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Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: I. Haemocyte changes following in vitro challenge

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Abstract

In microbial infections, the interaction between microorganisms and phagocytic cells is a crucial determinant in the outcome of the disease process. We used flow cytometry to study the in vitro interactions between *Vibrio tapetis*, the bacterium responsible for Brown Ring Disease (BRD) in the Manila clam *Ruditapes philippinarum*, and haemocytes from three bivalve species: the Manila clam (susceptible to BRD), the hard clam *Mercenaria mercenaria* and the eastern oyster *Crassostrea virginica* (both non-susceptible to BRD). Results demonstrated that *V. tapetis* cells and extracellular products elicit major changes in the haemocytes of *R. philippinarum*, including decreased viability and phagocytic activity, and altered size and internal structure. *V. tapetis* was able to kill haemocytes from *M. mercenaria* and *C. virginica* but to a far lesser extent than those of *R. philippinarum*. These results suggest that disease resistance is not solely dependent on a host activity against the pathogen, but is also a function and magnitude of the injury to the host cell by a given pathogen.

1. Introduction

Manila clam (*Ruditapes philippinarum*) culture in Western Europe has suffered a serious Vibriosis problem since the late 1980s. The condition, known as Brown Ring Disease (BRD), is caused by *Vibrio tapetis* and can be experimentally induced by injecting pathogen cells in the pallial (mantle) cavity of healthy clams [1]. This bacterium alters the periostracal lamina, an organic matrix secreted by the mantle to support shell growth, causing the deposition of brown organic material on the inner face of the shell. Although the pathogen is

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located mainly on and within the periostracal lamina, the organic deposit, and the mantle fluid, *V. tapetis* has been observed in mantle tissue and within digestive cells of severely infected clams [2,3]. When experimentally injected into the extrapallial space (between the mantle and the shell), the bacteria may penetrate the mantle via the epithelium, subsequently spreading throughout the body to cause severe systemic infection and death [4]. These results have led to a hypothesis that naturally infected clams die only after *V. tapetis* has penetrated from the extrapallial space (where the periostracal lamina and the organic deposit accumulate) into the mantle through lesions in the epithelium, and from there to infiltrate other parts of the body [4].

The establishment of in vitro models has led to major developments in the understanding of pathological processes in vertebrates and invertebrates. These models often use cultured host cell lines, but since no cell lines are available for bivalve molluscs, most in vitro experiments using bivalve species employ primary haemocyte cultures [5–10]. Haemocytes provide an excellent biological material to study host/pathogen interactions because the internal defence system of bivalves relies on these cells. Comparative studies of different host and pathogen pairs with differing resistance and virulence, respectively, provide additional insight into why particular hosts do or do not develop disease when challenged, or why certain microbes do or do not cause disease. For instance, the Manila clam is highly susceptible to BRD when experimentally challenged by *V. tapetis*, and in an earlier study, we demonstrated that haemocytes of this clam could be killed by contact with the pathogen [11]. In contrast, hard clams (*Mercenaria mercenaria*) and eastern oysters (*Crassostrea virginica*) are not susceptible to BRD [12–14]. To help explain this difference, we tested the hypothesis that following contact with *V. tapetis* cells and extracellular products, defence functions of Manila clam haemocytes would be impaired to a degree significantly greater than those of hard clams and eastern oysters. In the present work, we used flow cytometry to measure the in vitro effect of *V. tapetis* and two other bacteria with lower virulence (*V. splendidus* and *Listonella* (=*Vibrio*) *anguillarum*) on the viability and phagocytic activity of haemocytes of the three bivalve species in vitro. Since flow cytometry provides data on the morphology of analysed cells, we also measured changes in light scatter parameters that may reflect physiological disturbances in haemocytes (cell size and granularity) following contact with bacteria.

2. Materials and methods

2.1. Experimental animals

Manila clams (*Ruditapes philippinarum*, 40–46 mm shell length) were harvested from Puget Sound, WA, USA and shipped overnight to the laboratory, where they were held in quarantine. Specimens of the hard clam (*Mercenaria mercenaria*, 50–61 mm shell length) sampled near Atlantic City, NJ, USA, and eastern oysters (*Crassostrea virginica*, 60–70 mm shell length) collected from the Delaware Bay, NJ, USA, were brought to the laboratory on the same day. Animals were maintained in tanks of aerated seawater (32 ppt for clams and 26 ppt for oysters) at 13 °C. They were fed daily using a mixture of cultured algae until used for the experiments. Haemolymph was withdrawn from the adductor muscle (posterior muscle of clams) and samples were held on ice until assayed, typically within 10–20 min.

2.2. Bacteria

Vibrio tapetis (ATCC 4600), *Listonella* (=*Vibrio*) *anguillarum* (strain 775) [15] and *Vibrio splendidus* (ATCC 33125) were grown on marine agar (1 L distilled water, 15 g agar, 20 g sea salts [Sigma], 4 g peptone, 0.1 g $Fe(PO_4)_2$) or in marine broth (same composition as above but without agar), depending on the experiment, at 20 °C. The concentration of bacteria was estimated spectrophotometrically and adjusted before each experiment. The number of bacteria was approximately the same for each species used in a single experiment.

2.3. Effect of extracellular products of bacteria on phagocytic activity of haemocytes

The effect of extracellular products (ECP) of each bacterial species on the uptake of fluorescent latex beads (2.02 μm in diameter, Fluoresbrite Yellow-Green, Polysciences) by *R. philippinarum* haemocytes was assayed in vitro in 24-well microplates as previously described [11]. For each clam ($N=24$), eight wells were prepared, two for each of the bacterial species and two for control. Log-phase cultures of each bacterial species grown in marine broth for 36 h were adjusted spectrophotometrically to about 1×10^8 colony-forming units (CFU) mL^{-1} using sterile marine broth, then centrifuged ($3000\times g$, 15 min, 13 °C). The supernatants (containing ECP) were then filtered (0.22 μm) and used to prepare bead suspensions and to dilute haemolymph samples. Beads were diluted to provide working suspensions of 6×10^6 beads mL^{-1} in each bacterial supernatant or the sterile marine broth control. Two hundred microlitres of each working suspension (about 1.2×10^6 beads) were placed in each well of the microplate, which was centrifuged for 10 min at $200\times g$ at 20 °C to form a uniform monolayer of beads on the bottom of each well. Aliquots of haemolymph (100 μL) from individual clams were directly diluted (1:4) in each of the three bacterial supernatants and the marine broth control. Diluted haemolymph subsamples (200 μL) were added to two wells containing beads in the corresponding medium to give approximately a 1:10, cell:bead ratio. The volume of supernatant (=ECP) added per haemocyte was estimated to be equivalent to the volume of incubation medium containing 250 cfu (ECP-cfu equivalent). Cytochalasin B, which inhibits phagocytosis, was added (10 μg mL^{-1} final concentration) to one of the two wells to serve as a control for bead adherence without ingestion. After incubation for 30 min at 20 °C, test and control samples were processed for flow cytometry as previously described [11]. The percentage of phagocytic cells was measured on a Coulter EPICS flow cytometer and computed as the ratio of bead-associated haemocytes to total haemocytes×100 by bitmapping (electronic outlining) each of these two populations. The mean fluorescence intensity of each phagocyte (an index to the number of beads in each haemocyte or phagocytic index) was also calculated within each bitmap.

2.4. Effect of bacteria on haemocyte viability

The effect of each bacterial species on haemocyte viability in all three bivalves (*R. philippinarum*, *M. mercenaria*, and *C. virginica*) was assessed in vitro. Exponentially growing bacteria, cultured on marine agar for 72 h, were suspended in sterile seawater to yield about 1.5×10^9 cfu mL^{-1}. Haemolymph (400 μl) was withdrawn from each experimental bivalve ($N=18-20$ per species) and diluted directly in 800 μl cold (4 °C) Alsever's solution containing 10 μg mL^{-1} Cytochalasin B. Diluted haemolymph from each individual specimen was divided into four 300 μl aliquots in sterile polystyrene tubes. Three of the tubes each received a 100 μL suspension of one of the bacterial species to give a ratio of about 500 bacteria per haemocyte. The fourth tube received sterile seawater as a control. Following incubation for 2.5 h at 20 °C, the stain ethidium homodimer (2 μM), a red fluorescent dye that stains dead cells, was added and the tubes incubated for an additional 30 min. The percentage of red-fluorescing haemocytes was measured using the flow cytometer. A cursor was set at the upper limit of the red fluorescence signal for unstained haemocyte suspensions, which was used as the "zero" channel for the stained cells in treated samples. The percentage of dead cells was calculated as the ratio of cells above the "zero" channel to total haemocytes×100 [11].

2.5. Effect of V. tapetis culture fractions on haemocyte viability

In this experiment, the effect of different fractions of *V. tapetis* cultures on *R. philippinarum* haemocytes was evaluated. A log-phase culture of *V. tapetis*, grown in marine broth for 36 h, was divided into three aliquots. The first aliquot was centrifuged ($3000\times g$, 15 min, 13 °C), the supernatant was filtered (0.22 μm), and the pellet was washed twice in sterile marine broth, then resuspended in this medium. The second aliquot

was heated for 2 h at 60 °C. The third aliquot was used without manipulation to serve as a positive control. Negative controls were made using sterile marine broth. Haemolymph samples (500 μL, $N=18$), diluted in 2 mL Alsever's solution containing 10 μg Cytochalasin B mL^{-1}, were incubated (2.5 h at 20 °C) with each of these preparations to provide approximately a 1:500, haemocyte:bacteria or haemocyte:ECP-cfu equivalent ratio. Flow cytometric measurement of the percentage of dead haemocytes was made as described above.

2.6. Effect of bacteria on haemocyte light scatter parameters

Physiological changes may occur in haemocytes without affecting the uptake of vital stains. To document potential sublethal changes in haemocytes after in vitro contact with bacteria, we measured haemocyte size (forward light scatter or FS) and granularity (side light scatter or SS) of all three bivalve species before and after contact with *V. tapetis* and *V. splendidus*. Bacterial suspensions (1×10^8 cfu mL^{-1}) were made in sterile seawater using exponentially growing (72 h) bacteria incubated on marine agar. Haemolymph was withdrawn from each experimental bivalve ($N=10-18$ per species) and directly diluted in cold (4 °C) Alsever's solution (1:2 v:v) containing 10 μg mL^{-1} Cytochalasin B. Baseline light scattering by haemocytes and the percentage of granular and agranular haemocytes in each sample were measured immediately (Time 0) using a Coulter EPICS flow cytometer as previously described [16]. The diluted haemolymph was then divided into four aliquots (300 μl in each tube).

Two aliquots received 100 μl of each bacterial suspension, giving a ratio of about 40 bacteria per haemocyte. The third aliquot received a 100 μl suspension of latex beads (1 μm in diameter, Fluoresbrite Yellow-Green, Polysciences) in seawater (10^8 beads mL^{-1}) to compare the behaviour of haemocytes towards biotic and abiotic particles. The fourth tube contained sterile seawater and served as a control. Following incubation for 90 min at 20 °C, light scatters (FS and SS) were measured in granular and agranular haemocytes and compared to the Time 0 values. The comparison was made using the X/Y position, i.e. SS and FS channel number, of the peak in each haemocyte subgroup within each sample.

2.7. Statistics

Unless otherwise stated, a one-way general linear model ANOVA followed by a Fisher's PLSD post-hoc test was used to compare results from different treatments. Differences were considered significant at $\alpha=0.05$.

3. Results

3.1. Effect of extracellular products of bacteria on phagocytic activity of haemocytes

Extracellular products (ECP) from the different bacterial species significantly altered the phagocytosis of beads by *R. philippinarum* haemocytes (Tables 1 and 2). ECP from both *L. anguillarum* and *V. tapetis* caused a significant decrease in the phagocytic activity in comparison with controls, whereas *V. splendidus* induced a significant increase in uptake of beads. Products of *V. tapetis* and *L. anguillarum* also caused a decrease in the number of internalized beads in each phagocytic haemocyte, as reflected by low fluorescence intensities in comparison with controls and haemocytes incubated with extracellular products of *V. splendidus*.

3.2. Effect of bacteria on haemocyte viability

The percentage of dead haemocytes in *R. philippinarum* increased significantly, compared with controls, after incubation with each of the assayed bacteria (Tables 1 and 3). The highest percentage of dead

Table 1
Summary of experimental conditions and subsequent changes in haemocytes incubated with different bacterial strains

Experiment	Host species	Bacterial species	Haemocyte:bacteria	Results summary[a]	
1. Effect of ECP on phagocytosis	*R. philippinarum*	*V. tapetis* *L. anguillarum* *V. splendidus*	1:250 cfu equivalent ECP	% Phagocytosis ⇓ ⇓ ⇑	Phagocytic index ⇓ ⇓ ⇔
2. Effect of bacteria on haemocyte viability	*R. philippinarum* *M. mercenaria* *C. virginica*	*V. tapetis* *L. anguillarum* *V. splendidus*	1:500	Dead haemocyte counts *R. philippinarum*: ⇑ *M. mercenaria*: ⇑ *C. virginica*: ⇔	
3. Effect of *V. tapetis* culture fractions on haemocyte viability	*R. philippinarum*	Whole culture Washed cells ECP Heated cultures	1:500 (cfu equivalent for ECP)	Dead haemocyte counts ⇑ ⇑ ⇑ ⇔	
4. Effect of bacteria on light scattering by haemocytes	*R. philippinarum* *M. mercenaria* *C. virginica*	*V. tapetis* *V. splendidus*	1:40	Light scattering by granulocytes *R. philippinarum*: ⇓ *M. mercenaria*: ⇓ *C. virginica*: ⇔	

⇓, decrease; ⇔, stable; ⇑, increase; ECP, extracellular products.
[a] See text and following tables for more details.

cells (23%) occurred after incubation with *V. tapetis*, which is pathogenic to *R. philippinarum*. Lower percentages were found after incubation with *L. anguillarum* (19%) and *V. splendidus* (14%), respectively, but both were still significantly higher than controls (7%).

In *M. mercenaria*, the highest percentage of dead haemocytes was measured following incubation with *V. splendidus* (19%) (Tables 1 and 3). Somewhat lower percentages were found after incubations with *V. tapetis* (16%) and *L. anguillarum* (15%), but both figures were significantly higher than controls (9%).

The percentage of dead haemocytes in *C. virginica* was very low in comparison with both clam species, ranging from 3% after incubation with *V. splendidus* to 5% after incubation with *V. tapetis* (Tables 1 and 3). In oysters, differences were not significant between controls and haemocytes incubated with bacteria.

3.3. Effect of *V. tapetis* culture fractions on haemocyte viability

Both washed cells and ECP of *V. tapetis* were able to kill *R. philippinarum* haemocytes. The highest percentage of dead cells (21 ± 0.9, mean $\pm$ SEM) occurred after incubation with whole bacterial cultures.

Table 2
Effect of bacterial extracellular products (ECP) on in vitro phagocytosis of beads by haemocytes from *R. philippinarum* (mean $\pm$ SEM, $n=24$)

	Control	*V. splendidus*	*L. anguillarum*	*V. tapetis*
% Phagocytosis	15.9 ± 1.1^{a}	21.7 ± 1.6^{b}	8.8 ± 0.7^{c}	9.8 ± 1.2^{c}
Fluorescence intensity	25.2 ± 0.7^{a}	26.8 ± 0.9^{a}	21.3 ± 1.2^{b}	20.4 ± 1.0^{b}

For each parameter, letters (a, b and c) represent significant differences among different bacterial strains (Fisher's PLSD post-hoc test, $p<0.05$).

Table 3
Percentage of viable haemocytes following in vitro incubation with different bacterial strains (mean±SEM)

	Control	*V. splendidus*	*L. anguillarum*	*V. tapetis*
R. philippinarum ($n=20$)	7.0 ± 0.5^{a}	13.7 ± 1.1^{b}	18.8 ± 1.7^{c}	23.3 ± 1.2^{d}
M. mercenaria ($n=20$)	8.7 ± 0.5^{a}	19.3 ± 0.8^{b}	14.8 ± 1.1^{c}	15.5 ± 0.9^{c}
C. virginica ($n=18$)	$3.8\pm0.5^{a,b}$	3.0 ± 0.2^{a}	4.3 ± 0.4^{b}	4.7 ± 0.4^{b}

For each bivalve species, letters (a, b, c and d) represent significant differences among different bacterial strains (Fisher's PLSD post-hoc test, $p<0.05$).

Lower percentages were found after incubation with washed cells (18±1.0), and supernatants (15±1.0), respectively, but both were still significantly higher than values obtained for haemocytes incubated with the heated culture or the culture-medium control (9.3±0.8% and 9.1±0.8%, respectively).

3.4. Effect of bacteria on haemocyte light scatter parameters

The typical flow cytometric pattern of clam haemocytes displays two recognizable haemocyte groupings (Fig. 1A). The first group has relatively high forward scatter (FS) and side scatter (SS) signals and is mainly composed of granulocytes, while the second has relatively low FS and SS signals and is mainly made up of agranular cells [16]. Following incubation with bacteria, there was a notable shift of granular cells toward the agranular cell population, resulting in unimodal distributions of FS and SS for both clam species (Fig. 1B, Tables 1 and 4). Forward scatter signals occurring within the granulocyte bitmap decreased by up to 53% in haemocytes from both clam species incubated with either *V. splendidus* or *V. tapetis*, compared with untreated controls or those incubated with beads (Tables 1 and 4). A smaller decrease (14%), albeit statistically significant, was observed for side scatter signals (Tables 1 and 4). Neither forward nor side scatter signals of *C. virginica* granulocytes was affected by incubation with bacteria (Tables 1 and 4), and no significant changes in light scatter signals were measured for agranular haemocytes of any species incubated with any test particle.

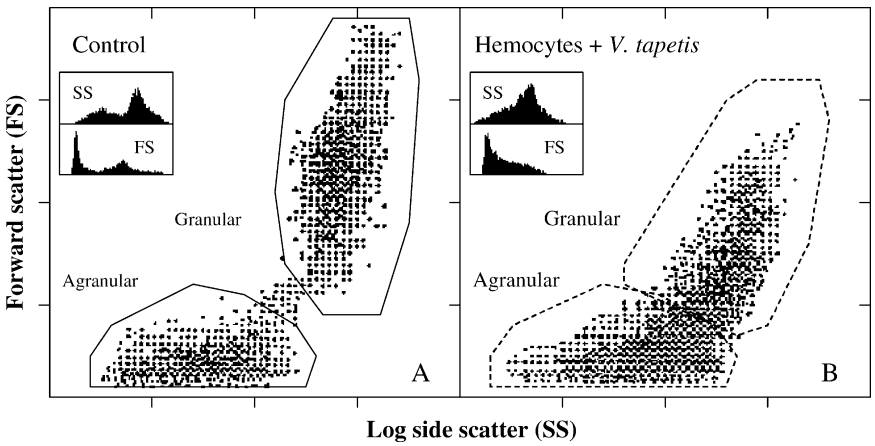

Fig. 1. Flow cytometry bivariate plots showing distributions of log side scatter (SS) and forward scatter (FS) of haemocytes in *Ruditapes philippinarum*. Two haemocyte subpopulations are recognizable in controls (A): granular and agranular cells. When incubated with bacteria (B), granulocytes shifted toward the agranular cell population, resulting in unimodal distributions of FS and SS.

Table 4
Forward and side scatter signals (mean channel number ± SEM) measured in granulocytes incubated with different test particles

	Control	Beads	*V. splendidus*	*V. tapetis*
Forward scatter (FS)				
R. philippinarum ($n=18$)	22.2 ± 1.6^{a}	20.1 ± 1.7^{a}	10.4 ± 0.3^{b}	13.4 ± 1.4^{b}
M. mercenaria ($n=12$)	43.1 ± 1.4^{a}	37.8 ± 3.6^{a}	23.8 ± 2.7^{b}	27.2 ± 3.8^{b}
C. virginica ($n=10$)	22.6 ± 1.0^{a}	21.9 ± 1.5^{a}	23.1 ± 0.6^{a}	23.1 ± 0.8^{a}
Side scatter (SS)				
R. philippinarum ($n=18$)	37.1 ± 0.3^{a}	36.9 ± 0.4^{a}	33.8 ± 0.4^{b}	32.2 ± 0.5^{b}
M. mercenaria ($n=12$)	45.1 ± 0.4^{a}	43.8 ± 1.6^{a}	38.7 ± 1.0^{b}	39.0 ± 1.4^{b}
C. virginica ($n=10$)	30.4 ± 1.1^{a}	33.2 ± 1.0^{a}	30.3 ± 0.5^{a}	30.7 ± 0.8^{a}

For each bivalve species, letters (a and b) represent significant differences among different treatments (Fisher's PLSD post-hoc test, $p<0.05$).

4. Discussion

The present work clearly demonstrates a close correspondence between the deleterious effect of three *Vibrio* species on the haemocytes of three marine bivalves in vitro and their reported pathogenic effects in vivo [14]. Specifically, whole cells and extracellular products of *V. tapetis*, which causes Brown Ring Disease (BRD) in the Manila clam, *Ruditapes philippinarum*, caused greater physiological disturbances and higher mortality of haemocytes of that species than of haemocytes of hard clams, *Mercenaria mercenaria*, and the eastern oyster, *Crassostrea virginica*. The latter two bivalves are not susceptible to BRD. Results underscore the fact that disease resistance is not solely dependent on host activity against the pathogen, but is also a function of the injury and its severity to the host cell by a pathogen.

In vitro incubation of *R. philippinarum* haemocytes with *V. tapetis* culture fractions induced severe changes in haemocyte structure (light scattering by haemocytes), and effected a decrease in haemocyte phagocytic activity and viability. Our results demonstrated that culture filtrate from *V. tapetis* was less toxic than an equivalent volume containing 2.5×10^{8} of washed bacteria. Heating *V. tapetis* cultures inactivated the toxic substance(s). Thus, *V. tapetis* seems to possess a thermosensitive cytotoxic factor(s) mediated by the intact bacterial cell, which is (are) present also as extracellular secretions. Similarly to most pathogenic vibrios, *Vibrio tapetis* produces several virulence factors potentially involved in its pathogenic capacity. These include haemolysins, cytotoxins, exotoxins and plasmids [17,18]. Previous work also reported that *V. tapetis* cells are able to kill cultured fish cells [17] and are associated with vibriosis in fish [19], confirming this bacterium's wide range of functionality. Protease production is widespread amongst members of the *Vibrionaceae* and could be involved in *V. tapetis* toxicity. Previous studies have shown that proteases produced by *Vibrio alginolyticus* have significant toxic activity against bivalve larvae [20,21]. Lambert et al. [22] described killing of haemocytes from the scallop *Pecten maximus* following contact with live bacteria and cytoplasmic extracts of *Vibrio pectinicida*, a pathogen of scallop larvae. These authors partially purified and identified the toxic substance as a heat-stable peptide named VHKT (for *Vibrio* haemocyte-killer toxin). The involvement of similar substances in the cytotoxic activity described in our work, although thermosensitive, cannot be rejected.

The mechanistic action of *V. tapetis* toxins remains unclear, as do those of other *Vibrionaceae* affecting marine invertebrates. However, our results clearly demonstrated that V. tapetis disturbs light scattering by clam granulocytes. Changes in cell shape following contact with bacteria can be discounted as a cause for changes in light scattering because the cytoskeleton inhibitor Cytochalasin B was added in test and control mixtures before incubation, so all mixtures contained round cells. Thus, the decrease in light scattering by granulocytes probably results from a loss of cytoplasmic granules in these cells and any associated cell shrinkage [23–25] following contact with live *V. tapetis* and *V. splendidus*. *Vibrio tapetis* (and to a lesser

extent *L. anguillarum*) toxins caused a decrease in haemocyte phagocytic activity in Manila clams. The increase in dead haemocytes alone does not necessarily explain the modulation of the phagocytic activity. For instance, both percentage of dead haemocytes and phagocytic activity increased when haemocytes were incubated with *V. splendidus* cells/ECP (Tables 2 and 3).

Processes associated with phagocytosis are severely altered by pathogenic microorganisms. Secretory-excretory products from digenean parasites significantly modulate, in a specific manner, haemocyte spreading and mobility in gastropods [26]. ECP from the protozoan pathogen *Perkinsus marinus* inhibited the motility of haemocytes from the host *Crassostrea virginica*, while cells or cell lysate of this pathogen stimulated haemocyte motility [27]. Similarly, random haemocyte motility significantly increases in mussels *Mytilus edulis* exposed to bacterial products [28]. Inhibitory effects of bacteria have also been reported. Bacterial cells and filtrate, including *V. alginolyticus* and *L. anguillarum*, caused the rounding and cessation of movement of haemocytes from *M. edulis* [9,29]. A more recent work demonstrated that *V. tapetis* decreases the ability of *R. philippinarum* haemocytes to adhere to plastic microplates and causes the rounding of these cells [10]. Rounding of haemocytes indicates a cessation of phagocytic activity since pseudopod formation is necessary for phagocytosis. Changes in phagocytic activity in haemocytes incubated with bacterial ECP reported in this study could be the result of modification in pseudopod formation and haemocyte motility, leading to changes in the probability for a haemocyte to encounter and/or to engulf test particles. Toxins active against the eukaryotic cytoskeleton have been described for several bacteria [30,31] and are probably involved in the present work.

In contrast with some other major diseases affecting bivalve molluscs (Perkinsiosis, Haplosporidiosis, Bonamiosis), haemocytes are believed to play a determinant role in the defence against BRD. There is corroborating evidence of heightened resistance against BRD in clams with high defence-related haemocyte activities [4,32]. During the early development of BRD, the pathogen colonizes and degrades the periostracal lamina, an organic matrix secreted by the mantle to support normal shell growth, causing the apparition of disease symptoms (brown deposit). At this site, *V. tapetis* is in direct contact with haemocytes initially present in the extrapallial fluid that bathes the periostracal lamina [32,33]. The result of the interactions between *V. tapetis* and these haemocytes appears to be a major factor in the outcome of the disease process. For instance, if haemocytes present in the extrapallial fluid are able to neutralize *V. tapetis* cells, BRD development remains low (light brown deposit at the inner face of the shell) or undetectable, as in resistant populations of *R. philippinarum* [32], *R. decussatus* [12,32,34], *M. mercenaria* and *C. virginica* [12,13]. The importance of this site is highlighted by the early mobilization of haemocytes toward the extrapallial space following experimental induction of BRD [35], and the high phagocytic rates of *V. tapetis* injected into the extrapallial space [4]. Indeed, *V. tapetis* cells are not inherently resistant to the internal defence system of the clam and are phagocytosed and digested when incubated in low numbers with haemocytes [4,32,36]. When injected in large numbers, the capacity of *V. tapetis* to kill and alter the phagocytic activity of *R. philippinarum* haemocytes may significantly impair the major defence mechanism that clams employ to protect themselves against the pathogen, leading to the development of BRD and subsequent physiological alterations. The fact that disease prevalence following experimental injection of *V. tapetis* cells into healthy clams appears to be dose-dependent is consistent with this hypothesis.

Our results also shed light on the cause of BRD-associated clam mortality. Previous studies demonstrated that *V. tapetis* is able to penetrate into tissues of clams with high bacterial loads in the pallial cavity. Contact between internal haemocytes and pathogens probably causes a weakening of internal defence in susceptible clams. For instance, increased numbers of dead haemocytes were observed in the haemolymph of diseased *R. philippinarum*, but not in *R. decussatus*, even though both species displayed disease signs [33]. Similarly, it has been demonstrated that phagocytic activity was severely reduced in haemocytes from diseased *R. philippinarum*, but increased significantly in haemocytes withdrawn from *R. decussatus* infected with *V. tapetis* [32]. An impaired immune system in diseased clams could lead to the establishment of secondary infections, or to the direct killing of these animals by *V. tapetis* following severe

physiological disturbances. Corroborating evidence is found in earlier work demonstrating that *V. tapetis* can induce, in a dose-dependent manner, severe septicaemia and clam death when injected inside tissue of *R. philippinarum*, but not in *R. decussatus*, where bacteria were rapidly eliminated [4]. *Listonella anguillarum* also caused clam death, but to a much lesser extent than *V. tapetis* [4], in accordance with results obtained here with the haemocytes/bacteria in vitro model.

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