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Gamma-proteobacteria detected in the digestive gland of the vent mussel *Bathymodiolus azoricus*

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Abstract: Bacterial endosymbiosis is widespread in hydrothermal vent bivalves, and is typically developed in gill bacteriocytes, while it has never been described in the digestive gland. Using ultrastructural examination and DNA amplification analyses, we provide evidence for the existence of a potentially new group of environmental bacteria in the digestive gland of *Bathymodiolus azoricus*, which are genetically distinct from sulphur-oxidiser endosymbionts in its gills. The 16S rRNA partial gene sequences showed 99% identity in the gill and the digestive gland. Our results indicate that the same bacteria phylotypes are associated with different tissues in the host, and also that they do not result from a random infestation by free-living forms.

Keywords: Hydrothermal vent • *Bathymodiolus azoricus* • Gamma-proteobacteria • Digestive gland • Gill endosymbiont

Introduction

Many macro-organisms from hydrothermal vents have developed symbiotic relationships with chemoautotrophic bacteria in order to utilize the chemical energy of this unique deep-sea environment (Won et al., 2003). The hydrothermal mussel *Bathymodiolus azoricus* von Cosel et al., 1999 is functionally dependent on its association with both sulphur-oxidizing and methanotrophic bacteria (Fiala-Medioni et al., 2002). Such a dual symbiosis in vent mytilids develops in specialized cells of the gill, named bacteriocytes, and may provide the bulk of the host's nutritional carbon requirement, as reported in *Loripes lucinalis* Lamarck, 1818 (Fiala-Medioni et al., 2002). The remaining energy source is obtained by classical filter feeding, i.e. ingestion of particulate organic matter, as well as absorption and incorporation of free amino acids (Page et al., 1991). Such mixotrophy allows these bivalves to have a broad distribution relative to the effluent compared to other vent species (Fisher et al., 1989) and may also be a basis for a non-obligate host-symbiont mutualism. Moreover mussels are able to survive for prolonged periods without their symbionts (over 12 months in our laboratory) most likely by switching to filter feeding (Page et al., 1991).

The endosymbiont transmission type in bivalves is family specific and has only been defined for few species (Won et al., 2003). From our post-capture experiments on *B. azoricus*, there is a strong indication for the environmental acquisition of symbionts which, however, does not clearly exclude the simultaneous existence of vertical transmission (Kádár et al., 2005).

One of the few studies that addressed assessment of micro-organisms in the digestive gland of vent molluscs was a health-assessment of mussels from petroleum seeps by Powell et al. (1999) that described inclusions in the digestive glands resembling Chlamydial/Rickettsial inclusions. Similar prokaryotic organisms have been reported in the digestive gland of the symbiont-bearing lucinid *Loripes lucinalis*, and suggestions have been made about the existence of a functional symbiosis between these microorganisms and their host (Johnson & Le Pennec, 1995). Further cases of non-gill symbioses were reported by Johnson & Fernandez (2001) as putative sulphur-oxidizing bacteria in the periostracal secretion of *L. lucinalis,* or by Streams et al. (1997) in the mantle and foot epithelium of seep mytilids.

The detection of the 16S rRNA gene as a molecular marker is a useful research strategy for identifying uncultivated prokaryotes and is now routinely performed in symbioses studies (Durand & Gros, 1996; Gros et al., 2003). Furthermore, comparisons of rRNA genes sequences have been particularly useful in the characterization of a range of chemoautotrophic endosymbionts whose investigation is hampered by their uncultivable nature.

Employing classical histology, electron microscopy and molecular biomarkers, we provide evidence for the presence of a novel bacterium in membrane-bound inclusions within the digestive gland tubules of the vent mussel *B. azoricus*. The biological implications of a potential endosymbiotic relationship are also discussed.

Materials and Methods

Sample collection

Samples were collected in August 2002 with the R/V "Atalante", using the robot arm of the ROV, Victor 6000. Mussels of different size ranges (4 ± 1 and 7 ± 1 cm shell length) together with sediment from their habitat were collected from Menez Gwen (-850 m, 31º31'W, 37º50'N). Mussels were measured, gills and digestive glands were dissected and fixed for light and electron microscopy. Additional tissue samples from three specimens were quick-frozen and stored at -80ºC until DNA extraction. Sediment samples were air-dried for later analyses. Gill endosymbionts were purified from ten mussels using the technique described elsewhere (Kadar et al., 2005) within 24 hours of being brought to the surface. Briefly, a gill homogenate was obtained by crushing the freshly removed gills with the addition of 3 mL IBS (imidazole-buffered-saline) buffer/1 g of wet weight tissue, on a laboratory tissue grinder (Ystral D-79282 Ballrechten-Dottingen), at the lowest speed, for 5 minutes. The homogenate was centrifuged with an equal volume of Percoll: $IBS_{2.5x}$ 6:4 mix in an Eppendorf swinging bucket rotor at 10300 rpm for 6 minutes at 4ºC. Bacterial pellets were recovered using Pasteur pipettes and kept frozen until analyses.

Tissue preparation for light and electron microscopy

Small (1 mm^3) tissue pieces from three mussels were fixed in modified Trumps fixative (3% glutaraldehyde and 3% paraformaldehyde in buffer containing: 0.15 M Na cacodylate (pH 7.8), 0.3 M sucrose, 0.2 M NaCl and 0.008 M $CaCl_2$. Following primary fixation, samples were washed in 0.1 M cacodylate buffer, post-fixed in 1% OsO_4 and embedded in Spurr resin (Sigma). Semi-thin (2 µm) sections were obtained using diamond knife on a LKB-BROMMA ultramicrotome and stained with methylene blue. Ultra-thin sections were mounted on copper grids, double-stained with uranyl acetate and lead citrate before examination with a JEOL 100CXII transmission electron microscope. Each grid supported approximately 10 ultra-thin sections, and 3 grids from each specimen were observed.

DNA extraction and PCR amplification

Nucleic acids from bacterial pellets from the gills and whole tissues from the digestive glands were extracted according to a method described elsewhere (Kádár et al., 2005). Briefly, an initial denaturation step at 94ºC for 2 min ("hot start") was followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 45 sec and extension at 68ºC for 45 sec, followed by a 7 min final extension at 72ºC. Proteinase K treatment was replaced by amylase in the case of the digestive gland tissues. Thermo-cycling conditions were performed according to standard conditions. Primers were designed based on nucleotide sequences available from the NCBI public database for the following genes with GenBank accession nos. AB073122, AY235678 and AB062137, respectively: *Bathymodiolus* sp. endosymbiont gene for 16S rRNA (P1, sense 5'AGAGTTTGTTCATGGCTCAGA3' and antisense 5'GAAGGCACC AATCCATCTC TG3'); *Bathymodiolus azoricus* thioautotrophic gill symbiont 16S ribosomal gene (P2, sense 5' CGGGTCTTGTACACACCGCCCG 3' and antisense 5' CAATGTGGTGGAGCCAGGGAGG 3') and *Bathymodiolus* symbiont methane monooxygenase subunit A gene (P3, sense 5' GGGGACTGGGACTTCTGGACA 3 and antisense 5' GAACGCTGAG AAGAACGCAGA 3'). Thus, P1 was chosen as general marker for any endosymbiont in *Bathymodiolus* sp., whereas P2 and P3 were expected to target thiotrophic and methanotrophic endosymbionts. The PCR products were examined on 0.8% agarose gel electrophoresis according to standard protocols.

The PCR products obtained with P1 were purified with

the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and submitted directly for nucleotide sequencing to the CRIBI sequencing facility (http://bmr.cribi.unip.it). The DNA sequence obtained with 924 base pairs is available in the public database (GenBank accession nº DQ054530). Database sequence homology searches were performed with the BLAST search program available at: http://www.ncbi. nlm.nhi.gov.

Results

The general macro anatomy of the digestive gland in *B. azoricus*, composed of blind-ended tubules made up by two classical cells types, digestive and secretory cells, was similar to those described in other mytilid bivalves (Fig. 1a). The presence in digestive cells of very large vesicles containing small rod-shaped bacteria that resemble in some aspects those present in the gill bacteriocytes was unexpected. These bacteria had an average diameter of approximatey 0.4 µm and a maximum length of 1.5 µm. The common morphological characteristic with thioautotrophs from the gill was their double membrane (gram negative) with DNA strands found in the centre of an electron-translucent area (Fig. 1 b and c). At the same time, there were distinctive features: bacteria-bearing vesicles were bigger in the digestive gland and contained a higher number of bacteria as compared to those in gills. Additionally, the lysosomal content in the digestive cells had a distinct appearance from that observed in gill lysosomes containing bacteria at different stages of digestion (Fig. 1c). In contrast, all digestive gland lysosomes were less compact and their membranous content hardly resembled bacterial membranes (Fig. 1c).

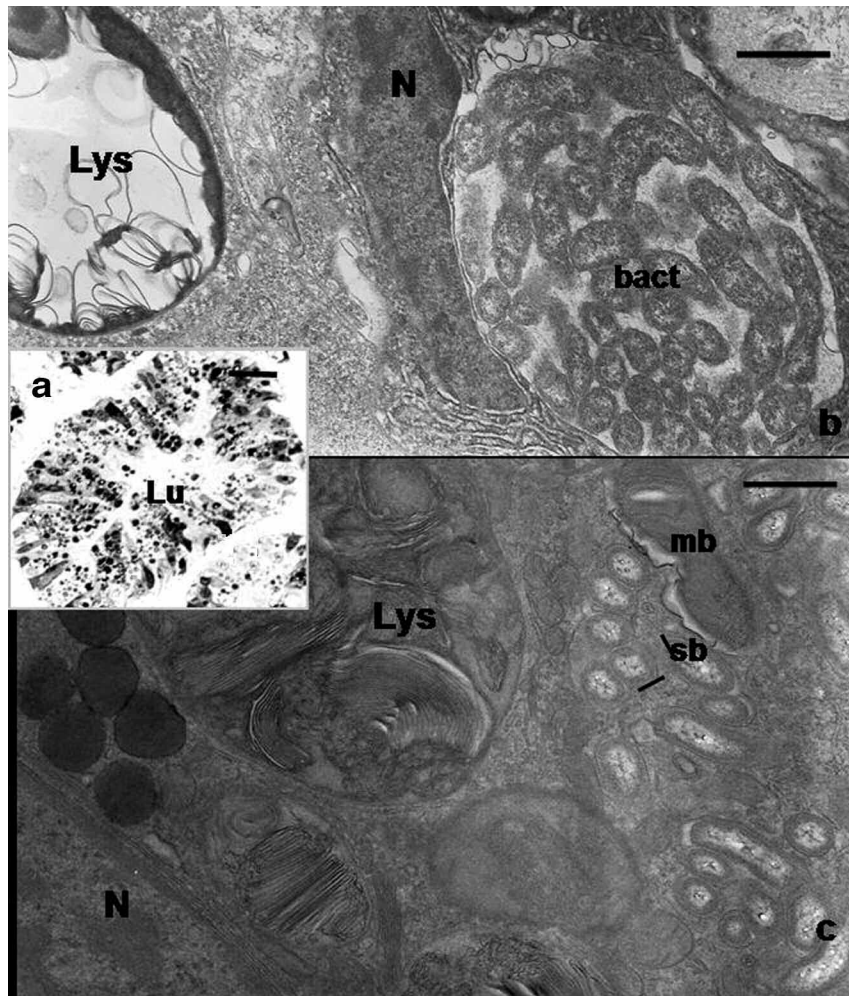

Figure 1. *Bathymodiolus azoricus.* Bacteria in the digestive gland and gill. **a.** Light micrograph showing cross-section of the typical mytilid digestive tubule surrounding the lumen (Lu). Scale bar: 50 µm. **b.** Digestive cell with a membrane-bound inclusion containing large number of bacteria (bact), a central nucleus (N) and large lysosomes (Lys). Scale bar: 1 µm. **c.** Gill bacteriocyte showing two types of endosymbiont bacteria: sulphur oxidizers (sb) and methanotrophs (mb). Note the distinct lysosomal content with membranous bacterial remains at different stages of degradation in the gill cell *versus* lysosomes with less compact structure in the digestive gland (Lys in **b** *versus* **c**). Scale bar: 1 µm.

Figure 1. *Bathymodiolus azoricus.* Bactéries dans la glande digestive et la branchie. **a.** Micrographie photonique montrant une section transversale d'un tubule digestif typique de moule entourant le lumen (Lu). Echelle : 50 µm. **b.** Cellule digestive : membrane entourant un grand nombre de bactéries (bact), noyau central (N) et des grands lysosomes (Lys). Echelle : 1 µm. **c.** Bactériocyte de la branchie : deux types de bactéries endosymbiontes, des sulphure-oxydants (sb) et des méthanotrophes (mb). Noter la différence entre le contenu du lysosome constitué de membranes bactériennes à différents stades de dégradation dans la cellule de la branchie et les lysosomes contenant une structure moins compacte dans la glande digestive (Lys dans **b** et **c**). Echelle : 1 µm.

The PCR reactions performed with three specific pairs of primers (sets P1, P2 and P3) designed to target *Bathymodiolus* endosymbiont genes generated single bands of the expected molecular size in the gill, demonstrating not only the presence of the 16S rRNA gene from the *Bathymodiolus* sp. endosymbiont, but also the sulfur-

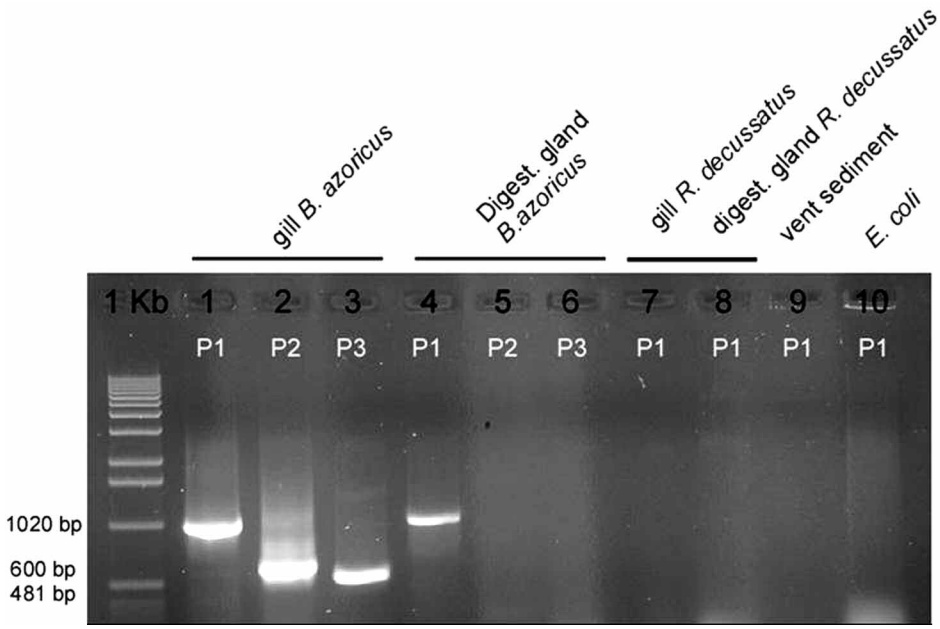

Figure 2. PCR detection of endosymbiotic bacterial genes. The presence of the 16S rRNA (primer set P1), sulfur-oxidizer 16S-23S rRNA (primer set P2) and methane monooxygenase subunit A (primer set P3) genes is detected in gills (lanes 1-3) but not in the digestive gland, where only the 16S rRNA gene is detected (lanes 4-6). DNA extractions from *Ruditapes decussatus* gill and the digestive gland are shown as negative controls (lanes 7 and 8, respectively). Free-living *Bathymodiolus* endosymbiotic bacteria were not detected with primer set 1 in mussel bed sediment (lane 9). *E. coli* colony PCR reaction also tested negative (lane 10). PCR product sizes are shown, as well as 1 Kb DNA ladder.

Figure 2. Détection par PCR des gènes bactériens endosymbiontes. La présence des gènes d'ARNr 16S (lot d'amorces P1), d'ARNr 16S-23S de sulphure-oxydants (lot d'amorces P2) et de la sous-unité A de la méthane monooxygénase (lot d'amorces P3) est détectée dans les branchies (lignes 1-3) mais pas dans la glande digestive où seul le gène de l'ARNr 16S est détecté (lignes 4-6). Les extractions d'ADN de branchie et de glande digestive de *Ruditapes decussatus* sont utilisées comme contrôle négatif (lignes 7 et 8, respectivement). Les bactéries endosymbiontes libres de *Bathymodiolus* ne sont pas détectées avec le lot d'amorces 1 dans le sediment de la moulière (ligne 9). La PCR effectuée sur une colonie d' *E. coli* est également negative (ligne 10). Les tailles des produits de PCR sont données, de même que le marqueur de taille de l'ADN 1Kb.

oxidizing and methane monooxygenase gill symbiont genes (Fig. 2, Lanes 1-3). In contrast, only the 16S rRNA gene was successfully detected in DNA extracts from the digestive gland (Fig. 2, Lanes 4-6). These results indicate that bacterial-symbiont DNA targets are not solely restricted to the symbiont-bearing gill tissues but are also found in the digestive gland as demonstrated by the DNA fragment generated with the 16S rRNA primer set (P1).

Escherichia coli Escherich, 1815 cells and DNA extracts from gills and digestive glands of the non-symbiotic shore-clam *Ruditapes decussatus* Linnaeus, 1758 were used as negative controls (Figure 2, Lanes 7, 8 and 10, respectively). The presence of free-living forms of *Bathymodiolus* endosymbiotic bacteria in vent sediments was not detected with primer set 1 (Figure 2, Lane 9). Furthermore, the sequence analysis of the 16S rRNA PCR fragment resulted in the greatest BLAST identity (99%) with uncultured gamma-proteobacterium partial 16S rRNA gene sequences from bacterial communities in ground waters of the deep well injection site (accession no AJ583184) and in deep sea sediment from the Western Pacific (AY375058), the insect midgut gamma-proteobacteria *Photorhabdus luminescens* Thomas & Poinar, 1979 (AY444555), and the gut bacteria from the pig gastrointestinal tract microbiota (AF371847). There was no significant similarity between this digestive gland bacterial 16S rDNA sequence and *Bathymodiolus* sp. endosymbiont sequences in GenBank (AB073122 or the more recently available DQ077893).

These results indicate the presence of yet another bacterium in the digestive gland tissues of the deep-sea vent mussel *B. azoricus* that is neither sulphur oxidising nor methanotrophic, but is related to deep-sea gammaproteobacteria and/or enterobacteria.

Discussion

Symbiotic relationships between marine invertebrates and bacteria are a prominent feature in hydrothermal vent

systems as a survival strategy (Won et al., 2003). In addition to extensive histological and biochemical investigations (Page et al., 1991; Fiala-Medioni et al., 2002), molecular methods have been employed to characterize the role of these endosymbiotic bacteria in vent bivalves (Durand & Gros, 1996; Gros et al., 2003). However, the nature of their symbiotic association in the case of non-gill symbiosis, whether it is nutritional, shelter or illness-related, is not completely elucidated to date.

The results presented in this study suggest that the *B. azoricus* digestive gland harbours bacteria that are distinct from the already-described sulphur- and methane-oxidising bacteria. In spite of the morphological resemblances (shape and size) of this putatively novel bacterium with sulphur oxidisers from gill bacteriocytes, our molecular evidence suggests that it is genetically distinct. Additionally, the bacteria within the digestive gland show a distinct distribution pattern where they are clustered within pocket–like vesicles, unlike in the gills, where sulphur oxidisers occupy a large volume of specialised cells. Also, the bacterial incidence in all digestive gland sections examined here appeared to be lower and more erratic than their incidence in the gills. Furthermore, the integrity of the bacteria and the absence of bacterial remains within the lysosomes may indicate that they have not undergone digestion and thus do not directly contribute to the nutritional supply of the host.

Our PCR amplifications using primer sets 2 and 3 (*B. azoricus* thioautotrophic gill symbiont 16S ribosomal gene and *Bathymodiolus* symbiont methane monooxygenase subunit A gene, respectively) in gill preparations are consistent with the earlier reported dual symbiosis in vent mytilids from the Mid Atlantic Ridge (Distel et al., 1995; Fiala-Medioni et al., 2002). Remarkably, successful detection of the 16S rRNA *Bathymodiolus* sp. endosymbiotic gene (P1, Fig 2) in both gill and digestive gland does not conform with earlier assumptions considering gill as the sole endosymbiont bacteria-bearing tissue. Although thiotroph- and methanotroph- specific primer sets failed to reveal the presence of gill-like symbionts in the digestive gland tissues, the presence of endosymbiotic bacteria was confirmed through the positive DNA amplification obtained with primer set 1 (lane 4, Fig 2), thus suggesting the occurrence of an undescribed proteobacterium. Moreover, the same bacterium seems to be present in various other tissues analysed, as PCR products corresponding to the 16S rRNA genes from gills, digestive gland and foot DNA preparations revealed 99% nucleotide identity when their sequences were aligned (data not shown).

The presence of a putative endosymbiont in the digestive gland tissues raises questions related to the host-bacteria reciprocal functions and to the transmission mode. Bacterial contribution to the host metabolism could take place in the digestive gland by means of the endosymbiotic production of extracellular enzymes that are otherwise not synthesized by the host, a widespread phenomenon among marine invertebrate symbioses (reviewed by Harris, 1993). In turn, the digestive gland, the site of nutrient absorption (Johnson & Le Pennec, 1995) would provide nutritional support and suitable settlement surface for the bacteria. Additionally, the digestive gland is the site where sequestration of toxic metallic elements takes place in *B. azoricus* (Kadar et al., 2006). Thus, endosymbiotic bacteria may be associated with a detoxification role via mechanisms well known in free living bacteria from extreme environments (Llanos et al., 2000). However, further in-depth biochemical investigations are needed to determine the exact role that symbionts play in vent bivalve digestive glands and ultimately in their host physiology.

The access of bacteria to the digestive gland is also a controversial issue. Recent evidence, based on FISH demonstrated that the sulphide-oxidising gill endosymbiont of *Codakia orbicularis* Linnaeus, 1758 is also present in the environment as a free-living form that suffers morphological transformations when entering host bacteriocytes and thus becoming endosymbiotic (Gros et al., 2003). However, this is unlikely the case in *B. azoricus*, since the bacterial 16S rRNA gene present in the digestive gland was not detected in the sediment surrounding the mussels. Instead, free-living forms in the water column could be the possible source of infection of digestive cells, whose pronounced endocytotic nature (Johnson & Le Pennec 1995) would make their presence sustainable. Similar unusual membrane-bound bacterial inclusions were previously referred to as Chlamydia/Rikettsia-like organisms in the digestive glands of the petroleum-seep *Bathymodiolus* sp. (Powell et al., 1999), and in *L. lucinalis* (Johnson & Le Pennec, 1995). According to these authors, at a certain stage the entire membrane-bound inclusion is released into the lumen and then may be phagocytosed by the gill bacteriocytes. A corresponding fate of bacterial inclusions described in this study in *B. azoricus* is possible, but needs to be confirmed. While our ultrastructural investigations did not provide evidence for such inclusion release, molecular evidence suggest the presence of a non-sulphur oxidising, non methanotrophic bacteria in both organs (gill and digestive gland) within the same organism. Our BLAST sequence analyses also suggest that these bacteria are not Chlamydia/Rickettsia like parasites.

In conclusion, we provide evidence for a novel bacterium in the digestive gland of *B. azoricus* that may play nutritional/detoxification roles, and could be therefore potentially endosymbiotic. However, the exact physiological mechanisms behind this putative mutualism, that is who is providing what to whom, deserves further in-depth biochemical investigations. The future use of *B. azoricus*

digestive gland as a symbiont-free tissue in biochemical and molecular studies should be avoided.

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