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MORPHOLOGICAL AND MOLECULAR EFFECTS OF *VIBRIO SPLENDIDUS* ON HEMOCYTES OF SOFTSHELL CLAMS, *MYA ARENARIA*

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ABSTRACT Hemocytes constitute the cellular part of the mollusc immune system and are involved in phagocytosis; the production of toxic oxygen radicals, antimicrobial peptides, opsonizing molecules, and lysozymes; digestion, excretion, and nutrient transport. In this study, we investigated the phenotypic response, phagocytosis, and respiratory burst activity in hemocytes of *Mya arenaria* exposed to the bacterium *Vibrio splendidus*. Exposure to *V. splendidus* led to a loss of pseudopodia and rounding of hemocytes. The phagocytic ability of hemocytes was significantly reduced in challenged hemocytes, as was the respiratory burst activity of hemocytes. The expression of actin and elongation factor 2 genes was measured to investigate a possible relation between phenotypic response of hemocytes exposed to *V. splendidus* and genes associated with cytoskeleton. Both actin and elongation factor 2 genes were upregulated in challenged hemocytes. Additional studies are underway to identify other genes of hemocytes whose expression is affected by exposure to *V. splendidus*.

KEY WORDS: hemocyte, gene expression, *Vibrio splendidus*, *Mya arenaria*, immune assay, green fluorescent protein, softshell clam

INTRODUCTION

The softshell clam *Mya arenaria* is a commercially important species of bivalve in the Maritime Provinces of Canada (DFO 2001) and Maine (Beal 2002) for its crucial economic contribution to livelihoods of the coastal communities. Clams live buried in the sediment, and their filter-feeding behavior and close association with the sediment expose them to various aquatic micro-organisms, including *Vibrio* species. Tubiash (1971) suggested that softshell clams are convenient laboratory animals that can be used to screen potential mollusc pathogens. Although there is no report that *V. splendidus* infects softshell clams, it is known to cause disease in other bivalve species such as oysters, *Crassostrea gigas* (Lacoste et al. 2001, Waechter et al. 2002); and scallops, *Pecten maximus* (Lambert et al. 1998, Lambert et al. 1999). Recently, *V. splendidus* has been increasingly used in various mollusc–bacteria challenge models (Lacoste et al. 2001, Waechter et al. 2002, Choquet et al. 2003, Allam & Ford 2006, Gonzalez et al. 2007, Parisi et al. 2008), providing valuable information on *in vitro* and *in vivo* interactions between bivalve hemocytes and *V. splendidus*. This is supported by significant efforts conducted to explore the *V. splendidus* genome and virulence mechanisms (Le Roux et al. 2007). The softshell clam offers many advantages in these host–pathogen models of interaction. It is a common species, it is easy to maintain under laboratory conditions, it offers an easy access for hemolymph collection, and it yields large quantities of hemolymph and hemocytes.

In vitro and *in vivo* interactions between bacteria and hemocytes have been investigated in different bivalve species: mussels, *Mytilus edulis* (Hernroth 2003, Mayrand et al. 2005); scallops, *Pecten maximus* (Mortensen & Glette 1996); oysters, *C. gigas* and *C. virginica* (Allam & Ford 2006, Labreuche et al. 2006a); and clam species, *Ruditapes philippinarum* and *Merccenaria mercenaria* (Allam & Ford 2006). Hemocytes placed on

flat surfaces tend to develop cytoplasmic extensions and adhere to surfaces (Labreuche et al. 2006b). However, when hemocytes associate with pathogenic bacteria (Labreuche et al. 2006b) or their extracellular products (Lane & Birkbeck 1999), they lose their pseudopodia and become round. Consequently their adhesion and phagocytic capability are reduced (Labreuche et al. 2006a). *In vitro* challenge of hemocytes from *R. philippinarum*, *M. mercenaria* and *C. virginica* with 3 different bacteria (*V. tapetis*, *V. splendidus*, and *V. anguillarum*) suggested that pathogenicity is host specific (Allam & Ford 2006).

Molluscs rely on innate immunity, which constitutes both cellular (hemocytes) and humoral components (see the review by Canesi et al. [2002]). Among other functions, hemocytes are involved in phagocytosis (Brousseau et al. 2000, Goedken & De Guise 2004, Allam & Ford 2006), production of reactive oxygen/nitrogen species (Buggé et al. 2007, Lambert et al. 2007a), and antimicrobial agents (Mitta et al. 2000, Cellura et al. 2007, Gonzalez et al. 2007). *In vitro* studies showed that mollusc hemocytes subjected to pathogens (Goedken & De Guise 2004, Allam & Ford 2006, Labreuche et al. 2006a) and high concentrations of heavy metals (Brousseau et al. 2000) have reduced phagocytosis. Lambert et al. (2007b) also reported inhibition of reactive oxygen species production in *C. gigas* subjected to *Vibrio* sp. (strain S322).

The immune system of molluscs is very complex and involves gene networks of different pathways (Huvet et al. 2004, Gestal et al. 2007). Some immune-related genes, which play an important role in pathogen recognition and destruction, have been identified in mussels (Mitta et al. 2000), oysters (Montagnani et al. 2001, Huvet et al. 2004, Gonzalez et al. 2007), scallops (Qiu et al. 2007), clams (Gestal et al. 2007), and abalone (Wang et al. 2008) subjected to bacteria and their components (such as lipopolysaccharide). In turn, bacteria have developed strategies to escape host defense mechanisms (Canesi et al. 2002). For instance, *V. aestuarianus* 01/32 and *V. anguillarum* A7 disturb cell cytoskeleton stability and cause hemocyte rounding in *C. gigas* and *M. edulis*, respectively, which hamper hemocytes

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adhesion (Lane & Birkbeck 1999) and phagocytosis (Labreuche et al. 2006a).

Unlike other mollusc species, the softshell clam is not known to be particularly affected by any major bacterial disease, both in the wild and under controlled conditions. Hence, understanding how immune mechanisms in the softshell clam afford protection from disease could be useful in unraveling bacterial disease processes in other mollusc species. The overall goal of our research is to describe hemocyte response to bacterial challenge. In this study, we examine the morphological, functional (phagocytosis and respiratory burst), and molecular responses of softshell clam hemocytes exposed to *V. splendidus* LGP32–green fluorescent protein (GFP).

MATERIALS AND METHODS

Experimental Animals and Bacteria

Healthy softshell clams, *Mya arenaria*, were collected from a pristine area (Magdalen Islands, Quebec, Canada) and acclimatized for several days in a recirculation system at the Atlantic Veterinary College (Prince Edward Island, Canada). Clams were maintained in a 300-L recirculation tank at a temperature of 16–17°C and a salinity of 30 ppt, and were fed an algae paste every other day. A total of 55 softshell clams (30 individuals in gene expression, 10 in challenge test for phenotypic observation, 15 in phagocytosis and respiratory burst assays) were used in this study.

V. splendidus strain LGP32 tagged with GFP was cultured in 2% NaCl tryptic soy broth for 12 h, harvested by centrifugation (5000 g, 4°C, 10 min), and washed with sterile seawater. Bacteria concentration was estimated using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA) and a spectrophotometer (UNICO spectrophotometer; Montreal Biotech, Inc.).

Hemolymph Collection

Hemolymph (0.3–2.0 mL from each individual) was collected from the adductor muscle of softshell clams using 3-mL syringes fitted to 25-gauge needles. The hemolymph quality of individual clams was checked using a phase contrast microscope (Axio Imager; ZEISS, Germany) at 400× magnification. It was passed through an 80-µm mesh to eliminate particles such as aggregated hemocytes, gametes, and other debris. In the different assays, hemolymph was pooled into one sterilized 30-mL glass tube held on ice.

Hemocyte–Vibrio splendidus Challenge

From the pool of hemolymph, 0.5 mL hemolymph (approximately 10⁶ hemocytes/mL) was allocated to separate test tubes labeled as controls (treated with filtered sterile seawater) or challenged (exposed to *V. splendidus*). Each treatment group was represented in triplicate. Hemocytes were challenged with 4 different ratios of hemocyte to bacteria (1:20, 1:10, 1:5, and 1:1). Hemocyte–*Vibrio* interaction was terminated by putting tubes in ice. Before transferring to slides for microscopic observation, samples were homogenized by vigorous pipetting to ensure that adherent hemocytes were resuspended. The morphological appearance of hemocytes was observed under phase contrast microscope (400×) 1, 2, and 3 h after the challenge. The percentage of rounded hemocytes (without pseudopodia) was

estimated by counting 6 different fields in each slide (each field contained from 10–35 hemocytes). Hemocytes subjected to *V. splendidus* for 2 h at a 1:1 ratio were used in the subsequent functional and gene expression assays. Hemocytes challenged in a 1:1 hemocyte-to-bacteria ratio for 3 h were also included in gene expression.

Phagocytosis Assay

The phagocytosis assay was adopted from Allam and Ford (2006) with some modifications. Briefly, 170 µL *V. splendidus* suspension (approximately 10⁶ cells/mL) was placed into 5-mL flow cytometry tubes, and an equal volume of hemolymph (to create a 1:1 ration of *Vibrio* to hemocyte) was added. The same volume of filtered seawater was used instead of *V. splendidus* suspension for the unchallenged (control) group, and each experimental condition had 6 replicates. Two hours after the challenge, 60 µL diluted suspension of phycoerythrin labeled (red) beads (2 µm in diameter; Polysciences Inc., Warrington, PA) was added to hemocytes in both the control and challenged samples to create a 40:1 bead-to-hemocyte ratio, and was incubated in the dark for an additional 2 h at room temperature on a gentle shaker. The internalization of the beads was terminated by placing the test tubes on ice. A FACSCalibur flow cytometer was used to estimate the percentage of hemocytes internalizing 2 or more beads by measuring red fluorescence (FL-2), which corresponds to the number of beads associated with hemocytes. Moreover, one group of hemocytes was treated with cytochalasin B (10 µg/mL final concentration; Sigma), an inhibitor of phagocytosis. Fluorescence (FL-2) in this group is a result of beads adhered to the hemocyte surface and not internalized. Fluorescence obtained in cytochalasin B-treated hemocytes was subtracted from that of *Vibrio*-challenged and control groups to exclude beads on hemocytes surfaces. Calcein AM (2.5 µM final concentration; Invitrogen, Carlsbad, CA) was added to hemocytes and incubated for 30 min at room temperature. Stained hemocytes were washed using filtered seawater and were observed using a laser scanning confocal microscope (LSM 510 META; ZEISS, Germany) to verify the internalization of beads. In this study we defined phagocytosis as the internalization of 2 or more beads, because laser scanning confocal microscope observation showed that hemocytes treated with cytochalasin B were able to internalize 1 bead.

Respiratory Burst

The respiratory burst assay was adapted from Goedken and De Guise (2004), and each experimental condition had 12 replicates. Briefly, equal volumes (197 µL each) of hemolymph and *V. splendidus* suspension were placed into 5-mL flow cytometry tubes (Falcon; BD Biosciences, San Jose, CA) in an experimental group, and filtered sterile seawater (FSSW) was used in controls. Hemocytes treated with phorbol 12-myristate 13 acetate (PMA; 10 µg/mL; Sigma, St. Louis, MO) served as a positive control. Another group of hemocytes was exposed to *V. splendidus* and PMA simultaneously. Nonfluorescent 2',7'-dichlorofluorescein diacetate (DCFH-DA; 10 µM; Sigma) was added to the samples. DCFH-DA diffuses into the cells and is oxidized by hydrogen peroxide or nitric oxide to DCF, which gives green fluorescence. The samples were incubated in the dark for 2 h at room temperature, and green fluorescence (FL-1) was measured using flow cytometry. *V. splendidus* LGP32-GFP

is tagged with GFP, which could contribute to the fluorescence measured in *Vibrio*-challenged hemocytes. Hence, respiratory burst activity of challenged hemocytes was measured as a difference in fluorescence between *Vibrio*-challenged hemocytes treated with DCFH-DA and *Vibrio*-challenged hemocytes without DCFH-DA.

Gene Expression

Total RNA was extracted from challenged hemocytes and their respective controls (each condition with 6 replicates) using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol and were treated with DNase-1 (Sigma). Total RNA concentration and integrity were analyzed using the Bioanalyzer (Agilent Technologies, Santa Clara, CA). First-strand cDNA was synthesized following the protocol of the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen) and real-time quantitative polymerase chain reaction (PCR) was performed. Briefly, total RNA in a 20- μ L final volume was reverse transcribed, and SYBR Green I (Invitrogen) technology-based real-time PCR (final volume 25 μ L) was performed using the Rotor Gene RG-3000 (Corbett Research, Australia). The PCR conditions for all genes were 10 min at 95°C followed by 40 cycles (95°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec) of amplification. The primers used to amplify the target (actin and elongation factor [EF] 2) and housekeeping genes (EF-1, S-18, and ubiquitin), and their respective efficiencies are listed in Table 1. Gel pictures (single band of expected size) and melting curves were examined to verify that a set of primers were amplifying only the gene of interest.

The relative expression of genes (actin and EF-2), which are associated with cytoskeleton filament stability, were quantified. In our previous study, reference genes that are suitable for this *in vitro* challenge model were selected (Araya et al. 2008). The

housekeeping genes were used to normalize the expression of the target genes at 2 h and 3 h postchallenge following the instructions in the geNorm manual (Vandesompele et al. 2002).

Data Analysis

Phagocytosis, respiratory burst, and relative gene expression data were analyzed using Student's *t*-test and 1-way analysis of variance (ANOVA; SigmaStat statistical software, version 2) to determine the significance between the different experimental conditions in each assay.

RESULTS

Morphological Response

The morphological appearance of both challenged and unchallenged hemocytes was observed microscopically at 1, 2, and 3 h postchallenge. These morphological data were taken as a marker to select the optimal hemocyte-to-*Vibrio* ratio in the subsequent studies. We have observed that hemocytes subjected to *V. splendidus* lose their pseudopodia and become rounded. Especially at high *V. splendidus*-to-hemocyte ratios, the percentage of round hemocytes increased rapidly. For instance at 1:20, 1:10, and 1:5 hemocyte-to-*Vibrio* ratios, the percentage of round hemocytes rose rapidly from 8–20% in 1 h to more than 80% and 100% in 2 h and 3 h postchallenge, respectively (Table 2). The percentages of round hemocytes in 1:1 were 4.2%, 44.3%, and 92% at 1, 2 and 3 h, respectively. Consequently, the gradual change of morphology at a 1:1 ratio is more suitable for our study compared with the others ratios. The hemocytes in the control group maintained their pseudopodia and appeared to be attached to the glass slides. The percentage of round hemocytes in the control group was significantly low (<10% after 3 h).

TABLE 1.

List of primers used to amplify target genes (actin and EF-2) and housekeeping genes (EF-1, S-18, and ubiquitin), their expected amplicon length, and respective PCR efficiency.

Gene Type	Gene Name	Primers	Sequence (5'-3')	Tm	Amplicon (pb)	Efficiency (%)	
2-H Challenge	Target genes	Forward	GCGAAAATACTCCGTCTGGA	60	179	100	
		Reverse	GCAGGTACGATCACAAGCAA				
		Forward	CTACAAGCCTGGCTCAAAGG	60	218	100	
		Reverse	TGACAAGTGGGCTGACAGAG				
	Reference genes	Elongation factor 1	Forward	GGTGGCTGTTGGTGTTCATC	60	158	100
			Reverse	GGCCTAGGTGTTTTCCATGA			
	Ubiquitin	Forward	TCGCTAAGGAGCTGGACATT	60	194	95	
		Reverse	ACCGTCGCTCCTTGTACATC				
	Ribosomal protein S-18	Forward	AAGATTCCCGACTGGTTCCT	60	189	90	
3-H Challenge	Target genes	Forward	GCGAAAATACTCCGTCTGGA	60	179	101	
		Reverse	GCAGGTACGATCACAAGCAA				
	Reference genes	Elongation Factor 1	Forward	GGTGGCTGTTGGTGTTCATC	60	158	104
			Reverse	GGCCTAGGTGTTTTCCATGA			
		Ubiquitin	Forward	TCGCTAAGGAGCTGGACATT	60	194	101
			Reverse	ACCGTCGCTCCTTGTACATC			
		Ribosomal protein S-18	Forward	AAGATTCCCGACTGGTTCCT	60	189	99
			Reverse	GCCGGTTGTCTTTGTATGCT			

PCR samples were obtained from hemocytes challenged with *V. splendidus* LGP32-GFP for 2 h and 3 h.

TABLE 2.
Percentage of rounded hemocytes of softshell clams
challenged at different hemocyte-*V. splendidus* ratios
for 1, 2, and 3 h (mean \pm SEM).

No. of hours	Hemocyte-to- <i>V. splendidus</i> Ratio				
	1:20	1:10	1:5	1:1	Control
1	20.76 \pm 2.8	8.47 \pm 2.1	8.89 \pm 2.6	4.17 \pm 1.3	0 \pm 0
2	94.41 \pm 4.9	86.11 \pm 12.7	80.37 \pm 2.8	44.29 \pm 5.2	2.38 \pm 4.1
3	100 \pm 0	100 \pm 0	100 \pm 0	92 \pm 1.2	9.09 \pm 2.4

In the current study, a 1:1 hemocyte-to-*Vibrio* ratio was chosen as an optimal ratio for the *in vitro* challenge model because, unlike the higher ratios, at 1:1 the percentage of round hemocytes increased gradually. Within the 1:1 ratio, hemocytes subjected to *V. splendidus* for 2 h (44.3% round cells) and 3 h were chosen mainly for gene expression purposes. However, phagocytosis and respiratory burst activity were carried out in hemocytes exposed to *V. Splendidus* for 2 h to link gene expression with immune functional assays.

Phagocytosis Assay

The effect of *V. splendidus* on hemocyte ability to phagocytose was investigated by providing fluorescent beads to hemocytes challenged with *V. splendidus* for 2 h at the 1:1 ratio. Multiple beads were observed internalized within unchallenged and challenged (exposed to *V. splendidus*) hemocytes. Although determining whether *M. arenaria* hemocytes could phagocytose *V. splendidus* was not the aim of this study, *Vibrio* were also observed inside hemocytes (Mateo et al. 2009) and appeared to be motile.

The percentage of hemocyte phagocytosing beads in the unchallenged groups was significantly higher than in hemocytes subjected to *V. splendidus* and cytochalasin B (Fig. 1). Moreover, the percentage of phagocytosing hemocytes in *V. splendidus*-treated groups was also significantly high compared with hemocytes subjected to cytochalasin B. After data were nor-

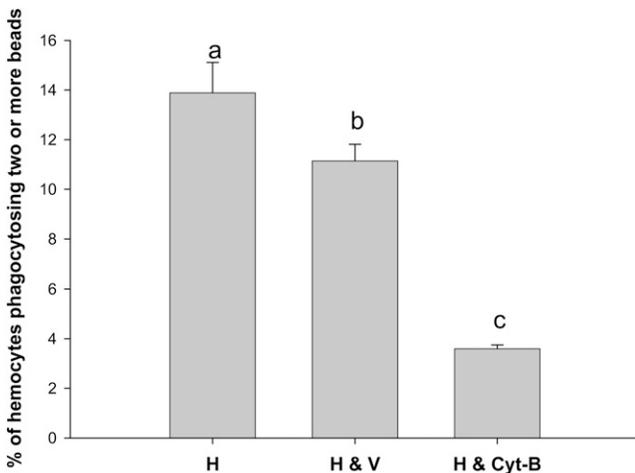


Figure 1. Effect of *V. splendidus* on phagocytosis of phycoerythrin-labeled (red) beads by hemocytes of softshell clam (mean \pm SEM). Letters indicate significant difference between treatments (ANOVA, $P < 0.001$; $n = 5$). Cyt-B, cytochalasin B; H, hemocyte; V, *V. splendidus*.

malized to the percentage of phagocytosing hemocytes exposed to cytochalasin B, the percentage of hemocytes internalizing 2 or more beads in challenged and unchallenged hemocytes was 8.5% and 11%, respectively.

Respiratory Burst Assay

Hemocytes treated with PMA, a chemical that stimulates respiratory burst activity and was used as a positive control, showed significantly higher respiratory burst activity compared with other treatment groups (Fig. 2), demonstrating that softshell clam hemocytes are able to produce toxic radicals. However, when hemocytes were exposed to *V. splendidus* alone, they generated the same level of response seen in negative controls (unchallenged hemocytes). When hemocytes were subjected to *V. splendidus* and PMA simultaneously, respiratory burst activity remained at the level of negative control and *Vibrio*-only challenged hemocytes.

Gene Expression

The quality/integrity of total RNA used to synthesize cDNA for gene expression was determined using a Bioanalyzer. Quantitative real-time PCR was performed for actin and EF-2 genes, which are actively involved in cytoskeleton filament stability. Their relative expression was calculated by normalizing the data to 3 stable housekeeping genes (EF-1, S-18, and ubiquitin). Actin and EF-2 genes were expressed significantly higher in *V. splendidus*-challenged hemocytes than in unchallenged groups (Fig. 3), being upregulated 3.5- and 2-fold, respectively. The relative expression of actin was also significantly upregulated in hemocytes exposed to *Vibrio* for 3 h (Fig. 4). However, hemocytes challenged for 3 h had significantly reduced actin expression compared with hemocytes challenged for 2 h.

DISCUSSION

Aquatic bacteria, including the genus *Vibrio*, are able to persist within bivalve tissues and fluids (Murphee & Tamplin 1995). These opportunistic *Vibrio* species can become pathogenic whenever the host weakens and its immune system is depressed.

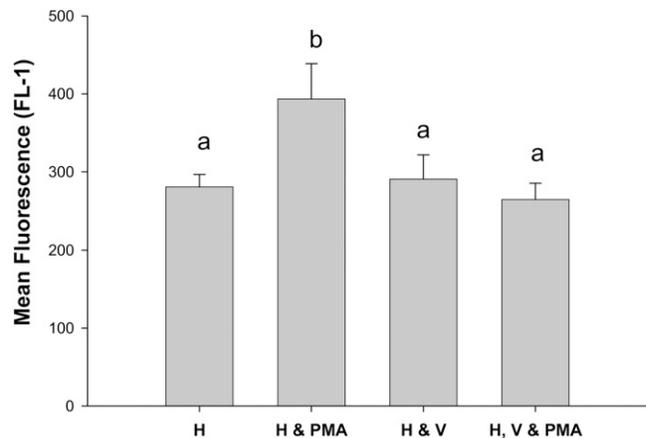


Figure 2. Respiratory burst activity in hemocytes incubated with filtered seawater (control), *V. splendidus*, PMA and *V. splendidus*, and PMA (mean \pm SEM). Letters indicate significant difference between treatments (ANOVA, $P < 0.001$; $n = 12$). H, hemocyte; PMA, phorbol 12-myristate 13 acetate; V, *V. splendidus*.

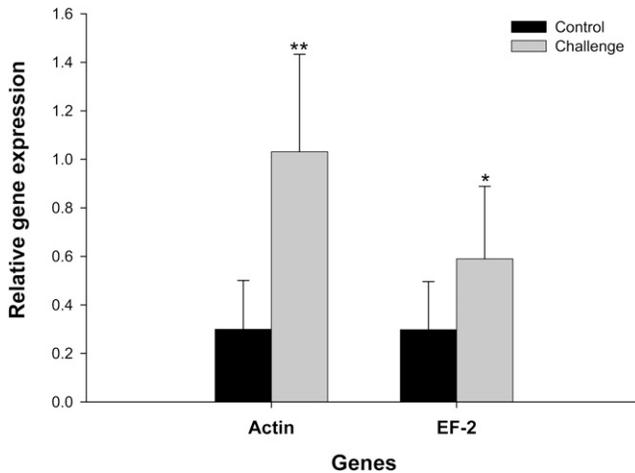


Figure 3. Relative expression of actin and EF-2 genes in control and challenged hemocytes (mean \pm SEM) (*t*-test, * $P < 0.05$; ** $P < 0.001$, $n = 6$).

The high mortality rates recently reported in juvenile Pacific oysters, *C. gigas* (Lacoste et al. 2001, Waechter et al. 2002); and adult scallops, *Pecten maximus* (Lambert et al. 1999) could be related to such a process. Hence, during the past decade several studies focusing on the interaction between mollusc hemocytes and *V. splendidus* have been performed to elucidate mollusc immune defense system against *V. splendidus* (Choquet et al. 2003, Allam & Ford 2006, Parisi et al. 2008). Here we report our results on the interaction between hemocytes and the *V. splendidus* strain LGP32-GFP. *V. splendidus* does not occur as a natural pathogen to softshell clams. Nevertheless, our study has shown that this strain has the ability to impair some of the normal functions of hemocytes, including loss of pseudopodia and reduced phagocytosis and respiratory burst activity.

Hemocytes from softshell clams were subjected to *V. splendidus* LGP32-GFP at different ratios for 1, 2, and 3 h. When compared with a ratio of 1:1, hemocytes challenged at high

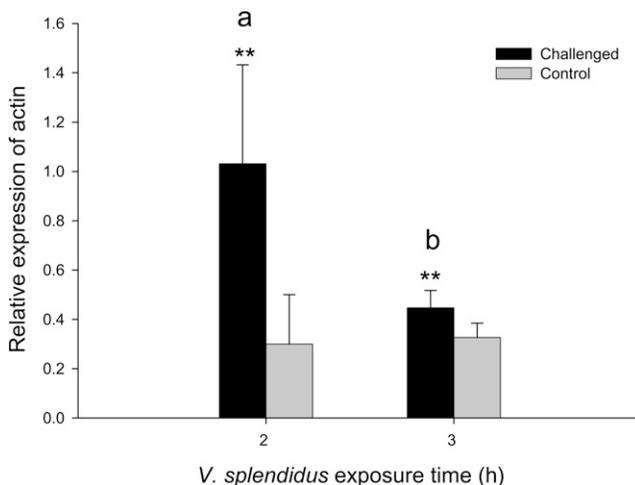


Figure 4. Comparison of actin expression in hemocytes of *M. arenaria* challenged with *V. splendidus* LGP32-GFP for 2 and 3 h (mean \pm SEM). The letters indicate a significant difference ($P < 0.05$) of actin expression between the challenged group at 2 and 3 h whereas the asterisk indicates significance between the control and challenged groups at each time (*t*-test, $P < 0.001$, $n = 6$).

ratios (1:20, 1:10, and 1:5 hemocyte-to-*Vibrio* ratio) rapidly lost their pseudopodia and had a high percentage of rounded cells. However, exposing hemocytes at a 1:1 ratio for 3 h also produced a high percentage (92%) of round hemocytes. Similar to our findings, a high percentage of rounded hemocytes was also reported in bivalve hemocytes exposed to *V. anguillarum* A7 (Lane & Birkbeck 1999). In human cells, *V. cholerae* toxin (MARTX_{vc}) covalently binds to G-actin and F-actin, and causes actin depolymerization, which leads to cell rounding (Kudryashov et al. 2008). Recently, a metalloprotease gene, *vsm*, an important cytotoxicity factor from the extracellular product of *V. splendidus* LGP32 was identified (Le Roux et al. 2007). This toxin was shown to cause rounding of mollusc cell lines (Le Roux et al. 2007) and may be responsible for the loss of pseudopodia (rounding) in *M. arenaria*. Although hemocyte rounding is well documented as a cellular response to bacterial challenge, the factors responsible for this response are still unknown.

Phagocytosis is a major hemocyte function in molluscs (Allam et al. 2001, Labreuche et al. 2006a), including softshell clams (Brousseau et al. 2000). In the current study, we showed that hemocytes are able to internalize beads, supporting results by Brousseau et al. (2000). However, *Vibrio*-challenged hemocytes showed lower capacity to phagocytose beads. The reduced phagocytosis in challenged hemocytes could be the result of the loss of pseudopodia resulting from the toxic effect of extracellular product (*vsm*) produced by *V. splendidus*. This cytotoxic extracellular product causes loss of pseudopodia (Le Roux et al. 2007) which is very important during phagocytosis of pathogens and other nonself cells (Labreuche et al. 2006a). *V. tapetis* also caused hemocyte rounding in the Japanese carpet clam *R. philippinarum*, which leads to decreased phagocytosis (Allam & Ford 2006). Loss of phagocytosis was also reported in round hemocytes induced by disseminated neoplasia in *M. arenaria* (Beckmann et al. 1992) and *M. edulis* (Kent et al. 1989).

The respiratory burst activity of softshell clam hemocytes was also investigated. We observed that respiratory burst activity of hemocytes treated with PMA was significantly higher than in control and *V. splendidus*-challenged groups. This suggests that either *V. splendidus* lacks a receptor that triggers respiratory burst activity or it actively suppresses the hemocyte response. When one group of hemocytes was treated with PMA and *V. splendidus* simultaneously, the respiratory burst in this group remained significantly lower compared with the PMA-treated group. This suggests that the low respiratory burst response of hemocytes challenged with *V. splendidus* is not the result of the lack of molecules on the surface of *V. splendidus* (receptors), which might activate a hemocyte response. Instead, it appears that *V. splendidus* could have the capability to inhibit respiratory burst activity in hemocytes of softshell clams. Bramble and Anderson (1998) reported the inhibition of toxic radicals in *C. virginica* by the antioxidase activity of another Vibrionaceae, *V. (Listonella) anguillarum*. Canesi et al. (2002) reviewed the different mechanisms involved in pathogens escaping from the host immune system. These include destabilizing the structural integrity of the cytoskeleton by producing toxins, forming capsules to prevent opsonization and to resist degradation, lacking receptors (which triggers the host immune system), lacking substrate susceptible to lysosomes, and producing antioxidant compounds (which inhibit respiratory burst activity of the host). In addition to phagocytosis and respiratory burst activity, mollusc hemocytes are also involved in the

production of antimicrobial peptides (Mitta et al. 2000), lysozymes (Labreuche et al. 2006a), inflammation, wound repair, and encapsulation (Pipe 1990).

There are 2 main pathways in respiratory burst (Dröge 2002). The NADPH-oxidase and NO-synthase pathways, which lead to the production of reactive oxygen species (H_2O_2 , O_2^-) and nitrogen reactive species (NO, ONO_2^-), respectively. In the current study, we have not been able to determine whether the response was from one pathway or a combination of both, because the probe (DCFH-DA) can detect both NO and H_2O_2 . Hence, further investigation is needed to determine the responsible pathway in hemocytes of softshell clams. In *C. gigas*, both pathways have been reported but the NADPH-oxidase and NO-synthase pathways were dominant in granulocytes and hyalinocytes, respectively (Lambert et al. 2007a). Molluscs without respiratory burst activity have also been reported (Lopez et al. 1994, Anderson et al. 2003). However, the low respiratory burst activity reported in those molluscs may have resulted from using inappropriate probes. In mollusc studies, only DCFH probe is used to detect the respiratory burst activity. Walrand et al. (2003) demonstrated that reactive oxygen species/reactive nitrogen species measurement in polymorphonuclear neutrophils depends on the selected probe, and recommended the use of dihydrorhodamine 123, DCFH, and hydroethidine to detect H_2O_2 , NO, and O_2^- production, respectively.

To link the morphological modifications of hemocytes to gene expression, relative expression levels of actin and EF-2 transcripts were quantified in hemocytes exposed to *V. splendidus* (1:1 ratio) for 2 and 3 h. Both genes are associated with cytoskeleton filament stability (Polard & Cooper 1986, Bektas et al. 2004). Actin is an abundant cellular protein that is involved in cell division, locomotion, cytoskeleton structure formation, and organelle reorganization (Walker & Garrill 2006). In addition, actin plays an important role in phagocytosis, encapsulation, and nodule formation (Takai et al. 2001), and may facilitate the clearance of microbes from the host (He et al. 2004). On the other hand, EF-2 is mainly involved in the elongation phase of protein synthesis in eukaryotes (Riis et al. 1990). EF-2 is also an actin binding protein, which plays a key role in the regulation of intracellular actin level (Bektas et al. 1994). Bektas et al. (2004) reported that cytoskeleton disruption leads to reduced protein synthesis, suggesting a link between actin and EF-2. Our results showed upregulation of actin and EF-2 2 h postchallenge. Two hours after the *Vibrio* challenge, nearly half (44.3%) of the hemocytes were round; however, the rest maintained pseudopodia. Hence, to determine which of the hemocytes (the ones that still maintain pseudopodia or the ones that lost it) contributed to the increased transcript level, the expression of actin was measured in hemocytes exposed for 3 h, in which almost all (92%) became round. Actin expression at 3 h postchallenge was significantly upregulated. However, it was

significantly reduced compared with the hemocytes challenged for 2 h. This suggests that hemocytes maintaining their pseudopodia after challenge contribute to the expression of actin. Actively phagocytosing hemocytes need to maintain their pseudopodia to adhere and internalize particles (Lane & Birkbeck 1999; Labreuche et al. 2006a). Similarly, an upregulation of actin and EF-2 genes was observed in carpet-shell clams, *R. decussatus*, challenged by a mixture of dead bacteria including *V. splendidus* (Gestal et al. 2007). The upregulation of these 2 genes in challenged cells could suggest that hemocytes were trying to maintain the cytoskeleton structural integrity, which plays a key role in phagocytosis. In eukaryotes, actin is involved in T-cell activation and plays a very crucial role in immune signaling cascades, such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) (see review by Kustermans et al. [2008]). Because those pathways are conserved between vertebrates and molluscs, the upregulation of actin in softshell clam hemocytes could also have a similar role, which is to trigger the immune signaling cascades to fight *V. splendidus* infection. MAPK and NF- κ B pathways are also reported in the sea mussel *Mytilus galloprovincialis* (Betti et al. 2006) and the hydrothermal vent mussel *Bathymodiolus azoricus* (Bettencourt et al. 2007), respectively.

CONCLUSION

When hemocytes were challenged with *V. splendidus* strain LGP32-GFP, the percentage of round cells (without pseudopodia) increased significantly with time of exposure and concentration of bacteria. Hemocytes exposed to *V. splendidus* at a ratio of 1:1 for 2 h lost their pseudopodia and showed reduced phagocytosis and respiratory burst activity. During this challenge, actin and EF-2 (2 cytoskeleton-associated genes) were upregulated, which could be related to the morphological changes observed. Further studies are needed to explore this mechanism and to unravel molecular pathways possibly involved in softshell clam hemocyte response to bacterial challenge.

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