

Pathogenicity of *Vibrio tapetis*, the etiological agent of brown ring disease in clams

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ABSTRACT: Brown ring disease (BRD) causes high mortalities in the introduced Manila clam *Ruditapes philippinarum* cultured in western Europe. The etiological agent of BRD, *Vibrio tapetis*, adheres to and disrupts the production of the periostracal lamina, causing the anomalous deposition of periostracum around the inner shell. Because the primary sign of BRD is found outside the soft tissues, the processes leading to death are not as obvious as those for internal pathogens. This study was designed to evaluate the pathogenicity of *V. tapetis*, in an attempt to help explain the mechanisms of mortality. We found high mortalities (up to 100%) for clams following the inoculation of *V. tapetis* into the extrapallial space (between mantle and inner shell) or the posterior adductor muscle of healthy *R. philippinarum*. Microscopy and immunological detection methods showed that the pathogen was rapidly eliminated from tissues and hemolymph of animals that survived the inoculation. In clams that died, the bacteria were found to have proliferated, resulting in severe tissue disruption. Bacteria were able to penetrate into tissues from the extrapallial space through the external epithelium of the mantle. In contrast, no mortalities were observed following injection of *V. tapetis* in the native European clam *Ruditapes decussatus*, which is resistant to BRD. This clam rapidly eliminated the bacterium from hemolymph and soft tissues. Clam mortality associated with BRD in the field is likely to result from the penetration of *V. tapetis* into the clam's extrapallial space through the disrupted periostracal lamina and from there into the soft tissues through the irritated mantle epithelium. Some bacteria also penetrate through the digestive epithelia. In either case, bacteria proliferate rapidly in the soft tissues, causing severe damage and subsequent death.

KEY WORDS: Bivalve · Bacteria · Mortality · Disease progression · Immunofluorescence · Enzyme-linked immunosorbent assay

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INTRODUCTION

In Europe, brown ring disease (BRD) affects the clam *Ruditapes philippinarum*, a species introduced for aquaculture in the 1970s. The disease was first recognized in 1987 in France, causing high mortalities and severe losses in cultured stocks. The etiological agent of BRD, a pathogenic bacterium named *Vibrio tapetis*, invades the clam's pallial (mantle) cavity, where it adheres to the periostracal lamina at the growing edge of the shell (Paillard et al. 1994, Borrego et al. 1996a).

Subsequent disruption of periostracal deposition allows the bacterium to penetrate into the extrapallial space and results in a brown organic deposit on the inner shell, which gives the disease its name. In severely diseased clams, *V. tapetis* has been detected using immunological methods within cells of the digestive diverticula (Plana 1995), in the extrapallial fluid (Allam et al. 2000b), and within the connective tissue of the mantle (Santamaria et al. 1995). These observations were associated with degeneration of the digestive diverticula and hypertrophy of the mantle (Plana & Le Pennec 1991, Paillard 1992, Plana et al. 1996). However, the process leading to the death of diseased

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clams is still not well understood and the relationship between the presence of the pathogen within tissues and clam death has not been established.

In experimental pathology, inoculation directly into tissues is a useful way to test the pathogenicity of microbes to mollusks (Tubiash 1971). This method allows the pathogen to avoid external defense factors of the host at the shell and epithelial levels (biological, physical, and chemical defenses). Once bacteria are present within host tissues, one of the following conditions can ensue: (1) bacteria are able to avoid the defense system of the host, proliferate, and, by means of their toxicity factors, threaten the vital processes of the host; or (2) the host is able to neutralize and eliminate bacteria using its humoral and cellular defense capacities (Chu 1988, Millar & Ratcliffe 1994). This elimination process is primordial in all invertebrates since it preserves animal integrity and restricts damages caused by microbes (Bayne 1983, Millar & Ratcliffe 1994).

Generally, the ability of the internal defense system of invertebrates to eliminate bacteria is inversely correlated with the pathogenicity of these microorganisms (Hartland & Timoney 1979, De La Pena et al. 1995). Thus, virulent strains proliferate in the circulatory compartment, whereas others are eliminated in relatively short time (a few hours to a few days). In the case of BRD, the injection of *Vibrio tapetis* directly into the clam's internal (soft tissue) and pseudo-internal (extrapallial, between mantle and inner shell) compartments could give information on the pathogenicity of this strain and on the efficiency of the neutralization and elimination mechanisms of the host.

This paper describes results obtained following injection of *Vibrio tapetis* into the posterior adductor muscle, the extrapallial space (between the shell and outer mantle epithelium), and the pallial (mantle) cavity of *Ruditapes philippinarum*. The parameters investigated were clam survival, *in vivo* bacterial clearance, *in vitro* phagocytosis, and damage observed in tissue sections. The pathogenicity of *V. tapetis* was compared with that of *V. anguillarum*, a pathogen of bivalve larvae. The response of the native clam *R. decussatus*, known to be highly resistant to BRD (Maes & Paillard 1992), was compared with that of *R. philippinarum*.

MATERIALS AND METHODS

Clams and bacteria. Adult (34.2 ± 0.8 mm) *Ruditapes philippinarum* and *R. decussatus* with no clinical signs of BRD were harvested from the Bay of Brest (France) and maintained in aerated marine aquaria at 14°C throughout the experiments. They were fed cultured algae daily throughout the study. *Vibrio tapetis* (ATCC

4600) had been previously isolated from diseased clams (isolate P16; Paillard et al. 1997). *V. anguillarum* (ATCC 12336) was kindly provided by the Micromer Laboratory (Brest, France). An unidentified non-Vibrionaceae bacterial strain (R2) frequently encountered in apparently healthy clams was isolated and used as a control in injection experiments to compare its effects with those of both *Vibrio* sp.

Injection experiments. Injection of different clam species with different bacterial species: Bacteria, cultured on marine agar (1 l distilled water, 15 g agar, 20 g sea salts [Sigma], 4 g peptone, 0.1 g $\text{Fe}(\text{PO}_4)_2$) and reaching the exponential phase of growth (typically 60 to 72 h at 20°C), were suspended in sterile seawater (SSW; filtered at 0.22 μm then autoclaved) and adjusted spectrophotometrically to about 5×10^8 colony-forming units (CFU) ml^{-1} . Four different batches of *Ruditapes philippinarum* ($n = 180$ clams batch^{-1}) were separately inoculated within the posterior adductor muscle with 0.1 ml (5×10^7 CFU) of different bacteria (live *Vibrio tapetis*, dead *V. tapetis*, live *V. anguillarum*, and live R2). Dead *V. tapetis* were obtained by heating the bacterial suspension to 60°C for 2 h. A fifth batch of 180 clams was injected with SSW as a control. Two batches of *R. decussatus* of 60 clams each were separately injected with live *V. tapetis* or SSW. Each batch of clams was maintained at 14°C in a separate aquarium at about 180 clams m^{-2} . Clams (10 *R. philippinarum* and 6 *R. decussatus*) were sampled at time intervals of 5 min, 2 h, 6 h, and 1, 3, 7, and 14 d following challenge. Hemolymph was withdrawn from the adductor muscle of each clam as previously described (Allam & Paillard 1998). Bacterial clearance from the hemolymph was measured using an enzyme-linked immunosorbent assay (ELISA) (see below). Dead clams were immediately removed and recorded. Cumulative mortality was calculated over the 2 week experiment from the number of remaining clams after each sampling.

Injection with different doses of *Vibrio tapetis*: Another experiment was performed to measure the effect of the dose of injected live *V. tapetis* on the survival of *Ruditapes philippinarum*. Six batches of clams ($n = 50$ clams batch^{-1}) were prepared. Three batches were injected with 0.1 ml of suspensions containing the following doses of bacteria: 10^9 CFU, 5×10^7 CFU, and 10^6 CFU clam^{-1} . A fourth batch was injected with SSW. The remaining 2 batches were injected with 10^9 or 10^6 CFU clam^{-1} , labeled according to dose, and incubated together in the same tank to see whether resulting mortality was strictly related to the inoculated dose of the pathogen or affected by bacteria in the surrounding seawater. Dead clams were immediately removed and counted, and cumulative mortality was calculated over the 2 wk experiment.

Injection with extracellular products of *Vibrio tapetis*: A culture of *V. tapetis* was grown in marine broth (1 l distilled water, 20 g sea salts [Sigma], 4 g peptone, 0.1 g $\text{Fe}(\text{PO}_4)_2$). Cells were harvested in the exponential phase, adjusted to about 5×10^8 CFU ml^{-1} , and divided into 2 subcultures. One of the subcultures was filtered through a 0.2 μm polycarbonate membrane. Culture filtrate, containing extracellular products of the pathogen, was inoculated (100 μl clam $^{-1}$) into the adductor muscle of 50 *Ruditapes philippinarum*. Another batch of 50 clams was injected with unfiltered culture (bacteria plus medium, 100 μl clam $^{-1}$). A control batch of 50 clams was injected with sterile culture broth (100 μl clam $^{-1}$). Dead clams were immediately removed and counted, and cumulative mortality was calculated over the 2 week experiment.

Injection within the extrapallial space: Five hundred *Ruditapes philippinarum* were separated into 10 equal batches of 50 clams each. The first batch was inoculated with live *Vibrio tapetis* in the extrapallial space as follows: a hole was made using a round dental burr in the central part of the shell, without cutting the mantle (Allam & Paillard 1998). Bacteria (5×10^7 CFU in 0.1 ml clam $^{-1}$) were injected into the extrapallial space between the mantle and the shell. The hole in the shell was then sealed with a piece of sterile glass coverslip and dental paste. The same *V. tapetis* inoculum was injected into the posterior adductor muscle or the pallial (gill) cavity of 2 additional batches. Similarly, 2 sets of control batches were made. The first set (3 batches) was inoculated with live cells of the non-pathogenic strain (R2, 5×10^7 CFU in 0.1 ml clam $^{-1}$) in the extrapallial compartment, the adductor muscle, or the pallial cavity, whereas the second set (3 batches) was injected with 0.1 ml SSW in to the same locations. The last batch was not treated to provide a non-disturbed control. Each batch was incubated in a separate tank. Dead clams were immediately removed and counted, and cumulative mortality was calculated over the 2 week experiment.

Detection of *Vibrio tapetis*. Histology: Histological analysis was designed to detect *V. tapetis* in tissues and bodily fluids of contaminated and control *Ruditapes philippinarum* and *R. decussatus*. (Allam et al. 1996a). Particular attention was paid to the examination of moribund and non-moribund clams in contaminated batches in order to attempt to understand the causes of clam death. After fixation in fresh aqueous Bouin's solution for 1 week, the tissues were dehydrated in a graded ethanol and xylene series, and embedded in paraffin. Paraffin sections (5 μm thick) were stained according to Brown & Hopps (1973) to detect Gram-positive and -negative bacteria or subjected to the immunofluorescence detection technique described below.

Indirect immunofluorescence: An indirect immunofluorescence method using an anti-*Vibrio tapetis* rabbit serum was used to identify *V. tapetis* in cytospin-prepared hemolymph smears and clam (*Ruditapes philippinarum* and *R. decussatus*) tissue sections (Allam et al. 1996a). Preparations were rinsed in 0.1 μM phosphate buffered saline (PBS; pH 7.4) and covered with a normal goat serum (20% in PBS, 30 min at room temperature). After rinsing with PBS, the primary antibody (rabbit anti-*V. tapetis*, 0.5% in PBS) was applied to the slides, which were placed in a moist chamber. After incubation for 1 h at 30°C, preparations were abundantly rinsed with PBS, then covered by 1% fluorescein isothiocyanate-conjugated goat anti-rabbit antibody in PBS containing 1% bovine serum albumin for 45 min at 30°C. The preparations were then rinsed 3 times with PBS and mounted in non-fluorescent microscopic oil before examination under epifluorescence illumination. Smears of both *V. tapetis* and other *Vibrio* sp. were subjected to the same treatments to constitute positive and negative controls, respectively.

ELISA: An ELISA was used to measure *Vibrio tapetis* burdens in the hemolymph of *Ruditapes philippinarum* and *R. decussatus* previously injected in the adductor muscle with various bacteria. Only clams that did not present signs of weakness (mainly difficulty in closing their valves) were analyzed. After hemolymph collection, plasma and cell pellets were immediately separated after gentle centrifugation (200 $\times g$ for 20 min at 4°C). Pellets were rinsed 3 times with an EDTA-PBS (1 l distilled water, 14.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.6 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 25 g NaCl, 10 g EDTA, pH 7.4) to detach adhered bacteria from the hemocytes. Cells were then lysed by adding Triton X-100 (0.1% in SSW). The bacterial burdens in the plasma and the cell lysate were determined using an ELISA previously developed for the detection of *V. tapetis* (Noël et al. 1996) and modified by Allam (1998). Samples were dispensed in triplicate into wells of 96 well microtiter plates and incubated overnight at 4°C. Unbound material was removed by washing the wells 5 times with PBS. Then PBS containing 1% Tween and 1% bovine serum albumin was added to the wells and the plates were incubated for 1 h at 37°C. After abundant rinsing with PBS, mouse anti-*V. tapetis* monoclonal antibody 18F10 (Noël et al. 1996) was added to each well and incubated for 1.5 h at 37°C. Plates were then washed 3 times with PBS, and the secondary antibody (goat anti-mouse immunoglobulins linked with biotin) was added and incubated for 1.5 h at 37°C. Plates were then washed as before and incubated with ExtrAvidine (Sigma) labeled with horseradish peroxidase for 1.5 h at 37°C. After rinsing 3 times with PBS, 100 μl of the substrate solution (0.067% *o*-phenyldiamine di-HCl in citric acid-phosphate buffer (pH 5) added with 0.1%

H₂O₂) was added. The reaction was allowed to proceed for 15 min at room temperature and then stopped by adding 100 µl of sulfuric acid (2 N) to each well. The optical density of each sample was read at 492 nm using a microplate reader and related to a standard curve made with known concentrations of *V. tapetis*.

In vivo phagocytosis of *Vibrio tapetis*. *V. tapetis* was grown in marine broth for 12 h, then labeled with the vital dye cyanoditoyl tetrazolium chloride (CTC; 1 mM) as previously described (Allam et al. 1996b). Approximately 5×10^7 labeled bacteria in 100 µl SSW were injected into the posterior adductor muscle (n = 70) or the extrapallial space (n = 36) of each *Ruditapes philippinarum*. Control animals were injected with SSW (n = 34 and 18, respectively). Test and control clams were maintained in marine aquaria and sampled at intervals from 5 min to 7 d. Hemolymph and extrapallial fluid were withdrawn and immediately fixed (v/5v) in formalin-SSW solution (3% formalin final concentration). Hemocytes and fluorescent bacteria were counted using a Coulter EPICS C flow cytometer (Model 752) equipped with an argon laser (emission at 488 nm). Forward light scatter (FLS) and red fluorescence signals were collected for each of 10 000 particles. Free bacteria (red fluorescent particles having a low FLS) and bacteria-associated hemocytes (red fluorescent, high FLS) were gated electronically and their percentage was calculated.

RESULTS

Adductor-muscle injection

Effect of different bacteria

Dead *Ruditapes philippinarum* appeared after the 5th day post inoculation in the group injected with *Vibrio tapetis* in their posterior adductor muscle (Fig. 1). In this batch, which had been injected with 5×10^7 CFU clam⁻¹, the percentage of dead clams increased rapidly until Day 10, then stabilized and was about 57% at Day 14. In contrast, mortality in clams injected with *V. anguillarum* was about 15% at Day 14, and final mortality was less than 6% in *R. philippinarum* injected with dead *V. tapetis*, the non-pathogenic strain R2, or SSW. Injection of *R. decussatus* with live *V. tapetis* did not induce any deaths. BRD signs were not observed in any of the challenged or control batches of either clam species.

Effect of *Vibrio tapetis* dose

There was a clear dose-response effect in *Ruditapes philippinarum* injected with *Vibrio tapetis* (Fig. 2).

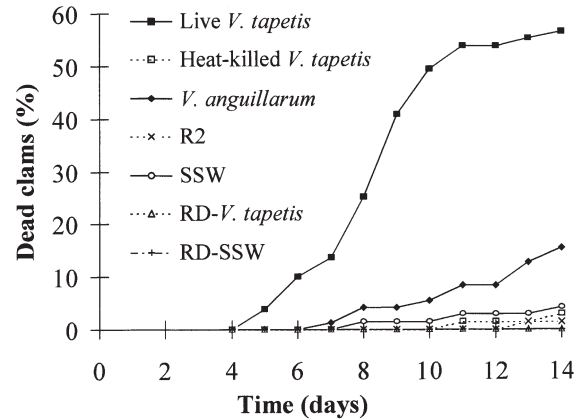


Fig. 1. *Ruditapes philippinarum*. Cumulative mortality following injection of different bacterial strains into the posterior adductor muscle. R2: non-Vibrionaceae bacterial strain; RD-SSW: *R. decussatus* injected with sterile seawater; RD-*V. tapetis*: *R. decussatus* injected with live *Vibrio tapetis*

Cumulative mortality after 2 wk reached a mean of about 80% in clams injected with 1×10^9 CFU ind.⁻¹, 48% in those inoculated with 5×10^7 CFU ind.⁻¹, and only 2% in those injected with 1×10^6 CFU ind.⁻¹, including the batch that had been incubated in the same aquaria as clams inoculated with 1×10^9 CFU ind.⁻¹ (Fig. 2). Mortality of clams injected with SSW was zero. None of the clams developed BRD signs.

Effect of extracellular factors

Ruditapes philippinarum inoculated with whole culture of *Vibrio tapetis* displayed about 60% mortality at

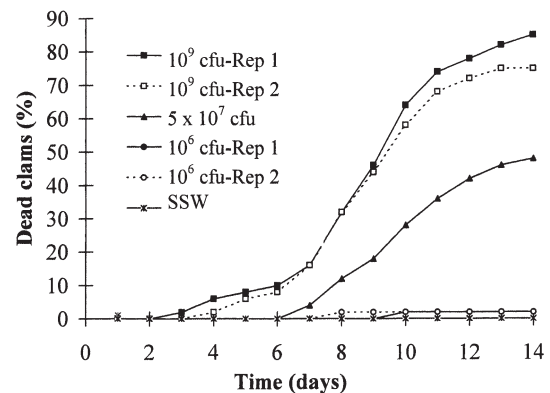


Fig. 2. *Ruditapes philippinarum*. Cumulative mortality following injection of different doses of *Vibrio tapetis* into the posterior adductor muscle. *R. philippinarum* of the second repetition (Rep) injected with 10^9 colony-forming units (CFU) ind.⁻¹ (10^9 CFU-Rep 2) and those inoculated with 10^6 CFU ind.⁻¹ (10^6 CFU-Rep 2) were incubated in the same aquaria. SSW: sterile seawater

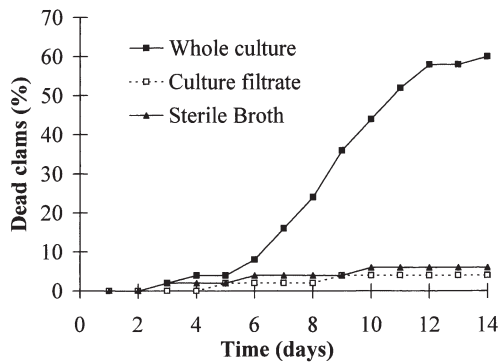


Fig. 3. *Ruditapes philippinarum*. Cumulative mortality following the injection into the posterior adductor muscle of different fractions of a culture of *Vibrio tapetis* in marine broth

Day 14 (Fig. 3). In contrast, clams injected with culture filtrate and sterile culture broth experienced only 4 and 6% mortality, respectively.

Comparison of adductor muscle, extrapallial space, and pallial cavity injection

The first dead *Ruditapes philippinarum* appeared at day 2 and day 4 in the batches injected with *Vibrio tapetis* within the adductor muscle and the extrapallial compartment, respectively (Fig. 4). Cumulative mortality increased rapidly, reaching 100% at Days 12 and 14, respectively. Mortality did not exceed 8% in the other test and control batches, including those injected in the pallial cavity. Clinical signs of BRD appeared

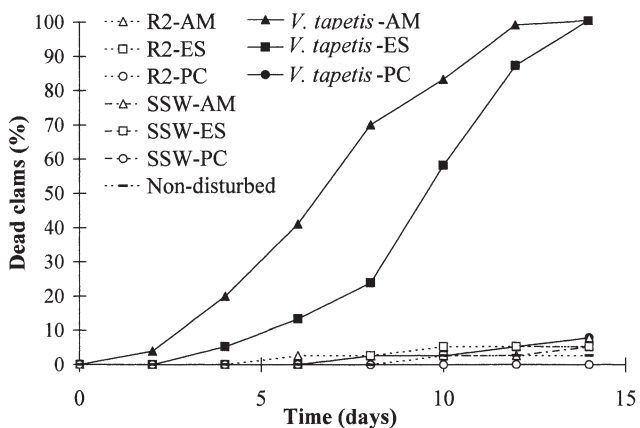


Fig. 4. *Ruditapes philippinarum*. Cumulative mortality following the inoculation of *Vibrio tapetis*, the non-Vibrionaceae strain (R2), or sterile seawater (SSW) into the adductor muscle (AM), the extrapallial space (ES) or the pallial cavity (PC) of *R. philippinarum*. The 10th batch was not injected to provide a non-disturbed control

only in clams inoculated with *V. tapetis* within the pallial cavity. The percentage of clams showing BRD signs was 42% at Day 3 and 92% at Day 14.

Bacterial burdens in body fluids

Plasma burdens measured by ELISA

As measured by the ELISA assay, bacterial densities in the plasma of *Ruditapes philippinarum* injected with live or dead *Vibrio tapetis* had decreased to less than 0.01% of the inoculum within 2 h of injection, although there was considerable individual variability (Fig. 5a,b). Much of this apparent disappearance was undoubtedly due to dilution within the circulatory system. After

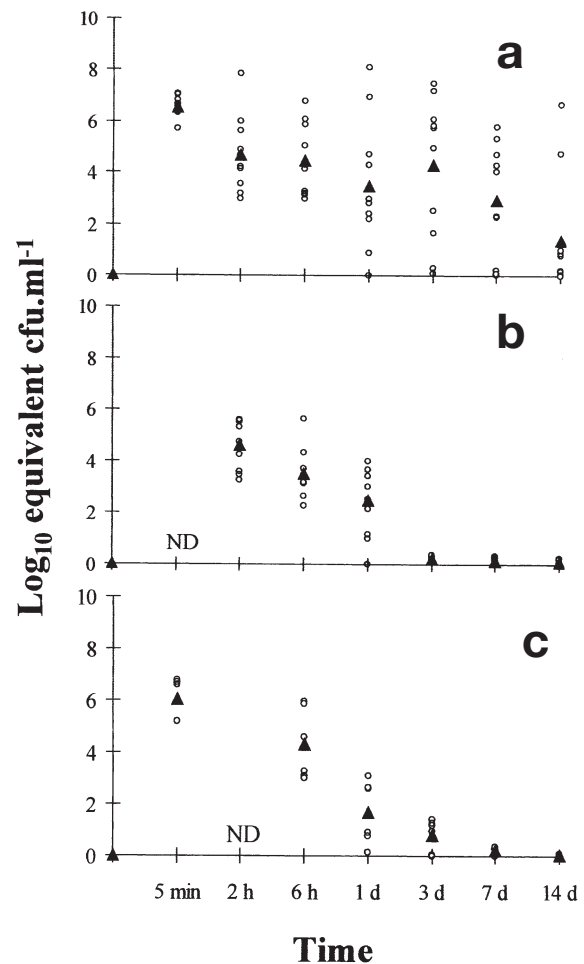


Fig. 5. Bacterial burdens, detected by enzyme-linked immunosorbent assay, in the plasma of individual *Ruditapes philippinarum* ($n = 10$ ind. sample⁻¹) injected in the adductor muscle (a) with live *Vibrio tapetis* and (b) heat-killed *V. tapetis*, and (c) of *R. decussatus* ($n = 6$ ind. sample⁻¹) injected with live *V. tapetis*. Solid triangles represent geometric means. ND: not determined

24 h, distinct differences between clam species, and between live and killed bacteria, were apparent (Fig. 5a–c). The mean density in the hemolymph of *R. philippinarum* injected with live *V. tapetis* was an order of magnitude greater than that of the same species injected with dead bacteria or of that in *R. decussatus* injected with live *V. tapetis*. Bacterial densities in *R. philippinarum* injected with live bacteria remained stable between Days 1 and 3, then decreased progressively until the end of the experiment. However, in 2 of 10 clams, the pathogen was completely eliminated by Day 3, and 5 of 10 clams had lost all detectable *V. tapetis* by Day 14 (Fig. 5a). In contrast to the persistence of live bacteria in *R. philippinarum*, detectable heat-killed bacteria had disappeared from the plasma by Day 3 (Fig. 5b). Live bacteria were cleared from the plasma of *R. decussatus* at approximately the same rate; very low densities were found at Day 3 and none thereafter (Fig. 5c).

Phagocytosis and adherence measured by ELISA

The number of *Vibrio tapetis* detected using ELISA in hemolymph cell lysates of *Ruditapes philippinarum* inoculated with live bacteria followed the same pattern as in the plasma but with a time shift in the appearance of maximum quantities. Bacteria were found in the lysates within a few minutes of injection (Fig. 6). Live *V. tapetis* associated with hemocytes increased with time and reached maximum values 2 to 6 h following challenge (Fig. 6a). The pathogen was still detected in hemocyte lysates from 7 of 10 clams at Day 7, and from 5 of 10 clams at Day 14. Positive responses were no longer detected by Day 7 in clams injected with heat-killed *V. tapetis* (Fig. 6b). The pathogen was not detected in cell lysates from the native species *R. decussatus* by 3 d post challenge (Fig. 6c).

Phagocytosis and adherence measured by flow cytometry

In hemolymph, the uptake of CTC-labeled *Vibrio tapetis* by *Ruditapes philippinarum* hemocytes, as measured by flow cytometry, was observed only a few minutes following the injection (Fig. 7a). The percentage of cells with ingested or adhering bacteria was about 20% at 30 min, then decreased to zero by Day 3. The number of bacteria not associated with hemocytes decreased rapidly during the first hours following injection and none were detected by Day 3 (Fig. 7b).

In the extrapallial fluid, the percentage of hemocytes with ingested or adhering bacteria was already about 60% 2 h following challenge (Fig. 7c). It decreased

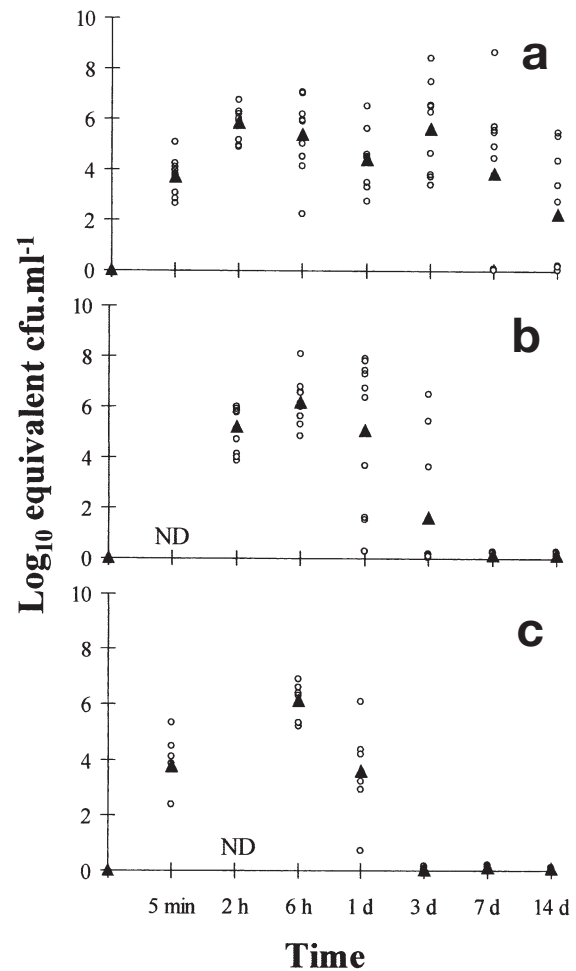


Fig. 6. Bacterial burdens, detected by enzyme-linked immunosorbent assay, in hemocyte lysates of individual *Ruditapes philippinarum* ($n = 10$ ind. sample⁻¹), (a) injected in the adductor muscle with live *Vibrio tapetis* and (b) heat-killed *V. tapetis*, and of *R. decussatus* ($n = 6$ ind. sample⁻¹), (c) injected with live *V. tapetis*. Solid triangles represent geometric means. ND: not determined

rapidly to 14% after 24 h, to 7% at Day 3, and to zero at Day 7. The number of free bacteria also decreased rapidly and had completely disappeared 7 d following challenge (Fig. 7d). No fluorescent particles were detected in hemolymph or extrapallial fluid of clams injected with SSW.

Microscopic detection of *Vibrio tapetis*

Numerous Gram-negative bacteria were observed in histological sections of moribund *Ruditapes philippinarum* that had been inoculated with live *Vibrio tapetis* in the adductor muscle or extrapallial space. These bacteria were identified as *V. tapetis* using immunofluorescence (Fig. 8). Clams that had received

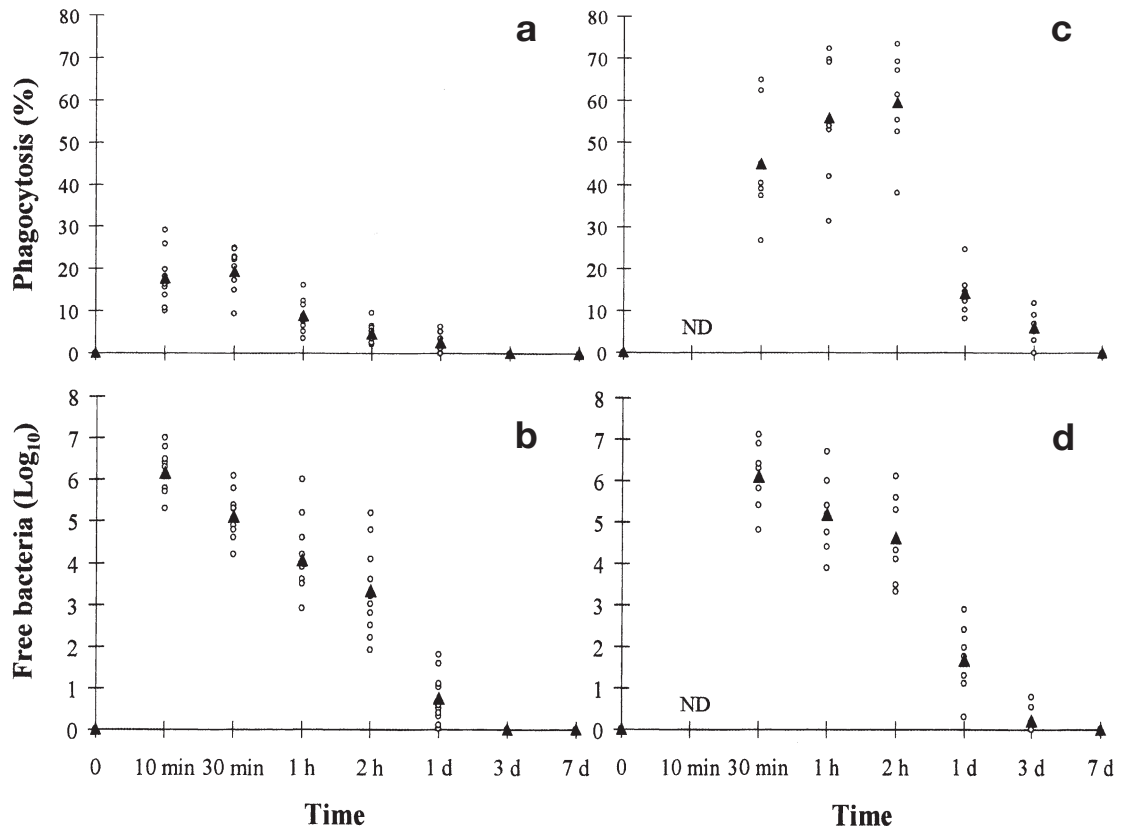


Fig. 7. Clearance of dye cyanoditolyl tetrazolium chloride (CTC)-labeled *Vibrio tapetis* in *Ruditapes philippinarum* injected in the adductor muscle (a and b, $n = 10$ ind. sample⁻¹) or the extrapallial space (c and d, $n = 6$ ind. sample⁻¹). a and c: percent phagocytosis by hemocytes; b and d: free bacteria (log_{10} bacteria ml^{-1}) in hemolymph and extrapallial fluid, respectively. Solid triangles represent geometric means. ND: not determined

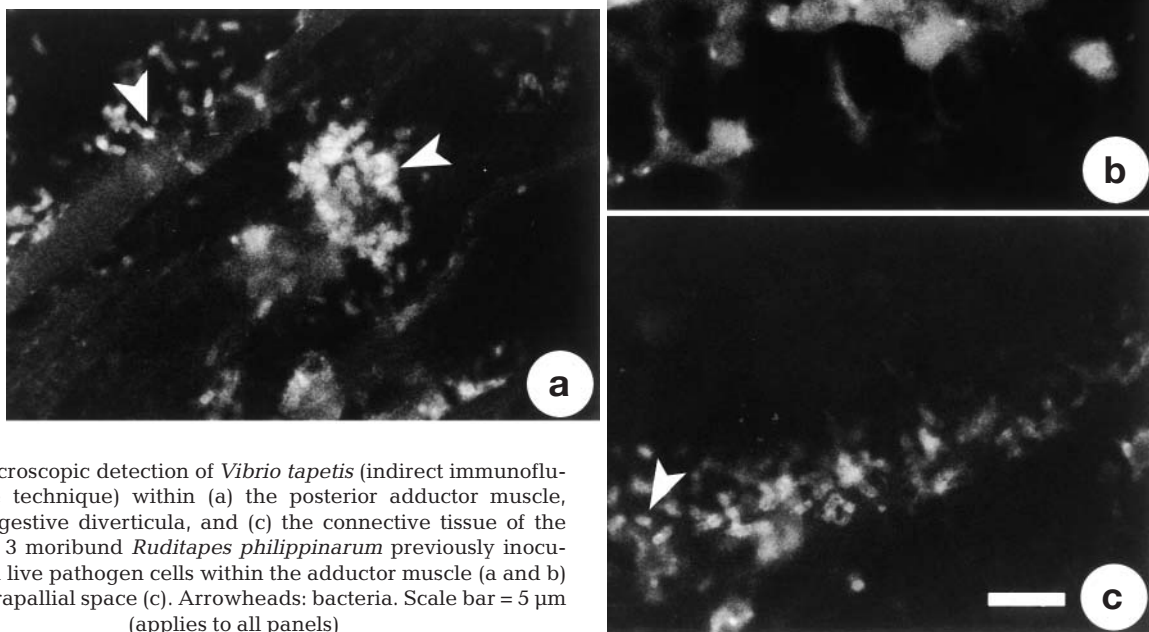


Fig. 8. Microscopic detection of *Vibrio tapetis* (indirect immunofluorescence technique) within (a) the posterior adductor muscle, (b) the digestive diverticula, and (c) the connective tissue of the mantle of 3 moribund *Ruditapes philippinarum* previously inoculated with live pathogen cells within the adductor muscle (a and b) or the extrapallial space (c). Arrowheads: bacteria. Scale bar = 5 μm (applies to all panels)

an injection into the muscle showed numerous *V. tapetis* not only in the sinuses between muscle filaments (Fig. 8a) but also inside epithelial and subepithelial cells of the digestive diverticula (Fig. 8b) and gills and within the connective tissue of the mantle (Fig. 8c). Hemolymph smears from moribund clams contained a high number of *V. tapetis* and much cell debris. In clams injected with live *V. tapetis* in the extrapallial space (between the mantle and shell), the epithelium of the mantle was disrupted and eroded. The underlying connective tissue was particularly affected (Fig. 8c). In these moribund animals, numerous *V. tapetis* were also observed in the digestive diverticula and gills. In addition, pathogens were abundant in the extrapallial fluid. Very few *V. tapetis* were detected in sections of 2 out of 10 individual non-moribund clams; no bacteria were detected in the remaining clams.

Extremely high numbers of *Vibrio tapetis* were found in the connective tissue of various organs, especially the mantle and digestive diverticula, of dead clams that had been injected with live *V. tapetis* into the muscle or the extrapallial space, and extensive lysis and necrosis of tissues were observed.

Vibrio tapetis was not detected in clams injected with *V. anguillarum*, R2, or SSW, including the few dead or moribund clams present in these batches.

DISCUSSION

To counter pathogens, bivalves have physical barriers, such as the epithelium, and biological factors, including the internal defense system, to combat pathogens that breach external barriers. Indeed, the success or failure of the host in neutralizing such invading microorganisms depends on both the virulence of the pathogens and the performance of the internal defense factors (Martinez-Manzanares et al. 1991). Results presented here show that both types of defense mechanisms are involved in fighting the etiological agent of BRD, *Vibrio tapetis*. Histopathological changes and mortalities of the introduced clam, *Ruditapes philippinarum*, reached 100% in groups injected directly in the adductor muscle or in the extrapallial space, whereas animals injected in the pallial cavity suffered insignificant mortality. These results indicate the importance of the epithelium and the pallial muscle attachment as external barriers. The comparison between BRD-susceptible *R. philippinarum* and the more resistant native clam *R. decussatus* illustrates the importance of the internal defense system in combating bacteria that are able to breach the external barriers. *R. philippinarum* often failed to eliminate injected *V. tapetis*, allowing the pathogen to proliferate

within its tissues and to kill it following inoculation into the adductor muscle or the extrapallial space. In contrast, *R. decussatus* successfully eliminated injected pathogens within a few days. The injection in *R. philippinarum* of heat-killed bacteria and extracellular extracts of *V. tapetis* did not cause death, showing that clam death is related to the activities of live bacteria rather than extracellular virulence factors alone.

BRD is one of the few reported bacterial diseases of marine bivalves. Adult bivalves rarely succumb to bacterial infection in nature; however, experimental challenges resulting in high mortality have been reported for many species: pearl oysters *Pinctada maxima*, injected in the pallial cavity or the extrapallial space with *Vibrio harveyi* or *Pseudomonas putrefaciens* (Dybdahl & Pass 1985, Pass et al. 1987); oysters *Crassostrea gigas* or *C. virginica*, immersed in or injected in the pallial cavity with *Nocardia crassostreae*, *V. salmonicida* or *Aeromonas* sp. (Friedman & Hedrick 1991, Olafsen et al. 1993, Friedman et al. 1998); clams *Mya arenaria*, and oysters *C. virginica*, injected directly in their soft tissues with *V. anguillarum* (Tubiash 1971, Tubiash et al. 1973); and mussels *Dreissena polymorpha*, injected with *Aeromonas* sp. (Maki et al. 1998). The deaths of bivalves in these experiments were associated with massive invasion of tissues and hemolymph by the bacteria. As in our study, the experimental conditions (injection of high bacterial doses, direct injection in tissues or extrapallial space, shell damage) were abnormal. Other studies underscore the resistance of adult bivalves when the contact with bacteria was under more 'normal' conditions such as immersion, even in high concentrations of bacteria, and even when strains known to be pathogenic for larvae are used (e.g. *V. anguillarum*, *V. alginolyticus*, several other *Vibrio* sp., and *Aeromonas* sp.) (Tubiash et al. 1965, Martin 1976). Similarly, the injection of *V. tapetis* into the pallial cavity of *Ruditapes philippinarum* does not induce death, although it causes the development of BRD signs (Paillard et al. 1994, Allam et al. 2000a, present study). The ability of many bacteria to induce death when external barriers are breached suggests that these barriers are an important element in the ability of adult bivalves to resist bacterial diseases.

In many challenge studies, the delivery of very high numbers of bacteria to experimental animals also causes disease and death. In our investigations, mortalities of clams were directly related to the injected dose of *Vibrio tapetis*. According to Sindermann (1990), the outcome of microbial infections is related to the balance between infection pressure of pathogens and the neutralization processes of the host. According to this author, successful establishment of a microbial infection generally results when a pathogen (1) survives the phagocytosis process, (2) develops mecha-

nisms to avoid phagocytosis, or (3) simply overwhelms the host by its capacity to proliferate faster than it can be eliminated. The relationship between clam mortalities and the inoculated doses of *V. tapetis* (2% and 80% mortality for doses of 1×10^6 CFU and 1×10^9 CFU ind.⁻¹, respectively) is in accordance with Sindermann's third hypothesis of an overwhelming number of pathogens that saturate the host's defense capacity. When the number of inoculated bacteria is low, most clams, even the susceptible *Ruditapes philippinarum*, are able to eliminate pathogens from soft tissues and hemolymph and repair damage caused by the infection. Conversely, when clams are injected with large quantities of bacteria, their defense system is simply unable to cope with the overwhelmingly large number of *V. tapetis*, which cause irreversible damage that leads to death. *In vitro* studies support this argument: during *in vitro* contact between various *Vibrio* sp. and mussel hemocytes, Lane & Birkbeck (1999) and Nottage & Birkbeck (1990) showed that the percentage of hemocytes killed increased significantly as the ratio of bacteria to hemocytes increased. Similar results were also observed during a preliminary study on *in vitro* interactions between *R. philippinarum* hemocytes and *V. tapetis* (B. Allam unpubl.).

Virulence factors allow pathogens to invade and proliferate in their hosts, and are an important cause of disease and mortality (Toranzo et al. 1983, Ellis 1991). In pathogenic *Vibrio* sp., virulence factors include adhesins, proteinases, endo- and exotoxins (hemolysins, cytotoxins, ciliostatic factors, etc.), and plasmids coding for iron chelators (Horne 1982, Toranzo et al. 1983, Moustafa et al. 1984, Nottage & Birkbeck 1986). *V. tapetis* factors include hemolysins, cytotoxins, exotoxins, and plasmids (Borrego et al. 1996b, Castro et al. 1997). *In vitro* experiments have shown that *V. tapetis* cells are able to kill cultured fish cells and hemocytes of *Ruditapes philippinarum* (Borrego et al. 1996b, Lane 1997, B. Allam unpubl.). Nevertheless, our results show that extracellular virulence factors alone are not involved in clam mortality; rather, the intact bacterium was needed to cause death. In addition to the production of toxic molecules, several other virulence factors are associated with intact *V. tapetis*. For instance, the bacterium displays a high adhesion capacity associated with the presence of pili (Paillard et al. 1994). Further, the cell wall contains smooth lipopolysaccharides that, according to Bradley (1979), play an important role in virulence by helping the penetration of pathogens into host tissues. They also allow bacteria to better resist the defense system of clams, particularly phagocytosis by hemocytes (Borrego et al. 1996b). In the present study, we noted the persistence of bacteria in clams without obvious signs of weakness (clams sampled for ELISA) and found high concentrations,

with severe pathological changes, in moribund clams. The histological results suggest that host death was the result of damage inflicted on host tissues, including the hemocyte components of the defense system, by high numbers of *V. tapetis*.

Given the ability of *Vibrio tapetis* to proliferate rapidly in the tissues of *Ruditapes philippinarum* and its failure to do so in *R. decussatus*, it is instructive to compare the internal defense system activities of the 2 species, starting with bacterial clearance rates. In the present study, only 2 of 10 *R. philippinarum* had cleared all detectable *V. tapetis* from their plasma by Day 3 post challenge, while all of the *R. decussatus* had done so. In fact, half the *R. decussatus* had no detectable bacteria after only 1 d. Using ELISA and flow cytometry, our experiments quantifying the uptake of *V. tapetis* by hemocytes suggest that phagocytosis is the major process used by clams to eliminate bacteria from their hemolymph. Hemocytes of both clam species ingested the bacteria within minutes of injection; however, those of *R. decussatus* were much more effective in killing or otherwise eliminating the bacteria than were hemocytes of *R. philippinarum*. Half of the latter still had bacteria associated with hemocytes 14 d after injection, whereas no bacteria were detected in hemocyte lysates of *R. decussatus* after only 3 d. In fact, bacteria disappeared from the plasma of live *V. tapetis*-injected *R. decussatus* as rapidly as they did from *R. philippinarum* injected with dead bacteria. Non cellular factors, such as lectins (or agglutinins), present in bivalve plasma may also play a role in the clearance process. They are involved in non-self recognition and opsonization and display differential reactivity toward various bacterial strains (Tamplin & Fisher 1989, Olafsen et al. 1993, Olafsen 1996). The presence of such agglutinins in *R. philippinarum* has not yet been shown, although clumped *V. tapetis* have been observed microscopically in hemolymph smears. Further, *in vitro* phagocytosis of *V. tapetis* by *R. philippinarum* and *R. decussatus* hemocytes do not seem to be affected by the presence or the absence of soluble hemolymph proteins of each species (Lopez-Cortes et al. 1999).

The differences in phagocytic and clearance rates between the 2 clam species disappeared when heat-killed or CTC-labeled bacteria were injected. These were rapidly and completely eliminated from hemolymph and extrapallial fluid of *Ruditapes philippinarum*, without causing significant mortality. We previously showed that CTC significantly inhibits the mobility and the growth of *Vibrio tapetis* (Allam et al. 1996b). Thus, *R. philippinarum* had no trouble eliminating weak or dead *V. tapetis* from its tissues. These results emphasize the specificity of interactions between *V. tapetis* with *R. philippinarum* or *R. decussatus*.

The molluscan digestive tract is often considered to be the major portal of entry for many pathogens, which accumulate in the connective tissue surrounding digestive organs and can subsequently proliferate in the host and provoke death (Lauckner 1983). In the case of BRD-affected clams, the extrapallial space may constitute an additional entry point. This compartment is bacteriologically sterile in healthy animals (Allam & Paillard 1998) but contains *Vibrio tapetis* cells in heavily diseased clams (Allam et al. 2000b). The extrapallial space of clams is physically separated from the pallial cavity and the external medium by a periostracal layer, secreted by the mantle edge and extending to the outer edge of the shell. A further barrier to the central extrapallial compartment is formed by the attachment of the pallial muscle. *V. tapetis* that enter the pallial cavity adhere to and colonize the periostracal lamina of *Ruditapes philippinarum*. The normal deposition process is disrupted and the periostracal lamina becomes severely disorganized, resulting in the characteristic organic deposit ringing the inner shell. The disruption of the periostracal membrane allows penetration of bacteria into the peripheral extrapallial space, from which they enter the central extrapallial space after disruption of the pallial attachment. Once in the extrapallial space, as the present study shows, *V. tapetis* can readily penetrate the external mantle epithelium of *R. philippinarum* and proliferate to lethal densities in the soft tissues. A similar disease progression was described during vibriosis in larvae of the eastern oyster *Crassostrea virginica* (Elston & Leibovitz 1980) and in juvenile Pacific oysters *C. gigas*, affected by extrapallial abscesses caused by an unknown bacterium (Elston et al. 1999). In addition, *V. tapetis*, abundant in the pallial cavity fluid of diseased animals (Maes 1992, Allam et al. 1996a), is likely to be ingested in large quantities and may also penetrate into the soft tissues through digestive epithelia, especially of the digestive diverticula. *R. philippinarum* experimentally injected in the pallial cavity with *V. tapetis* develop clinical signs of BRD, indicating that the bacteria have entered the extrapallial space, but they rarely die (Paillard et al. 1994, present study). Thus, the ability of clams to control *V. tapetis* at the extrapallial fluid level appears to be a major factor in the outcome of the disease process. The importance of this site is highlighted by the very high early phagocytic rates of *V. tapetis* injected into the extrapallial space. An infection that remains 'controlled' within the extrapallial space is not lethal even though an extensive brown ring may be deposited. For this disease, bacterial elimination from the hemolymph is a second line of defense. The development of BRD signs in *R. decussatus* injected in the pallial cavity is much less than in *R. philippinarum* (Paillard et al. 1994). The resistance of *R. decussatus* to BRD and subsequent death (Maes &

Paillard 1992, Allam 1998) is primarily the result of a strong barrier to infection at the periostracal membrane level and efficient neutralization of any *V. tapetis* that enter the extrapallial cavity; however, any bacteria that might penetrate the soft tissues would also encounter a very effective internal defense system.

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