Applied and Environmental Microbiology, June 2009, p. 3513–3521 Vol. 75, No. 11
0099-2240/09/$08.00+0 doi:10.1128/AEM.00002-09

Inter- and Intraspecific Variations of Bacterial Communities Associated with Marine Sponges from San Juan Island, Washington[▽]

On On Lee, Yue Him Wong, and Pei-Yuan Qian*

KAUST Global Academic Partnership Program, Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China

Received 1 January 2009/Accepted 31 March 2009

This study attempted to assess whether conspecific or congeneric sponges around San Juan Island, Washington, harbor specific bacterial communities. We used a combination of culture-independent DNA fingerprinting techniques (terminal restriction fragment length polymorphism and denaturing gradient gel electrophoresis [DGGE]) and culture-dependent approaches. The results indicated that the bacterial communities in the water column consisted of more diverse bacterial ribotypes than and were drastically different from those associated with the sponges. High levels of similarity in sponge-associated bacterial communities were found only in *Myxilla incrustans* and *Haliclona rufescens*, while the bacterial communities in *Halichondria panicea* varied substantially among sites. Certain terminal restriction fragments or DGGE bands were consistently obtained for different individuals of *M. incrustans* and *H. rufescens* collected from different sites, suggesting that there are stable or even specific associations of certain bacteria in these two sponges. However, no specific bacterial associations were found for *H. panicea* or for any one sponge genus. Sequencing of nine DGGE bands resulted in recovery of seven sequences that best matched the sequences of uncultured *Proteobacteria*. Three of these sequences fell into the sponge-specific sequence clusters previously suggested. An uncultured alphaproteobacterium and a culturable *Bacillus* sp. were found exclusively in all *M. incrustans* sponges, while an uncultured gammaproteobacterium was unique to *H. rufescens*. In contrast, the cultivation approach indicated that sponges contained a large proportion of *Firmicutes*, especially *Bacillus*, and revealed large variations in the culturable bacterial communities associated with congeneric and conspecific sponges. This study revealed sponge species-specific but not genus- or site-specific associations between sponges and bacterial communities and emphasized the importance of using a combination of techniques for studying microbial communities.

Marine sponges (phylum Porifera) harbor a remarkable array of microorganisms, including bacteria (51, 54), unicellular algae (50), cyanobacteria (45, 48), dinoflagellates (14), zoochlorellae (58), and members of the domain *Archaea* (33). Of these microorganisms, bacteria are the most dominant group of microbial associates in sponges and can account for up to 40 to 50% of a sponge's biomass (17). The density of bacteria can be up to 10^8 to 10^{10} bacteria per g (wet weight) of sponge (18). The great abundance of bacteria in sponges caused workers to coin the term "bacteriosponges" (35) and has attracted much research interest in the role and specificity of the sponge-bacterium association.

There are generally two pathways by which sponges may acquire their bacterial associates. The first pathway is filter feeding and selective retention of bacteria (44). Bacteria in the surrounding seawater can be captured by sponges when sponges filter the food particles out of the water column. The bacteria that resist digestion by sponge choanocytes and archaeocytes can survive and live inside the sponges. Sponge-associated bacterial communities acquired via this pathway are therefore heavily influenced by the type of bacteria in the water column. The second pathway is vertical transmission of bacterial associates from adult sponges to their progeny (10, 40, 49). Bacteria acquired through this pathway may exhibit specificity for genera or species of sponges due to coevolution (for a review, see reference 44).

Sponge-bacterium symbioses are often mutualistic; while bacteria may benefit from the favorable nutritional conditions in sponges (18, 44), some bacterial associates may help their hosts eliminate metabolic waste (4), stabilize the sponge skeleton (36), and defend against pathogens, predators, or competitors via the production of bioactive secondary metabolites (5, 21, 39, 47). In addition, some cyanobacterial symbionts are a source of nutrients for their hosts because of their photosynthetic and nitrogen-fixing abilities (3, 57).

There is a large body of knowledge concerning the possible roles of bacterial associates in sponges, but whether sponges harbor specific bacterial communities deserves more detailed study. Many previous investigations demonstrated that associated bacterial communities in certain species of sponges were highly similar and consistently different from the bacterial communities in the ambient environment (6, 22, 46). For instance, Hentschel et al. (16) showed that there were uniform microbial communities in the marine sponges *Aplysina aerophoba*, *Rhopaloeides odorabile*, and *Theonella swinhoei* from different geographic regions that were distinct from those in the water column or in sediments. In addition, Friedrich et al. (12) demonstrated that the composition of sponge-associated

* Corresponding author. Mailing address: KAUST Global Academic Partnership Program, Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China. Phone: (852) 2358-7331. Fax: (852) 2358-1559. E-mail: boqianpy@ust.hk.

▽ Published ahead of print on 10 April 2009.

TABLE 1. Sponge samples collected near San Juan Island, WA, in April 2005[a]

Collection site	Location	Sponge no.	Sponge species	Reference	Sample designation
1	Roche Harbor (48°36′35″N, 123°08′56″W)	1	*Haliclona rufescens*	Lambe 1892	HaR-1
		2	*Halichondria panicea*	Pallas 1766	HP-1
		3	*Myxilla incrustans*	Johnston 1842	MyI-1
		4	*Mycale adhaerens*	Lambe 1894	MA-1
2	Friday Harbor (48°32′7″N, 123°01′52″W)	5	*Myxilla incrustans*	Johnston 1842	MyI-2
3	Cattle Point (48°25′00″N, 122°59′17″W)	6	*Haliclona foraminosa*	Thiele 1905	HaF-3
		7	*Clathria pennata*	Lambe 1895	CP-3
		8	*Halichondria panicea*	Pallas 1766	HP-3
4	San Juan Channel (48°37′24″N, 122°59′17″W)	9	*Myxilla incrustans*	Johnston 1842	MyI-4
		10	*Xestospongia hispida*	Ridley & Dendy 1886	XH-4
5	Deadman Bay (48°35′46″N, 123°08′14″W)	11	*Mycale loveni*	Fristedt 1887	ML-5
		12	*Haliclona rufescens*	Lambe 1892	HaR-5
		13	*Halichondria panicea*	Pallas 1766	HP-5

[a] Water samples (W-1) were collected at site 1.

bacterial communities was resistant to environmental perturbations resulting from transplantation to different habitats. These studies suggest that there is a stable, specific, and perhaps mutualistic relationship between the two types of organisms (44). However, some bacterium-sponge symbioses do not appear to be consistent. For instance, Wichels et al. (56) demonstrated that the bacterial communities associated with the North Sea sponge *Halichondria panicea* varied substantially over time. Qian et al. (34) and Lee et al. (25) showed that the congeneric *Callyspongia* and *Mycale* sponges from different biogeographic regions had different bacterial associates. Although the discrepancies may be attributed to the methods employed in different studies, whether consistent sponge-bacterium associations occur in different species or genera of sponges remains unclear. Furthermore, most of the previous studies focused on only one sponge species or involved a few sponges from geographically separated regions. So far, there has been no large-scale study comparing the bacterial communities associated with sponges of different genera and species.

In this study, we compared the bacterial communities associated with marine sponges around San Juan Island, Washington, in order to investigate the specificity of congeneric and conspecific sponge-associated bacterial communities. Most previous studies have relied on only one method to assess the bacterial communities, limiting the resolution of community assessments. In addition, different studies have used different approaches to address the same question, creating uncertainties and making generalizations difficult. Here, we employed both culture-independent and -dependent approaches to compensate for the limitations of different methods and to obtain a more reliable assessment of the associated bacterial communities. We used two DNA fingerprinting techniques, terminal restriction fragment length polymorphism (TRFLP) (27) and denaturing gradient gel electrophoresis (DGGE) (11). TRFLP is an effective, sensitive, high-throughput technique that differentiates bacterial community structures, while DGGE allows subsequent identification of bacteria of interest by excision and sequencing of specific bands. Both techniques have been successfully and widely used for characterization of bacterial communities in marine samples (13, 26, 28, 43, 56). In addition, bacteria associated with sponges were isolated using cultivation methods and identified by comparative analysis of 16S rRNA gene sequences. Phylogenetic affiliations of the isolates were determined and compared for different samples in order to study the specificity of the sponge-associated culturable bacterial communities.

MATERIALS AND METHODS

Sample collection. Bacterial communities associated with 39 sponge specimens belonging to eight different sponge species were collected (Table 1) from five locations which were at least 10 km apart near San Juan Island, Washington (48.55°N, 123.01°W), in April 2005. Some sponge species were collected from more than one location. The sponges were identified by Rob von Soest using scanning electron microscopic analysis of sponge spicules, as well as fatty acids profiles for chemotaxonomy. For each sponge species collected at each location, three different individuals (three independent replicates) were retrieved from seawater less than 3 m deep and rinsed with autoclaved 0.22-μm-filtered seawater (AFSW) to remove loosely attached bacteria. Approximately 2 cm^3 of sponge tissue from each individual (replicate) was cut into small pieces with sterile razor blades and divided into two equal portions; one portion was immediately frozen in 0.8 ml of extraction buffer (100 mM Tris-HCl, 100 mM Na_2-EDTA, 100 mM Na_2HPO_4, 1.5 M NaCl, 1% of cetyltrimethylammonium bromide; pH 8) for bacterial community DNA extraction, and the other was put in an Eppendorf tube containing 1 ml of AFSW for bacterial isolation (except for *Mycale loveni* due to the limited amount of tissue collected). The two portions were then transported to the laboratory on ice. Samples of the bacterial communities in in situ seawater approximately 2 m from the sponges sampled were collected at site 1 in triplicate by filtering 1 liter of seawater obtained with a Niskin bottle onto 0.22-μm polycarbonate membranes (Osmonics, United States). The membranes were then divided in half, and the pieces were either frozen in extraction buffer or put in AFSW as described above.

Extraction of bacterial community DNA and PCR for fingerprinting analyses. Bacterial cells in the samples used for DNA extraction were lysed using two freeze-thaw cycles with liquid nitrogen and a 65°C water bath. Total DNA was extracted and purified using the sodium dodecyl sulfate-based method described by Zhou et al. (59). Purified DNA was dissolved in 50 μl of double-distilled H_2O (ddH_2O) and kept at −20°C until it was used. The 16S rRNA genes in the crude DNA extracts were amplified by PCR using the universal primers 341F and 926R-Fam for the TRFLP analysis and primers 341F-GC and 907R for the DGGE analysis (Table 2). For TRFLP, each PCR mixture (total volume, 25 μl) contained 2 μl of DNA template from a replicate, 1.25 U of *Taq* polymerase (Amersham Biosciences), 0.25 mM of deoxynucleoside triphosphates, 0.1 μM of each primer, and 1× PCR buffer. For DGGE, a 50-μl PCR mixture was prepared proportionally using the same recipe but with different primers and with replicate

TABLE 2. Universal primers used for PCR amplification in this study

Use	Primer	Sequence (5′–3′)	Reference
TRFLP	341F	CCT ACG GGA GGC AGC AG	27
	926R-Fam[a]	CCG TCA ATT CCT TTR AGT TT	27
DGGE	341F[b]	CCT ACG GGA GGC AGC AG	30
	907R	CCG TCA ATT CMT TTG AGT TT	30
	GC clamp	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G	
Sequencing	26F	AGA GTT TGA TCC TGG CTC A	38
	1055R	CAC GAG CTG ACG ACA GCC AT	2

[a] Fluorescently labeled at the 5′ end with 6-carboxyfluorescein.
[b] Carrying a GC clamp at the 5′ end.

DNA extracts that were pooled and used as the template. PCR was performed with a thermal cycler (MJ Research, United States) using the following conditions: 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^{-1}) 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).

TRFLP analysis of bacterial communities. PCR products were digested with 10 U of the restriction enzyme MspI at 37°C for 6 h and then purified by ethanol precipitation. Ten microliters of purified products was mixed with 0.5 μl of an internal size standard (ET-550R; Amersham Biosciences), denatured at 95°C for 2 min, snap cooled on ice, and subjected to electrophoresis with a MegaBACE genetic analyzer (Amersham Biosciences) operated in the genotyping mode. After electrophoresis, the sizes of the fluorescently labeled terminal restriction fragments (TRFs) were determined by comparison with the size standard using the software Genetic Profiler (Amersham Biosciences).

DGGE analysis of bacterial communities. PCR products were mixed with loading buffer and loaded onto a 6% acrylamide gel with a 35 to 70% denaturing gradient using urea-formamide as the denaturing agent. Electrophoresis was performed using the D-Code system (Bio-Rad, United States) in 1× TAE (20 mM Tris base, 10 mM sodium acetate, 0.5 mM EDTA) at a constant temperature of 60°C and a voltage of 125 V for 18 h. The gel was stained with 1× SYBR Gold (Molecular Probes, United States) for 15 min and photographed with the Alpha Imager 2200 gel documentation system (Alpha Innotech).

Excision and sequencing of DGGE bands. Bands of interest were excised from the gel with sterile razor blades, washed twice with 100 μl of autoclaved ddH_2O, and incubated in 50 μl of autoclaved ddH_2O for 24 h at 4°C. DNA dissolved from the gel sections was used as the template for PCR amplification with the 341F and 907R primers. The PCR mixture and conditions were the same as those mentioned above. PCR products were purified using a PCR purification mini kit (Watson Biotechnologies, China) according to the manufacturer's manual. The 16S rRNA gene amplicon of each band was sequenced from both ends using primers 341F and 907R. Each cycle sequencing mixture contained 5.5 μl of purified DNA, 4 μl of DYEnamic ET dye terminator (Amersham Biosciences), and 0.05 μM of one of the primers. Cycle sequencing was performed with the thermal cycler using the following conditions: 24 cycles of denaturing at 96°C for 30 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. Cycle sequencing products were purified by ethanol precipitation and subjected to electrophoresis with a MegaBACE genetic analyzer (Amersham Biosciences) operated in the sequencing mode. Nucleotide sequences obtained with the two primers were assembled using the computer software Sequencher (Gene Codes Corporation) and then compared with the DNA sequences deposited in the NCBI (National Center for Biotechnology Information) web server using BLAST (Basic Local Alignment Search Tool).

Bacterial isolation and identification. Samples used for bacterial isolation were diluted 1:10 and 1:100 with AFSW. Two hundred microliters of each dilution was spread on plates containing 1× nutrient agar (3 g of yeast, 5 g of peptone, and 12 g of bacteriological agar in 1 liter of AFSW), 0.1× nutrient agar (0.3 g of yeast, 0.5 g of peptone, and 12 g of bacteriological agar in 1 liter of AFSW), and tryptone soya agar (30 g of tryptone soya broth and 12 g of bacteriological agar in 1 liter of AFSW) and incubated at 22°C with a photoperiod consisting of 15 h of light and 9 h of darkness. Bacterial colonies were examined with a dissecting microscope (at a magnification of ×10) to determine morphological characteristics, including color, shape, size, and surface texture. Colonies with morphological differences were isolated and purified. The same morphological differentiation standard was applied to all species and replicates. DNAs were extracted from pure bacterial colonies using the method of Valsecchi (52). The 16S rRNA genes in the crude extracts were amplified by PCR using primers 26F and 1055R (Table 2). PCR was carried out using a mixture (total volume, 25 μl) containing 2 μl of DNA extract, 1 U of *Taq* polymerase (Amersham Biosciences), 0.25 mM of deoxynucleoside triphosphates, 0.1 μM of each primer, and 1× PCR buffer under the following conditions: 95°C for 8 min, followed by 37 cycles of 95°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min and then a final extension at 72°C for 5 min. PCR products were purified and sequenced as described above for the sequencing of DGGE bands except that the primers used (primers 26F and 1055R) were different. The closest phylogenetic affiliation for each isolate was obtained by comparing the assembled nucleotide sequences with those deposited in the GenBank database using BLAST. The phylogenetic affiliation was verified by using RDP classifier (53).

Statistical analysis. The average numbers of TRFs generated from TRFLP analysis of different samples ($n = 3$) were calculated and compared by using one-way analysis of variance, followed by a Dunnett test. The similarity of the TRF profiles for different samples was determined using cluster analysis based on the presence or absence of TRFs that were different sizes in each replicate. Although the relative signal intensities of TRFs may reflect the relative abundance of different PCR amplicons (i.e., different bacterial types), they were not used in the statistical analysis because of the likelihood of preferential PCR amplification of numerically dominant bacterial types in the samples (hence, the signal intensity may not be a true reflection of the relative bacterial abundance) (42). TRFs that accounted for <0.01% of the total fluorescence intensity, TRFs that were <35 bp long, and TRFs that were >500 bp long were excluded from the statistical analysis to screen out background noise, to avoid pseudo-TRFs from primers, and to avoid inaccurate size determinations, respectively. The Pearson coefficient was calculated based on the total number of TRFs observed in all samples and the presence of these TRFs in individual replicates to produce a similarity matrix. Agglomerative hierarchical clustering based on the matrix was performed using the PRIMER v3.1 program (Plymouth Marine Laboratory, United Kingdom) to generate a dendrogram showing the similarity among samples (7). For DGGE analysis, the DGGE band position and intensity were determined by the GELCOMPAR II software (Applied Maths), with manual modifications. Band matching was performed with 1% position tolerance and 1% optimization. A similarity matrix was calculated based on the Pearson coefficient and used to construct a dendrogram (7). Since DNAs extracted from replicate samples were pooled for DGGE analysis, no replicates were shown in the dendrogram. For isolation of bacteria, the occurrence of different bacteria differentiated by comparative analysis of the 16S rRNA gene sequences in replicates was recorded.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in this study have been deposited in the GenBank database under accession numbers FJ596288 to FJ596553.

RESULTS

TRF profiles of bacterial communities. With the primers and restriction enzyme used in this study, the average number (three replicates per sample) of TRFs derived from the sponge-associated bacterial communities ranged from 4 to 22; the lowest number of TRFs were derived from the sponge *Myxilla incrustans* at site 4, and the highest number of TRFs were derived from the sponge *Haliclona foraminosa* at site 3 (Fig. 1). The bacterial communities in the water column at site 1 yielded an average of 17 TRFs and harbored distinct 65-, 67-, 91-, 133-, and 207-bp TRFs that were found exclusively in the water column. Some TRFs were consistently found only in sponge samples and not in the water column. For instance, for all of the *M. incrustans* samples 233- and 420-bp TRFs were obtained, and for all of the *Haliclona rufescens* samples 40-, 174-, and 205-bp TRFs were obtained, irrespective of the sampling site. In contrast, no *H. panicea*-specific, *Mycale* sp.-specific, or site-specific TRFs were found.

The comparison of the TRFLP patterns for the bacterial community samples using cluster analysis indicated that all three replicate samples containing a particular bacterial com-

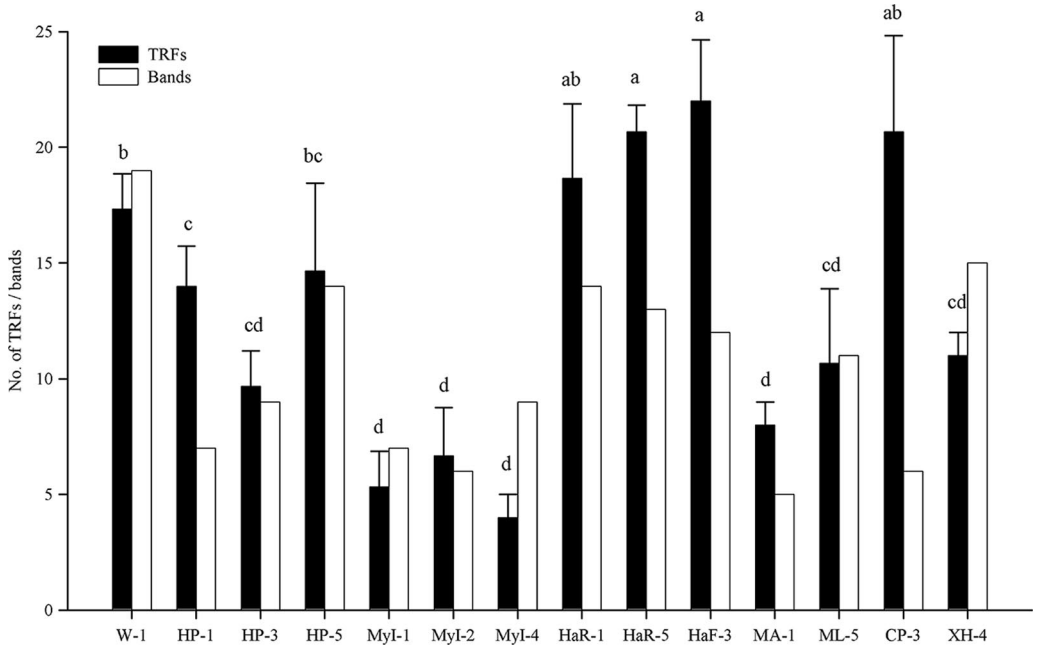

FIG. 1. Numbers of TRFs (three replicates) and DGGE bands generated using 16S rRNA amplicons from the bacterial communities associated with different sponges and in the water column. Samples for the water column analysis were collected at site 1 (Roche Harbor). Data that are significantly different at $\alpha = 0.05$ in a one-way analysis of variance followed by a Dunnett test are indicated by different letters above the filled bars. Table 1 explains sample abbreviations.

munity fell into one cluster, except for those derived from the sponge *M. incrustans* (Fig. 2). The bacterial communities associated with *M. incrustans* from different sites were closely related to each other, forming a cluster with >60% similarity. Although the six replicate samples from the two *H. rufescens* sponges were clearly separated by site into two clusters, the two clusters shared >60% similarity. In contrast, the bacterial communities associated with *H. panicea* from different locations fell into different clusters with <40% similarity. Bacterial communities from congeneric sponges (*Haliclona* spp. and *Mycale* spp.) did not cluster together (Fig. 2). Similarly, neither did bacterial communities from sponges at a specific site (Fig. 2).

DGGE profiles of bacterial communities. Since the bacterial communities in replicates were highly similar, as shown by TRFLP analysis, and the number of samples that can be loaded into a DGGE gel is limited, the replicate crude DNAs were pooled for DGGE analysis. The number of bands detected by the software ranged from 5 to 19; the fewest bands were obtained from the bacterial community associated with *Mycale adhaerens* collected at site 1, and the most bands were obtained from the seawater collected at site 1 (Fig. 1). Cluster analysis, which was performed based on the band patterns on the DGGE gel, indicated that the sponge-associated bacterial communities did not show spatial specificity, as the communities from one location fell into different clusters, which was in good agreement with the results of the TRFLP analysis (Fig. 2 and 3). Likewise, the cluster analysis based on the DGGE gel indicated that most of the sponge-associated bacterial communities, even those obtained from sponges at site 1, were substantially different from the communities in the water column, which was also in good agreement with the results of the TRFLP analysis. In contrast to the variation in the bacterial communities of *H. panicea* from different sites (HP-1, HP-3, and HP-5), conspecific *M. incrustans* (MyI-1, MyI-2, and MyI-4) and *H. rufescens* (HaR-1 and HaR-5) communities clustered closely together by species regardless of the site (Fig. 3); this result is similar to the results of the TRFLP analysis. In addition, neither the bacterial communities of congeneric *Mycale* spp. (MA-1 and ML-5) nor the bacterial communities of *Haliclona* spp. (HaR-1 and HaF-3) showed high similarity; rather, they were separated into distinct clusters. Again, this was shown by TRFLP analysis.

In order to identify possible sponge-specific bacteria, the nine dominant bands that were found only in the sponge samples (and not in the seawater sample) were excised from the DGGE gel for direct sequencing. Bands a and b were found in all specimens of the sponge *M. incrustans*, irrespective of the sampling site (Fig. 3), and their sequences were affiliated with an uncultured alphaproteobacterium from the sediment of a salt marsh in the United States and with a culturable *Bacillus* sp. from the seawater of Kievka Bay in Russia, respectively (Fig. 4). Similarly, all samples of *H. rufescens* shared the same dominant band, band c, which was affiliated with an uncultured gammaproteobacterium associated with the Mediterranean sponge *Axinella verrucosa*. Some other unique bands were found in other sponge samples, and all of them except band f were bands for uncultured *Proteobacteria* from various environments (Fig. 4). The sequences of five of the nine bands were closely related to sequences recovered from other sponges, including *A. verrucosa*, *Ircinia dendroides*, *Stylinos* sp., *Microciona* sp., and *Mycale acerata*.

Bacterial isolates from sponges and the water column. Altogether, 397 isolates were obtained from the sponge and water samples. The largest portion (53 isolates) was isolated from

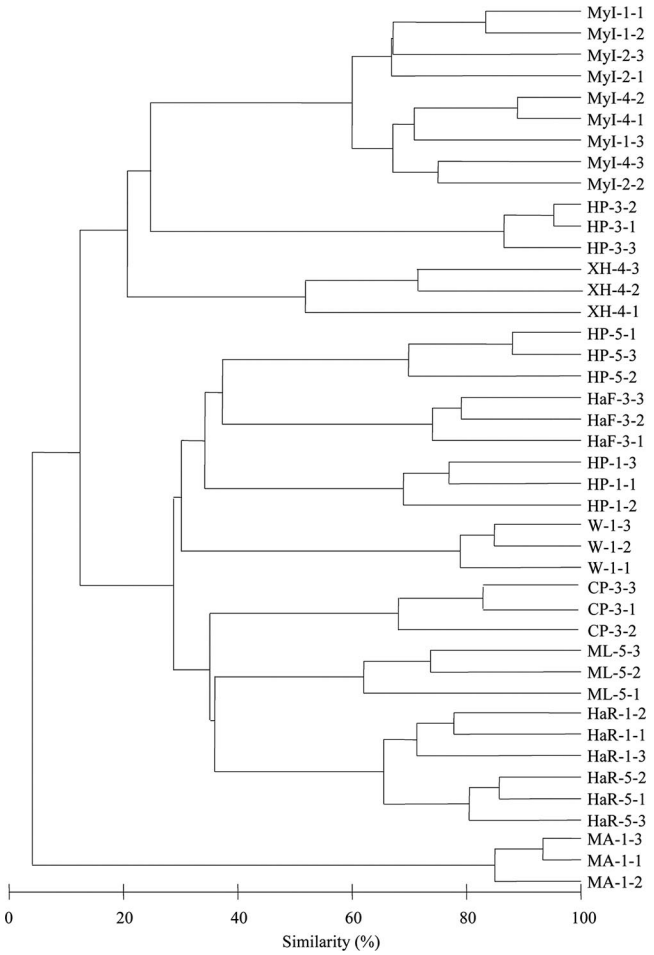

FIG. 2. Dendrogram derived from the TRFLP analysis, showing the relatedness of bacterial communities (three replicates each) associated with different sponges and in the water column. The dendrogram was constructed by using the Pearson correlation of similarity. Table 1 explains sample abbreviations.

the seawater samples, and 14 of the isolates, the smallest portion, were obtained from the sponge *M. incrustans* at site 2. Analysis of the partial 16S rRNA gene sequences of all the isolates (length of the aligned sequences, ~1,000 bp) using BLAST and the RDP classifier placed these isolates into 45 different genera in four phyla, the *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Fig. 5). Of the four phyla, *Proteobacteria* and *Firmicutes* were the most dominant groups, accounting for 71% (in MyI-2) to 100% (in XH-4) of the culturable bacterial communities, but the relative percentages varied substantially among samples (Fig. 5). For instance, >80% of bacteria isolated from the seawater samples belonged to *Proteobacteria*, but only 17% of them belonged to *Firmicutes*. In contrast, *Firmicutes*, especially the genus *Bacillus*, was the most dominant group in the sponge samples and could account for up to 82% of the culturable bacterial communities (in XH-4), and the percentage of *Proteobacteria* could be as low as 2% (in MA-1). In general, the number and type of bacteria isolated from conspecific or congeneric sponges varied substantially.

DISCUSSION

This study was the first study that compared the bacterial communities associated with different sponges collected around San Juan Island, Washington, using both culture-independent and -dependent methods in order to reveal the specificity of the sponge-bacterium association. The bacterial community in the water column was also studied at one site to illustrate the difference between the planktonic bacterial community and the sponge-associated communities. Generally, the bacterial community in the water column contained more diverse bacterial types and was substantially different from the sponge-associated bacterial communities. This was evident from the relatively large numbers of TRFs, DGGE bands, and isolates (Fig. 2 and 5) and from the distantly related clusters

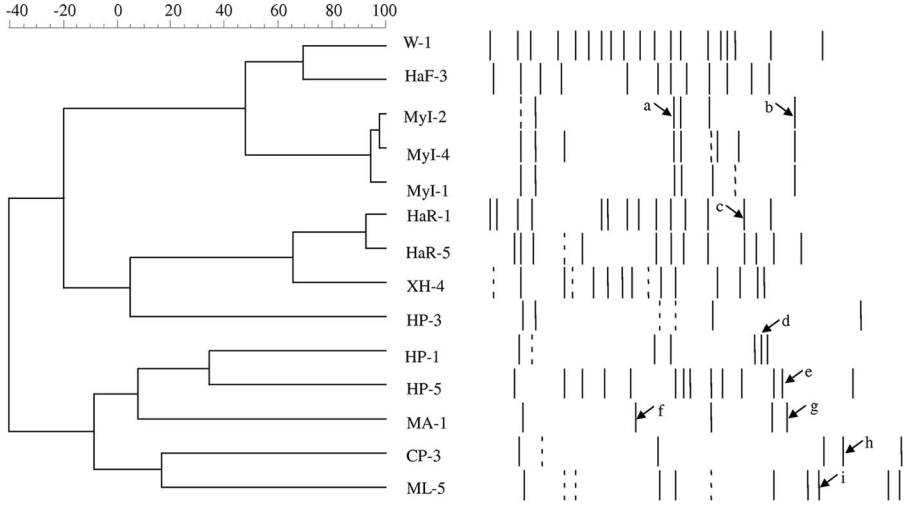

FIG. 3. Dendrogram (left panel) derived from DGGE profiles (right panel) of PCR-amplified 16S rRNA fragments from the bacterial communities associated with different sponges and in the water column at site 1. A solid line in the DGGE profiles indicates a band with a strong signal detected by the software, while a dashed line indicates a band with a weak signal. Arrows a to i indicate prominent and unique bands that were found in the sponge samples but not in the seawater sample. These bands were excised and sequenced for identification of bacteria (Fig. 4). The dendrogram was constructed by using the Pearson correlation of similarity. Table 1 explains sample abbreviations.

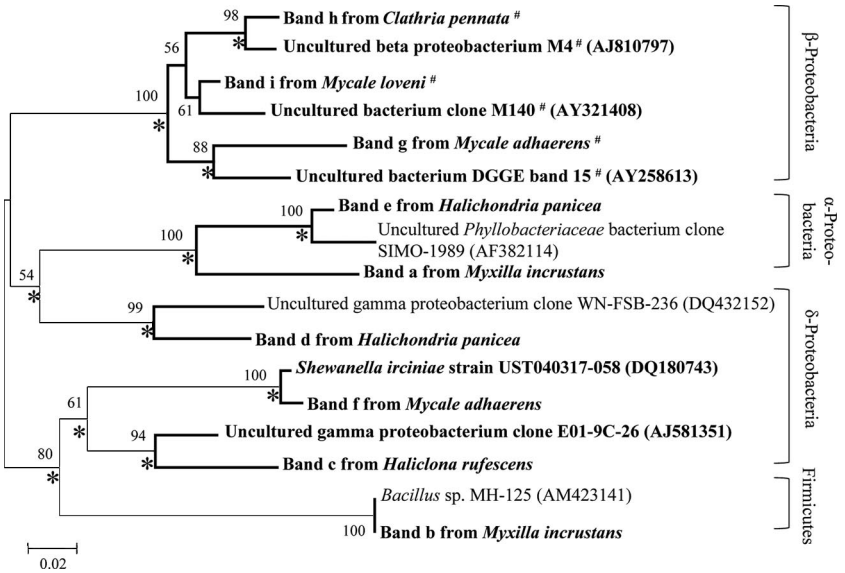

FIG. 4. Neighbor-joining tree based on 16S rRNA gene sequence comparisons, showing the estimated phylogenetic relationships between sequences retrieved from the DGGE bands (Fig. 3) and their closest relatives. Asterisks indicate nodes that are also present in a maximum-parsimony tree; the bold lines indicate branches that are also present in a tree constructed by the unweighted-pair group method using average linkages. Bootstrap values greater than 50%, based on 1,000 replicates, are indicated at the nodes. GenBank accession numbers are in parentheses. Sequences that fell into the sponge-specific clusters defined by Taylor et al. (44) are indicated by pound signs. Sequences retrieved from sponge-associated bacteria are indicated by bold type, while sequences retrieved from bacteria in seawater or sediments are indicated by light type. Bar, 2 nucleotide substitutions per 100 nucleotides.

shown by TRFLP and DGGE analyses (Fig. 3 and 4). Similarly, higher diversity of bacterioplankton was recorded in the Sargasso Sea and the waters around Hawaii, Concepción, and Fiji than in sponge-associated bacterial communities (15, 32). It is not surprising that, in general, the water column harbors a more diverse bacterial community than sponges harbor as it serves as both a sink and a source of bacteria for various microhabitats, such as sediment and biofilm, in the marine environment.

The number of bacterial types (or ribotypes) in the sponge samples, as evaluated by the number of TRFs and DGGE bands, varied among species (Fig. 2); *H. rufescens* and *M. incrustans* had the highest and lowest diversity of ribotypes, respectively, and *H. panicea* had various numbers of TRFs and DGGE bands depending on the site. At site 1, the sponge-associated bacterial communities were different in different species and were distinct from those in the water column (Fig. 3 and 4). Similarly, distinct bacterial communities in sponges and the ambient water have been found in previous investigations (18, 19, 37, 55). It is generally believed that sponge-associated microbes benefit from an enriched nutrient environment due to the release of secondary metabolites from their sponge hosts (8, 18). However, this benefit may result in an increase or decrease in bacterial diversity depending on how the microbes utilize the metabolites and how they interact with each other.

Close associations of a variety of microorganisms, especially bacteria, are found in many sponges. So far, 14 bacterial phyla from sponges have been recognized, and the most frequently recovered sequences belonged to the phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* (44). In our study, sequences belonging to the phyla *Proteobacteria* and *Firmicutes* and isolates belonging to the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* were retrieved from the sponge samples (Fig. 5), indicating that these phyla were present in our sponge-associated bacterial communities. Five of the nine bands selected from our sponge samples were affiliated with bacteria whose sequences were retrieved from other sponges. Phylogenetic analysis showed that three of these five bands (bands g, h, and i) fell into the sponge-specific sequence clusters defined by Taylor et al. (44). Our results further support the argument that some sponges from geographically separated regions share some specific bacteria and sponge-specific clusters do exist (16, 44). In addition, the low level of similarity of some of our sequences with sequences in the GenBank database indicated that there is an unexplored bacterial group in sponges and the potential of identifying novel bacteria. In fact, we have already identified two novel bacteria isolated from *M. incrustans* (23) and *H. panicea* (24).

When the bacterial communities in conspecific sponges were compared, highly similar bacterial communities were observed for certain sponge species. We demonstrated for the first time that both *H. rufescens* and *M. incrustans* from different locations harbored highly similar bacterial communities, as shown by both TRFLP and DGGE analyses (Fig. 3 and 4). This is the first study of the bacterial communities in these two species, while only one previous study examined the fungal community in *M. incrustans* (31). In contrast to the great consistency in the bacterial communities associated with these two species at different sites, the bacterial communities associated with individuals of *H. panicea* from different sites varied substantially (Fig. 3 and 4). Bacterial communities in *H. panicea* have been investigated in a number of studies (1, 20, 29, 41, 56). A group

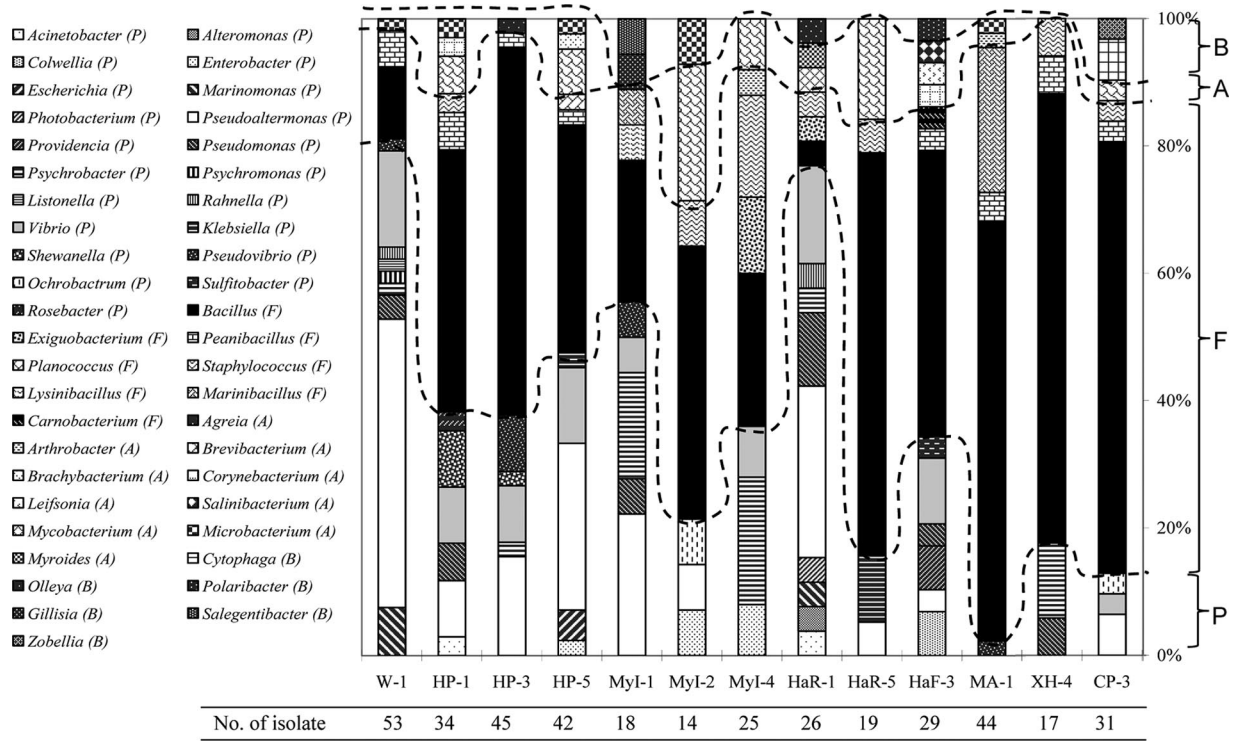

FIG. 5. Percentages of different genera recovered from the sponge and water samples. The phylogenetic affiliations of bacteria were determined by comparing the 16S rRNA gene sequences of the isolates with those deposited in the GenBank database. The letters in parentheses and on the right indicate phyla, as follows: P and *P*, *Proteobacteria*; F and *F*, *Firmicutes*; A and *A*, *Actinobacteria*; and B and *B*, *Bacteroidetes*. Dashed lines separate different phyla. Table 1 explains sample abbreviations.

of bacteria with 16S rRNA sequences closely related to sequences of the bacterial group *Roseobacter* was consistently recovered from geographically separated *H. panicea* sponges, yet different bacterial copopulations also occurred seasonally or on a small geographical scale with occasional dominance in this species. The two sequences retrieved from *H. panicea* in our study did not resemble any of the sequences previously recovered from this species. These findings indicated that there are considerable variations in the bacterial communities associated with *H. panicea* from different locations, and the previous results are in good agreement with our observations for the *H. panicea* samples.

It is not known why consistent bacterial communities were found in the sponges *M. incrustans* and *H. rufescens* but not in the sponge *H. panicea*. The type of bacteria present in a sponge depends on both environmental and sponge-related factors, such as structural organization and life history strategy (47). *M. incrustans* and *H. rufescens* are encrusting sponges commonly found on the surface of scallops that live in a defined geographical range and have a stable environment, while *H. panicea* occurs in a wide range of forms and locations and can be difficult to identify morphologically (R. von Soest, personal communication; our observations). Evolutionary history may lead to different degrees of specificity in the types of bacterial associates that a sponge can host. It is generally believed that there may have been three different evolutionary scenarios for the sponge-bacterium association, as summarized by Taylor et al. (44): vertical transmission, horizontal transmission, and environmental acquisition. At site 1, the sponge-associated bacterial communities varied among sponge species and were all different from the indigenous planktonic bacterial community; thus, neither purely environmental acquisition nor horizontal transfer between sponges can explain this observation perfectly. Vertical transmission of bacterial symbionts may explain our observations for *M. incrustans* and *H. rufescens* but not for other sponges. However, since the reproductive biology of these sponges is poorly understood and the host sponge phylogeny is still not clearly resolved, information on whether the sponge hosts and bacterial associates have coevolved is necessary to obtain further evidence of the symbiont transmission modes of these sponges.

As determined by both TRFLP and DGGE analyses, the bacterial communities in congeneric sponges (*M. adhaerens* and *M. loveni*; *H. rufescens* and *H. foraminosa*) were in different clusters (Fig. 3 and 4), indicating that there are substantial variations in the bacterial communities in the two genera. This is in good agreement with other studies which demonstrated that there are distinct and diverse bacterial communities in congeneric *Mycale* or *Haliclona* spp. (9, 25), providing further evidence that bacterial communities do not remain consistent in these genera.

In this study, both culture-dependent and culture-independent methods were employed to study the sponge-associated bacterial communities. TRFLP and DGGE allow easy and quick comparison of community profiles for microbial assemblages. Although these two methods resulted in slightly different clustering patterns (Fig. 3 and 4), the different patterns did not affect our broad overall conclusion that members of certain

sponge species from different locations shared highly similar and consistent bacterial communities, suggesting that some sponges might exhibit specificity in their associated bacterial communities. There were even larger discrepancies when the results obtained with culture-dependent and -independent methods were compared (Fig. 3 to 5). None of the sequences retrieved from excised DGGE bands resembled the sequences from our bacterial isolates. In addition, the sequences of only two isolates from our study most closely matched other sponge-derived sequences from previous studies. The limitations of the culture-dependent methodology (2, 20) may explain the observed differences for the methods used. However, using the same medium selective pressure, we showed that the culturable bacterial communities in the sponges were dominated by *Firmicutes*, especially *Bacillus*, and that they were very different from the culturable bacterial communities in the water column, in which *Proteobacteria*, especially *Pseudoalteromonas*, was the major group (Fig. 5). This supported our observation resulting from TRFLP and DGGE analyses that the bacterial community in the water column was very distinct from the bacterial communities associated with the sponges. One advantage of the culture method is that it allows isolation of pure strains for further experiments. It should be noted that, although clone libraries may help to identify sponge-specific bacteria, considering the scope of this study, the enormous work involved, and the complex communities analyzed, such libraries were not used in this study.

This study provided information on variations in bacterial communities within and between a variety of sponge species. Clearly, generalizations concerning the specificity of sponge-associated bacterial communities are risky since the degrees of specificity may be different for different sponges. Our results indicated that the bacterial communities in certain species of sponges, including *M. incrustans* and *H. rufescens*, from different locations were highly similar, suggesting that these sponges consistently host specific bacterial communities. The mechanism that the sponges employ to maintain this consistent association and over what scales the associations are maintained are still not known. Further studies of whether these associations are consistent for larger geographic scales, as well as studies of the mode of symbiont transmission, are currently under way.

ACKNOWLEDGMENTS

We thank Rob von Soest, Zoological Museum, University of Amsterdam, for identification of sponges, Y. K. Tam for assistance with TRFLP analysis, V. Thiyagarajan for comments on the manuscript, Shawn Arenallo for editing the manuscript, and Friday Harbor Laboratories for providing research facilities and technical support for field work and preliminary laboratory analysis.

This study was supported by a grant from the China Ocean Mineral Resources Research and Development Association (COMRRDA06/07.SC02) and the KAUST Global Academic Partnership Program (KAUST005-CML.07/08) to P.-Y. Qian.

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