Epsilonproteobacteria as gill epibionts of the hydrothermal vent gastropod *Cyathermia naticoides* (North East-Pacific Rise)

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Abstract

Molluscs, and particularly gastropods, are one of the major taxonomic groups at vents. In these ecosystems, devoid of light, chemoautotrophic bacteria are at the base of the food web, and symbiotic association between metazoa and these bacteria are numerous. Nevertheless, apart few "large size" well known species, the "small size" gastropods (shell < 5mm), although very abundant, remain poorly studied regarding symbioses. We investigated here *Cyathermia naticoides* (Warén and Bouchet 1989), a small coiled gastropod found in abundance on the East Pacific Rise among *Riftia pachyptila* tubes and usually inferred to graze on tubeworms bacterial cover, and/or filter feeding. Among molluscs, symbioses are well known in large species and almost exclusively rely on sulfide or methane-oxidizing Proteobacterial endosymbionts, occurring within the host tissues in gill epithelial bacteriocytes. Combining several approaches (molecular biology, microscopy, stable isotopes analyses), we described here an unusual symbiosis, where autotrophic filamentous Epsilonproteobacteria are located extracellularly, at the base of hosts gill filaments. Numerous endocytotic lysosome-like structures were observed in the gill epithelium of the animal suggesting bacteria may contribute to its nutrition through intracellular digestion by gill cells. Additional food source by non-symbiotic Proteobacteria grazed on *R. pachyptila* tubes could complete the diet. The possible role of temperature in the selection of Epsilon *vs* Gamma proteobacterial partners is discussed.

Introduction

To date, about 600 metazoan species have been reported at hydrothermal vents, belonging to 12 phyla. Among those, 150 species of Mollusca and more than 100 species of Gastropoda have been described (Desbruyères et al. 2006), making them one of the major taxonomic groups at vents. Gastropod feeding habits are extremely diverse, although most species make use of a radula in some aspect of their feeding behavior (see review in Kohn 1983). Grazers can be herbivorous, rasping either micro- or macroalgae, or predators, rasping on encrusting invertebrates such as hydroids, sponges, cnidarians or ascidians. Herbivorous may also swallow sand containing algae. And carnivores may also hunt their prey and use their radula to drill mollusc shells or calcareous echinoids test, or perforate prey soft tissues (polychaetes, fishes,…). Some predators have lost the radula and engulf animal prey whole. Various feeding modes, using no radula, are also encountered in gastropods. In filter feeders, hypertrophy of the ctenidium as a ciliray-mucous food collecting device is used as a trap to capture and sort particles suspended in seawater. Other feeding strategies include parasitic species, devoid of radula, that feed on body fluids thank to a sucker, and establishment of nutritional symbioses. Some herbivorous species can suck algal cell content and establish symbioses with chloroplastes (family Elysiidae) or zooxanthellae (family Aeolidae). Chemoautotrophic symbioses also occur in a wide range of habitats, including cold seeps, whale and wood falls, shallow-water coastal sediments and continental margins (Dubilier et al. 2008).

In the hydrothermal vent environment, chemosynthetic production by bacteria is the main food source of primary consumers (Felbeck and Somero 1982). The majority of hydrothermal gastropods are thus grazers or filter feeders that appear to feed on free-living bacteria (Bates 2007a). Another widespread strategy at vents is symbiotic association with chemoautotrophic bacteria. Up to now, such symbioses have been demonstrated in at least 7 different phyla (Dubilier et al. 2008), including molluscs, the most famous examples being described in gills of bivalves (Bathymodiolinae and Vesicomyidae) and involve sulfur-oxidizing Gammaproteobacteria endosymbionts. But it also exists among gastropods. The best known examples are *Alviniconcha hessleri* and *Ifremeria nautilei*, found in the western Pacific (Windoffer and Giere 1997; Borowski et al. 2002; Suzuki et al. 2005a, 2005b, 2006; Urakawa et al. 2005; Saito and Hashimoto 2010). Most of the examples described for these two species also rely on sulfur-oxidizing Gammaproteobacteria gill endosymbionts. But recently, Epsilonproteobacteria were described as gill endosymbionts in some species of Provannidae (Urakawa et al. 2005; Suzuki et al. 2006, Beinart et al. 2013). Symbioses in smaller gastropod species remain poorly studied and the presence of bacteria as symbionts has only been

documented in *Lepetodrilus fucensis* from the Juan de Fuca Ridge (Bates 2007a), in which a nutritional role has been suggested.

In this study, we investigate a coiled gastropod, the Neomphalina *Cyathermia naticoides* (Warén and Bouchet 1989). Not much is known about it, despite it is a common species, found in abundance among *Riftia pachyptila* clumps (Mills et al. 2007), and in lower abundances among *Alvinella pompejana* and *Bathymodiolus thermophilus* (Warén et al. 2006). Up to now, *C. naticoides* was inferred to graze on tubeworms bacterial cover, but also to use filter feeding, based on its very large bipectinate gill (Warén and Bouchet 1989). A distinct labial notch described by Warén and Bouchet (1989) in the shell morphology is interpreted as an adaptation to allow the gill to be extended outside the shell even when the snail is resting on the substrate, partially retracted into the shell (Warén and Bouchet 1989; Sasaki et al. 2010). Here we investigate an additional hypothesis as feeding strategy in *C. naticoides*. Our study describes a unusual symbiosis, where epibiotic autotrophic Epsilonproteobacteria are endocytosed within the gill filaments of the animal. The large size of the gill, the recurrent observation of endocytosis and lysis of bacteria, and the stable isotopes results advocate for a nutritional symbiosis.

Material and methods

Animal collection and conditioning. *Cyathermia naticoides* specimens were collected, among *Riftia pachyptila* tubes, using the DSV Nautile during the Mescal 2010 cruise (East Pacific Rise, 2,500 m depth), on two different sites : 9°50'N (Bio9 site) and 12°50'N (Genesis site). Once on board, the entire specimens were fixed (operculum removed) in: 1) 2.5% glutaraldehyde (for light and electron microscopies), 2) ethanol (for DNA extraction), 3) 2-4% formaldehyde (for Fluorescent In Situ Hybridization, FISH) and 4) liquid nitrogen (for stable isotope analyses and chitinase activity assays).

Light and Electron Microscopies. Gut and gill tissues of 3 specimens of *C. naticoides* from 9°50'N were dissected. Two other specimens were embedded whole. Samples were post-fixed in osmium tetroxide 1%, dehydrated in increasing ethanol series (50, 70, 95 and 100%) and embedded in Epon resin (48 h, 60°C). Sections were cut using a Reichert–Jung ultramicrotome. Semi-thin (600 nm) sections were stained with toluidine blue and observed with an Olympus BX 61. Thin (60 nm) sections were mounted on copper grids, contrasted using uranyl acetate and observed using a HITACHI H-7100 transmission electron

microscope, operated at 80 kV.

Fluorescence In situ hybridization (FISH)

Four specimens from 9°50'N and 2 from 12°50'N were used. Specimens were pulled out their shells, and after 2-4 hours in 4% formaldehyde, were rinsed and dehydrated in 50, 70 and 96% ethanol. They were then embedded whole in polyethylene glycol (PEG) distearate: 1-hexadecanol (9:1). Sections of 7-10 µm were cut using a Jung microtome and deposited on Superfrost Plus slides. Wax was removed and tissue rehydrated in decreasing ethanol series. Sections were hybridized as described in Zbinden et al. (2010), using 30% formamide for 3 hours at 46°C, rinsed (15 min, 48°C), covered with SlowFade containing DAPI, and examined under an Olympus BX-61 epifluorescence microscope (Olympus, Japan). Following probes, labeled with Cy-3 and Cy-5, were used: Eub-338 (5'-GCTGCCTCCCGTAGGAGT-3', Amann et al. 1990), Gam-42 (5'-GCCTTCCCACATCGTTT-3', Manz et al. 1992), Del-495a (5'- AGTTAGCCGGTGCTTST – 3', Loy et al. 2002), Epsy-549 (5'-CAGTGATTCCGAGTAACG-3', Manz et al. 1992) and Arc-94 (5'- TGCGCCACTTAGCTGACA – 3', Moreno et al. 2003). Phylotypes targeted by the different probes are indicated in Table 1.

DNA extraction and 16S rRNA amplification

DNA was extracted from 3 specimens from 9°50'N and 3 from 12°50'N. Specimens were dissected as follows: at 9°50'N, gill was extracted from two of the specimens (9-1-Gi and 9-2-Gi), while the visceral mass (i.e. the rest of the animal containing the digestive tract and the heart, gonad, digestive gland, liver, and excretory organs), was treated separately (9-1-VM and 9-2-VM). A third specimen (9-3) was treated as a whole. DNA was also extracted from the empty shell of specimen 2 (9-2-Sh). At 12°50' N, DNA was extracted separately from the gill and visceral mass of three specimens (12-1, 12-2, 12-3). Extractions were performed using the DNA Tissue Kit (Qiagen). A ~1500bp fragment of the 16S rRNA-encoding gene was amplified by PCR using primers 27F and 1492R, over 32 cycles. Three PCR products were pooled together for each sample, to reduce PCR bias. Fragments were cloned using a TOPO TA Kit (Invitrogen, CA). 11 to 32 clones were successfully sequenced from each sample by GATC Biotech (Table 1).

Genes encoding for key enzymes of sulphur oxidation (*aprA*) and autotrophic carbon fixation (*aclB*) were sought. Fragments of the *aprA* gene encoding APS (adenosine 5′-phosphosulfate) reductase and the *aclB* gene encoding ATP Citrate Lyase (the key enzyme in reverse tricarboxylic acide (rTCA) cycle) were amplified using primer sets aps1F/aps4R and

892F/1204R, respectively as described previously and using 32 to 35 PCR cycles (Campbell et al. 2003, Meyer and Kuever 2007). Obtained PCR products were cloned and inserts were sequenced (Table 2).

Gene sequence analyses

Chromatograms were checked for quality. For each sample, sequences were aligned, and grouped in Operational Taxonomic Units (OTUs) when >97% of the nucleotide positions were identical. For the 16S rRNA-encoding genes, overall 11 OTUs were present in more than a single sample. Sequences were compared with databases using BLAST (Altschul et al. 1990; Cole et al. 2009), and the 8 OTUs for which full sequences were available were included in a dataset with their best hits and reference sequences, and aligned using SINA Web Aligner (Pruesse et al. 2007). Alignment was manually checked, and phylogenetic relationships were inferred by using the Maximum Likelihood (ML) method. For the reconstruction, a General Time Reversible model, with a Gamma distribution of evolutionary rates among sites was used (5 categories and invariant sites). For *aprA* and *aclB* genes, recovered sequences were compared to the database using BlastX (Table 2). For *aclB*, best hits and representative sequences were included in a dataset, and phylogenetic reconstruction was based on a 100 aa-long fragment using a ML approach, a JTT model of amino acid substitution and a Gamma distribution of evolutionary rates among sites (tree in Fig. S1). All analyses were conducted using MEGA6 (Tamura et al. 2013).

Stable isotope analysis

Frozen *Cyathermia naticoides* (n = 4 for $\delta^{13}C$ and $\delta^{15}N$, n = 10 for $\delta^{34}S$ for 9°50'N and n = 5, only $\delta^{13}C$ and $\delta^{15}N$, for 12°50'N) were dissected under a dissecting microscope to remove the shell. Specimen tissues were rinsed in distilled water, dried (3 days, 60°C) and then reduced into powder. To avoid significant changes in $\delta^{15}N$ isotopic composition, no HCl was used to remove carbonates (Kaehler and Pakhomov 2001). About 1 mg (± 0.1 mg) of dried tissues (except 8 mg for sulfur stable isotopes; pool of 10 specimens) were analyzed by a GV IsoPrime (UK) stable isotope mass spectrometer (Iso-Analytical, Crewe, UK). Values of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ were determined and expressed as relative per-mil (‰) differences between samples and Pee Dee Belemnite (PDB) for carbon, air N_2 for nitrogen and Canyon Diablo Troilite for sulfur according to the following equation:

$$\delta(X) = \left\lfloor \left(\frac{R_{sample}}{R_{standard}}\right) - 1 \right\rfloor * 1000$$

where X (‰) is ^{13}C, ^{15}N or ^{34}S abundance and R is the $^{13}C/^{12}C$, $^{15}N/^{14}N$ or $^{34}S/^{32}S$ ratios.

Chitinolytic activity assays.

Chitinolytic activity was determined using a modification by Gutowska et al. (2004) of the standard procedure of Jeuniaux (1966), which measures the production of N-Acetyl glucosamine (NAG). Five specimens from 12°50'N were removed from their shell and ground together in liquid nitrogen. The powder was homogenized in 0.15 M citric acid, 0.3 M Na_2HPO_4 buffer (pH = 5 and pH = 7). The homogenates were then centrifuged at 2000 g for 10 min at 4°C, the supernatants were recovered and assayed for their chitinolytic activity. The standard mixture consisted of 2 vol. of tissues extract, 1 vol. of chitobiase and 1 vol. of chitin suspension (5 $mg.ml^{-1}$). Two control assays were added, with either the tissues extract or the chitin solution replaced by distilled water. For comparison, assays with commercial *Streptomyces griseus* chitinase (Sigma C6137) were also conducted in parallel. They were all incubated at 37°C, and aliquots were taken after t = 0 min, t = 90 min and t = 180 min. Chitinolytic reaction was stopped by mixing 1 vol. of the reaction medium with 1 vol. of boiling water. The mix was placed 10 min at 100 ° C, then centrifuged at 2000 g for 10 min. The supernatants were used for NAG measurements by adding $K_2B_4O_7$ 0.8M, and further incubating for 3 min at 100°C. p-dimethylaminobenzaldehyde (DMAB) was added and after 20 min at 37°C, the concentration of NAG released was determined by comparing each sample's absorbance at 585 nm to NAG standard curves. The activity was expressed as µg of NAG released per gram protein per hour.

Results

Morphology and ultrastructure of gut and gill

Cyathermia naticoides is a gastropod with a regularly coiled shell (diameter up to 7 mm), with a deep notch at basal side of the outer lip. This gastropod possesses a very large bipectinate gill, which occupies most of the anterior part of the animal (Fig. 1a, b). Semi-thin sections revealed an abundant bacterial community associated with the gill (Fig. 1c, d). These gram-negative filamentous bacteria (0.5 to 0.6 µm in diameter and up to 5.8 µm in length, Fig. 2a, b) were located extracellularly, between the gill filaments, mainly at their base. Some of the bacteria were free in the inter-lamellar space, but many have also been observed trapped in lysosome-like structures in the gill epithelium (see Fig. 1d and 2a), and appeared to undergo different stages of degradation (Fig. 2b). Bacterial colonization and endocytosis occurred on each animal and all sections observed.

The digestive tract contained pieces of *Riftia pachyptila* tubes (not shown), recognizable by chitin microfibrils organized in parallel bundles with various orientations (Gaill and Shillito

1995). No bacteria similar to those present on the gill were observed in the gut contents.

Fluorescence microscopy observations

Probes Eub-338 and Arc-94 yielded strong signals in regions of the gill filaments of *Cyathermia naticoides* from both 9°50'N and 12°50'N (Fig. 1e, f). Cy-3 labeled probe Epsy-549 yielded weaker signal, but signal-to-noise ratio was greatly improved when letting tissue autofluorescence decrease under the laser for 30 seconds. Signals from the three probes fully overlapped in gills (not shown). Hybridized objects corresponded to thin filamentous bacteria. Parts of the gill filaments were free of bacteria and did not display any signal, suggesting that the distribution of bacteria was not homogeneous (Fig. 1f). Probes Gam-42 and Del-495a did not display any signal in the gills. No FISH signal was observed from the gut epithelium or content.

Chitinolytic activity

Chitinase activity was assayed on crude extracts from whole specimens. The measured activity (20 µg NAG released g^{-1} protein h^{-1}) was very weak when compared to the reference sample (*Streptomyces griseus* chitinase, 6500 µg NAG released g^{-1} protein h^{-1}). Another gastropod (*Lepetodrilus elevatus*) found on *R. pachyptila* tubes was also analyzed for comparison, and showed a two-fold higher activity (42 µg NAG released g^{-1} protein h^{-1}), which was still very weak when compared to *S. griseus*.

Bacterial communities associated with digestive tract and gill

Out of 295 sequences obtained, 244 (83%) belonged to one of the 11 OTUs (defined as groups of sequences displaying above 97% identical positions) that were present in more than a single sample (see Table 1). The remaining sequences corresponded to single reads occurring in a single sample. Above 78.0% of total sequences, and 7 of the 11 OTUs (94.3% of the OTUs-assigned sequences) were assigned to the Epsilonproteobacteria. Five of the OTUs (1, 3, 8, 11, 16-p), including the 4 most abundant and representing 90% of the OTU-assigned sequences, were present at both sampling sites 9°50'N and 12°50'N.

OTUs 3, 1 and 8 dominated clone libraries, representing 23.1, 20.0 and 19.3% of the total sequences, respectively (Table 1). OTU 3 was present in gills and visceral mass at 9°50N and gill at 12°50N. This sequence displayed above 98% identity and was most closely related to sequences from an epibiont of the gastropod *Lepetodrilus fucensis*, and to various *Arcobacter* from the EPR (Fig. 3). OTU 1 was present in all samples from 9°50'N, and in visceral mass samples from two specimens at 12°50’N. The sequence was closely related and highly similar

(>98%) to sequences from bacteria associated with the tube of *Ridgeia piscesae* on the EPR. The third, OTU 8, was present in gill, visceral mass and shell at all sites and displayed above 98% identity with several sequences related to the sulfur-oxidizing chemolithotroph *Sulfurovum* from the Brothers Seamount (Kermadec Arc) and vents.

Functional gene analysis

Overall 70 *aprA* sequences were obtained from the visceral mass of three specimens, and none from the gill tissue. Six distinct nucleotide sequences were obtained, 4 of which were related to Gammaproteobacteria and represented above 94% of total sequences (Table 2). The dominant sequence, clone 761, was 96% similar (amino acids) to a sequence from an epibiont of the Yeti crab *Kiwa hirsuta*. Other sequences were similar to a sequence from a bacterium associated with the oligochete *Tubificoides benedii* and from the tube of the siboglinid annelid *Lamellibrachia anaximandri* (Table 2).

PCRs on the *aclB* gene yielded faint bands from the visceral mass of specimen 9-1 and 9-2-, and from the gills of specimen 9-2. Out of 28 sequenced clones from each of these 3 samples, only 35 good quality sequences were obtained. The majority (24) were from the gill, of which 19 corresponded to a single sequence, clone 765, and were related to various sequences of Epsilonproteobacteria from hydrothermal vents and to epibionts from the gill chamber of the vent shrimp *Rimicaris exoculata* (around 98% amino acid similarity, Table 2 and Fig S1).

Stable isotope composition

The $\delta^{13}C$ values of *Cyathermia naticoides* varied following vent sites, with -9.0‰ (± 0.3‰) for 9°50'N and -10.8‰ (± 0.4‰) for 12°50'N (Fig. 4). These stable isotopic ratios of carbon fall after correction of fractionation (1‰ for $\delta^{13}C$ and 3.3‰ for $\delta^{15}N$) into the range of stable isotopic ratios of carbon of Epsilonproteobacteria (range between -8 and -12‰; Campbell et al. 2003) (Fig. 4). Regarding $\delta^{15}N$, value of both sites was 6.8‰ (± 0.5‰). This nitrogen stable isotopic ratio is typical of a primary consumer at vents (between 4 and 8‰). Isotopic signatures of $\delta^{34}S$ measured in a pool of *C. naticoides* (n = 10) was 5.5‰.

Discussion

Grazing vs. filter-feeding

Gastropods have two main feeding strategies: i.e. grazing, using their radula to rasp various kinds of substrates, or filter feeding, using their gills as a trap to capture and sort particles suspended in seawater. At hydrothermal vents, where the organic matter synthesis relies on chemoautotrophic bacteria, most gastropods appear to feed on free-living bacteria (Bates et al.

2007b). Up to now, *Cyathermia naticoides* was inferred to graze on tubeworms bacterial cover, but also to use filter feeding, based on its very large bipectinate gill (Warén and Bouchet 1989). Grazing on tubeworms bacterial cover is congruent with our observations, as we noted the occurrence of *Riftia pachyptila* tube pieces in the gut content. Conversely, filter feeding on bacteria is not well-supported by our data. The position of the filamentous bacteria, deep between the filaments, and the large number of endocytosed and lysed bacteria advocates for a stronger association than a classic trapping through filter feeding mechanism for transport to the gut. Nevertheless, filter feeding on particulate organic matter cannot be discarded.

A diet based on bacteria

Despite *R. pachyptila* tube pieces, which contain up to 25% of chitin (Ravaux et al. 1998), are rasped and ingested by *C. naticoides*, a weak chitinolytic activity was measured for this species, when compared to the reference chitinase of *Streptomyces griseus*, or to values obtained for chitin degrading animals, such as fishes feeding on crustaceans (Gutowska et al. 2004). This suggest a minor nutritional input of chitin and that *C. naticoides* rather grazes on *R. pachyptila* tubes for feeding either on proteins contained therein (representing 37-41% of the tube, Ravaux et al. 1998) or on the bacterial biofilm. López-García and collaborators (2002) observed dense microbial populations on *R. pachyptila* tubes, with very diverse 16S rRNA phylotypes, belonging mostly to Epsilon-, but also to Delta-, Alpha- and Gamma-proteobacteria. Interestingly, among our recovered bacterial phylotypes, OTUs 1 and 11 are closely related to several sequences from the tubes of *Ridgeia piscesae* and *Riftia pachyptila* (Fig. 3; Forget and Juniper 2013; López-García et al. 2002). These could be bacteria ingested alongside with tube fragments. Indeed, of the 59 sequences of OTU 1 for example, 36 were from the visceral mass of *C. naticoides* (3.4 to 63% of the sequences depending on the sample). This sequence displays 5 and 1 mismatches with FISH probes Arc94 and Epsy549 and thus does not hybridize with them. It is thus surely not the sequence from the bacteria located in the gills, which respond to both probes. Sequences encoding APS reductase were successfully amplified from the visceral mass of all tested specimens. Related sequences were typically associated with the tube or cuticle of protostomes. These might again correspond to sequences of bacteria ingested with scrapings of tubes. The dense colonies of filamentous Epsilonproteobacteria observed on *C. naticoides* gill surface could be another nutritional pathway. Many are indeed endocytosed within lysosomes, arguing for a internal digestion of bacteria in the gill epithelium.

Vent gastropods at hydrothermal vents are considered as primary consumers feeding both on free-living bacteria of different origins but also on particulate organic matter (Limen et al.

2007), resulting in a range of stable isotopes nitrogen ratio between 4 and 8 ‰ (Bergquist et al. 2007; Limén et al. 2007; Gaudron et al. 2012), which includes our values for *Cyathermia naticoides*. Several species of *Lepetodrilus* are known to be also primary consumers (heterotrophic) such as *L. elevatus*, *L. pustulosus* and *L. ovalis*, displaying the same range of stable isotopic nitrogen values (Fig. 4). However *L. fucencis* known to harbor symbiotic bacteria within its gills also has a similar stable isotopic nitrogen value (7‰, Bates et al. 2011), as well as others larger symbiotic gastropods (*Alviniconcha* spp) harboring Epsilonproteobacteria (Fig. 4), meaning that heterotrophic and symbiotic diet may co-occur in *C. naticoides* and cannot be easily distinguished based on nitrogen isotopes.

In the previously studied symbioses involving Epsilonproteobacteria, a trophic role has been suggested based on carbon stable isotope signatures of hosts ($\delta^{13}C$ values between -11 and -10.7‰ for *A.* aff *hessleri* ; -12.8 and -11.2‰ for *Alvinella pompejana* and -12 to -10‰ for *Rimicaris exoculata*, Suzuki et al. 2005b; Desbruyères et al. 1998; Polz et al. 1998). These values indeed fall within the range of typical values measured in Epsilonproteobacteria which use the reverse TCA cycle for autotrophic carbon fixation (-12 to -8‰, Campbell et al. 2006; Sievert and Vetriani 2012). Similar values are measured in *C. naticoides* (-10.84 ± 0.58‰; Fig. 4), suggesting that similar bacteria may significantly contribute to the hosts diet, either those grazed on tubes or those endocyted in the gill. López-García and collaborators (2002) identified that most bacteria (68%) present on *R. pachyptila* tube surface belonged to the Epsilonproteobacteria ($\delta^{13}C$ value for scrappings from *R. pachyptila* tubes are -12,5‰). In *Cyathermia*, this is further supported by the identification of ATP Citrate Lyase-encoding genes from the visceral mass of specimens which confirm the presence of rTCA. On the other hand, a single dominant ATP Citrate Lyase sequence is also identified from gill-associated bacteria, which supports the hypothesis of a significant contribution of the gill bacteria to the host carbon nutrition. The next step will be to quantify the respective roles of gill-associated versus ingested bacteria.

The vast majority of symbiotic bacteria described at present in molluscs are chemoautotrophic sulfur-oxidizing bacteria. The $\delta^{34}S$ value of an animal indicates the origin of the assimilated sulfur. Marine invertebrates for which the sulfur source comes from chemosynthetic sulfur-oxidation have values lower than 5 ‰. $\delta^{34}S$ of *C. naticoides* (5‰) is at the upper end normally measured into thiotrophic metazoans, which is between -25 to 5‰ (Vetter and Fry 1998), allowing to suppose that some sulfur absorbed by the animal comes from chemosynthetic sulfur-oxidizers. If evidence for thiotrophic metabolism has been shown through sequencing of APS reductase in the visceral mass (possibly coming from the bacteria

in the gut, rasped on *R. pachyptila* tubes), no positive PCR result was obtained from gills. We cannot thus confirm the thiotrophic metabolism of gill-associated bacteria.

Symbiosis

A widespread feeding strategy at hydrothermal vents is to obtain organic carbon through symbiotic associations. Among molluscs, symbioses are well known and described in large species, such as the mussels and clams (Mytilidae and Vesicomyidea, Dubilier et al. 2008) or the large gastropods *Ifremeria nautilei* and *Alviniconcha hessleri* (Provannidae, Borowski et al. 2002; Suzuki et al. 2005a, b). In all these symbioses, sulfide-oxidizing Gammaproteobacterial symbionts are endosymbionts, occurring within the host tissues in gill epithelial bacteriocytes. The hosts are fuelled by by-products of bacterial metabolism (ultimately relying on sulfide oxidation) or intracellular bacterial digestion (Bates 2007b). Here we described the occurrence of a dense population of filamentous bacterial located extracellularly at the base of the gill filaments. Some are free in the inter-lamellar space, but many have also been observed trapped in lysosome-like structures, in the gill epithelium. At hydrothermal vents, epibiotic symbioses have been described in only a few groups: Ciliophora (Kouris et al. 2007), annelids (*Alvinella pompejana*, Haddad et al. 1995; Cary et al. 1997; Bright and Giere 2005) and crustaceans (*Rimicaris exoculata*, Segonzac et al. 1993; Zbinden et al. 2008; Petersen et al. 2010; the galatheid crabs *Kiwa hirsuta* (Macpherson et al. 2005; Goffredi et al. 2008), *Kiwa puravida* (Thurber et al. 2011), and *Shinkaia crosnieri* (Miyake et al. 2007). In Molluscs, only very few examples are known : in Aplacophora (Katz et al. 2006) and in Gastropoda (*Lepetodrilus fucensis*, Bates 2007a, b). The kind of symbiosis described in *L. fucensis* is the closest to what we observed in *Cyathermia naticoides*, with a few exceptions. *L. fucensis* hosts dense colonies of filamentous bacteria on its gill surface, where bacteria are found partially embedded in the host's gill epithelium and extend into the fluid circulating between the lamellae (de Burgh and Singla 1894 ; Bates et al. 2007a, b). Frequent endocytosis was observed in the epithelium (de Burgh and Singla 1984). Observed residual bodies of lysosome-like organelles, with concentric membrane stacks, mirror our observations. The main difference between *L. fucensis* and *C. naticoides* is that most abundant *L. fucensis* epibionts are Gammaproteobacteria (Bates et al. 2011), and those of *C. naticoides* belong to Epsilonproteobacteria. Furthermore, stables isotopes analyses ($\delta^{13}C$ = -19.5 to -14.8‰ and $\delta^{15}N$ = 2.5 to 5‰) situate *L. fucensis* within a group of known deposit feeding invertebrates at the Juan de Fuca Ridge vents (Fox et al. 2002), whereas *Cyathermia* values fall within the range of typical values measured in Epsilonproteobacteria, and in

organisms living in symbiosis with these bacteria (-12 to -8‰, Campbell et al. 2006; Sievert and Vetriani 2012).

As suggested by de Burgh and Singla (1984) and Bates (2007a), there are 3 ways in which the gill bacteria may contribute to the organic carbon of the host : 1) the bacteria may be farmed and ingested; 2) dissolved organic molecules, byproduct of the bacterial metabolism, may pass from the bacteria to the host through the epithelium, as it was suggested for *Alvinella pompejana* and evidenced for the shrimp *Rimicaris exoculata* and its epibionts (Ponsard et al. 2013); 3) bacteria may be endocytosed in the gill epithelium and digested within lysosomes. For *L. fucensis*, Bates (2007b) argues that endocytosis of bacteria by the gill epithelium followed by lysosomal digestion (de Burgh and Singla 1984) may not be an important feeding mechanism. In our case, the huge number of lysosome-like structures observed, with bacteria at different stages of degradation conversely rather advocates for the third hypothesis. Nevertheless, additional contribution by the two other ways cannot be discarded.

These gill bacteria likely correspond to our OTU 3, which is the predominantly associated with gill samples. Indeed, 43 of the 68 sequences were from gill samples, representing between 22 to 100% of sequences in the various gill samples. Besides, OTU 3 was present in gills of all specimens at both sites, and far less abundant in visceral mass samples, representing only from 0 to 22% of the sequences. Futhermore, it responds to both Arc-94 and Epsy-549 probes, as do gill bacteria observed using FISH. Finally, it is closely related to one of the documented gill epibionts of *Lepetodrilus fucensis*. OTU 3 might be a widespread epibiont of gastropod gills.

The third most abundant OTU identified in our clone libraries, namely OTU 8, related to *Sulfurovum* also responds to both FISH probes. However, only 10 of the 57 recovered sequences were from gill tissue, representing 0 to 29% of sequences depending on gill sample, while 14 were found on the shell analyzed (45%) and 33 in the visceral mass. Closest relatives do not include any reported symbiont. This bacterium is thus most probably an environmental bacterium, although this cannot be ascertained using FISH probes from this study.

As seen above, the large majority of the mollusc-associated symbionts from chemosynthetic environments are Gammaproteobacteria (in the Thyasiridae, Lucinidae, Solemyidae, Vesicomyidae, Mytilidae, and some Provanidae). But recently (Suzuki et al. 2005a, 2005b), Epsilonproteobacteria were described as symbionts (and as endosymbionts) in some species of Provannidae (*Alviniconcha* sp.). *A. hessleri* from the Mariana Trough, *Alviniconcha* sp. type 1 from Manus Basin and Fiji, and *Alviniconcha* sp. from Lau Basin harbors sulfur-oxidizing chemoautotrophic Gammaproteobacterial endosymbionts that mediate the Calvin-

Benson cycle to fix CO_2. Whereas *Alviniconcha aff. hessleri* from the Central Indian Ridge and *Alviniconcha* sp. type 2 from Manus Basin and Fiji harbors chemoautotrophic Epsilonproteobacterial endosymbionts that mediate the reductive tricarboxylic acid (rTCA) cycle for CO_2 fixation (Urakawa et al. 2005; Suzuki et al. 2006). A fragment of the gene encoding ATP Citrate Lyase was identified in the gill and visceral mass of *C. naticoides*. In particular, the most abundant sequence in the gills was related to sequences from various vent bacteria including gill epibionts of the vent shrimp *R. exoculata*. This advocates for the presence of this pathway in gills of *C. naticoides*. This finding is congruent with the dominance of Epsilonproteobacteria in the gill, and it is possible that the dominant *aclB* sequence (clone 765) is indeed associated with the dominant 16S rRNA OTU 3, or one of the other dominant gill-associated phylotypes.

Symbiotic association with filamentous Epsilonproteobacteria have been described, but mostly as ectosymbioses, as in the crustaceans *R. exoculata*, *K. hirsuta*, *K. puravida* or *S. crosnieri* and in the annelid *A. pompejana* (see references above). The *C. naticoides* symbiosis described here thus represents a unusual type of association in the long list of symbiosis within Molluscs, with Epsilonproteobacteria as ectosymbionts being the first exemple of this combination in molluscs, to our knowledge.

Epsilon- versus Gammaproteobacteria : an issue with temperature ?

Urakawa and collaborators (2005) suggest that thermal gradient may affect the acquisition and evolutionary selection of either Epsilon- or Gammaproteobacterial symbionts. Vent hosts harboring Epsilonproteobacterial symbionts such as shrimps or polychetes, usually live at higher temperatures than those harboring Gammaproteobacteria, such as clams or vestimentiferans. Indeed, the two Provannidae gastropods, *Alviniconcha* spp. and *Ifremeria nautilei*, studied by Urakawa, co-occur at the same sites in the Manus Basin, the former harboring Epsilonproteobacterial symbionts living at higher temperatures than *I. nautilei* which harbors Gammaproteobacteria. This could be congruent with our example, as *C. naticoides* that lives on tubes of *Riftia pachyptila* may in fact live in a warmer microhabitat that the tubeworm itself and its Gammaproteobacterial endosymbionts, the latter being protected by the chitinous tube. *C. naticoides* lives in sympatry with another small gastropod, *Lepetodrilus elevatus* on the tube of *R. pachyptila*, where a vertical microzonation has been observed. Individuals of *C. naticoides* cluster at the base of the tubes, where temperatures up to 25°C were measured (Sarradin et al. 1998), whereas *L. elevatus* rather graze higher up the tubes (P. Tyler, pers. obs. cited in Mills et al. 2007), where temperatures ranged between 1.6 and 10°C (Sarradin et al. 1998). So *C. naticoides* is associated with the warmer part of *R.*

pachyptila tube, and is also sometimes found among *A. pompejana* tubes (Desbruyères et al. 2006; Mills et al. 2007), which live on the chimney walls at even higher temperatures (up to 50°C was measured at 2-5 cm within the tube assemblages, Le Bris et al. 2005). Temperature can also be put forward to explain the different bacterial partners in *C. naticoides* (Epsilonproteobacteria) et *L. fucensis* (Gammaproteobacteria) ectosymbioses. Indeed *Lepetodrilus fucensis* was reported (Bates et al. 2005) to be abundant in fluids with temperature between 4 and 10°C, and to be absent where maximum fluid temperature reached 18°C. Although precise temperatures have not been reported in literarture, *C. naticoides* is probably exposed to temperatures exceeding 20°C in the habitats its occupies (base of *R. pachyptila* tubes or *A. pompejana* clumps).

This selection of either Epsilon- or Gammaproteobacterial symbionts which seem to be affected by temperature, could also be linked to oxygen availability (both being negatively correlated). Sulfur metabolism pathways are indeed not the same in Epsilon- and Gammaproteobacteria. Both of the pathways used by deep-sea hydrothermal Gammaproteobacteria (the reverse sulfate reduction and the Sox multienzyme system) require O_2 as a terminal electron acceptor in most cases. This indicates that a relatively O_2-depleted environment is less suitable for their growth (Yamamoto and Takai, 2011). Thus, it is predicted that the metabolically habitable niches for deep-sea chemoautotrophic Gammaproteobacteria strictly require co-existence of reduced sulfur compounds and O_2. Besides oxygen, some Epsilonproteobacteria are also able to use sulfur compounds as electron acceptors (Yamamoto and Takai, 2011), which may allow them to tolerate and colonize O_2-depleted and warmer niches within the mixing zone, closer to the reducing hydrothermal fluid.

Conclusion

Cyathermia naticoides harbors dense populations of filamentous Epsilonproteobacteria in its gill which may contribute to their nutrition through intracellular digestion by gill cells. OTU 3 was identified as a probable candidate dominant gill bacterium. Yet, the diet could be mixotrophic, an additional food source being the bacteria grazed on *R. pachyptila* tubes. OTUs 1 and 11 identified here are likely siboglinid tube-associated Epsilonproteobacteria that may be significant food sources on this route. Novel for molluscs by the combination of the location (ectosymbionts) and bacterial phylotype (Epsilonproteobacteria) encountered and the feeding mechanism, the symbiosis of *C. naticoides* represents an unusual type of association in the already long list of molluscan symbioses, of which more await characterization in particular in smaller-sized species.

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Ethical standards

The authors declare that the experiments comply with the current laws of the country they were performed (France).

The authors declare that they have no conflict of interest.

Figure Legends

Fig. 1 *Cyathermia naticoides* ctenidium. **a**) Specimen outside its shell showing the extansion of the ctenidium (ct). **b**) Semi-thin section of the ctenidium. **c**) Basal part of the gill filaments showing the accumulation of bacteria (b) between the gill filaments (gf) and in lysosome-like structure (ly). **d**) Close-up of bacteria in the inter-filament space (ifs) and in lysosome-like structure (ly). **e**) Transverse section through the gill, displaying bacterial filaments in white, hybridized with the FISH probe Arc-94. **f**) Transverse section through the gill displaying animal cells (DAPI-labelled nuclei in blue) and bacteria labelled with probes Eur-338 (Cy3, red) and Arc-94 (Cy5, green), overlying signals resulting in a yellow color. Scale bars : a = 1 mm, b = 100 µm, c = 20 µm, d = 10 µm, e = 50 µm, f = 100 µm

Fig. 2 Transmission electron micrographs of the gill (A, B) of *C. naticoides*. **a**) Bacteria are endocyted in the gill epithelium and **b**) progressively degraded. Scale bars : a, b = 2,5 µm

Fig 3 Phylogenetic tree based on the analysis of 16S rRNA-encoding gene sequences. The tree with the highest log likelihood (-13800) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Scale bar represents the number of substitutions per site. Full (>1400 bp) sequences from this study appear in bold and an asterisk indicates an OTU that is present at both 9°50'N and 12°50'N. Sequences corresponding to confirmed metazoan symbionts or epibionts are underlined. All positions with less than 95% site coverage were eliminated. There were a total of 1302 positions in the final dataset, and partial sequences were excluded

Fig. 4 $\delta^{13}C$ and $\delta^{15}N$ values of *Cyathermia naticoides* and other heterotrophic and symbiotic vent gastropods. Triangle up are isotopic ratios of *Cyathermia naticoides*, with dark blue symbols, those sampled at EPR 9°50'N (9°) and light blue symbols, those sampled at EPR 12°50'N (13°). Triangle down, displayed previous isotopic values respectively, after correction using trophic step fraction of 1.0‰ for $\delta^{13}C$ and 3.3‰ for $\delta^{15}N$. The rectangle with dashed lines represents the stable isotopes ratios of ε–proteobacteria from Campbell et al. (2003). $\delta^{13}C$ and $\delta^{15}N$ values of other gastropods are from: Levesque et al. (2006) for *Lepetodrilus fucencis*; Gaudron et al. (in revision) for *L. elevatus* (9° and 13°), *L. pustulosus, L. ovalis* and *Eulepetopsis vitrea*; Henry et al. (2008) for *Ifremeria nautilei*; Goffredi et al. (2004) for the Scaly snail; Beinart et al. (2013) for $\delta^{13}C$ for *Alviniconcha hessleri* dominated by ε–proteobacteria (*Alviniconcha* (Epsilon)) and for *Alviniconcha hessleri* dominated by γ–proteobacteria (*Alviniconcha* (Gamma)). $\delta^{15}N$ of *Alviniconcha hessleri* is not documented, except for *Alviniconcha* sp. from Henry et al. (2008), which was used in this study

Table 1 Number of sequences representing each identified OTU in each sample investigated in this study. OTU names in bold correspond to full length sequences included in the phylogeny, the suffix '-p' indicates a partial sequence. Affiliation based on best BLAST hits: Epsilonproteobacteria (E), Deltaproteobacteria (D), Mollicutes (M) and Bacteroides (B). Names of samples are indicated by site (9 or 12), specimen ID (1 to 3) and tissue type (Gi-gill, VM-visceral mass, Sh-shell) as follows: site-specimen-tissue. Sum of sequences per OTU and percentage of total sequence counts are indicated. Finally, the two bottom rows indicate whether the OTU has no mismatch (+), a single mismatch (1mis) or more (-) to FISH probes Arc-94 and Epsy-549

Table 2: analysis of fragments of functional genes encoding APS reductase (aprA) and ATP citrate Lyase (ACL), their length, the identity of the represenative sequence, GENBANK accession number, percentage out of 70 (aprA) and 35 sequences (ACL) , number of specimens in which the sequence occurred out of 3 (aprA) and 2 (AclB), tissue occurrence (G: gill, R: visceral mass), and best hit according to BLASTX translated nucleotide sequence analysis

Supplementary material Fig S1: Phylogenetic reconstruction based on a 100 aa-long fragment of the *aclB* gene. A Maximum Likelihood approach using the JTT matrix-based model and a discrete Gamma distribution of evolutionary rates with a proportion of invariant sites was used. All bacteria from the ingroup are Epsilonproteobacteria, and the tree is rooted on two Aquificales. Boostrap percentage values based on 500 replicates are displayed. Scale bar corresponds to 10% estimated sequence divergence

Figure 1
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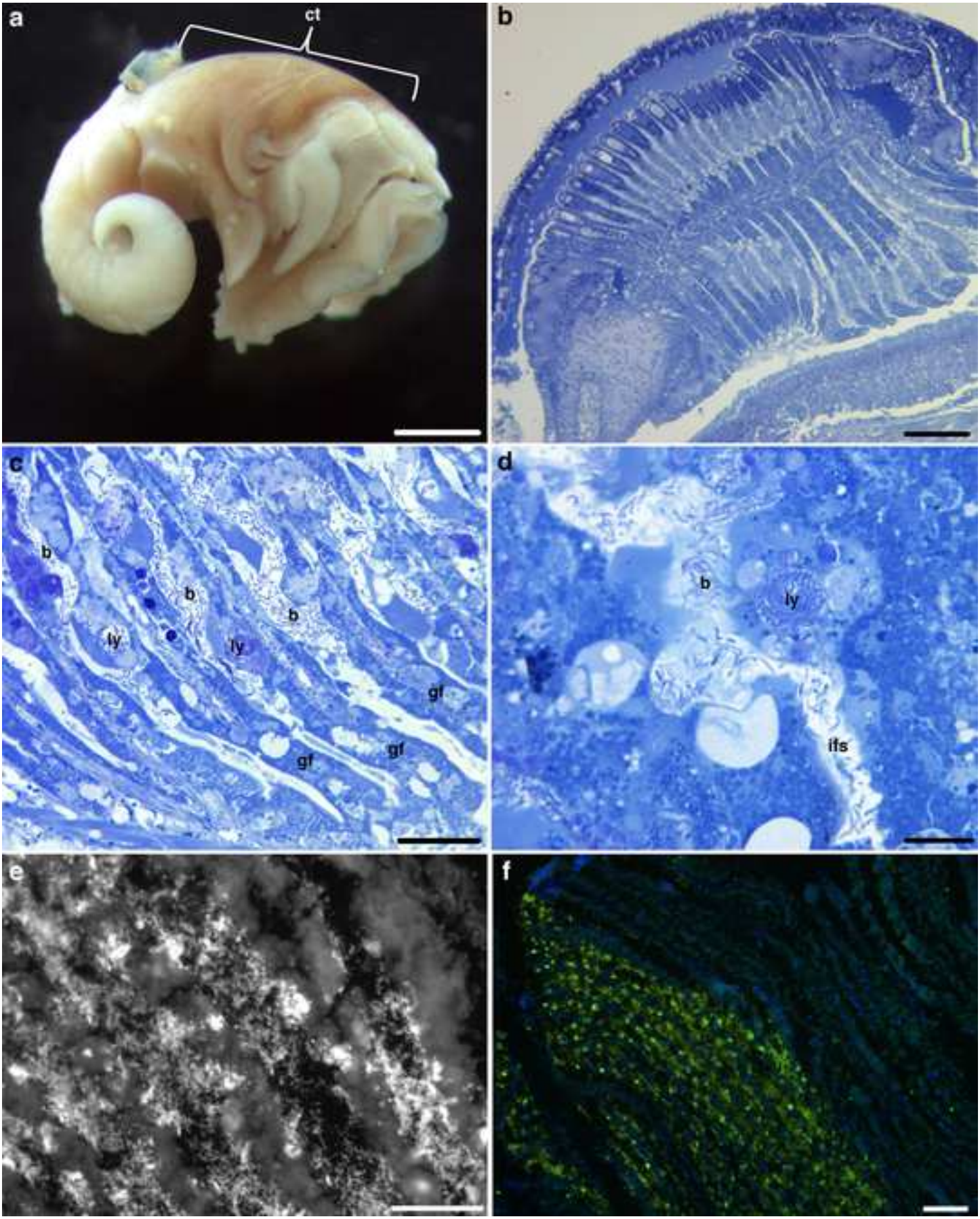

Figure 2
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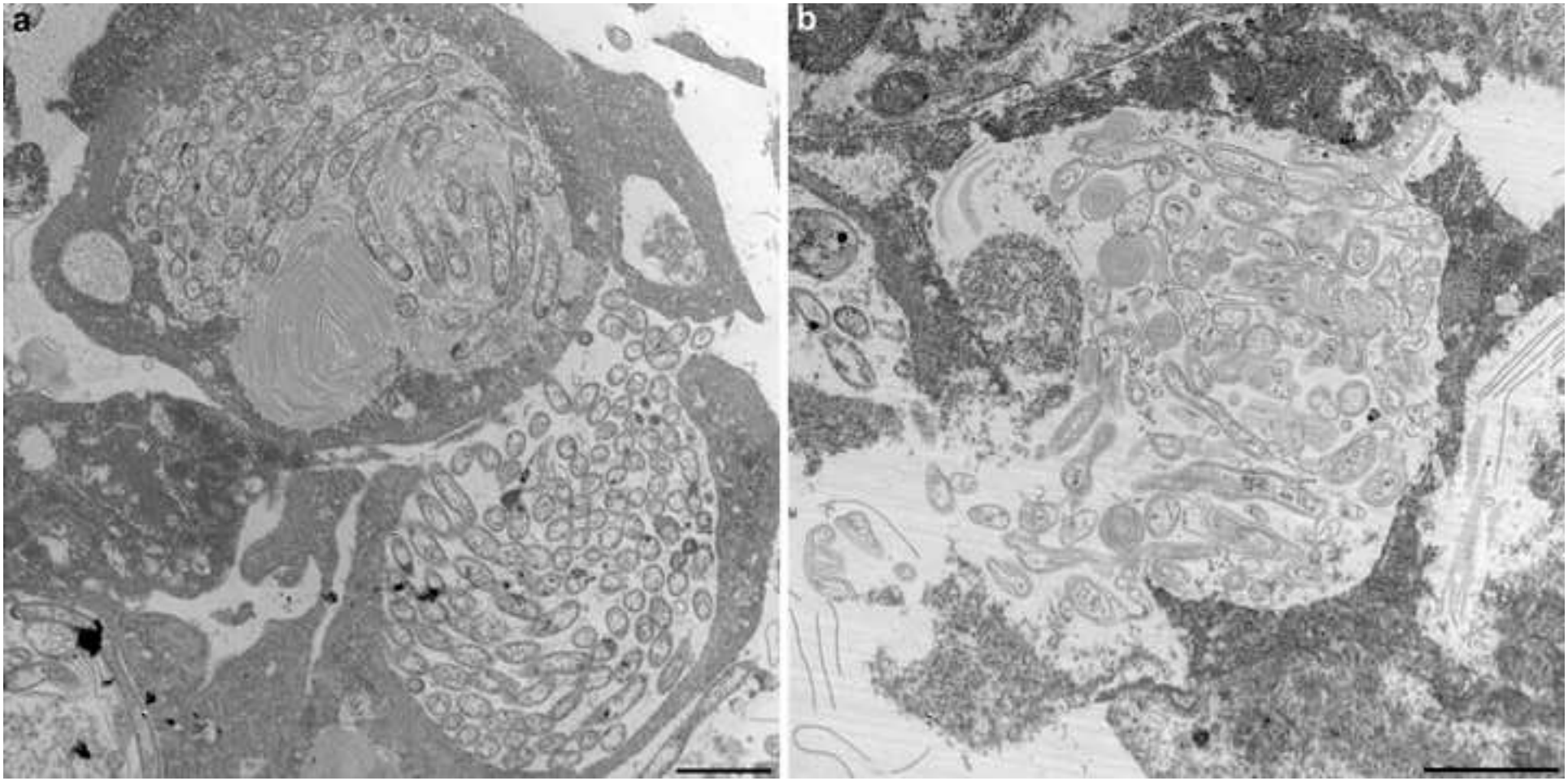

Figure 3

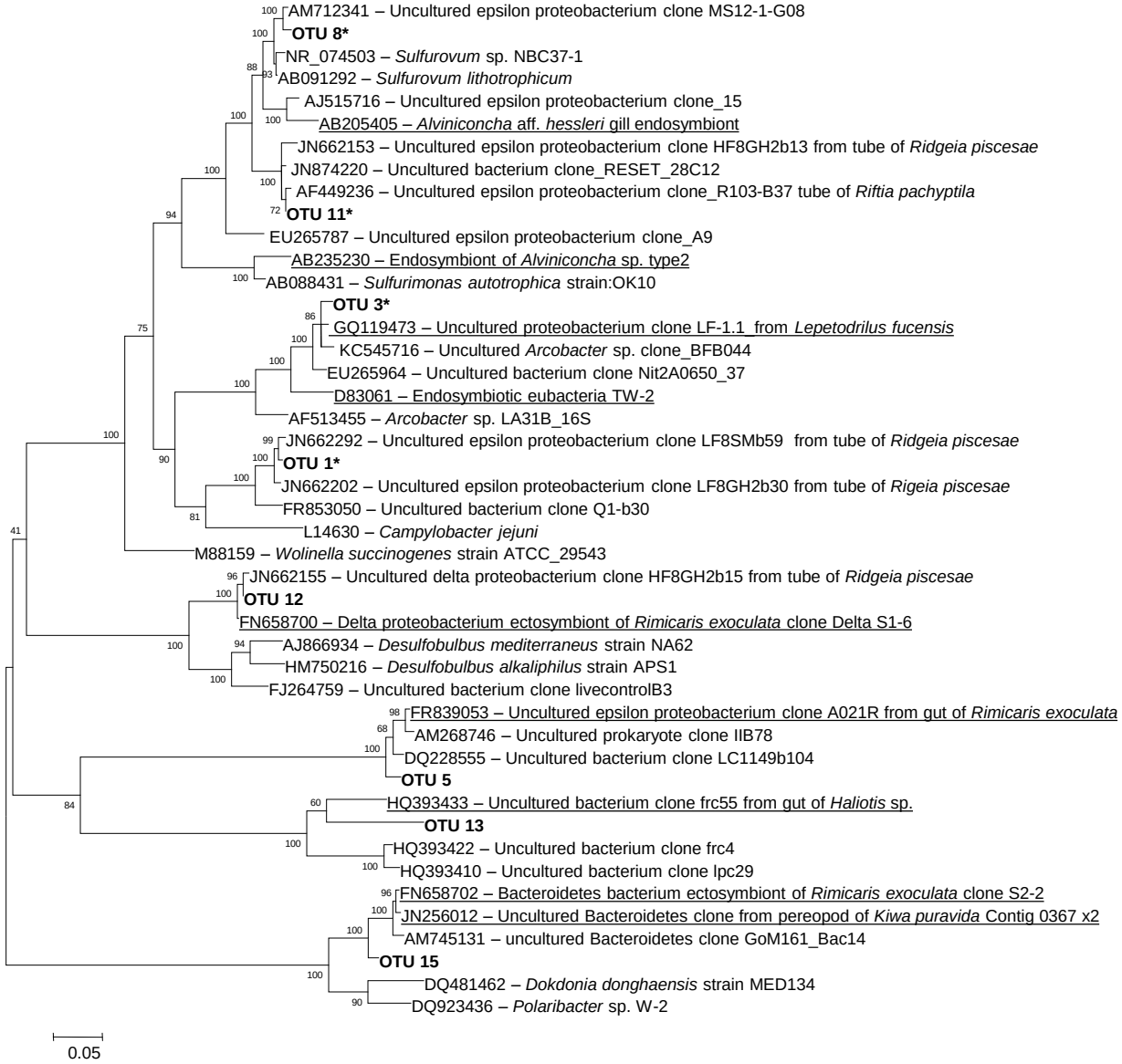
AM712341 – Uncultured epsilon proteobacterium clone MS12-1-G08
OTU 8*
NR_074503 – Sulfurovum sp. NBC37-1
AB091292 – Sulfurovum lithotrophicum
AJ515716 – Uncultured epsilon proteobacterium clone_15
AB205405 – Alviniconcha aff. hessleri gill endosymbiont
JN662153 – Uncultured epsilon proteobacterium clone HF8GH2b13 from tube of Ridgeia piscesae
JN874220 – Uncultured bacterium clone_RESET_28C12
AF449236 – Uncultured epsilon proteobacterium clone_R103-B37 tube of Riftia pachyptila
OTU 11*
EU265787 – Uncultured epsilon proteobacterium clone_A9
AB235230 – Endosymbiont of Alviniconcha sp. type2
AB088431 – Sulfurimonas autotrophica strain:OK10
OTU 3*
GQ119473 – Uncultured proteobacterium clone LF-1.1_from Lepetodrilus fucensis
KC545716 – Uncultured Arcobacter sp. clone_BFB044
EU265964 – Uncultured bacterium clone Nit2A0650_37
D83061 – Endosymbiotic eubacteria TW-2
AF513455 – Arcobacter sp. LA31B_16S
JN662292 – Uncultured epsilon proteobacterium clone LF8SMb59 from tube of Ridgeia piscesae
OTU 1*
JN662202 – Uncultured epsilon proteobacterium clone LF8GH2b30 from tube of Rigeia piscesae
FR853050 – Uncultured bacterium clone Q1-b30
L14630 – Campylobacter jejuni
M88159 – Wolinella succinogenes strain ATCC_29543
JN662155 – Uncultured delta proteobacterium clone HF8GH2b15 from tube of Ridgeia piscesae
OTU 12
FN658700 – Delta proteobacterium ectosymbiont of Rimicaris exoculata clone Delta S1-6
AJ866934 – Desulfobulbus mediterraneus strain NA62
HM750216 – Desulfobulbus alkaliphilus strain APS1
FJ264759 – Uncultured bacterium clone livecontrolB3
FR839053 – Uncultured epsilon proteobacterium clone A021R from gut of Rimicaris exoculata
AM268746 – Uncultured prokaryote clone IIB78
DQ228555 – Uncultured bacterium clone LC1149b104
OTU 5
HQ393433 – Uncultured bacterium clone frc55 from gut of Haliotis sp.
OTU 13
HQ393422 – Uncultured bacterium clone frc4
HQ393410 – Uncultured bacterium clone lpc29
FN658702 – Bacteroidetes bacterium ectosymbiont of Rimicaris exoculata clone S2-2
JN256012 – Uncultured Bacteroidetes clone from pereopod of Kiwa puravida Contig 0367 x2
AM745131 – uncultured Bacteroidetes clone GoM161_Bac14
OTU 15
DQ481462 – Dokdonia donghaensis strain MED134
DQ923436 – Polaribacter sp. W-2
0.05

Figure 4

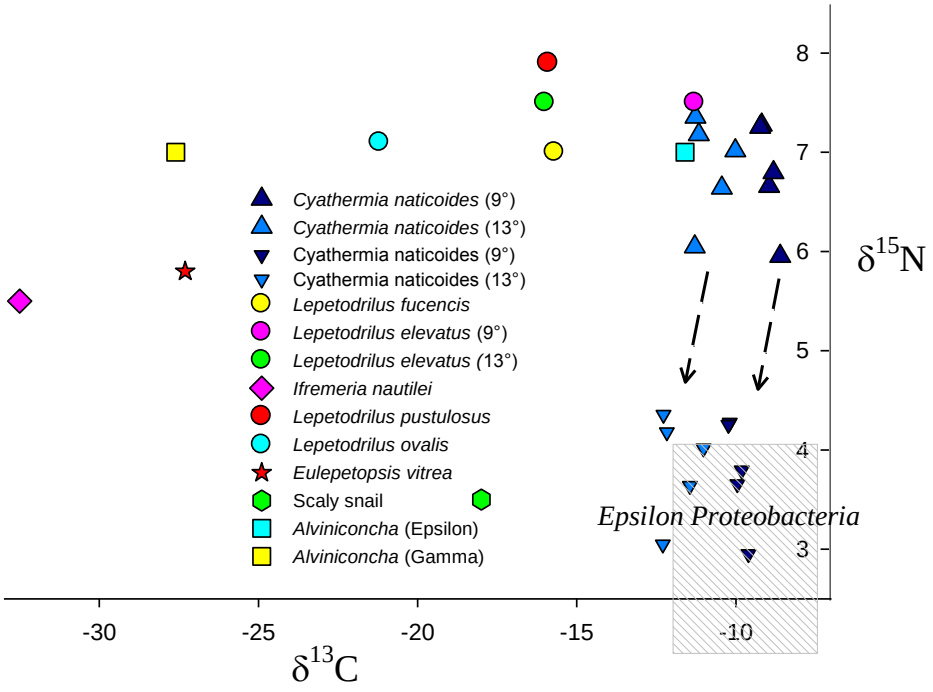
8
7
6
5
4
3
δ15N
-30
-25
-20
-15
-10
δ13C
Cyathermia naticoides (9°)
Cyathermia naticoides (13°)
Cyathermia naticoides (9°)
Cyathermia naticoides (13°)
Lepetodrilus fucencis
Lepetodrilus elevatus (9°)
Lepetodrilus elevatus (13°)
Ifremeria nautilei
Lepetodrilus pustulosus
Lepetodrilus ovalis
Eulepetopsis vitrea
Scaly snail
Alviniconcha (Epsilon)
Alviniconcha (Gamma)
Epsilon Proteobacteria

Table 1

OTU ID	3	1	8	11	5	2-p	16-p	12	13	4-p	15	Sum
Accession	KM213004	KM213002	KM213007	KM213008	KM213006	KM213003	KM213012	KM213009	KM213010	KM213005	KM213011	
Affiliation	E	E	E	E	E	E	E	D	M	M	B	
9-1-Gi	7	7			1	4				1		30
9-1-VM	3	20	3		1	3				1		32
9-2-Gi	14	7	3	1	5							32
9-2-VM	7	7	3		5			4	1		1	32
9-2-Sh		9	14	2				1			1	31
9-3	15	6	2		1		1	1	2	1		32
12-1-Gi	11											11
12-1-VM			11	7								19
12-2-Gi	5		5	2								17
12-2-VM		2	3	3								13
12-3-Gi	6		2	1			3					17
12-3-VM		1	11	5			1					29
Sum per OTU	68	59	57	21	13	7	5	6	3	3	2	244
Percentage	23,05	20	19,32	7,12	4,41	2,37	1,69	2,03	1,02	1,02	0,68	82,71
Arc-94	+	-	+	+	-	1 mis	+	-	-	-	-	
Epsy-549	+	1 mis	+	+	-	+	+	-	-	-	-	

Table 2

Fragment	Approx length	Clone ID	Accession number	%/total	Specimen occurrence	Tissue occurrence	Best BLAST hit (BlastX)
aprA	365 nt	761	KP115589	40.0	3	R	96% EU265804 Epibiont of the vent crab *Kiwa hirsuta* (Gammaproteobacteria)
		843	KP115590	24.3	2	R	91% GU197406 Bacterium associated with the Oligochete *Tubificoides benedii* (Gammaproteobacteria)
		144	KP115591	10.0	2	R	90% GU197406 Bacterium associated with the Oligochete *Tubificoides benedii* (Gammaproteobacteria)
		820	KP115592	18.6	2	R	100% FM165456 Bacterium associated with the tube of *Lamellibrachia anaximandri* (Gammaproteobacte
		786	KP115593	4.3	1	R	96% EF633097 Bacterium associated with *Echinocardium cordatum* (Deltaproteobacteria)
		827	KP115594	1.4	1	R	97% AM234053 *Olavius algarvensis* Delta-4 endosymbiont (Deltaproteobacteria)
AclB	305nt	765	KP115581	54.3	2	G	98% FN659794 bacterium from branchial chamber of *Rimicaris exoculata* (Epsilonproteobacteria)
		782	KP115582	11.4		G, R	98% FN908920 bacterium from hydrothermal fluid, Clueless (Epsilonproteobacteroa)
		847	KP115583	2.9	1	R	99% FR670537 bacterium from Lucky Strike (Epsilonproteobacteria)
		766	KP115584	2.9	1	R	98% FN659786 branchial chamber of *Rimicaris exoculata* (Epsilonproteobaceria)
		805	KP115585	2.9	1	R	98% FN908920 bacterium from hydrothermal fluid, Clueless (Epsilonproteobacteria)
		808	KP115586	17.1		R, G	99% FN562694 bacterium from the Irina II vent, Logatchev (Epsilonproteobacteria)
		840	KP115587	2.9	1	R	99% FN908925 Bacterium from the Logatchev vent field (Epsilonproteobacteria)
		163	KP115588	5.8	1	R	97% FN562694 bacterium from the Irina II vent, Logatchev (Epsilonproteobacteria)