

RESEARCH ARTICLE

Diversity and function in microbial mats from the Lucky Strike hydrothermal vent field

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Abstract

Diversity and function in microbial mats from the Lucky Strike hydrothermal vent field (Mid-Atlantic Ridge) were investigated using molecular approaches. DNA and RNA were extracted from mat samples overlaying hydrothermal deposits and *Bathymodiolus azoricus* mussel assemblages. We constructed and analyzed libraries of 16S rRNA gene sequences and sequences of functional genes involved in autotrophic carbon fixation [forms I and II RuBisCO (*cbbL/M*), ATP-citrate lyase B (*aclB*)]; methane oxidation [particulate methane monooxygenase (*pmoA*)] and sulfur oxidation [adenosine-5'-phosphosulfate reductase (*aprA*) and *soxB*]. To gain new insights into the relationships between mats and mussels, we also used new domain-specific 16S rRNA gene primers targeting *Bathymodiolus* sp. symbionts. All identified archaeal sequences were affiliated with a single group: the marine group 1 *Thaumarchaeota*. In contrast, analyses of bacterial sequences revealed much higher diversity, although two phyla *Proteobacteria* and *Bacteroidetes* were largely dominant. The 16S rRNA gene sequence library revealed that species affiliated to *Beggiatoa* *Gammaproteobacteria* were the dominant active population. Analyses of DNA and RNA functional gene libraries revealed a diverse and active chemolithoautotrophic population. Most of these sequences were affiliated with *Gammaproteobacteria*, including hydrothermal fauna symbionts, *Thiotrichales* and *Methylococcales*. PCR and reverse transcription-PCR using 16S rRNA gene primers targeted to *Bathymodiolus* sp. symbionts revealed sequences affiliated with both methanotrophic and thiotrophic endosymbionts.

Introduction

Microbial mats – layered biofilms containing different types of cells – are complex systems in which representatives of various groups of organisms co-occur in various types of ecosystems. These mats are good models to study biogeochemical processes, such as chemical element cycles, in which a variety of microorganisms cooperate and interact in complex ways (Seckbach & Oren, 2010).

Recent studies on microbial mat communities from chemosynthesis-based ecosystems such as cold-seep areas (Arakawa *et al.*, 2006; Gilhooly *et al.*, 2007), mud volcanoes (Heijs *et al.*, 2005; Omeregíe *et al.*, 2008) or sulfide-rich caves (Engel *et al.*, 2004; Dattagupta *et al.*, 2009) have shown they may be host to a huge diversity of bacterial species,

dominated by *Proteobacteria*, including filamentous species belonging to the order *Thiotrichales*.

White filamentous mats have also been observed in various oceanic hydrothermal vent fields; large bacteria, morphologically affiliated with the genera *Beggiatoa* and *Thiothrix*, are the dominant morphotypes (Jannasch & Wirsén, 1981; Jacq *et al.*, 1989; Nelson *et al.*, 1989; Kalanetra *et al.*, 2004). These organisms, members of the order *Thiotrichales* (*Gammaproteobacteria*), are known for their ability to form biofilms on oxic/anoxic interfaces, using dissolved free oxygen to oxidize reduced sulfur compounds (Teske & Nelson, 2006).

Despite this fact, only a few studies have attempted to characterize microbial communities of deep-sea hydrothermal vent mats, i.e. along the Mariana Arc (Davis & Moyer,

2008), at the Loihi Seamount (Hawaii) (Moyer *et al.*, 1995), at the 17°S East Pacific Ridge (EPR) hydrothermal vent field (Longnecker & Reysenbach, 2001), the Lost City vent fields [Mid-Atlantic Ridge (MAR)] (Gerasimchuk *et al.*, 2010) and at the Fryer and Kaiko hydrothermal areas in the southern segment of the Mariana Trench (Kato *et al.*, 2009). In the present study, microbial mats were sampled from the Tour Eiffel edifice located on the Lucky Strike (LS) hydrothermal vent field on the MAR (37.29°N; 32.28°W) (Sarradin *et al.*, 1999). The LS vent field spreads around a central lava lake and where both high-temperature, active black smokers (324 °C) and lower-temperature diffuse flow areas (170 °C) are observed. The Eiffel Tower is a 12-m-high sulfide edifice located at 1650–1700 m depth. At this edifice, the vast majority of fluid-exposed hydrothermal deposits and *Bathymodiolus azoricus* mussels are covered by white filamentous mats whose role is still not understood. *Bathymodiolus azoricus* mussels dominate the megafauna and form large assemblages in low-temperature flow areas around the central edifice (De Busserolles *et al.*, 2009). Although the environment near mussel communities undergoes a temporal variation, it is characterized by a pH of 5.9–7.3 and sulfide and methane concentrations that allow chemosynthetic activity (De Busserolles *et al.*, 2009; Cuvelier *et al.*, 2011).

Bathymodiolus azoricus mussels harbor both thiotrophic and methanotrophic symbionts within their gills (Duperron *et al.*, 2006). Two species, *B. azoricus* and *Bathymodiolus puteoserpentis*, found in the northern MAR (Menez Gwen, LS, Rainbow and Logatchev vent fields), share the same two dominant bacterial symbiont phylotypes that belong to the *Gammaproteobacteria* (Duperron *et al.*, 2006). These chemosynthetic microorganisms enable the host to colonize sulfide- and/or methane-rich environments. The acquisition pathways of symbionts are not yet fully understood, although some studies provide support for the environmental transmission of thiotrophic symbionts (Won *et al.*, 2003). To date, no free-living forms of the symbionts have ever been described in this environment.

To better understand the function of microbial mats in the LS ecosystem and their possible relationships with other biological communities, we characterized microbial mats using molecular approaches. To do so, both DNA and RNA were extracted from mat samples overlaying hydrothermal deposits or *B. azoricus* assemblages. 16S rRNA gene-based phylogeny coupled with functional gene analyses can provide a better understanding of the microbial contribution to chemosynthetic ecosystems (Elsaied & Naganuma, 2001; Elsaied *et al.*, 2007). We therefore used universal primers to amplify the 16S rRNA gene from both DNA and RNA extracts, as well as the functional genes implicated in sulfur (*aprA*, *soxB*), methane (*pmoA*) and carbon (*cbhM/L*, *aclB*) metabolism. Moreover, primers specific to *Bathymodiolus*

sp. symbionts were used to screen for free-living symbiont-like phylotypes (Duperron *et al.*, 2006).

Materials and methods

Sampling site and procedure

The LS vent field (1700 m depth) is located at 31°17'N, 32°16'W on the MAR. Microbial mat filaments covering *B. azoricus* assemblages and hydrothermal deposits were sampled on the eastern and southern sides of the Tour Eiffel edifice during the French oceanographic cruises EXOMAR (2005), MoMARETO (2006) and MoMAR-08 (2008). Mat samples were retrieved from mussel assemblages 1 and 2b and substratum 1b, as defined by Cuvelier (Supporting Information, Table S1) (Cuvelier *et al.*, 2009). Using a water-pumping device on the ROV Victor 6000, samples were collected in 5-L pouches or by scraping mussel shells brought to the surface in previously decontaminated boxes. Before the dive, the tubes of the water-pumping device were washed twice with Desibac HPSTM (7 d'Armor), rinsed with sterile water, then with alcohol and finally filled with sterile seawater. Once on board (2–5 h after sampling), the contents of the pouches were filtered through 0.2 µm pore-size filters. Both types of samples (filters and scrapings) were transferred into sterile 2 mL Nunc CryotubesTM filled with sterile seawater or RNAlaterTM and immediately frozen at –80 °C.

DNA extraction, primer selection and PCR amplification

Total DNA was extracted using the FastDNA[®] Spin kit for soil (Qbiogene Inc., CA) protocol as modified by Webster and Roussel (Webster *et al.*, 2003; Roussel *et al.*, 2009).

For phylogenetic analyses, amplifications of 16S rRNA genes were performed using universal primers for *Bacteria* and *Archaea*: U1492R as reverse primer and E8F for *Bacteria* or A8F for *Archaea* as a forward primer (all the primers used in this study are listed in Table 1). Given the chemical composition of the fluids in LS, we focused our study on the main chemoautotrophic metabolisms that may occur within microbial mat communities. The presence of sulfur oxidizers was investigated using two pairs of primers (APS1F/APS4R and Sox432F/Sox1446R) specific to the adenosine-5'-phosphosulfate (APS) reductase α -subunit gene (*aprA*) and to the *soxB* component of the multienzyme thiosulfate-oxidizing complex gene (*soxB*), respectively. *soxB* and *aprA* genes constitute efficient functional markers for the two major biochemical pathways of bacterial sulfur oxidation (Meyer & Kuever, 2007a). To study methane oxidation, the primer pair A189F/MB661R was used; it is specific to the particulate methane monooxygenase gene (*pmoA*), which has been reported in all methanotrophs, except the genus

Table 1. Primers used in the study

Designation	Specificity	Primer sequence (5'–3')	References
U1492R	Universal 16S rRNA gene	GTT-ACC-TTG-TTA-CTA-CTT	Lane (1991)
E8F	Bacterial 16S rRNA gene	AGA-GTT-TGA-TCA-TGG-CTC-AG	
A8F	Archaeal 16S rRNA gene	CGG-TTG-ATC-CTG-CCG-GA	Kolganova et al. (2002)
907R	Universal 16S rRNA gene	CCG-TCA-ATT-CMT-TTG-AGT-TT	Lane et al. (1985)
Meth138F	16S rRNA gene methanotrophic <i>Bathymodiolus</i> sp. symbiont	TCT-GCC-TAT-TAG-TGG-GGG-ACA-ACA-TGG-T	Duperron et al. (2006)
Meth845R		GCT-CCG-CCA-CTA-AGC-CTA-TAA-ATA-GAC-C	
Sulfo193F	16S rRNA gene thiotrophic <i>Bathymodiolus</i> sp. symbiont	CTC-TAT-GGA-GTA-AAG-TGG-AGG-ACC-TTC-G	
Sulfo642R		CCT-ATA-CTC-TAG-TTT-GCC-AGT-TTC-AA	
<i>cbbL</i> _1b F	RuBisCO form I	CAC-CTG-GAC-CAC-VGT-BTG-G	Blazejak et al. (2006)
<i>cbbL</i> _2c R	RuBisCO form I	CGG-TGY-ATG-TGC-AGC-AGC-ATI-CCG	
<i>cbbM1</i> _Els F	RuBisCO form II	ATC-ATC-AAR-CCS-AAR-CTS-GGC-CTG-CGT-CC	
<i>cbbM</i> _2b R	RuBisCO form II	MGA-GGT-SAC-SGC-RCC-RTG-RCC-RGC-MCG-RTG	
<i>cbbM2</i> _Els R	RuBisCO form II	MGA-GGT-GAC-SGC-RCC-GTG-RCC-RGC-MCG-RTG	
<i>acIB892F</i>	ATP-citrate lyase β -subunit	TGG-ACM-ATG-GTD-GCY-GGK-GGT	Campbell et al. (2003)
<i>acIBR</i>	ATP-citrate lyase β -subunit	ATA-GTT-KGG-SCC-ACC-TCT-TC	
APS1F	Adenosine-5'-phosphosulfate reductase α -subunit	TGG-CAG-ATC-ATG-ATY-MAY-GG	Meyer & Kuever (2007b)
APS4R		GCG-CCA-ACY-GGR-CCR-TA	
<i>soxB432F</i>	<i>soxB</i> component of the periplasmic thiosulfate-oxidizing Sox enzyme complex	GAY-GGN-GGN-GAY-CAN-TGG	Petri et al. (2001)
<i>soxB693F</i>		ATC-GGN-CAR-GCN-TTY-CCN-TA	
<i>soxB1164R</i>		AAR-TTN-CCN-CGN-CGR-TA	
<i>soxB1446R</i>		CAT-GTC-NCC-NCC-RTG-YTG	
A189F	Particular pMMo	GGN-GAC-TGG-GAC-TTC-TGG	Holmes et al. (1995)
MB661R	Particular pMMo	CCG-GMG-CAA-CGT-CYT-TAC-C	

Methylocella (Dedysh et al., 2000). *pmoA* gene phylogeny is congruent with 16S rRNA gene phylogeny (Bourne et al., 2001); thus, *pmoA* is the preferred molecular marker to study the diversity of methane oxidizers (Fuse et al., 1998; Shigematsu et al., 1999). For autotrophic carbon fixation, we amplified forms I and II of RuBisCO (D-ribulose-1,5-bisphosphate carboxylase-oxygenase) using, respectively, *cbbLF/cbbLR* and *cbbMF/cbbMR/cbbMR* primer combinations, plus the ATP-citrate lyase β -subunit gene with *acIB892F/acIBR* primers. The Calvin–Benson–Basham (CBB) and the reductive tricarboxylic acid (rTCA) cycles are the two main pathways for carbon dioxide assimilation. *CbbL/M* are the usual markers for forms I and II RuBisCO (Badger & Bek, 2008); *acIB* is a gene encoding a key enzyme in the rTCA cycle, which appears to be used by a variety of deep-sea *Epsilonproteobacteria* (Campbell & Cary, 2004; Takai et al., 2005).

Bulk DNA was amplified in a 25 μ L reaction mix containing 5 μ L of 5 \times GoTaq[®] Flexi DNA polymerase buffer (Promega), 1.5 μ L of 25 mM MgCl₂ solution, 0.2 μ L of 10 mM dNTP solution, 0.1 μ L of each primer at 100 pM and 0.12 μ L of 5 U μ L^{−1} GoTaq[®] Flexi DNA polymerase (Promega). Domain-specific 16S rRNA gene primers targeting *Bathymodiolus* sp. symbionts, which are based on specific FISH probes (Duperron et al., 2007a, b), were tested. For methanotrophic symbionts, Meth138F and Meth845R

(5'-GCT-CCG-CCA-CTA-AGC-CTA-TAA-ATA-GAC-C-3') primers were used. For thiotrophic symbionts, Sulfo193F and Sulfo642R were used.

PCR conditions were the same as those described in the references cited for each primer pair.

RNA extraction and reverse transcription (RT)-PCR amplification

Total RNA was extracted using the FastRNA[®] Pro Soil Direct kit (Qbiogene Inc.) according to the manufacturer's instructions. To digest trace amounts of DNA, the extraction products were incubated for 1 h at 37 °C with 1 \times TURBO DNase[®] buffer and 18 U of TURBO DNase[®] (Ambion[™]). The digestion was stopped by adding EDTA to a final concentration of 15 mM and heating for 10 min at 65 °C before a purification step using the RNeasy minikit (Qiagen[™]) following the manufacturer's instructions. The absence of DNA was tested by direct PCR; all were negative.

RNA reverse transcriptions, followed by DNA amplification (RT-PCR) were performed using the OneStep RT-PCR kit (Qiagen[™]). Amplifications were performed using the same primer sets as for PCR amplifications, except to amplify the 16S rRNA gene, where the primers 907R and E8F were used for *Bacteria* and 915R and A8F were used for *Archaea* (Table 1).

The PCR conditions were the same as those indicated in the references for each primer pair with an additional reverse-transcription step (30 min at 50 °C) and an initial PCR activation step at 95 °C for 15 min before the standard cycles.

Clone library construction and sequencing

Before cloning, all PCR products were purified using the Qiaquick® Gel Extraction kit (Qiagen™) according to the manufacturer's instructions. Clone libraries were constructed by transforming *Escherichia coli* TOP10F' using the TOPO XL Cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid extraction, purification and sequencing were carried out by the OUEST-Genopole® sequencing facilities at the Roscoff Biological Station (France). For each gene library, mat samples from different assemblages (1 and 2b) and from substratum 1b were used. As we evidenced no difference between the obtained libraries, they were pooled for phylogenetic analysis.

Phylogenetic analysis

Sequence alignments, edition and analysis were performed using both BIOEDIT 7.0.9 (<http://www.mbio.ncsu.edu/BioEdit/page2.html>) and MUSCLE (Edgar, 2004) software. Phylogenetic analyses of the 16S rRNA gene sequences were performed using PHYLO_WIN (Galtier *et al.*, 1996) to construct trees based on the neighbor-joining algorithm and Kimura 2-parameter correction. Phylogenetic analyses of the other gene sequences were performed on amino acid-deduced sequences using the same software and algorithm, but with PAM correction. Robustness was tested by bootstrap resampling (1000 bootstrap samples). Sequences displaying more than 97% similarity were clustered within the same operational taxonomic unit (OTU). Rarefaction curves at 97% sequence similarity levels were performed using the DOTUR program (Schloss & Handelsman, 2005).

Nucleotide sequence accession numbers

For each OTU, one sequence was deposited in the GenBank database under accession numbers FR670348–FR670522 for 16S rRNA gene sequences and FR670535–FR670580 for functional gene sequences.

Results

Macroscopic and microscopic description

At the LS vent field, the microbial mats appeared as attached white filaments covering a large part of the mussel assemblages and fluid-exposed hydrothermal deposits (Fig. 1). Light microscopy observations revealed a wide diversity of morphology, both in size and shape, among microbial populations. We observed various kinds of filaments that resembled

filamentous bacterial populations reported at White Point, CA vent fields (Jacq *et al.*, 1989; Kalanetra *et al.*, 2004), in addition to numerous coccoid- and rod-shaped cells. Large, vacuolated filaments (14–65 µm in diameter), containing inclusions that could be sulfur granules, were the dominant morphotype in the mat communities, but thinner filaments (2–6 µm in diameter), probably nonvacuolate, and rosettes or tangles of smaller filaments, were also observed (Fig. 1). Most of the filament morphotypes (including the large dominant ones) hybridize with the GAM42a TRITC-labeled fluorescent probe, specific to the *Gammaproteobacteria*.

Archaeal and bacterial diversity in LS hydrothermal microbial mats

We identified 61 archaea-related sequences from the 16S rRNA gene library. They were distributed in only four OTUs defined at a similarity threshold of 97%. The archaeal 16S RNA transcript library was even less diversified, with only one OTU clustering the 22 sequences obtained.

From the bacterial 16S rRNA gene library, we retrieved 323 bacterial-related sequences. They were distributed in 163 OTUs defined at a similarity threshold of 97% (Table 2). The bacterial RNA transcript library was less diversified, with 76 sequences distributed in only three OTUs.

The diversity and representativeness of the microbial communities were estimated using rarefaction curves and diversity and coverage indices (Table 2 and Fig. S1). Coverage rates were high and curves reached saturation for the archaeal 16S rRNA gene and transcript libraries and also for the bacterial RNA transcript library. In contrast, for the bacterial 16S rRNA gene library, the coverage rate was only 55% and curves did not reach saturation, indicating that the molecular analysis only retrieved the dominant phyla. Simpson indices confirmed that the bacterial diversity was high, even if the active members of this community seemed limited to a few species.

Archaeal community structure

Archaeal diversity, according to the retrieved 16S rRNA gene sequences, was restricted to the marine archaeal group 1 *Thaumarchaeota* lineage. Almost all sequences were closely related (99% similarity) to the *Nitrosopumilus maritimus* 16S rRNA gene sequence and also, but more distantly (97% similarity), to species *Candidatus* Giganthauma karukerense and *Candidatus* Giganthauma insulaporcus (Fig. 2). The same *N. maritimus*-related sequence was also the only OTU identified in the 16S RNA transcript library.

Bacterial community structure

Analysis of the 323 bacterial 16S rRNA gene sequences indicated that mats were made up of a phylogenetically diverse bacterial population (Table 3 and Figs S2 and S3).

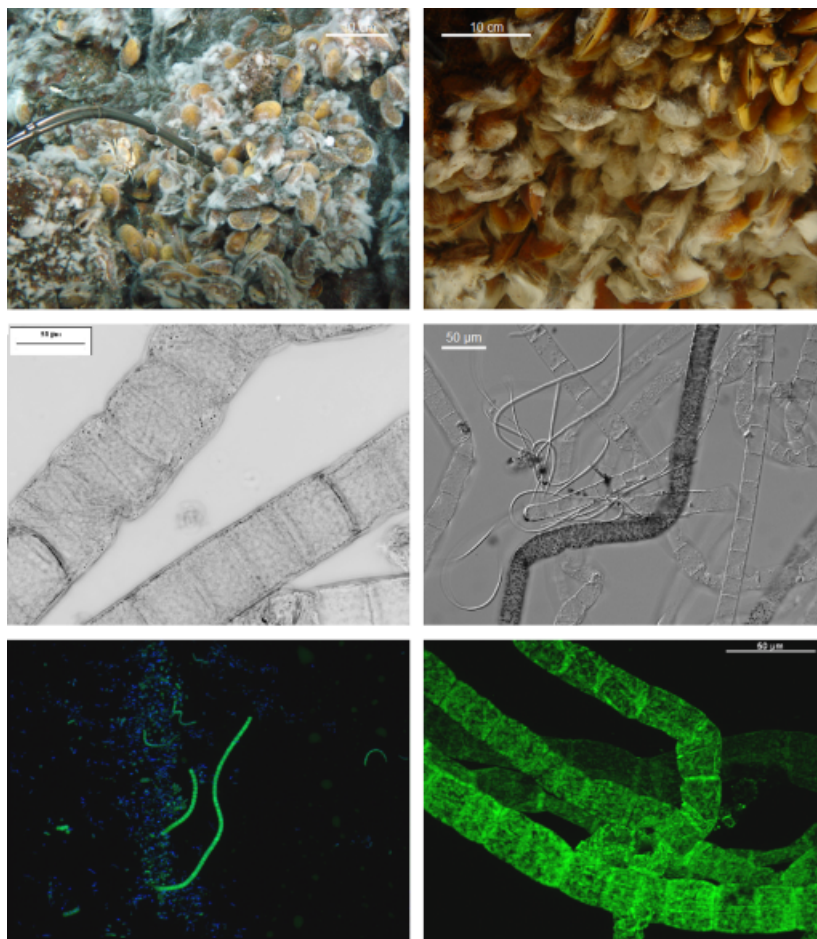


Fig. 1. White filamentous mats on *Bathymodiolus azoricus* mussels from the LS vent field (top left and right panels). Scale bar = 10 cm [IFREMER[ ], EXOMAR (2005) and Bathyluck (2009) cruises]. Light and differential interference contrast micrographs of large mat filaments found on the LS vent field (middle left and right panels, respectively). Scale bar = 50 µm. Whole-cell hybridization of various bacteria with a TRITC-labeled fluorescent probe (GAM42a) *Gammaproteobacteria* (in green) (bottom left and right panels, respectively). Scale bar = 50 µm (same for both pictures).

Table 2. Analysis of bacterial and archaeal diversity in LS microbial mats

Library	Number of clones	Number of OTUs (with ≥97% similarity)	Good's coverage (%)	1 – H_{Simpson}^*
Archaeal	61	4	96.7	0.157
Archaeal transcripts	22	1	100	0
Bacterial	323	162	55.1	0.988
Bacterial transcripts	76	3	97.4	0.152

*Simpson index values are presented as 1 – D for better readability, diversity ranges from 0 (one species) to 1 (maximal diversity).

Retrieved bacterial sequences were affiliated with seven phyla, but among them, the phyla *Proteobacteria* and *Bacteroidetes* were clearly dominant. Within *Proteobacteria*, various OTUs were related to genera implied in sulfur oxidation such as *Roseobacter*, *Sulfitobacter* and *Thalassobacter* for the *Alphaproteobacteria*, *Beggiatoa* and *Leucothrix* for the *Gammaproteobacteria* and *Sulfurimonas* and *Sulfovorum* for the *Epsilonproteobacteria*. A few *Gammaproteobacteria* sequences related to methane oxidizers were also retrieved with affiliations with the genera *Methylobacter* and *Methylo-*

monas (Costello & Lidstrom, 1999; Hirayama et al., 2007); moreover, two sequences were closely affiliated (99.7% similarity) with the *B. azoricus* methanotrophic symbiont (Duperron et al., 2006). Most of the OTUs observed were affiliated with clones from various chemosynthetic ecosystems including hydrothermal-fauna-associated clones and microbial mats from the Milano mud volcano (Heijs et al., 2005), cold seeps in the Sea of Japan (Arakawa et al., 2006), shallow White Point (CA) hydrothermal vents (Kalanetra et al., 2004) and Okinawa (East China Sea) and Lost City (North Atlantic Ocean) vent fields (Hirayama et al., 2007; Brazelton et al., 2010).

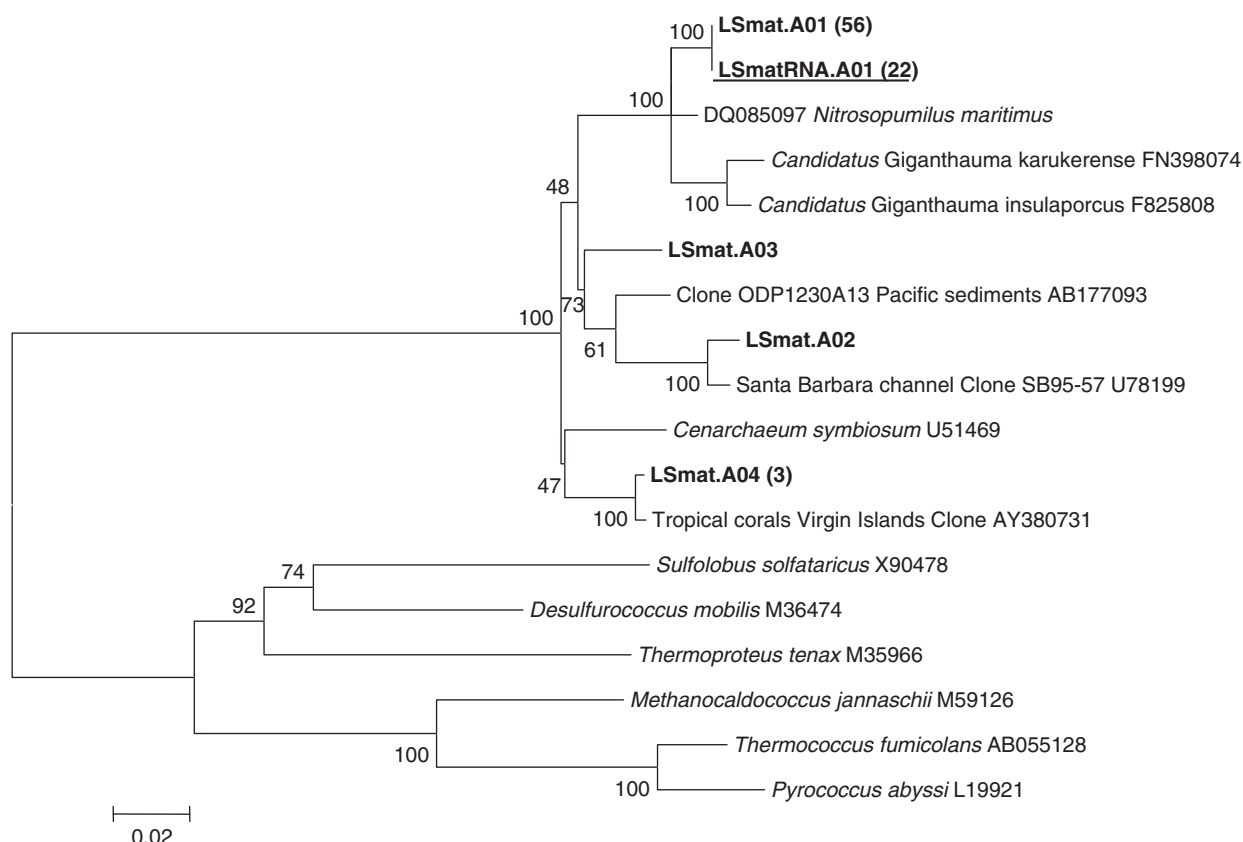


Fig. 2. Neighbor-joining phylogenetic tree showing the relationship of 16S rRNA genes sequences obtained with universal *Archaea* 16S rRNA gene primers. Bootstrap values > 60% (from 1000 bootstrap samples) are indicated near their corresponding nodes. Representative sequences obtained in this study are in bold and all RNA transcript sequences are also underlined. Numbers in parentheses indicate the number of identical clones.

Analysis of the 16S RNA transcript library indicated that the active bacterial community was restricted to three OTUs. Almost all sequences were closely affiliated (99% similarity) with the 16S rRNA gene sequence of a filamentous *Beggiatoa* from White Point, also retrieved by direct PCR (Table 4 and Fig. S2). This vacuolated, filamentous, sulfur-oxidizing bacterium has been described as attached to diverse biotic and abiotic substrates in the shallow White Point hydrothermal vents (Kalanetra *et al.*, 2004). The other two sequences were affiliated, respectively, with an uncultivated *Gammaproteobacteria* *Methylococcales* from the Lost City vent field (Brazelton *et al.*, 2006) and with an *Alphaproteobacteria* from the EPR (Santelli *et al.*, 2008).

Presence of *Bathymodiolus* symbiont 16S DNA and RNA

Two sequences from our 16S DNA library were closely affiliated (> 99% similarity) with the monophyletic methanotrophic *B. azoricus* and *B. puteoserpentis* symbiont (*Gammaproteobacteria*) from the MAR, but none were affiliated to the thiotrophic symbiont. To confirm this result and explore the possibility of a free-living mussel symbiont, we used new

16S rRNA gene primers specific to *Bathymodiolus* sp. methanotrophic or thiotrophic symbionts (Duperron *et al.*, 2006).

Eleven 16S DNA clone sequences and 10 RNA clone sequences were retrieved with the Meth138F/845R primer pair targeted to methanotrophic symbionts. All sequences were identical to each other and closely matched (> 99% similarity) the monophyletic methanotrophic *B. azoricus* and *B. puteoserpentis* symbiont sequence from the MAR (Duperron *et al.*, 2006) (Fig. 3a).

Twelve 16S DNA clone sequences and 10 RNA clone sequences were retrieved with the Sulfo193F/642R primer pair targeted to thiotrophic symbionts. Three OTUs were detected in both gene DNA and RNA transcript sequences; two of them were related (93.7% and 100% similarity) to *B. azoricus* thioautotrophic symbionts from LS (Duperron *et al.*, 2006; Won *et al.*, 2008) (Fig. 3a).

Characterization of the chemolithoautotrophic populations

Six DNA and four RNA transcript clone libraries targeting functional genes were constructed. Although we failed to

Table 3. Phylogenetic positioning and abundance of bacterial OTUs in LS microbial mats based on the 16S gene

Phylogenetic group	Number of related clones	Number of OTUs ($\geq 97\%$ similarity)	% of OTUs
<i>Alphaproteobacteria</i>	36	21	13
<i>Rhodobacterales</i>	27		
<i>Rhizobiales</i>	7		
Other α clones	2		
<i>Gammaproteobacteria</i>	88	44	27
<i>Thiotrichales</i>	10		
<i>Methylococcales</i>	5		
<i>Chromatiales</i>	9		
<i>Oceanospirillales</i>	3		
<i>Alteromonadales</i>	5		
<i>Pseudomonadales</i>	3		
<i>B. azoricus</i> methanotrophic symbiont	2		
Other γ clones	51		
<i>Deltaproteobacteria</i>	13	8	5
<i>Desulfurobacterales</i>	2		
<i>Mycococcales</i>	6		
Other δ clones	5		
<i>Epsilonproteobacteria</i>	81	29	18
<i>Helicobacteraceae</i>	16		
Group F	65		
<i>Bacteroidetes</i>	82	44	27
<i>Flavobacteriales</i>	72		
<i>Sphingobacteriales</i>	10		
<i>Planctomycetes</i>	8	7	4
<i>Chloroflexi</i>	7	6	4
<i>Actinomycetes</i>	4	2	1
<i>Chlorobi</i>	2	1	< 1
<i>Verrucomicrobia</i>	2	1	< 1
Total	323 (100%)	163 (100%)	100

Table 4. Phylogenetic positioning and abundance of bacterial clones sequenced from the 16S RNA transcript library

Clone designation	Number of clones	Phylogenetic group	Nearest relative (NCBI GenBank)	Similarity (%)
LSmat_RNA.B01	74	<i>Gammaproteobacteria Thiotrichales</i>	Filamentous bacterium from White Point (AY496953)	99
		<i>Gammaproteobacteria Thiotrichales</i>	Filamentous bacterium from Escanaba Trough (AY883934)	99
LSmat_RNA.B02	1	<i>Gammaproteobacteria (Methylococcales)</i>	Kazan mud volcano clone (FJ712402)	97
			<i>Methylomonas</i> sp. (AB01603)	93
LSmat_RNA.B03*	1	<i>Alphaproteobacteria (Rhizobiales)</i>	Bacterium clone EPR3968-O8a-Bc54 (EU491721)	95

*Partial sequence (200 bp).

amplify transcripts using Sox432F/Sox1446R and *aclB*892F/*aclB*R primer pairs in RT-PCR, successful PCR amplifications indicated at least the presence of microorganisms having *soxB* and *aclB* genes in the community.

Sulfur oxidation

Forty clones from the DNA library and 16 clones from the RNA library were retrieved using APS (*aprA*) primers (Fig. 4c). Clone sequences were distributed between sulfur-oxidizing bacteria lineages I and II, and were all affiliated with sequences related to *Gammaproteobacteria*. Most transcript and gene sequences were related to a *Siboglinidae* tubeworm

symbiont from eastern Mediterranean cold seeps. One transcript sequence related to a bacterial endosymbiont of the bivalve *Idas* sp. (Duperron *et al.*, 2008) was also identified. Among the gene sequences, some were related to clones associated with symbionts of tubeworms such as *Sclerolium contortum* and *Oligobranchia haakonmosbiensis* from H  kon Mosby mud volcano (L  sekann *et al.*, 2008) and others with symbionts of species such as *Asterechinus elegans*, an echinoderm associated with wood falls (Becker *et al.*, 2009), or *Kiwa hirsuta*, a galatheid collected from hydrothermal vents in the Pacific-Antarctic Ridge (Goffredi *et al.*, 2008).

We retrieved 31 gene clones using *soxB* primers, but no transcripts were amplified (Fig. 4a). All retrieved sequences



Fig. 3. Neighbor-joining phylogenetic tree showing the relationship of 16S rRNA gene sequences obtained with domain-specific primers targeted to *Bathymodiolus* sp. symbionts in PCR and RT-PCR (a). Neighbor-joining phylogenetic tree constructed using PAM correction of the amino acid sequences deduced from a fragment of the gene encoding for the particulate methane monooxygenase gene (LSmat.pmoA#) and for the APS reductase α -subunit gene (LSmat.aprA#) from LS mats (b and c, respectively). Bootstrap values > 60% (from 1000 bootstrap samples) are indicated near their corresponding nodes. Representative sequences obtained in this study are shown in bold and all RNA transcript sequences are also underlined. Numbers in parentheses indicate the number of identical clones.

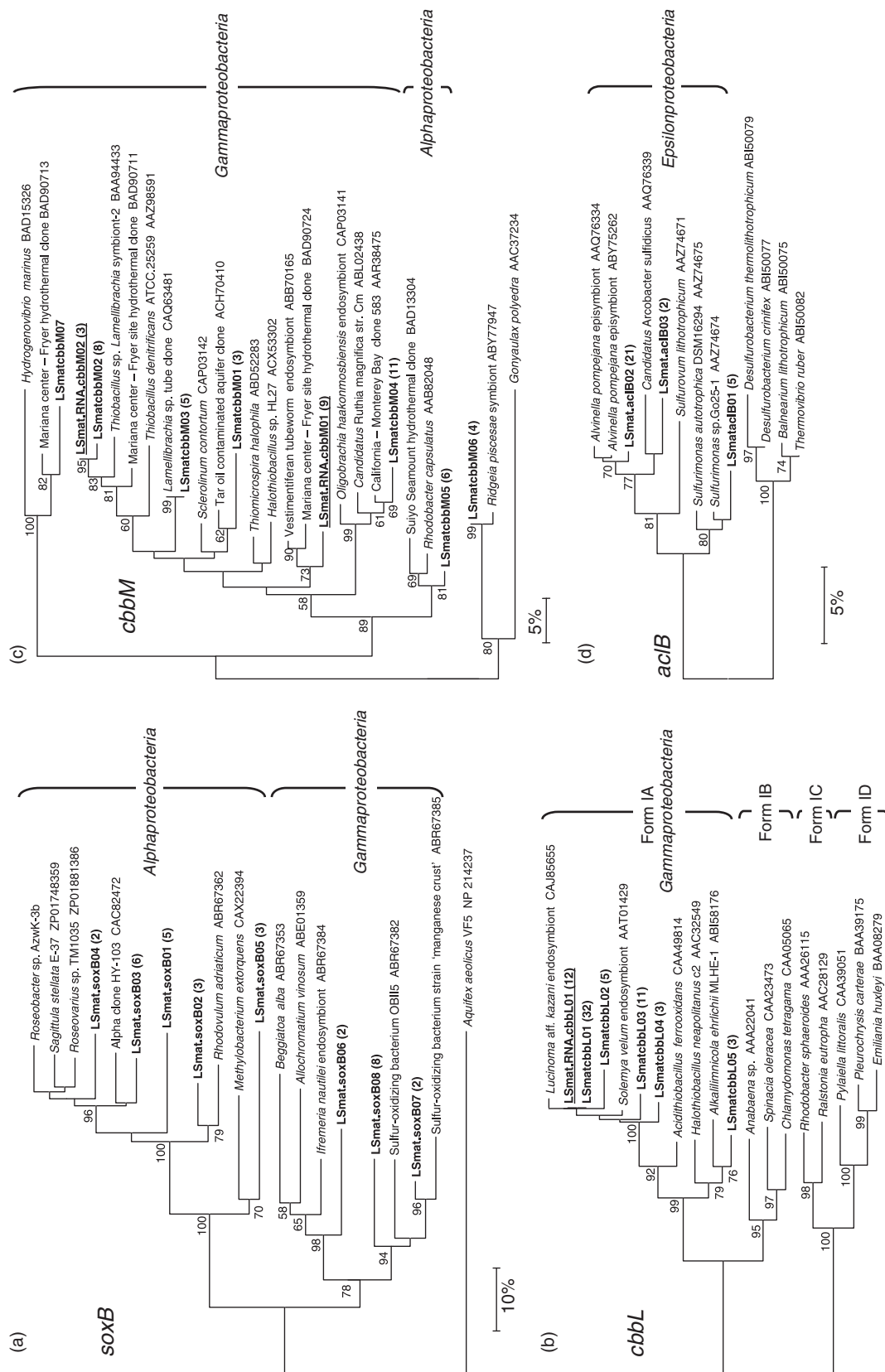


Fig. 4. Neighbor-joining phylogenetic tree constructed using PAM correction of amino acid sequences deduced from a fragment of the gene encoding for an element of the periplasmic thiosulfate-oxidizing Sox enzyme complex (LSmat.soxB#), the form I and II Rubisco (LSmat.cbbL/M#) and the β -subunit of ATP-citrate lyase (LSmat.acIB#) (a, b, c and d, respectively). Bootstrap values > 60% (from 1000 bootstrap samples) are indicated near their corresponding nodes. Representative sequences obtained in this study are in bold and all RNA transcript sequences are also underlined. Numbers in parentheses indicate the number of identical clones.

were related to *Alphaproteobacteria* and *Gammaproteobacteria* sulfur oxidizers (Mukhopadhyaya *et al.*, 2000; Meyer *et al.*, 2007).

Methane oxidation

We obtained 33 clones of particulate methane monooxygenase (*pmoA*) genes and 15 transcript sequences using *pmoA* primers (Fig. 3b). This transcript library confirmed the presence of active methane oxidizers, all affiliated with *Gammaproteobacteria* in the microbial mat community. They were related to uncultured bacteria from Pacific Northwest marine sediments (Nold *et al.*, 2000) or the Rainbow vent field (MAR) (Nercessian *et al.*, 2005) and to *Bathymodiolus childressi* symbionts (Duperron *et al.*, 2007a,b). In addition, sequences related to *B. azoricus* symbionts, *Methylococcales* *Methylohalobius crimeensis* (Heyer *et al.*, 2005), *Idas* sp. symbionts (Duperron *et al.*, 2008) and uncultured bacteria from methane seep habitats at the Kuroshima Knoll in the southern Ryukyu Arc (Inagaki *et al.*, 2004b) were identified in the *pmoA* gene library.

Carbon fixation

We retrieved 54 form I RuBisCO (*cbbL*) genes and 12 transcript sequences, all related to *Gammaproteobacteria* (Fig. 4b). Several clones from both libraries were affiliated with a *Lucinoma* aff. *kazani* endosymbiont (Duperron *et al.*, 2007a,b) and *Solemya velum* gill sulfur-oxidizing symbionts (Schwedock *et al.*, 2004). In the *cbbL* gene library, sequences distantly affiliated with *Chromatiales* *Alkalilimnicola ehrlichii* MLHE-1 and *Halothiobacillus neapolitanus* (Baker *et al.*, 1998) were also identified.

We retrieved 38 form II RuBisCO (*cbbM*) genes and 12 transcript sequences (Fig. 4c). Most were related to *Gammaproteobacteria*. Sequences related to a *Thiobacillus* sp. *Lamellibrachia* symbiont (Elsaied & Naganuma, 2001) and to a vestimentiferan tubeworm symbiont (Vrijenhoek *et al.*, 2007) were identified both in gene and in transcript libraries. In the gene library, sequences related to tubeworm symbionts of *Lamellibrachia* from the Håkon Mosby mud volcano (Lösekann *et al.*, 2008) were also retrieved. Additionally, sequences related to the nitrogen-fixing *Alphaproteobacteria* *Rhodobacter capsulatus* (Larimer *et al.*, 1995) were identified, as well as sequences related to organisms from diverse marine environments.

Using *aclB* primers, 28 ATP-citrate lyase B gene sequences were obtained, but we failed to construct a transcript library (Fig. 4d). All *aclB* gene sequences were affiliated with *Epsilonproteobacteria*, including relatives of the genera *Sulfurimonas* and *Arcobacter* (Campbell *et al.*, 2003; Takai *et al.*, 2005) and hydrothermal invertebrate epibionts associated

with *K. hirsuta* (Goffredi *et al.*, 2008) and *Alvinella pompejana* (Campbell *et al.*, 2003).

Discussion

Limits of the molecular approaches

Phylogenetic analysis of 16S rRNA gene sequences from the LS microbial mats revealed the presence of a highly diversified bacterial community, but a relatively low diversity of archaeal taxa. Because of low Good's coverage values (55.1%, Table 2) for the bacterial 16S rRNA gene sequence library, and due to biases associated with PCR, DNA/RNA extraction and cloning (Wintzingerode *et al.*, 1997), the abundance of a particular OTU in a library does not necessarily reflect its real abundance in the original sample (Teske & Sorensen, 2008). Furthermore, free-living microorganisms from the surrounding environment not directly involved in mat structure and activity may also have been trapped in the mat and thereby contribute to an inaccurate picture of microbial diversity.

In addition, our results underline the need for specific primers to amplify 16S rRNA genes of poorly represented species or specific groups, which cannot be retrieved with universal primers. For example, we noted that although we identified some *Planctomycetes*-related sequences, none of them were affiliated with anaerobic ammonium oxidation (anammox) clones already retrieved from the same mat samples analyzed in a previous study (Byrne *et al.*, 2008). In this study, the use of domain-specific primers revealed 16S thiotrophic symbiont signatures, although universal 16S rRNA gene primers failed to detect them.

mRNA has a short intracellular lifetime and therefore directly represents active metabolic processes. mRNA molecules are therefore more suitable as microbial activity indicators than 16S rRNA gene molecules, which can persist for up to a few days in the environment (Chin *et al.*, 2008; Lloyd *et al.*, 2010). In this study, both approaches were combined. In our RNA studies, we attempted to minimize as much as possible the lapse of time between sampling and on-board treatment (2–5 h) to avoid the rapid degradation of mRNA.

Finally, it should be noted that phylogenetic assignment and the metabolic role of bacterial communities were difficult to determine due to the presence of numerous bacterial 16S rRNA gene sequences that had sequence similarities of < 94% with their closest cultured relatives. Many sequences retrieved in this study may therefore represent new, undiscovered genera or families. Conferring a possible phenotype to the organisms detected in this study must therefore be carried out with caution. This is particularly crucial for the phylogenetic groups that contain phenotypically diverse organisms, such as the *Proteobacteria*. The analysis of

functional gene libraries is even more complicated due to the lack of data and the over-representation of symbiont-related sequences in gene banks.

Prevalence of sulfur oxidizers among microbial populations

Low, but constant levels of sulfides surrounding the mat samples (0.8–2 μM) and the presence of oxygen (230–240 μM) (Sarradin *et al.*, 2009; Cuvelier *et al.*, 2011) make the mats a good habitat for sulfur-oxidizing populations (Fig. 5). The highest number of clone sequences retrieved in our 16S rRNA gene bacterial library (Table 3) was affiliated with the *Gammaproteobacteria*. Most of them were closely related to environmental sequences identified from deep-sea sediments and some clustered within groups containing sulfur-/methane-oxidizing isolates and gill symbionts. Functional gene libraries were also dominated by clone sequences affiliated with similar populations: the nine OTUs detected using primers based on the *aprA* gene all belonged to the *Gammaproteobacteria* symbiont cluster (Meyer & Kuever, 2007a,b) (Fig. 3c). Likewise, among the eight OTUs detected with the *soxB* gene, three were related to *Gammaproteobacteria* sulfur oxidizers (Fig. 4a). In addition, form I and II RuBisCO gene sequences confirmed the

presence of chemoautotrophs belonging to this group (Fig. 4b and c).

However, despite this high taxonomic diversity, the 16S rRNA gene transcript library was dominated by sequences affiliated with filamentous *Beggiatoa* species. This may indicate that this genus dominates active microbial communities. However, because it was not possible to construct a *soxB* transcript library, we cannot conclude as to *Beggiatoa* sulfur-oxidizing activity. Furthermore, this pathway may be present, but not active at the time of sampling or its mRNA may be more sensitive than others, degrading more rapidly when the samples were recovered. On the contrary, in the analysis of the transcript library, the use of *aprA* gene primers detected three OTUs belonging to the *Gammaproteobacteria* symbiont cluster that includes active sulfur oxidizers (Fig. 3c). Form I and II RuBisCO gene transcript libraries also indicated active carbon fixation for three OTUs related to potential *Gammaproteobacteria* symbionts (Fig. 4b and c). No transcripts belonging to *B. azoricus* thiotrophic symbionts were found in the *aprA* gene library despite the symbionts' presence in both 16S rRNA gene libraries (RNA and DNA) constructed with symbiont-specific primers. This result can be attributed to either a low symbiont cell concentration in the sample or to the absence of sulfur-oxidizing activity at the time of sampling.

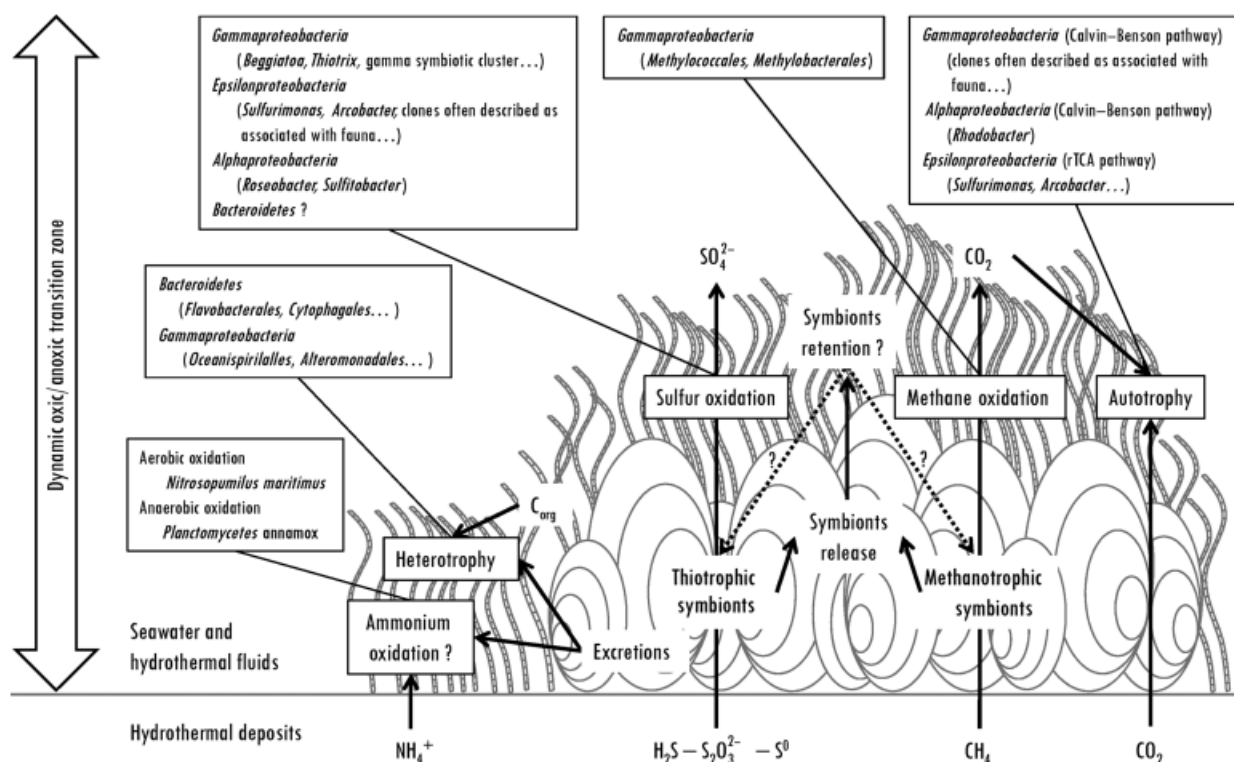


Fig. 5. Detected metabolisms occurring in LS microbial mats and hypothetical relationships with mussels *Bathymodiolus azoricus*. Main orders or genera for each metabolism are indicated in parentheses. Groups detected as active in RT-PCR are underlined. Data on *Planctomycetes anammox* are from a previous study (Byrne *et al.*, 2008).

Alternatively, the symbionts may be in a dormant form, or may have a different metabolism, before they are trapped in the gill cells of *Bathymodiolus* sp.

Oxidation of reduced sulfur compounds as a possible prevailing microbial metabolism in the mats was also consistent with the finding of sequences clustering among the *Epsilonproteobacteria*, *Alphaproteobacteria* and *Bacteroidetes* groups in the 16S rRNA gene library (Table 3, Figs S2 and S3). Several recent molecular studies have demonstrated the presence and the dominance of the *Epsilonproteobacteria* as both free-living organisms or in association with metazoans at deep-sea hydrothermal vents (Corre *et al.*, 2001; Campbell *et al.*, 2006; Zbinden *et al.*, 2008). Many sequences from the 16S rRNA gene library were affiliated with *Epsilonproteobacteria*, including OTUs previously identified from vent epibionts and environmental sequences from deep-sea cold seeps and hydrothermal vents (Corre *et al.*, 2001). The phylogenetic analysis of the rTCA gene library also revealed OTUs related to known *Epsilonproteobacteria*. Surprisingly, no sequences in the *soxB* clone library matched known *Epsilonproteobacteria* sequences, although sulfur oxidizers of this group are known to use the SOX pathway and were well represented in the *aclB* gene library. This may indicate PCR biases or the predominance of *Gammaproteobacteria* in sulfur-oxidizer populations. Indeed, despite the fact that the *Epsilonproteobacteria* are known for their metabolic and thermal versatility, they appear to be well adapted to microaerobic or anaerobic conditions and also to high sulfide concentrations (Engel *et al.*, 2003; Inagaki *et al.*, 2004a; Campbell *et al.*, 2006; Takai *et al.*, 2006). The low sulfide (0.8–2 μM) and high oxygen (230–240 μM) concentrations measured in the mats might actually inhibit *Epsilonproteobacteria* and generally favor *Gammaproteobacteria*. In addition, it is possible that the *Epsilonproteobacteria* are active below the seafloor, where conditions are more suitable, and become inactive when washed out with the hydrothermal fluids and being exposed to microbial mat conditions.

The *Alphaproteobacteria* and the *Bacteroidetes* groups are ubiquitous in diverse marine environments, including surface and deep waters, sediments (Lopez-Garcia *et al.*, 2001; Kirchman, 2002) and deep-sea hydrothermal vents (Reysenbach *et al.*, 2000; Alain *et al.*, 2002; Lopez-Garcia *et al.*, 2002; Huber *et al.*, 2003). The presence of sequences belonging to *Alphaproteobacteria* in the 16S rRNA gene library as well as in the *soxB* and form II RuBisCO gene libraries (Fig. 4a and c) seems to demonstrate its importance in LS mats, even if no transcript sequences were obtained. The *Bacteroidetes* bacteria are usually aerobic chemoorganotrophic or lithotrophic microorganisms and exhibit diverse metabolic capabilities (Kirchman, 2002) such as oxidation of reduced sulfur compounds, although they do not depend on this reaction for growth (Teske *et al.*, 2000).

Presence of active methane oxidizers

Methane concentrations in LS smokers were 0.68 mM and 0.5–40 μM in the mussel assemblages (Table S1). Methane oxidation-based metabolism is thus a possible pathway utilized by species in our mat samples (Fig. 5). Few sequences in the 16S rRNA gene library were affiliated with the orders *Methylobacterales* and *Methylococcales* (*Gammaproteobacteria*) (Fig. S2). In addition, one sequence in the 16S rRNA gene transcript library was related to a methanotrophic uncultured clone, perhaps representative of aerobic methane-oxidizing activity. The analysis of the *pmoA* gene-based libraries provides support for this interpretation, as eight OTUs, including three in RNA transcript library, were detected (Fig. 3b). There are therefore potential active methane oxidizers in our mats such as *Gammaproteobacteria*, including *Methylococcales*. The presence (but not the activity) of methanotrophic *B. azoricus* symbionts among the mat microbial community is also confirmed. As surmised above for the thiotrophic symbiont, this may indicate that the symbiont-like microorganisms are not active in their free-living state and may be in a dormant form.

Other metabolic pathways in LS mats

Apart from methane and sulfur oxidizers, the geochemistry of the vent fluids at LS provides favorable conditions for other chemoautotrophic metabolisms based on ammonium, hydrogen, metals, etc. (Salerno *et al.*, 2005; Duperron *et al.*, 2007a,b; Sarradin *et al.*, 2009). Our study did not focus on these pathways, but analysis of the 16S rRNA gene libraries provided some information (Fig. 5). A phylotype related to *N. maritimus* dominated the archaeal 16S clones and constituted probably the main active *Archaea* present in the collected mats (Fig. 2). These ubiquitous, low-temperature *Thaumarchaeota* grow chemolithoautotrophically via the aerobic oxidation of ammonium to nitrite (Konneke *et al.*, 2005). Their presence in LS microbial mats, as the presence of other marine archaeal group 1-related sequences, may reflect aerobic ammonium-oxidizing activity (Walker *et al.*, 2010), which is consistent with the ammonia concentrations observed in the LS hydrothermal fluid (i.e. 8–10 $\mu\text{mol L}^{-1}$) and in the mussel bed environment (< 2 $\mu\text{mol L}^{-1}$) (Sarradin *et al.*, 1999). Nevertheless, the recent discovery of giant *Thaumarchaeota* species (Muller *et al.*, 2010) with 16S rRNA gene sequences closely related to *N. maritimus*, but morphologically and metabolically different (as they do not seem to have *AmoA* genes), makes it difficult to conclude that the *Thaumarchaeota* detected in this study are ammonia oxidizers at all.

Heterotrophs also seemed to be present, with a significant number of OTUs affiliated with phylum *Bacteroidetes* in the 16S rRNA gene library (Table 3 and Fig. S3). Their ability to degrade diverse organic molecules also explains the diversity

of this group in rich organic material systems such as microbial mats (Kirchman, 2002; Stevens *et al.*, 2005).

Other sequences in this library were related to members of the *Deltaproteobacteria* with species affiliated with *Myxococcales* and *Desulfobacterales* (Table 3 and Fig. S2). *Myxococcales* including various aerobic heterotrophs and *Desulfobacterales* have been described as being able to reduce sulfate (Rabus *et al.*, 2006). However, despite the fact that APS reductase is used in both the reductive and the oxidative pathways of sulfur metabolism, we did not identify any sequence affiliated with sulfate reducers (Fig. 3c). However, we cannot reject the possibility of anaerobic microenvironments within the mats where sulfate reducers could be active. For example, form II RuBisCO is only functional under anaerobic conditions (Haygood, 1996; Elsaied *et al.*, 2007) and we detected substantial taxonomic diversity for this gene. Biases in PCR and the possibility that environmental free-living microorganisms – some of them being anaerobic – may aggregate in mats may also partially explain these results.

Identification of the filamentous bacteria and possible interactions with mussels

While our data demonstrate the existence of a highly diverse microbial community within microbial mats, the combination of light micrograph observations (Fig. 1) and molecular results indicates the dominance of a large filamentous phylotype, probably involved in the sulfur cycle. The 16S rRNA gene transcript sequences were affiliated with those of a large marine, sulfur-oxidizing *Beggiatoa* species (order *Thiotrichales*, class *Gammaproteobacteria*) isolated in the shallow White Point hydrothermal system, confirming the morphological observations (Jacq *et al.*, 1989; Kalanetra *et al.*, 2004). Although these sequences dominated our RNA transcript library, only a few copies were present in our gene library, suggesting possible biases in lysis efficiency, low 16S rRNA gene copy number, difficulties in PCR amplification and other factors already mentioned in the literature (Heijs *et al.*, 2005). *Beggiatoa*-like filaments may also have proportionally more RNA compared with other cells.

It is difficult to affiliate the other observed morphologies (cells 2–6 µm in diameter or smaller filaments) with clone sequences. However, preliminary FISH results (Fig. 1) showed that they mostly belonged to *Gammaproteobacteria*. In addition, the presence of one clone closely affiliated (99%) with an uncultured *Leucothrix* sp. in our 16S rRNA gene library suggested that at least another member of the order *Thiotrichales* may be present (Fig. S2).

The relationships between mussels and mats are still poorly understood and studied, but the results obtained here lend themselves to several hypotheses. It has been

demonstrated previously that dense clusters of *B. thermophilus* can disperse the hydrothermal fluids laterally over distances of several meters. This dispersion increases the redox transition zone area, where both dissolved oxygen and hydrogen sulfide are available. As a result, faunal communities can occupy areas that would not otherwise provide adequate reduced substrates (Johnson *et al.*, 1994). Large filamentous bacteria composing mats may benefit from this extended redox transition zone for growth. The relationship does not seem mutual because only scarce bacterial populations have been observed in *B. azoricus* digestive tracts (V. Crépeau, unpublished data), suggesting low bacterial consumption. Competition for hydrogen sulfide and methane or a detoxication role does not seem plausible either. Healthy mussel assemblages covered by microbial mats coexist with mussel assemblages free of microbial mats, and to date, there is no significant physiological or toxicological evidence that the associated sulfur-oxidizing filamentous bacteria affect the mussels in the LS area (Cuvelier *et al.*, 2009; Martins *et al.*, 2009). A commensal relationship is the most convincing scenario: sulfur and methane oxidizers benefit from fluid dispersion by mussels and numerous heterotrophs such as *Bacteroidetes* can degrade the organic material that is released.

Are mats implicated in symbiont transmission?

Two sequences in our 16S DNA library and one in our *pmoA* DNA library were affiliated with genes from *B. azoricus* methanotrophic symbionts. Moreover, using domain-specific primers, we obtained clone sequences closely associated (> 99.5%) with both thiotrophic and methanotrophic symbionts of *B. azoricus* (Fig. 3a and b). In addition, the presence of 16S RNA transcript clones indicated that, in addition to the presence of these symbionts, they may be metabolically active. However, the absence of sequences related to methane or sulfur oxidizers from *B. azoricus* symbionts in the *pmoA* and *aprA* transcript libraries may indicate that even if free-living symbionts are present in the environment, their methane- or sulfur-oxidation activity only occurs inside the mussel bacteriocytes.

Mats may be involved in the symbiont transmission process. Their structure and localization in diffuse-flow areas offer a favorable environment for maintaining both *B. azoricus* symbionts. This is consistent with an optimal larval strategy involving the local retention of larvae within environments where symbionts can thrive and contribute to larval and or postlarval infection and then promote host growth (Van Dover *et al.*, 2001). Mats may help mussels retain their symbionts and postlarvae and may therefore participate in the maintenance of mussel assemblages. Hence, our work supports the assumption of the environmental acquisition of thiotrophic endosymbionts by vent

mussels from the MAR (Won *et al.*, 2003) and may indicate a similar transmission for methanotrophic symbionts.

Taken together, our results suggest that there may be mutualisms between mats and *Bathymodiolus* mussel patches, whereby the mats facilitate larval and postlarval infection, as well as larval settlement. In return, *Bathymodiolus* sp. may furnish organic matter, ammonium and promote fluid diffusion, resulting in a long-term association.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction curves of the diversity determined for the bacterial and archaeal 16S rRNA gene and transcript libraries defined at 97% similarity.

Fig. S2. Neighbor-joining phylogenetic tree showing the relationship of 16S rRNA gene sequences from *Proteobacteria* obtained with universal 16S rRNA gene primers specific to *Bacteria*.

Fig. S3. Neighbor-joining phylogenetic tree showing the relationship of 16S rRNA genes sequences obtained with universal 16S rRNA gene primers specific to *Bacteria*.

Table S1. Mean value of temperature (*T*), sulfur total concentrations (σ S), methane concentrations (CH_4), and pH and their SDs measured or estimated on different habitats identified at the Eiffel Tower hydrothermal edifice: assemblage 1: dense *Bathymodiolus azoricus* beds (the mussels are of the large size class, in general > 4 cm), occasionally patches of microbial mats are present; assemblage 2b: *Bathymodiolus azoricus* clumps (in this assemblage, the mussels are almost always < 4 cm in length) separated by visible microbial mats; substratum 1b has bare dark brownish, sometimes slightly reddish, surfaces with visible microbial mats.

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