Changes of gill and hemocyte-related bio-indicators during long term maintenance of the vent mussel Bathymodiolus azoricus held in aquaria at atmospheric pressure

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A B S T R A C T

The deep-sea hydrothermal vent mussel Bathymodiolus azoricus has been the subject of several studies aimed at understanding the physiological adaptations that vent animals have developed in order to cope with the particular physical and chemical conditions of hydrothermal environments. In spite of reports describing successful procedures to maintain vent mussels under laboratory conditions at atmospheric pressure, few studies have described the mussel's physiological state after a long period in aquaria. In this present study, we investigate changes in mucocytes and hemocytes in B. azoricus over the course of several months after deep-sea retrieval. The visualization of granules of mucopolysaccharide or glycoprotein was made possible through their inherent auto-fluorescent property and the Alcian blue-Periodic Acid Schiff staining method. The density and distribution of droplets of mucus-like granules was observed at the ventral end of lamellae during acclimatization period. The mucus-like granules were greatly reduced after 3 months and nearly absent after 6 months of aquarium conditions. Additionally, we examined the depletion of endosymbiont bacteria from gill tissues, which typically occurs within a few weeks in sea water under laboratory conditions. The physiological state of B. azoricus after 6 months of acclimatization was also examined by means of phagocytosis assays using hemocytes. Hemocytes from mussels held in aquaria up to 6 months were still capable of phagocytosis but to a lesser extent when compared to the number of ingested yeast particles per phagocytic hemocytes from freshly collected vent mussels. We suggest that the changes in gill mucopolysaccharides and hemocyte glycoproteins, the endosymbiont abundance in gill tissues and phagocytosis are useful health criteria to assess long term maintenance of B. azoricus in aquaria. Furthermore, the laboratory set up to which vent mussels were acclimatized is an applicable system to study physiological reactions such as hemocyte immunocompetence even in the absence of the high hydrostatic pressure found at deep-sea vent sites.

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1. Introduction

The hydrothermal vent mussel Bathymodiolus azoricus is commonly found in dense populations around vents on the Mid-Atlantic Ridge and south of the Azores. Other Bathymodiolid species are also found in dense communities associated with deep-sea hydrothermal vents or cold-water sulfide/hydrocarbon seeps throughout the Atlantic, Pacific and Indian oceans, which reflects the functional adaptability of the genus to these extreme environments (Von Cosel et al., 1999; Gustafson et al., 1998).

The presence of both thiotrophic and methanotrophic bacterial symbionts in specialized epithelial cells of B. azoricus gill tissues is believed to provide substantial nutritional advantage to the mussel allowing it to obtain energy from both sulfide and methane at the vent sites (Distel et al., 1995; Fiala-Médioni et al., 2002; Duperron et al., 2006). Moreover, the ability of housing symbiotic intracellular bacteria appears to be a general feature among bivalves which have adapted very efficiently to living from organic matter generated from chemosynthetic processes (Fiala-Médioni and Felbeck, 1990). In spite of detailed studies describing the intracellular co-existence of methanotrophic and thiotrophic bacteria in gill bacteriocytes (Distel et al., 1995; Fiala-Médioni et al., 2002; Duperron et al., 2006), little is known concerning the fate of endosymbiont bacteria while vent mussels are maintained under experimental conditions in acclimatized aquaria. In the absence of adequate methane and sulfide supply, it is assumed that the number of endosymbiont bacteria is frequently reduced until apparent total disappearance from gill tissue of mussels.
maintained for at least 2–3 weeks in plain sea water and exposed to atmospheric pressure. Nonetheless, there is evidence that some endosymbiotic bacteria remain in gill tissues after months in sea water aquaria supplemented with methane and sulfide and subjected to atmospheric pressure conditions (Dando et al. and Colaço et al. unpublished results).

The maintenance of live vent mussels in our laboratory has been a key factor in gaining insights into the physiology of vent mussels. It has prompted us to investigate cellular and molecular mechanisms of adaptation in *B. azoricus* during long term post-capture aquaria conditions (Kadar et al., 2005; Bettencourt et al., 2007). The presence of gill-associated mucopolysaccharides, changes in hemocyte glycoproteins, endosymbiotic content of gill tissues and phagocytosis were selected as biological indicators of *B. azoricus* physiological state under long term aquarium conditions. The decrease of dense droplets of mucus-like granules at the ventral end of lamellae of gill filaments transverse sections was followed over a period of 6 months. In addition, the immunocompetence of *Bathymodiolus* hemocytes was assessed by phagocytosis after long term maintenance in aquarium conditions at atmospheric pressure.

2. Methods and materials

2.1. Animal collection, maintenance, feeding regime and tissue preparation

The present study was carried out with mussels collected from the hydrothermal vent field Menez Gwen (37°50.8–37°51.6N; 31°30–31°31.8W), on the Mid-Atlantic Ridge (MAR), with the French R/V *Pourquoi pas?* using the ROV Victor 6000 (MoMARETO cruise (August 6th–September 6th 2006). Some mussels were placed in cages over the vents and recovered by acoustic release at intervals between

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Fig. 1. Detection of methanotrophic and thiotrophic bacterial endosymbionts in gill tissues. Fluorescent probes were used to target methanotrophic (panel A, upper panels) and thiotrophic (panel A, lower panels) bacterial symbionts in fluorescence in-situ hybridization (FISH) experiments. Differential interference contrast (DIC) visualization is shown (panel A, left upper and lower panels). Elliptical lines indicate the endosymbionts confinement to the bacteriocytes (specialized endothelial cell) boundaries. Scale bar (panel A, 5 μm) and original objective magnifications are indicated. Panel B represents images of FISH experiment showing the variation of methanotrophic bacterial density in animals maintained for 4 weeks in plain sea water and at atmospheric pressure. Gill samples were analyzed at weekly intervals (i–iv, scale bar 10 μm).
October 2006 and May 2007. In the LabHorta aquarium, mussels were kept at 7–8 °C in plastic containers filled with 20 L of sea water and aerated to give an oxygen saturation of 10–50%. The stocking density was 1 animal/L of sea water, which was changed every other day to keep a pH of 7–8 (Kadar et al., 2005). Each container was supplied every other day, between sea water replenishments, with 5 mL of a food mixture consisting of freeze dried ocean plankton enriched with vitamins (Ocean Plankton, Hikari BIO-PURE® FD). The mixture was prepared by suspending 6 g of freeze dried product in 40 mL of sterile sea water and homogenized with a sample preparation homogenizer (Heidolph Instruments, Germany). Gill tissues were dissected immediately after removing the animals from aquaria at intervals and preserved for histological observations according to standard protocols using 10% buffered formalin and 70% ethanol solutions. As with hemocytes, gills were also used fresh, in conformity with cellular studies. Gill tissues were sectioned through the ventral end of gill filaments, showing their frontal surface.

2.2. Detection of carbohydrate moieties in gill filaments and hemocytes of B. azoricus

The combined Alcian blue and Periodic-Acid Schiff (PAS) staining method (Woods and Ellis, 1994–96) was utilized to determine the distribution of glycoproteins in paraffin tissue sections of gills and in hemocytes of B. azoricus. In brief, after deparaffinization and serial rehydration, tissues sections were transferred to distilled water and then stained with Alcian blue for 20 min. After rinsing with distilled water, the sections were treated with periodic-acid for 10 min and subsequently stained with Schiff’s reagent for 20 min. The sections were finally rinsed thoroughly with distilled water, dehydrated and mounted for light microscopy.

Fig. 2. Visualization of mucus-like granules in gill tissues. The auto-fluorescent granules were readily observed at the ventral end of the lamellae in freshly collected animals (panels B and D). If primarily stained with the AB-PAS method (panel A) visualization of glycosylated granules (panel A, red arrow) is still observed under UV light (panel B). However, the auto-fluorescence intensity is not as strong as when the gill tissues sections were not stained (panels C and D). Panels E and F represent higher magnifications of broken tissue revealing individualized glycosylated and auto-fluorescent granules (white arrows) in the same lamellar region as in A and C (scale bar, 10 μm). Inset represents a higher magnification (63×) of the boxed area corresponding to glycosylated granules (blue and magenta colored granules) visualized in Fig. 2A. Digital images were captured with DIC (panels A, C and E) and epifluorescence microscopy (panels B, D and F). Scale bar (A-D, 20 μm) and original objective magnifications are shown.
microscopy. After the Alcian-PAS staining and in order to enhance the color of acidic (blue) and neutral (magenta) carbohydrate moieties, the nuclei staining with haematoxylin was minimal (1–2 min) in those tissue sections not meant to be visualized under fluorescent light. For the detection of glycoproteins in hemocytes, mussel hemolymph was collected directly onto a positively charged microscope slide (Super-Frost® Plus). Hemocytes attached immediately to the glass slide and were treated as for gill tissues, after a short fixation with 10% buffered formalin.

2.3. Fluorescence in situ-hybridization (FISH)

The presence of methanotrophic and thirotrophic bacterial endosymbiont in *B. azoricus* gill tissue was determined according to Duperron et al. (2005) with slight modifications. Gill tissues were fixed in 10% buffered formalin and processed for paraffin embedding according to standard protocol. Transverse sections (7 μm thick) were subjected to deparraffinization and rehydration in a decreasing ethanol series, permeabilized with proteinase-K (10 μg/ml) for 10 min, rinsed with distilled water and then incubated with Phosphate buffered saline solution (PBS buffer) for 5 min. Prior to hybridization, tissue sections were pre-treated for 15 min with hybridization solution and then subjected to hybridization solution containing the specific symbiont probes. The *Alexa Fluor*-488 GCTCAGCGAATGCTA and *Alexa Fluor*-532 CGAAGGTCCCTCAGCTTTA fluorescent probes were used to target respectively methanotrophic and thirotrophic bacterial symbionts in fluorescence in-situ hybridization (FISH) experiments. (Duperron et al., 2005). The fluorochromes Alexa 488 and Alexa 532 were from Molecular Probes, Invitrogen. Gill filaments were visualized under fluorescent light and differential interference contrast (DIC) microscopy using a Leica DM6000 digital microscope (Leica Microsystems CMS GmbH, Germany).

2.4. In vitro phagocytosis assays

The phagocytosis assays were performed on monolayer preparations after hemolymph withdrawal and direct attachment of live hemocytes to microscope slides (SuperFrost Plus®). A suspension of the yeast derivative, zymosan A (from *Saccharomyces cerevisiae*) conjugated with *Alexa Fluor* 488 (Molecular Probes®, Invitrogen) was used at a concentration of 1μg/μl in sterile sea water and incubated with the hemocytes already attached to a glass slide, in a moist chamber, for 30 min at 4 °C. For each slide/individual, the percentage of phagocytic hemocytes was recorded after the examination of a minimum of 500 hemocytes. A phagocytic hemocyte was considered to be active if it contained at least one or two fluorescent yeast particle.

3. Results

The maintenance of live vent mussels in our laboratory has prompted us to investigate the physiology of *B. azoricus* under aquarium conditions. Thus, health-related biological indicators were assessed during long term experimental acclimatization. Gill tissues were taken from freshly collected animals immediately after deep-sea retrieval; therefore, bacterial detection corresponds to natural abundance of endosymbionts. After 2–3 weeks of acclimatization in plain sea water aquarium the density of bacterial symbionts was reduced (Fig. 1). However, individual variations may have accounted for differences observed during the FISH experiments. In some cases, methanotrophic bacteria were still seen up to 4 weeks in mussels kept in plain sea water and at atmospheric pressure (Fig. 1B, iv).

The presence of dense glycosylated granules was detected by means of light and epifluorescent microscopy and the Alcian blue-Periodic Acid Schiff staining method (AB-PAS). In gill tissues from animals immediately retrieved from vents, the goblet mucus cells contained granules that fluoresced under UV light (Fig. 2). These granules were visible in the gill filaments for several months until complete disappearance from the distal ends of the lamellae (Fig. 3). Some granules appear to be stained with the Alcian blue and Periodic-Acid Schiff stain, which indicates that they contained proteoglycans or glycoproteins (Figs. 2 and 3) although the staining is also shown in the main cell content (Fig. 2A, inset). A higher magnification of a broken lamellae revealed that the granules appear to be spherical and approximately 2 μm diameter and colored magenta and dark blue, revealing thus their neutral and acidic polysaccharide nature respectively (Fig. 2E). After 3 months maintenance in aquaria, the granules were no longer visible, under UV light.

After the mussels had been kept for 3 and 6 months in the aquaria (Fig. 3C and E, respectively), acidic polysaccharides (stained blue) were still observed in the epithelial surface of the middle part of the filament. This is an area corresponding to the bacterial zone. Staining procedures similar to those used on gill sections were also applied to detect glycoproteins in hemocytes from *B. azoricus*. Hemocytes were withdrawn from both freshly collected and from vent mussels held in aquaria. Glycoproteins were detected in all hemocyte preparations regardless the mussel’s condition (Fig. 3). Hemocytes withdrawn from mussels kept for 3–6 months stained less strongly than did the cells from fresh mussels. In addition, the dominant sugar moiety, as indicated by the color resulting from the Alcian-blue PAS staining (magenta), is the one corresponding to neutral mucin staining (Woods and Ellis, 1994–96) although some blue-stained granules appear in the freshly collected hemocytes. Thus, the diffuse staining in hemocytes could indicate a gradual consumption of intracellular carbohydrate reserves in aquaria specimens (Fig. 3). Despite the reduction of glycoprotein, the general appearance of the granulocytes remained basically unchanged after 6 months.

To evaluate the animals’ health under such laboratory conditions and after total loss of mucus-like droplets from the goblet mucus cells in their gills, we conducted phagocytosis experiments using fluorescent yeast particles. Hemocytes withdrawn from freshly collected mussels revealed higher number of engulfed fluorescent yeast per granulocyte (Fig. 4A, A’ and 4B, B’). Furthermore, granulocytes were still capable of phagocytizing yeast particles although the number of engulfed particles had greatly diminished after 6 months of aquaria conditions (Fig. 4C, C’). Thus, from a cellular immunity point of view, we concluded that animals seem to be capable of mounting cellular immune defenses even after months of physiological endurance to aquarium conditions under atmospheric pressure. However, the number of engulfed foreign particles by hemocytes withdrawn from long term aquaria animals suggests that the cellular immune responses might not be as robust as in deep-sea freshly retrieved animals.

4. Discussion

Most bivalves are suspension-feeders and rely on their large gills for particle capture and transport to the peribuccal organs and mouth. Mucus production is a key factor in the suspension-feeding process of bivalves for ingestion and transport of nutrient particles (Beninger and St-Jean, 1997) and also for decreasing the resistance of water flow across the gills (Beninger et al., 1997). Mucus is produced not only as a barrier to the peribuccal region but also in the gill where the abundance and distribution of mucus-producing cells have been studied (Beninger and Dufour, 1996). The mytilid mussels of the genus *Bathymodiolus* are biomass dominants at many known deep-sea hydrothermal vent and cold seep habitats. Whereas vent mussels probably obtain some nutrition by suspension feeding (Le Pennec et al., 1990; Page et al., 1991) their filter-feeding capabilities make them one of the last survivors of the vent fauna at dying vents (Hessler et al., 1988). Yet, *Bathymodiolus* species have attained a further level of nutritional specialization utilizing sulfur-oxidizing and/or methane-oxidizing bacterial symbionts within bacteriocytes in their gills (Childress and Fisher, 1992; Fisher et al., 1993; Fiala-Médioni et al., 2002).

Previous work conducted in our aquarium system has demonstrated the usefulness of the strategies applied to ensure the survival of mussels for months under experimental conditions (Kadar et al.,
2005). Such strategies may include the use of methane and hydrogen sulfide as supplement or simply plain sea water replenished at regular intervals. The appearance of the mucus-producing goblet-like cells may be regarded as a possible indicator of the vent mussel’s condition while adapting to a feeding regime based on particulate food nourishment. Glycoprotein granules seem to persist for long periods of time, however, after 3 months in aquaria, the mucus-like granules were not so abundant and very few were visible at the abfrontal part of the gill filament (Fig. 3). Moreover, we have not found histological evidence to support a continuous production or turn-over of glycosylated granules during acclimatization to aquarium conditions. Yet, more mucus production from these mucus-producing cells was expected as an adaptation to greater reliance on particulate feeding. Throughout the different periods of acclimatization, acidic glycoproteins were stained blue in the middle part of the lamellae (Fig. 3 insets, red star). This suggests that acidic glycoproteins are still produced in this area of the gill filament. However, it is also possible that glycoproteins did not metabolize during long term maintenance in aquaria rather than a continual turn-over of acidic polysaccharides took place. Two types of storage cells have been previously reported in the mantle connective tissue of *B. azoricus*. One type corresponds to the adipogranular cells and the second type was described as containing large lysosomes and lipid droplets (Lobo-da Cunha et al., 2006). However, glycogen storage was not detected in vesicular connective tissue cells. The reserves accumulated in the two types of storage cells could be utilized by hydrothermal vent mussels when coping with sulfide and/or methane shortages (Lobo-da Cunha et al., 2006). Additionally, mucopolysaccharide storage in gill tissue could serve as an instant source of energy when sulfide and/or methane supply is affected by irregular venting activity.

A gradual diminution of methanotrophic bacteria from gill tissues was observed in our studies. This rather swift phenomenon was evident following the two initial weeks of mussel’s acclimatization to plain sea water and atmospheric pressure. Thus, it is probable that the depletion of bacterial endosymbionts from bacteriocytes would shorten the survival of these mussels. Even so, mussels seem to endure...
changes incurred from adaptations to atmospheric pressure under aquarium conditions while subjected to a wholly particulate diet and an unnatural food regime. Consequently, we investigated the physiological fitness of vent mussels kept under aquarium conditions by means of phagocytosis. An evaluation of cellular reactions during experimental acclimatization could, thus, help in establishing a connection between mussel’s long term endurance in aquaria and the deterioration of the animal’s living conditions or susceptibility to infectious diseases.

Although we considered in this study that a phagocytic cell would bear at least one yeast particle, the number of engulfed particles per phagocytic cell decreased substantially over time. After 6 months, the percentage of granulocytes with two or more engulfed yeast particles was markedly reduced when compared to granulocytes from freshly collected mussels (Fig. 4D, grey bars). Although capable of phagocytosis, the granulocytes thus appear affected by long term maintenance in aquaria at atmospheric pressure. In spite of unaltered general hemocyte morphology, we concluded that cellular defense reactions may be adversely affected by long term maintenance in aquaria conditions and a solely particulate diet. For that reason, the investigation of cellular immunity in B. azoricus should be considered within the first months after animal retrieval from deep-sea.

Fig. 4. In vitro phagocytosis assay. The phagocytosis assays were performed with fluorescent zymosan A as the phagocytic particle. The number of hemocytes containing fluorescent particles was recorded from a minimum of 500 hemocytes per individual/slide. Digital images of granulocytes (gr), containing yeast particles, were captured on DIC (panels A, and A'; B; C and C') and on fluorescent microscopy (A", B" and C"). Panels A" and C" are combined images from DIC and fluorescent microscopy. The percentage of hemocytes containing at least one or more (white bars) and two or more phagocytized yeast particles (grey bars) are shown in panel D. Results are from 3 individuals per each acclimatization period using hemocytes from different preparations. gr, granulocytes; hy, hyalinocytes. Scale bar=20 μm.
The only mytilid in which mucocytes, mucus distribution and type has been comprehensively studied, is Mytilus edulis. It appears that in B. azoricus, the mucocytes distribution, as far as it is shown here, is similar to that found in M. edulis (Beninger et al., 1993), but with higher density of mucocytes toward the distal end of the lamellae. SEMs of the frontal face at the base of the filaments showed, as in Mytilus, that mucocytes were not present in the food groove but were present just dorsal to the groove (Dando et al. unpublished results). Different types of mucopolysaccharides are supposed to have different functions according to their carbohydrate moieties, resulting in different mucus viscosities (Beninger et al., 1993). Adding to their normal role in facilitating water flow across the lamellae and particle capture and transport (Beninger et al., 1997), it is also possible that B. azoricus mucocytes have a storage function and thus our observations would correspond to a starvation response with reserves being mobilized. This is further suggested by the weakened magenta staining of hemocytes from animals kept for prolonged periods of time suggesting a consumption of intracellular carbohydrate reserves. Interestingly, mucus production does not appear to be prevented in mussels kept up to 6 months in aquaria, as shown by the typical staining of acidic polysaccharides (blue color) in the middle zone of the lamellae. However, the degree of staining has decreased between 3 and 6 months of maintenance in aquaria as mucus-producing cells appeared smaller (Fig. 3C and E, insets). Seemingly, a change in mucus composition resulting from long term acclimatization could affect mucus viscosity in aquarium animals and thus affecting mucociliary transport mechanisms along epithelial surfaces.

In conclusion, direct visualization of intrinsic auto-fluorescent mucus-like granules at the ventral end of the lamellae provides a means to monitor vent mussels during aquarium acclimatization. The present study also raises new interesting hypothesis regarding the possibility that vent mussels may use carbohydrate reserve products stored in gill tissues and hemocytes in order to cope with experimental acclimatization. Our study also supports B. azoricus as a viable model organism to tackle the molecular and cellular mechanisms involved in physiological alterations during experimental acclimatization of vent mussels to atmospheric pressure. The observed decrease of phagocytosis during long term maintenance in aquaria may be explained in part due to an attenuation of cellular immune competence resulting from intrinsic energy demands and metabolic adjustments of B. azoricus to new feeding regimes.

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