, and *V. vulnificus*, possess VtpA homologs in their genome (Hasegawa et al. 2008). Culture supernatants of these species were all toxic to oyster larvae and their effects were strongly reduced in the presence of EDTA, indicating that secreted metalloproteases are involved in these observed toxicities to oyster larvae. On the other hand, culture supernatants of *V. alginolyticus* and *V. parahaemolyticus* did not produce high levels of azocasein activities and no toxicity, further supporting the concept that metalloproteases are mostly responsible for larval toxicity. However, these results do not exclude the possibility that other strains of these species are toxic to larvae. Interestingly, culture supernatants of a metalloprotease (elastase) producer, *Pseudomonas aeruginosa*, caused only minimal larval mortality, although they stopped the overall movement of the larvae. Culture supernatants of an elastase (LasB) deficient mutant of *P. aeruginosa* also caused this effect on the larvae, whereas a quorum sensing (LasR) deficient mutant did not. LasR is a transcriptional regulator which controls a number of virulence factors (Juhas et al. 2005), indicating that this effect of *P. aeruginosa* supernatants on the larvae is not due to the metalloprotease elastase, but to other factors controlled by quorum sensing in *P. aeruginosa*, such as pyocyanin. Together, these results indicated that culture supernatants of many *Vibrio* species exhibited potentially high toxic effects to oyster larvae, though those of *P. aeruginosa* did not.

Our previous findings revealed that culture supernatants of a VtpA mutant of *Vibrio tubiashii* showed much reduced toxicity to oyster larvae (Hasegawa et al. 2008). However, since the previous VtpA minus strain was an insertional mutant, a new stable deletion mutant was created in this study. In addition, isogenic mutants carrying deletions in metalloprotease genes of *V. cholerae* and *V. splendidus* were analyzed. Culture supernatants of not only the VtpA mutant but also *V. cholerae* and *V. splendidus* metalloprotease mutants showed a marked loss of toxicity compared to their respective wild type strains, further implying that the metalloproteases from other *Vibrio* species are toxic to oyster larvae. Our previous sequence analyses revealed that these metalloproteases share high levels of similarity, in particular, throughout the mature pro-

Table 4. *Vibrio* spp. and *Crassostrea gigas.* Mortality of different ages of oyster larvae induced by *V. tubiashii* protease A (VtpA). A_{420}: absorbance at 420 nm. Data are means ± SD based on at least 3 independent experiments

VtpA concentration (ng ml^{-1})	Calculated proteolytic units (A_{420})	Mortality (%) of VtpA to 6 d old oyster	16 d old oyster
500	0.543	96.3 ± 4.4	91.6 ± 8.3
250	0.272	95.9 ± 5.3	88.1 ± 4.7
125	0.136	90.2 ± 14.2	78.3 ± 6.2
50	0.054	86.5 ± 10.3	50.8 ± 9.1[a]
25	0.027	71.2 ± 8.9	28.5 ± 12.5[a]

[a]Data for larval mortalities were evaluated by Student's *t*-test ($p < 0.01$ compared with the 6 d old larvae)

tease region (Hasegawa et al. 2008), suggesting that these metalloproteases target a similar substrate that results in killing the oyster larvae. It appears that the elastase of *Pseudomonas aeruginosa* does not utilize the putative target protein(s). Amongst the tested supernatants, differences in substrate specificity were noted that did not match the observed toxicity. Consistent with previous observation (Binesse et al. 2008), cytotoxicity assays using Vero cells showed strong toxic effects of *V. tubiashii* supernatants as well as a purified VtpA protein (authors' unpubl. data). Future study will focus on identifying the oyster proteins targeted by these toxic metalloproteases.

In this study, we have conclusively demonstrated that the VtpA metalloprotease is a structural toxin against oyster larvae. However, it is interesting to note that the prototype metalloprotease, thermolysin, did not efficiently kill larvae, again suggesting differences in substrate specificity for these different metalloproteases. Importantly, these results also conclusively show that the VtpA alone can kill oyster larvae. The importance of proteases, in particular metalloproteases, in the pathogenicity of fish and shellfish diseases caused by marine bacteria has been widely reported (Nottage & Birkbeck 1987, Norqvist et al. 1990, Thompson et al. 2005, Labreuche et al. 2006a, Le Roux et al. 2007, Binesse et al. 2008, Hasegawa et al. 2008, Valiente et al. 2008).

Clearly, bacterial proteases produced by various marine *Vibrio* species can play important roles in shellfish disease. However, it is not clear how specific these enzymes are for their respective targets found on different shellfish. To compare the relative toxicity of the *V. tubiashii* protease to those produced by other *Vibrio* species, the doses of protease required to kill 50 and 20% of oyster larvae, respectively, were determined for *V. tubiashii* and *V. cholerae* supernatants. In addition, purified VtpA protein and thermolysin were compared. Overall, no major difference was observed for the 2 *Vibrio* species proteases, whereas thermolysin required a much higher dose to produce equivalent larval mortality. We conclude that the majority of the toxicity observed in culture supernatants of *V. tubiashii* can be attributed to the direct activity of VtpA on the larvae. Moreover, it appears that, at least for *V. cholerae*, other metalloproteases are just as efficient at killing larvae as the *V. tubiashii* metalloprotease.

It has been reported that sensitivity of different stages of shellfish development to vibriosis varies, with larval and juvenile stages being more susceptible than adult oysters (Elston & Leibovitz 1980, Gómez-León et al. 2008). Consistent with these reports, our results showed that resistance to VtpA significantly increased with oyster age. Our studies further revealed that the previously known age-dependent sensitivity to vibriosis may in fact be due to the activities of the metalloprotease. Moreover, these differences indicate that the putative targets degraded by the metalloprotease are modified or differentially expressed in oysters as they develop with ages. Additionally, differences in larval sensitivity might be due to changes in the accessibility to the protease targets, or due to active production by oyster larvae of molecules counteracting or inhibiting protease activity. For future study, we plan to utilize our purified VtpA to determine the exact nature of the oyster proteins affected by the metalloprotease.

In this report, we conclusively showed, for the first time, that the *Vibrio tubiashii* metalloprotease itself is directly toxic to oyster larvae. We also observed that a variety of *Vibrio* species produce related metalloproteases that can potentially kill oyster larvae. These highly similar proteases appeared to be equally efficient at inducing larval toxicity. However, not all metalloproteases are toxic, with the *Pseudomonas aeruginosa* elastase and *Bacillus thermoproteolyticus* thermolysin being virtually non-toxic. Age of the oyster larvae affected their susceptibility to the toxic metalloprotease, with older larvae being more resistant. Further studies will be required to fully understand the underlying mechanisms of oyster toxicity produced by various metalloproteases.

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