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Extracellular bacterial association in gills of «wood mussels»

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Abstract: Four «morphotypes» of Mytilidae were collected from wood falls in the west Pacific (Vanuatu islands) and in the Bohol sea (Philippines) between 300 and 1800 metre depth. Results from our study demonstrated the existence of extracellular bacteria located between microvilli at the apical surface of the cells all along the lateral zone of the gill filaments in the four mytilid morphotypes analyzed. Based on TEM observations, these Gram-negative bacteria were not methanotrophic due to the lack of concentric stacking of intracellular membranes in their cytoplasm. Based on FISH experiments, these bacteria belong to γ-Proteobacteria. These preliminary observations described gill symbiosis-like interactions in metazoan species associated with sunken wood, strengthening the hypothesis that decomposing wood may serve as steps for the introduction of symbiotic Mytilidae to vents and seeps. Phylogenetic analysis of these extracellular bacteria are in progress in our lab in order to compare their 16SrDNA sequences to that of other marine invertebrate symbionts described to date.

Résumé : *Association bactérienne extracellulaire dans les bactéries de moules associées aux bois coulés.* Quatre morphotypes de Mytilidae ont été récoltés sur des bois coulés dans le Pacifique ouest (Iles Vanuatu) et dans la Mer de Bohol (Philippines) entre 300 et 1800 mètres de profondeur. Les résultats de notre étude montrent la présence de bactéries extracellulaires localisées entre les microvillosités sur la surface apicale des cellules le long de la zone latérale des filaments branchiaux sur les quatre morphotypes analysés. D'après les observations réalisées au microscope électronique à transmission, ces bactéries Gram négatives ne sont pas des bactéries méthanotrophes en raison de l'absence de membranes intracellulaires concentriques dans leur cytoplasme. D'après les hybridations *in situ* effectuées (technique FISH), ces bactéries appartiennent aux γ-protéobactéries. Ces observations préliminaires suggèrent des interactions de type symbiotique au niveau des branchies de métazoaires associés à des bois coulés. Cela renforce l'hypothèse que les bois coulés pourraient représenter une étape de l'introduction des Mytilidae symbiotiques au niveau des sources hydrothermales et des suintements froids. L'analyse phylogénétique de ces bactéries extracellulaire est en cours dans notre laboratoire afin de comparer les séquences de leur ADNr 16S à celles d'autres symbiotes d'invertébrés marins déjà décrits.

Keywords: Extracellular bacteria ● Mytilidae ● Wood falls ● Ultrastructure ● Symbiosis

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Introduction

Gill-symbiosis in hydrothermal vent mussels was first described in *Bathymodiolus thermophilus* which harbors bacterial endosymbionts (Fiala-Médioni et al., 1986). Since that symbiont-containing mussels are commonly found at hydrothermal vents and cold seeps throughout the world (for review, see Duperron et al. 2005), and small symbiotic mussels belonging to the genus *Idasola* were also reported to be associated with whale bones (Deming et al., 1997). Distel et al. (2000) have suggested, based on phylogenetic analyses of various mytilid species, that decomposing organic substrates such as wood and bone may serve as steps for the introduction of mytilids into vent and seep environments. According to the fact that sulfur-oxidizing gill-endosymbionts were already reported from *Idas (Idasola) washingtonia* (Bernard, 1978) individuals collected from whale falls, they made the hypothesis that mussels found in wood falls may harbor also chemoautotrophic bacterial symbionts.

The aim of this study was to look for bacterial interactions in the gill tissues of mytilid specimens collected from wood falls in order to validate the hypothesis of bacterial symbiosis in such metazoan fauna.

Material and methods

Sampling

Samples were collected during the BOA0 (Vanuatu islands) and Panglao (Bohol sea) cruises in November 2004 and May 2005 respectively, using a beam trawl between depths of 150 to 1800 meters from wood falls. Mytilids, representing four morphotypes (Fig. 1), were recovered attached to small pieces of submerged woods. Samples were processed on the ship within 1 hour after collection. Only one individual per morphotype was available for this study.

For each single individual freshly collected, gills were dissected; one gill was used for TEM analysis while the other one was used for FISH experiment.

Transmission Electron Microscopy (TEM) preparation

Gills were prefixed on ship for one hour at 4°C in 2.5% glutaraldehyde in 0.1M pH 7.2 caccodylate buffer, adjusted to 900 mOsM with NaCl and $CaCl_2$ in order to improve membrane preservation. Gills were briefly rinse in the same buffer before to be stored in the same buffer at 4°C until they were brought to the laboratory 2-3 weeks after. Samples were fixed for 45 minutes at room temperature in 1% osmium tetroxide in the same buffer, then rinsed in distilled water and post-fixed with 2% aqueous uranyl acetate for one hour before embedding, sectioning (60 nm thick), and observation with a H-8000 microscope at 100kV.

Fluorescent in situ hybridization

Gills, and the remaining individual bodies, were fixed for 1-3 hours at 4°C in 4% paraformaldehyde either in 1xPBS buffer or in seawater. The specimens were then washed three times for 10 minutes each at 4°C in 1xPBS, dehydrated in an ascending series of ethanol, stored in 100% ethanol at 4°C until they were embedded in paraplast. Four micrometer-thick sections were placed on precoated slides from Sigma before hybridization. Five oligonucleotide probes were used: Eub338 (5'-GCTGCCTCCCGTAGGAGT-3'), targeting most members of the Eubacteria (Amann et al., 1990), and probes as ALF968 for α-Proteobacteria (5'-GGTAAG-GTTCTGCGCGTT-3'), GAM42 for γ-Proteobacteria (5'-GCCTTCCCACATCGTTT-3'), BET42a for β-Proteobacteria (5'-GCCTTCCCACTTCGTTT-3'), and NON338 (5'-ACTCCTACGGGAGGCAGC-3') as a negative control. Hybridization conditions were similar to those previously described by Dubilier et al. (1995).

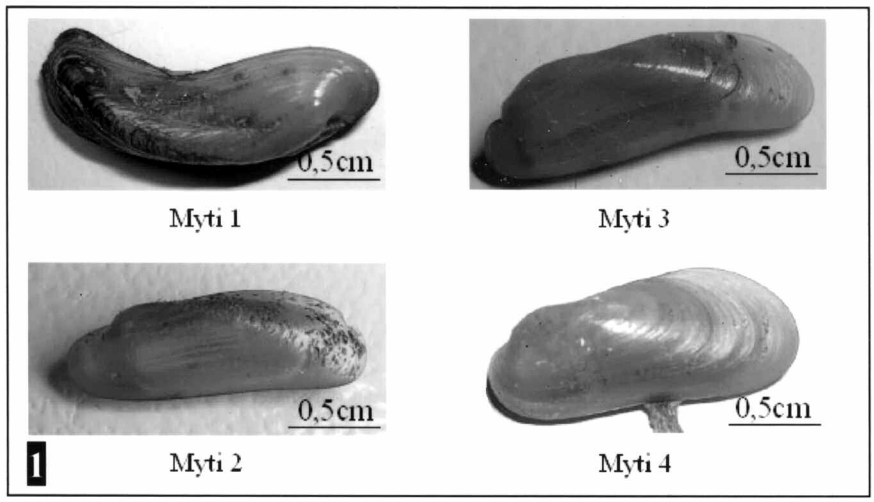
Results

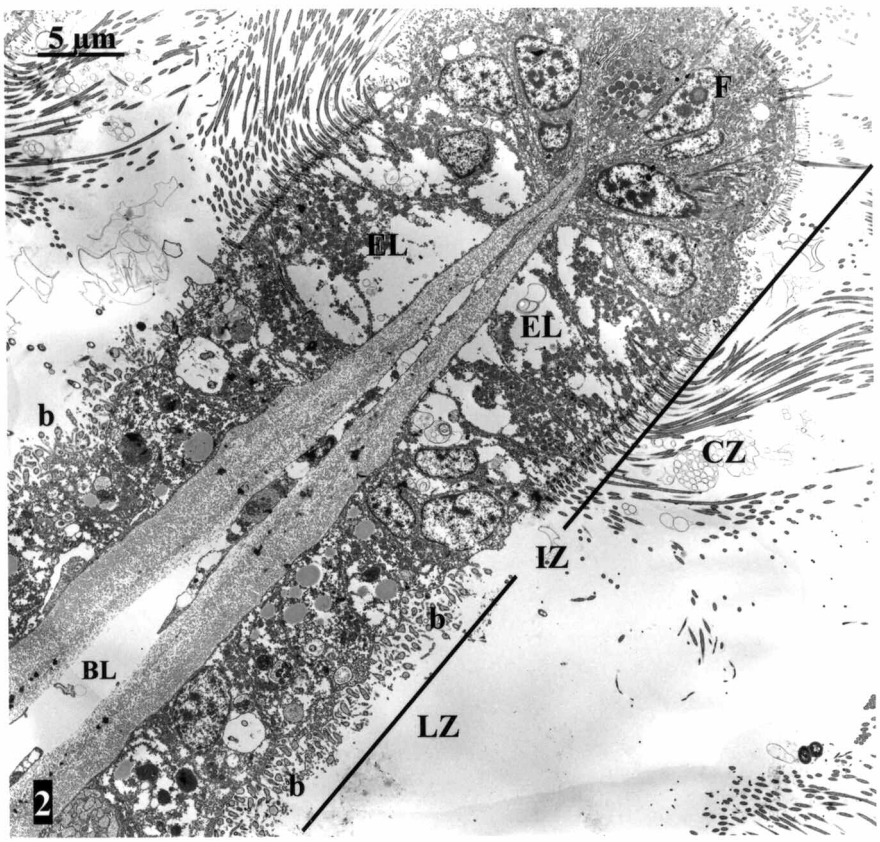
Each individual analysed had gills characterized by two demi-branchs, each of which consisting of an ascending and a descending lamella. The gill filaments were weakly united by ciliary discs that were arranged at regular interval as previously described in the Mytilidae.

Gill filament ultrastructure

The ciliated zone looked similar to that of previously described symbiont-bearing bivalves (Fig. 2) and appeared devoid of bacteria. The intermediary zone was short as the first cell in contact with the last eulateral cell from the ciliated zone contained bacteria on its surface for all the samples studied (Fig. 2).

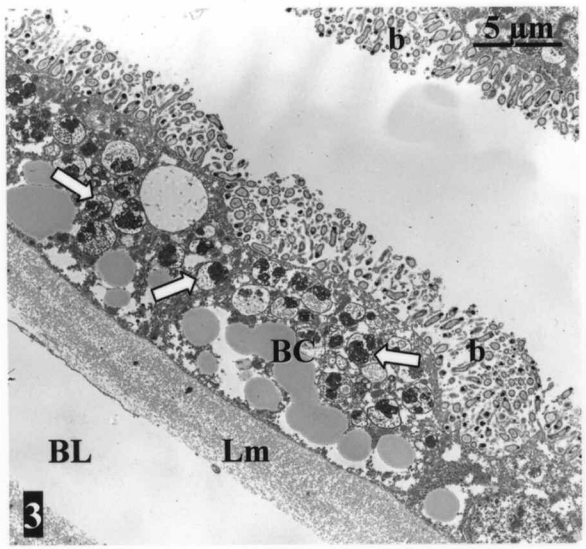
The lateral zone was mostly composed of a prevalent cell type harboring bacteria on its apical surface (Figs 3-5). The thickness of the extracellular bacterial layer depended on the specimens analyzed and varied from 1 micrometer (Fig. 5) to 10 µm (Fig. 4). Such a proximity, associated with the bacterial density, led us to define these bivalve cells as bacteriocytes, similarly to those harboring endocellular bacteria in other bivalve families (Fisher, 1990; Dufour, 2005). The bacteriocytes, which had a basally-located nucleus, were much wider than high and usually possessed long microvilli, among which bacteria are located (Figs 3-5). The bacteriocyte organelles consisted of few mitochondria and numerous lysosome-like structures (Figs 3 & 5) depending on the specimen analysed. No relationship was established between bacterial density (layer thickness) and





Figures 1 & 2. 1. General view of the four morphotypes analysed in this study. **2.** TEM view of the ciliated and lateral zones. Frontal (F) and eulateral (EL) ciliated cells are the main cell types of the ciliated zone (CZ) which is devoid of bacteria. The intermediary zone (IZ) is short and devoid of bacteria. On the other hand, the cells from the lateral zone (LZ) contain numerous bacteria (b) on their apical surface. BL: blood lacuna.

Figures 1 & 2. 1. Vue générale des quatre morphotypes analysés dans cette étude. **2.** Vue en microscopie électronique à transmission des zones ciliées et latérales. Les cellules ciliées frontales (F) et latérales (EL) sont les principaux types cellulaires de la zone ciliée (CZ) qui est dépourvue de bactéries. La zone intermédiaire (IZ) est courte et dépourvue de bactéries. En revanche, les cellules de la zone latérale (LZ) contient de nombreuses bactéries (b) sur leur surface apicale. BL : lacune sanguine.


b
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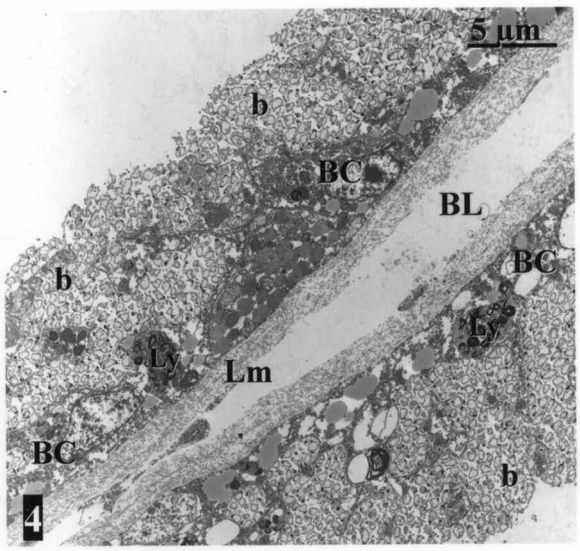
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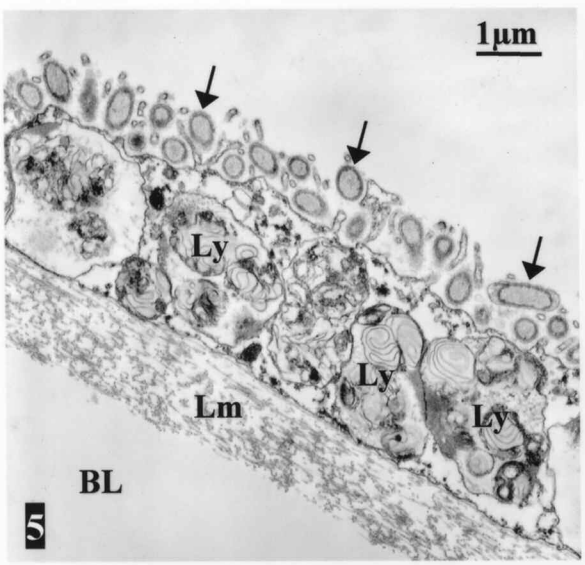
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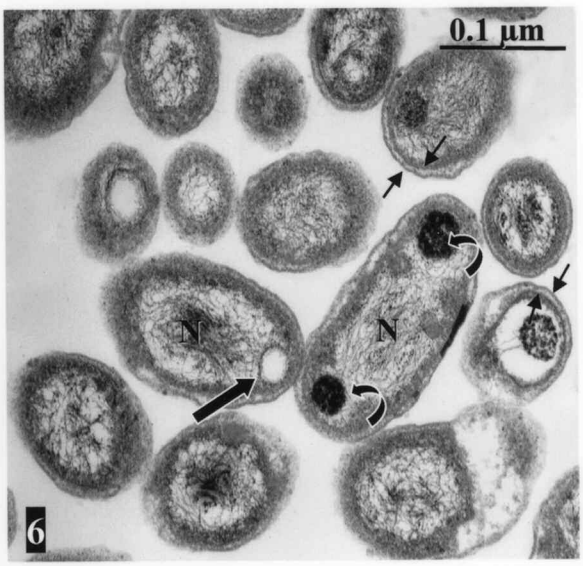
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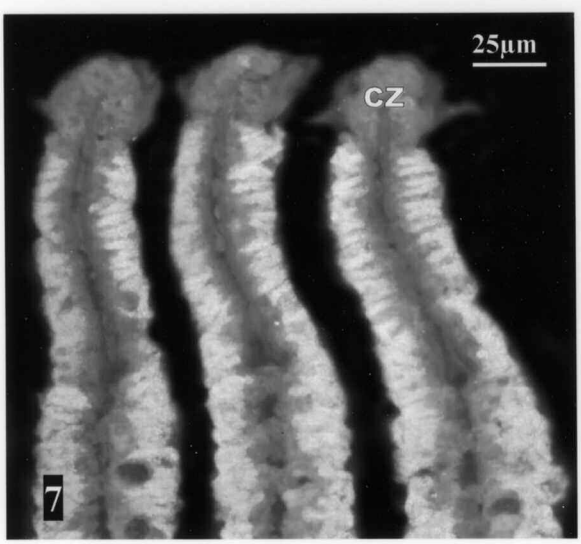
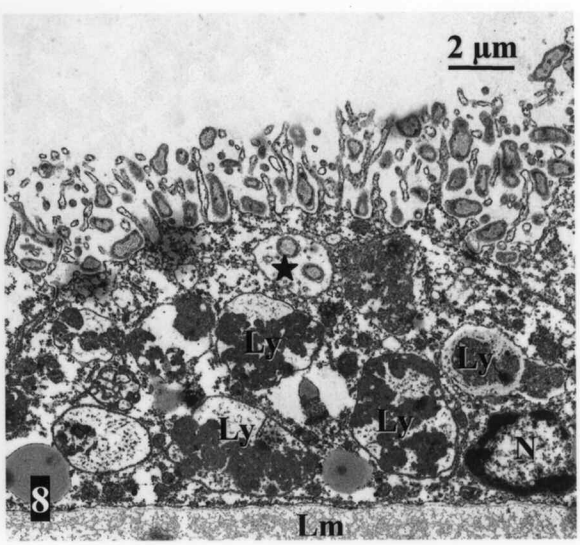

25µm
CZ
7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8
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Figures 3-8: 3. TEM view of a gill filament of the lateral zone from morphotype 3 collected at 1764 metre depth. Bacteriocytes (BC) harbour few layers of extracellular bacteria (b). In the bacteriocyte cytoplasm, numerous small lysosomes (arrows) are seen. BL: blood lacuna; Lm: basal lamina. **4.** TEM view of the lateral zone of a gill filament from a mussel (morphotype 1) collected at 400 metre depth. The thickness of the extracellular bacteria above the apical pole of the bacteriocytes (BC) reaches 10 µm. The bacteriocytes contain lysosome-like structure (Ly) in their cytoplasm. BL: blood lacuna; Lm: basal lamina. **5.** TEM view of the lateral zone of a gill filament from a mytilid collected at 550 meter depth. Bacteriocyte cytoplasm is filled with secondary lysosomes (Ly), characterized by their heterogeneous aspect and whorls of membranes (asterisks). Extracellular symbionts (arrows) are located on the apical surface of the bacteriocytes in contact with microvilli. BL: blood lacuna; Lm: basal lamina. **6.** Electron micrograph of the extracellular symbionts. They possess a double membrane (small arrows) typical of Gram negative bacteria. The DNA (N) occupies most of the volume of the bacterial cytoplasm, which also contains osmiophilic dense granules (curved arrows) in addition of numerous ribosomes or glycogenic storage. Electron lucent granules located in the periplasmic space (straight arrow) look like elemental sulfur granules observed in most of thioautotrophic gill-endosymbionts. **7.** FISH. Positive hybridization (white color) with the GAM42 probe (specific for γ-Proteobacteria) all along the lateral filament of the gill from morphotype 1. The ciliated zone (CZ) and the middle part of the lateral zone remain negative (gray color). **8.** Bacteriocyte cytoplasm of morphotype 3 filled with secondary lysosomes (Ly), characterized by their heterogeneous aspect. Extracellular symbionts are located on the apical surface of the bacteriocytes in contact with microvilli. Two envacuolated bacteria are seen inside a phagosom (star) prior to addition of lysosomal enzymes. Such structure indicates that ectosymbionts might be phagocytosed by bacteriocytes. Lm: basal lamina; N: nucleus of the bacteriocyte.

Figures 3-8. 3. Vue en microscopie électronique à transmission de la zone latérale du morphotype 3 récolté à 1764 mètres de profondeur. Les bactériocytes (BC) abritent quelques couches de bactéries extracellulaires (b). Dans le cytoplasme des bactériocytes, de nombreux petits lysosomes (flèches) sont visibles. BL : lacune sanguine ; Lm : lame basale. **4.** Vue en microscopie électronique à transmission de la zone latérale d'un filament branchial du morphotype 1 récolté à 400 mètres de profondeur. L'épaisseur des bactéries extracellulaires au-dessus du pôle apical des bactériocytes (BC) atteint 10 µm. Les bactériocytes renferment des structures de type lysosome (Ly) dans leur cytoplasme. BL : lacune sanguine ; Lm : lame basale. **5.** Vue en microscopie électronique à transmission de la zone latérale d'un filament branchial d'une moule récoltée à 550 mètres de profondeur. Le cytoplasme du bactériocyte est rempli de lysosomes secondaires (Ly), caractérisés par leur aspect hétérogène et par des enroulements des membranes (astérisques). Les symbiotes extracellulaires (flèches) sont localisés sur la surface apicale des bactériocytes en contact avec les microvillosités. BL : lacune sanguine ; Lm : lame basale. **6.** Micrographie électronique des symbiotes extracellulaires. Ils possèdent une double membrane (petites flèches) typique des bactéries Gram négatives. L'ADN (N) occupe la plus grande partie du volume du cytoplasme bactérien, qui contient également des granules denses osmiophiles (flèches incurvées) en plus de nombreux ribosomes et des réserves de glycogène. Les granules transparents aux électrons localisés dans l'espace périplasmique (flèche droite) ressemble aux granules de soufre élémentaire observés dans la plupart des endosymbiotes thioautotrophes de branchies. **7.** FISH. Hybridation positive (couleur blanche) obtenue avec la sonde GAM42 (spécifique des γ-Protéobactéries) le long du filament latéral de la branchie du morphotype 1. La zone ciliée (CZ) et la partie médiane de la zone latérale restent négatives (couleur grise). **8.** Cytoplasme du bactériocyte du morphotype 3 rempli de lysosomes secondaires (Ly), caractérisés par leur aspect hétérogène. Les symbiotes extracellulaires sont situés sur la surface apicale des bactériocytes en contact avec les microvillosités. Deux bactéries envacuolées sont visibles à l'intérieur d'un phagosome (étoile) avant l'ajout d'enzymes lysosomales. Une telle structure indique que les ectosymbiotes peuvent être phagocytés par les bactériocytes. Lm : lame basale ; N : noyau du bactériocyte.

host morphotype, depth, and wood status.

Bacterial symbionts were usually small and rod shaped (1 µm long, 0.3 µm wide), with the typical double-membrane of Gram-negative bacteria (Figs 5-6). The ovoid-shaped figures are probably due to transverse sections. As evidenced by TEM observations, these symbionts are not methanotrophic bacteria due to the fact that their cytoplasm lacks the concentric stacks of intracellular membranes typical of methanotrophs (Fig. 6). The bacterial cytoplasm contains essentially DNA and ribosomes (Fig. 6) but electron-dense, non-membrane bound granules can also be observed (Figs 3 & 6). Such granules could be some polyphosphate compounds as described in bacteria from *Riftia pachyptila* tube wall (Lechaire et al., 2002). Electron lucent granules observed in the periplasmic space (Fig. 6) could represent sulfur granules as commonly observed in gill-endosymbionts encountered in marine invertebrates.

In situ hybridization

Extracellular bacteria were positively hybridized with the probes Eub338 and GAM42a (Fig. 7) indicating that the bacteria observed are γ-Proteobacteria. No hybridization was obtained with either other host tissues or with the oligonucleotide probes against α- β- and δ-Proteobacteria. Similar results were obtained with all the samples analysed in this study.

Discussion

Wood falls represent a massive input of organic matter at depths where food is typically scarce due to the absence of sunlight. Wood debris can provide a huge amount of organic matter that could be used by microbial mats (Palacios et al., 2006) or by eukaryotic macrofauna directly (i.e.

xylophagous organisms, Distel & Roberts, 1997) or indirectly (heterotrophic organisms feeding on the microbial mats degrading the wood; Pailleret et al., 2007). Moreover, Leschine (1995) has shown that sulfide represents the principal product of the cellulose degradation in the marine environment. Such a production of hydrogen sulfide could thus also support chemosynthetic communities as previously described with bacterial decomposition of lipids from whale bones in the deep sea (Deming et al., 1997).

The bacteria described in the Mytilidae presented here were located extracellularly while remaining in contact with the apical pole of gill cells along the entire lateral zone of the gill filaments. The lateral zone consisted of a thin, simple epithelium, suggesting that exchanges can occur between the bacteriocytes and the environment. Thus, bacteria live in close contact with host cells and with the flow of water circulating through the lateral zone of gill filaments. Because of their particular location, such bacteria could protect host cells by taking up reduced sulfur compounds from the environment. The variability of bacterial number could depend either on the environment where the bivalve lives (the concentration of sulfide could vary according to the decomposing status of the wood) or on the host species. We need to get more individuals of the same mytilid morphotype from different environments to conclude about that.

Both, the distribution of the bacteria throughout the lateral zone of each gill filament and their specific location between microvilli below the glycocalyx strongly suggest that these bacteria are not ordinary fouling microorganisms that could be found on any inert surface associated with sunken wood environment. They have established particular relationships with the bivalve, which are probably symbiotic, as described in marine invertebrates colonizing sulfide-rich habitats.

TEM analysis revealed that some bacteriocytes contained either early phagosomes containing bacteria or lysosome-like structures as large as nuclei. These structures are usually considered to be lysosomes involved in the digestion of a limited portion of bacterial endosymbionts (Frenkiel et al., 1996). Thus, the ectosymbionts might be phagocytosed by bacteriocytes and digested inside lysosomal structures providing the host cells with an input of organic compounds as previously described in various marine symbiosis models. In our case, they may be involved in the digestion of the extracellular bacteria after their phagocytosis by host cells.

This is a preliminary study to document bacterial association in gills of marine bivalves associated with sunken woods. Mytilidae from cold seeps or hydrothermal vents usually harbor gill endosymbionts which are either sulfur-oxidizing (Fiala-Médioni et al., 1986; Distel & Cavanaugh, 1994; Fujiwara et al., 2000) or methanotrophic bacteria (Fujiwara et al., 2000), while in some cases a dual symbiosis has been reported, with both methanotrophic and thiotrophic bacteria within the same bacteriocyte (Fisher et al., 1993; Duperron et al., 2005). Our TEM observations rule out the fact that the extracellular bacteria observed on the gill epithelium are methanotrophic while these ultrastructural observations did not allow us to go further about the metabolic pathways used by the bacteria. Moreover, some of these bacteria present in their cytoplasm, in addition of the classical sulfur granules which appear empty with conventional TEM analysis, dense granules similar to those described in bacteria from the tube wall of *Riftia pachyptila* (Lechaire et al., 2002). Such granules will be analysed by EFTEM analysis to demonstrate if they correspond to polyphosphate.

Recently, Mc Kiness et al. (2005) have described extracellular bacterial symbionts in a bathymodioline mussel collected at 2200 m depth at the Juan de Fuca hydrothermal vents. The 16S rDNA sequence obtained from the gills clustered with other mussel chemoautotrophic symbionts previously described. However, due to the poor quality of the fixation of the gill tissue, the real location of the bacteria remained unclear and these authors did not conclude for sure. Chemoautotrophic bacteria also occur as ectosymbionts in most of the symbiotic thyasirid bivalves (Dufour, 2005).

In conclusion, this study has shown strong bacterial relationships occurring in metazoan species associated with wood falls, strengthening the hypothesis that these sites could serve as steps for the colonization of chemo-symbiotic species into seeps and vents. However, new investigations are needed to improve our knowledge of such bacterial relationships, especially regarding symbiont phylogeny.

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