Microbial community analysis in the gills of coastal shellfish and molecular identification of the potentially dominant epsilonproteobacterium on the gills of *Haliotis gigantea*

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Abstract

Gills are important organs for aquatic invertebrates in deep-sea hydrothermal vents and cold seeps because they harbor chemosynthetic bacteria in their gills, which fix inorganic carbon and provide their hosts with organic compounds. Recently, it has been reported that chemosynthetic bacteria in the gills of some shallow-water bivalves have the ability to fix nitrogen carbon, and synthesize amino acids for their hosts. Therefore, this study investigated the community structure of microbes associated with the gills of various coastal invertebrates, especially gastropods, using next-generation sequencing. Molecular identification of representative bacterial sequences was performed using cloning, nested PCR, and fluorescence in situ hybridization (FISH) analysis with specific primers or probes. We examined four gastropod species, namely *Haliotis gigantea*, *H. discus*, *H. diversicolor* and *Turbo cornutus*, and two species of bivalves, namely *Meretrix lusoria* and *Cyclina sinensis*. Microbiome analysis suggested that the gills of *H. gigantea*, *M. lusoria* and *C. sinensis* each have unique bacterial

community structures that differ from those in the surrounding environment. Gills of *M. lusoria* and *C. sinensis* were dominated by unclassified members of the Spirochaetaceae. On the other hand, the dominant bacterium in some *H. gigantea* was a previously unknown epsilonproteobacterium species, which accounted for as much as 62.2% of microbial reads obtained from the gills. Phylogenetic analysis performed using almost the full-length of the 16S rRNA gene placed the uncultured epsilonproteobacterium species within the same cluster as Helicobacterceae spp. Further, the uncultured epsilonproteobacterium was commonly detected from gill tissue rather than from gut and foot tissue using a nested PCR assay with uncultured epsilonproteobacterium-specific primers. FISH analysis with the uncultured epsilonproteobacterium-specific probe revealed that probe-reactive cells in *H. gigantea* had a coccus-like morphology and formed microcolonies on gill tissue. This is the first report to show that epsilonproteobacterium has the potential to be the dominant bacterium in the gills of the coastal gastropod, *H. gigantea*.

Introduction

Some aquatic invertebrates, especially those that inhabit deep-sea hydrothermal vents or cold seeps, are characterized as having chemosynthetic epi- or endo-symbionts in their gills (Dubilier et al. 2008). Symbiotic relationships between marine invertebrates and bacteria are characterized by the exchange of chemical molecules, such as carbon dioxide, oxygen, hydrogen, hydrogen sulfide, nitrogen or methane, from the hosts, through the seawater to symbionts. The symbionts then take up these molecules and provide their hosts with organic compounds. Consequently, many studies of marine invertebrates in these environments are focused on gills, as these are the primary organs that receive these geochemical fluids. It has previously been reported that bivalves inhabiting hydrothermal vents, such as *Bathymodiolus* spp. and *Calyptogena* spp., harbor symbionts in their gills (Duperron et al. 2006, Kuwahara et al. 2007 and Newton et al. 2007). These symbiotic bacteria are referred to as nutritional symbionts as they provide nutrients to their hosts. In addition, the gastropod, *Ifremeria nautilei* (Borowski et al. 2002), and members of the shrimp family, Alvinocarididae (Zbinden et al. 2008 and Apremont et al. 2018) have chemosynthetic bacteria in their gills; these bacteria are also considered to be nutritional symbionts. While most of the studies on the microbial communities in invertebrate gills have focused on deep-sea floor vent and cold seep environments, symbionts have also been

found in the gills of shallow-water marine bivalves (Duperron et al. 2012). For example, while some members of the Solemyidae inhabit deep-sea floor environments, most studies have focused on coastal species. Although the members of Solemyidae are capable of suspension feeding, they derive more than 97% of their carbon from symbiotic sulfur-oxidizing Gammaproteobacteria. Indeed, this relationship is evidenced by the drastic reduction or total absence of a digestive system and labial palps in *Solemya velum* and *S. reidi*, (Conway et al. 1989, Krueger et al. 1992). Some members of Thyasiridae harbor sulfur-oxidizing symbionts in their gill filaments (Dando and Spiro 1993, Dufour and Fel-beck 2003, Roeselers and Newton 2012 and Duperron et al. 2013), making them capable of living deeper in sediments than species without symbionts. These species use their super-extensile foot to mine for sulfide-rich pockets in the sediment, creating a network of tunnels (Dufour and Fel-beck 2003). Although members of the Lucinidae have retained the ability to filter feed, these bivalves also harbor chemoautotrophic Gammaproteobacteria in their gill epithelial cells; it is considered possible that some lucinid species may digest these symbionts (Frenkiel and Moueza 1995 and Duplessis et al. 2004). For example, in addition to carbon fixation and sulfur-oxidization, symbionts associated with two species in Lucinidae*, L. lucinalis* and *C. orbiculate,* have been reported to fix atmospheric nitrogen and synthesize amino acids for their hosts (Petersen et al. 2016 and König et al. 2016). Thus, as in deep-sea floor environments, many invertebrate taxa in shallow waters also have symbionts in their gills. However, the gill microbiota of shallow-water invertebrates, especially gastropods, has not been studied as extensively as the fauna from deep-sea environments, primarily because many of shallow-water invertebrates have a developed digestive system.

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In this study, we analyzed the microbial communities associated with the gills of economically important gastropod molluscs, such as *Haliotis gigantea*, *H. discus*, *H. diversicolor* and *Turbo cornutus*, as well as the bivalves, *Meretrix lusoria* and *Cyclina sinensis*, in shallow-water environments. Additionally, this study investigated the phylogenetic characteristics and placement of the bacteria in the gills of *H. gigantea*.

Materials & Methods

Sample preparation and DNA extraction

Five cultured giant abalones, *Haliotis gigantea*, were collected from the Minami-ise Farming Fishery Center (Minami-ise, Mie, Japan) in October 2016 (sample code: Hgig1). Other abalone specimens (*H. gigantea, H. discus* and *H. diversicolor*) and *Turbo cornutus* were obtained from a fish-market in Mie, Japan, from April 2017 to May 2019.

Gill tissues from *H. discus* (n=3), *H. diversicolor* (n=5), and *T. cornutus* (n=3) specimens were pooled into three tubes, one for each species (sample code: Hdis1, Hdiv1, and Tcor1). Gill tissues from other *H. gigantea* and *H. discus* specimens were prepared individually (sample code: Hgig2-6, Hdis2-4). Specimens of the two bivalve species, *M. lusoria* (n=3) and *C. sinensis* (n=5), were collected from coastal areas in Mie, Japan (Tsu city, 34°44'08.5"N 136°31'30.7"E). These gill tissues were also pooled into separate tubes, one for each species (sample code: Mlus1, Csin1). Gut and foot tissues were also collected from all *H. gigantea* specimens, excluding Hgig1 from which no foot tissues were obtained. For all other specimens, only gill tissues were used.

The collected gill, gut or foot tissues were then homogenized in sterile artificial seawater using a bead beater homogenizer (4200 rpm, 30 seconds; Tietech Co., Nagoya, Japan) followed by a previously described method (Tanaka et al. 2004). Host tissues were removed from the homogenate by a quick centrifugation step (1 s, 8000 g), and the supernatant was transferred to new tubes and centrifuged for 20 min at 15000 g to recover bacterial cells. Seawater and stone samples were collected from Minami-ise. Fifty milliliters of seawater was filtered by passing through 0.22 μm filter paper and resuspended in sterile phosphate-buffered saline (PBS: 130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4; pH 7.4) (sample code: SW). Stones were shaken in sterile PBS (sample code: ST) and bacterial cells were recovered from the SW and ST PBS samples by centrifuging for 20 min at 15000 g. Bacterial genomic DNA from each bacterial pellet was extracted using a Promega DNA purification system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

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PCR amplification, Illumina MiSeq sequencing, and Sequence processing

All gill, SW, and ST samples were used for the analysis of microbial communities. The V1-V2 region of 16S rRNA genes was amplified using Ex Taq (TaKaRa Biotechnology Corp., Kyoto, Japan). The first PCR step was performed using primers 27F-mod (5'-

ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGRGTTTGATYMTGGCTCAG-3') and 338R (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT-3'). The amplification conditions were as follows: initial denaturation of 2 min at 94°C followed by 24 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, and primer extension at 72°C for 30 s. The amplified PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega Corp.) and used for the second PCR step, which was performed using primers with a tag sequence. After the second PCR, the products were once again purified using a Wizard SV Gel and PCR Clean-Up System before sequencing on a MiSeq platform (Illumina Inc., San Diago, CA, USA).

The raw paired-end FASTQ reads were demultiplexed using the Fastq barcode splitter (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, ver. 2019.7, https://qiime2.org/). Demultiplexed reads were quality filtered, denoised, chimera checked and dereplicated using a DADA2 denoise-paired plugin (Callahan et al. 2016). To be equal to sampling-depth, sequences were rarefied at 23,665 reads using qiime feature-table rarefy (Weiss et al. 2017). Next, the align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin was used to perform multiple sequence alignment, remove phylogenetically uninformative or ambiguously aligned sequences, and to generate unrooted and rooted phylogenetic trees (Lane 1991, Price et al. 2010, Katoh and Standley 2013). Diversity metrics were calculated using the core-metrics-phylogenetic pipeline from the diversity plugin on QIIME2. For alpha diversity, Shannon index (Shannon and Weaver, 1949), observed OTU number (DeSantis et al. 2006), Chao 1 index (Chao 1984), and Good's coverage (Good, 1953) were calculated using the diversity alpha command. PCoA plots were visualized using unweighted UniFrac data in the R packages "qiime2R", "tidyverse", "phyloseq" (McMurdie and Holmes, 2013) and "ggplot2" (Wickham, 2016) (R 3.5.0). Taxonomic assignments were performed using the qiime feature-classifier classify-sklearn on Greengenes v_13.8 (McDonald et al. 2012). Taxa bar plots were constructed using the plugin qiime taxa bar plot. All of the data were deposited at the Sequence Read Archive (SRA) under the accession number PRJDB8953.

Obtaining the 16S rRNA gene sequences of the uncultured epsilonproteobacterium by cloning

PCR amplification of the bacterial 16S rRNA gene was performed using primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3', Lane 1991) and primer 1492R (5'-GGTTACCTTGTTACGACTT-3', Lane 1991). The bacterial 16S rRNA gene clone library was constructed using PCR amplicons obtained from Hgig1. PCR reaction mixtures contained 1× PCR reaction buffer, 200 µM dNTP, 5 pmol of each primer, 2.5 units Ex Taq polymerase (TaKaRa Biotechnology Corp.), and 10 – 100 ng of community DNA in a total volume of 50 µl. PCR reactions were performed on a thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA) using the following amplification conditions: initial denaturation of 4 min at 95°C followed by 25 cycles of denaturation for 30 sec at 95°C, primer annealing for 30 sec at 55°C, and primer extension at 72°C for 1.5 min. This was followed by a final extension reaction at 72°C for 7 min. PCR product was ligated into the TOPO TA cloning vector (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturers' instructions. Ligation products were transformed into *Escherichia coli* One Shot TOP10 cells (Invitrogen Corp.) and clones were amplified by PCR using vector-specific primers. Plasmid DNA with insertions was sequenced using primer 27F. Partial sequencing was performed using the BigDye terminator cycle sequencing method, and an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The resulting chromatograms of DNA sequences were examined using Chromas 2.33. Homology searches were performed using sequences and close relatives were identified using a BLAST search of the GenBank database on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree of representative members of Epsilonproteobacteria inferred from 16S rRNA gene sequences was estimated by the Maximum Likelihood method using MEGA 7.0 (Kumar et al. 2016).

Nested PCR analysis and sequencing of the uncultured epsilonproteobacterium

The nested PCR assay targeted 356 bp of the uncultured epsilonproteobacterial 16S rRNA gene using bacterial universal primers 27F and 1492R, as well as the uncultured epsilonproteobacterium-specific primers, Eps222F (5'-CGCTAAGAGATTGGACTATAT-3') and Eps578R (5'-GACTTAATAGGACACCTACATACC-3') (designed in this study). DNA samples extracted from gill, gut or foot tissue of each *H. gigantea* specimen were used as DNA

templates. The first-round master mixture contained the following: primers (27F and 1492R, Lane 1991) at 0.2 µM (each), 25 µl volume of EmeraldAmp® PCR Master Mix (TaKaRa Biotechnology Corp.), approximately 50 ng of DNA, and up to 50 µl with distilled H_2O. Distilled H_2O was used as the template for negative controls. Cycle parameters were 98°C for 10 sec; 10 cycles of 98°C for 10 sec, 55°C for 30 sec, and 72°C for 90 sec; and 72°C for 5 min. PCR reactions were performed using an iCycler (Bio-Rad Laboratories). A 2.5-µl aliquot of the amplified PCR product was transferred to a new master mixture containing primers (Eps222F and Eps578R) at 0.2 µM (each) in a 25 µl volume of EmeraldAmp® PCR Master Mix (TaKaRa Biotechnology Corp.) made up to a volume of 50 µl with distilled H_2O. Cycle parameters were 98°C for 10 sec; 25 cycles of 98°C for 10 sec, 57°C for 30 sec, and 72°C for 60 sec; and 72°C for 5 min. A 5-µl aliquot of the amplified PCR product was analyzed by agarose (1.5% [wt/vol] prepared in TAE buffer) gel electrophoresis and Midori Green Direct (Nippon Genetic Europe GmbH, Düren, Germany) staining, and the gel was photographed under UV light. The presence of a band at around 350 bp was interpreted as a positive result. Sequencing and phylogenetic analysis were performed as previously described in the subsection "Obtaining the 16S rRNA gene sequences of the uncultured epsilonproteobacterium using cloning method" above.

Fluorescence in situ hybridization localization of the uncultured epsilonproteobacterium

Fluorescence in situ hybridization (FISH) was performed on the Hgig4 sample using a previously described method (Tanaka et al. 2016). Gill tissue was obtained from Hgig4 and placed in 75% ethanol at -30°C before being fixed in 4% (v/v) paraformaldehyde/PBS at 4°C overnight. Following fixation, specimens were rinsed and dehydrated in a 50, 70, 80. 85, 90, 95, and 100% ethanol series, followed by 100% xylene. The fixed gill specimens were then embedded in paraffin and sliced into 10-µm transverse sections using a microtome (RV240, Yamato, Japan), before being placed on APS-coated microscope slides (Matsunami, Japan), and stored in slide boxes at room temperature until deparaffinization. Wax was removed, and tissue was rehydrated in a decreasing ethanol series. The sections were dewaxed and hybridized at 47°C for 3 h using a solution containing 20% formamide, 0.9 M NaCl, 20 mM Tris–HCl (pH 7.4), 0.1% SDS, and 0.5 µM FITC-labeled Eub338 probe (5'-GCTGCCTCCCGTAGGAGT-3', Amann et al. 1990) or 0.5 µM TAMRA-labeled Eps222 probe (5'-CGCTAAGAGATTGGACTATAT-3'), which was designed to specifically target the 16S rRNA

gene of the uncultured epsilonproteobacterium (designed in this study). After hybridization, the sections were washed twice with washing buffer containing 20 mM Tris-HCl (pH 7.4), 180 mM NaCl and 0.01% SDS for 30 min at 48°C, and the sections were then rinsed with ddH_2O and air-dried. An epifluorescence light microscope (Eclipse 400; Nikon Instech., Tokyo, Japan), was used to observe the stained cells.

Results

Microbial Community Analysis by next-generation sequencing (NGS)

The microbial communities on the gills of various marine invertebrates were characterized by sequencing the V1-V2 region of the 16S rRNA gene. A total of 669,727 quality-filtered sequence reads were obtained from 16 samples (PRJDB8953). Alpha diversity index values for each sample are shown in Table 1. For all of the samples in this study, observed OTUs and Chao1 were approximately the same value, and Good's coverage value was higher than 99.99%, indicating that sequencing depth sufficient for capturing all bacterial species and for downstream analysis. We identified 454 OTUs from 16 phyla, which we taxonomically assigned with the Greengenes database. At the phylum level, the microbial taxonomic composition of most samples, excluding Hgig5, Tcor1, Mlus1, and Csin1, had a high relative abundance of Proteobacteria (67.8% on average). Hgig5, Mlus1, and Csin1 showed almost complete dominance of Spirochaetes (72.0% on average), while Tcor1 showed a dominance of Tenericutes (56.4%).

In Proteobacteria, sequence reads were related to Alphaproteobacteria, Gammaproteobacteria or Epsilonproteobacteria (Figure 1). Alphaproteobacteria was dominant in Hgig1, Hgig5, Hdis2, Hdis4, SW and ST, (11.9-40.0%, Figure 1). The most abundant Alphaproteobacteria in abalone specimens were aligned with unclassified Rickettsiales, while unclassified Rhodobacteraceae and *Nautella* spp. were the most abundant Alphaproteobacteria-related sequences in the ST and SW samples, respectively. The relative abundance of Gammaproteobacteria-related sequences was more than 10% in all samples, except Hgig2, Mlus1 and Csin1. In the Gammaproteobacteria, *Vibrio* spp. were observed in all samples except Mlus1. In addition, the relative abundance of the Gammaproteobacteria was more than 10% in SW and all *H. discus* specimens, especially in Hdis4 (58.3%). In Hdiv1, unclassified Endozoicimonaceae accounted for 25.1% of the relative abundance and were more frequently

recovered than *Vibrio* spp. Epsilonproteobacteria-related sequences were dominantly recovered from SW and all *H. gigantea* specimens, except Hgig5 (18.3-62.2%). Members of the class Epsilonproteobacteria in SW were affiliated with the genus *Arcobacter*. On the other hand, a previously unknown epsilonproteobacterium affiliated with the uncultured epsilonproteobacterium accounted for a total of 59.0% of all sequences in *H. gigantea*; however, no related sequences were ever detected in SW and ST. Spirochaetes-related sequences in Hgig5 were aligned with unclassified Spirochaetaceae. These sequences were observed in all abalone specimens, but were not dominant, except in Hgig5 (68.4%). The relative abundance of the unclassified Spirochaetaceae was high in Hdis1, Hdiv1, Mlus1, and Csin1 (19.6-47.9%). Another unclassified Spirochaetaceae was detected only in Csin1 (14.7%). In addition to these Spirochaetes, unclassified Brachyspiraceae sequences were obtained from both bivalve specimens, Mlus1 and Csin1 (19.4 and 40.7%). No Spirochaetes-related sequences were detected in SW and ST. All members of the phylum Tenericutes were affiliated with 1 OTU in the genus *Mycoplasma*. *Mycoplasma* sp. was present in every invertebrate sample except Csin1, and most prevalent in Tcor1 (56.4%), followed by Hdis1 (22.1%), Hdis3 (21.4%) and Mlus1 (21.2%). In contrast, the sequences related to *Mycoplasma* were never observed in Csin1, SW, and ST.

The similarity in the microbiota was supported by principal coordinates analysis (PCoA, Figure 2). The microbiota in each invertebrate differed from those in the environment, such as from the seawater and stones. The microbial community structure of *T. cornutus* was similar to that *H. gigantea*, which in turn was similar to *H. discus*. The microbiota from both bivalve species, *M. lusoria* and *C. sinensis*, maintained a divergent cluster pattern away from those of the gastropods and environmental sources.

Phylogenetic analysis of 16S rRNA gene of the uncultured epsilonproteobacterium

Cloning and sequencing of the long 16S rRNA gene sequence of the uncultured epsilonproteobacterium found in *H. gigantea* were performed to clarify the phylogenetic position of this bacterium. Four out of sixteen cloned sequences were closely related to the sequence of the uncultured epsilonproteobacterium obtained from the 16S rRNA gene amplicon sequencing undertaken in this study. The longest sequence length was 1436 bp (LC511979).

BLAST analysis of the uncultured epsilonproteobacterial sequence revealed that the sequence could be assigned to various described Epsilonproteobacteria species, such as

Arcobacter canalis strain F138-33 (87.43% identity), *Arcobacter marinus* strain CL-S1 (87.19% identity), *Sulfurovum lithotrophicum* strain 42BKT (87.01% identity) and *Helicobacter pullorum* strain ATCC51801 (86.78% identity), but these identity scores were low. Among the environmental clones in public databases, the uncultured epsilonproteobacterium specimen was most closely related to an uncultured clone sequence found in the gill tissue of a wood-feeding gastropod in genus *Pectinodonta* (96.12-96.78% identity, Zbinden et al., 2010), followed by an uncultured clone sequence from gill tissue of thyasirid bivalves from a cold seep in the Eastern Mediterranean (94.86% identity, Brissac et al., 2011). Although the sequence of the uncultured epsilonproteobacterium was assigned to Campylobacterales in the NGS analysis, phylogenetic analysis with Maximum Likelihood method clustered the sequence in the Helicobacteraceae group (Figure 3).

Detection of the uncultured epsilonproteobacterium from each part of *H. gigantea*

Results of the 16S rRNA gene amplicon sequencing analysis revealed that the uncultured epsilonproteobacterium was dominant only in the gill tissues of *H. gigantea*. Therefore, in order to determine whether the uncultured epsilonproteobacterium was restricted to the gill tissues, a PCR assay of gill, gut and foot tissues from *H. gigantea* using uncultured epsilonproteobacterium-specific primers was performed. PCR products were obtained from gill tissue from Hgig2, Hgig3 and Hgig6 (Table 2). Amplified products were also obtained from the gill tissue from Hgig1, Hgig4, gut tissues from Hgig3 and foot tissues from Hgig4, but band visibility was low. All amplification product sequences matched that of the uncultured epsilonproteobacterial sequence obtained by cloning.

Localization of the uncultured epsilonproteobacterium on gill tissue of *H. gigantea*

In semi-thin section FISH analysis, Eps222-positive bacteria were observed on the gill surface of Hgig4 (Figure 4) where they formed microcolonies on gill tissue (Figure 4C). Eps222-positive bacteria showed a coccus-like morphology and measured ~1.0 μm in diameter (Figure 4F).

Discussion

306 In this study, we analyzed the microbiota in the gills of six species of coastal shellfishes. It
307 was previously considered that the bacterial community in the gills would be similar to that in
308 seawater because the gill filaments of aquatic organisms are exposed to seawater, particularly
309 during respiration. However, PCoA analysis suggested that the microbial composition and
310 characteristics of shellfishes differed from those of seawater and/or stone. In addition, the
311 distribution of gill microbiota from gastropods and bivalves were distinct from each other. If we
312 look at the bacterial community structure in detail, the dominant bacterial species were different
313 for each host species, even among congeneric hosts, for example, *H. gigantea* and *H. discus*.
314 The members of class Molicutes were detected in all of the invertebrate samples examined,
315 while *Mycoplasma* spp. in the class was frequently detected in *T. cornutus* (56.4%). The
316 *Mycoplasma* samples obtained in this study showed 99.7% homology identity with uncultured
317 clones that were dominant in the gut of *H. d. hannai* (Tanaka et al., 2004). *Mycoplasma* spp.
318 were also expected to inhabit not only the gill tissues, but also gut tissues, and it is considered
319 that these bacteria may be prevalent in a variety of marine taxa. However, the associations
320 between these potential symbiotic bacteria and their hosts is still unknown.
321 The sequence of one of the unclassified members of Spirochaetaceae in the class
322 Spirocaetia, which were dominant in one *H. gigantea*, *M. lusoria* and *C. sinensis*, were closely
323 related to the sequence detected in the cold-water coral *Lophelia pertusa* in the northeastern Gulf
324 of Mexico (91.2% identity, Kellogg et al. 2009). The relationship between uncultured
325 *Spirochaeta* and *L. pertusa* was unknown, but spirochetes have been reported to catalyze the
326 synthesis of acetate from H_2 and CO_2 in the termite gut (Leadbetter et al. 1999), as well as being
327 a symbiont of the gutless worm, *Oligochaeta loisae*, in the Australian Great Barrier Reef
328 (Dubilier et al., 1999). Additionally, *Spirochaeta* symbionts have detected in the gill tissues of
329 cold-seep clams (Lucinidae) where it is presumed that they are involved in sulfide oxidation
330 and/or autotrophy (Duperron et al. 2007). In this study, sulfide concentrations were not measured
331 at the sampling site where the two bivalve species were collected, but the sediments clearly
332 smelled of sulfide. The unclassified bacteria belonging to Spirochaetaceae in *M. lusoria* and *C.*
333 *sinensis* may therefore be a symbiont that detoxified sulfide in the same way that *Spirochaeta*
334 spp. detoxified sulfide in Lucinidae.
335 Gammaproteobacteria was the dominant class of bacteria in *H. discus,* and most of these
336 bacteria were *Vibrio* spp. Non-motile *Vibrio* spp., called non-motile fermenters (NMF) have

been reported to be symbionts of abalones (Sawabe et al. 2003). These authors reported that they fermented organic acids, especially acetate, from alginate of brown algae in the abalone gut and provide the resulting organic acids to their hosts. However, since it cannot be assumed that there is always alginate around the gills of abalone specimens, it is proposed that *Vibrio* spp. on the gills originated in the gut and then adhered to the gills. Alternately, the *Vibrio* spp. found in the gills have different ecological roles from those found in the gut.

The uncultured epsilonproteobacterium was dominant in all of the *H. gigantea* samples, except Hgig5, by NGS analysis. Interestingly, this bacterium was not dominant in the congeneric species, *H. discus* and *H. diversicolor*. The relative abundance of the uncultured epsilonproteobacterium in Hgig6 was slightly lower than that in the other *H. gigantea* samples. The high Shannon index value obtained for Hgig6 and the detection of less common bacterial species in other *H. gigantea*, such as *Polaribacter* spp. and unclassified Flavobacteriaceae species, indicates that the abundance of the uncultured epsilonproteobacterium in Hgig6 was relatively low compared to that found in other *H. gigantea* specimens. This assumption was also supported by the finding that the gills of Hgig6 gave a clearer band than the gills of Hgig4 in the PCR assay using the same concentration of DNA template.

The class Epsilonproteobacteria in terrestrial environments is widely known for its pathogenic genera; *Campylobacter*, *Helicobacter,* and to a lesser extent *Arcobacter,* (Gilbreath et al. 2011). Some of the members of this class also inhabit at hydrothermal vents, where they often associate with invertebrate hosts as ecto-, endo- or epi-symbionts; for example, polychaete worm (*Alvinella pompejana*) (Haddad et al. 1995), shrimp (*Alvinocaris longirostris*) (Tokuda et al. 2008), crab (*Shinkaia crosnieri*) (Fujiyoshi et al. 2015) and gastropods (*Alviniconcha* spp.) (Suzuki et al. 2005 and 2006, Urakawa et al. 2005). The members of this group are considered to be symbionts that provide their hosts with organic carbon compounds by carbon fixation and detoxify hydrogen sulfide. Although the 16S rRNA gene sequence of the uncultured epsilonproteobacterium obtained from *H. gigantea* in this study formed a cluster in phylogenetic analysis with members of Helicobacteraceae, the similarity score to described species was as high as 87.43%. Thus, it is considered that the uncultured epsilonproteobacterium bacterium obtained in this study could be, at least, a novel genus. BLAST analysis comparing the uncultured epsilonproteobacterium with other uncultured bacterial clones revealed that they were closely related to clones detected from the gills of a wood-feeding gastropod, *Pectinodonta* sp.

(Patellogastropoda, Mollusca) (96.12-96.78%, identity). Although the relationship between these clones and their host was unknown, the epsilonproteobacterial clone sequence obtained from the gills was not detected from the digestive system or foot of *Pectinodonta* sp. (Zbinden et al., 2010). The uncultured epsilonproteobacterium is therefore considered to specifically inhabit gill tissues.

Results of the microbial community analysis revealed that the uncultured epsilonproteobacterium was dominant in the gills of the gastropod, *H. gigantea*. However, it was surmised that the bacterium was also presents in other body parts. Like the gills, the foot of *H. gigantea* in in direct contact with seawater. Compared to gills or the foot, the gut is also a closed space in which bacteria can be easily retained. The gut environment is also microaerobic, which means that it is well suited for the growth of some members of Epsilonproteobacteria, such as *Campylobacter* and *Arcobacter* (On et al., 2017). Therefore, PCR using a specific primer for the uncultured epsilonproteobacterium was performed to confirm whether the bacterium also inhabited tissues other than the gills. PCR amplification products of the uncultured epsilonproteobacterium were obtained from all gill tissues from *H. gigantea*, but never or very little, from the gut and the foot tissue, even when different tissues from the same individual were used. These findings therefore suggested that the uncultured epsilonproteobacterium is generally restricted to gill tissues. In addition, semi-thin section FISH analysis revealed micro-colonies of the uncultured epsilonproteobacterium on *H. gigantea* gills. It is necessary to culture these bacteria in order to better clarify their metabolism and the biological interactions that exist between the uncultured epsilonproteobacterium and *H. gigantea*.

Conclusions

In this study, we assayed the microbiota that colonize the gills of shallow-water invertebrates such as *H. gigantea*, *H. discus*, *H. diversicolor, T. cornutus*, *M. lusoria* and *C. sinensis*, using 16S rRNA amplicon sequencing. The findings suggested that the gills of the giant abalone, *H. gigantea*, and the bivalves, *M. lusoria* and *C. sinensis*, support specific bacterial communities. Additionally, an uncultured epsilonproteobacterium, which dominant only in *H. gigantea*, formed micro-colonies on the gills of this species, but not in its gut or foot. This uncultured epsilonproteobacterium specifically inhabits the gills of the shallow-water gastropod, *H. gigantea*.

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General comments:

1. Consistency of cited reference (i.e. authors and year of publication)
2. More detail information for legend of table and figure

Detail comments:

Line 36: Helicobacterceae spp. or Helicobacter spp. or Helicobacteraceae?

Line 59-61: Dupperon et al 2012 is not available in the reference list. If it is the one in 2007 0r 2013 is not the most appropriate for this situation since they informed symbionts of the deep-sea clams or bivalves. Please explain which reference and why you used this reference.

Line 62-82: Most of cited references informed symbionts of deep-sea bivalves. It is necessary to add information from previous studies on shallow water bivalves which are still limited in the current introduction section. In addition, the English language should be improved to ensure that an international audience can clearly understand the text. The current phrasing makes comprehension difficult to support justification of the necessity of current study, for example what is the linkage between a developed digestive system and the gill microbiota of shallow water invertebrates?

Line 75-77: Both references mentioned symbionts which are capable of nitrogen fixation only.

Line 76: C. orbiculate should be *Codakia orbicularis*.

Line 180-181: Specific primer for uncultured epsilonproteobacterium? How do you check the specificity? It is necessary to test the primer over Campylobacterales since the sequence of the primer is also matched 16S rRNA gene of Campylobacterales.

Line 200-2018: What are the positive and negative controls? Sequence has a high degree similarity to Campylobacterales and Bdellovibrio.

Is there (an) other reason(s) out of the highest proportion of epsilonproteobacterium which may lead the authors to further investigate this bacterial group in the gill tissues of H. gigantea?

Line 271-273: the result was positive for 4 out of 16. Does it mean that the primer is not specific for epsilonproteobacterium?

Line 283-285: Campylobacterales in the NGS analysis and phylogenetic analysis with Maximum Likelihood method clustered the sequence in the Helicobacteraceae means order and family

Line 288-297: Provide gel picture please!

Line 332-334: How does the detoxified sulfide mechanism occurs in Lucinidae?

Line 341: Alternatively instead of Alternately?

Figure 1: Are they symbionts in gills only?

Figure 2: Legend in circle form may make the readers confused either as species or as bivalves. In addition for term class and species, are they referred to taxonomy identity? If not, please use proper term, for examples: sample types instead of class and erase species. Bivalve and gastropods are gill samples? How do you distinguish Hgig1 with other Hgig samples in PCoA plot?

Figure 4: Indication of epsilonproteobacterium cells is missing.