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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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Abstract:

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 the 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.

Keywords: Mucopolysaccharide; Alcian blue-Periodic acid Schiff staining; Phagocytosis; Aquarium acclimatization; *Bathymodiolus azoricus*; Hydrothermal vent; Vent

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 Bathymodiolus 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 chemoautotrophic processes (Fiala-Médioni & Felbeck 1990). In spite of detailed studies describing the intracellular co-existence of methanotrophic and chemoautotrophic 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 endosymbiont 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. 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 *Bathymodiolus 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, endosymbiont 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 transverse sections of gill filaments 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 (37º50,8-37º51,6N; 31º30-31º31,8W), 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 October 2006 and May 2007. In the LabHorta aquarium, mussels were kept at 7- 8 ºC in plastic

containers filled with 20 liters of sea water and aerated to give an oxygen saturation of 10-50%. The stocking density was 1 animal/liter 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 5ml 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 6g of freeze dried product in 40ml of sterile sea water and homogenized with a sample preparation homogenizer (Heidolph Instruments GmbH & Co.KG). 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 Bathymodiolus azoricus

The combined Alcian blue and Periodic-Acid Schiff (PAS) staining method (Woods & Ellis, 1994-96) was utilized to determine the distribution of glycoproteins in paraffin tissue sections of gills and in hemocytes of *Bathymodiolus azoricus*. In brief, 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. 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 (SuperFrost® 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 thiotrophic 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 deparrafinization 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 Fluo-488 GCTCCGCCACTAAGCCTA and Alexa Fuo-532 CGAAGGTCCTCCACTTTA fluorescent probes were used to target respectively methanotrophic and thiotrophic bacterial symbionts in fluorescence in-situ hybridization (FISH) experiments. (Duperron et al. 2005). The fluochromes 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 Fluo 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 ºC. 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 *Bathymodiolus 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 aquaria 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 (Fig. 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 *Bathymodiolus 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 & 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 & St-Jean 1997) and also for decreasing the resistance of water flow across the gills (Beninger et al. 1997). Mucus is produced not only from the peribuccal region but also in the gill where the abundance and distribution of mucus producing cells have been studied (Beninger & 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 & 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 turnover of acidic polysaccharides took place. Two types of storage cells have been

previously reported in the mantle connective tissue of *Bathymodiolus 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 *Bathymodiolus azoricus* should be considered within the first months after animal retrieval from deep-sea.

The only mytilid in which mucocytes, mucus distribution and type has been comprehensively, studied is *Mytilus edulis*. It appears that in *Bathymodiolus 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 mucopolysaccharide 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 3E, 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 *Bathymodiolus 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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329 **References**

330 Bettencourt, R., Roch, P., Stefanni, S., Rosa, D., Colaço, A., Santos, R.S., 2007. Deep
331 sea immunity: unveiling immune constituents from the hydrothermal vent mussel
332 *Bathymodiolus azoricus*. Marine Environmental Research 64, 108-27.
333 Beninger, P.G., St-Jean, S., Poussart, Y., Ward, J.E., 1993. Gill function and mucocytes
334 distribution in *Placopecten magellanicus* and *Mytilus edulis* (Mollusca: Bivalvia):
335 the role of mucus in particle transport. Marine Ecology Progress Series 98, 275-282.
336 Beninger, P.G., Dufour, S.C., 1996. Mucocyte distribution and relationship to particle
337 transport on the pseudolamellibranch gill of Crassostrea virginica (Bivalvia:
338 Ostreidae). Marine Ecology Progress Series 137, 133-138.
339 Beninger, P.G., St-Jean, S.D., 1997a. The role of mucus in particle processing by
340 suspension-feeding marine bivalves: unifying principles. Marine Biology 129, 389-
341 397.
342 Beninger, P.G., Lynn, J.W., Dietz, T.H., Silverman, H., 1997b. Mucociliary Transport
343 in Living Tissue: The two-Layer Model Confirmed in the Mussel *Mytilus edulis* L.
344 Biological Bulletin 193, 4-7.
345 Childress, J.J., Fisher, C.R., 1992. The Biology of hydrothermal vent animals:
346 physiology, biochemistry, and autotrophic symbioses. Oceanography and Marine
347 Biology, Annual Review 30, 337-441.
348 Distel, D.L., Lee, H.K.W., Cavanaugh, C.M., 1995. Intracellular coexistence of
349 methano- and thioautotrophic bacteria in a hydrothermal vent mussel. Proceedings

of the Natural Academy of Sciences of the United States of America 92, 9598-9602.

Duperron, S., Nadalig, T., Caprais, J.C., Sibuet, M., Fiala-Médioni, A., Amann, R., Dubilier, N., 2005. Dual symbiosis in a *Bathymodiolus sp*. Mussel from a Methane Seep on the Gabon Continental Margin (Southeast Atlantic): 16S rRNA Phylogeny and Distribution on the Symbionts in Gills. Applied Environmental Microbiology 71, 1694-1700.

Duperron, S., Bergin, C., Zielinski, F., Blazejak, A., Pernthaler, A., McKiness, Z.P., DeChaine, E., Cavanaugh, C.M., Dubilier, N., 2006. A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge. Environmental Microbiology 8, 1441-1447.

Fiala-Médioni, A., Felbeck, H., 1990. Autotrophic processes in invertebrate nutrition: bacterial symbiosis in bivalve molluscs. In: Mellinger J (Ed.) Animal nutrition and Transport Processes. 1. Nutrition in Wild and Domestic Animals. Comparative Physiology 5, 49-69.

Fiala-Médioni, A., McKiness, Z.P., Dando, P., Boulegue, J., Mariotti, A., Alayse-Danet, A.M., Robinson, J.J., Cavanaugh, C.M., 2002. Ultrastructural, biochemical and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis. Marine Biology 141, 1035-1043.

Fisher, C.R., Brooks, J.M., Vodenichar, J.S., Zande, J.M., Childress, J.J., Burke Jr, R.A., 1993. The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidising bacterial symbionts in a deep-sea mussel. Marine Ecology 14, 277-289.

374 Gustafson, R.G., Turner, R.D., Lutz, R.A., Vrijenhoek, R.C., 1998. A new genus and
375 five new species of mussels (Bivalvia, *Mytilidae*) from deep-sea
376 sulfide/hydrocarbon seeps in the Gulf of Mexico. Malacologia 40, 63-112.
377 Hessler, R.R., Smithey, W.M., Boudrias, M.A., Keller, C.H., Lutz, R.A., Childress, J.J.,
378 1988. Temporal changes in megafauna at the Rose garden hydrothermal vent
379 (Galapagos Rift; eastern tropical Pacific). Deep-Sea Research 35, 1681-1709.
380 Kadar, E., Bettencourt, R., Costa, V., Santos, R.S., Lobo-da-Cunha, A., Dando, P.,
381 2005. Experimentally induced endosymbiont loss and re-acquirement in the
382 hydrothermal vent bivalve *Bathymodiolus azoricus*. Journal of Experimental Marine
383 Biology and Ecology 318, 99-110.
384 Le Pennec, M., Donval, A., Herry, A., 1990. Nutritional strategies of the hydrothermal
385 ecosystem bivalves. Progress in Oceanography 24**,** 71–80.
386 Lobo-da Cunha, A., Kadar, E., Serrão Santos, R., 2006. Histochemical and
387 ultrastructural characterisation of mantle storage cells in the hydrothermal-vent
388 bivalve *Bathymodiolus azoricus*. Marine Biology 150, 252-260.
389 Page, H.M., Fiala-Medioni, A., Fisher, C.R., Childress, J.J., 1991. Experimental
390 evidence for filter-feeding by the hydrothermal vent mussel, *Bathymodiolus*
391 *thermophilus.* Deep-Sea Research A 38**,** 1455–1461.
392 Von Cosel, R., Comtet, T., Krylova, E.M., 1999. *Bathymodiolus* (Bivalvia: Mytilidae)
393 from hydrothermal vents on the Azores triple junction and the Logatchev
394 hydrothermal field, Mid Atlantic Ridge. Veliger 42, 218-248.
395 Woods, A.E., Ellis, R.C., (eds) 1994-96. Laboratory histopathology: a complete
396 reference. Vols. 1,2. Edinburgh: Churchill Livingstone.
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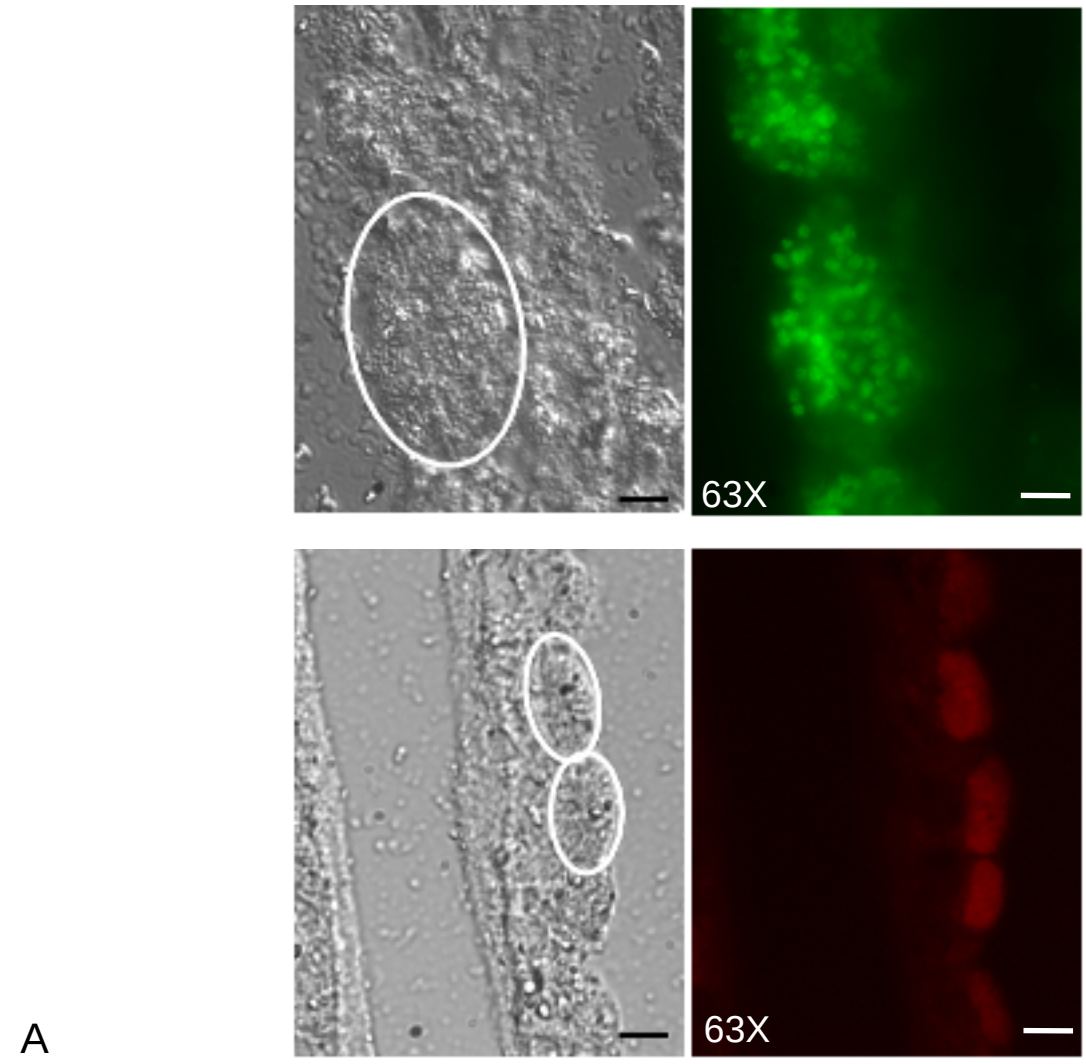
FIGURE LEGENDS

Fig.1 Detection of methanotrophic and thiotrophic bacterial endosymbionts in gill tissues. Fluorescent probes were used to target methanotrophic (Fig 1A, upper panels) and thiotrophic (Fig 1A, lower panels) bacterial symbionts in fluorescence in-situ hybridization (FISH) experiments. Differential interference contrast (DIC) visualization is shown (Fig 1A, left upper and lower panels). Elliptical lines indicate the endosymbionts confinement to the bacteriocytes (specialized endothelial cell) boundaries. Scale bar (Fig 1A, 5 µm) and original objective magnifications are indicated. Fig 1B represent 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 during (i-iv).

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 fluorescent granules (white arrows) in the same lamellar region as in A and C (scale bar, 10µm). Inset represents a higher magnification (63X) 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

423 microscopy (panels B, D and E). Scale bar (A-D, 20 µm) and original objective
424 magnifications are shown.
425
426 Fig. 3 Detection of carbohydrate moieties in gill filaments and hemocytes of
427 *Bathymodiolus azoricus*. The carbohydrate granules were examined at the beginning of
428 acclimatization to aquaria at atmospheric pressure (A, blue and red arrows) and after 3
429 and 6 months (C and E, respectively). After 6 months of maintenance, mucus-like
430 granules are no longer detected (panel E) whereas the acidic mucin-like (blue) staining
431 is still visualized on the ventral edges of gill epithelia from 3 months and 6 months
432 acclimatized animals (C and E, respectively, white arrows). Acidic mucin-like staining
433 was also observed in an area corresponding to the bacterial zone or bacteriocytes area.
434 This staining was more pronounced in 3 months (inset in Fig. 3C, red stars) than in 6
435 months acclimatized animals (inset in Fig. 3E, red star). The presence of glycosylated
436 proteins or carbohydrate moieties was observed inside granulocytes withdrawn from
437 freshly collected mussels (panel B) and from mussels kept for 3 and 6 months in aquaria
438 (panels D and F, respectively).
439
440 Fig. 4 In vitro phagocytosis assay. The phagocytosis assays were performed with
441 fluorescent zymosan A as the phagocytic particle. The number of hemocytes containing
442 fluorescent particles was recorded from a minimum of 500 hemocytes per
443 individual/slide. Digital images of granulocytes (gr), containing yeast particles, were
444 captured on DIC (panels A, and A'; B; C and C'') and on fluorescent microscopy (A'',
445 B' and C'). Panels A' and C'' are combined images from DIC and fluorescent
446 microscopy. The percentage of hemocytes containing at least one or more (white bars)
447 and two or more phagocytized yeast particles (grey bars) are shown in D. Results are

448 from 3 individuals per each acclimatization period using hemocytes from different
449 preparations. gr, granulocytes; hy, hyalinocytes. Scale bar = 20 µm..



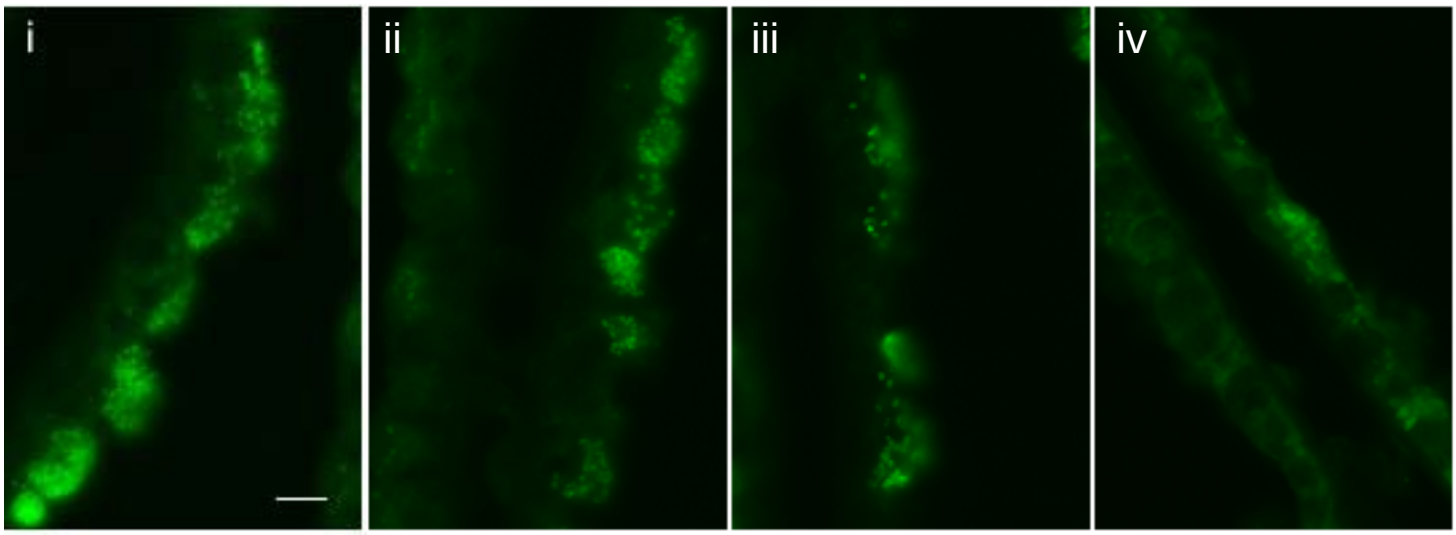


Fig 1 Bettencourt et al. 2007

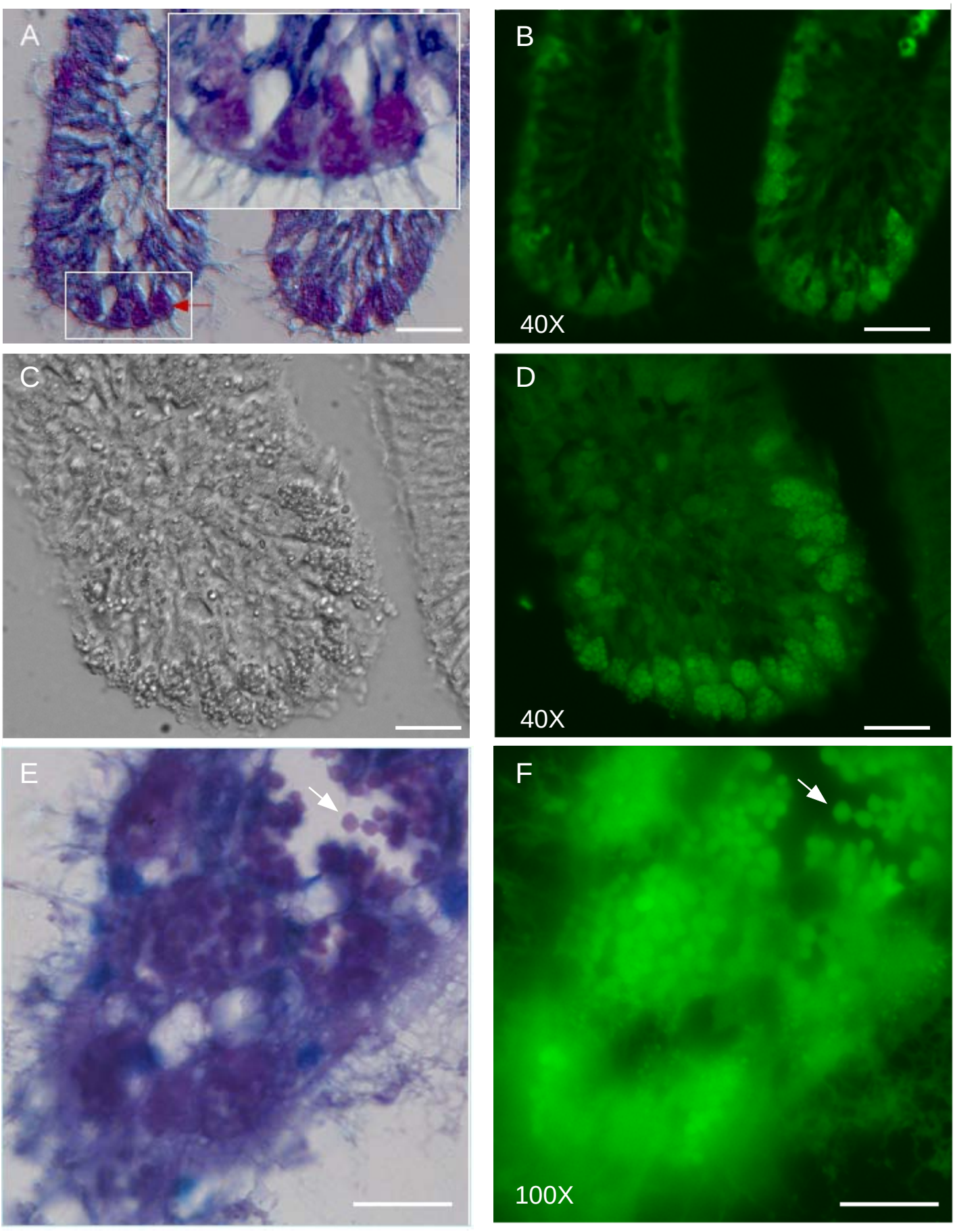


Fig 2 Bettencourt et al 2007

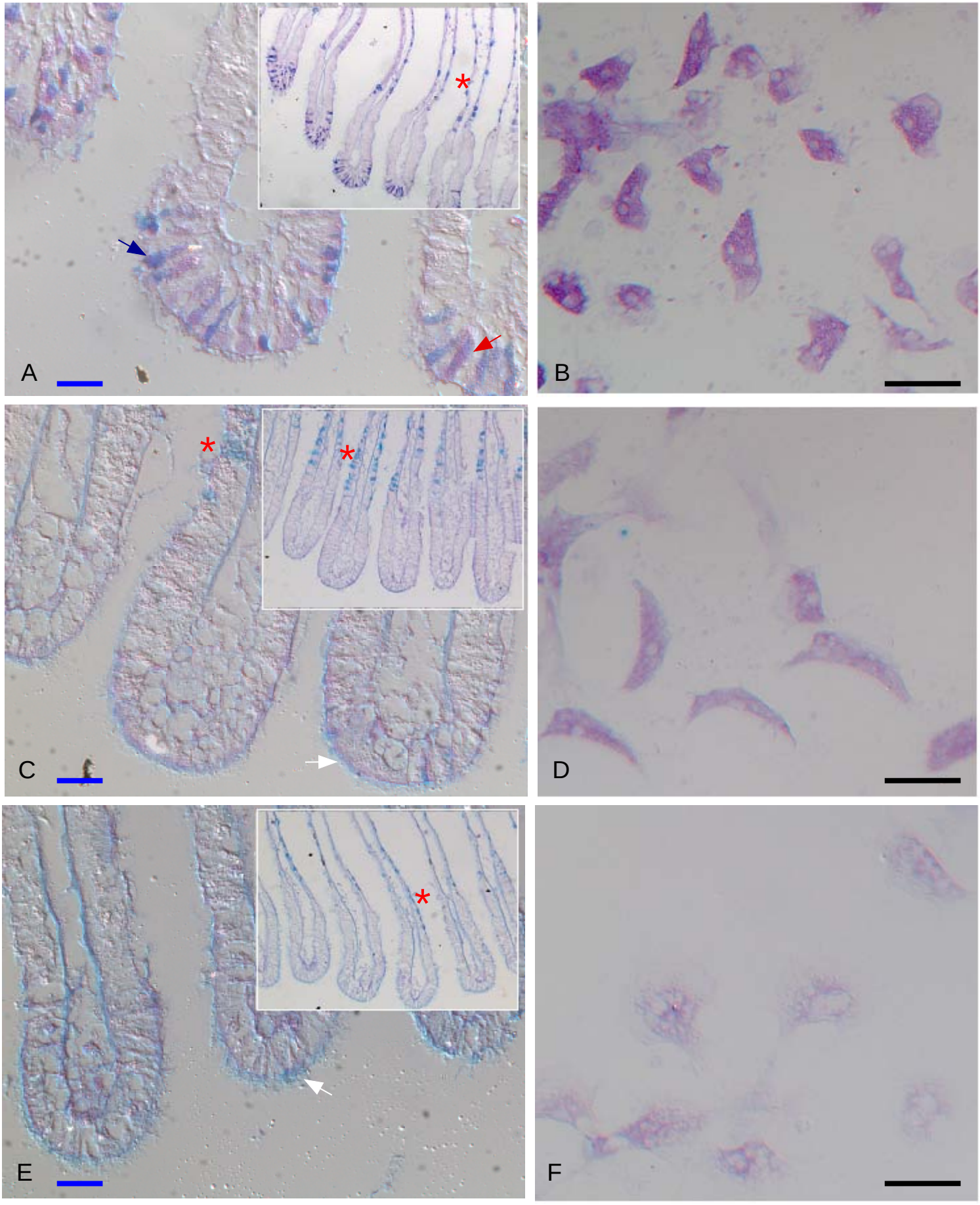
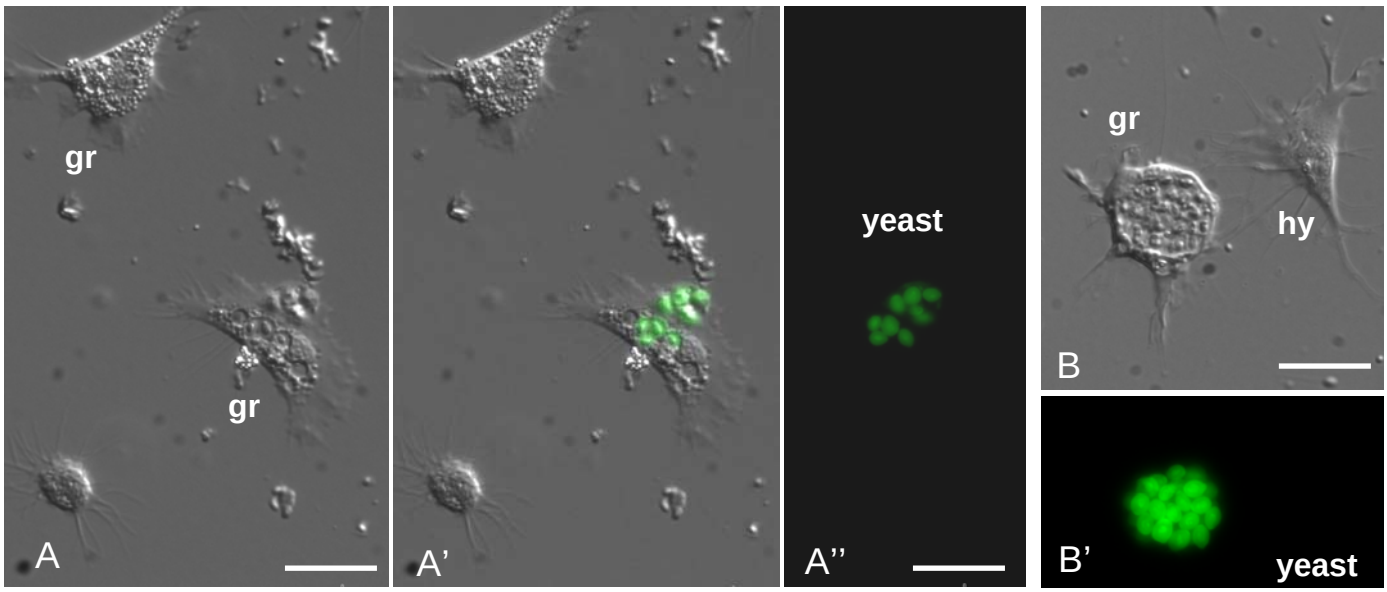
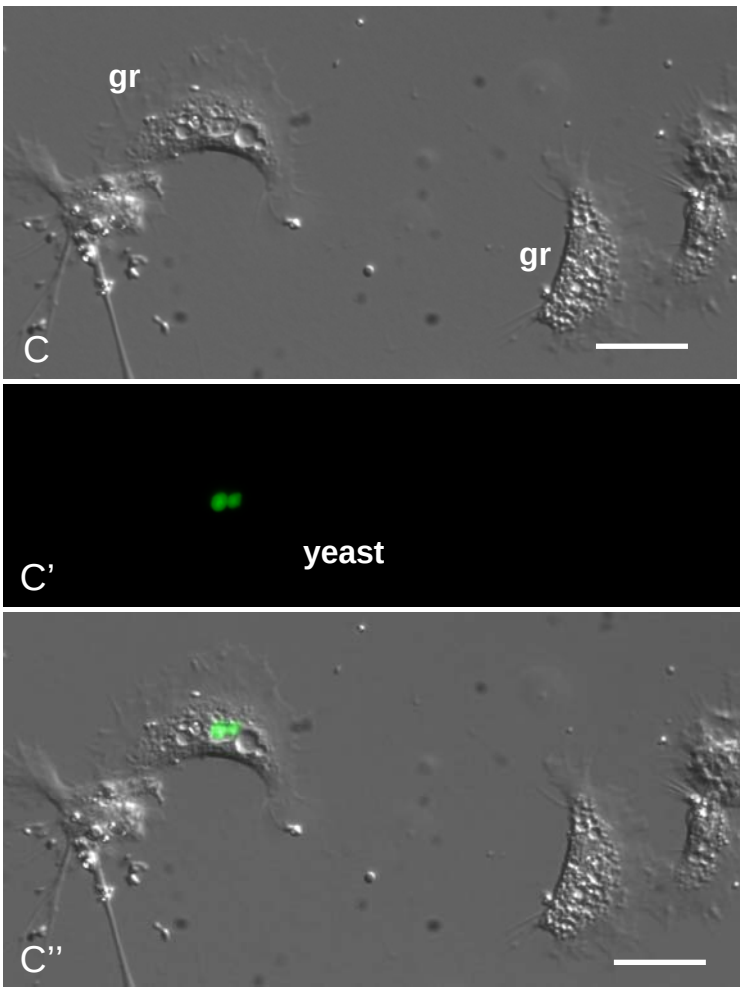


Fig 3 Bettencourt et al 2007





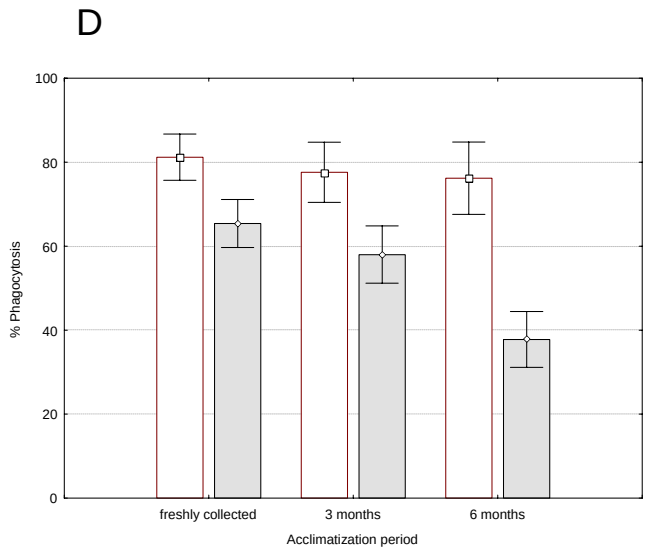


Fig. 4 Bettencourt et al. 2008