RESEARCH ARTICLE

Molecular characterization of bacteria associated with the trophosome and the tube of *Lamellibrachia* sp., a siboglinid annelid from cold seeps in the eastern Mediterranean

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Abstract

Specimens of *Lamellibrachia* (*Annelida*: *Siboglinidae*) were recently discovered at cold seeps in the eastern Mediterranean. In this study, we have investigated the phylogeny and function of intracellular bacterial symbionts inhabiting the trophosome of specimens of *Lamellibrachia* sp. from the Amon mud volcano, as well as the bacterial assemblages associated with their tube. The dominant intracellular symbiont of *Lamellibrachia* sp. is a gammaproteobacterium closely related to other sulfide-oxidizing tubeworm symbionts. *In vivo* uptake experiments show that the tubeworm relies on sulfide for its metabolism, and does not utilize methane. Bacterial communities associated with the tube form biofilms and occur from the anterior to the posterior end of the tube. The diversity of 16S rRNA gene phylotypes includes representatives from the same divisions previously identified from the tube of the vent species *Riftia pachyptila*, and others commonly found at seeps and vents.

Introduction

Symbioses between chemoautotrophic bacteria and metazoans are the basis of high biomass communities occurring at deep-sea hydrothermal vents and cold seeps. Such benthic communities, dominated by chemosynthetic symbioses, have only recently been identified in the eastern Mediterranean (Olu-LeRoy *et al.*, 2004). They populate diverse settings such as mud volcanoes (MVs) and carbonated areas covering reduced sediments (Bayon *et al.*, 2009). The eastern Mediterranean chemosynthetic communities display low densities of megafauna compared with other cold seep sites such as in the Gulf of Mexico (GoM) and the Gulf of Guinea (GoG), but members of the same taxa are found, i.e. siboglinid tubeworms (annelids) and mytilid, lucinid, thyasirid and vesicomyid bivalves (Olu-LeRoy *et al.*, 2004). The occurrence of chemosynthetic symbionts in the lucinids and mytilids from the eastern Mediterranean was demonstrated previously (Duperron *et al.*, 2007, 2008).

Tubeworms of the genus *Lamellibrachia* were reported from the Anaximander MV (Olu-LeRoy *et al.*, 2004), resembling seep species from the GoM according to their morphology. *Lamellibrachia* species have been thoroughly studied, in particular *Lamellibrachia luymesi* from the GoM, and have been shown to harbor dense populations of sulfide-oxidizing gammaproteobacterial endosymbionts in their trophosome, an organ derived from the mesoderm, which develops during early life stages and replaces the digestive tract that displays no mouth or anus in adults (Jones & Gardiner, 1988; Southward, 1988; Bright & Sorgo, 2003; Pflugfelder *et al.*, 2005; Nussbaumer *et al.*, 2006). As for the well-studied vent tubeworm *Riftia pachyptila*, endosymbionts perform primary production through the oxidation of reduced sulfur compounds, which are transported to the trophosome, along with oxygen, via specialized hemoglobins (Stewart & Cavanaugh, 2005). Sulfide is present in the vicinity of the *R. pachyptila* plume, while *L. luymesi* obtains sulfide from the sediment using the buried, permeable posterior region of its tube named the 'root' and transports it to the trophosome (Freytag *et al.*, 2001). Preliminary work on specimens from the eastern Mediterranean suggested that the siboglinids host

sulfide-oxidizing symbionts, but no molecular data on the identity and function of the symbionts are available as yet (Olu-LeRoy *et al.*, 2004). In *R. pachyptila*, free-living forms of bacterial symbionts were shown to occur in the environment, and bacteria are acquired through the epidermis during larval settlement (Nussbaumer *et al.*, 2006; Harmer *et al.*, 2008). The mode of infection and transmission of endosymbionts is likely also horizontal for *Lamellibrachia* (Tyler & Young, 1999). Molecular phylogenetic analyses suggest that siboglinid hosts and their symbionts do not cospeciate, but instead certain bacteria available in the local environment seem to be able to associate with several species, and several symbiont 16S rRNA gene phylotypes seem to co-occur at least in some species (McMullin *et al.*, 2003). Compared with the Pacific or the GoM species, siboglinids from the western Atlantic and its marginal basins (Mediterranean and GoG) have not yet been studied in detail, although a comparison would be highly valuable for a better understanding of the species biogeography and phylogeny (see Andersen *et al.*, 2004; Lösekann *et al.*, 2008).

Furthermore, although some studies indicate the presence of bacterial communities colonizing the tubes of *R. pachyptila*, even in juveniles, and point to their potential role (Lechaire *et al.*, 2002; Lòpez-Garcìa *et al.*, 2002; Nussbaumer *et al.*, 2006), no data are as yet available regarding bacteria associated with tubes of cold seep siboglinids. Tubes of vestimentiferans constitute an important interface between the animal and its environment. They provide a structural support allowing tubeworms to maintain their position at the oxic–anoxic interface, and a shelter from predators. Tubes also represent suitable surfaces for colonization by microorganisms from the environment. Because seep tubeworms are apparently long lived compared with their vent relatives (Fisher *et al.*, 1997), stable and complex bacterial communities could establish on their tubes and influence the symbiotic system over long time periods.

In this study, we provide evidence for thioautotrophic symbiosis in a new species of *Lamellibrachia* from the eastern Mediterranean, and the first characterization of the bacterial community associated with the tube of a cold seep siboglinid, in an attempt to gain a more integrated overview of the system formed by hosts, symbionts, tubes and associated bacteria. Specimens of a large (1 m long, 0.5 cm in diameter) tubeworm, tentatively assigned to the genus *Lamellibrachia* (A. Andersen, pers. commun.), were recovered from reduced muds flowing from a fault system west of the Amon MV (Nile deep-sea fan) at a depth of 1157 m during the Bionil cruise (RV METEOR M70/2 Nov 2006). Several specimens were collected to identify their bacterial symbionts using molecular methods (16S rRNA and functional gene sequence analysis), FISH and electron microscopy (EM). Tube samples were collected from the same specimens to characterize associated bacterial communities using similar techniques.

Materials and methods

Samples

Tubeworms belonging to the genus *Lamellibrachia* were sampled from the active, gas-emitting Amon MV during the 2006 BIONIL cruise (RV METEOR M70/2, chief scientist A. Boetius) to the eastern Mediterranean cold seeps of the Nile deep-sea fan. The Amon MV is located east of the Nile deep-sea fan. During the BIONIL cruise, a small seep system was discovered in the southwestern fault surrounding the Amon MV, characterized by a band of sulfidic blackish sediments, partially covered by whitish bacterial mats, flowing from the base of the MV. Tubeworm specimens were collected during dive 123-12 using the ROV *Quest* (MARUM) from a single tubeworm patch, a few meters large, located in an area covered with black anoxic sediment at a depth of 1157 m (32°22.054′N, 31°42.274′E). The tubes were lying atop the sediment, with only the most anterior part positioned vertically upward and their root anchored in the sediment (Supporting Information, Fig. S1). Upon recovery, four complete live specimens were transferred to an aquarium to test the effect of sulfide and methane compounds on their oxygen uptake. Other specimens were dissected. Animal tissues and tubes were separated and rinsed carefully. From each sample, fragments were stored in ethanol for DNA analyses, and fixed for FISH and EM [scanning electron microscopy (SEM) and transmission electron microscopy (TEM)].

Sulfide uptake experiment

Four live specimens with their tubes, each roughly 1 m long, were curled and placed together in a 2-L cylinder-shaped aquarium (a polycarbonate tube closed at the bottom with a rubber stopper) containing bottom seawater and kept in a dark room cooled at sea bottom temperature (14 °C, pH 8.2 at 1157 m depth, measured by an ROV probe). Water was aerated before each experiment. The cylinder was sealed by a thin foil (kitchen wrap) over the water surface, taking care that no air was entrapped. The tank was stirred with a magnetic bar, and microsensors for oxygen and H_2S were pushed through the foil. The microsensors, having a tip diameter of 30 μm, were prepared and calibrated as described previously (Revsbech, 1989; Jeroschewski *et al.*, 1996). The signal obtained from the water column was continuously monitored using a custom-built data acquisition system. Na_2S was added to defined end concentrations (20, 40 and 80 μM). The pH was measured after each sulfide addition, and was found to remain constant. Then the kinetics of oxygen and sulfide uptake were followed during

a total of 12 h. The sulfide concentration at the start of each experiment was measured from water samples fixed in 5% ZnAc for later analysis using the Cline (1969) method. Oxygen consumption by worms was calculated as the difference between consumption measured in the presence and absence of tubeworms in the cylinder $d[O_{2\ TW}]/dt - d[O_{2\ clear\ water}]/dt$. Incubations were then repeated with 40 and 80 μM thiosulfate and with 20 and 60 μM methane. After the end of all experiments, worms were weighed. For control, heated (60 °C for 15 s), presumably dead, tubeworms were placed back into the aquarium, reduced sulfide was injected as previously (20, 40 and 80 μM) and the same measurements were performed.

DNA extraction, gene amplification, cloning and sequencing

DNA was extracted from the trophosome of four *Lamellibrachia* sp. specimens (V1, V2, V3 and V11) and from a section of the middle region of the tube from specimens V1 and V2, using the standard phenol–chloroform–isoamylalcohol procedure, after sonication of the tissue or tube. Bacterial 16S rRNA-encoding genes were amplified from all samples as described previously using primers 27F and 1492R, and 27 PCR cycles (Duperron *et al.*, 2008). Fragments of functional genes were amplified using primer couples cbbm1_Els/cbbm_2b (RubisCO form II), APS1-FW/APS4-RV (APS reductase) and A189F/MB661R (particulate methane monooxygenase or pmoA) according to the protocols described previously (Holmes *et al.*, 1995; Blazejak *et al.*, 2006; Meyer & Kuever, 2007) with 25 (APS and RubisCO) to 30 (pmoA) PCR cycles. Two parallel PCR reactions were run for every sample and gene, and pooled before purification using a QIAQUICK kit (Qiagen, Germany). Purified products were ligated into a plasmid and used to transform competent *Escherichia coli* cells using the TA cloning Kit (Invitrogen, CA). Positive clones were picked, cultured overnight, their plasmids were extracted and their inserts were sequenced using vector-specific primers M13F and M13R. Few sequences of good quality were obtained from clone libraries constructed for RubisCO and pmoA. The number of sequences per specimen and gene are summarized in Table 1.

Gene sequence analysis

Sequences encoding bacterial 16S rRNA gene recovered from animal and tube samples were compared with the GenBank database using the 'classifier' function from Ribosomal Database Project II (Cole *et al.*, 2007; Wang *et al.*, 2007). Best hits as well as sequences representative of bacterial groups were included in datasets for phylogenetic analysis. Alignments were performed using SINA web aligner (http://www.arb-silva.de/aligner/) and manually checked using BIOEDIT (Hall, 2001). The presence of chimeric sequences was investigated using BELLEROPHON (Huber *et al.*, 2004). Phylogenetic relationships within each group were estimated based on a Maximum Likelihood (ML) algorithm and a General Time Reversible model using TREEFINDER, which was also used to compute bootstrap values based on 500 ML replicates (Jobb, 2003). Rarefaction analyses were performed for the sequences associated with the tubes using DOTUR (Schloss & Handelsman, 2005).

Sequences encoding fragments of RubisCO form II, APS reductase and particulate methane monooxygenase were translated into amino acids and compared with the GenBank database using BLAST. Datasets were built including best blast hits as well as representative amino acid sequences. Phylogenetic relationships were estimated using a Bayesian algorithm run for 500 000 or more generations under a GTR model, using the software MBAYES v. 3.12 (Huelsenbeck & Ronquist, 2001).

FISH

FISH was performed on tissue and tube fragments from the same specimens used for molecular characterization (V1, V2, V3 and V11). Animal tissues and tube fragments were

Table 1. PCR primer sequences, annealing temperature, numbers of cycles and number of sequences obtained for genes investigated in this study

Gene fragment (bp)	Annealing temperature (°C) (cycles)	Primer name	Primer sequence (5′–3′)	Number of sequences							
				V1	V2	V3	V11	Sum	V1t	V2t	Sum
16S rRNA gene (c. 1500)	45 (27)	27F	AGA GTT TGA TCA TGG CTC AG	12	12	11	10	45	51	70	121
		1492R	GTT ACC TTG TTA CGA CTT								
APS reductase (c. 390)	58 (25)	APS1-FW	TGG CAG ATC ATG ATY MAY GG		8	7		15		6	6
		APS4-RV	GCG CCA ACY GGR CCR TA								
RuBisCO II (c. 390)	62 (25)	cbbm1_Els	ATC ATC AAR CCS AAR CTS GGC CTG CGT CC		4	2		6		6	6
		cbbm2_Els	MGA GGT GAC SGC RCC GTC RCC RGC MCG RTG								
Pmoa (c. 500)	55 (30)	A189F	GGN GAC TGG GAC TTC TGG		–	–		–	5		5
		M661R	CCG GMG CAA CGT CYT TAC C								

V1, V2, V3 and V4 correspond to trophosome tissue, V1t and V2t to tubes of specimens V1 and V2.

fixed immediately after recovery in 4% formaldehyde (4 °C, 2–3 h), rinsed in 22-μm filtered seawater and dehydrated in increasing ethanol series. Fragments from the anterior and posterior region of the trophosome of all four specimens were embedded in polyethylene glycol distearate : hexadecanol-1 (9 : 1), and transverse sections 4–7 μm thick were cut using a microtome (Jung, Germany) (Duperron *et al.*, 2008). Before hybridization, wax was removed and samples were rehydrated in decreasing ethanol series. One-centimeter-long fragments of tubes were cut every 5 cm over the whole length of the tube of specimens V1, V2, V3 and V11. Each fragment was embedded in LR White and 1-μm-thick transverse sections were cut using an Ultracut R^{TM} (Reichert Jung, Germany). Hybridizations were performed on sections without removal of LR White.

FISHs were performed at 46 °C for 3 h according to the protocol described previously using several general probes (Duperron *et al.*, 2008). The probes and formamide concentrations are summarized in Table 2. Probes LaSp60 and LaSp640 were designed based on probes from Lösekann *et al.* (2008) (Table 2). Each probe targeted both tubeworm endosymbiont sequences as well as environmental clones (63 and 51 hits among *Gammaproteobacteria*, respectively, no hit in common between the two probes), but only the two phylotypes from *Lamellibrachia* sp. endosymbionts recovered in this study were targeted by both the probes simultaneously. Probes were tested against bacteria from the gill symbiont of the lucinid clam *Myrtea* sp., which displays three and two mismatches in its 16S rRNA gene sequence, respectively. At 30% formamide, both probes hybridized successfully on *Lamellibrachia* sections and not on sections from *Myrtea* sp. Positive and negative controls were performed using probes EUB338 and NON338 (antisense of EUB338), respectively (Amann *et al.*, 1990). Cy3- or Cy5-labelled probes were ordered from Eurogentec (Liège, Belgium). After hybridizations, slides were mounted using SlowFade or ProlonGold containing 4′,6′-diamino-2-phenylindole (DAPI) (Invitrogen) and a coverslip and observed using an Olympus BX 61 epifluorescence microscope (Olympus, Japan).

EM

Fragments of tubes from three specimens (V1, V2 and V3) were cut every 5 cm over the whole length of the tube, and fixed and dehydrated as for FISH. The inner and the outer surface of tube fragments were dissected (1) from the most apical part of specimens (anterior end), (2) from a region situated 30 cm below (specimens V1 and V2) and (3) from the posterior end of the tube corresponding to the buried part (all three specimens). For a better contrast for TEM and SEM observations, samples were first rehydrated and post-fixed in 1% osmium tetroxide. For SEM, samples were then dehydrated through an ethanol series and dried in a critical-point dryer (CPD7501). They were coated with gold (scancoat six sputter coater) and observed under a SEM (Cambridge S260 at 10 kV). For TEM and light microscopy, samples were dehydrated in ethanol and propylene oxide series and then embedded in an epoxy resin (Serlabo). Semi-thin and ultrathin sections were cut using an ultramicrotome. Semi-thin sections were stained with toluidine blue for observations by light microscopy (using a BX61 microscope, Olympus). Ultrathin sections, 75-nm thick, were contrasted with uranium acetate and lead citrate, and observed on a LEO 912 electron microscope (LEO Electron Optics GmbH, Oberkochen, Germany) equipped with an LaB6 source, and operated at 80 kV.

Results

In vivo experiments

All four tubeworms, weighing between 5.3 and 10.2 g wet weight excluding the tube (total weight 31.5 g), remained alive for the whole duration of the experiment, as seen by

Table 2. Oligonucleotide probes used in this study, position in the 16S rRNA gene of *Escherichia coli* and % of formamide used

Probe	Sequence (5′–3′)	Position	%Formamide	Target	References
EUB338	GCTGCCTCCCGTAGGAGT	338	20–40	Most eubacteria	Amann *et al.* (1990)
LaSp60	CCATCGTTACCGTTCGAC	60	30	Tubeworm endosymbionts, uncultivated *Gammaproteobacteria*	Modified from Lösekann *et al.* (2008)
LaSp640	CACACTCTAGTCAGGCA	640	30	Tubeworm endosymbionts, uncultivated *Gammaproteobacteria*	Modified from Lösekann *et al.* (2008)
DEL495a	AGTTAGCCGGTGCTTSTT	495	30	Some *Delta-*, *Beta-* and *Gammaproteobacteria*, some *Actinobacteria*	Loy *et al.* (2002)
GAM42	GCCTTCCCACATCGTTT	42	30	*Gammaproteobacteria*	Manz *et al.* (1992)
EPSY549	CAGTGATTCCGAGTAACG	549	50	*Epsilonproteobacteria*	Manz *et al.* (1992)
ALF968b	GGTAAGGTTCTKCGCGTT	968	20	Various bacteria (not highly specific)	Neef (1997)
CF319	TGGTCCGTGTCTCAGTAC	319	35	*Bacteroides*	Manz *et al.* (1996)
ImedM	ACCATGTTGTCCCCCACTAA	138	40	Methanotrophic symbionts of mussels	Duperron *et al.* (2008)

Target refers to the main groups targeted by probes as obtained from a 'probe match' search in RDP.

movements of the plume in and out of the tubes. A basal consumption rate of oxygen of 0.42 μmol g^{-1} h^{-1} (wet weight) was estimated in unamended seawater (with tubeworms, no sulfide added). When corrected for the microbial consumption of oxygen in ambient seawater without tubeworms in a cleaned cylinder, we measured 0.18 μmol g^{-1} h^{-1}. Upon injection, sulfide disappeared within minutes, and an increase in oxygen consumption was observed lasting roughly 1 h after injection before returning to the basal consumption rate (Fig. 1a). The initial oxygen uptake rate was higher when more sulfide was added in the range of concentrations tested (Fig. 1b), with estimated rates of 0.49 μmol g^{-1} h^{-1} (in the presence of 20 μM sulfide), 1.03 μmol g^{-1} h^{-1} (40 μM) and 1.33 μmol g^{-1} h^{-1} (80 μM). Mean uptake of an extra 0.51 molecule of O_2 per molecule of H_2S, was estimated, after correction for the basal oxygen consumption measured in the absence of sulfide (Fig. 1). No increase in oxygen uptake occurred when sulfide was added to the aquarium containing no tubeworms (Fig. 1b). Injection of thiosulfate (40–80 μM) as well as methane (20–60 μM) did not have any significant effect on the rates of oxygen consumption by live specimens (data not shown).

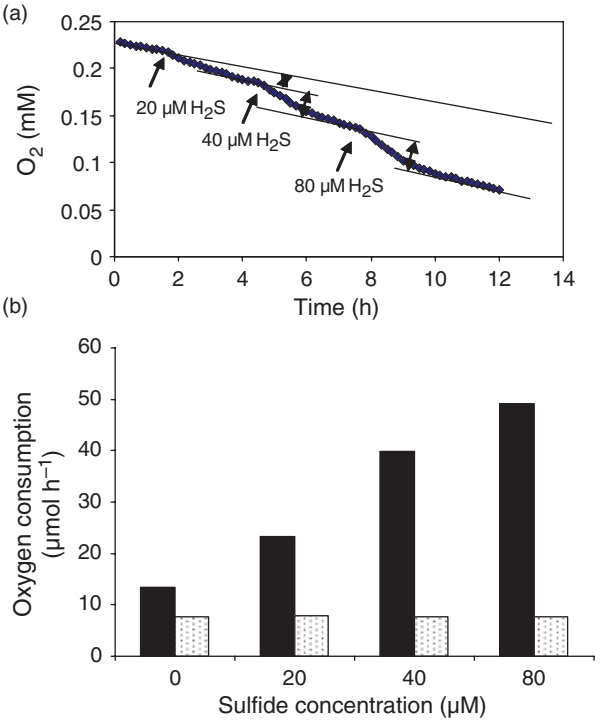

Fig. 1. (a) Oxygen concentrations monitored during an *in vivo* experiment on tubeworms. Simple arrows indicate when sulfide was injected. Lines represent basal uptake in the experiment. Double arrows indicate the amount of extra oxygen consumed besides normal consumption. (b) Histogram representing oxygen consumption (in μmol h^{-1}) as a function of sulfide concentrations (in μM) in the presence (black bars) and absence (dotted bars) of the four tubeworms. Values correspond to the consumption measured right after sulfide addition.

Molecular characterization of tubeworm endosymbionts

Two distinct 16S rRNA gene phylotypes were obtained out of 45 sequences from the trophosome of four specimens of *Lamellibrachia* sp. The two phylotypes displayed 28 consistent differences from one another (*c.* 2% divergence, including 22 differences between positions 992 and 1038). Phylotype 1 was recovered from all specimens, while phylotype 2 was only recovered from specimen V11, in which both phylotypes were present in the clone library. Both sequences clustered within clades comprising sequences from gammaproteobacterial endosymbionts of various seep-associated *Escarpia*, *Lamellibrachia* and *Seepiophila* tubeworm species (98–99% similarity to several sequences, Fig. 2), corresponding to hydrocarbon seep groups 2 and 3 from Thornhill *et al.* (2008). Two APS reductase sequences differing by three nucleotide positions (two synonymous substitutions and one resulting in an aminoacid substitution) were identified in both specimens V2 and V3, closely related to the sequence from the endosymbiont of *R. pachyptila* (93% identical aminoacid positions, Fig. 3a, only one sequence displayed). Form II RubisCO sequences from specimen V2 and V3 were all identical, displaying 95% identical aminoacid positions with that of an uncultured groundwater bacterium (Fig. 3b). Form I RubisCO and pmoA could not be amplified from the animal tissue.

FISH on trophosome

Transverse sections of both the anterior and the posterior part of the animal (V1, V2, V3 and V11) displayed abundant bacteria occupying most of the space within host cells localized within the trophosome (Fig. 4). Bacteria hybridized with the EUB338 and GAM42 probes. All bacteria also hybridized with both LaSp60 and LaSp640 probes (Fig. 4b). Each probe has extra hits besides the two *Lamellibrachia* sp. phylotypes (see Table 2), but only these two phylotypes 1 and 2 are the targets for both probes, indicating that bacteria located in the trophosome most likely correspond to the 16S rRNA gene phylotypes 1 and 2 recovered in clone libraries. Hybridized bacteria displayed darker spots, possibly indicating the localization of sulfur granules observed under the light microscope (see Fig. S2). Bacteria of various sizes were observed, many being moderately large (up to 5-μm diameter) (Fig. 4c, SM2). To distinguish between the two recovered phylotypes (1 and 2), probes targeting the region between positions 992 and 1038, which includes 22 differences between the two phylotypes, were designed, but did not hybridize successfully.

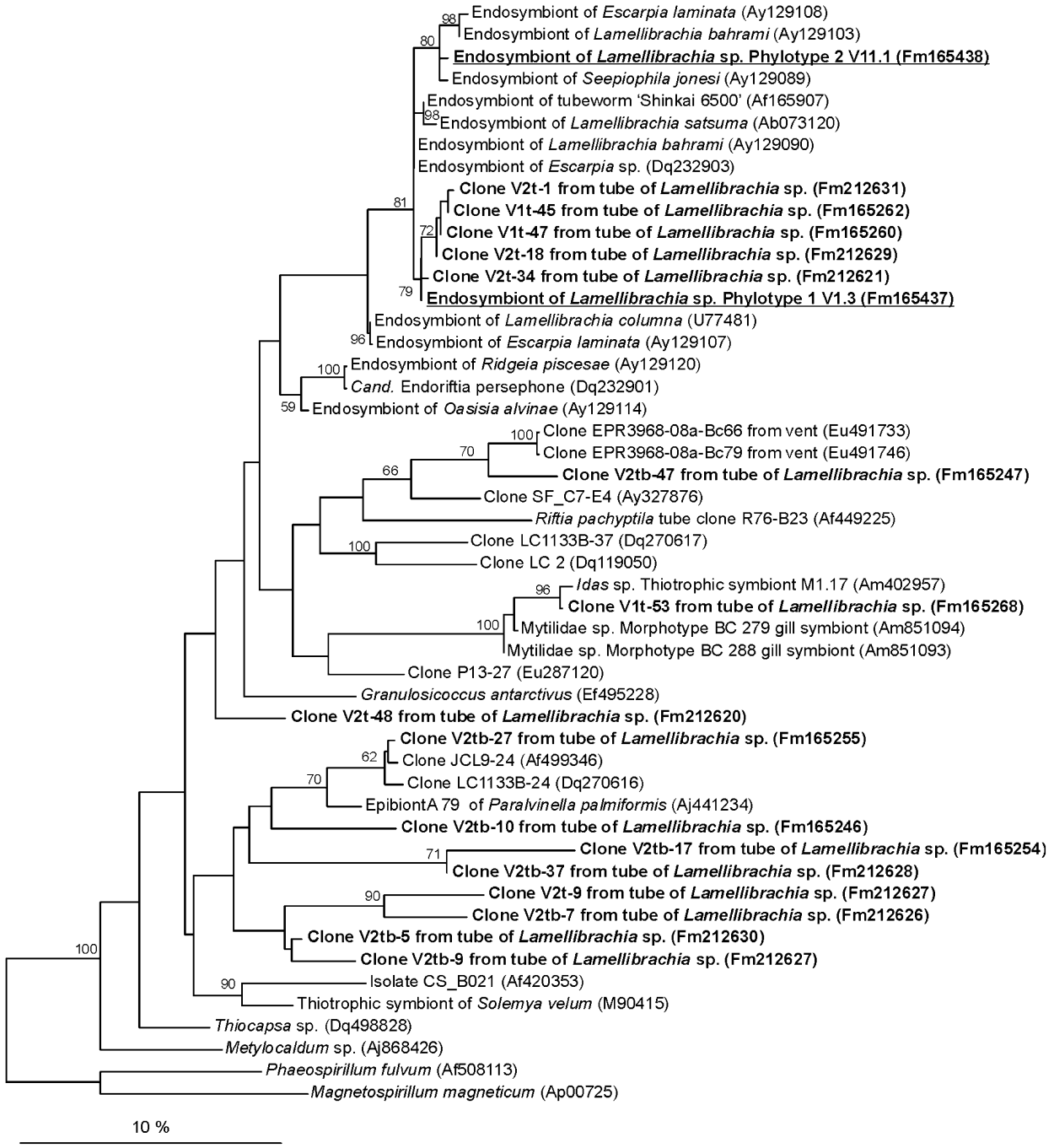

Fig. 2. Tree displaying the phylogenetic position of *Gammaproteobacteria*-affiliated 16S rRNA gene sequences recovered from the trophosome (underlined bold) and tubes (bold) of *Lamellibrachia* sp. Tree was reconstructed using a ML algorithm and a GTR model. Values at nodes represent bootstrap percentages (500 replicates, > 50% shown). Scale bar corresponds to estimated 10% divergence. Two *Alphaproteobacteria* are used as an outgroup.

Molecular characterization of bacteria associated with the tube

A total of 121 16S rRNA gene sequences were recovered from the tube of specimens V1 and V2. Sequences belong to nine bacterial divisions, and three sequences could not be assigned to any known division with certainty. *Epsilon-* and *Gammaproteobacteria* were represented by 36 and 33 sequences (32% and 30% of clones), *Deltaproteobacteria* by 17 sequences (15%) and all other divisions by seven or less sequences (< 6%) (Fig. 5a). Rarefaction curves with a 1%

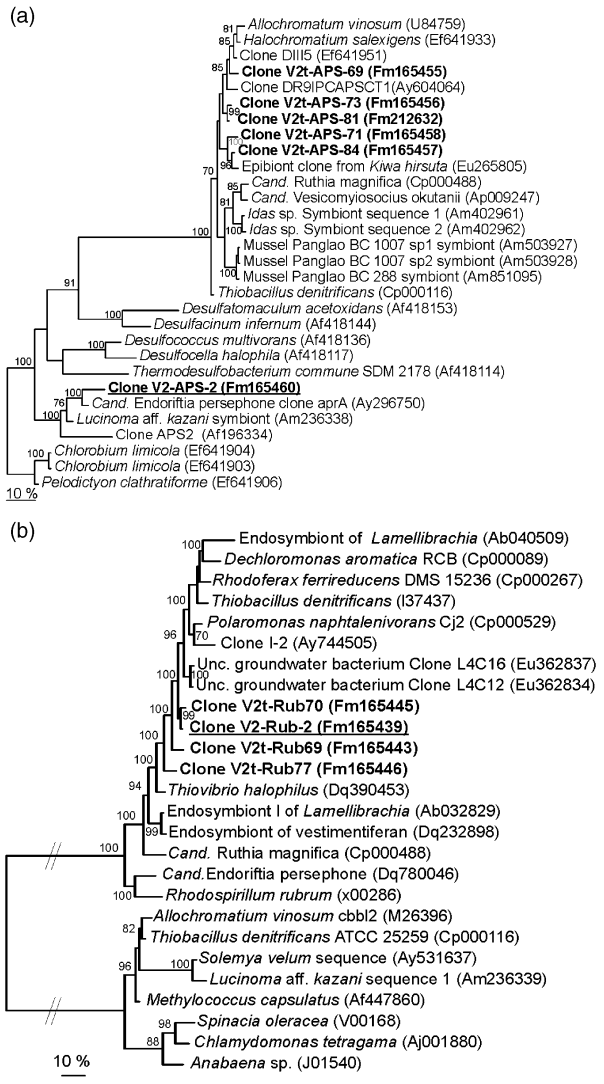

Fig. 3. Phylogenies of functional genes identified from the trophosome (underlined bold) and tube (bold) of *Lamellibrachia* sp. (a) APS reductase (aprA) (*Chlorobia* sequences used as an outgroup). (b) RubisCO tree, all sequences cluster within the RubisCO form II clade (forms IA and IB used as outgroups). Branches to the root of the tree are shortened for clarity; 122 aminoacid positions were used. Both trees were reconstructed using a Bayesian algorithm run for 500 000 generations with a GTR model. Percentages at nodes represent posterior probabilities calculated from the 100 best trees. Scale bar represents 10% estimated aminoacid substitution.

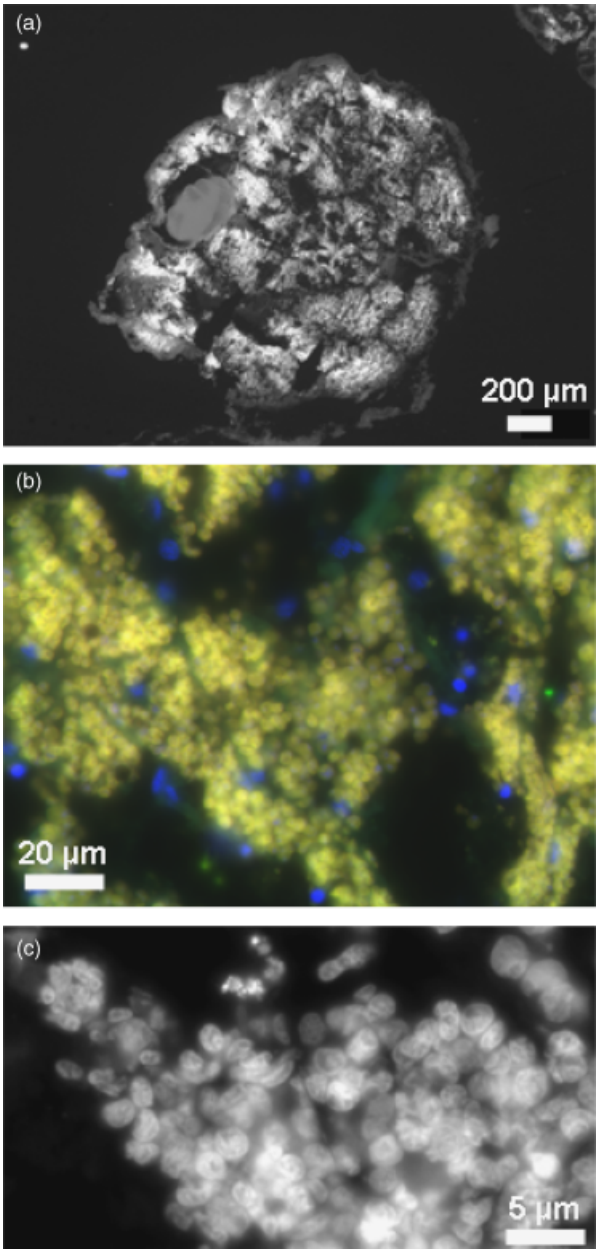

Fig. 4. (a) An overview of a section of tubeworm hybridized with the GAM42 probe. (b) Transverse section of the trunk of *Lamellibrachia* sp. Yellow color results from the superimposition of the green channel (bacteria hybridized with Cy3-labeled LaSp60) and the red channel (bacteria hybridized with Cy5-labeled LaSp640), blue DAPI signal is associated with nuclei of host cells (blue) and bacteria (DAPI signal is then hard to see because of the brighter FISH signal). (c) Detail displaying large bacteria of various sizes.

or a 3% difference threshold between sequences show a clear inflexion, but do not reach a plateau, confirming that the sequencing effort was not sufficient to exhaustively describe bacterial diversity (Fig. 5). This is supported by the observation that five of the nine bacterial groups were represented by less than five sequences, and that very few sequences were shared between the two specimens. A total of 54 unique 16S rRNA gene sequences were recovered and their phylogenetic relationships were assessed.

Epsilonproteobacteria were the most abundant group in clone libraries from tubes. Phylotypes were closely related to various phylotypes previously recovered from vent habitats, from the tube of *R. pachyptila*, and to epibionts from the scaly snail (Fig. 6a) (Lòpez-Garcìa *et al.*, 2002; Goffredi *et al.*,

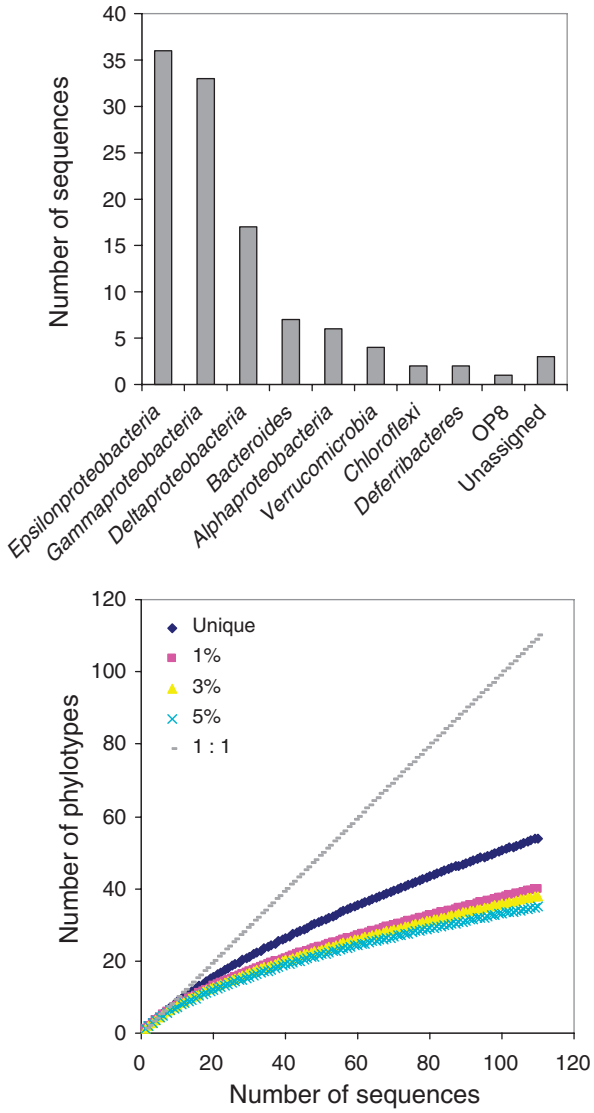

Fig. 5. Upper panel, composition of 16S rRNA gene clone libraries built from tubes: number of sequences affiliated with each bacterial group; lower panel, rarefaction curves obtained from clone libraries from tubes. Sequences are grouped as 'unique' and at the 1%, 3% and 5% divergence levels. The regression curve (1 : 1) is shown for convenience.

2004; Pernthaler *et al.*, 2008). Among the gammaproteobacterial phylotypes, close relatives of the symbiont phylotype 1 were identified, although always displaying at least 0.5% distinct positions. Clone V1t-53 appeared to be closely related to thiotrophic symbionts of *Idas* sp. (Duperron *et al.*, 2008), and clone V2tb-27 related to epibionts of the annelid *Paralvinella palmiformis* (Alain *et al.*, 2002) (Fig. 2). Clone V2tb-47 clustered with vent-associated phylotypes, and several phylotypes such as V2t-9 clustered close to each other with no close relatives. *Deltaproteobacteria* were represented by phylotypes related to sequences from reduced habitats such as hypersaline habitats, mangrove and sediment associated with gas hydrates (Fig. 6b) (Knittel *et al.*, 2003; Ley *et al.*, 2006). Two sequences, V1tb-34 and V1t-39, were closely related to the strict anaerobic sulfate-reducing (and dehalogenating) bacterium *Desulfomonile limimaris* (Sun *et al.*, 2001). No recovered deltaproteobacterial sequence was closely related to sequences from the *Riftia* tube. *Bacteroidetes*-related sequences were closely related (Fig. 6c) to sequences from chemosynthesis-based ecosystems such as the Lost City hydrothermal vent, cold seeps at the Cascadian margin and MVs in the GoM (Knittel *et al.*, 2003; Brazelton *et al.*, 2006; Martinez *et al.*, 2006). Clone V2tb-48 from this study was closely related to bacterial epibionts identified in the scaly snail from the Indian ocean (Goffredi *et al.*, 2004). The three distinct alphaproteobacterial phylotypes were related to two vent phylotypes and to Arctic sediment bacteria (Fig. 6d). Other divisions (*Chloroflexi*, *Deferribacteres*, candidate division OP8) were represented by few sequences clustering with sequences recovered from reduced habitats including MVs, sediment around gas hydrates and mangrove (Fig. 6b) (Knittel *et al.*, 2003; Ley *et al.*, 2006). Sequence V1t-43 clustering within the *Verrucomicrobia* was related to sequences from the *Riftia* tube from the East Pacific Rise (Lòpez-Garcìa *et al.*, 2002). Sequences V1t-44 and V1tb-44 could not be assigned with certainty to existing divisions (Fig. 6b).

Despite several attempts, very few good-quality sequences were obtained for functional genes. Only six, six and five usable sequences were obtained for APS reductase, RubisCO form II and methane monooxygenase, respectively. Five distinct APS sequences were recovered, none being identical to those recovered from the trophosome. All clustered together with sequences from bivalve thiotrophic symbionts and epibionts of the galatheid *Kiwa hirsuta* (Fig. 3a). Of the three distinct RubisCO form II aminoacid sequences recovered, V2t-Rub70 was almost identical to that identified in the trophosome. The other two belonged to the same clade, including environmental sequences from the Lost City vent field and a *Thiovibrio* species (Fig. 3b). Three distinct pmoA aminoacid sequences were recovered, which clustered within the group of type I methanotrophs (Fig. S3). Closest relatives include cold seep- and hydrothermal vent-associated clones, as well as cultivated *Methylocaldum*.

Microscopic observation of the anterior end of the tubes

Under the transmission electron microscope, the inner and outer surface of the tube displayed abundant microbial communities organized in dense mats covering large areas, including small irregularities of the surface of the tube as well as areas covered with mineral precipitates (Fig. 7a–c). Diverse shapes were observed including cocci, rods and filaments covered with ramified 'web-like' material perhaps

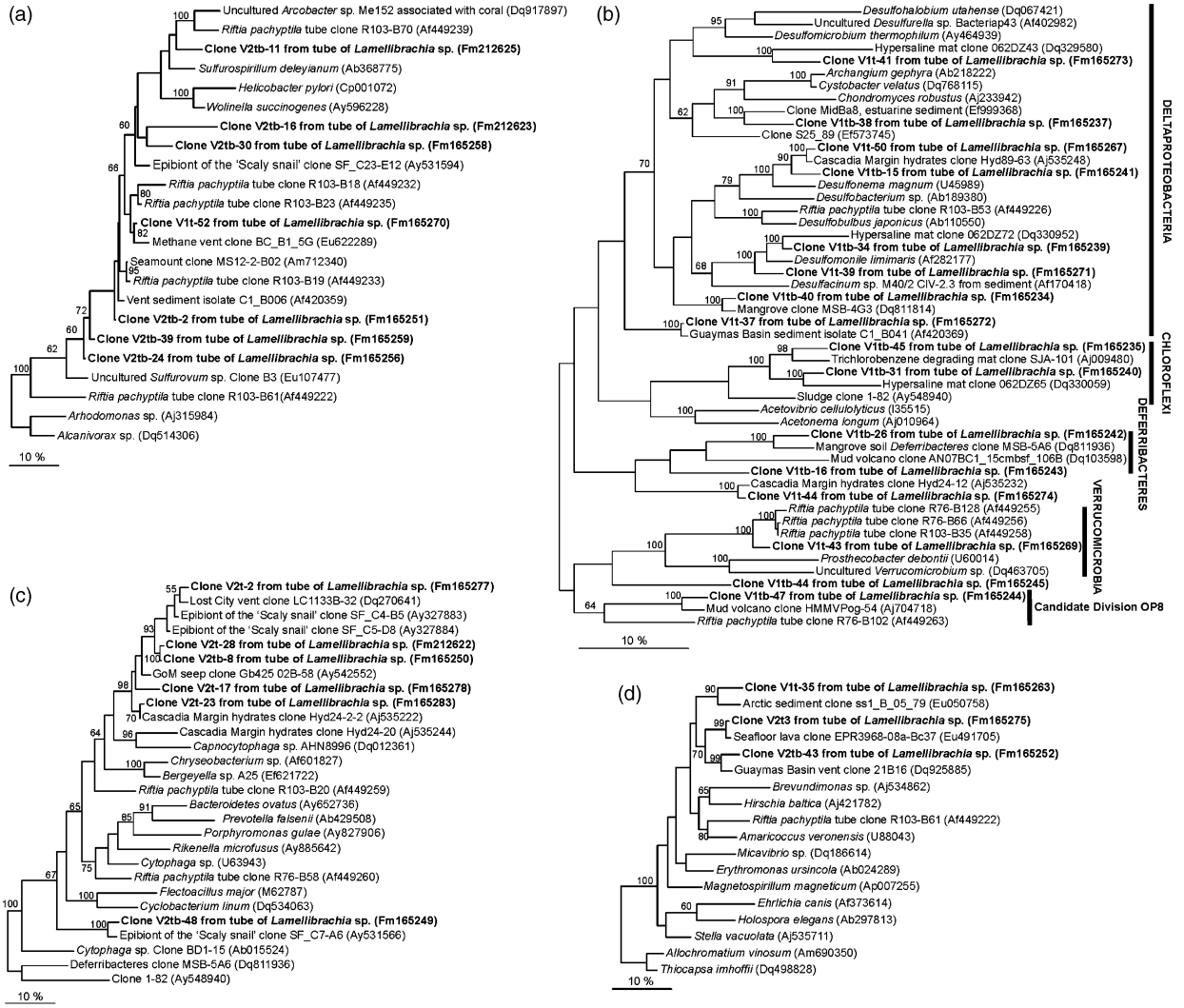

Fig. 6. Trees displaying the phylogenetic position of 16S rRNA gene sequences recovered from the tubes of specimens 1 (V1) and 2 (V2). Trees were reconstructed using a ML algorithm and a GTR model. Values at nodes represent ML bootstrap percentages (500 replicates, > 50% shown). Scale bar corresponds to estimated 10% divergence. (a) Sequences affiliated with *Epsilonproteobacteria*; two *Gammaproteobacteria* are used as outgroups. (b) Sequences affiliated with *Deltaproteobacteria*, *Chloroflexi*, *Defferibacteres*, *Verrucomicrobia*, OP8 and no known division. (c) *Bacteroides*-affiliated 16S rRNA gene sequences. *Deferribacteres* are used as an outgroup. (d) Sequences affiliated with *Alphaproteobacteria*; two *Gammaproteobacteria* are used as an outgroup.

consisting of polysaccharides or a glycoprotein matrix (Fig. 7b). Some areas of the tube were not covered by a bacterial film, and only a few isolated cells were attached to the tube. Very large filaments (> 200 μm long, 5 μm in diameter, Fig. 7a) were seen in the anterior end of one of the tubes (specimen V2). Mats were also abundant on the inner surface of the tube, and filaments were observed (Fig. 7d). No very large filament as in Fig. 7a was seen, but filaments were larger than those displayed in Fig. 7b and c. Diverse shapes were also observed including rods, cocci, stalked prokaryotes resembling *Hyphomicrobium* or *Caulobacter* and organisms clustering in groups of two or tetrads of coccoid-shaped cells in a manner similar to that of *Deinococcus* or *Sarcina* (Fig. 7c).

DAPI-stained sections displayed layers of microorganisms covering most of the inner and outer sides of the tube in the anterior end. A microbial layer up to 8-μm thick was observed in the upper part of the tube (Fig. 8a). Microorganisms were also seen sometimes between distinct layers of the tube (Fig. 8b). Most DAPI-stained microorganisms hybridized with the general eubacterial probe EUB338, indicating that most of the observed community consisted of bacteria. General probes targeting members of the *Epsilon-*, *Gamma-* and *Deltaproteobacteria* (probes EPSY549,

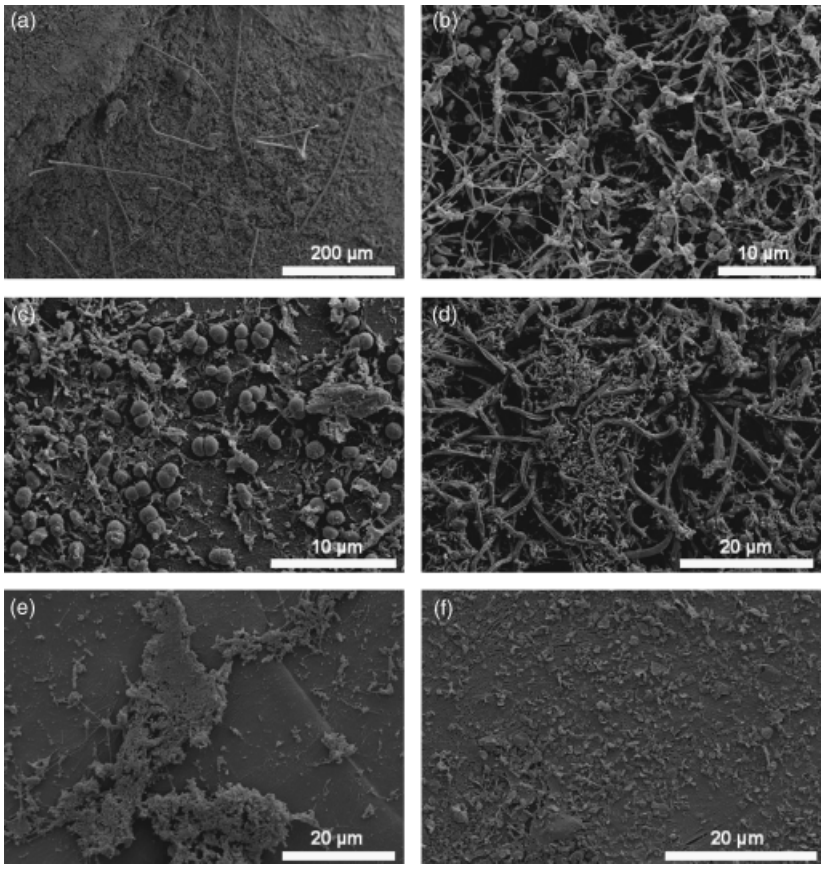

Fig. 7. Scanning electron micrographs from the surface of tubes. (a)–(c) Apical part of the tube, external side. (a) Large microbial filaments on individual V1. (b) Microbial mat, individual V1. (c) *Sarcina*-like morphologies. (d) Apical part, internal side, large filaments and small rods in the tube of individual V1. (e, f) Basal part of the tube (e) Few small aggregates of bacterial cells (individual V1, internal side). (f) Bacteria associated with small mineral precipitates in the internal side of the tube, individual V4.

GAM42 and DEL495A) yielded unambiguous positive signals on most sections from both the inner and the outer side of the tubes (Fig. 8c and d). However, strong autofluorescence of the tube may have precluded the detection of some weak positive signals. Each probe targeted bacteria with diverse morphologies. Methanotroph-specific probe ImedM-138 hybridized with large bacteria located in the inner side of the tube (Fig. 8c). Each probe hybridized with only a fraction of EUB338-labelled bacteria, indicating that no bacterial group was overwhelmingly dominant in the community. Unfortunately, autofluorescence discussed above prevented quantification of the different groups. Probes ALF968B and CF319 did yield ambiguous signals despite several attempts.

Microscopic observation of the posterior end of the tube

The most posterior part of the tube, corresponding to the buried part of the tube, displayed much lower abundances of microorganisms compared with the anterior end. Dense, but small and sparse, patches of microbial cells were observed, covered with organic material, and mostly associated with detached scraps of tube material or mineral precipitates (Fig. 7e). In other areas, only a few isolated microorganisms were found attached to the tubes (Fig. 7e and f). Shapes were diverse with cocci, rods and short filaments present. FISH confirmed the apparently lower abundances of microorganisms, as very few signals were seen on tube sections compared with sections from the anterior end. Furthermore, signals tended to be weaker than in the apical part. Bacteria targeted by the EPSY549 and DEL495A probes were seen, mostly located between chitin layers. CF319, GAM42 and ALF968B yielded few positive signals consisting of rod- and filament-shaped bacteria.

Discussion

Bacterial symbiosis in *Lamellibrachia* sp.

Lamellibrachia sp. specimens recovered from the Amon MV measure up to 1 m long, with a diameter of around 0.5 cm, which decreases toward their posterior part. Their morphology resembles that of tubeworms previously collected at the Anaximander MV, in the same area of the eastern Mediterranean, and that are currently being described by taxonomists (Olu-LeRoy *et al.*, 2004). *Lamellibrachia* sp. harbors gammaproteobacterial symbionts in its trophosome, which

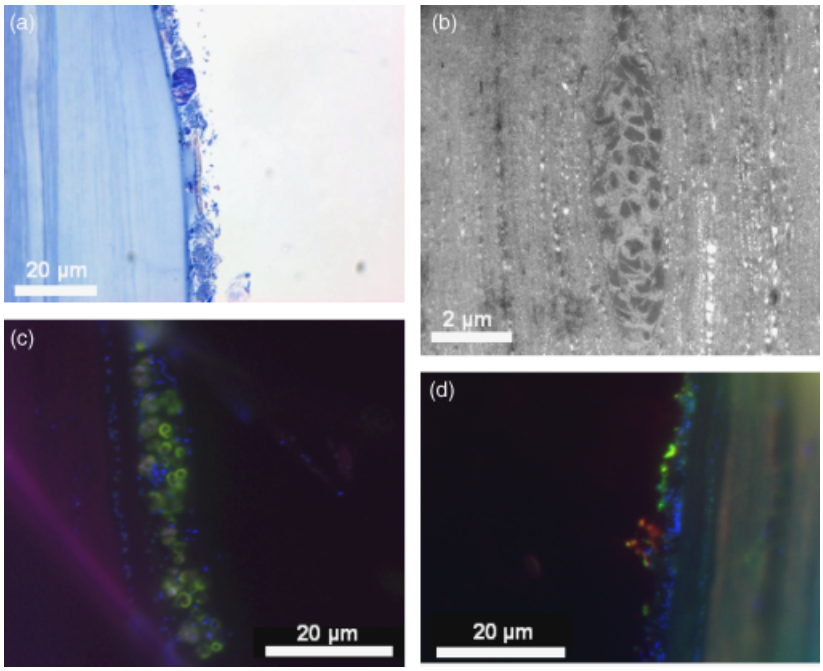

Fig. 8. Light and transmission electron microscopies and FISHs. DAPI signal is in blue; (a) semi-thin section of the apical part of the tube displaying the various shapes of microorganisms coating the external side, and possible mineralizations in orange. (b) TEM view of a transverse section of the apical part of the tube displaying chitin layers and a dense aggregation of prokaryotes occupying an interstice between chitin layers. (c, d) Hybridizations on transverse section of the tube. (c) Bacteria hybridized with the methanotroph-specific probe ImedM138 in green and EUB338 signal in red, on the internal side of the apical part of the tube. (d) External side of the apical part of the tube EUB338 signal in green and Gam42 signal in red.

are closely related to symbionts of other cold seep tubeworms. Although bacteria located within the trophosome vary considerably in shape and size, they seem to represent only one or two closely related 16S rRNA gene phylotypes. Co-occurrence of two phylotypes, differing by 2–3%, in the same region as phylotypes 1 and 2 from this study, was previously documented in several vestimentiferans including the siboglinid *Oligobrachia haakonmosbiensis* (Lösekann *et al.*, 2008). Regarding the latter species, authors used FISH but were not able to demonstrate whether both phylotypes corresponded to distinct copies of the 16S rRNA gene within a single bacterium, or to sequences belonging to distinct symbiont strains present in the trophosome, because they could not obtain good probes to discriminate between the two phylotypes (Lösekann *et al.*, 2008). We faced the same problem in the present study. Hybridizations using probes LaSp60 and LaSp640 simultaneously confirmed that most, if not all, bacteria located within the trophosome correspond to one, the other or both identified 16S rRNA gene phylotypes. Bacteria occupy most of the trophosome on hybridized sections, which is typical for large seep and vent vestimentiferans, but different from smaller tubeworms such as frenulates (Lösekann *et al.*, 2008). Based on symbiont phylogeny and the presence of APS reductase- and form II RubisCO-encoding genes related to sequences from tubeworm symbionts, the symbiont of *Lamellibrachia* sp. investigated here is suggested to be a chemoautotrophic sulfide-oxidizing bacterium.

The presence of sulfide-oxidizing symbionts is supported by the results of the *in vivo* incubation experiment carried out on a batch of four freshly sampled tubeworms (Fig. 1). The addition of sulfide induced a rapid, although temporary, increase in the respiration rate of *Lamellibrachia* sp. possibly due to the aerobic oxidation of sulfide by symbionts. The estimated uptake of an extra 0.5 mol O_2 mol^{-1} sulfide in addition to the basal oxygen consumption fits the stoichiometry of aerobic sulfide oxidation to elemental sulfur (S^0) by thiotrophic bacteria: $0.5O_2+1H_2S=S^0+H_2O$ (Kelly *et al.*, 1997; Madigan *et al.*, 2002). Sulfur granules were observed within the symbiont cells under the light and electron microscope (SM2). Symbionts are thus likely to oxidize excess sulfide to S^0, to store energy in the absence of a regular supply of sulfide. Although thiosulfate is an intermediate in the aerobic oxidation pathway of sulfide, it did not induce an increase in oxygen consumption when added to the aquaria with the living tubeworms. This is congruent with results from numerous studies indicating that tubeworm symbionts get reduced sulfur through host blood, which displays very low thiosulfate concentrations, and binds only HS^- thanks to the hemoglobins (Childress *et al.*, 1991; Flores *et al.*, 2005). Besides the question of availability, Wilmot & Vetter (1990) have also shown that thiosulfate was not utilized (and not produced) by homogenized trophosome and isolated bacteria from *R. pachyptila* . The addition of methane did not lead to an increase in respiration rates, confirming the absence of methane-oxidizing symbionts in *Lamellibrachia* trophosome. Rates of oxygen uptake measured in the present study ranged from 0.18 to 1.33 µmol g^{-1} h^{-1} depending on the sulfide concentration (from 0 to 80 µM). With rates of 1.4 ± 0.59 µmol g^{-1} h^{-1} in

the absence of reduced sulfur compounds (Girguis *et al.*, 2002), and of 5.3–10.4 μmol g^{-1} h^{-1} in the presence of 218–431 μM H_2S around their root (Freytag *et al.*, 2001), *L. luymesi* from seeps in the GoM displayed *c.* fivefold higher respiration rates than that reported here. Such a difference could suggest a lower metabolic rate in our specimens, despite their adaptation to the 14 °C temperature of the eastern Mediterranean deep waters, but replicate experiments would be needed to conclude.

Bacterial communities associated with the tube

The internal and external surfaces of the tubes harbor dense and diverse microbial communities. Although not quantified, it appears from our observations that the abundance of microbial cells was much higher at the anterior end than at the posterior end of the tube, and that the surface of the tube is suitable for microbial colonization (Lòpez-Garcìa *et al.*, 2002). Prokaryotes were also distributed patchily within the tube walls, between successive layers such as described in other species (Lechaire *et al.*, 2002).

Most 16S rRNA gene phylotypes recovered were closely related to environmental phylotypes previously identified in chemosynthesis-based ecosystems or reduced habitats, such as hydrothermal vents, cold seeps and hypersaline habitats. Whether colonizing bacteria are a subset of environmental bacteria or specific associates remains to be determined. Representatives of the seven bacterial divisions previously identified in the tube of the East Pacific Rise hydrothermal vent siboglinid *R. pachyptila* were obtained in our study, although within each division, sequences were not closely related, except in the case of *Epsilonproteobacteria* and *Verrucomicrobia* (Lòpez-Garcìa *et al.*, 2002). The two groups identified as dominant in our clone libraries, i.e. *Epsilon-* and *Gammaproteobacteria*, were confirmed to occur in both the anterior and the posterior part of the tubes based on FISH, along with less represented groups such as *Bacteroides*. Because of the low specificity of probe DEL495a (Table 2), support for the presence of *Deltaproteobacteria* is weaker. The diverse morphologies of bacteria hybridized with each specific probe appeared to mirror the diversity found in 16S rRNA gene libraries. Although positive signals were quite abundant on some sections, no group seems to dominate the microbial community associated with the tube, and representatives of diverse groups and morphologies were mixed in close association.

Although metabolic hypotheses must be considered with caution when based on 16S rRNA gene phylogenies, several possible bacterial metabolisms can be suggested to occur on the tube. The *Epsilonproteobacteria*, known to be early colonists of available surfaces at vents, include many bacteria with sulfur-linked metabolisms (Polz & Cavanaugh, 1995; Campbell *et al.*, 2001; López-Garciá *et al.*, 2003; Suzuki *et al.*, 2005). Similarly, the *Gammaprotebacteria* from this study display close phylogenetic relationships with sequences from free-living and symbiotic sulfur-oxidizers. The presence of autotrophs and sulfur-oxidizers is further supported by the identification of type II RubisCO and APS-reductase sequences. Close relatives, but no exact match to the *Lamellibrachia* sp. symbiont phylotype 1, were recovered from tube samples. Similarly, López-Garcia and colleagues did not recover the *Riftia* symbiont sequence from *Riftia* tubes, even though free-living forms were recently demonstrated to occur in the environment (Harmer *et al.*, 2008). The occurrence of *Deltaproteobacteria* related to phylotypes from reduced habitats suggests the presence of sulfate reducers. Members of the *Bacteroides* and *Verrucomicrobia* could represent heterotrophs, for which chitin could be a carbon source. Based on FISH using a methanotroph-specific probe and on the recovery of pmoA sequences, the presence of methanotrophs is probable despite the absence of characteristic 16S rRNA gene sequences.

The abundant and diverse bacteria found on tubes, in particular, in the anterior part, probably influence chemical conditions in the immediate vicinity of animals, and could have consequences for tubeworms. In *L. luymesi*, for example, sulfide uptake occurs through a permeable 'root' at the posterior end of the tube, where protons and sulfate are also eliminated (Julian *et al.*, 1999; Freytag *et al.*, 2001; Cordes *et al.*, 2005; Dattagupta *et al.*, 2006). Sulfur compounds are thus available for microbial communities located on the inner surface of the tube, perhaps explaining the presence of bacteria with potentially sulfur-oxidizing and sulfate-reducing metabolisms. The microbial community associated with the tube could thus consume or emit H_2S. Experimental data are needed to evaluate this hypothesis.

Conclusion

Lamellibrachia sp., a tubeworm species morphologically resembling the well-studied species *L. luymesi* from the GoM, occurs at cold seeps in the eastern Mediterranean. It harbors a thioautotrophic symbiosis functionally and phylogenetically very similar to that displayed by its relative. A bacterial community consisting of representatives of more than nine divisions occurs on the tube, which appears as a complex habitat with various microniches suitable for different bacterial groups, and possibly different metabolisms.

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Supporting Information

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

Fig. S1. An overview of the sampled tubeworm patch, located at the Amon MV.

Fig. S2. Upper picture shows bacterial cells observed from homogenized trophosome tissue under the light microscope. Lower picture: TEM micrograph of a section through the trophosome, displaying numerous bacteria with empty vacuoles likely corresponding to the localization of intracellular sulfur granules.

Fig. S3. Tree displaying the phylogenetic position of particulate methane monooxygenase (pmoA) sequences recovered from the tube of specimen #1.

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