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Differential *in vivo* response of soft-shell clam hemocytes against two strains of *Vibrio splendidus*: Changes in cell structure, numbers and adherence

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ABSTRACT

Host–pathogen interaction models in aquatic species are useful tools for understanding the pathogenicity of diseases in cultured and wild populations. In this study we report the differential *in vivo* response of soft-shell clam (*Mya arenaria*) hemocytes against two strains of *Vibrio splendidus*. Responses were measured 24 h after injecting into the posterior adductor muscle either an endemic wild-type strain (7SHRW) or a strain associated with oyster mortalities (LGP32-GFP). Changes in hemocyte structure (percentage of rounded cells) were assessed microscopically. Changes in adherence and hemocyte numbers were analyzed by flow-cytometric cell counting. Increased percentages of rounded cells were found in response to both strains. However, values from the group infected with LGP32-GFP were significantly higher ($p < 0.01$) than with 7SHRW. The cell adherence was markedly diminished ($p < 0.001$) by LGP32-GFP whereas 7SHRW did not change it significantly. Increased numbers of hemocytes ($p < 0.001$) were induced by LGP32-GFP, while no significant changes were found after infection with 7SHRW. These results show the regulatory capacity of soft-shell clams hemocytes to perform specific responses against different strains of *V. splendidus*.

1. Introduction

Host–pathogen interaction models are gaining more attention in aquatic species since they are useful tools for understanding the pathogenicity of diseases in cultured and wild populations. In bivalve molluscs, hemocytes play a major role in the host defence as mediators of cellular and, indirectly, of humoral defences. Hence, host–pathogen interaction studies have focused their attention on hemocytic responses against pathogens.

Common responses attributed to bacterial challenge are changes in (total and differential) hemocyte numbers, viability, structure, adherence, phagocytosis, production of reactive oxygen intermediates (ROIs), and enzymatic activities (Nottage and Birkbeck, 1990; Allam et al., 2001, 2006; Choquet et al., 2003; Lambert et al., 2003; Allam and Ford, 2006; Labreuche et al., 2006a,b; Parisi et al., 2008). Although the innate immune systems of bivalves are known to be less complex and specific than the adaptive systems of vertebrates their hemocytes have the capacity to discriminate microorganisms (Bachère et al., 2004). Thus, several of these responses have been shown to be specific to certain bacterial species or strains. In soft-shell clams, however, there is no information on hemocytic responses induced by bacterial infections.

Mya arenaria is an important resource for coastal communities of North-Eastern North America with potential for aquaculture (Chevarie et al., 2005). This species has also been deemed to be a convenient bivalve for screening bacterial pathogenicity (Tubiash, 1971). Although bacterial outbreaks are rare in soft-shell clams (Kaneko et al., 1975) their vulnerability to hemic neoplasia, a common disorder that considerably affects some populations in Atlantic Canada (McGladdery et al., 2001) and the US (Farley et al., 1991), might render them susceptible to opportunistic bacteria (Kent et al., 1989). Among the most common opportunistic bacteria are vibrios, normally found in marine and eurohaline environments (Huq and Colwell, 1995). While most *Vibrio* spp. outbreaks are associated with mortalities of bivalve larvae in hatcheries, few species are deemed to be primarily pathogenic to adult or juvenile bivalves (Paillard et al., 2004). Among them, some strains of *Vibrio splendidus* have been associated with the "summer mortalities" syndrome of juvenile oysters *Crassostrea gigas* in France (Lacoste et al., 2001; Waechter et al., 2002).

Given the importance of both, host and pathogen, we are developing a model of interaction between soft-shell clams and two strains of *V. splendidus*. In this study, we report differential changes

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in hemocyte structure, numbers, and adherence induced by a pathogenic and a wild-type strain of *V. splendidus*.

2. Materials and methods

2.1. Experimental animals and conditioning

Wild soft-shell clams of 4–6 cm in size and an average weight of 21.5 ± 3.1 g, shipped from the Iles-de-la-Madeleine (Gulf of Saint Lawrence, Canada), were used in our experiments. This site was chosen due to its minimal level of pollution. The clams sampled have reduced environmental bacterial loads and undetected levels of hemic neoplasia. These factors have been shown to have detrimental effects on hemocytic parameters (Kent et al., 1989; Fournier et al., 2002; Mayrand et al., 2005; Gagnaire et al., 2007). Likewise, wild bacteria might have an agonist/antagonist effect to the experimental bacteria (Gay et al., 2004a,b).

On arrival at our facility, clams were held within 300 L tanks with recirculating artificial sea water (Instant Ocean®). During the acclimation period (at least 1 week before trials were performed) the water temperature was kept at 16 °C and the salinity at 30 ppt. During this period, clams were fed with an algae paste every other day.

2.2. Bacterial strains, growth conditions and suspension preparation

V. splendidus LGP32-GFP has been associated with the occurrence of "summer mortalities" syndrome in juvenile oysters *C. gigas* in France (Gay et al., 2004a,b). To facilitate its traceability this strain has a GFP gene insertion that confers green fluorescence when excited with UV light. *V. splendidus* 7SHRW is a wild strain isolated from sediments from Hillsborough River, Prince Edward Island (Gulf of Saint Lawrence, Canada). This strain was identified as *V. splendidus* based on conventional biochemical tests and BIOLOG automated identification. Both strains, 7SHRW and LGP32-GFP, form green colonies in Thiosulfate Citrate Bile Sucrose agar, produce catalase and oxidase, and cannot utilize citrate, neither can degrade urea and aesculin. They can produce acid from glucose but not from lactose and are able to grow up to 28 °C in blood agar. The strain 7SHRW is hemolytic and sensitive to the vibriostatic agent O/129 while LGP32-GFP is not. Although the strain 7SHRW could be identified as *V. splendidus* based on the utilization of 96 carbon sources, the strain LGP32-GFP could not.

Both strains were cultured overnight to reach exponential growth phase in Trypticase Soy Broth (TSB, BD-Bacto™) supplemented with 2% NaCl at 16 °C in 250 mL Erlenmeyer flasks, shaken at 100 rpm. Bacteria were collected by centrifugation at 5000*g* and washed twice with filtered (0.22 μm) sterile seawater (FSSW). The bacterial concentration was determined by optical density measured with a spectrophotometer (2802 UV/VIS Unico) at 600 nm, and adjusted to approximately 4.8×10^8 bacteria/mL in FSSW according to 1 $OD_{600nm} = 4 \times 10^8$ bacteria/mL as estimated by flow-cytometry cell counting.

2.3. Clam hemocytes pre-screening

Clams were pre-screened through microscopic observation of their hemolymph immediately before experiments. Hemolymph was extracted as described in the next section. A drop of hemolymph from each sample was placed on Snow coat X-tra™ polylysine-coated slides, cover slipped and placed in a humid chamber for 20 min to allow hemocytes to adhere to the glass surface. Samples were analyzed with an Axio Imager A1 (Carl Zeiss) fluorescent light microscope with phase contrast (400×). The numbers of stretched (normal) and rounded hemocytes were counted in a total of five fields per sample. The percentage of rounded hemocytes per sample was estimated by calculating the average of five measurements. Samples whose percentage of rounded hemocytes was higher than 5% on average were excluded from the experiment as we previously established that higher values are related to abnormal levels of stress (data not shown). Likewise, those clams whose hemolymph showed obvious presence of bacteria were excluded to avoid interference.

2.4. Clam infection and hemolymph withdrawal

Approximately 4.5×10^6 bacteria of either strain per gram of clam, contained in 200 μL of bacterial suspension, were injected into the posterior adductor muscle. FSSW was injected into the control group clams. After injection, clams were kept out of the water for 1 h, to assure the bacterial suspension was retained prior to the clams being transferred to containers with non-circulating artificial seawater at 16 °C for 24 h.

Hemolymph was withdrawn from the posterior adductor muscle of each clam with a 3 mL syringe fitted with a 25-gauge needle. For the microscopic assessment of the percentage of rounded hemocytes and the flow-cytometric assessment of the number of hemocytes, hemolymph was collected 4 d before and 24 h after injection. In the former assessment one drop was extracted while in the latter assessment 300 μL was required. For the adhesion assay, 400 μL of hemolymph was withdrawn per clam 24 h after injection. In all cases, hemolymph was screened through an 80 μm mesh to avoid large particles. The hemolymph used in flow-cytometric assessments was immediately placed on ice to prevent formation of aggregates of hemocytes.

2.5. Phase contrast and confocal microscopy

Samples were analyzed as described above for the hemocyte pre-screening. Samples were also examined with a Zeiss 510 Meta laser confocal microscope to monitor internalization of phagocytosed bacteria after infection.

2.6. Adhesion assay

The capacity of the hemocytes to adhere after injection was estimated using a modification of the method described by Choquet et al. (2003). Briefly, a 200 μL aliquot of hemolymph was taken into a control tube and mixed with 200 μL of 6% formalin in FSSW for fixation and flow-cytometric assessment. Another 200 μL sample of hemolymph was taken into a 48-well microplate and incubated at 16 °C for 3 h. After incubation, 200 μL of fixative was added. The supernatant was then analyzed using a FACSCalibur flow cytometer (BD Biosciences). The proportion of adhered and non-adhered cells was estimated in relation to the total number of hemocytes from the control tube.

2.7. Hemocyte counts

Hemocyte counts, before and after injection, were determined by using a 300 μL hemolymph sample read through a FACSCalibur flow cytometer according to gates previously established to discriminate bacteria from other particles. Readings of the number of events were obtained after 30 s under low flow rate.

2.8. Statistical analysis

Statistical analysis was done using the MINITAB 15.1.0.0 statistical software. The differences of the percentages of rounded hemocytes before and after infection were tested by using the non-parametric sign test, and the differences between groups