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Reduction of adhesion properties of *Ruditapes philippinarum* hemocytes exposed to *Vibrio tapetis*

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ABSTRACT: *Vibrio tapetis* is the causative agent of brown ring disease (BRD), which affects a species of clam, *Ruditapes philippinarum*. After incubation with *V. tapetis*, hemocytes lose filopods and become rounded, indicating cytotoxic activity of the bacterium. To rapidly quantify this cytotoxicity, a flow-cytometry test was developed based on the capacity of *V. tapetis* to inhibit adhesion of clam hemocytes to plastic. Several bacteria:hemocyte ratios, the cytotoxicity of other *Vibrio* spp. pathogenic to bivalves, and that of various *V. tapetis* isolates were tested. Inhibition of adherence is detectable with as few as 5 bacteria per hemocyte. The greater cytotoxic activity of *V. tapetis* compared to that of *V. splendidus* and *V. pectenicida* suggests a specific pathogenicity of *V. tapetis* to *R. philippinarum* hemocytes. Although all *V. tapetis* isolates inhibited adhesion, significant variations in cytotoxicity among isolates was demonstrated.

KEY WORDS: Cytotoxicity · Flow cytometry · *Vibrio tapetis* · *Ruditapes philippinarum* · Bivalve hemocytes · Pathogenicity

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INTRODUCTION

Vibrio tapetis is the bacterium responsible for brown ring disease (BRD), which affects the Japanese clam *Ruditapes philippinarum* (Paillard & Maes 1990, Borrego et al. 1996a). This species of clam was introduced into France as an aquaculture species in the 1970s. The disease appeared in 1987 in Brittany. It is characterized by a brown conchiolin deposit between the edge of the shell and the pallial line (Paillard et al. 1989, 1994). The major site of infection of *V. tapetis* is in the extrapallial space, and the bacterium is mainly located on the surface of the mantle epithelium and on the periostracal lamina (Paillard & Maes 1995). The effects of inoculation of *V. tapetis* into the pallial cavity on immune defense parameters have been well studied in both *R. philippinarum* and *R. decussatus*. The immune responses to challenge by pathogenic bacteria were first characterized in the hemolymph of *R. philippinarum* (Oubella et al. 1993, 1994, 1996), whereby hemocyte concentrations increased over a 2 wk period. Thereafter, concentrations decreased and remained low for an additional period of 7 wk. Allam & Paillard (1998) demonstrated the presence of active hemocytes in the extrapallial fluids, and proposed that these hemocytes are likely to be involved in the immune response to the presence of *V. tapetis* in this compartment. Total hemocyte counts, differential hemocyte counts, phagocytic capacities of hemocytes and enzymatic activities were shown to be sensitive to *V. tapetis* inoculation (Allam & Paillard 1998, Allam et al. 2000, 2001). For instance, hemocyte concentrations and lysozyme activity increased significantly not only in the hemolymph, but also (and especially), in the extrapallial fluid.

Recently, a rapid toxic action of *Vibrio tapetis* against *Ruditapes philippinarum* was observed after inoculation of a bacterial suspension (5×10^7 bacteria per clam) directly into the adductor muscle, resulting in clam death within a few days (Allam et al. 2002). When this inoculation procedure is used, *V. tapetis* can avoid

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external defense factors, such as phagocytosis by hemocytes in the pallial fluid as well as the epithelium.

However, mechanisms of interactions between *Vibrio tapetis* and clam hemocytes remain poorly understood. Measurement of cytotoxicity of *V. tapetis* against hemocytes could be a good indicator of the virulence properties of this bacterium. In the literature, 2 assays using bacteria-hemocyte interaction have already been described: one was direct and employed microscopic observation, the other was indirect and used a chemiluminescence assay. The first used a time-lapse video recording to determine the toxic effect of *V. anguillarum* and *V. alginolyticus* on mussel *Mytilus edulis* hemocytes (Lane & Birkbeck 1999). These 2 *Vibrio* species cause cell-rounding of the hemocytes. The most toxic bacterium, *V. anguillarum*, was able to induce rounding in 50% of the hemocytes at a ratio of only 1 bacterium per hemocyte. Differences in cell-rounding percentages permitted quantification of cytotoxicity. The second method was used to examine the effects of *V. pectenicida* and of its cytoplasmic extract on the respiratory burst of *Pecten maximus* hemocytes (Lambert & Nicolas 1998, Lambert et al. 2001). This indirect method used chemiluminescence to quantify production of reactive oxygen intermediates (ROIs). *V. pectenicida*, and in particular the cytoplasm extract, were responsible for the inhibition of chemiluminescence activity in oyster and scallop hemocytes.

To better understand the cellular effects of *Vibrio tapetis* toward hemocytes, the present study was designed first to develop a cytotoxicity test using flow cytometry, and second to compare the pathogenicity of different *V. tapetis* strains on clam and oyster hemocytes.

MATERIALS AND METHODS

Bacteria. The bacteria used in this study were generally isolated from BRD-diseased clams and asymptomatic cockles at various times and places (Table 1). Strain L2, isolated from a fish (*Symphodus melops*) in Norway, was kindly provided by Jensen et al. (2003). Isolates were characterized by phenotypic tests and/or molecular characteristics. As negative controls, we used 2 other species of *Vibrio*: a strain of *V. pectenicida* (CIP 105229^T), a pathogen of larvae of the scallop *Pecten maximus* (Lambert et al. 1998); and a strain of *V. splendidus* (ATCC 25914), a species generally associated with juvenile bivalve mortalities (Lacoste et al. 2001, Waechter et al. 2002). Cells were cultured overnight at 18°C in 5 ml Zobell's medium. They were harvested by centrifugation at 1500 × *g* for 15 min at 4°C and washed twice with filtered (0.22 µm) sterile seawater (FSSW). Bacterial concentration was determined as optical density measured with a spectrophotometer at 492 nm. The bacteria: hemocyte ratio for each experiment was achieved by diluting the bacterial suspension with FSSW. Bacteria were heat-killed when necessary (50°C, 1 h).

Hemolymph. Clams *Ruditapes philippinarum* (length 25 to 35 mm) and oysters *Crassostrea gigas* were maintained in aquaria with aerated seawater. Clams were supplied by the SATMAR Company (Marennes) or collected from natural beds in the Gulf of Morbihan; 2 yr old oysters were supplied by the hatchery of IFREMER (Argenton). For clams, hemolymph was obtained from the posterior adductor muscle using a 1 ml syringe fitted with a 25-gauge needle inserted through the hinge. For oysters, hemolymph was withdrawn from the adductor muscle through a notch previously (24 h earlier) ground in the oyster shell. Prior to use, individual hemolymph samples were maintained in a 1.5 ml microtube on ice. Hemolymph samples were observed by microscopy to verify that they were free of any contaminating particles (ciliates or large pieces of debris). Hemolymph samples were pooled to provide the final volume required for each experiment. Pooled hemolymph samples were passed through 80 µm mesh screen to eliminate cell aggregates. A subsample of each pooled sample was fixed with 6% formalin solu-

Table 1. Bacterial isolates tested, showing date, location, and species from which isolate was obtained. Isolates were classified as *V. tapetis* strain because of phenotypical and/or molecular characteristics

Strain	Date	Species	Origin	Source
CECT4600	Oct 1990	*Ruditapes philippinarum*	France, Landeda	Paillard & Maes (1995), Borrego et al. (1996a)
IS1	Sep 1988	*Ruditapes philippinarum*	France, Landeda	Borrego et al. (1996a)
IS5	Oct 1991	*Ruditapes philippinarum*	France, Landeda	Borrego et al. (1996a)
IS7	Apr 1990	*Ruditapes philippinarum*	France, Quiberon	Borrego et al. (1996a)
IS8	Apr 1990	*Venerupis aurea*	France, Quiberon	Borrego et al. (1996a)
IS9	Apr 1990	*Cerastoderma edule*	France, Quiberon	Borrego et al. (1996a)
P16B	Sep 1995	*Ruditapes philippinarum*	France, Golfe du Morbihan	Allam et al. (2002)
UK6	Jun 1996	*Ruditapes philippinarum*	United Kingdom, Poole Harbour	Allam et al. (2000b)
L2	Sep 1999	*Symphodus melops*	Norway	Jensen et al. (2003)
GM4	Jul 1999	*Ruditapes philippinarum*	France, Golfe du Morbihan	

tion (v/v) in FSSW to determine the cell concentration. These hemocyte concentrations were used to prepare the various bacterial dilutions required.

Hemocyte counting by flow cytometry. All samples were analyzed with a FACS-Calibur™ flow cytometer (Becton Dickinson). In order to determine the cell concentration of each sample, flow rate of the cytometer was controlled for each experiment by weighing a tube containing distilled water before and after analysis to determine the volume analyzed over a known time (Marie et al. 1997). Cells were detected with FSC (forward scatter) as the primary parameter, since this parameter is related to the size of the cells. Cells were stained with Sybr Green I (final conc. = 10^{-3} of commercial solution: Molecular Probes), a DNA-binding fluorochrome, to verify that counted events corresponded to cells (Marie et al. 1997). Indeed, only DNA-carrying particles have to be counted as cell.

Cytotoxicity assay. In each cytotoxicity assay, contact between hemocytes and bacteria was established in 24-well microplates. Subsamples of each hemocyte pool were exposed to both washed bacteria and FSSW at 18°C. After incubation, 6% formalin (v/v) was added to stop the interaction, and supernatants were transferred to cytometry tubes. The hemocyte number present in bacteria- or FSSW-exposed supernatants was determined by flow cytometry.

Results are expressed as a non-adherent cell ratio, i.e. the number of non-adherent cells incubated with bacteria divided by the number of cells incubated with FSSW. A non-adherent cell ratio above 1 represents a cytotoxic effect of the tested bacteria.

Different incubation times (1 to 6 h) were tested using a ratio of 25 bacteria:1 clam hemocyte. Several ratios (5, 10, 25, 50, and 100) were also tested with 3 h incubation. Heat-killed bacteria were also tested.

The cytotoxicity of *Vibrio tapetis* was compared to that of *V. pectenicida* and *V. splendidus* using 3 ratios of bacteria:clam hemocyte (25, 50, and 100). Incubation time was 3 h.

Finally, the cytotoxicity of 10 isolates of *Vibrio tapetis* was tested using a bacteria:clam hemocyte ratio of 25 with an incubation time of 3 h. The cytotoxicity of these strains was also tested on oyster hemocytes.

Variability in hemocyte adhesion. To estimate the variability of hemocyte adhesion capacities as a function of collection date, different pools of clam hemocytes were tested on the following dates in 2001: April 26, May 14, May 28, June 6, July 21, September 19, November 21, December 19. Non-adherent hemocytes, present in the supernatant after 3 h of incubation with FSSW, were removed and fixed with 6% formalin and cell number was determined by flow cytometry. To determine the percentage of non-adherent cells in each assay, the number of cells in the supernatant was compared to the initial total hemocyte count of the tested pool.

Statistical analysis. Data were tested by ANOVA. Differences were considered to be statistically significant at $\alpha = 0.05$. When necessary, data were classified with Fisher's least significant difference multiple-range test.

RESULTS

Effect of *Vibrio tapetis* on *Ruditapes philippinarum* hemocytes

After 3 h, clam hemocytes incubated in FSSW appeared healthy, with cytoplasmic extensions (Fig. 1A) as did those incubated with *Vibrio splendidus*, a nega-

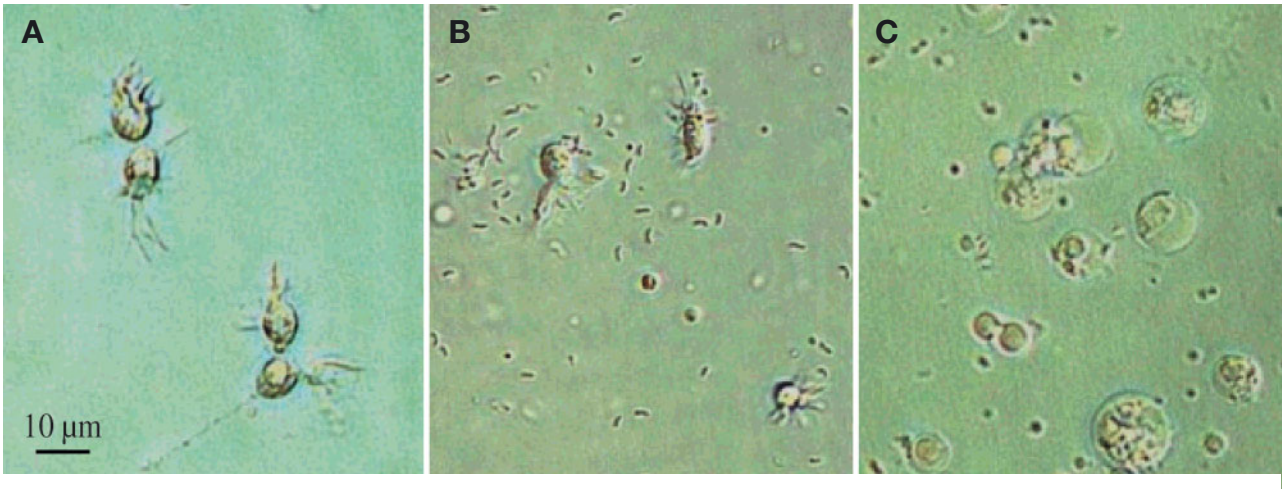

Fig. 1. *Ruditapes philippinarum*. Hemocytes after incubation with *Vibrio* sp. for 3 h at 18°C. (A) Control: incubation of hemolymph with filtered sterile seawater (FSSW); (B) hemolymph + *V. splendidus* in FSSW; (C) hemolymph + *V. tapetis* in FSSW

tive control (Fig. 1B). When hemocytes were incubated with *V. tapetis* for 3 h (Fig. 1C), they became swollen, rounded and lost filopods. Further, they lost their capacity to attach to the microplate plastic. Hemocytes incubated with *V. splendidus* (Fig. 1B) retained the same adherence capacity as in FSSW.

Variability of *Ruditapes philippinarum* hemocyte adhesion capacities

Throughout the series of experiments, hemocyte pools obtained at different times displayed different adhesion capacities (Fig. 2). When incubated in FSSW, the percentage of cells in the supernatant varied from 4 to 12%. This result was probably affected by the physiological state of the clams at the time of year of sampling, but since the non-adherent cell ratio for each experiment was calculated using the number of non-adherent cell in FSSW as the divisor, the results from different experiments can be compared.

Incubation time

When hemocytes were incubated at a ratio of 25 bacteria:1 hemocyte for 1 to 6 h, there was an increase in the non-adherent cell ratio from 1.5 to 6 within 3 h (Fig. 3). After 3 h incubation, there was no further increase; thus the incubation time used in subsequent experiments was 3 h.

Dose effect

The non-adherent cell ratio increased linearly with increasing number of bacteria per hemocyte from 5 to 50 (Fig. 4). The ratio with 50 bacteria:1 hemocyte reached a maximum of about 12 and did not increase with a ratio of 100 bacteria:1 hemocyte. Cytotoxicity was already significant at a bacteria:hemocyte ratio as low as 5:1, with a non-adherent cell ratio of 4. The reduction in adhesion properties occurred only with live bacteria. Dead bacteria tested at the same ratios did not inhibit adhesion (data not shown).

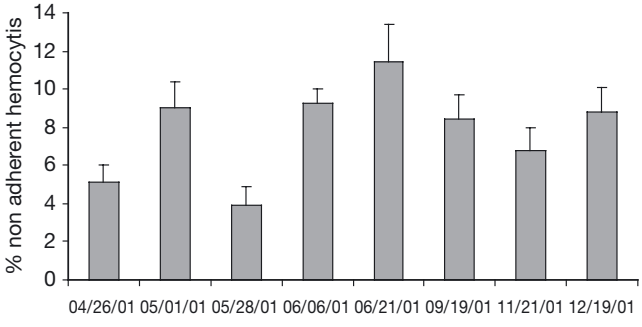

Fig. 2. *Ruditapes philippinarum*. Percentage of non-adherent hemocytes collected at various times of the year. Incubation time = 3 h; temperature = 18°C. Hemocytes were incubated with FSSW. Error bars = SD. Dates presented as mo/d/yr

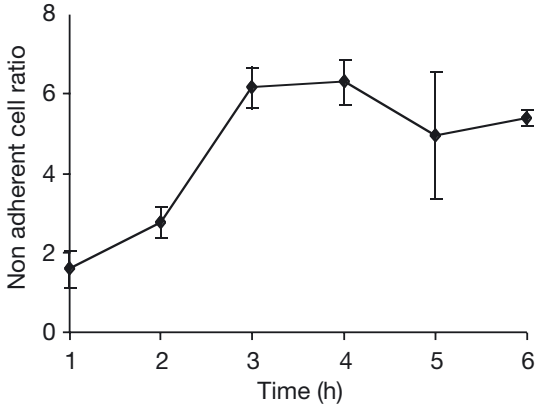

Fig. 3. *Ruditapes philippinarum*. Effect of incubation time on non-adherent cell ratio. Temperature = 18°C; 25 bacteria per hemocyte. Error bars = SD

Specificity of *Vibrio tapetis* cytotoxicity

At each bacteria:hemocyte ratio tested, *Vibrio tapetis* showed the greatest capacity to inhibit hemocyte adhesion. Indeed, the non-adherent cell ratio was 6 for 25 bacteria, 8 for 50 bacteria and 8.5 for 100 bacteria (Fig. 5). Moreover, whatever the ratio tested, cytotoxic activity of *V. tapetis* was at least 6 times higher than that of *V. splendidus*, which showed no significant cytotoxicity (i.e. the non-adherent cell ratio was ~1). Although *V. pectenicida* had a cytotoxic effect above a ratio of 50 bacteria:1 hemocyte, the non-adherent cell ratio obtained with this *Vibrio* sp. was always significantly lower than that of *V. tapetis*.

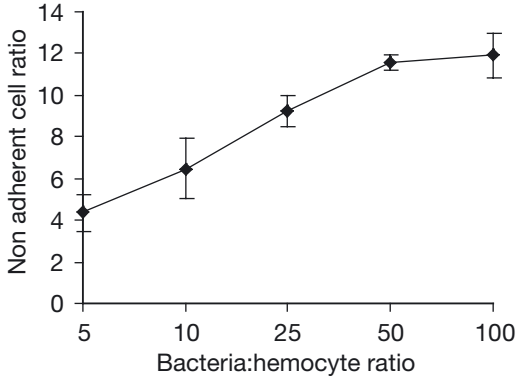

Fig. 4. *Ruditapes philippinarum*. Effect of number of bacteria per hemocyte on non-adherent cell ratio. Incubation time = 3 h; temperature = 18°C. Error bars = SD

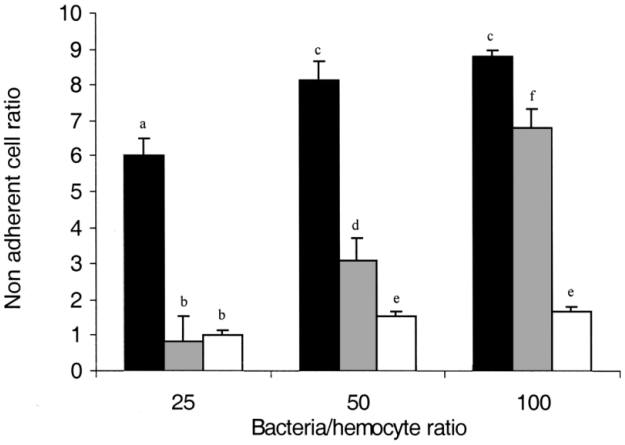

Fig. 5. *Ruditapes philippinarum*. Effect of 3 species of *Vibrio* on non-adherent cell ratio. (Black bars: *V. tapetis*, grey: *V. pectenicida*, white: *V. splendidus*). Incubation time = 3 h; temperature = 18°C. Letters represent significant differences (Fisher's least significant difference multiple-range test, $p < 0.05$). Error bars = SD

Cytotoxicity of different isolates

We also tested 10 bacterial isolates (Table 1) obtained since the discovery of BRD in 1987, at a bacteria:hemocyte ratio of 25:1. We conducted 3 independent experiments, each employing all isolates, to measure their cytotoxicity. The mean non-adherent cell ratio of each isolate for all 3 experiments was calculated, and isolates were classified from lowest to highest cytotoxic activity (Fig. 6). Strains IS8, CECT-4600 and IS9 had the highest cytotoxic activities (in increasing order of toxicity). The maximum non-adherent cell ratio (8) was obtained with Strain IS9. Strain GM4 was remarkable in that its activity was as low as that of *Vibrio splendidus*, which was used as a negative control. The other strains (P16B, L2, IS5, UK6, IS7 and IS1) displayed intermediate activity (non-adherent cell ratios between 3 and 6).

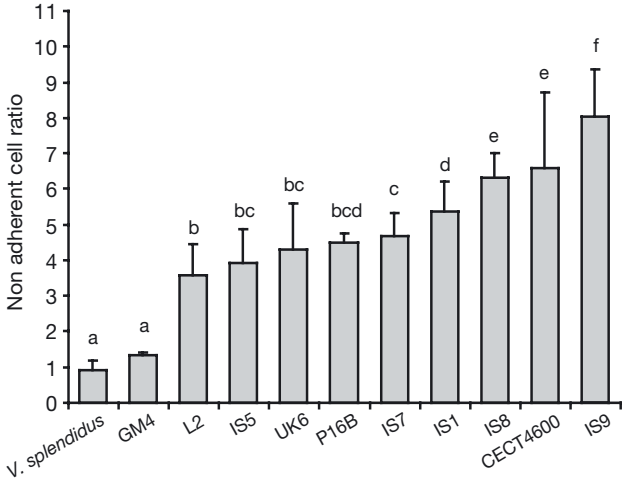

Fig. 6. *Ruditapes philippinarum*. Effect of *Vibrio tapetis* and related strains on non-adherent cell ratio of hemocytes. Incubation time = 3 h; 25 bacteria per hemocyte; temperature = 18°C. Graph summarizes results for 3 independent experiments on May 28, June 6 and December 19, 2001. Letters represent significant difference (Fisher's least significant difference multiple-range test, $p < 0.05$). Error bars = SD. *Vibrio* strain origins are detailed in Table 1; strains presented here in order of increasing cytotoxicity

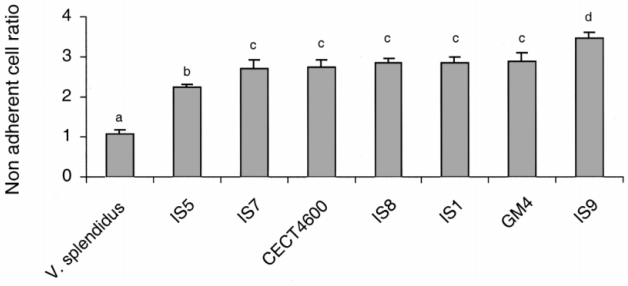

Fig. 7. *Crassostrea gigas*. Effect of different strains of *Vibrio* on non-adherent cell ratio of oyster hemocytes. Incubation time = 3 h; 25 bacteria per hemocyte; temperature = 18°C. Letters represent significant difference (Fisher's least significant difference multiple-range test, $p < 0.05$). Error bars = SD. *Vibrio* strain origins are detailed in Table 1; strains presented here in order of increasing toxicity

Specificity of cytotoxicity of *Vibrio tapetis* for *Ruditapes philippinarum* hemocytes

Some isolates of *Vibrio tapetis* were tested for their cytotoxicity to both oyster and clam hemocytes. All tested strains presented cytotoxic activity against oyster *Crassostrea gigas* hemocytes (Fig. 7). However, except for Strain GM4, the cytotoxicity to oyster hemocytes for all the strains tested was 1.7 to 2.4 times lower than that to clam hemocytes. The maximum non-adherent cell ratio was about 4 for oyster hemocytes, while it was 8 for clam hemocytes. Moreover, the differences in cytotoxicity among isolates for oyster hemocytes were very much less marked than those for clam hemocytes (ANOVA, $n = 4$, $p < 0.05$).

DISCUSSION

Vibrio tapetis, a pathogen of *Ruditapes philippinarum*, affects clam hemocytes by causing cell-rounding, loss of cytoplasmic extension and, as a consequence, loss of adherence capacity. Some authors have previously observed similar cytotoxic effects on molluscan hemocytes of *Vibrio* strains. Cell-rounding caused by *V. tapetis* on *R. philippinarum* hemocytes was described by Lane & Birkbeck (2000). Other *Vibrio* spp. are able

to provoke cell-rounding of hemocytes. For example, rounding of *Mytilus edulis* hemocytes in contact with *V. alginolyticus* and *V. anguillarum* was previously described by Nottage & Birkbeck (1990) and Lane & Birkbeck (1999). For *Vibrio* spp. pathogenic to shrimp, cytopathic effects, including the obliteration of cellular boundaries and the disturbance of chromatin, were observed in cell monolayers derived from hemocytes (Goarant et al. 2000). However, microscopic observations of bivalve hemocyte alterations caused by bacterial pathogens are rare. Usually, assays are made with cell lines. For instance, the toxicity of *V. tapetis*, Strain *Vibrio* P1 (subsequently named CECT4600), was tested on a fish cell line developed from fibroblasts of gilthead seabream fins (Borrego et al. 1996b). Effects of *Vibrio* P1 extra-cellular products (ECP) were vacuolization of the cells after 18 h incubation, morphological alterations such as rounding, shrinking, detaching and finally monolayer destruction. *V. vulnificus*, which is a human pathogen responsible for gastroenteritis, also displayed cytotoxicity to salmon, trout and carp fish cell lines (Biosca & Amaro 1996). The observed effects were characterized by cell elongation and rounding, which appeared after 3 or 4 h of contact and was followed by total detachment of cells in the 3 fish lines. For *V. cholerae*, similar morphological and cytoskeletal changes were observed in human cell lines, 1 intestinal (Int 407) and 1 non-intestinal (HeLa) (Basu et al. 1999). Although usage of cell lines comprises an interesting tool for studying cytotoxicity of vibrios, the use of cell lines from different species may not accurately reflect bacterial effects on the species to which it is pathogenic. This is the case for a *Vibrio* strain pathogenic to shrimp, which was cytotoxic for shrimp hemocytes but not for the fish cell line epithelioma papulosum cyprini (EPC) originating from carp (Goarant et al. 2000).

There are no cell lines available for marine bivalves, but hemocytes make an attractive alternative since they can be easily obtained and kept *in vitro* for many hours. Furthermore, they are the principal cells of the internal defense system and it was the main interest of the present study to observe and quantify the direct impact of a pathogenic bacteria on defense cells. For this purpose, we developed an *in vitro* assay based on the counting of non-adherent hemocytes by flow cytometry. This instrument permits fast enumeration of various types of cells (Marie et al. 1997, Gunasekera et al. 2000). Flow cytometry was used here to quantify easily the cytotoxic effect of *Vibrio tapetis* and other *Vibrio* spp. on bivalve hemocytes. Inhibition of adhesion appeared within 3 h with as few as 5 bacteria per hemocyte. These results are comparable to those of Lane & Birkbeck (1999) for mussels, who observed a cell-rounding effect in 2 to 3 h with 1 bacteria: 1 hemocyte.

Our biotest also revealed host-specificity of *Vibrio tapetis* cytotoxicity for *Ruditapes philippinarum*, compared to oyster *Crassostrea gigas* hemocytes, which were much less sensitive. Specific actions of *V. pectenicida* against scallop hemocytes and of Strain S322 against oyster hemocytes have also been demonstrated using a chemiluminescent test by Lambert & Nicolas (1998). These 2 bacteria are known to be pathogenic to scallop and oyster larvae respectively (Lambert & Nicolas 1998, Lambert et al. 1998, 2001).

Also, this bioassay permitted us to compare different isolates of *Vibrio tapetis* and to classify them from lowest to highest cytotoxic activity. The 5 most cytotoxic were isolated before 1991, and corresponded to the first years of the disease and to 'intense' outbreaks. Moreover, these 5 isolates were obtained not only from the clam *Ruditapes philippinarum* but also from other bivalves, including the cockle *Cerastoderma edule* and the clam *Venerupis aurea*. Similarly, Lane & Birkbeck (1999) demonstrated differences of cytotoxicity among strains of *Vibrio anguillarum* and *V. alginolyticus* in *Mytilus edulis* hemocytes. Among the strains tested in the present study, 2 are of particular interest: GM4 and L2. The isolate GM4 is considered to be a variant strain of *V. tapetis*, but its toxic activity was as small as that of the negative control *V. splendidus*. This results could have 2 explanations: (1) GM4 is not a true *V. tapetis* variant but merely closely related, (2) GM4 is a true *V. tapetis* variant but is not cytotoxic. The test was sensitive enough to discriminate very closely related strains. The Strain L2 is interesting for its relatively low cytotoxicity among the isolates tested and for its geographical and host origin. It was isolated from a fish in Norway and re-infection experiments have confirmed its ability to cause vibriosis in fish (Jensen et al. 2003). The ability of our bioassay method to classify different isolates based on their cytotoxic activity without long *in vivo* experiments suggests that would be useful in a number of ways. Thus, it could be applied to (1) measurement of cytotoxic activity of new isolates to identify potential pathogens, (2) comparison of cytotoxic activity under different culture conditions of the pathogen and (3) evaluation of host susceptibility as a function of physiological status, which varies with season, genetic background, and food availability.

The cytotoxicity of *Vibrio tapetis* is now fairly well-established, but its toxicity mechanism remains poorly understood. It is difficult to identify the factor responsible. However, some bacterial toxins have been identified, notably in *Vibrio* spp. One important class of toxins are hemolysins, which are able to lyse erythrocytes. This class of toxins has been found not only in humans pathogens such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Chang et al. 1997, Fabbri et al. 1999, Coelho et al. 2000), but also in *V.*

anguillarum and *V. harveyi*, i.e. pathogens of marine animals (Zhang et al. 2001). In *V. tapetis*, the presence of hemolysin activity was described by Borrego et al. (1996b). Some classes of hemolysin are responsible for cytotoxic activities, such as cell vacuolation, apoptotic cell death or morphological changes in cultured cell lines (Fabbri et al. 1999, Coelho et al. 2000, Figueroa-Arredondo et al. 2001, Naim et al. 2001).

Our observation of hemocyte-rounding caused by *Vibrio tapetis* leads us to believe that this effect is directly or indirectly related to rearrangement of the cytoskeleton. A cell-rounding factor called NMDCY (non-membrane-damaging cytotoxin) is produced by clinical strains of *V. cholerae* Non-O1, usually in association with sporadic cases of gastroenteritis (Saha et al. 1996). This extracellular factor causes cell-rounding of the Chinese hamster ovary (CHO), and of Int 407 (human intestinal) and HeLa (human non-intestinal) cell lines without cell-membrane disruption and without cell death (Saha et al. 1996, Mitra et al. 1998, Basu et al. 1999). NMDCY toxin-treated cells exibited important changes in morphological and cytoskeletal structures. Furthermore, another toxin RTX, repeats in toxin, has been discovered in *V. cholerae*. This toxin is responsible for the rounding of epithelial cells associated with actin depolymerization (Lin et al. 1999, Fullner & Mekalanos 2000). Some bacterial toxins responsible for cytoskeleton perturbation have also been studied in other species such as *Clostridium difficile* (ToxA; Ernst 2000), *Salmonella typhimurium* (SptP; Fu & Galan 1998) and *Yersinia pestis* (Bliska 2000).

The importance of the cytoskeleton in phagocytosis is well known. All phagocytic processes are driven by a rearrangement of the actin cytoskeleton (May & Machesky 2001). Disruption of this rearrangement by a pathogen might shield it from being ingested and destroyed by the phagocytic cell. Additional experiments examining cytoskeleton polymerization and phagocytosis in clam hemocytes are necessary in order to better understand the process used by *Vibrio tapetis* to overcome the cellular defense of *Ruditapes philippinarum*.

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