APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2002, p. 4431–4440 Vol. 68, No. 9
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.9.4431–4440.2002


# Molecular Evidence for a Uniform Microbial Community in Sponges from Different Oceans

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Received 13 March 2002/Accepted 28 June 2002

**Sponges (class Porifera) are evolutionarily ancient metazoans that populate the tropical oceans in great abundances but also occur in temperate regions and even in freshwater. Sponges contain large numbers of bacteria that are embedded within the animal matrix. The phylogeny of these bacteria and the evolutionary age of the interaction are virtually unknown. In order to provide insights into the species richness of the microbial community of sponges, we performed a comprehensive diversity survey based on 190 sponge-derived 16S ribosomal DNA (rDNA) sequences. The sponges *Aplysina aerophoba* and *Theonella swinhoei* were chosen for construction of the bacterial 16S rDNA library because they are taxonomically distantly related and they populate nonoverlapping geographic regions. In both sponges, a uniform microbial community was discovered whose phylogenetic signature is distinctly different from that of marine plankton or marine sediments. Altogether 14 monophyletic, sponge-specific sequence clusters were identified that belong to at least seven different bacterial divisions. By definition, the sequences of each cluster are more closely related to each other than to a sequence from nonsponge sources. These monophyletic clusters comprise 70% of all publicly available sponge-derived 16S rDNA sequences, reflecting the generality of the observed phenomenon. This shared microbial fraction represents the smallest common denominator of the sponges investigated in this study. Bacteria that are exclusively found in certain host species or that occur only transiently would have been missed. A picture emerges where sponges can be viewed as highly concentrated reservoirs of so far uncultured and elusive marine microorganisms.**

Sponges (class Porifera) form one of the deepest radiations of the Metazoa, whose origins date back to the Precambrian more than 600 million years ago. Today, an estimated 9,000 living sponge species are found mostly on tropical reefs but also at increasing latitudes (8). Functionally, sponges share many features with unicellular protozoa, particularly with respect to nutrition, cellular organization, gas exchange, reproduction, and response to environmental stimuli (6, 8). Instead of organs or tissues, sponges possess amoeboid cells that move freely through the three-dimensional sponge matrix, termed the mesohyl. Nevertheless, sponges are true metazoans that can reach considerable size (1 m or more in height), particularly in tropical waters. Sponges are filter feeders that pump large volumes of water through a unique and highly vascularized canal system, leaving the expelled water essentially sterile (32, 51). Nutrients are acquired by phagocytosis of bacteria that are removed from the water column.

In addition to a transient seawater population serving as a food source, sponges harbor large amounts of bacteria in their tissues that can amount to 40% of their biomass (43, 44), exceeding that of seawater by two to three orders of magnitude (9). This population consists mostly of extracellular bacteria that are enclosed within the mesohyl matrix and that are physically separated from the seawater by contiguous host membranes, called the pinacoderm. Microorganisms are removed from the seawater passing through the canal system and transferred into the mesohyl interior. The anatomical structure of sponges demands that bacteria be transported through a host barrier (8, 52). Because sponge-bacteria interactions are widely distributed and, in some cases, specific to the host, it is generally believed that symbiotic interactions exist between sponges and microorganisms (11, 16, 27). Symbiotic functions that have been attributed to microbial symbionts include nutrient acquisition (45, 52), stabilization of the sponge skeleton (33), processing of metabolic waste (5), and secondary metabolite production (7, 37, 42). The latter aspect is of particular pharmaceutical and biotechnological interest, as many sponge-derived natural products may in fact be of microbial origin (16, 24). Several studies have examined the diversity of sponge-associated microbial communities by using cultivation-based approaches and revealed that the microbial communities can be quite different (35, 48, 53, 55). To date, only one study has employed 16S ribosomal DNA (rDNA) library construction to assess microbial diversity in sponges independent of the culturability of the associated microorganisms (49). Additionally, few eubacterial and archaeal sponge-derived 16S rDNA sequences have been deposited in public databases (1, 27).

With the availability of molecular tools for community anal-

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TABLE 1. Compilation of sponges from which 16S rDNA sequences have been recovered

| Sponge species | Taxonomic affiliation (Family/Order/Subclass)[a] | Depth and collection site (latitude; longitude) | GenBank accession no. | Reference |
|---|---|---|---|---|
| *Aplysina aerophoba* | Aplysinidae/Verongida/ Ceractinomorpha | 7–15 m, Banyuls sur Mer, France (42°29′ N; 03°08′ E) | AJ347025–AJ347088 | This study |
| *Theonella swinhoei* | Theonellidae/Lithistida/ Tetractinomorpha | 20–30 m, Western Caroline Islands, Palau (07°23′ N; 134°38′ E) | AF186410–AF186459 | This study |
| | | 5 m, Eilat, Israel (31°35′ N; 34°54′ E) | AF434939–AF434963 | This study |
| | | 15 m, Hachijo-jima Island, Japan (33°38′ N; 139°48′ E) | AF434964–AF434986 | This study |
| *Rhopaloeides odorabile* | Spongiidae/Dictyoceratida/ Ceractinomorpha | 13 m, Davies Reef, Australia (18°49′ S; 147°38′ E) | AF333519–AF333552 | 49 |
| *Halichondria panicea* | Halichondriidae/Halichondrida/ Ceractinomorpha | 15 m, Limski Canal, Croatia (45°07′ N; 13°39′ E) | Z88580–Z88591 | 1 |
| *Axinella mexicana* | Axinellidae/Halichondrida/ Ceractinomorpha | 10–20 m, Santa Barbara, Calif. (34°25′ N; 119°57′ E) | AY029297–AY029298,[b] U51469[b] | 27 |

[a] All sponges belong to the class Demospongiae.
[b] Archaeal sequences.

yses in microbial ecology, the area of sponge microbiology has gained new momentum. It is now possible to obtain phylogenetic information on complex microbial consortia, including those that have so far eluded cultivation efforts (2, 17, 19, 36). With this study we aim to provide general insights into the identity, diversity, and distribution patterns of sponge-associated microbes. The sponges *Theonella swinhoei* (order Lithistida) and *Aplysina aerophoba* (order Verongida) were chosen because they are phylogenetically only distantly related, have geographically restricted nonoverlapping distribution patterns, and contain different host-specific secondary metabolite profiles. The results presented herein surprisingly reveal a uniform, yet phylogenetically complex, microbial population in sponges from different oceans.

## MATERIALS AND METHODS

**Sponge collection.** *A. aerophoba* and *T. swinhoei* are both found on open reef bottoms and form morphologically similar colonies of individual, upright tubes. Specimens of the Mediterranean sponge *A. aerophoba* were collected by scuba diving at depths of 5 to 15 m off Banyuls sur Mer, France, in May 2000. *T. swinhoei* was collected by scuba diving at depths of 20 to 30 m off the Western Caroline Islands in the Republic of Palau in September 1998. Individual specimens were placed separately into plastic bags to avoid contact with air and brought to the surface. *T. swinhoei* from Japan and the Red Sea were provided by S. Matsunaga (University of Tokyo, Tokyo, Japan) and M. Ilan (Tel Aviv University, Tel Aviv, Israel), respectively, as ethanol-preserved samples (Table 1).

**16S rDNA library construction.** The sponges were kept individually in plastic bags containing natural seawater in the cold (4°C) until processing within a few hours after collection. Tissue samples were removed from the center with a sterilized cork borer (11 mm in diameter), and the exposed surface tissues were removed with a sterile scalpel blade. The tissue was rinsed three times in autoclaved artificial seawater (22). Additional cell separation was performed on *T. swinhoei* from Palau by a modified procedure of Bewley et al. (7). After removal of the surface layers with a sterilized scalpel, the endosome of a single specimen was processed with an Omega 1000 juicer. The sponge pulp was suspended in artificial seawater and sequentially filtered through a 500-μm-pore-size metal sieve and a 42-μm-pore-size nylon mesh (Tetko). Unicellular and filamentous bacteria were separated by repeated differential centrifugation in artificial seawater. Processed whole-sponge tissue, ectosomal tissue, and sorted unicellular and filamentous bacteria were subjected to DNA isolation as described below. Moreover, liquid chromatography-mass spectrometry analysis of an ethyl acetate extract of *T. swinhoei* verified the presence of swinholide A and theopalauamide (37).

Genomic DNA was extracted from liquid nitrogen-frozen sponge tissues by using the QIAamp tissue kit (Qiagen) and the Fast DNA Spin kit for soil (Q-Biogene, Heidelberg, Germany). Amplification of rDNA was performed with the eubacterial primers 27f and 1385r from *A. aerophoba* and with the eubacterial primers 27f and 1492r from *T. swinhoei* (20). The PCR cycling conditions for both primer pairs were as follows: initial denaturation (2 min at 95°C) followed by 30 cycles of denaturation (1 min at 95°C), primer annealing (1 min at 50°C), and primer extension (1.5 min at 72°C) and a final extension step (10 min at 72°C). DNA was ligated into the pGEM-T-easy vector (Promega) and the TA cloning kit (Invitrogen) and transformed in $CaCl_2$-competent *Escherichia coli* DH5α. Plasmid DNA was isolated by standard miniprep procedures, and the correct insert size was verified by using agarose gel electrophoresis following restriction digestion (34).

**Sequencing and phylogenetic analysis.** Sequencing was performed on a LiCor 4200 automated sequencer (LiCor, Inc., Lincoln, Nebr.) and on an ABI 377XL automated sequencer (Applied Biosystems) with the M13universal and M13reverse sequencing primers and the 16S rDNA-specific primers 519f and 907r. Sequence data were edited with Chromas, version 1.51 (Technelysium), and ABI Prism Autoassembler, version 2.1 (Perkin Elmer), software and checked for possible chimeric origins (CHECK_CHIMERA software of the Ribosomal Database Project). Phylogenetic analyses were performed with the ARB software package (www.arb-home.de). Complete sequences of the 16S rDNA fragments were determined for representative clones selected on the basis of initial neighbor-joining trees. Initially, trees were calculated with 16S rDNA sequences (>1,000 bp in length only) by using the neighbor-joining (Jukes-Cantor correction), maximum parsimony, and maximum likelihood methods implemented in ARB. Partial sequences were added subsequently to the respective trees without changing their topology by use of the ARB parsimony interactive method. A selection of (at least) 145 near full-length 16S rDNA sequences representing all bacterial and archaeal phyla was used as the outgroup in all tree calculations. Taxonomic nomenclature was used according to *Bergey's Manual of Systematic Bacteriology* (5a).

**Nucleotide sequence accession number.** The 16S rRNA gene sequences were deposited in GenBank (Table 1) and given accession numbers AF186410 to AF186459, AF434939 to AF434986, and AJ347025 to AJ347088.

## RESULTS

**16S rDNA diversity within sponges.** Altogether, 160 clone sequences were recovered in this study from the sponges *A. aerophoba* collected from the Mediterranean (64 clone sequences with the prefix TK) and *T. swinhoei* collected from Palau (51 clone sequences with the prefix PA), the Red Sea (25 clone sequences with the prefix RS), and the coast of Japan (20 clone sequences with the prefix JA). Three sequences were discarded as chimeras. Additional sponge-derived 16S rDNA sequences from *Rhopaloeides odorabile* (with the prefixes R [49] and NWCu [50]) and *Halichondria panicea* (1) were included for comparison from the GenBank database. Figure 1 provides an overview of the phylogenetic relationships of

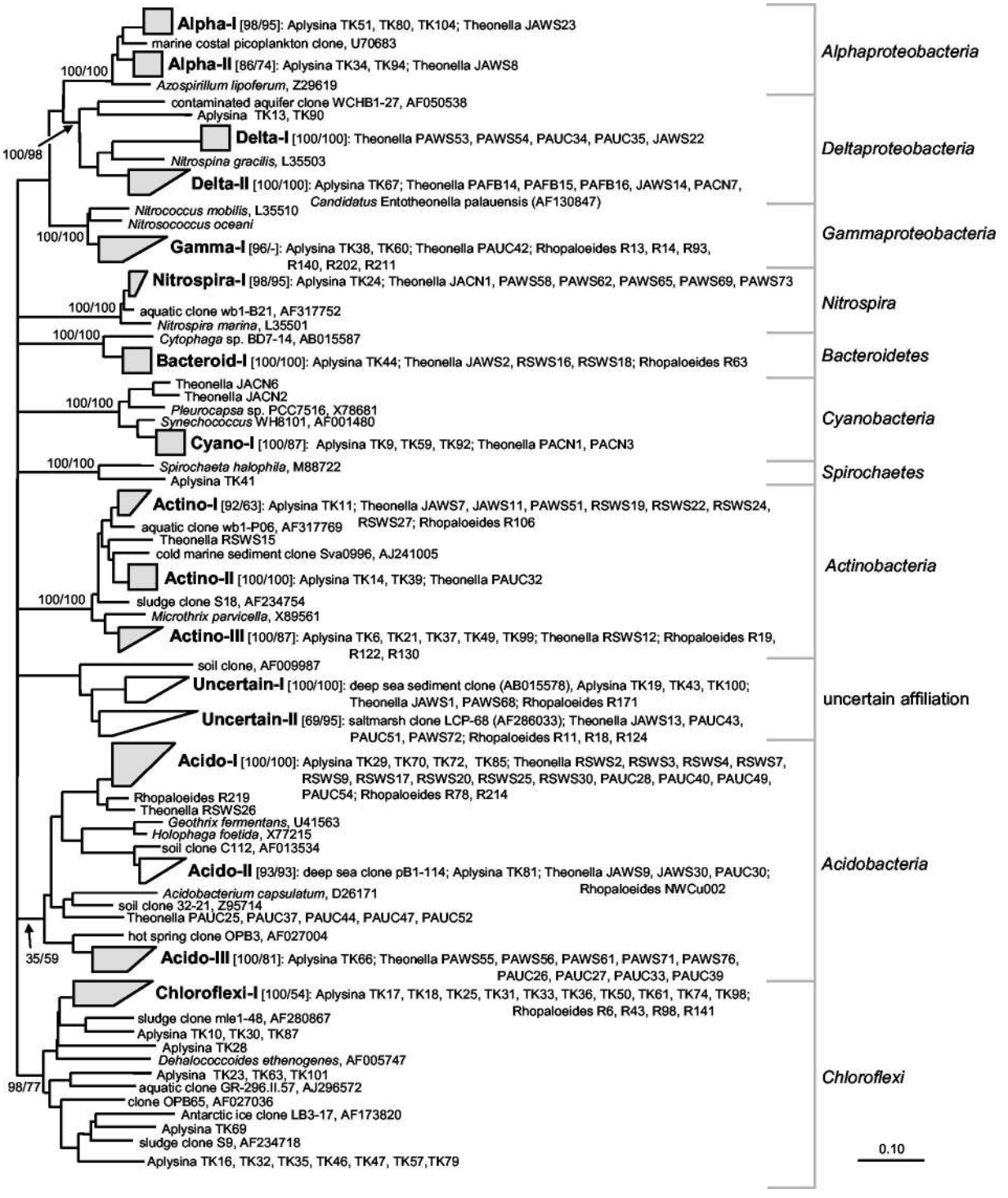


FIG. 1. Phylogenetic dendrogram calculated with all publicly available 16S rDNA sequences that were recovered from marine sponges. Multifurcations indicate that the respective branching order could not be unambiguously resolved by different treeing methods. Parsimony and neighbor-joining bootstrap values are provided for relevant groups and the sponge-specific clusters (square brackets). The scale bar indicates 10% sequence divergence. The tetragons (squares and fans) depict monophyletic clusters (shaded shapes) and those that contain additional environmental sequences (open shapes). The differences in the lengths of the horizontal lines of each tetragon represent the degree of sequence divergence within each phylogenetic cluster.

sponge-associated bacteria while Fig. 2 to 6 show individual division-level trees. The majority of all sponge-derived sequences are related to the *Acidobacteria* division ($n = 44$; 23%) and the *Chloroflexi* ($n = 42$; 22%). Clones affiliated with the *Actinobacteria* ($n = 24$; 12%), *Alphaproteobacteria* ($n = 13$; 7%), *Gammaproteobacteria* ($n = 20$; 10%), *Deltaproteobacteria* ($n = 15$, 8%), *Cyanobacteria* ($n = 7$; 4%), and the phylum *Nitrospira* ($n = 7$; 4%) were also abundant. Sequences related

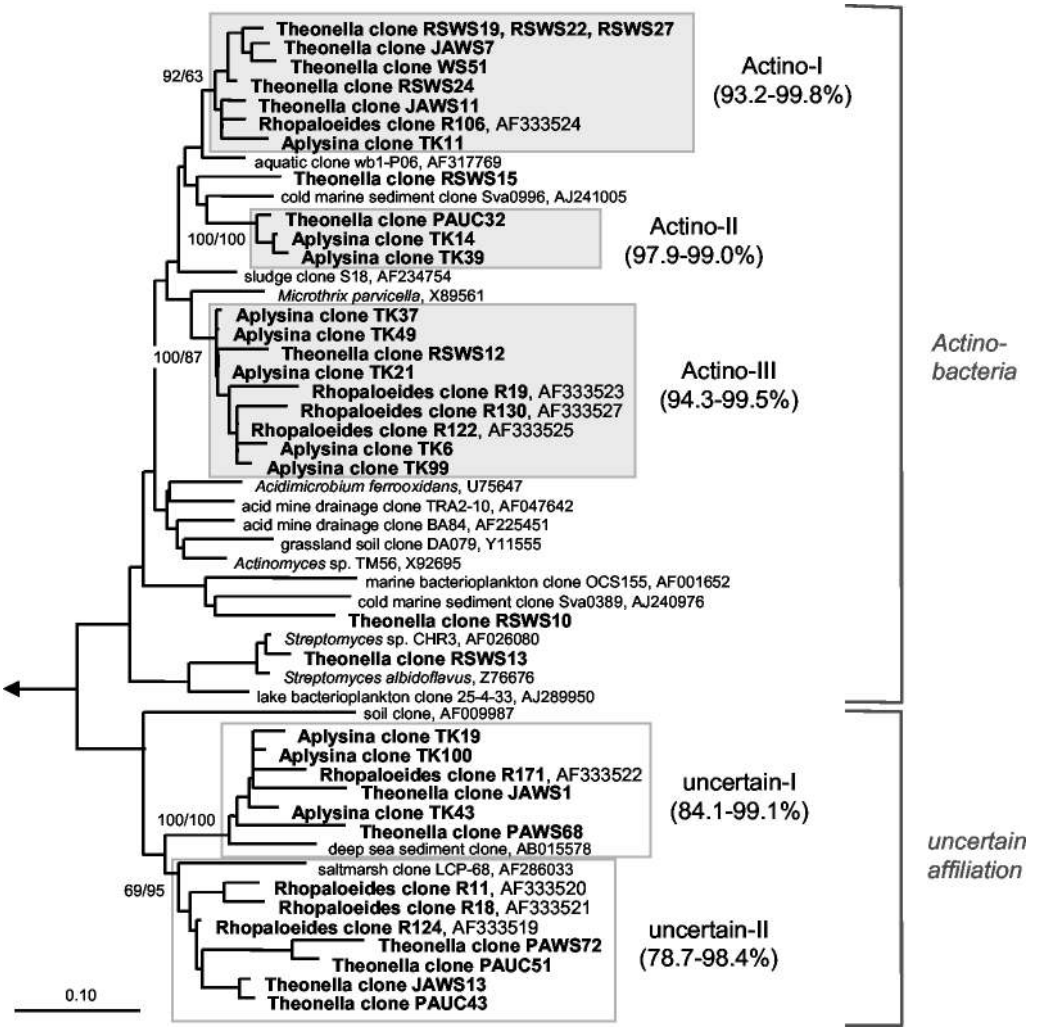


FIG. 2. Phylogenetic dendrogram calculated with 16S rRNA sequences affiliated with the phylum *Actinobacteria* and sequences of uncertain affiliation that were recovered from marine sponges. The boxes depict monophyletic sequence clusters (shaded boxes) and those that contain additional environmental sequences (open boxes). Parsimony and neighbor-joining bootstrap values are given for sponge-specific clusters. The scale bar indicates 10% sequence divergence. Arrow, to outgroup.

to the *Bacteroidetes* ($n = 5$; 3%) and the class *Spirochaetes* ($n = 1$; 0.5%) were only minor components of the gene libraries. The affiliation of several deep-branching clones belonging to the domain *Bacteria* ($n = 13$; 7%) could not be resolved unambiguously. Coverage estimates with a 95% 16S rDNA sequence similarity threshold for the definition of an operational taxonomic unit revealed that approximately 60 and 58% of the diversity in the gene libraries of *A. aerophoba* and the Palauan *T. swinhoei*, respectively, were harvested (38). More than two thirds of all sponge-derived 16S rDNA sequences (68%) showed less than 90% homology to their nearest sequence relatives from nonsponge sources, indicating the occurrence of many previously unrecognized bacteria within these animals.

**Sponge-specific 16S rDNA sequence clusters.** In this study, a sponge-specific, monophyletic 16S rDNA cluster is defined by the following criteria: a group of at least three sequences that (i) have been recovered from different sponge species and/or from different geographic locations, (ii) are more closely related to each other than to any other sequence from nonsponge sources, and (iii) cluster together independent of the treeing method used. Altogether, 70% of all sponge-derived 16S rDNA sequences belong to a phylogenetic cluster. Some clusters have high intracluster similarities exceeding 98% (*Nitrospira*-I and Cyano-I) while others show intracluster similarities below 85% (Gamma-I, Delta-I, Delta-II, and Chloroflexi-I). Most of the *Actinobacteria*, *Cyanobacteria*, *Acidobacteria*, and *Deltaproteobacteria* and all of the *Nitrospira* and *Bacteroidetes* sequences belong to sponge-specific clusters. In contrast, only about half of the *Gammaproteobacteria* and the *Chloroflexi* sequences are affiliated with sponge-specific clusters. Five sequence clusters are present in each of the 16S rDNA clone libraries from *A. aerophoba*, *T. swinhoei*, and *R. odorabile* (Fig. 7).

## DISCUSSION

The implementation of the 16S rDNA approach has revolutionized the field of microbial ecology. With the use of the

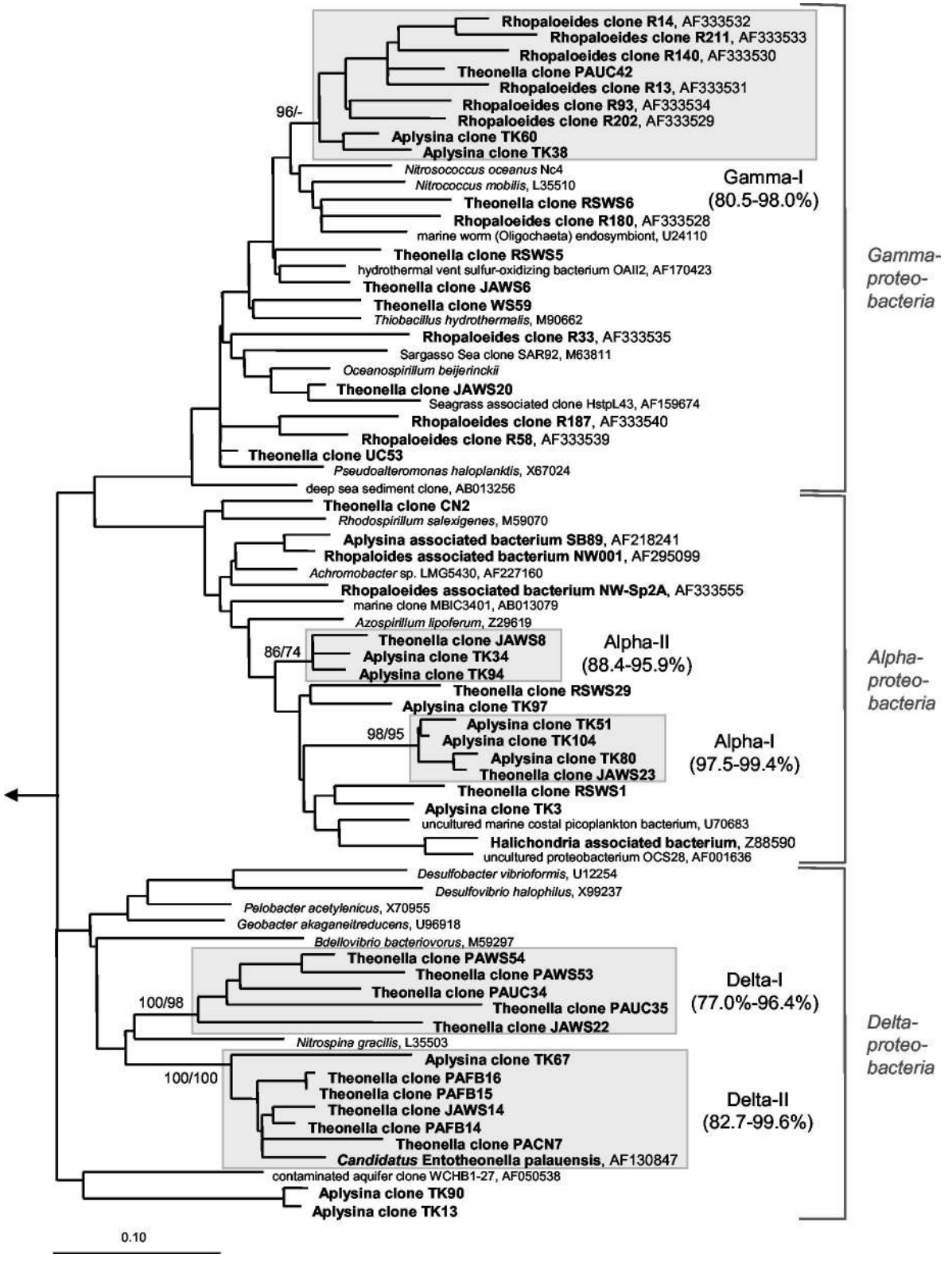


FIG. 3. Phylogenetic dendrogram calculated with 16S rRNA sequences affiliated with the phylum *Proteobacteria* that were recovered from marine sponges. The boxes show monophyletic sequence clusters. Parsimony and neighbor-joining bootstrap values are given for sponge-specific clusters. The scale bar indicates 10% sequence divergence. Arrow, to outgroup.

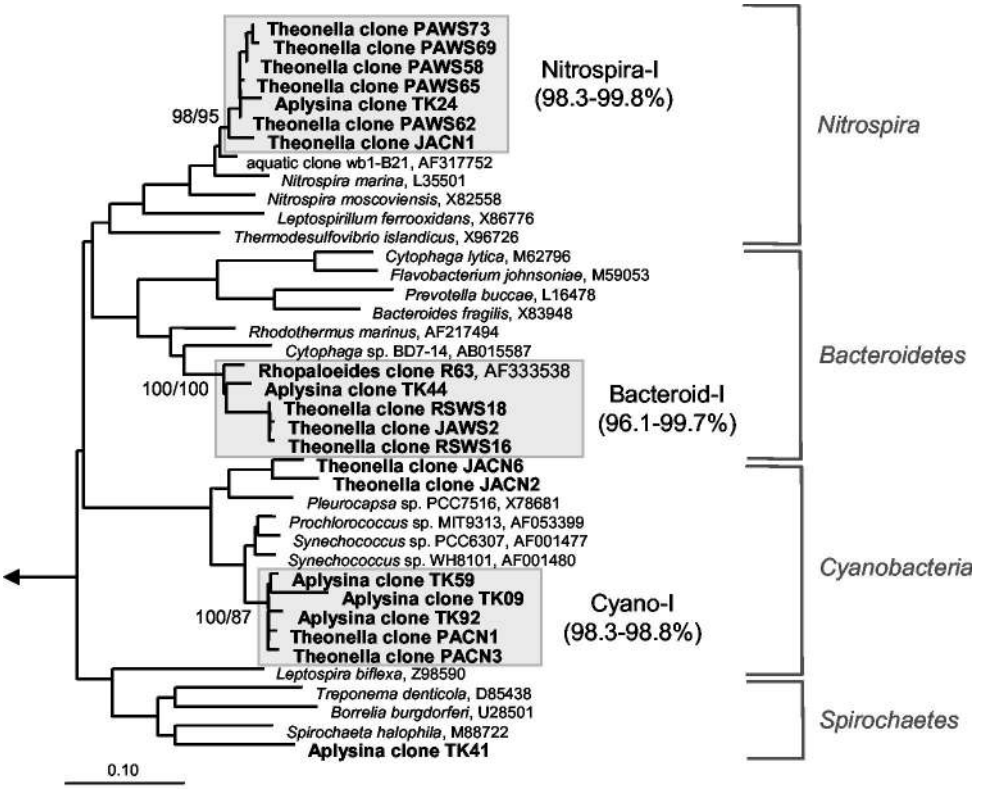


FIG. 4. Phylogenetic dendrogram calculated with 16S rRNA sequences affiliated with *Nitrospira*, *Bacteroidetes*, *Cyanobacteria*, and *Spirochaetes* that were recovered from marine sponges. The boxes show monophyletic sequence clusters. Parsimony and neighbor-joining bootstrap values are given for sponge-specific clusters. The scale bar indicates 10% sequence divergence. Arrow, to outgroup.

16S rDNA gene as a phylogenetic marker, it has become possible to determine the precise phylogenetic position of environmental bacterial populations in the evolutionary tree of life independent of their culturability and to trace them in complex ecosystems. Taking the inherent limitations of the PCR-based approach into account (47), it still represents a powerful tool for assessing the phylogenetic diversity of a complex microbial assemblage. The application of these techniques to environmental samples revealed a previously unseen microbial diversity (18, 25) that encompasses an estimated >99% of the total microbial community of a given habitat (2). The discovery of this large pool of yet uncultured bacteria in environmental samples is considered a milestone of environmental microbiology.

One of the surprising findings that has come out of this study is the discovery of a sponge-specific, yet phylogenetically diverse, microbial community (Fig. 1 to 6). The phylogenetic signature of the sponge-associated microbial consortium is distinctly different from that of typical seawater (13, 14, 29, 30). Considering that more than 600 16S rDNA sequences have been recovered from seawater, making this probably the largest environmental 16S rDNA database available, the apparent lack of overlap with sponge clone libraries is striking. Evidently, the sponge environment must impose strong selective pressures on the microbial community to account for the differences to planktonic bacteria. In contrast to what one might have anticipated from classical symbioses, the number of potential symbionts exceeds those of typical symbiotic interactions and has an impressive diversity. Altogether, 14 different, monophyletic, sponge-specific sequence clusters belonging to seven bacterial divisions were discovered. Members of five of these clusters are present in each of the three sponge species from which 16S rDNA libraries have been constructed (Fig. 7). With regard to complexity, the microbiology of the ecosystem sponge resembles more adequately the beneficial assemblages of rumen (40), the mammalian gut (28), or the squid nidamental gland (3) than those of the intimate symbioses commonly observed with invertebrate hosts (reviewed in reference 39).

In searching for commonalities, the smallest common microbial denominator of the sponges investigated was identified. This does not preclude the existence of bacteria that are specific to certain host sponges or those that occur only transiently or seasonally. For example, many of the *Chloroflexi* sequences recovered from *A. aerophoba* are not shared with any of the sponges investigated. It is conceivable that these sequences are specifically associated with *A. aerophoba* or with *Aplysina* sponges. Preliminary analysis of *A. aerophoba* tissue sections by fluorescent in situ hybridization reveals that bacteria belonging to the *Chloroflexi* are very abundant in *A. aerophoba* tissues. More-detailed studies are currently under way to evaluate the quantitative contribution of specific sequence clusters to the total microbial population of host sponges.

The question arises as to which evolutionary mechanism results in the formation of sponge-specific microbial communities. Since sponges form one of the earliest radiations of metazoan evolution, sponge-bacteria interactions could princi-

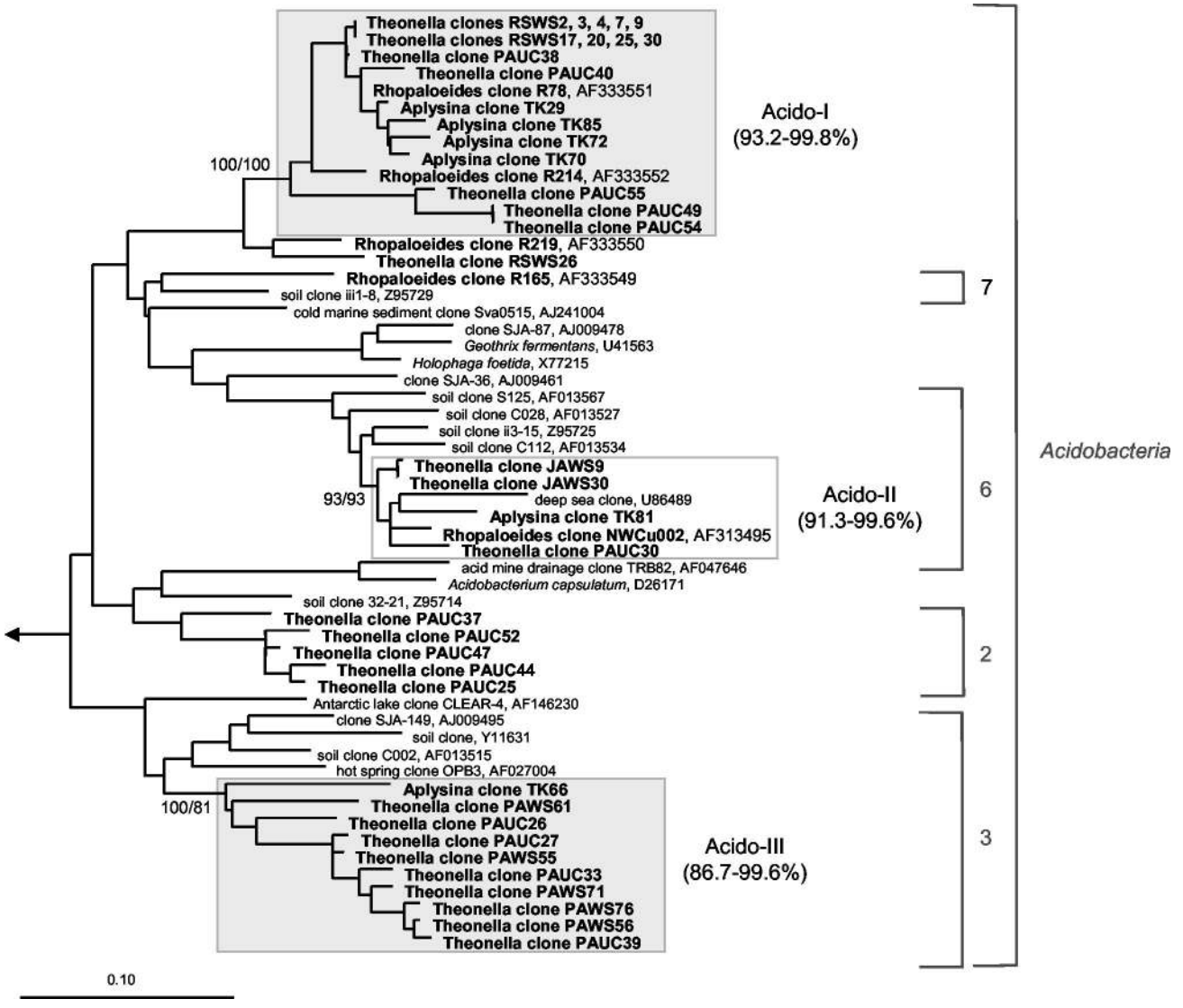


FIG. 5. Phylogenetic dendrogram calculated with 16S rRNA sequences affiliated with *Acidobacteria* that were recovered from marine sponges. The boxes depict monophyletic sequence clusters (shaded boxes) and those that contain additional environmental sequences (open boxes). Parsimony and neighbor-joining bootstrap values are given for sponge-specific clusters. The scale bar indicates 10% sequence divergence. Arrow, to outgroup.

pally result from an evolutionarily ancient multiple symbiotic integration event. As such, sponges could be reservoirs of evolutionarily ancient bacteria. The data retrieved in this study do not support this hypothesis since the sponge-specific sequence clusters are generally not deeply branching within their divisions. One exception is the Delta-II sequence cluster which represents an comparatively early separation within the *Deltaproteobacteria* (Fig. 3). This might reflect a long-standing existence within sponge tissues. This cluster also contains the 16S rDNA sequence of the filamentous candidate bacterium *Entotheonella palauensis*, which is visually abundant in the tissues of *T. swinhoei* (37). Most other sponge-specific sequence clusters, such as *Nitrospira*-I, have only recently separated from their free-living relatives (Fig. 4). It is also possible that the sponge microbial consortium contains a mixture of evolutionarily ancient, permanently associated bacteria and those that are acquired horizontally from the water column.

Symbioses in general can be identified by certain unifying features. Coevolution between a host and symbionts is a parameter that is particularly evident in ancient symbioses (26; reference 4 and references cited therein). In these model systems, a given host generally houses a single or a few symbiotic species. With regard to host specificity in sponge-microbe interactions, the presented data do not conform to the paradigm of a classical symbiosis. An explanation for the observed lack of specificity may lie in the particular reproduction of sponges, which includes both sexual and asexual strategies (8). Bacteria have in fact been observed in the reproductive stages, such as the oocyte stage (12, 21, 45), which is generally considered an indicator for symbiosis. However, sponges are also capable of

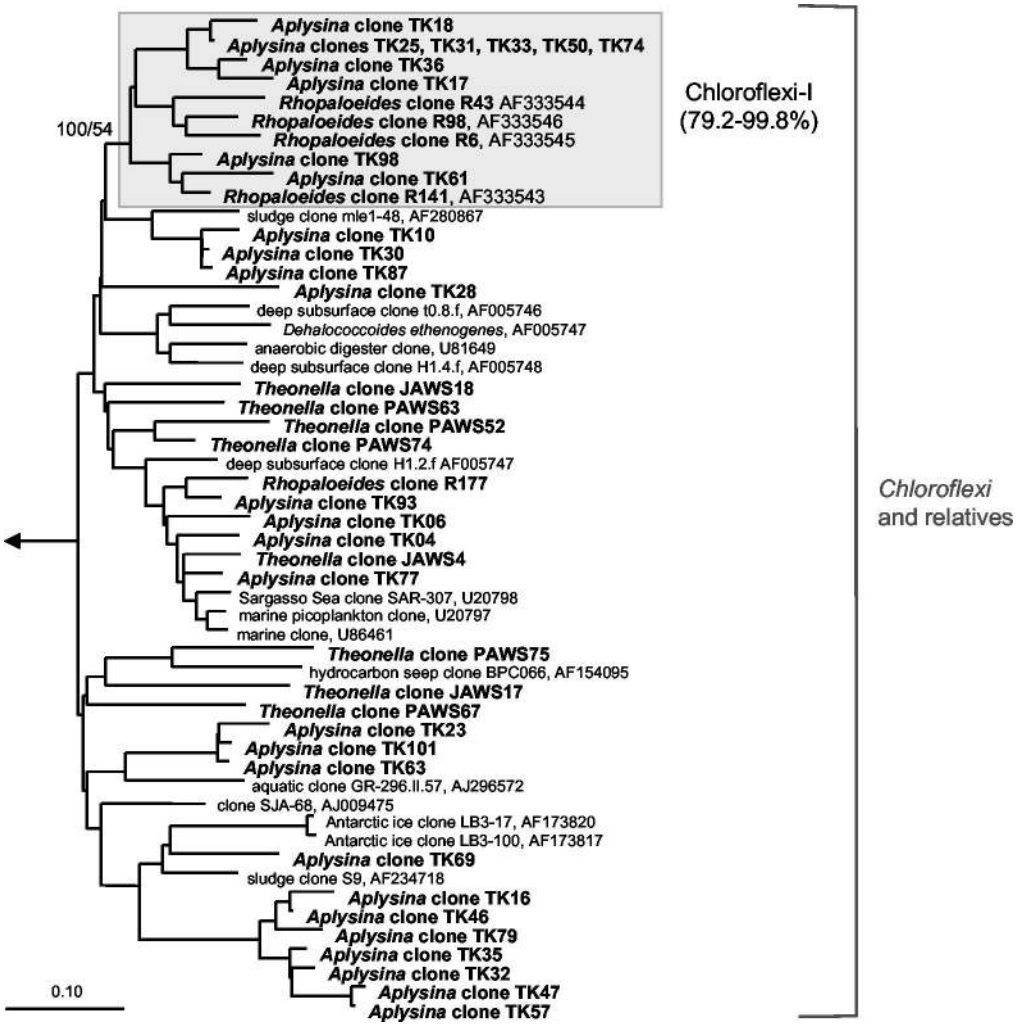


FIG. 6. Phylogenetic dendrogram calculated with 16S rRNA sequences affiliated with *Choroflexi* that were recovered from marine sponges. The box shows a monophyletic sequence cluster. Parsimony and neighbor-joining bootstrap values are given for sponge-specific clusters. The scale bar indicates 10% sequence divergence. Arrow, to outgroup.

asexual reproduction via the formation of gemmules, buds or branches that can develop into viable adults elsewhere. Asexual reproductive stages may act as vehicles by which multiple bacteria are transmitted vertically from generation to generation without being exposed to the stringent conditions that accompany transmission via the germ lines. It is also conceivable that convergent evolution played a role in shaping the microbial community of sponges. Convergent evolution defines the development of similar structures in phylogenetically unrelated organisms as a result of adapting to the same environment. Accordingly, if the sponge-specific bacteria of different phylogenetic divisions have populated the mesohyl for long periods of time, similar structures may have evolved in distantly related phylogenetic clusters to accommodate their existence in sponge tissues.

As an alternative explanation to evolution, the sponge-specific clusters may result from selective enrichment of specific bacterial types from the marine environment. Sponges are known for their immense filtration capacities. A specimen of 1 kg is capable of filtering 24,000 liters (24 $m^3$) of seawater per day, an accomplishment which is unsurpassed in the animal kingdom (46). If the isolates of sponge-specific clusters occur in the environment, they must be widespread but probably occur only at very low abundances, which could explain why they have been missed in seawater clone libraries. Because monophyletic 16S rDNA sequence clusters have also been documented in seawater (23, 29, 30), freshwater lakes (15), and marine sediments (31), the presence of monophyletic lineages in sponges can be principally explained without the necessity of host contact.

The mechanisms that may promote selective enrichment in sponges are intriguing. So far there is no evidence that the characteristic secondary metabolite profiles of the sponges *A. aerophoba* (brominated alkaloids), *T. swinhoei* (peptides and polyketides), and *R. odorabile* (diterpenes) have an effect on that fraction of the microbial community that is shared by different sponge species. Selective filtration by the sponge should be considered, since parameters such as the size of the ingested particles affect their clearing rates (41, 51). The fate of the microorganisms will also be determined by their turnover

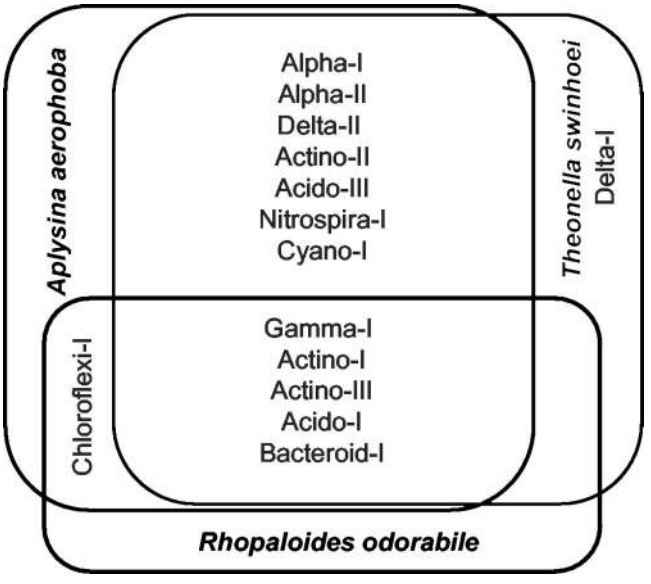


FIG. 7. Distribution of monophyletic 16S rDNA sequence clusters between three marine sponges.

rates in the mesohyl tissue. Electron microscopy revealed that the most abundant bacterial morphotypes contain thickened cell walls, multiple membranes, and slime capsules, which probably serve as barriers and shields to prevent phagocytosis by sponge archaeocytes (10, 54). In addition to resisting clearance, certain bacteria may be able to take advantage of this niche, for example, if syntrophic interactions exist between different bacteria or if the sponge provides specific nutrients that are lacking in the oligotrophic tropical waters.

A picture emerges in which sponges can be viewed as reservoirs that are highly concentrated in yet uncultured, elusive marine microorganisms. While it is generally believed that symbiotic interactions exist between sponges and specific microorganisms, alternative explanations, such as selective enrichment of ubiquitous seawater bacteria, should be considered. Nevertheless, highly specific selective pressures, possibly the resistance to digestion, must exist to account for the uniform composition patterns of the microbial communities present in sponges that have otherwise few commonalities. With the comparative 16S rDNA approach, global, ocean-spanning, sponge-specific microbial communities were discovered.

## ACKNOWLEDGMENTS

We are indebted to D. J. Faulkner and his group (Scripps Institution of Oceanography) for valuable assistance and helpful discussions, to S. Matsunaga (University of Tokyo) and M. Ilan (Tel Aviv University) for providing *T. swinhoei*, and to T. Bickert (University of Würzburg) for assistance in 16S rDNA library construction from *A. aerophoba*. Field work was assisted by the staff of the Coral Reef Research Foundation (Palau) and the Laboratoire Arago (France).

This research was generously supported by grants to B.S.M. (Washington and the National Sea Grant programs, project no. R/B-28 and R/B-39), U.H. (BMB+F 03F0239A and SFB567), and M.W. (WA1047/2-2) and by postdoctoral fellowships to J.N.H. (DFG and DAAD).

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