Microb Ecol
DOI 10.1007/s00248-012-0130-y

INVERTEBRATE MICROBIOLOGY

Deep Sequencing of *Myxilla* (*Ectyomyxilla*) *methanophila*, an Epibiotic Sponge on Cold-Seep Tubeworms, Reveals Methylotrophic, Thiotrophic, and Putative Hydrocarbon-Degrading Microbial Associations

Shawn M. Arellano · On On Lee · Feras F. Lafi · Jiangke Yang · Yong Wang · Craig M. Young · Pei-Yuan Qian

Received: 8 March 2012 / Accepted: 26 September 2012

Abstract The encrusting sponge *Myxilla* (*Ectyomyxilla*) *methanophila* (*Poecilosclerida*: *Myxillidae*) is an epibiont on vestimentiferan tubeworms at hydrocarbon seeps on the upper Louisiana slope of the Gulf of Mexico. It has long been suggested that this sponge harbors methylotrophic bacteria due to its low $\delta^{13}C$ value and high methanol dehydrogenase activity, yet the full community of microbial associations in *M. methanophila* remained uncharacterized. In this study, we sequenced 16S rRNA genes representing the microbial community in *M. methanophila* collected from two hydrocarbon-seep sites (GC234 and Bush Hill) using both Sanger sequencing and next-generation 454 pyrosequencing technologies. Additionally, we compared the microbial community in *M. methanophila* to that of the biofilm collected from the associated tubeworm. Our results revealed that the microbial diversity in the sponges from both sites was low but the community structure was largely similar, showing a high proportion of methylotrophic bacteria of the genus *Methylohalomonas* and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria of the genera *Cycloclasticus* and *Neptunomonas*. Furthermore, the sponge microbial clone library revealed the dominance of thioautotrophic gammaproteobacterial symbionts in *M. methanophila*. In contrast, the biofilm communities on the tubeworms were more diverse and dominated by the chemoorganotrophic *Moritella* at GC234 and methylotrophic *Methylomonas* and *Methylohalomonas* at Bush Hill. Overall, our study provides evidence to support previous suggestion that *M. methanophila* harbors methylotrophic symbionts and also reveals the association of PAH-degrading and thioautotrophic microbes in the sponge.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-012-0130-y) contains supplementary material, which is available to authorized users.

Shawn M. Arellano and On On Lee equally contributed in this manuscript.

S. M. Arellano · O. O. Lee · J. Yang · Y. Wang · P.-Y. Qian (✉)
KAUST Global Collaborative Research Program, Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay,
Hong Kong, SAR, China
e-mail: boqianpy@ust.hk

F. F. Lafi
Computational Bioscience Research Center, King Abdullah University of Science and Technology,
Thuwal, Jeddah 23955-6900, Kingdom of Saudi Arabia

C. M. Young
Oregon Institute of Marine Biology, University of Oregon,
P.O. Box 5389, Charleston, OR 97420, USA

Present Address:
S. M. Arellano
Department of Biology, Woods Hole Oceanographic Institution,
Woods Hole, MA 02536, USA

Introduction

Microorganisms, including bacteria [1, 2], archaea [3], algae [4], dinoflagellates [5], and fungi [6], can constitute up to 60 % of the volume of sponge tissues [7, 8] and serve a variety of functions. Besides using microorganisms directly as sources of food [9], sponges are known to use bacterial symbionts for metabolic activities such as carbon metabolism via photosynthesis [10, 11], taking up dissolved organic carbon [12], recycling insoluble proteins [13], and processing metabolic wastes [14, 15].

Published online: 11 October 2012

At deep-sea hydrothermal vents and cold seeps, fixation of inorganic carbon by endosymbiotic chemoautotrophic bacteria accounts for the successful colonization of these habitats by many of the foundation macroorganism species. Although sponges are well-known to take advantage of bacterial associations for multiple metabolic functions and, in many cases, sponges harbor unique bacterial associations that may themselves be sponge-specific [16–19], sponges generally are not a large part of the dense invertebrate assemblages that constitute deep-sea chemosynthesis-based ecosystems, with a few notable exceptions. For example, carnivorous cladorhizid sponges associated with methane-oxidizing bacteria are abundant around deep-sea (~4,900 m) mud volcanoes near the Barbados accretionary prism [20–22]. In addition, thiotrophic symbioses were recently described for three deep-sea sponges: *Characella* sp. (*Pachastrellidae*), collected at a hydrothermal vent in the Sumisu Caldera, Japan (686 m depth), and *Pachastrella* sp. (*Pachastrellidae*) and an unidentified poecilosclerid sponge, both collected near cold seeps (572 m depth) in the Gulf of Mexico [23].

Besides the sponges analyzed by Nishijima et al. [23], one of the most conspicuous sponges found at the cold seeps on the upper Louisiana slope of the Gulf of Mexico is *Myxilla* (*Ectyomyxilla*) *methanophila* (Maldonado and Young, 1998) (Fig. 1). This sponge is epibiotic on the vestimentiferan tubeworms *Lamellibrachia luymesi* and *Seepiophila jonesi* (=*Lamellibrachia* sp. and *Escarpia* sp., respectively, in [34]) and has long been suspected to harbor methylotrophic bacteria [24, 25]. Initial descriptions of these cold-seep communities reported *M. methanophila* to have depleted $\delta^{13}C$ values, which suggest methylotrophic nutrition [26]. The suspected methylotrophic symbiosis was further corroborated when Harrison et al. [24] reported that this sponge showed significant activities of methanol dehydrogenase, an enzyme diagnostic of methylotrophic bacteria, and low activities of RuBP carboxylase/oxygenase, an enzyme diagnostic of bacterial autotrophy in deep-sea organisms. Nevertheless, full characterizations of the bacterial associations in *M. methanophila* or any other sponges from deep-sea, chemosynthesis-based ecosystems have remained incomplete.

Here, we used massively parallel tag pyrosequencing along with traditional 16S rRNA gene cloning and sequencing, as well as transmission electron microscopy (TEM), to answer several questions. Because thiotrophic symbionts have been identified in other sponges from the same sites [23], we asked: Does *Myxilla methanophila* harbor methylotrophic, thiotrophic, or both bacterial symbionts and what are the phylogenetic relationships of these bacteria? Furthermore, *M. methanophila* is known exclusively as an epibiont on tubeworms inhabiting areas of seepage of crude oil, methane, and other hydrocarbons in the Gulf of Mexico [25]—conditions that may require special physiological adaptations. Thus, we asked whether there are other microbial associations that may contribute to the success of these sponges in these harsh ecosystems. Finally, we collected sponges from two sites and microbial samples from the biofilms developed on the host tubeworms in order to investigate the specificity of the microbial associations with the sponges.

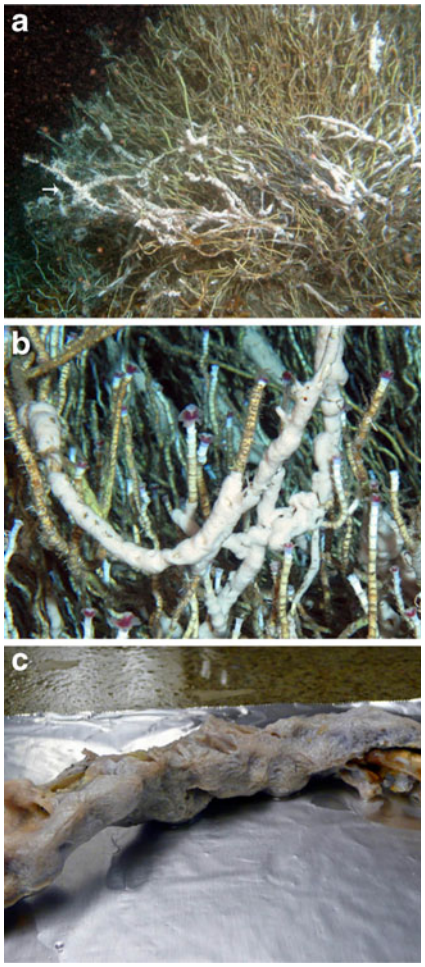

Fig. 1 *M. methanophila* sponges on a bush of *L. luymesi* tubeworms **a** at the Bush Hill cold seep in the Gulf of Mexico; **b** is a close-up view of the sponge in situ, while **c** is a close-up taken in the laboratory

Materials and Methods

Specimen Collection and Shipboard Sample Processing

Tubeworms with sponges were collected in September 2009 using the manipulator arm of the *Johnson Sea Link* manned submersible at two different cold-seep locations on the upper Louisiana slope in the Gulf of Mexico: Bush Hill (BH; 27°47′ N, 91°30′24″ W) and a site in the Green Canyon oil leasing block known as GC234 (27°44.4′ N,

91°13.3′ W). Both sites lie at approximately 550 m depth with an ambient temperature of ~7 °C and are about 28.5 km apart from one another. Tubeworms with sponges were recovered to the surface in a thermally stable acrylic box. Upon recovery, tubeworms and sponges were maintained alive in separate containers of filtered seawater at ambient temperature until sample processing.

Each sample sponge was processed for both genetic material and microscopy. Additionally, the adjacent biofilm from each associated tubeworm was also scraped for microbial community analysis. Five sponge and biofilm samples were taken from each site, but only two samples from each site were processed for pyrosequencing. Samples are labeled as BH-2 and BH-4 (sponge) or BHBio-2 and BHBio-4 (biofilm samples) from Bush Hill and GC234-2 and GC234-4 (sponge) or GC234Bio-2 and GC234Bio-4 (biofilm samples) from GC234.

Excess seawater was gently squeezed from each sponge and then the sponges were rinsed with 0.22 μm filtered seawater before a thin layer of the epidermis was removed with a sterile razor blade and discarded. Two small samples (~5 mm^3) of the mesohyl were removed and either stored at −20 °C in DNA extraction buffer (100 mM of Tris–HCl, 100 mM of Na_2-EDTA, 100 mM of Na_2HPO_4, 1.5 M of NaCl, 1 % of CTAB; at pH 8) or fixed for TEM. Samples for TEM were fixed overnight at 4 °C in 0.2 % glutaraldehyde/4 % paraformaldehyde and then stored at 4 °C in Millonig's phosphate buffer until electron microscopy processing.

Before sampling the biofilm on the surface of the tubeworm adjacent to each sponge, the tube of the tubeworm was gently rinsed with 0.22 μm filtered seawater to remove any loosely attached material. The rinsed tube was swabbed with a sterile cotton tip swab, immersed in DNA extraction buffer, and stored at −20 °C until processing. Because we could not easily swab a standard area of the tubes, biofilms were instead swabbed until one cotton tip swab was saturated.

Transmission Electron Microscopy

Samples were frozen in a high-pressure freezer (Leica EM PACT 2, Leica Microsystems) and cryosubstituted at −85 °C for 2 days in 1.5 % osmium tetroxide, 0.5 % uranyl acetate, and 5 % water in acetone. They were then warmed to 20 °C over 15 h (7 °C/h) and kept at 20 °C for 1 h. All the cryosubstitution processing was done in a Leica AFS Freeze Substitution System. They were subsequently washed in acetone and infiltrated with Epon resin, which was polymerized at 60 °C for 2 days. Ultrathin sections (60 nm) were cut using a Leica UltraCut T Ultramicrotome. The sections were mounted on poliform-coated copper grids and stained with 5 % uranyl acetate in 5 % methanol and Reynolds lead citrate. Viewing was performed in a JEOL 1010 transmission electron microscope operated at 80 kV at the Centre for Microscopy and Microanalysis, University of Queensland.

DNA Extraction, PCR Amplification, and Pyrosequencing of Barcoded Amplicons

Total genomic DNA from the sponge samples was extracted and purified using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Bacterial cells in the biofilm samples were lysed by three freeze–thaw cycles with liquid nitrogen and a 65 °C water bath before the total genomic DNA was extracted according to the modified SDS-based method described by Zhou et al. [27]. The quality and quantity of the DNA samples were checked with a NanoDrop Spectrophotometer (ND-1000, NanoDrop, USA). Purified DNA samples were stored at −20 °C for future use.

The 16S rRNA gene for each sample was amplified by polymerase chain reaction (PCR) for pyrosequencing using barcoded primers. A set of primers targeting the hypervariable V6 region of the 16S rRNA gene from bacteria and archaea were designed by adding a 6-nucleotide barcode (Table 1) to the universal forward primer U789F (5′-TAGA TACCCSSGTAGTCC-3′) and the reverse primer U1068R (5′-CTGACGRCRGCCATGC-3′) [28]. A 100-μl PCR mixture contained 5 U of *Pfu* Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA), 1× *Pfu* reaction buffer, 0.2 μM of dNTPs (TaKaRa, Dalin, China), 0.1 μM of the appropriate barcoded primer, and 20 ng of genomic DNA template. PCR was performed with a thermal cycler (Bio-Rad, USA) under the following conditions: initial denaturation for 5 min at 94 °C followed by 28 cycles of denaturation for 50 s at 94 °C, annealing for 50 s at 55 °C, and extension at 72 °C for 50 s, then a final extension step for 6 min at 72 °C. PCR products were purified using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, China) and quantified with the NanoDrop Spectrophotometer. A mixture of PCR products was prepared by mixing 200 ng of the purified 16S rRNA gene amplicons from each sponge and biofilm sample and then pyrosequenced on the Roche 454 FLX Titanium Platform (Roche, Basel, Switzerland) at the National Human Genome Centre of China at Shanghai, China, according to the manufacturer's protocol.

Taxonomic Classification and Community Analyses of Pyrosequencing Reads

The pyrosequencing reads were deposited in the NCBI Sequence Read Archive database with the accession number SRA048681. Downstream analysis of pyrosequencing data was processed through the QIIME pipeline [29]. First, raw pyrosequencing reads were split into different libraries according to their barcodes (Table 1) and the low-quality reads (i.e., reads that were <100 bp or larger than 1,000 bp,

Table 1 Similarity-based OTUs, species diversity, and richness estimates of the sponge-associated and tubeworm biofilm microbial communities revealed by 16S rRNA gene pyrosequencing and clone library

Sample ID	Source	Pyrosequencing						Clone library		
		Barcode	No. of reads	OTU	Chao1	Shannon	Simpson	No. of clones	OTU	Shannon
GC234-2	Sponge	CTACTG	10,564	144 (144)	187 (184)	4.1 (4.1)	0.90 (0.90)	80	8	1.17
GC234-4	Sponge	CTACAC	10,196	87 (86)	116 (115)	3.8 (3.8)	0.89 (0.89)	80	6	1.15
BH-2	Sponge	TGATAT	9,808	70 (70)	137 (137)	2.8 (2.8)	0.76 (0.76)	80	5	0.97
BH-4	Sponge	TGATCA	9,856	67 (67)	96 (96)	2.7 (2.7)	0.77 (0.77)	80	5	1.23
GC234Bio-2	Biofilm	TGACAC	13,950	935 (801)	1,420 (1,266)	5.9 (5.9)	0.95 (0.95)	–	–	–
GC234Bio-4	Biofilm	TGAGCG	13,846	760 (664)	1,039 (955)	5.3 (5.2)	0.88 (0.88)	–	–	–
BHBio-2	Biofilm	ACATCA	14,658	720 (607)	1,024 (941)	4.9 (4.9)	0.86 (0.86)	–	–	–
BHBio-4	Biofilm	ACACTG	14,039	424 (364)	570 (562)	4.3 (4.3)	0.87 (0.87)	–	–	–

Values in parentheses are based on the datasets normalized by the smallest dataset (i.e., 9,808 reads). Clone library was only constructed for the sponge samples but not the biofilm samples. Values were determined based on a dissimilarity level of 3 %

had quality score windows <50, had more than 3 nucleotide mismatches in the primer, or had ambiguous nucleotides) were removed. The dataset was further denoised by Donoiser [30] and chimeric sequences were identified by ChimeraSlayer [31] and removed. Qualifying reads with their barcodes truncated were assigned into operational taxonomic units (OTUs) at a 3 % dissimilarity level based on UClust [32] and one representative sequence from each OTU was picked for multiple alignments by MUSCLE [33]. Rarefaction curves were generated and the alpha diversity (Shannon, Simpson, Chao1) was computed at a 3 % dissimilarity level. The dataset was rarified to remove sampling heterogeneity before computing the beta diversity. The similarity among the microbial communities in sponges and biofilms from different sites was determined by using unweighted UniFrac [34, 35] and a jackknifed hierarchical cluster was generated based on UPGMA clustering method. The assignment of qualified representative reads into different taxa at different taxonomic levels was performed with the RDP classifier against the greengenes database at a confidence level of 50 %.

Full-Length 16S rRNA Gene Clone Library Construction and Analysis

The 16S rRNA genes in the crude DNA extracts from the sponge samples were PCR-amplified using the universal primers 8F (5′-AGAGTTTGATCCTGGCTC-3′) [36] and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [37]. Each 50-μl reaction contained 1 U of rTaq polymerase (TaKaRa, China), 1× PCR buffer, 0.2 mM of dNTPs (TaKaRa, China), 0.1 μM of each primer, and 10 ng of genomic DNA template. Thermocycling conditions were as follows: 95 °C for 2 min (initial denaturation); 10 touchdown cycles of 95 °C for 1 min (denaturation), 65 °C (reduced to 55 °C in increments of 1 °C/cycle) for 1 min (annealing), and 72 °C for 1 min (extension); 15 cycles with a constant annealing temperature of 55 °C; and 72 °C for 5 min (final extension). PCR products were purified by the PCR Clean-up Gel Extraction Kit (Macherey-Nagel, Germany) and quantified by the NanoDrop Spectrophotometer. Purified PCR products (250 ng) were cloned into a pCR2.1-TOPO vector and then transformed into *Escherichia coli* competent cells by using the TOPO TA Cloning Kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Eighty positive colonies were selected from each sample and Sanger-sequenced bidirectionally with the vector primers M13F and M13R on the ABI 3730l DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at BGI in Shenzhen, China, according to the manufacturer's protocols.

Chimeras and vector contaminations were detected and removed by the Pintail algorithm [38] and the VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen), respectively. Nearly full-length 16S rRNA gene sequences were obtained by assembling sequences from the two directions using the software Sequencer 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA) with manual modification. The 16S rRNA gene sequences were deposited in GenBank under the accession numbers JQ862015 to JQ862038. Multiple alignments of the clone sequences were performed with MUSCLE [33] and then inputted into DNAdist in the PHYLIP package (version 3.6) to produce a distance matrix. This matrix served as the input to DOTUR by using furthest neighbor assignment algorithm [39] to assign the sequences into OTUs, to generate rarefaction curves, and to calculate the Shannon diversity index at a 3 % dissimilarity level. Clone sequences were aligned with closely related sequences from the NCBI database using ClustalW 1.6 in the software MEGA 4.1 and phylogenetic trees were

constructed using the neighbor-joining, maximum likelihood, and maximum parsimony methods. A bootstrap test of 1,000 replications was performed to examine the reliability of tree topologies.

LIBSHUFF analysis [40] was applied to determine whether there were significant differences between different clone libraries. To obtain an experiment-wide false detection rate of 0.05, a critical p value of 0.0043 (employing the Bonferroni correction) was necessary for a LIBSHUFF pairwise comparison of four libraries for the sponge samples. For each pairwise comparison, if the lower of the two p values calculated by LIBSHUFF was less than or equal to the critical p value, then there was a significant difference, with a confidence of 95 %, among libraries.

Results

Diversity and Species Richness Estimators of Sponge and Biofilm Microbial Communities

To get an indication of the morphology of symbiotic microorganisms, one sample from each location was analyzed with TEM (Fig. 2). In both sponge samples, TEM revealed one dominant morphotype—about 80 % of the cells were coccoid. These coccoid cells were about 0.2–0.5 μm in diameter with large nucleoids (Fig. 2c) and were evenly distributed in mesohyl tissues of the sponges. Both samples were densely packed with coccoid cells, with a higher density in the sample from Bush Hill. In addition, some rod-shaped cells were occasionally observed (Fig. 2d).

Pyrosequencing and clone libraries also indicated low diversity in the sponge samples (Table 1). Approximately 200,000 raw pyrosequencing reads of 16S rRNA gene spanning the hypervariable region V6 (average read length= 237 bp) were obtained from the sponge and biofilm samples. After quality filtering, denoising, and removing the chimeric sequences, 96,917 reads (average read length= 287 bp) qualified for subsequent analysis to determine their diversity and taxonomic classification (Table 1). Rarefaction analysis suggested there was sufficient coverage to estimate microbial diversity of the sponge samples, but more sequencing effort may be necessary to achieve a complete representation of microbial diversity in the biofilm samples (Fig. 3a). Table 1 lists the alpha diversity estimators (OTUs, Chao1, Shannon, and Simpson indices) at a 3 % dissimilarity level. Species richness was much higher in the biofilm samples than in the sponge; Chao1 estimators were up to tenfold higher in the biofilm samples than the sponge sample and far more OTUs were detected from the biofilm samples (424–935 OTUs) than from the sponge samples (67–144 OTUs). Shannon index also suggests higher

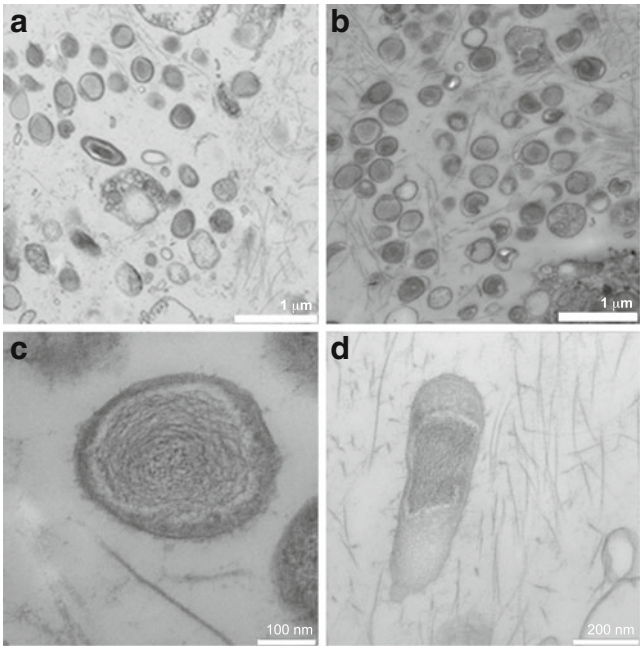

Fig. 2 Transmission electron micrographs of the mesohyl of *M. methanophila* from **a** GC234 and **b** Bush Hill. Close-ups of the two most common morphotypes **c** cocci and **d** rods are also shown. Note that both sponges have similar dominant bacterial morphotypes, but there is a greater density in the sponge from Bush Hill

diversity in the biofilm samples than in the sponge samples, while the Simpson index suggested similar evenness among the samples (Table 1).

To gain more insights into the types of bacteria that are associated with the sponge, a clone library was constructed of the nearly full-length 16S rRNA gene for each of the four sponge samples. Rarefaction analysis based on sequencing of 80 clones from each library indicated good coverage (Fig. 3b). Again, low bacterial diversity was observed; at a 3 % dissimilarity level, fewer than ten OTUs were detected with Shannon diversity index ranging from 0.97 to 1.23 (Table 1).

Sponge and Biofilm Microbial Communities Revealed by Pyrosequencing

At a confidence threshold of 50 %, 81,498 (81,443 bacteria, 55 archaea) out of the 96,917 qualified reads (84.1 %) could be assigned to a known phylum. Altogether, 21 phyla were recovered from the biofilm samples but only 8 (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, and *Proteobacteria*) were found in the sponge samples (Fig. 4, Table S1). A majority of the classified reads (>82 %) belonged to *Proteobacteria* (particularly *Gammaproteobacteria*, comprising >80 %). *Alphaproteobacteria* was the second dominant group following *Gammaproteobacteria* in the sponge samples, yet this group greatly diminished in the biofilm samples where *Bacteroidetes*

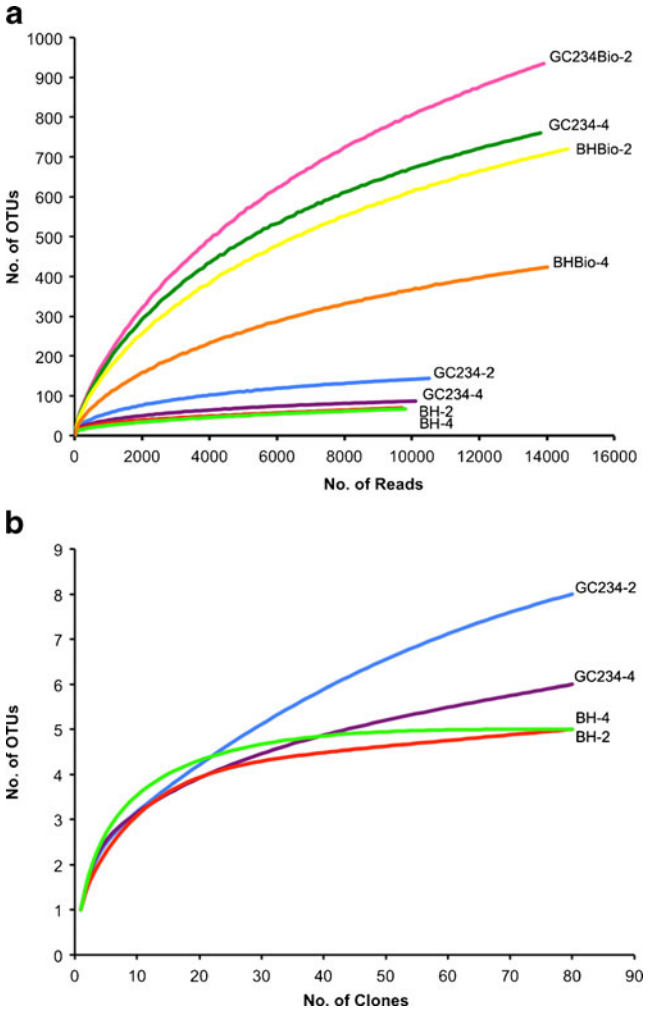

Fig. 3 Rarefaction analysis of microbial communities revealed by **a** 16S rRNA gene pyrosequencing and **b** clone library

and *Planctomycetes* constituted a significant proportion. The number of reads that could be classified (i.e., the proportion of classified reads) reduced gradually at each successive taxonomic level (Fig. S1). An average of 19 % of qualified reads from the sponge samples and 44 % from the biofilm samples could be classified to the genus level. Among these classified reads, those assigned to *Methylohalomonas* were dominant (ranging from 27 to 88 %) in all four sponge samples and the two biofilm samples from Bush Hill (Fig. 4b). The second and third most dominant groups in the sponges were *Cycloclasticus* and *Neptunomonas*, while the biofilms from Green Canyon were dominated by *Moritella*. In addition, the percent assigned to minor groups (i.e., with relative abundance of <0.5 % in all samples) was higher in the biofilm than in the sponge samples.

None of the qualified archaeal reads from the sponge samples could be assigned to any known archaeal phylum, and only a very small number of *Crenarchaeota* and *Euryarchaeota* reads could be found in the biofilms samples (Table S1). Among these reads, *Crenarchaeota* could be further classified into the class *Thermoprotei* and *Euryarchaeota* could be classified into the classes *Thermoplasmata* and *Methanomicrobia*, but not further down to the family or genus level.

Visual comparison of microbial community structure revealed by pyrosequencing of the 16S rRNA genes from the sponge and biofilm samples showed large differences between the two types of samples from different locations, yet replicated samples were similar (Fig. 4). This was supported by jackknifed clustering analysis, which grouped the communities from the sponge and biofilms into two distinct clusters (Fig. 5). The clusters formed by the four biofilm samples and the four sponge samples were each further divided into two well-defined groups, each comprising the two replicates from one particular sampling location (i.e., clustered with respect to sampling site).

Sponge Microbial Communities Revealed by Clone Library

Phylogenetic analysis of the ten OTUs from the sponge clone library indicated that they fell into two phyla, with 94.7 % of the clones affiliated with *Proteobacteria* and the remaining 5.3 % with *Bacteroidetes* (Fig. 6). Within the phylum *Proteobacteria*, clones belonged to *Alphaproteobacteria* (29.7 %), *Gammaproteobacteria* (60.3 %), and *Epsilonproteobacteria* (4.7 %). Nearly all of the gammaproteobacterial clones resembled sequences from thiotrophic endosymbionts of two mytilid mussels, *Bathymodiolus* spp. and *Idas* spp., and the sponge *Characella* from cold-seep and deep-sea areas. Three of the gammaproteobacterial clones from the GC234 sponges were affiliated with *Cycloclasticus* from sediments enriched with hydrocarbons, and two others were affiliated with *Pseudoalteromonas* and *Moritella* from deep-sea and Arctic seawater, respectively (Fig. 6). All the alphaproteobacterial clones fell into one cluster sharing similarity with *Ahrensia* sp. from Yellow Sea water and uncultured bacteria from arctic surface sediment, abalone gut, Caribbean coral, and brittle stars (Fig. 6). All of the epsilonproteobacterial clones except one were affiliated with *Arcobacter* from abalone gut and Arctic sediment or uncultured bacteria from Arctic or hydrocarbon-enriched sediment (Fig. 6), and the only exception also clustered with uncultured bacteria from either cold water or methane-seep sediment. Most of the *Bacteroidetes* clones were closely related to *Maribacter* from Arctic seawater and sediments and one clone fell into a cluster with sequences retrieved from uncultured bacteria from Arctic seawater, coral, and sponge.

With a critical p value of 0.0043 (employing the Bonferroni correction), LIBSHUFF pairwise comparisons of the four

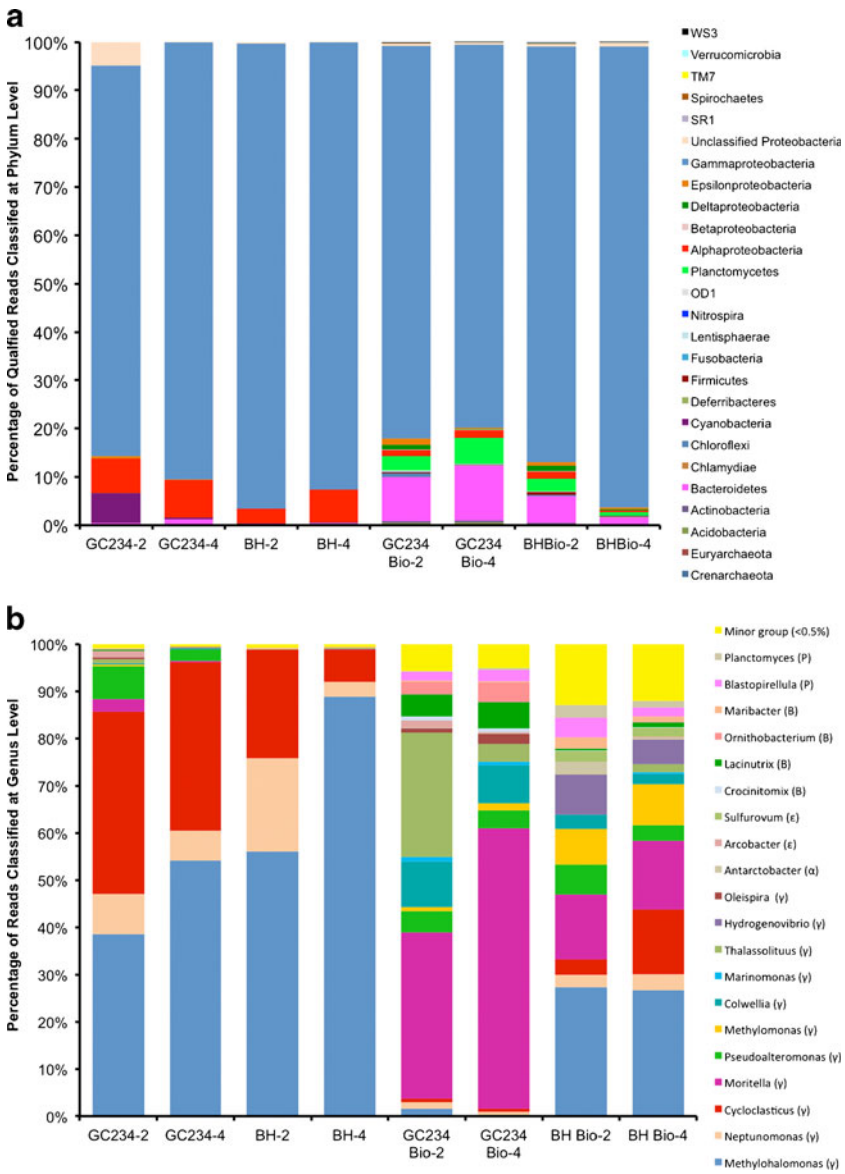

Fig. 4 Taxonomic classification of pyrosequencing reads retrieved from different sponge and biofilm samples into **a** phylum and **b** genus levels using the RDP classifier. A confidence threshold of 50 % was applied for classification and only the classified reads were shown. The proportions of unclassified reads at the genus level are shown in Supplementary Fig. S1. *Letters in parentheses* represent different phyla: *P* for *Planctomycetes*, *B* for *Bacteroidetes*, ε for *Epsilonproteobacteria*, α for *Alphaproteobacteria*, and γ for *Gammaproteobacteria*. *Minor group* represents a collection of genera with relative abundance of <0.5 % in all samples

sponge clone libraries indicated that the BH2, GC234-2, and GC234-4 libraries were not significantly different from each other, but significant differences were found in BH4 library when compared with BH2 and GC234-4 (Table 2).

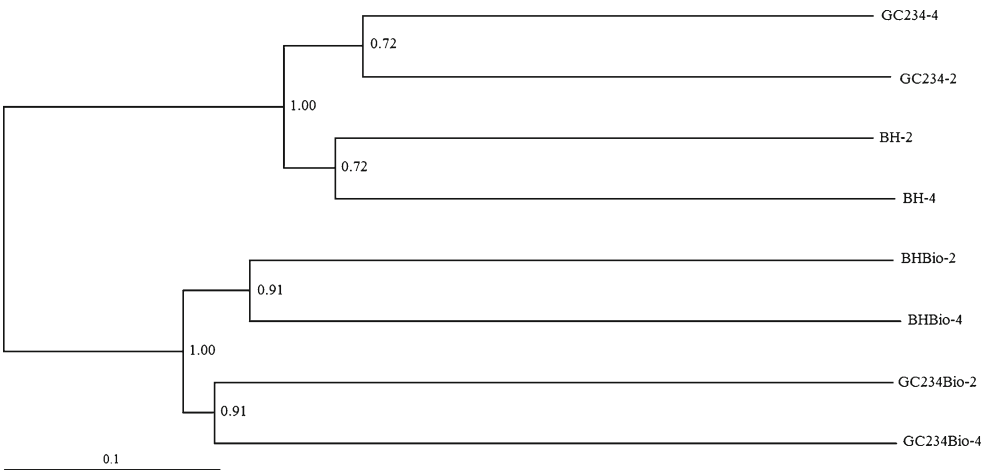

Fig. 5 Jackknifed cluster analysis of microbial communities in the sponge and biofilm samples from different locations

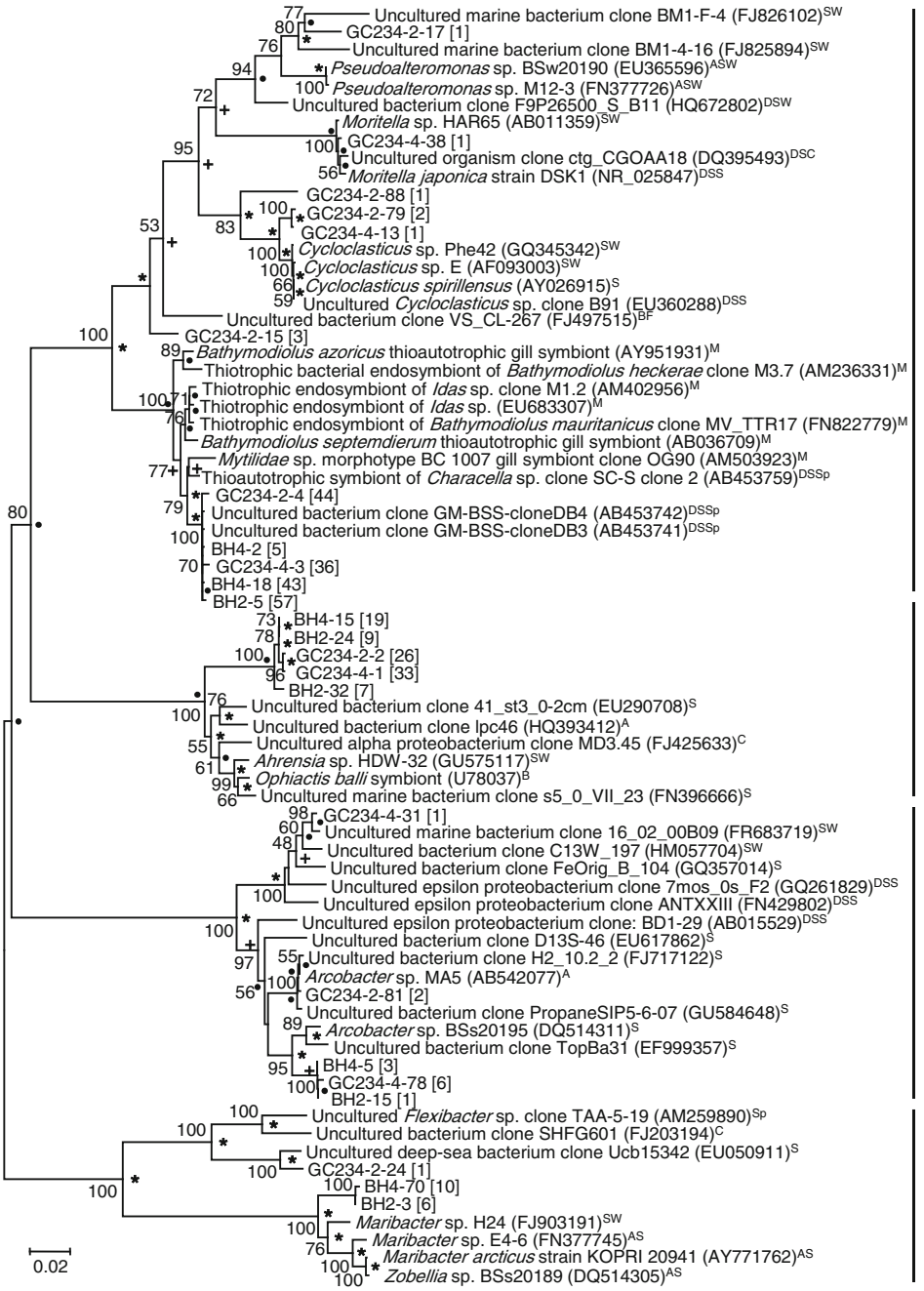

Fig. 6 16S rRNA gene-based phylogenetic tree showing the genetic distances among clones retrieved from the sponges from different locations in reference to members of *Alphaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria*, and the phylum *Bacteroidetes*. The tree was constructed based on the neighbor-joining method. Nodes observed in both of the trees constructed with the maximum likelihood (ML) and the maximum parsimony (MP) methods are denoted by *asterisks*, while those observed in either the ML tree or the MP tree are denoted by *filled circles* and *plus sign*, respectively. Nearly complete 16S rRNA gene sequences obtained from clone libraries of different sponge samples are prefixed with sample ID (for details, see Table 1) followed by clone number and the number of clones belonging to the same OTU are given in *brackets*. Reference sequences from closest relatives are retrieved from NCBI with the accession number shown in *parentheses*. Their sources were indicated by superscripts *SW* seawater, *ASW* Arctic seawater, *DSW* deep-sea water, *DSS* deep-sea sediment, *DSC* deep-sea coral, *DSSp* deep-sea sponge, *AS* Arctic sediment, *Sp* sponge, *M* mussel, *A* abalone, *C* coral, *S* sediment, *B* brittle star, and *BF* biofilm. The *scale bar* represents percent substitutions per nucleotide position. Bootstrap values of >50 % based on 1,000 resamplings are indicated by the numbers at the nodes

Discussion

Microorganisms can constitute a large part of sponges, up to 60 % of the tissues of some sponge species [7, 8] and can exceed more than 10^9 cells/ml of sponge tissue [41]. Such sponges generally have large numbers of extracellular bacteria populating the mesohyl matrix and have been termed either "bacteriosponges" [42] or "high-microbial-abundance sponges" [7, 43]. In this study, we found a relatively high density of cocciform and some bacilliform bacteria in the mesohyl as indicated by TEM (Fig. 2). Similarly, Harrison et al. [24] reported numerous bacilliform bacteria in the mesohyl of *M. methanophila*, occupying up to 21 % of the volume, although our TEMs suggest our samples contained dominantly coccoid cells (Fig. 2). Besides high microbial density, high-microbial-abundance sponges generally host a diverse community of microbial associates [7, 43] and recent high-throughput sequencing studies have generally revealed a high diversity of sponge microbial associates [18, 44–46]. Interestingly, although the abundance of microbes in our *M. methanophila* samples was high, the diversity was relatively low when compared with other high-microbial-abundance sponges (Tables 1 and S1; [25, 48]). Similarly, in a PCR-denaturing gradient gel electrophoresis (DGGE) study of the

Table 2 LIBSHUFF comparisons of the 16S rDNA clone libraries of sponge-associated bacterial communities

Comparison	ΔC score	p value	Significant?
BH2 vs BH4	4.401	0.004	Yes
BH4 vs BH2	1.130	0.065	
BH2 vs GC234-2	0.478	0.052	No
GC234-2 vs BH2	0.943	0.009	
BH2 vs GC234-4	1.517	0.020	No
GC234-4 vs BH2	1.125	0.006	
BH4 vs GC234-2	0.411	0.118	No
GC234-2 vs BH4	3.727	0.005	
BH4 vs GC234-4	0.460	0.110	Yes
GC234-4 vs BH4	3.578	0.004	
GC234-2 vs GC234-4	0.480	0.007	No
GC234-4 vs GC234-2	0.235	0.041	

Data presented are LIBSHUFF [ΔC]; critical p value is 0.0043

16S rRNA gene in two sponges collected near our sampling sites, Nishijima et al. [23] found only a single major band and several minor bands in each of the two sponge species.

Although the low diversity of microbes shown in *M. methanophila* is not typical of other high-microbial-abundance sponges, the major phylotypes comprising the *M. methanophila* microbial community were typical of other sponges. *Proteobacteria* dominated the *M. methanophila* sponge microbial community, making up the majority of the classified reads (mean=97.7±2.9 % SD, n=4). In a recent meta-analysis, Webster and Taylor [47] examined the phylogenetic distribution of more than 11,000 16S rRNA gene sequences of sponge-associated bacteria found in GenBank. Of these, *Proteobacteria* comprised about half, with at least 16 other phyla making up the other half of the sequences. While our sponge samples contained many of the other phyla that are common sponge associates, such as *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* [45, 47], we did not detect any of the "rare" phyla (e.g., *Deferribacteres*, *Tenericutes*, WS3, and *Chlamydiae*) that have been revealed by pyrosequencing in other studies [18, 45, 46]. However, some of these "rare" phyla, including *Deferribacteres*, WS3, and *Chlamydiae*, were identified at low abundance (<1, 15, and 3 %, respectively) in the reads from the associated tubeworm biofilms.

Our *M. methanophila* samples had a conspicuous lack of *Poribacteria*, a candidate phylum with cosmopolitan geographic distribution in demosponges [48, 49] that has only rarely been detected in seawater surrounding sponges [46, 50]. So far, *Poribacteria* have only been found in shallow-water sponge species with high microbial diversity and abundance, although it should be reemphasized that studies of sponge associates in deep-sea species are rare. The 16S rRNA gene-based diversity analysis of the deep-sea sponge *Polymastia* cf. *corticata* from the Caribbean Sea revealed a complex and diverse community; although 56 % of the phylotypes identified fit into "sponge-specific" 16S rRNA gene sequence clusters that Meyer and Keuver [51] compiled from sequences in other sponge studies, they could not identify *Poribacteria* in their sponge samples. Similarly, although Olson and McCarthy [52] reported that *Scleritoderma cyanea* collected at 242 m depth off the coast of Curacao and *Scleritoderma* sp. collected from 255 m depth off Bonaire hosted microbial communities most similar to uncultivated microbes retrieved from the shallow-water sponges, they did not detect any *Poribacteria*. While the lack of *Poribacteria* in these sponges may simply be due to the specificity of primers used, which failed to detect this particular phylum, there is clearly a need for more studies on deep-sea sponge microbial communities in general and further scrutiny into the depth distribution of the seemingly cosmopolitan sponge-associated candidate phylum *Poribacteria*.

Our pyrosequencing results showed that the majority (39–88 %) of classified OTUs within *M. methanophila* sponge samples belonged to the methylotrophic genus *Methylohalomonas*. Methylotrophic symbiosis is known in mussels in chemosynthetic ecosystems [53], including within "*Bathymodiolus*" *childressi* [54], which are found at both GC234 and Bush Hill cold-seep sites. As the species name —*methanophila*—suggests, this sponge has been suspected to rely on methane as a significant carbon source since it was first described [25]. Isotopic analyses revealed that the sponge had a very light (−32.8) δ^{13}C value, which is consistent with methylotrophy. Furthermore, a preliminary follow-up study reported significant dehydrogenase activity and low RuBP carboxylase/oxygenase activity, which together suggested that the sponges harbor methylotrophic bacteria [24].

Conversely, our library of 80 clones from *M. methanophila* did not contain sequences from methylotrophic bacteria. The lack of methylotrophic-like clones recovered in our library was likely due to the inherent limitation of this technique to recover nondominant groups. Moreover, primer bias may further affect recovery of less common groups; in fact, when the available *Methylohalomonas* sequences from GenBank were aligned with our clones, they do not reach the target binding sites for the two primers we used. Instead, our clone library contained six OTUs that were related to thioautotrophic symbionts from *Bathymodiolus* mussels and the thioautotrophic bacteria cloned from other deep-sea sponges in the Gulf of Mexico (Fig. 6). However, sequences of these thioautotrophic symbionts were not recovered by pyrosequencing. Again, this is largely due to primer specificity; these thioautotrophic bacterial sequences do not have a binding site for the V6 primers we used for pyrosequencing (see Supplementary Table S2). In a DGGE study that examined five other sponges, including a different poecilosclerid sponge

around the cold seeps in the deep Gulf of Mexico, Nishijima et al. [23] found primarily thioautotrophic clones in addition to several methylotrophic-like clones. As Nishijima et al. [23] pointed out, dual symbiosis with both methylotrophic and thioautotrophic bacteria has been reported before in bathymodiolin mussels [55, 56]. Thus, it is not unusual that these sponges possess both types of chemosynthetic symbionts and both may play a nutritional role for this sponge.

Overall, the microbial communities in the sponges were substantially different from the biofilm communities, suggesting that there were probably specific communities associated with the sponge samples. Although it should be noted that we used different methods to extract DNA from the sponge and biofilm samples, which may lead to small variations in the results, the biofilm communities were more diverse and species rich than the sponge-associated communities and the major groups of microbes varied between the two types of samples. The communities within each sample type clustered by site (Fig. 5), but the percentages of reads assigned to the three most abundant genera varied substantially between the sponges and biofilm and between sites in the biofilms (Fig. 4). At GC234, 38 and 54 % of the classified sponge reads aligned with *Methylohalomonas*, but the biofilm samples at the same site contained <1 % *Methylohalomonas* in the classified reads. Conversely, the percentage of reads classified as *Methylohalomonas* was much higher in the biofilms (mean=27±0.5 % SD of the classified reads; n=2) at Bush Hill than at GC234, but the percentage of *Methylohalomonas* reads in the sponges was high at both sites (GC234: mean=72±23 % SD of the classified reads; n=2). While *Cycloclasticus* comprised 36 and 39 % of the classified reads in the two sponges at GC234, <~0.7 % of the OTUs from the biofilm samples there belonged to *Cycloclasticus*. On the other hand, there was high variation in the percentage of OTUs from the biofilms that were assigned to *Cycloclasticus* at Bush Hill (6 and 23 % of the classified OTUs). Finally, the percentage of classified OTUs in the sponges assigned to *Neptunomonas* ranged from 3 to 20 %, while the percentage in the biofilm samples was consistently <2 %. The variation in microbial composition between the samples and sites suggests that, although the types of the most abundant sponge microbes remain consistent between sponges and sites, their relative proportions in the community vary depending on environmental conditions. Unfortunately, water chemistry around the sponges was not determined in this study.

Cycloclasticus and *Neptunomonas* may play important roles as symbionts in these hydrocarbon-seep communities. *Cycloclasticus* spp., which were also identified in our clone library (Fig. 6), are widely known as major players in the degradation of polycyclic aromatic hydrocarbons (PAHs) in the marine environment [57–60] and *Neptunomonas* spp. and *Pseudoalteromonas* spp. are also known to degrade a diversity of PAHs [61–63]. Bush Hill and GC234 are both "petroleum-dominated" seep sites [64]. The sediment and organisms around these sites are visibly stained with oil (Arellano, personal observation) and numerous PAHs have been reported from both sites [65]. So far, *Neptunomonas* spp. have only been reported in creosote-contaminated marine sediments (*Neptunomonas napthovorans*) [61], marine sediments adjacent to a whale carcass (*Neptunomonas japonica*) [66], and Antarctic marine sediments (*Neptunomonas antarctica* sp. nov.) [63]. Similarly, although *Cycloclasticus* has been found in marine sediments onshore and offshore in the Gulf of Mexico [58], it has never been reported as a symbiont in sponges or any other invertebrate. Recent deep-sequencing studies on sponges have led to the identification of several taxa recorded from sponges for the first time [44–46], but these "new" taxa are generally found in low abundance. In this case, the percentage of classified reads belonging to *Cycloclasticus* and *Neptunomonas* was high in *M. methanophila*. Additionally, *Pseudoalteromonas*, strains of which have also been shown to contribute to degradation of PAHs [62], were also present in lower abundances in the sponges (Fig. 4). These novel associations within the sponge may contribute to their ability to specialize in such oil-rich and potentially toxic environments. Whether PAH-degrading bacteria may act as detoxifiers or even contribute to the nutrition of the sponge via degradation of aromatic hydrocarbons remains unclear. Further studies to determine the function of these bacteria and determine the nature of their associations with this sponge, as well as to examine other invertebrates for associations with PAH-degrading microbes in oil-seep or contaminated environments, should yield interesting results.

In conclusion, our study revealed associations of a complex community of methylotrophic, thiotrophic, and hydrocarbon-degrading microbes in *M. methanophila*. While our study suggests that these communities were consistent between individual sponges and two sites, further studies may reveal whether these bacterial communities are stable associations and how they may contribute to the success of this sponge living in such unique ecosystems.

Acknowledgments We gratefully acknowledge Mr. Rick Webb, at the Centre for Microscopy and Microanalysis (University of Queensland), who performed the TEM. We also thank Dr. Salim Bougouffa (Hong Kong University of Science and Technology) for the useful advice in bioinformatics and data analysis. Ship time and submersible dives were funded by grant number OCE-0527139 from the National Science Foundation to CMY. This research was supported by the National Basic Research Program of China (973 Program, No. 2012CB417304) and an award (SA-C0040/UK-C0016) made by the King Abdullah University of Science and Technology to PYQ. SMA was supported by a Woods Hole Oceanographic Institution postdoctoral scholarship during manuscript preparation.

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