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Ultrastructural and molecular evidence for potentially symbiotic bacteria within the byssal plaques of the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*

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Abstract This study reports on the presence of a putatively symbiotic bacterial flora within the byssus plaque of the deep sea hydrothermal mussel *Bathymodiolus azoricus*, contributing to metal sequestration/deposition and testing positive to methane oxidizing symbiont-specific fluorescent probes. Combining an array of approaches including histology, electron microscopy, X-ray microanalysis, analytical chemistry, and microbiology we provide evidence for the frequently assumed, but rarely shown influence of prokaryotes on the biogeochemical cycling of metals as well as inorganic C sources (i.e., methane) at deep sea hydrothermal vents. Our results indicate that in spite of its antibacterial protective sheath, the byssus plaque gives access to a whole range of prokaryotic organisms which may be responsible for the extremely high concentration of metallic elements (Fe, Cu, Zn, Mn, Co, Mo, Cd, Pb and Hg) measured in this attachment organ. The very high levels of metals in byssus, together with its frequent renewal rate due to the dynamic nature of the habitat, suggest that intra-byssal bacteria may have a major influence on biomineralisation/deposition of metals. The presence of a methanotroph morphotype within the byssus plaque was confirmed by FISH and TEM. The implications of the biogeochemical cycling of metals and methane at hydrothermal vents are discussed.

Keywords Hydrothermal vent · *Bathymodiolus azoricus* · Biomineralisation · Byssus · Endosymbiont · Fe-oxidising bacteria

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Introduction

Biogeochemical processes resulting from microbial metabolic activities are thought to be important at deep sea hydrothermal vents (Emerson and Moyer 2002; Edwards et al. 2003; Little et al. 2004; Glynn et al. 2006), where chemo- litho-autotrophic bacteria are sufficiently abundant to sustain thriving ecosystems as the primary producers (Desbruyères et al. 2000). As a consequence, the symbiotic associations between chemosynthetic bacteria and invertebrates as the emblematic feature of vent habitats have been widely studied during the past 30 years of vent research (Cavanagh et al. 1981; Childress et al. 1986; Fiala-Medioni et al. 1987; Fisher 1990; Duperron et al. 2005; Kadar et al. 2005a, 2006c, e, f). Metal-micro organism interactions within vent macro-invertebrates however, remain a largely unexplored field, in spite of few studies that have already pointed out their putative role in metal detoxification and thus prevention of uptake by the host (Zbinden et al. 2003, 2004; Kadar et al. 2005a, 2006a, b, c). This partly

due to the limited sample availability, but more importantly, it is also because of the difficulty of maintaining vent macro-invertebrates in the laboratory (reviewed in Kadar and Powell 2006d).

Bathymodiolid bivalves are biomass dominant species at many deep-sea hydrothermal vent ecosystems of the Mid-Atlantic Ridge (MAR), where *Bathymodiolus azoricus* (Von Cosel et al. 1999) can reach densities as high as 700 individuals per m^2 forming "mussel beds" through byssal attachment to each other and/or rocky surfaces (Desbruyères et al. 2000). They rely nutritionally on endosymbionts belonging to sulphide-oxidising chemoautotrophs and methanotroph bacteria harboured in their gill epithelium (Fiala-Medioni et al. 1987; Kádár et al. 2005a). A number of intra- and extra-cellular prokaryotic organisms have been found in various organs, although their physiological roles are often unclear (Kadar and Azevedo 2006e; Kadar et al. 2006f). Allegedly parasitic prokaryotes within the byssus threads were previously reported (Kadar and Azevedo 2006e), while gill endosymbionts were found to contribute significantly to metal detoxification (Kadar et al. 2006a, b). This fragmentary literature suggests the need for further interdisciplinary investigations in order to understand the role of the bacterial flora associated with vent macro-organisms.

The structure of *B. azoricus* attachment organ has previously been described and reported to be similar to that of other mytilids (Kadar and Azevedo 2006e). Briefly, it is composed by the stem emerging from the byssal gland and continuing by the proximal and distal parts of the thread and ending in the adhesive plaque that is the bonding site to hard surfaces (Lucas et al. 2002). In spite of being anchored by byssal threads, bathymodiolids are capable of migrating towards the nutrient flow (Desbruyeres et al. 2000) that can be very variable given the dynamic nature of the vent ecosystem (Kádár et al. 2005c). Undisturbed byssogenesis therefore, is a means of survival. Byssus ultra structure also deserves closer scrutiny given the extreme environmental conditions under which it forms. Byssus threads are extra cellular collagenous structures secreted by the byssus gland of the foot, and are a target for positively charged metallic elements such as Zn^{2+}, Cu^{2+} and Fe^{2+} (Gundacker 1999). Essential metals were reported to play an important role in the structural integrity and normal functioning of this attachment organ in littoral mytilids (Lucas et al. 2002).

This study examines the possible reasons for unprecedented metal concentrations detected analytically in the byssus of the vent mussel. Applying electron microscopy, both descriptive and semi quantitative and molecular approach (FISH) evidence are provided to the presence of a series of prokaryotic organisms with potential role in metal biomineralisation and/or methane oxidizing. The biological implications of these associations within the host's attachment organ are discussed.

Materials and methods

Sample collection

Mussel samples were collected from two vent sites: Menez Gwen (−850 m, 37°50' N, 31°31' W) (Charlou et al. 2000) and Lucky Strike (−1700 m, 37°18' N, 32°16' W) (Langmuir et al. 1997) along the Mid-Atlantic Ridge, during various cruises (summer 2002, SEAHMA; summer, 2005 EXOMAR) or via acoustically retrievable mussel cages (spring, 2007). Sampling was made by the telemanipulated ROV arm using the Remotely Operated Vehicle (ROV) Victor 6000 of R/V "Atalante". Individual *B. azoricus* were measured and byssus fragments were fixed for light, transmission-, and scanning-electron microscopy ("Tissue preparation for light and electron microscopy"), frozen for later dehydration and acid digestion for metal analysis ("Tissue preparation and metal analysis"), and fixed in 10% saline buffered formol for later FISH ("Tissue preparation for fluorescence in situ-hybridization"), according to the methods described in detail below.

Tissue preparation for light and electron microscopy

Byssal fragments from the three distinct regions of *B. azoricus* (adhesive plaque, distal portion and proximal portion) were fixed in modified Trump's fixative (3% glutaraldehyde and 3% paraformaldehyde made up with a fixation buffer containing: 0.15 M Na cacodylate, 0.3 M sucrose, 0.2 M NaCl and 0.008 M $CaCl_2$) according to Distel and Felbeck (1987).

Following primary fixation, samples were washed in 0.1 M cacodylate buffer (pH 7.8), post-fixed in 1% OsO_4, dehydrated in an ascending ethanol series and embedded in Epon (Sigma).

Semi-thin (2 μm) sections were stained with methylene blue, while ultra-thin sections were double stained with uranyl acetate and lead citrate before examination with a JEOL 100CXII transmission electron microscope operated at 60 KV.

Additional thick (5 μm) sections were cut and air-dried onto polyethylene slides, carbon-coated (FINE-COAT Ion sputter JFC-1100) and glued to aluminum stubs for SEM observations/elemental analysis using JEOL JSM-35C scanning electron microscope.

Tissue preparation and metal analysis

Whole byssus threads were acid digested prior to analysis as previously described (Jugdaohsingh et al. 1998). Briefly, 0.5–1 g of dry tissue (previously lyophilised using a Savant refrigerated vapour trap system overnight) was digested with equal volumes (1 ml/0.1 g dry weight) of Aristar grade concentrated (69% v/v) nitric acid and 30% hydrogen peroxide for 24 h. Digested samples were diluted 1:4 with high purity deionised water prior to ICPMS analysis using an Elan DRC ICP-MS system (Perkin-Elmer, Beaconsfield, UK). Standards were prepared using a commercially available multi-element standard added to blank digests, and ranged between 0 and 2,000 μg ml^{-1} for all metals of interest except for Fe and Zn that were between 0 and 20 μg ml^{-1}. The 0 μg ml^{-1} solution was used as a blank.

For total Hg analysis, digested tissue samples were diluted 12 times with high purity deionised water prior to analysis using Cold Vapour Atomic Fluorescence Spectrometry (PSA Millenium Merlin System) using a tin (II) reductant (Millenium Merlin method for determination of total mercury in mussel homogenate—*Mytilus edulis*, Application note 019). Five standards, ranging between 0 and 200 μg l^{-1}, were prepared in 4% HNO_3 using 1,000 μg ml^{-1} Hg standard solution (Merck).

Accuracy of the analytical methods was monitored by analyzing two different certified reference materials: CRM 414 (plankton) and CRM 278R (mussel tissue) and the measured values for Zn, Cu, and Cd were within 12% variation in both types of biological matrixes, while Al is not available and Co is only given as "not certified" in CRM 414. Total Hg concentrations in the same reference materials: CRM 414 (plankton) and CRM 278R (mussel tissue) were within 10% variation from the certified values, and sample spikes and blank spikes recoveries were between 90 and 116%.

Tissue preparation for fluorescence in situ-hybridization (FISH)

The presence of methanotrophic and thiotrophic bacterial endosymbiont in *B. azoricus* byssus plaque was tested using FISH according to Dupperron et al. (2005) with slight modifications: byssus plaques were fixed in 10% saline buffered formol and processed for paraffin embedding according to standard protocol. Transverse sections (thickness, 7 μm) were then subjected to deparrafination and rehydration in a decreasing ethanol series, permeabilized with proteinase-K (10 μg/ml) for 10 min, rinsed with distilled water and Phosphate buffered saline. Prior to hybridization, sections were pre-treated for 15 min with hybridization solution and then subjected to hybridization solution containing either of the specific methanotrophic or the thiotrophic simbiont probes (Dupperron et al. 2005). The fluochromes Alexa 488 and Alexa 532 (Molecuar Probes™, Invitrogen) were used to label the methanotrophic bacteria and thiotrophic bacteria probes respectively. Byssus plaques and intrabyssal bacteria were visualized under epi-fluorescent light and differential interference contrast (DIC) microscopy using a Leica DM6000 digital microscope (Leica Microsystems CMS GmbH, Germany).

Results

Metal content of byssus in *B. azoricus*

High concentration of the essential metals Fe, Cu and Zn were found in the byssus threads of mussels from both hydrothermal vent sites, reaching values over 65,000 μg g^{-1} Fe and 7,000 μg g^{-1} Cu in byssal threads of mussels from Lucky Strike, while Zn concentrations in byssus have reached over 2,000 μg g^{-1}at Menez Gwen (Fig. 1). Average concentrations

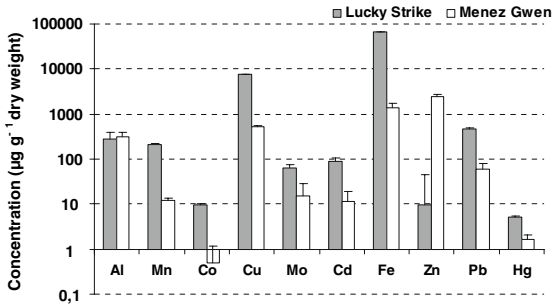


Fig. 1 Average concentration of metallic elements (μg g^{-1} dry weight) in the byssus threads of *B. azoricus* from Lucky Strike and Menez Gwen hydrothermal vent sites of the Mid-Atlantic Ridge. Vertical bars represent means ± SEM, $N = 3$

of non-essential metals were one order of magnitude lower than those of essential metals and decreased from 450 to 5 μg g^{-1} dry weight following the order Pb > Al ≥ Mn > Cd > Mo > Co > Hg. However, when considering molar concentrations, which is a better indicator of an element's prevalence, the non-essential metal concentration-order was changed to Al > Mn > Pb > Cd > Mo > Co > Hg.

Byssus morphology in *B. azoricus*

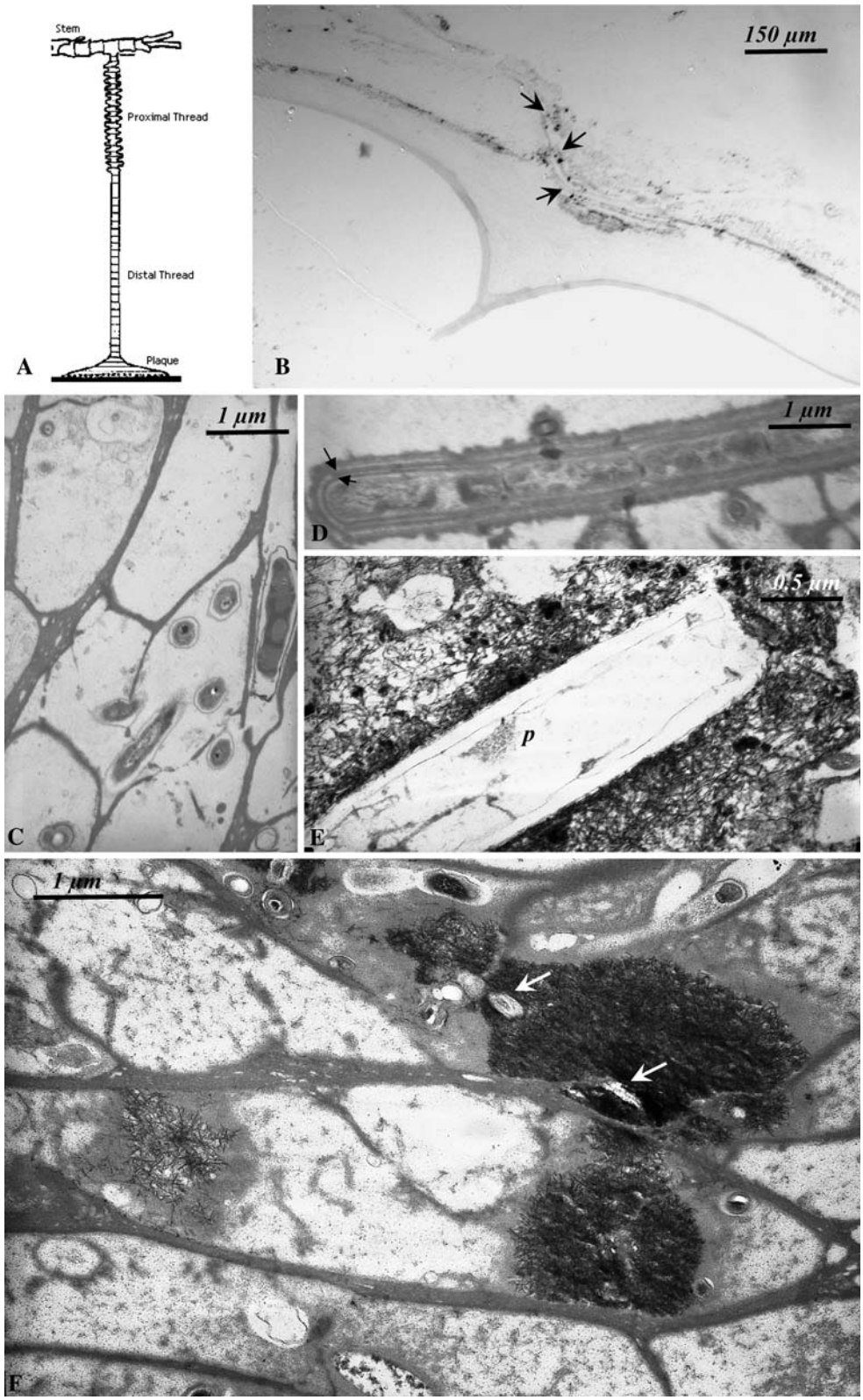
In order to elucidate the origin of such unusually high metal concentrations in byssus, we have started a more in depth investigation on its morphology and ultra structure. The general macro-anatomy of the byssus thread of *B. azoricus* is similar to that of other mytilid bivalves (Kadar and Azevedo 2006e), and is composed by the stem emerging from the byssal gland, continuing by the proximal and distal parts of the thread, and the adhesive plaque at its extremity (Fig. 2A). The adhesive plaque is covered by a protective coating of about 3 μm thickness that covers the internal spongy matrix (Fig. 2B and Fig. 3).

It was interesting to note the occurrence of unusual prokaryotic organisms embedded within the spongy matrix of the adhesive plaques in all byssus samples analysed in mussels from Lucky Strike vent field (Fig. 2C and D). Observed under TEM, at favourable sectioning angles, these organisms appeared to be filamentous and were made up of several cells. Each cell was enclosed in a double membrane indicating that it is probably a Gram negative bacterium. The uniform matrix of the byssus plaque was frequently disturbed by the presence of these cells and their putative metabolic products (Fig. 2). Several stages of disturbance could be seen, the simple entanglement of fibres around the filamentous bacteria (Fig. 2C); the appearance of mineral deposits at the surface of dead bacteria (Fig. 2D and E) that seemingly constitute surface for mineralization; appearance of electron-dense mineral deposits (Fig. 2F).

Fig. 2 Byssal threads of the vent mussel *B. azoricus*: (**A**)▶ schematic presentation of the byssus thread consisting of the stem emerging from the gland, the proximal- and distal-regions of the thread and the adhesive plaque that comes in contact to the attachment surface (adapted from Herbert Waite with his kind permission); (**B**) Light micrograph showing the adhesive plaque covered by a protective coating (asterisks) and several electron dense granules; (**C**) Electron micrograph showing membrane-bound prokaryotic organisms within the spongeous collagen matrix of the byssus; (**D**) longitudinal section through the string formed by the several cells covered by a double membrane (arrows). Note individual cells with DNA strands in the centre of an electron-translucent area (asterisks); (**E**) Higher magnification of one dead prokaryote (P) showing electron dense deposition surrounding the cell; (**F**) Electron dense deposition in a more advanced phase of biomineralisation within the collagenous net of byssus. Note remains of bacterial cells at the origin of each deposition (arrows)

Intra-byssal localization of metals in *B. azoricus*

In order to better understand the intra-byssal localization of these metallic elements and mechanism of their incorporation within the collagenous matrix of the plaque, semi-thin sections were prepared for scanning electron microscopy and observed using back scattered imaging (Fig. 3). Electron lucent areas, indicative of heavy elements, were then scanned for elemental composition (Fig. 3, Z1–Z13). Clear C, O and S peaks obtained when scanning areas within the protective layer of the byssus plaque (Z1 on Fig. 3) are consistent with the composition of the organic matrix, while peaks of Mn, Fe, Si, Al, Ba, S and P in electron-lucent granules (Z8–Z13) indicate the presence of hydrothermal elements found in the water column surrounding the mussels at Lucky Strike (Kadar et al. 2005c). Electron dense smears within the byssus network (Z5–Z7) showed distinct Mn and S peaks.


Stem
Proximal Thread
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Plaque
A
150 µm
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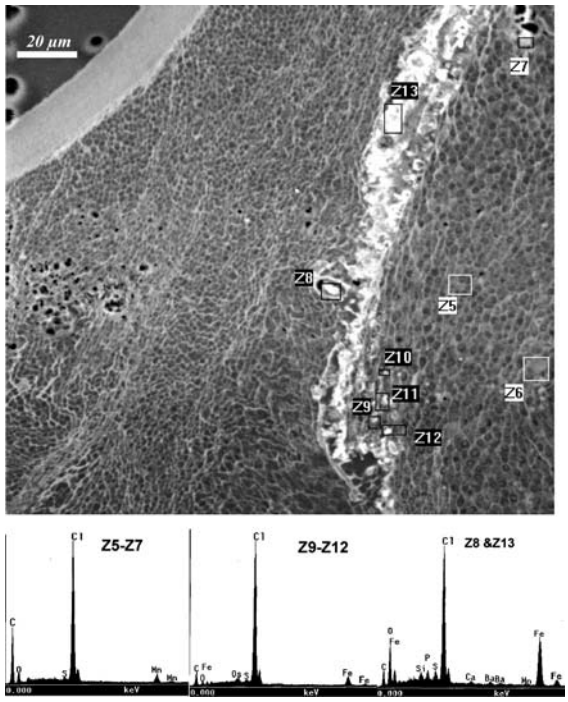

Fig. 3 Back scatter SEM image of a semi-thin (5 µm) section through the adhesive plaque showing the outer protective coat (oc) overlaying the spongeous collagen matrix (scm) containing various types of electron-lucent areas corresponding to site of heavy element localisation. Typical formations that were scanned for elemental composition were: Z5–Z17 electron-dense smears, Z9–Z12 more electron-dense granules and Z8&Z13 electron lucent aggregations; Z5&Z6&Z7: typical spectra obtained for metallic depositions surrounding sheath-forming individual cells within the collagen network of the byssus plaque, showing clear peaks for Mn; Z9–Z12: typical spectra obtained for spherical electron-dense depositions showing peaks for Fe and S; and Z8 & Z13: typical spectra obtained for electron-lucent metallic aggregations showing complex composition with peaks for the typical hydrothermal elements Fe, Ba, Mn, S, Si; High Cl peaks correspond to background levels in the plastic slide supporting the sections and Os peak is from the solution used for post-fixation

In situ hybridization of endosymbiont-specific probes by intra-byssal organisms

Byssus samples collected from mussels at Menez Gwen vent site indicated the presence of further morphotypes attached within the protective sheath and within the spongy network of the plaque (Fig. 4A, B, E and F). Hybridisation with these byssal plaque sections by using probes specific for endosymbiont bacteria found in gill bacteriocytes of *B. azoricus* (both sulphur oxidizer and methanotroph) confirmed the intra-byssal presence of only one morphotype present in the gills, i.e., the larger methane oxidizers, and the absence of the small, rod-shaped sulphur oxidisers. The probe specific to the thiotroph-related sequence hybridized to small bacteria found only on or within the protective sheet of the byssus plaque (Fig. 4C and D), while the probe specific for the methanotroph-related sequence hybridized to the large bacteria found both outside the protective layer and within the collagen matrix of the byssal plaque (Fig. 4G and H). This morphotype, although not identical, shared several common morphological features with methanotrophs described in the gill becteriocytes: had similar shape and size, and contained stacked internal membranes (Fig. 4 E and F). Similarly, the smaller morphotype showed analogous features with the sulphur oxidizers known in gill bacteriocytes: rod-shaped with clear double membrane (gram negative) with DNA strands found in the centre of an electron-translucent area (Fig. 4A and B).

Discussion

Unusually high metal concentration in byssus threads of *B. azoricus* have been previously reported in our lab (Kadar et al. 2005b, 2006a, b) and were considered a potential route of metal sequestration by binding negatively charged sites on its protein-made fibres. Additionally, the role of byssus threads in metal detoxification was also reported in other bivalves (Gundacker 1999; Yap et al. 2003). It may be possible that bivalent metals would occupy binding sites of essential metal ions that play significant roles in the structural integrity of the thread (Lucas et al. 2002), providing explanation to the high metal concentrations measured. This would imply that byssus threads of *B. azoricus* would be uniquely rich in metal binding sites. Alternatively, bacterial sequestration of metals within the thread would be a more likely explanation to the elevated concentrations measured. Indeed, our unusual finding of the ubiquitous presence of intra-byssal organisms, some of them apparently acting as organic matrix for Mn and Fe deposition (which maybe followed by co-deposition/adsorption of other metals) or methane oxidizers raises interesting questions related to the host-prokaryote reciprocal functions in the vent mussel.

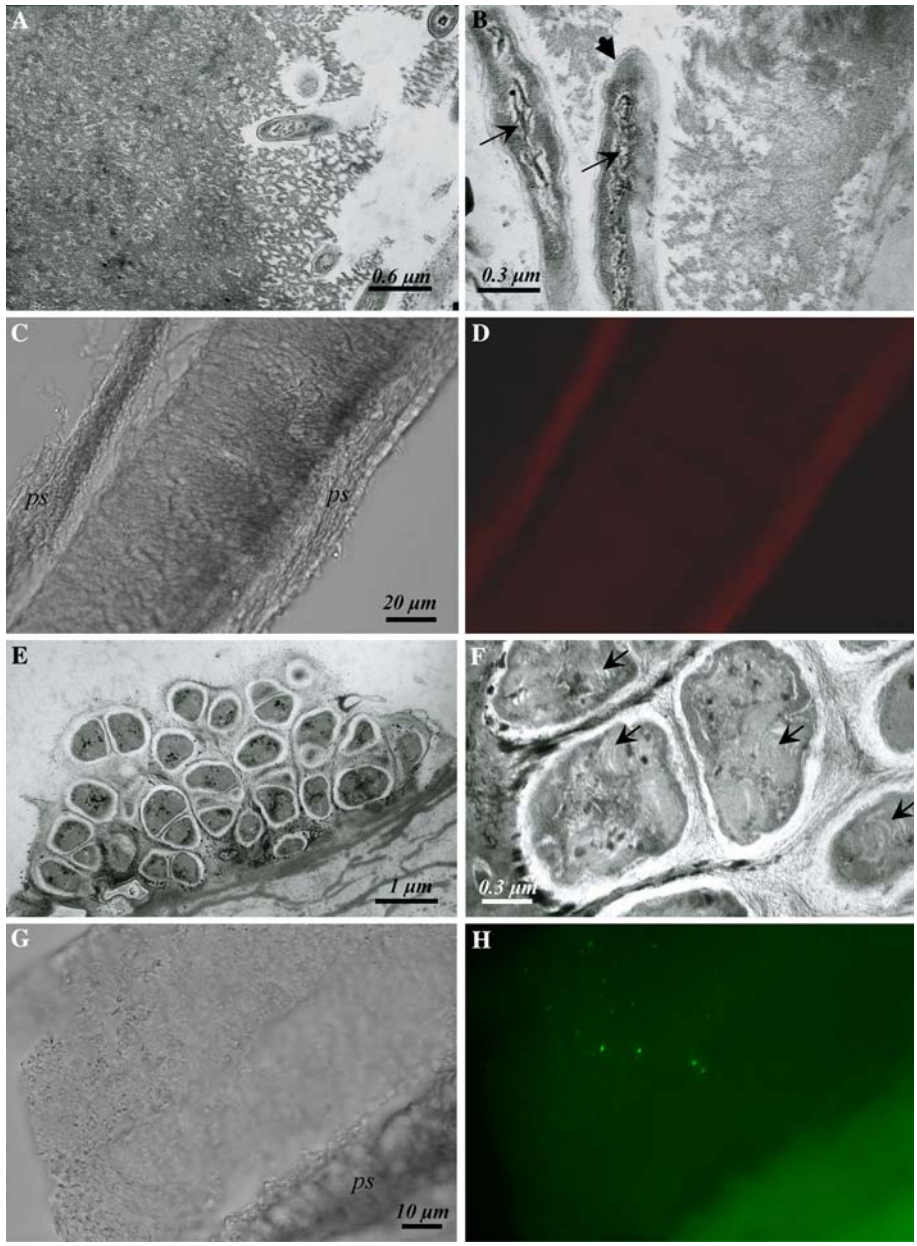

Fig. 4 Byssal threads of the vent mussel *B. azoricus* (**A**) Transmission electron micrograph showing intrabyssal organisms gaining access to the protective sheath of the byssus plaque; (**B**) higher magnification showing the intra-byssal organism resembling the sulphur oxidizer endosymbiont morphotype from gill bacteriocytes: rod shaped, has double cell membrane (Gram negative) (arrowheads) and a central clear zone with DNA strands visible (arrows); (**C**) Light micrograph on byssus plaque sections hybridized with the thiotrophic specific probe under bright light showing a portion of the collagenous byssus plaque covered by the protective sheath (*ps*); (**D**) FISH image on the same portion of plaque showing weak positive reaction only within the protective sheath of the plaque; (**E**) Transmission electron micrograph showing the larger intra-byssal organisms attached to the protective sheath of the byssus plaque; (**F**) higher magnification showing the intra-byssal organism resembling the methanotroph endosymbiont morphotype from gill bacteriocytes: large, coccoid, containing stacks of membranes (arrows); (**G**) Light micrograph on byssus plaque sections hybridized with the methantroph-specific probe under bright light showing a portion of the collagenous byssus plaque covered by the protective sheath (*ps*); (**H**) FISH image on the same portion of plaque showing strong positive reaction within the collagenous matrix of the plaque

In addition to the widely known gill endosymbioses, prokaryotes were also described in vent and seep mussels as non-gill symbioses and/or parasitoses (Johnson and Le Pennec 1995; Powell et al. 1999; Johnson and Fernandez 2001; Ward et al. 2004; Kadar et al. 2006f). 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.

Although some of the bacteria described here, i.e., the filamentous morphotype in mussels from Lucky Strike, share many morphological characteristics with the filamentous *Leptotrix* sp. (Tebo et al. 2004) regarding their dimension and cellular organization within the filament as well as the Mn oxide microcrystal accumulation at the cell surface, it is more likely that in the former metal sequestration/deposition is a passive process following the death of the bacteria. There is evidence of metal resistance in microorganisms isolated from deep-sea hydrothermal vents that suggests existence of an active mechanism responsible for immobilization of metallic ions (Cd and Zn) within the cell membrane and thus prevention of their toxicity (Llanos et al. 2000). Microorganisms that oxidize Mn (II) to Mn (III/IV) oxides are widespread in nature (Ghiorse 1984). The oxidation occurs on the outermost layer and Mn oxides accumulate on the extracellular organic matrix (glycocalyx) until the organism becomes encrusted (Tebo et al. 2004). It is unclear whether precipitation and mediation of Mn and sulphate minerals is a result of the metabolic activity of the bacteria, or a more passive process where functional groups on the bacterial surface react with positively charged ions (Emerson and Moyer 2002; Little et al. 2004). Whatever the mechanism, our observations may suggest that microbially mediated Fe/Mn oxidation at hydrothermal vents is a more common process than it was previously thought. Therefore, understanding the physiological role of these novel organisms within the byssus threads of *B. azoricus* is important to an understanding of energy flow and cycling of elements in the hydrothermal vent ecosystem.

Byssus is energetically costly (Papov et al. 1995), and has a very high energetic value that could provide nutritional support and suitable settlement surface to these organisms. They are clustered within the inner spongy net of the byssus string, and are exclusively found in the adhesive plack, i.e., the distal end of the byssus that is in contact with the rock surfaces. There were no indication as to how these organisms enter the thread despite its strong antibacterial activity that was demonstrated in modiolid byssus by Haug and co-workers (2004). Similar protective protein Mefp-1 (*Mytilus edulis* foot protein prevents threads from degradation by bacteria (Qin and Waite 1995). It is very likely, that free living forms are abundant and may enter the byssus string for shelter and nutritional support. Moreover, detection of methanotroph endosymbiont-specific hybridization products throughout the plaque may indicate that methane oxidizer endsosymbionts are hosted in the plaque in high number, and that they may also sequester metals in their cell wall, accounting for concentrations measured in byssus. In fact our earlier investigations on metal content in bacteria purified from gills of *B. azoricus* indicated that endosymbionts accounted for most of the metals accumulated in gills (Kadar et al. 2006a). Moreover, X-ray microanalysis on individual methanotroph bacteria within the gill bacteriocytes of mussels showed clear peaks for Cu, Fe, Cr, Ca, Al, and Si (Kádár et al. in press), indicating that they are indeed involved in accumulation of metals.

The presence of distinct species of bacteria that actively take up metals however needs to be confirmed, by for example, species-selective nucleic acid staining currently under development in our laboratory. Since Fe/Mn has been shown to be an important microbial energy source at vents, and it is predominant metal component of the byssus thread it is very likely that sheath-forming Fe-oxidizers co-exist with methanotroph endosymbionts here. Clearly, new nucleic acid sequences from additional free-living bacteria are needed to extend FISH analysis in order to fully resolve the nature of relationship between these bacteria and their host especially regarding their role in metal sequestration. The presence of methanotroph bacteria within the byssus plaque is an intriguing result from this study. These endosymbiont bacteria as we know them are harboured in the gills to provide their host with nutrients, which is quite unlikely in the case of byssus. It is interesting to speculate on their putative role in synthesis of organic molecules that may play a role in the adhesive properties of the plaque under such corrosive conditions as are typical at vents. However, such molecules are yet to be described.

These results demonstrate that in order to gain full understanding of the processes controlling metal cycling at vents, simultaneous micro-scale mineralogical and microbiological studies are needed in addition to chemical analysis to unravel the complex metal-organism interactions. This study has provided new insight into the frequently inferred, but rarely proven influence of prokaryotes on the biogeochemical cycling of metals at deep sea hydrothermal vents.

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