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Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential†

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INTRODUCTION

Marine sponges represent a significant component of benthic communities throughout the world, in terms of both biomass and their potential to influence benthic or pelagic processes (73, 74, 124, 220). Sponges (phylum Porifera) are among the oldest of the multicellular animals (Metazoa) and possess relatively little in the way of differentiation and coordination of tissues (26, 371). They are sessile, filter-feeding organisms which, despite a simple body plan, are remarkably efficient at obtaining food from the surrounding water (290, 308, 443). The more than 6,000 described species of sponges inhabit a wide variety of marine and freshwater (somewhat more restricted) systems and are found throughout tropical, temperate, and polar regions (167). Sponges have been the focus of much recent interest (Fig. 1) due to the following two main (and often interrelated) factors: (i) they form close associations with a wide variety of microorganisms and (ii) they are a rich source of biologically active secondary metabolites. This increasing research interest has greatly improved our knowledge of sponge-microbe interactions, and yet, as apparent throughout this article, many gaps remain in our knowledge of these enigmatic associations. For example, we still lack a clear picture of microbial diversity—and the factors which influence it—in these hosts. Similarly, the physiology of most sponge-associated microorganisms remains unclear, as do many fundamental aspects of sponge symbiont ecology. (Throughout this article, the terms "symbiont" and "symbiosis" are used in their loosest possible definitions, to refer simply to two [or more] different organisms that live together over a long period

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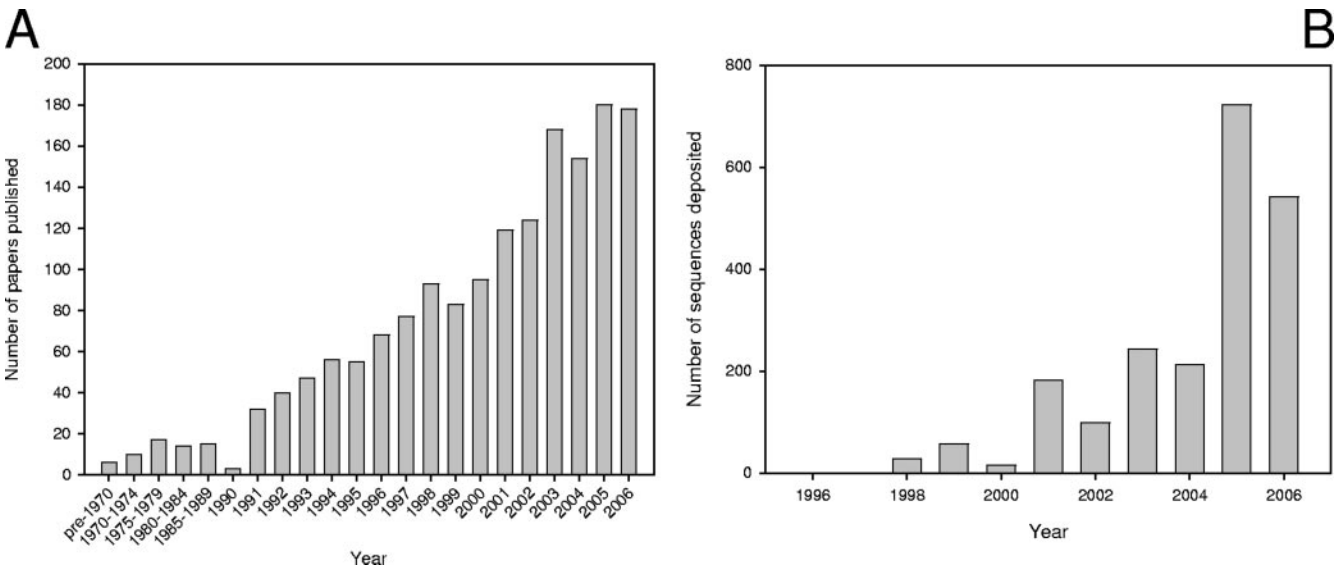

FIG. 1. Increasing research interest in marine sponge-microorganism associations. (A) Number of publications retrieved from the ISI Web of Science database by using the following search string: (sponge* or porifera* or demospong* or sclerospong* or hexactinellid*) and (bacteri* or prokaryot* or microbe* or microbial or microorganism* or cyanobacteri* or archaeon or archaea* or crenarchaeo* or fung* or diatom* or dinoflagellate* or zooxanthella*) not (surgery or surgical). (B) Number of sponge-derived 16S rRNA gene sequences deposited in GenBank per year. The 2006 value includes the 184 sequences submitted to GenBank from this article. The search string used to recover sequences was as follows: (sponge* or porifera*) and (16S* or ssu* or rRNA*) not (18S* or lsu* or large subunit or mitochondri* or 23S* or 5S* or 5.8S* or 28S* or crab* or alga* or mussel* or bivalv* or crustacea*).

of time, similar to the original de Bary definition. No judgment is made regarding benefit to either partner.) Here we aim to provide a comprehensive review of the current knowledge of the evolution, ecology, and biotechnological potential of sponge-microbe associations.

We begin with an introduction to the host organism. The phylum Porifera is a paraphyletic grouping consisting of three major sublineages (classes), namely, the Hexactinellida (glass sponges), Calcarea (calcareous sponges), and Demospongiae (demosponges), with the last group containing the majority of extant species (38, 167). Sponge architecture is unlike that for any other taxon, and sponge morphology greatly affects many aspects of sponge biology, including interactions with microorganisms. The basic body plan comprises several different cell layers (Fig. 2) (371). The outer surface, or pinacoderm, is formed by epithelial cells known as pinacocytes. Through pores

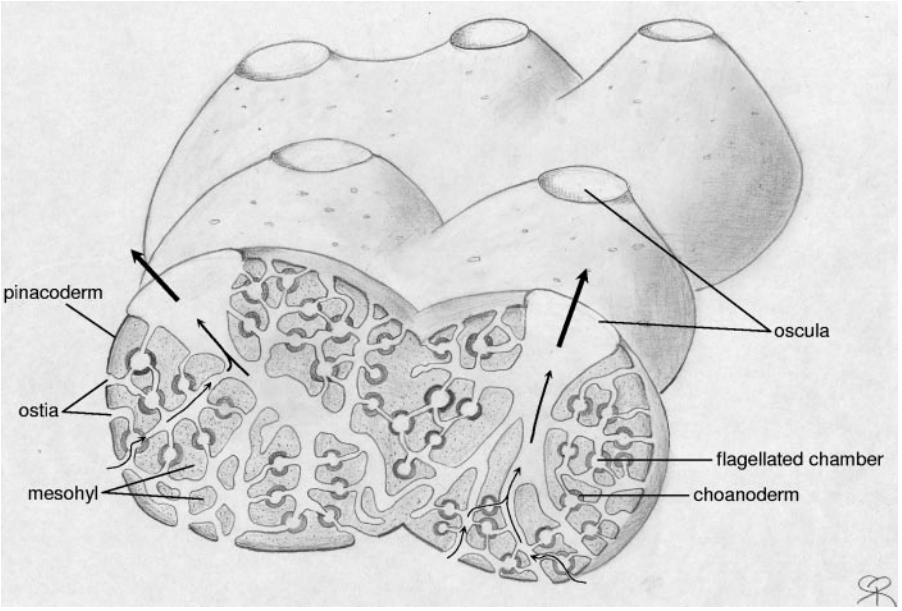

FIG. 2. Schematic representation of a sponge. Arrows indicate the direction of water flow through the sponge. (Adapted from reference 328 with permission of Brooks/Cole, a division of Thomson Learning.)

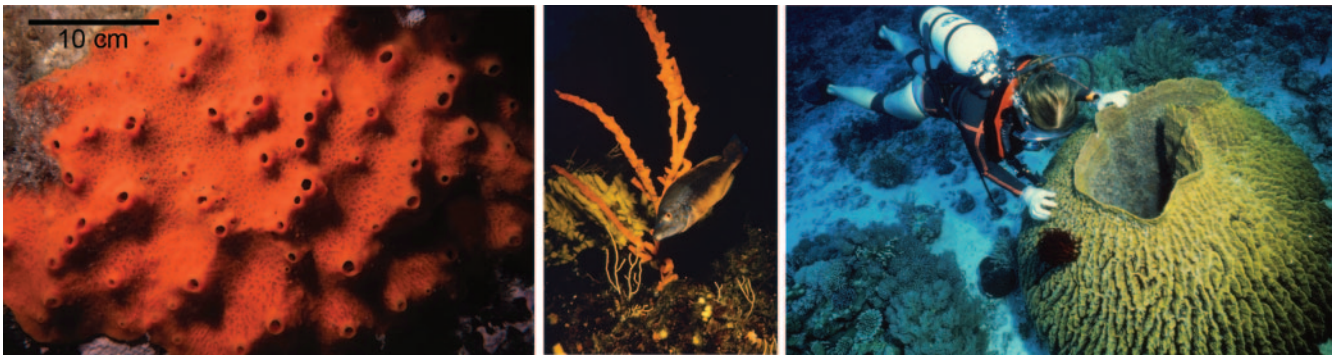

FIG. 3. Sponges of diverse size, shape, and color. The encrusting sponge *Tedania digitata* (left), the branching sponge *Axinella cannabina* (center), and the giant barrel sponge *Xestospongia testudinaria* (right) are shown. The last two images were kindly provided by Armin Svoboda (Ruhr-Universität, Bochum, Germany).

(ostia) on the sponge surface, these cells also extend along the interior canals which permeate the sponge. Inside the sponge, specialized flagellated cells (choanocytes) form a series of chambers where feeding takes place. In these chambers, collectively called the choanoderm, the flagellated choanocytes beat to pump water in through the ostia and along the often elaborate aquiferous systems within the sponge. Choanocytes also filter out food particles (including bacteria and microalgae) from the water, and these are transferred to the mesohyl, an extensive layer of connective tissue (Fig. 2). In the mesohyl, food particles are digested via phagocytosis by another group of sponge cells, the archaeocytes. These totipotent cells are capable of differentiating into any of the other sponge cell types. Also present in the mesohyl of many sponges are dense communities of microorganisms (106, 430, 471–473). The existence of these putative symbionts alongside bacterium-digesting archaeocytes is somewhat paradoxical and implies either recognition of different microbial types by the sponge cells or shielding of symbiont cells to prevent consumption (482). Once filtered in the choanocyte chambers, water is eventually expelled from the sponge via the exhalant opening, or osculum. It has been estimated that up to 24,000 liters of water can be pumped through a 1-kg sponge in a single day (443).

Beyond the basic body plan described above, sponge morphology is highly diverse. Inspection of any marine "sponge garden" will reveal a colorful array of encrusting, branching, cup-shaped, and massive (amorphous) types (Fig. 3), with individuals ranging in size from a few millimeters to more than a meter in diameter (328). Sponge morphology can also reflect ecological function, as seen in the many cyanobacterium-containing species whose flattened shapes allow optimal light reception for their photosynthetic symbionts (337, 474, 477). Structural integrity is conferred upon most sponges by siliceous or calcareous spicules (371), and these skeletal components are the basis for much of sponge biology and taxonomy. A wide range of spicule types are secreted, many of which are characteristic of particular taxa (167). Collagenous tissues, such as spongin, also play a role in providing structural support and, together with spicules, allow the development of very large individuals, such as those found among many tropical species.

Sessile organisms such as sponges and other marine invertebrates (including corals and ascidians) rely heavily on the production of chemicals as a form of defense against natural enemies, such as predators and competitors. Marine sponges have attracted particularly intense scrutiny in this regard, with a wide variety of sponge natural products characterized to date (see reference 32 and its preceding versions). More novel bioactive metabolites are obtained from sponges each year than from any other marine taxon, and a range of pharmacological properties have been demonstrated (32, 250). Various ecological roles have also been proposed for these compounds, including defense against predators (20, 55, 275), competitors (94, 395, 411), fouling organisms (363, 487), and microbes (19, 254, 398). Interestingly, in at least some cases, the compounds appear to be produced by associated microorganisms rather than by the sponge (27, 285, 351). Continued investigations of sponge-derived compounds and their biotechnological and ecological implications should guarantee vigorous interest in sponge-microbe associations for some time to come.

Interactions between sponges and microorganisms occur in many forms. To a sponge, different microbes can represent food sources (290, 307, 308), pathogens/parasites (16, 171, 199, 455), or mutualistic symbionts (474, 477). Microbial associates can comprise as much as 40% of sponge tissue volume (427), with densities in excess of 10^9 microbial cells per ml of sponge tissue (159, 453), several orders of magnitude higher than those typical for seawater. The diversity in types of interaction is matched by the phylogenetic diversity of microbes that occur within host sponges. It was already evident from early microscopy and culturing studies of sponge-associated microbes that high levels of morphological and metabolic diversity were present (62, 218, 336, 430, 471–473). The application of molecular tools over the past decade has greatly extended the known diversity of microorganisms within these hosts (100, 106, 146, 214, 294, 390, 458). Each of the three domains of life, i.e., *Bacteria*, *Archaea*, and *Eukarya*, are now known to reside within sponges. We now consider in detail this enormous diversity together with the evolutionary mechanisms driving its existence.

EVOLUTION AND DIVERSITY OF SPONGE-ASSOCIATED MICROORGANISMS

Marine sponges are widely considered the most primitive of the metazoans, arising at least as early as the Precambrian, some 600 million years ago (206). According to molecular

clocks, the divergence of sponges from the ancestors of other metazoans may have occurred even earlier, around 1.3 billion years ago (144). During subsequent periods of the Paleozoic era, sponges accounted for much of the biomass on marine reefs (167, 491). Today, they remain important members of both shallow- and deep-water communities, occupying as much as 80% of available surfaces in some areas (74). Such sustained evolutionary and ecological success is probably due, at least in part, to their intimate associations with microbial symbionts. However, unlike many other studied host-microbe associations, in which only a very small number of participants are involved (e.g., squid-*Vibrio fischeri* [258], amoeba-*Chlamydiae* [168], and *Bugula*-"*Endobugula*" symbioses [142, 210]), it is apparent that sponge-associated microbial communities can be highly diverse, with a range of different microorganisms consistently associated with the same host species. In this section, we describe the extent of this diversity, providing in-depth phylogenetic analyses of all known sponge-associated microorganisms. We summarize current evidence for the existence of sponge-specific microorganisms and conclude by considering whether sponge-microbe associations are evolutionarily ancient or are, instead, recently initiated relationships involving microorganisms which are present in the surrounding seawater.

Known Diversity of Microorganisms from Sponges

Prior to this review, the diversity of microorganisms known from sponges was categorized in 14 recognized bacterial phyla (and one candidate phylum), both major archaeal lineages, and assorted microbial eukaryotes (145, 148, 477). Sequences representing the following bacterial phyla have been recovered from 16S rRNA gene libraries and/or excised denaturing gradient gel electrophoresis (DGGE) bands: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Proteobacteria* (*Alpha*, *Beta*, *Delta*, and *Gammaproteobacteria*), *Spirochaetes*, and *Verrucomicrobia* (7, 95, 123, 146, 148, 151, 154, 214, 317, 342, 383, 390, 391, 396, 404, 407, 421, 452, 454, 458; S. R. Longford, N. A. Tujula, G. R. Crocetti, A. J. Holmes, C. Holmström, S. Kjelleberg, P. D. Steinberg, and M. W. Taylor, unpublished data). In addition, a seemingly sponge-specific candidate phylum, "*Poribacteria*," has also been reported for several sponges (100). The most frequently recovered sequences in general 16S rRNA gene surveys of sponges include those from the *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* (148). Members of several bacterial phyla, namely, the *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*, have also been isolated in pure culture from marine sponges (46, 47, 56, 81, 95, 147, 187, 188, 198, 202, 214, 235, 263, 264, 292, 334, 341, 365, 453, 458). Sequences from the *Chlorobi* (green sulfur bacteria) have not been obtained from sponges, although positive fluorescence in situ hybridization (FISH) signals were obtained from *Rhopaloeides odorabile* with a specific probe for this phylum (458). In contrast to the case for marine sponges, the (limited) available evidence for freshwater species suggests that bacterial diversity and abundance are both much lower. Only sequences from the *Actinobacteria*, *Chloroflexi*, and *Alpha*- and *Betaproteobacteria* were recovered in a recent 16S rRNA gene library constructed from the freshwater sponge *Spongilla lacustris* (123). Moreover, many of these sequences were highly similar to those known previously from freshwater habitats, suggesting that they may not represent true symbionts.

With a few exceptions in the *Euryarchaeota* (164, 456), archaea reported from marine sponges are members of the phylum *Crenarchaeota* (164, 200, 226, 294, 454, 456). Lipid biomarkers also suggested the presence of both crenarchaeotes and euryarchaeotes in a deep-water Arctic sponge, though no phylogenetic information was provided in that study (272). The group I.1A *Crenarchaeota* are extremely prevalent in marine environments (180), and almost all sponge-derived archaeal sequences are affiliated with this group. The best-studied sponge-associated archaeon is the psychrophilic crenarchaeote "*Candidatus* Cenarchaeum symbiosum," which comprises up to 65% of prokaryotic cells within the Californian sponge *Axinella mexicana* (135, 294, 343, 345).

Eukaryotic microbes also occur in sponges. Sponge-inhabiting dinoflagellates (120, 152, 153, 338, 339, 355, 382, 454, 477) and diatoms (16, 47, 51, 53, 65, 113, 305, 390, 409, 454) have been reported, with the latter seemingly most prevalent in polar regions (16, 51, 53, 113, 305, 409, 454). Freshwater sponges often contain endosymbiotic microalgae, primarily zoochlorellae (30, 108, 109, 331, 333, 475, 488). Two previous reports of cryptomonads in sponges were noted by Wilkinson (477), while marine sponge-derived fungi are receiving increasing attention due to their biotechnological potential (44, 163, 191). Interestingly, of 681 fungal strains isolated worldwide from 16 sponge species, most belonged to genera which are ubiquitous in terrestrial habitats (e.g., *Aspergillus* and *Penicillium*) (163). It thus remains unclear in most cases whether such fungi are consistently associated with the source sponge, or even whether they are obligate marine species. Compelling evidence for symbiosis of a yeast with sponges of the genus *Chondrilla* was obtained by extensive microscopy studies of both adult sponge tissue and reproductive structures, with strong indications of vertical transmission of the yeast symbiont (221).

Little is known about viruses in sponges, although virus-like particles were observed in cell nuclei in *Aplysina* (*Verongia*) *cavernicola* (432). It was suggested that these particles could be involved in sponge cell pathology. Infection of an *Ircinia strobilina*-derived alphaproteobacterium by a bacteriophage isolated from seawater has also been demonstrated (211), although the propensity of this siphovirus to infect the bacterium in nature is not known.

In addition to the realization of high microbial diversity per se, we are now beginning to recognize more subtle patterns of host-symbiont distribution. For example, it appears that a given species of sponge contains a mixture of generalist and specialist microorganisms (390) and that the associated microbial communities are fairly stable in both space and time (105, 390, 391, 454). One particularly interesting pattern to emerge is the apparent widespread existence of sponge-specific bacterial clusters, i.e., closely related groups of bacteria which are found only in sponges (146). In the following section, we examine the published evidence for such clusters.

Existing Evidence for Sponge-Specific Microorganisms

The notion that marine sponges might contain a specific microbiota arose some 3 decades ago from the seminal work of Vacelet et al. and Wilkinson et al. (427, 430, 469, 471–473, 483). Based on electron microscopy and bacterial cultivation studies, these pioneers of sponge symbiont research proposed the following three broad types of microbial associates in sponges: (i) abundant populations of sponge-specific microbes in the sponge mesohyl, (ii) small populations of specific bacteria occurring intracellularly, and (iii) populations of nonspecific bacteria resembling those in the surrounding seawater (427, 472). One type of bacterial isolate, regarded as a single species, was recovered from 35 taxonomically diverse sponges from several geographic regions, but never from seawater (469, 483). Immunological experiments in which these same isolates cross-reacted with other "sponge-specific" bacteria but not with seawater isolates were taken as further evidence of sponge specificity (469). Another significant advance came in 2002, when Hentschel and coworkers integrated these concepts into the molecular age (146). They defined sponge-specific clusters as sponge-derived groups of at least three 16S rRNA gene sequences which (i) are more similar to each other than to sequences from other, nonsponge sources; (ii) are found in at least two host sponge species and/or in the same host species but from different geographic locations; and (iii) cluster together irrespective of the phylogeny inference method used (146).

The hypothesis of widespread, sponge-specific microbial communities put forward by Hentschel and colleagues (146) was compelling and was constrained only by the limited data set available at that time. They performed phylogenetic analyses with the 190 publicly available sponge-derived 16S rRNA gene sequences, the majority of which were from *Aplysina aerophoba*, *Rhopaloeides odorabile*, and *Theonella swinhoei*. These three sponges are phylogenetically only distantly related and were collected from the Mediterranean Sea, the Great Barrier Reef, and Micronesia/Japan/Red Sea, respectively, yet they contained largely overlapping microbial communities. Together with the earlier work of Wilkinson and contemporaries (e.g., see reference 483), these remarkable results suggested that even unrelated sponges with nonoverlapping geographic ranges might share a common core of bacterial associates. Indeed, subsequent studies have lent further weight to this notion, with numerous reports of similar (and in some cases sponge-specific) bacteria found in different sponge species (100, 151, 154, 198, 235, 342, 404, 407). Furthermore, both cultivation-based and molecular methods have provided evidence for distinct microbial communities between sponges and the surrounding seawater (151, 265, 334, 391, 472). Taken together, these results appear to indicate that sponge-associated microbial communities are indeed unique and at least partially sponge specific, and the existence of sponge-specific microorganisms has consequently become something of a paradigm in this field.

A total of 14 monophyletic, sponge-specific sequence clusters were identified in the original study of Hentschel et al. (146). These occurred in the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, and *Proteobacteria* (*Alpha*, *Delta*, and *Gammaproteobacteria*) and, in most cases, were strongly supported by bootstrap analyses (in all cases, the clusters were found with three different tree construction methods). Three further clusters—each sponge specific, with the exception of a single nonsponge sequence—were also identified in the *Acidobacteria* and in a lineage of uncertain affiliation (later recognized as *Gemmatimonadetes* (146, 499). Overall, 70% of the 190 sponge-derived sequences available at the time fell into one of these monophyletic clusters or the other. Interestingly, within-cluster 16S rRNA sequence similarities ranged down to as low as 77% (146), often considered indicative of phylum-level differences (170). Several subsequent, mostly cultivation-independent studies have also led to the recovery of apparently sponge-specific sequences. Approximately 50% of 16S rRNA gene sequences in a gene library obtained from the unidentified Indonesian sponge 01IND 35 were most closely related to sequences derived from other sponges (154). These included members of the *Acidobacteria*, *Nitrospira*, *Bacteroidetes*, and *Proteobacteria*, as well as several sequences in a group of uncertain affiliation (our analyses indicate that these may be deltaproteobacterial sequences [see Fig. 8]). A similar situation was reported for *Discodermia dissoluta*, whereby three-quarters of 160 retrieved 16S rRNA sequences were most similar to other sponge-derived sequences (342). Conversely, of 21 unique sequences (each representing a unique restriction fragment length polymorphism [RFLP] type) obtained from the Caribbean sponge *Chondrilla nucula*, only 5 retrieved other sponge-derived 16S rRNA sequences during BLAST searches (although with the advantage of our larger data set, we found indications that several more of the *C. nucula* sequences are in fact from members of sponge-specific clusters) (151). Perhaps the most impressive sponge-specific cluster to be reported so far is the candidate phylum "*Poribacteria*" (100). Fieseler and colleagues found members of this lineage, which is moderately related to the *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* (446), in several sponges from geographically diverse locations, but never in adjacent seawater or sediment samples (100). It will be especially interesting to see whether "*Poribacteria*" sequences are recovered from other environments in the future.

The sheer number of reports dealing with sponge-specific microorganisms is compelling. However, one must be cautious when proposing a sponge-specific cluster. Of crucial importance is the selection of nonsponge reference organisms for phylogenetic analyses. In principle, any group of sequences can appear sponge specific if the most appropriate reference organisms (i.e., those that are most closely related to the sponge-derived sequences) are not also included. The length of analyzed sequences is also of concern, with the level of phylogenetic information obtainable increasing with sequence length. Every effort should be made to obtain at least one near-full-length sequence per sequence type (or operational taxonomic unit). Decreasing sequence costs render this eminently achievable, and in many cases, it would only involve performing a few additional sequencing reactions. These are not new ideas and we are certainly not the first to advocate the use of full-length sequences (e.g., see reference 216), but during our analyses of sponge-derived 16S rRNA sequences, it became apparent that many of these sequences are rather short and therefore phylogenetically not particularly informative. Indeed, we encountered many problems with inser-

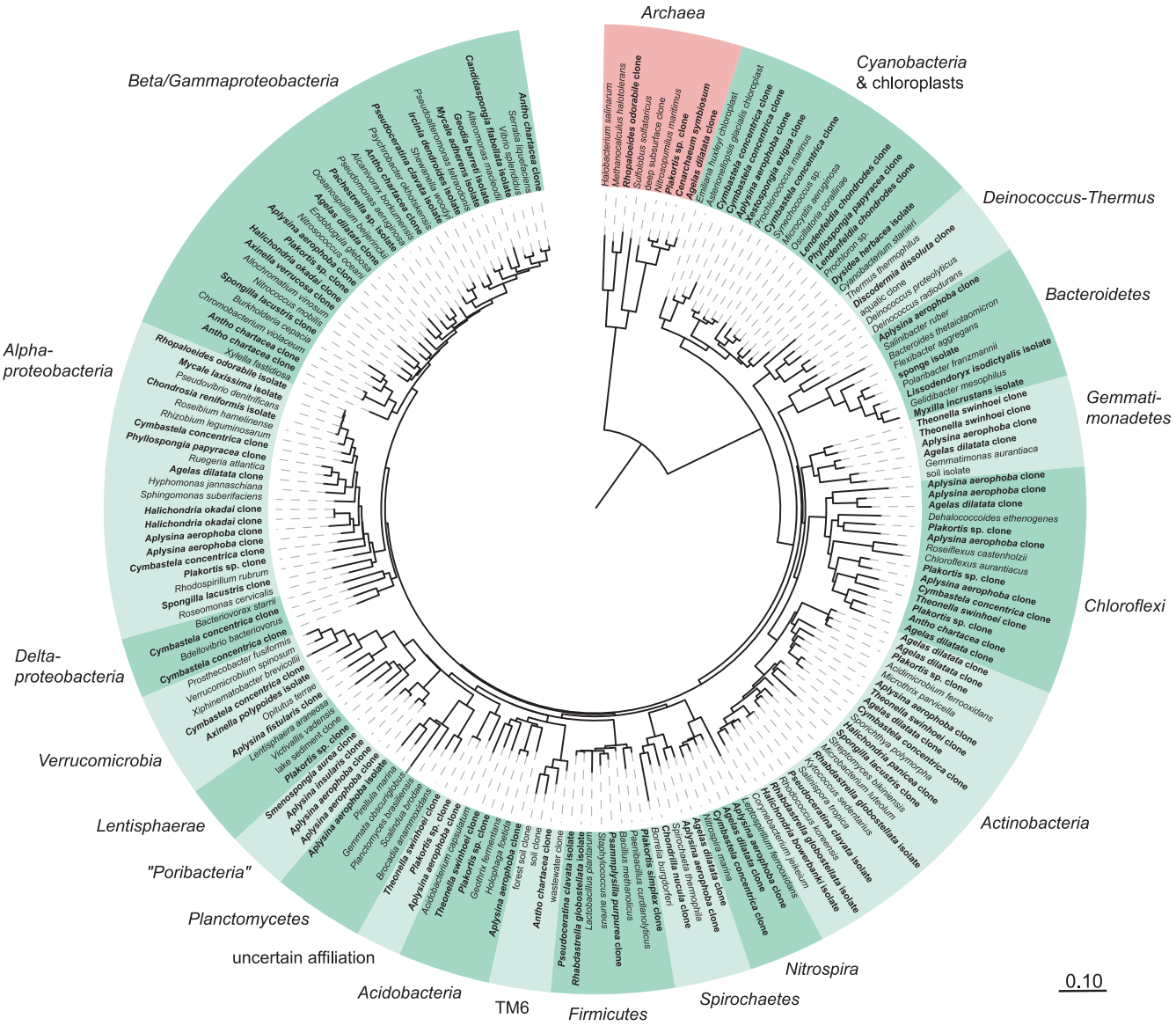

FIG. 4. 16S rRNA-based phylogeny showing representatives of all bacterial and archaeal phyla from which sponge-derived sequences have been obtained. Sponge-derived sequences are shown in bold, with additional reference sequences also included. The displayed tree is based on a maximum likelihood analysis. Bar, 10% sequence divergence.

tion of short sponge-derived sequences into our phylogenetic trees, and in some cases, we were not even certain of their phylum-level affiliation.

Census of Sponge-Associated Microorganisms

Increasing interest in sponge-microbe associations has resulted in a concomitant increase in the amounts of 16S rRNA sequence data obtained from sponges (Fig. 1B). There are currently ~1,500 sponge-derived 16S rRNA gene sequences available in GenBank (http://www.ncbi.nlm.nih.gov/), in contrast to only 190 such sequences available for the 2002 study by Hentschel et al. (146). We carried out an extensive phylogenetic analysis of all currently available sponge-derived 16S rRNA gene sequences, with two main objectives, as follows: (i) to provide an overview of microbial diversity in sponges and (ii) to critically assess the occurrence of monophyletic, sponge-specific sequence clusters. As mentioned above, such clusters are often discussed, yet their existence has not been reevaluated rigorously in light of the rapidly expanding 16S rRNA sequence databases. It is thus unclear whether these clusters are truly sponge specific or merely reflect a greater sampling effort for these communities than for others.

We began, using the ARB program package (217), by establishing an encompassing database that contains all sponge-derived 16S rRNA sequences which were available in GenBank on 28 February 2006. In addition to these 1,499 sequences (plus 11 18S rRNA sequences amplified from eukaryotic microbes in sponges), we contributed a further 184 bacterial and archaeal sequences from three hitherto unstudied sponges,

TABLE 1. Summary of all publicly available sponge-derived 16S rRNA sequence data (as of 28 February 2006) plus 184 bacterial and archaeal sequences contributed from this article

Phylogenetic affiliation	Total no. of sequences	No. of sequences of >1,000 bp	No. of sequences		% of sequences in clusters obtained from:		
			Uncultivated	Obtained from an isolate	Exclusively sponges[a]	Sponges and corals	Sponges and one nonsponge organism
Bacteria	1,630	592					
Acidobacteria	66	9	66	0	5	64	24
Actinobacteria	266	99	190	92	38 (45)	0	1
Bacteroidetes	77	20	46	31	0 (4)	0	10
Chloroflexi et al.	109	48	109	0	62 (75)	0	0
Cyanobacteria	119	68	111	7	79	0	0
Deinococcus-Thermus	2	0	2	0	0	0	0
Firmicutes	96	31	45	51	9	0	0
Gemmatimonadetes	16	5	16	0	25	56	0
Lentisphaerae	1	1	1	0	0	0	0
Nitrospira	14	6	14	0	57	29	0
Planctomycetes	11	4	9	2	0	0	0
"*Poribacteria*"	21	10	21	0	100	0	0
Proteobacteria							
Alpha-	311	125	196	115	14 (22)	0	4
Beta/Gamma-	430	114	298	134	34 (37)	0	0
Delta-	48	25	48	0	15	40	6
Spirochaetes	6	2	6	0	67	0	0
TM6	1	1	1	0	0	0	0
Verrucomicrobia	13	13	12	1	23	0	0
Uncertain affiliation	23	11	23	0	78	0	0
Archaea	44	10					
Crenarchaeota	43	10	43	0	28 (42)	0	0
Euryarchaeota	1	0	1	0	0	0	0
Eukarya[b]	20	6	18	2			
Total	1,694	608	1,259	435	546	74	43

[a] Numbers in parentheses are inclusive of clusters which are supported by only two of three tree construction methods.
[b] Includes both 18S rRNA- and 16S rRNA (plastid)-derived sequences.

namely, *Agelas dilatata*, *Plakortis* sp. (both from the Bahamas; kindly provided by U. Hentschel), and *Antho chartacea* (from southeastern Australia). Preliminary phylogenetic analyses identified members of putative sponge-specific clusters, and for each cluster, the most similar nonsponge sequences were retrieved by BLAST searches (from both regular NCBI and environmental genome databases) and imported into ARB for subsequent alignment (automatic and manual). The resulting ARB database, containing an alignment of all sponge-derived sequences and their nearest relatives (together with annotated information [e.g., host species and collection location] for the sponge sequences), is available upon request. Extensive phylogenetic analyses (see the supplemental material for details) were conducted, taking all (n = 1,694) sponge-derived sequences into account. In order to rigorously test the existence of monophyletic, sponge-specific sequence clusters, we employed multiple tree construction methods (maximum likelihood, neighbor joining, and maximum parsimony), together with the use of various sequence conservation filters and correction parameters.

In total, sequences representing 16 bacterial phyla and both major archaeal lineages (*Crenarchaeota* and *Euryarchaeota*) have been recovered from sponges (Fig. 4). In addition to those known prior to this study (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, "*Poribacteria*," *Proteobacteria* [*Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria*], *Spirochaetes*, *Verrucomicrobia*, and *Chlorobi* FISH signals), we report for the first time the presence in sponges of 16S rRNA sequences affiliated with the phylum *Lentisphaerae* and the candidate phylum TM6. The number of sequences representing each phylum varied widely, from single sequences for the *Lentisphaerae* and TM6 to more than 250 sequences for each of the *Actinobacteria*, *Alphaproteobacteria*, and *Beta/Gammaproteobacteria* (Table 1). The proportions of sequences derived from cultivated versus noncultivated microorganisms also varied greatly among phyla.

The phylogenetic analyses presented here strongly support the existence of monophyletic, sponge-specific 16S rRNA sequence clusters. These occurred in many of the bacterial and archaeal phyla found in sponges, with approximately one-third (32%) of all sponge-derived sequences falling into such clusters (Table 1; Fig. 5 to 15; also see the supplemental material). If sequences derived from cultured isolates are excluded, this figure rises to 42%. This result was expected since tightly linked symbionts—those presumed to occur in sponge-specific clusters—are likely difficult to cultivate and therefore underrepresented in culture collections. Several additional clusters each contained a single nonsponge sequence, with the extra sequences often, but not always, obtained from marine envi-

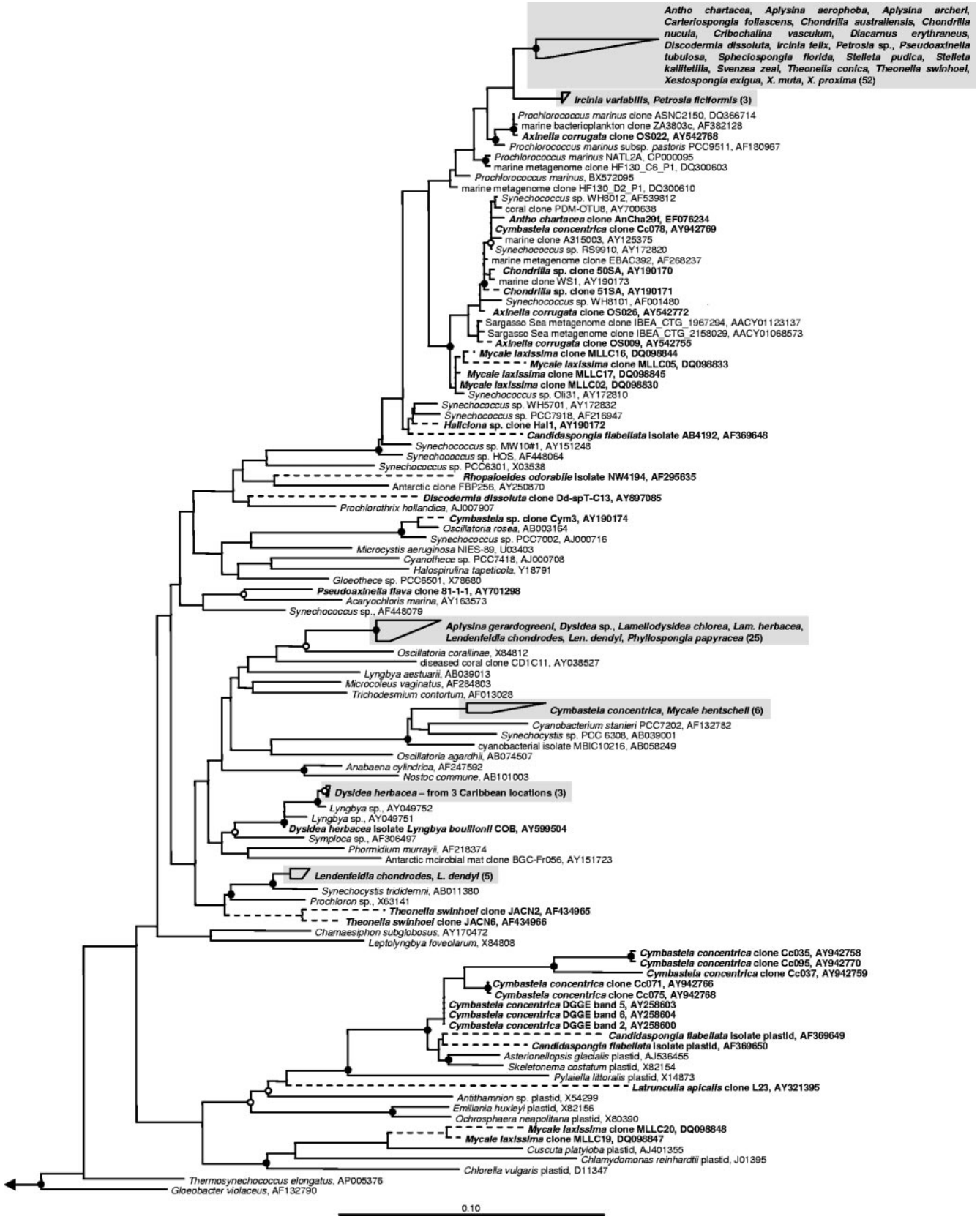

FIG. 5. 16S rRNA-based phylogeny of sponge-associated cyanobacteria and chloroplasts. Sponge-derived sequences are shown in bold. The displayed tree is a maximum likelihood tree constructed based on long (≥1,000 nucleotides) sequences only. Shorter sequences were added using the parsimony interactive tool in ARB and are indicated by dashed lines. Shaded boxes represent sponge-specific monophyletic clusters, as defined

ronments. It is also possible that sponge-specific microbes are more prevalent in those sponges which contain very dense microbial communities (Ute Hentschel, personal communication), i.e., the so-called bacteriosponges or high-microbial-abundance sponges (148, 430). Due to a lack of microbial abundance data for most host sponges, we did not attempt to take this factor into account during our analyses. Overall, while representation of sequences in sponge-specific clusters was quite high, it should be noted that the proportions of sequences falling within such clusters differed greatly among the various phyla.

More than three-quarters of the 119 available sponge-derived *Cyanobacteria* sequences fell into monophyletic, sponge-specific clusters (Table 1; Fig. 5). Most of these were in two clusters, with one comprising 25 sequences from at least 7 sponge species and the other comprising 52 sequences from 21 sponges. The latter cluster represents the recently described candidate species "*Candidatus* Synechococcus spongiarum" (426) and was the sole *Cyanobacteria* cluster in the study of Hentschel et al. (146), while the former corresponds to the filamentous cyanobacterium *Oscillatoria spongeliae* (39, 157). Sequences representing *O. spongeliae* were not available for the 2002 study. Several other, smaller clusters are also evident among the cyanobacteria (Fig. 5). Additionally, in a number of cases, microalgal plastids have also been amplified using 16S rRNA primers.

Another bacterial phylum containing many sponge-specific sequence clusters is the *Chloroflexi* (Table 1; Fig. 6). Of the 109 sponge-derived sequences analyzed, 62% comprised such clusters, while the occurrence of a further 13% of sequences in clusters was weakly supported. In the new analyses, all but one of the members of a sponge-specific cluster described by Hentschel and coworkers (146) remained in a cluster, although these sequences were now dispersed over four different clusters. Such movement of sequences was frequently observed and is not surprising given the much larger data set at our disposal now (i.e., many new related sequences, both sponge- and non-sponge-derived, were included in the phylogenetic analyses described here). None of the sponge-derived sequences were closely related to the few described *Chloroflexi* species, although many were similar to sequences from uncultivated organisms, particularly from marine environments (Fig. 6).

Interestingly, many sponge-derived 16S rRNA sequences formed exclusive monophyletic clusters with sequences obtained from corals (Table 1). This was particularly apparent for the *Acidobacteria* and *Deltaproteobacteria* (Fig. 7 and 8, respectively) but was also evident for the *Gemmatimonadetes* (Fig. 9) and *Nitrospira* (Fig. 10). No coral-derived sequences shared monophyletic clusters with sponge sequences in the original study of Hentschel et al. (146), no doubt reflecting the fact that most of the relevant coral sequences were deposited in GenBank since then. It is too early to speculate whether some sort of marine invertebrate-specific sequence cluster exists, but further sampling of taxa such as ascidians and bryozoans should help to resolve this issue. A study of two marine macroalgae and the cooccurring sponge *Cymbastela concentrica* gave no indication of specific clusters spanning these taxonomically disparate groups (Longford et al., unpublished data).

Sponge-specific sequence clusters were not prevalent for, among others, the *Bacteroidetes* (see Fig. S1 in the supplemental material) and *Firmicutes* (see Fig. S2 in the supplemental material), perhaps reflecting the relatively high proportions of sequences derived from cultivated organisms in these phyla.

We report for the first time the recovery of *Lentisphaerae* (Fig. 11) and candidate phylum TM6 (Fig. 12A) sequences from sponges. Each phylum was represented by a single 16S rRNA sequence, from the marine sponges *Plakortis* sp. and *Antho chartacea*, respectively, and it cannot be ruled out that these represent contaminating sequences from the surrounding environment (although arguably this also applies to many, more commonly recovered sequence types). The *Lentisphaerae* phylum comprises part of the so-called *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum (446), with sponge-derived sequences from the superphylum additionally being found in the *Verrucomicrobia*, *Planctomycetes*, and "*Poribacteria*" (Fig. 11). Members of the superphylum are frequently associated with eukaryotes. There is also a group of uncertain affiliation which falls near the PVC superphylum (but without strong bootstrap support) during phylogenetic analyses. This group includes sequences from many sponges, such as *Agelas dilatata*, *Aplysina aerophoba*, *Discodermia dissoluta*, and *Theonella swinhoei*. Those sequences most closely related to the sponge sequences are also from marine environments.

Several large sponge-specific clusters were found among the *Actinobacteria* sequences, particularly in the family *Acidimicrobiaceae* (Fig. 13). The largest comprised 54 sequences obtained from sponges from the Caribbean (*Agelas dilatata*, *Discodermia dissoluta*, *Plakortis* sp., and *Xestospongia muta*), Indonesia (*Xestospongia testudinaria*), the Red Sea (*Theonella swinhoei*), and the South China Sea (*Dysidea avara*). None of the sequences within this cluster were obtained from cultured bacteria, with the nearest (but still distantly related) cultured actinobacteria being the wastewater bacterium *Microthrix parvicella* and the acidophilic *Acidimicrobium* spp. (Fig. 13).

Although not representing a sponge-specific cluster, the group of sequences affiliated with the marine *Pseudovibrio* spp. within the *Alphaproteobacteria* deserves special mention (Fig. 14). Members of this genus are frequently found in sponge-

by Hentschel et al. (146), i.e., a group of at least three sponge-derived 16S rRNA gene sequences which (i) are more similar to each other than to sequences from other, nonsponge sources, (ii) are found in at least two host sponge species and/or in the same host species but from different geographic locations, and (iii) cluster together irrespective of the phylogeny inference method used (all clusters shown here also occurred in neighbor-joining and maximum parsimony analyses). Names outside wedges of grouped sequences represent the sponges from which the relevant sequences were derived; the number in parentheses indicates the number of sequences in that wedge. Filled circles indicate bootstrap support (maximum parsimony, with 100 resamplings) of ≥90%, and open circles represent ≥75% support. The outgroup (not shown) consisted of a range of sequences representing several other bacterial phyla. Bar, 10% sequence divergence.

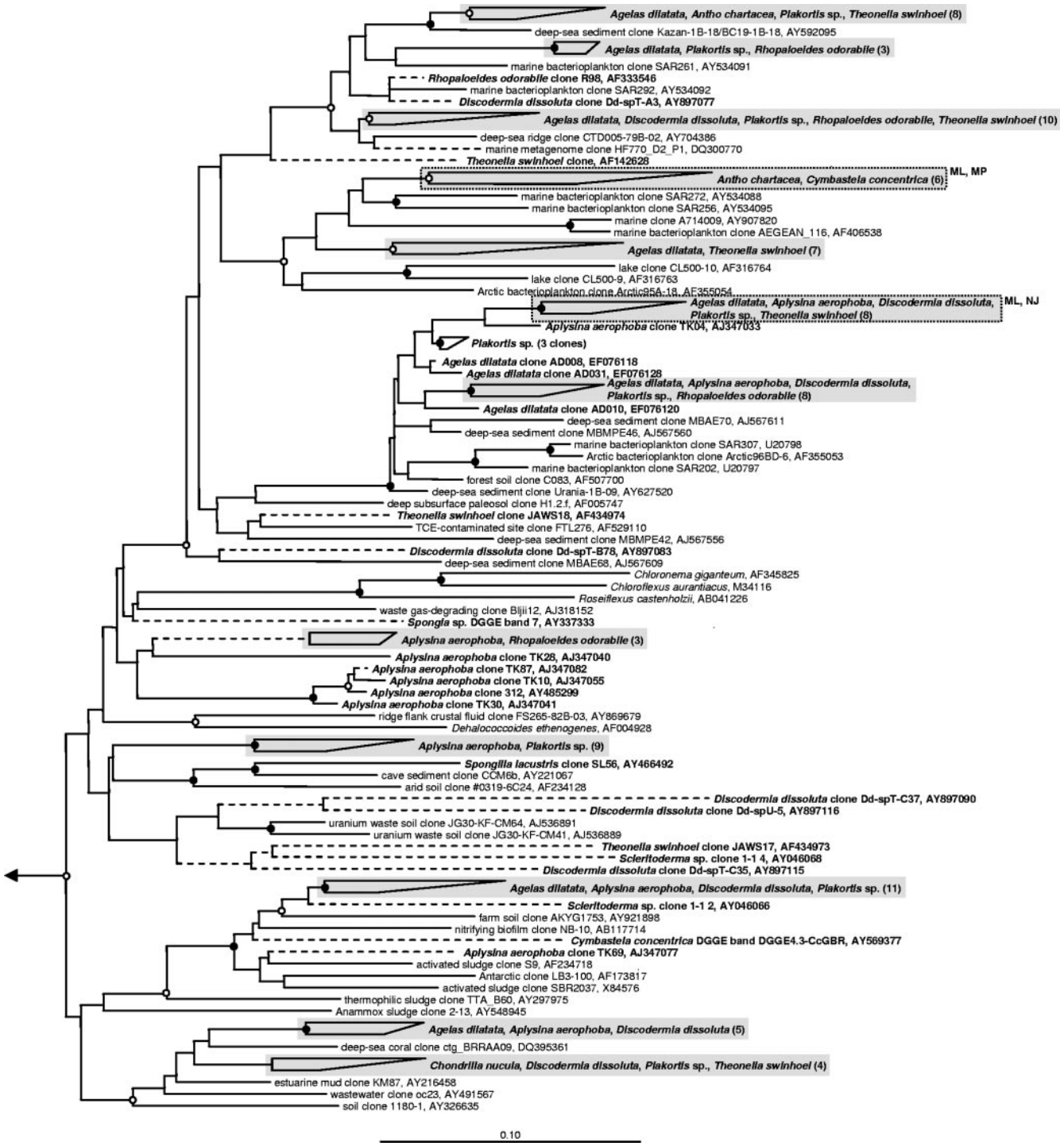

FIG. 6. 16S rRNA-based phylogeny of sponge-associated *Chloroflexi* organisms. Details are the same as those provided for Fig. 5, with the following additions. Shaded boxes contained within dotted lines represent sponge-specific clusters supported by only two tree construction methods (ML, maximum likelihood; MP, maximum parsimony; and NJ, neighbor joining), and new sequences from our laboratory have the prefix "AD" (for the sponge *Agelas dilatata*), "AnCha" (*Antho chartacea*), or "PK" (*Plakortis* sp.).

derived cultivation-based and molecular studies (95, 96, 147, 187, 198, 263, 453), and there is strong evidence for its being a true sponge symbiont (95, 453).

Only 28% of sponge-derived *Archaea* sequences fell into well-supported sponge-specific clusters (Fig. 15), although the fact that almost all of these were within the group I.1A *Crenarchaeota* bears testimony to their high degree of phylogenetic relatedness. The recently isolated ammonia-oxidizing archaeon "*Candidatus* Nitrosopumilus maritimus" (192) is the only cultivated member of this group, with the well-studied

FIG. 7. 16S rRNA-based phylogeny of sponge-associated *Acidobacteria* organisms. Details are the same as those provided for Fig. 5 and 6, with the following two additions. Open boxes represent monophyletic clusters containing sponge-derived sequences and a single, nonsponge origin sequence, and open boxes with asterisks outside them signify clusters containing only sponge- and coral-derived sequences (the number of asterisks corresponds to the number of coral-derived sequences within the cluster).

FIG. 8. 16S rRNA-based phylogeny of sponge-associated *Deltaproteobacteria* organisms. Details are the same as those provided for Fig. 5 to 7.

(but still uncultivated) archaeon "*Ca*. Cenarchaeum symbiosum" being the best known sponge-associated member. A genome project for the latter has recently been completed (134). At the time of sequence collection, 44 archaeal sequences had been recovered from sponges, all of which were marine sponges (Table 1; Fig. 15). All but one of these was from the *Crenarchaeota*, with a single *Euryarchaeota* sequence from the Great Barrier Reef sponge *Rhopaloeides odorabile* (456). An

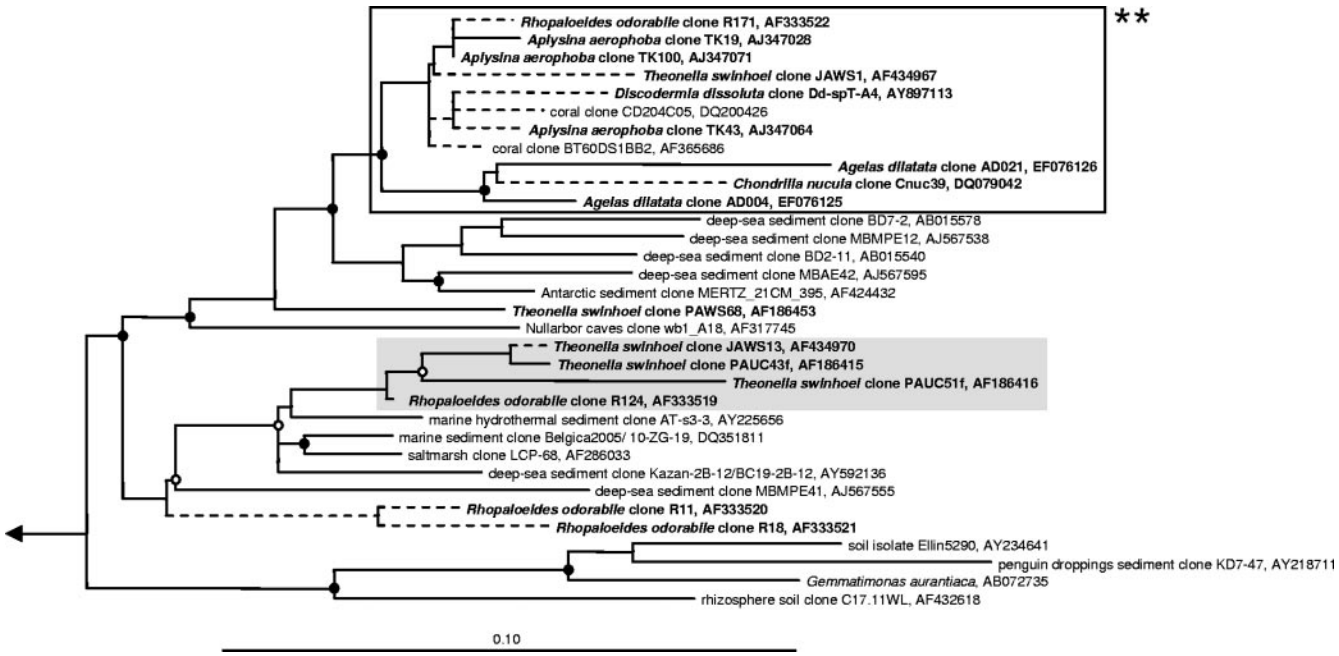

FIG. 9. 16S rRNA-based phylogeny of sponge-associated *Gemmatimonadetes* organisms. Details are the same as those provided for Fig. 5 to 7.

article which appeared in mid-2006 (whose sequences were not available on 28 February 2006 and were therefore not included in our study) reported more euryarchaeotal sequences from various sponges, although the majority of sequences in that study were still affiliated with the *Crenarchaeota* (164).

All sponge-derived 16S rRNA sequences available on 28 February 2006 were analyzed phylogenetically, but for practical reasons the larger trees are available only in the supplemental material. Broadly speaking, the results of our analyses are consistent with the earlier study by Hentschel et al. (146), with, for example, the *Actinobacteria*, *Nitrospira*, and *Acidobacteria* still well represented by sponge-specific microorganisms. As could be expected, some sponge-specific clusters from the 2002 study now form parts of several new clusters, while others do not comprise clusters at all in the new data set. Conversely, the addition of more sequences meant that many formerly single sequences are now in specific clusters with other sponge-derived sequences.

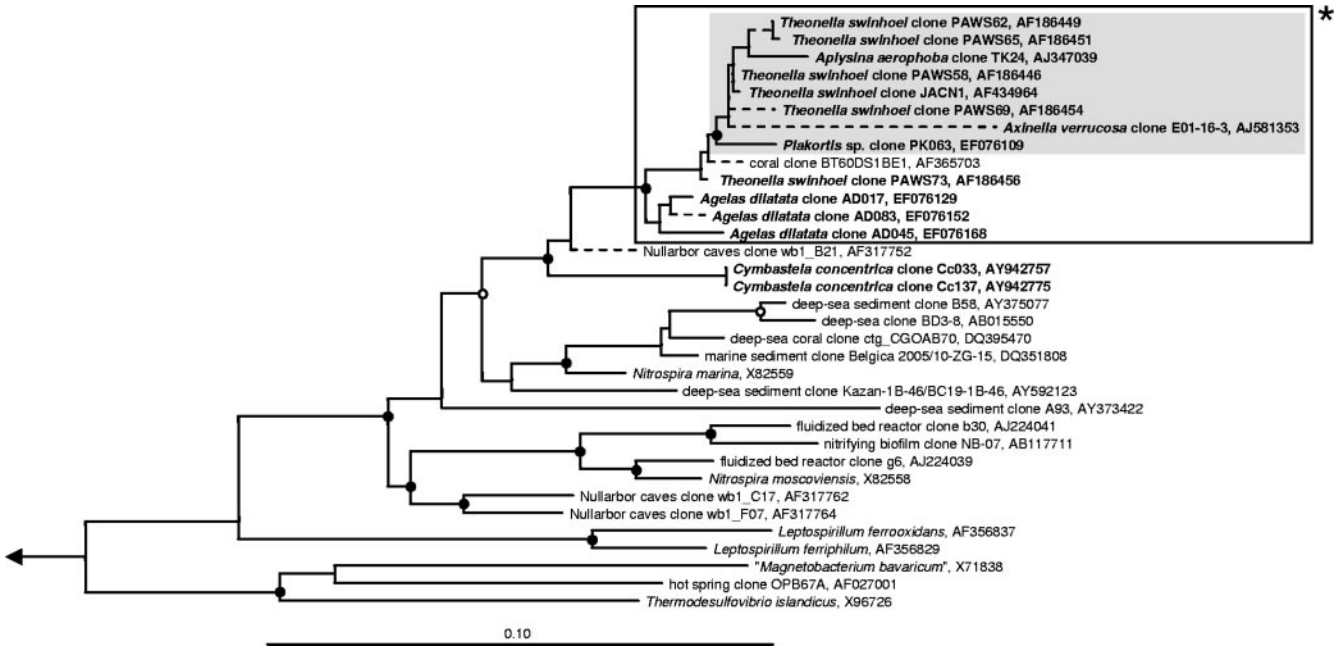

FIG. 10. 16S rRNA-based phylogeny of sponge-associated *Nitrospira* organisms. Details are the same as those provided for Fig. 5 to 7.

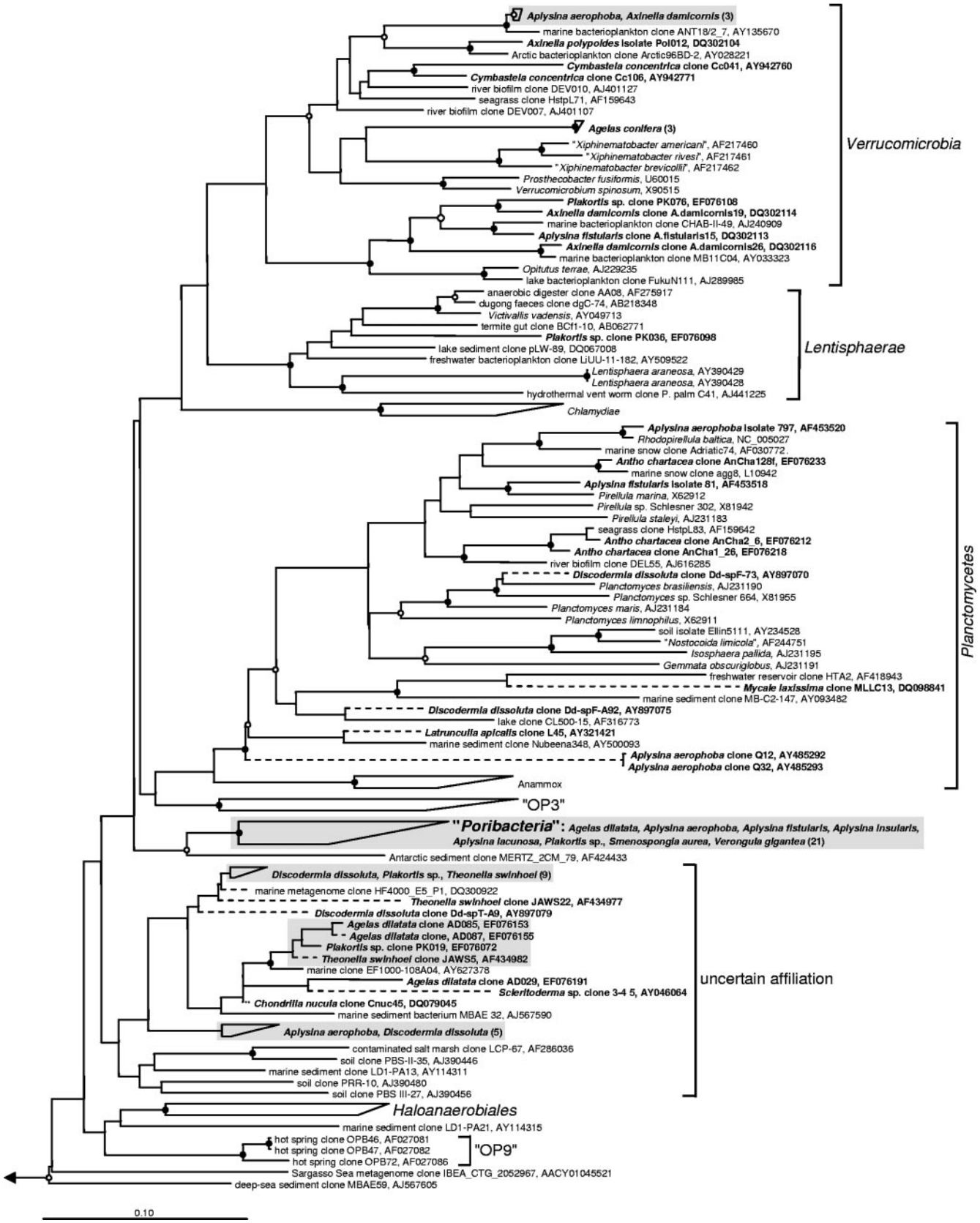

FIG. 11. 16S rRNA-based phylogeny of sponge-associated *Verrucomicrobia*, *Planctomycetes*, *Lentisphaerae*, and "*Poribacteria*" organisms and of a lineage of uncertain affiliation. These and associated lineages comprising the PVC superphylum (446) are shown. Details are the same as those provided for Fig. 5 to 7.

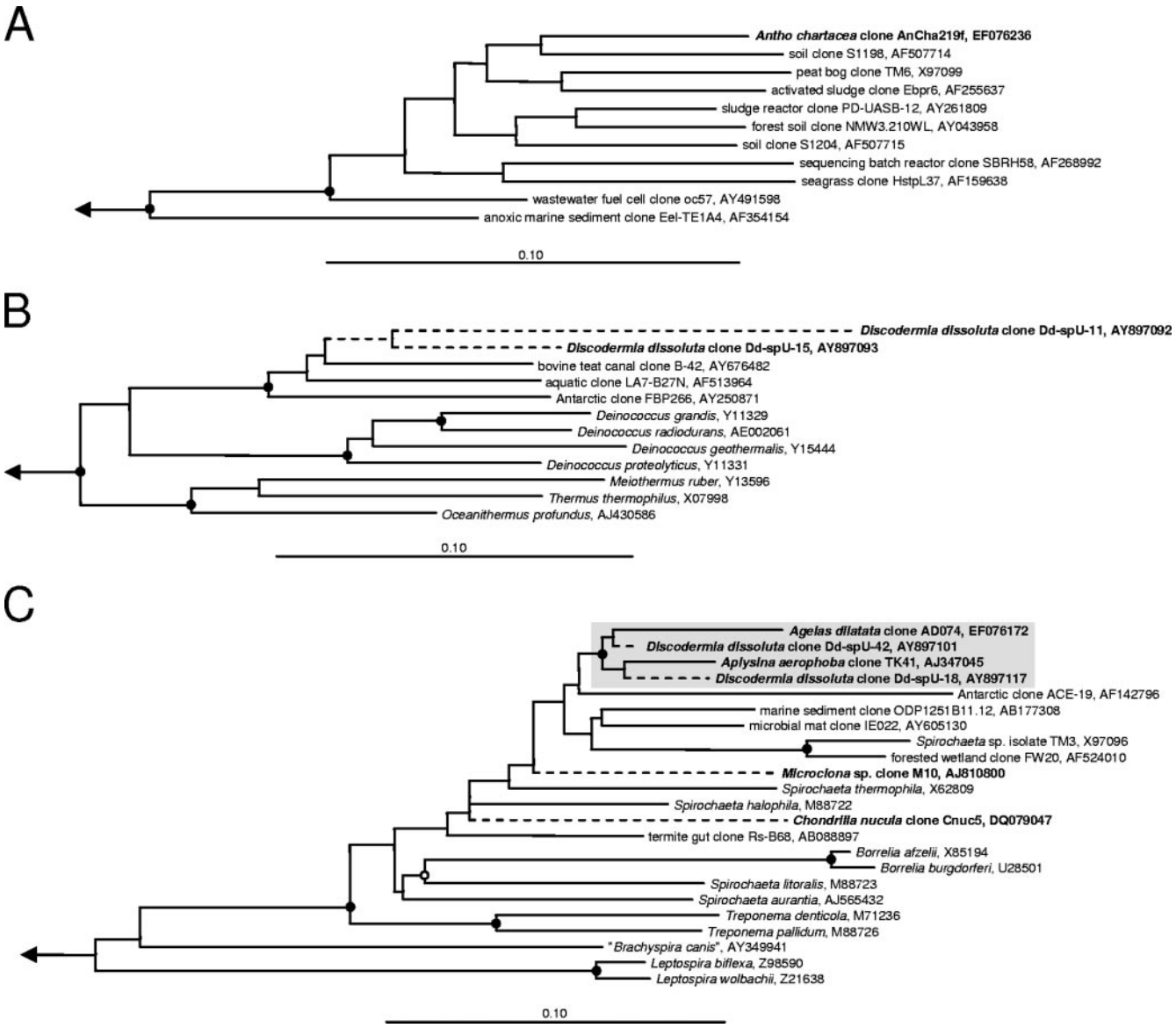

FIG. 12. 16S rRNA-based phylogeny of sponge-associated members of the candidate phylum TM6 (A), *Deinococcus-Thermus* organisms (B), and *Spirochaetes* organisms (C). Details are the same as those provided for Fig. 5 to 7. (B) In our analyses, the position of clone Dd-spU-11 (from the sponge *Discodermia dissoluta*) was not stable, and we are not certain of its phylogenetic affiliation.

Very few sequences were available from sponge-associated eukaryotic microbes at the time of database establishment (since then, some 45 18S rRNA sequences derived from sponge-associated fungi have been deposited in GenBank). Those that are included in our database include 9 16S rRNA sequences derived from diatom chloroplasts (Fig. 5) and 11 18S rRNA sequences obtained from diatoms and dinoflagellates. All but one of the 18S rRNA sequences were obtained from Antarctic sponges (454), with the remaining sequence representing a zooxanthella (*Symbiodinium* sp.) from the Palauan sponge *Haliclona koremella* (49).

We endeavored to be as thorough and as careful as possible throughout our analyses, yet there remain some caveats to our results. Despite extensive BLAST searches using members of all putative sponge-specific clusters, it is not inconceivable that we failed to include some key sequences which would have broken up otherwise specific sponge clusters. Another factor relates to the short lengths of many sponge-derived 16S rRNA sequences. We constructed our trees using only sequences longer than 1,000 bp, but more than two-thirds of all sponge-derived sequences are shorter than this (Table 1), and we added these via the parsimony interactive tool in ARB. In principle, this method allows the insertion of short sequences without changing tree topology (217). However, when many short sequences are added at once, they can influence each other's positioning (and potentially bias the analysis towards the formation of sponge-specific clusters). We attempted to gauge the severity of this problem by (for a selection of sequences) sequentially adding and removing individual short sequences and comparing their placement to the outcome

FIG. 13. 16S rRNA-based phylogeny of sponge-associated *Actinobacteria* organisms belonging to the family *Acidimicrobiaceae*. Other sponge-derived actinobacteria are shown in Fig. S3 in the supplemental material. Details are the same as those provided for Fig. 5 to 7.

when they were all added at once. The results were highly consistent, but it should not be assumed that this will always be the case. The alternative is to perform the entire phylogenetic analysis with short sequences and to truncate longer sequences to leave only the homologous region; this results in the loss of much phylogenetic information and is not recommended under any circumstances (216). Again, we reiterate the importance of obtaining at least one near-full-length sequence for each operational taxonomic unit obtained. This is not possible in some cases (e.g., excised DGGE bands) but is feasible in many others.

It is prudent to consider whether the apparent occurrence of sponge-specific sequence clusters could have a more dubious origin, namely, laboratory contamination. Theoretically, a 16S

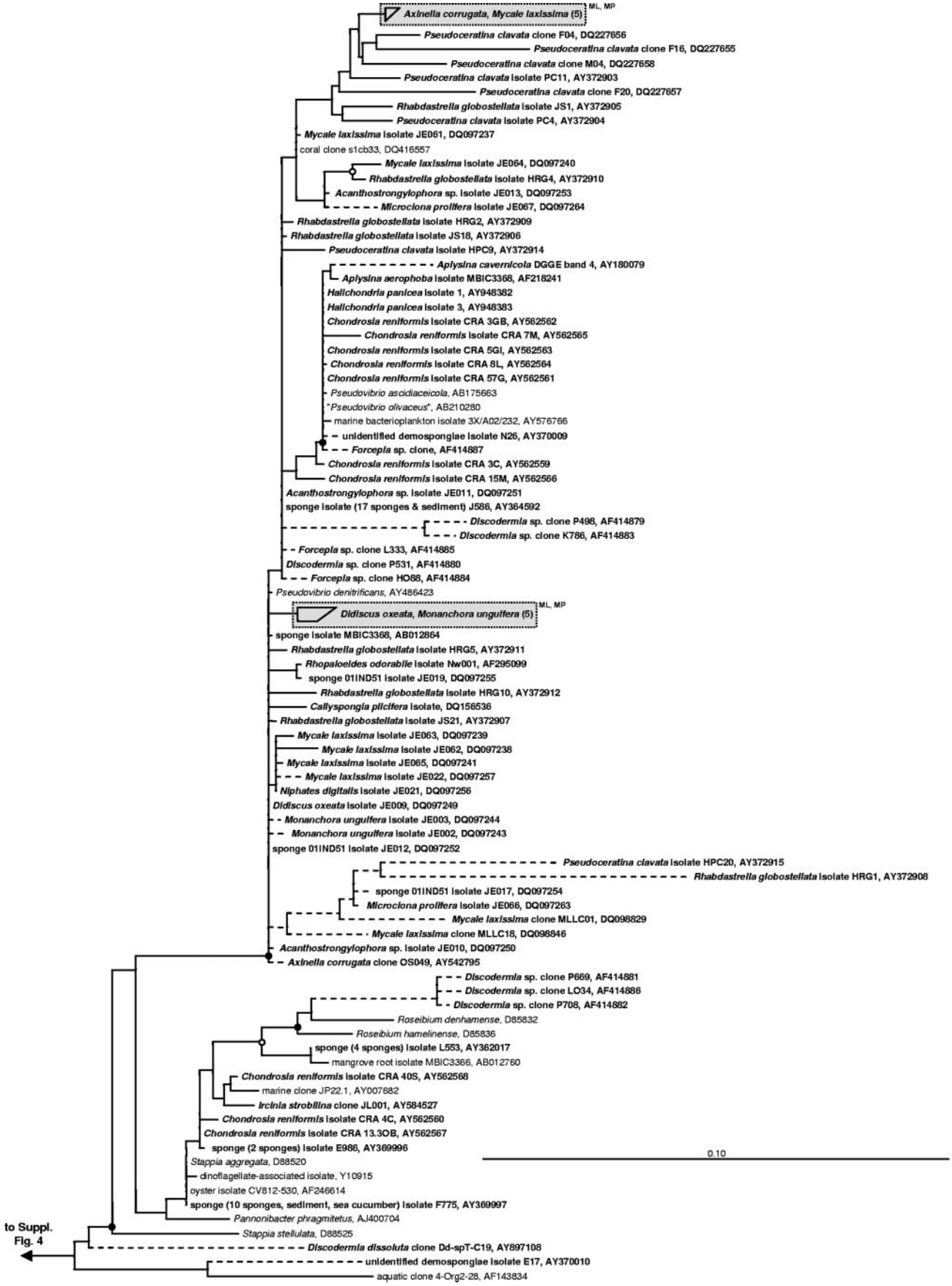

FIG. 14. 16S rRNA-based phylogeny of sponge-associated *Alphaproteobacteria* organisms affiliated with the genus *Pseudovibrio* and its relatives. Other sponge-derived alphaproteobacteria are shown in Fig. S4 and S5 in the supplemental material. Details are the same as those provided for Fig. 5 to 7.

FIG. 15. 16S rRNA-based phylogeny of sponge-associated archaeal organisms. Details are the same as those provided for Fig. 5 to 7.

rRNA gene-containing plasmid or PCR product could, if accidentally spread to DNAs from several sponges in the same laboratory, appear to form its own sponge-specific cluster. However, the available evidence strongly suggests that this is not the case, since many or most clusters contain sequences originating from several independent laboratories.

With almost 1,700 sponge-derived 16S rRNA sequences falling into some 16 or more bacterial and archaeal phyla, we sought to address the following question: how well sampled are marine sponge-associated microbial communities? If current studies are recovering mainly sequences which were previously obtained from sponges (as the presence of sponge-specific clusters might imply), then we may have already uncovered most of the microbial diversity in these hosts, suggesting that our current descriptive phase might be nearing its logical conclusion. Unfortunately, the available data are insufficient to satisfactorily address this issue for sponges. In a recent article in this journal, Schloss and Handelsman (348) used the program DOTUR to estimate richness at different levels of phylogenetic relatedness for each bacterial phylum represented in the Ribosomal Database Project (61). To perform an analogous study with the sponge symbiont data set, we were restricted to sequences which met the following criteria: (i) they were part of attempts at extensive microbial community surveys using general 16S rRNA gene primers for the construction of clone libraries; (ii) they overlapped a sufficient distance to be useful (*Escherichia coli* positions 100 to 500 would have been appropriate for a reasonable portion of the sponge data set); and (iii) they were not obtained from prescreened gene libraries (e.g., by RFLP analysis), as this would heavily bias results—thus, all collected sequences must have been deposited in GenBank. After applying these (in our eyes) minimal criteria, only 317 sequences (of 1,694) were deemed suitable for use with DOTUR or similar programs. For many phyla, only a few sequences were retained (e.g., for *Cyanobacteria*, 8 of 119 sequences were kept, and for *Alphaproteobacteria*, 21 of 311 sequences were kept), precluding meaningful analyses. Furthermore, even if 50 or more sequences were suitable (as in the case of the *Beta*/*Gammaproteobacteria*), these were not necessarily representative of the known (sponge-derived) diversity within that phylum, again preventing the drawing of meaningful conclusions. Although statistically robust analyses are therefore not possible at this stage, data from two recent studies can add greatly to this discussion. In the first, Lopez and colleagues at the Harbor Branch Oceanographic Institution (213) obtained more than 700 sequences from 20 different sponge-derived gene libraries by using general 16S rRNA primers, with the vast majority of these belonging to phyla already obtained from sponges, such as *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, *Planctomycetes*, and *Spirochaetes*. Of the recovered sequences, *Epsilonproteobacteria* was the only major taxonomic group not previously obtained from sponges. In another study, examining the Adriatic sponges *Chondrilla nucula* and *Tethya aurantium*, Thiel and coworkers recovered representatives of only known sponge-associated phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria* [*Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria*], *Spirochaetes*, and *Verrucomicrobia*) (404; V. Thiel, T. Staufenberger, and J. F. Imhoff, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006). The lack of new phyla in these data allow one to speculate that the majority of sponge-associated microorganisms may have already been encountered in gene libraries, at least at the phylum level. However, two major caveats exist. Although we may arguably be nearing the point of diminishing returns with respect to using current techniques to recover novel lineages from sponges (i.e., gene libraries constructed using general 16S rRNA primers), it is highly likely that the use of phylum-specific primers and/or metagenomic (i.e., PCR-independent) approaches will reveal phyla previously unknown to exist in these hosts or even unknown to science (e.g., "*Poribacteria*") (100). To our knowledge, there is no example of a sponge for which the results of general versus specific 16S rRNA gene libraries have been compared. A second point is that few gene libraries, including those from sponges, are sequenced to full coverage, and it is possible that the recurring sequences obtained from different sponges are merely those that are most abundant (or those that PCR is most biased toward) in each sponge, with the unsequenced remainder of the library potentially contributing new sequence types. The advent of high-throughput sequencing technologies (e.g., see reference 227) offers the potential to sequence gene libraries to much greater depth, illuminating the rare biosphere within sponges (376).

Statistical comparisons of microbial community compositions allow for the inclusion of more sequences (relative to species richness estimates via DOTUR) due to less stringent selection criteria. We thus used the so-called parsimony test, implemented in the program TreeClimber (347), to compare our three new gene libraries (from the sponges *Agelas dilatata*, *Antho chartacea*, and *Plakortis* sp.) with selected sponge-derived libraries from the literature and those deposited in GenBank. The parsimony test compares phylogenetic trees rather than sequence data per se (228, 347), and various tree construction algorithms can be employed. Our criteria for sequence inclusion were that (i) general 16S rRNA gene primers were used and (ii) at least 25 sequences were available from each library. The main caveats are that prescreening of clones (e.g., by RFLP analysis) with subsequent representation of each operational taxonomic unit by a single sequence prevents strict application of the parsimony test (347), while low sequencing coverage of some libraries may obscure true similarities or differences among libraries by missing overlapping or distinct sequences, respectively. With these considerations in mind, we compared the three libraries obtained from this study with those from the marine sponges *Theonella swinhoei* (146), *Aplysina aerophoba* (146), *Rhopaloeides odorabile* (458), *Cymbastela concentrica* (Longford et al., unpublished data), *Discodermia dissoluta* (342), and *Chondrilla nucula* (151) and the freshwater sponge *Spongilla lacustris* (123). An initial analysis comprising all 10 libraries yielded a highly significant ($P < 0.001$) result (i.e., the differences in sequence composition among the various libraries were not due to chance). Likewise, comparisons of the marine versus freshwater (*S. lacustris*) libraries, as well as comparisons among the marine libraries and among broad geographic locations, were all highly significant. The usefulness of such analyses should increase as more 16S rRNA gene libraries are sequenced from sponges (and with greater sequencing coverage), including multiple species from the same location and/or from the same genus or family.

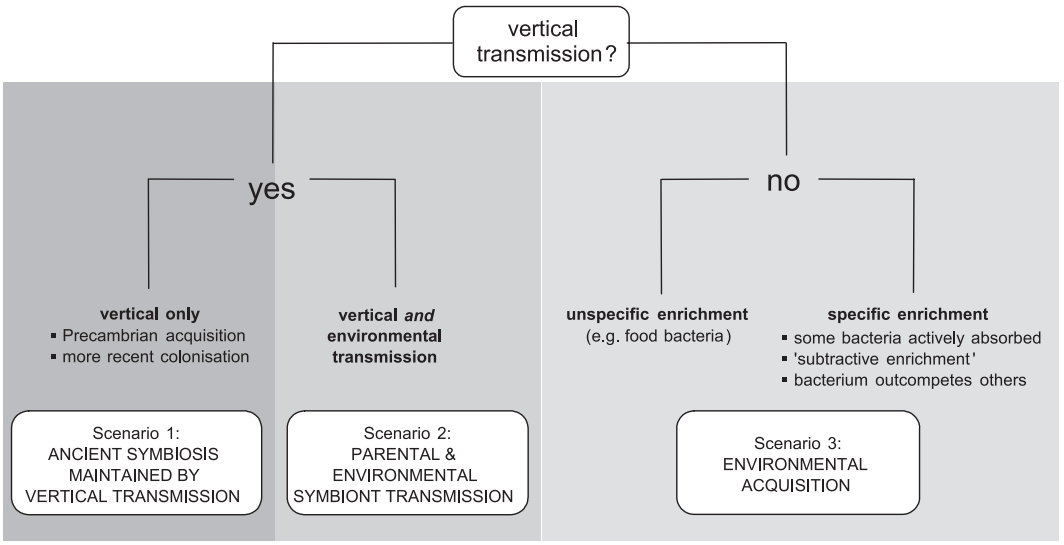

FIG. 16. Summary of various evolutionary scenarios for sponge-microorganism associations.

Sponge-Associated Microorganisms: Ancient Partners or Recent Visitors That Have Come To Stay?

Based largely upon arguments centering on immunological evidence dating back to the 1980s (469), it is often stated that sponge-bacterium symbioses have existed for as long as 600 million years. This would date such associations back to the Precambrian, prior to the bulk of taxonomic radiation in sponges. Moreover, given the likely basal position of sponges in the metazoan phylogenetic tree (38, 133), this would presumably make sponges and microorganisms the most ancient of all metazoan-microorganism associations. So what is the evidence for this oft-cited ancient symbiosis? In his 1984 study, on which the majority of these arguments rest, Wilkinson used a collection of 296 sponge isolates which, on the basis of morphological and physiological characteristics, comprised one bacterial species (469). In addition, 128 seawater and nonspecific sponge isolates were included as control strains. It is important to note that the sponge-specific isolates were obtained from phylogenetically distant sponges in widely separated geographical regions. From seven of the specific isolates and five of the others, Wilkinson prepared antisera and performed agglutination tests. Many of the "sponge-specific" strains reacted positively in these tests to one or more of the antisera derived from sponge-specific bacteria, but none of them reacted with sera derived from non-sponge-specific strains, nor did cross-reactions occur between the 128 non-sponge-specific strains and the sera prepared from sponge-specific bacteria. The implication of these results was that the studied, widespread, sponge-specific bacterium did indeed form a single species group distinct from isolates found in the surrounding seawater (469). According to Wilkinson, the most logical explanation for the occurrence of this specific bacterial type in such diverse hosts and locations was that these bacteria became associated with an ancestral sponge prior to the evolution of current sponge classes (i.e., during the Precambrian). One should bear in mind, however, that the enormous complexity of microbial communities in seawater could have led to this bacterium being missed in Wilkinson's culture libraries.

In the 22 years since the Wilkinson study, a wealth of molecular data has become available for sponge-associated microorganisms, ranging from sequences of single genes to an entire genome (for the archaeon "*Candidatus* Cenarchaeum symbiosum") (134). Here we ponder whether such data can be exploited to address the issue of the ancientness (or otherwise) of sponge-microbe associations. First, we consider some of the many possible evolutionary scenarios (summarized in Fig. 16), as follows.

Scenario 1: Ancient symbioses maintained by vertical transmission. A given sponge-specific cluster in the phylogenetic tree of life may contain 16S rRNA gene sequences derived from distantly related, geographically disparate sponge species. If the microorganisms represented by these sequences do not occur outside sponges today, then the ancestral strain (the future symbiont) may have first inhabited a sponge during one or several colonization events prior to sponge speciation (~600 million years ago) (the Precambrian acquisition hypothesis of Wilkinson [469]). Such a symbiosis could have been maintained in the intervening years via vertical transmission (see "Establishment and Maintenance of Sponge-Microbe Associations"), and the microbes evolved to become sponge (or even species) specific. A related but subtly different hypothesis is that an association could still be ancient but not predate the bulk of sponge speciation. In this scenario, it is conceivable that one sponge could have been colonized very early on, resulting in the evolution of a sponge-adapted microorganism. Millions (or hundreds of millions) of years later, this microbe could have spread across the oceans and, upon encountering other sponges, colonized them. Perhaps it is no longer present in seawater, or perhaps it is still there but in very small numbers. Yet another scenario is that today's sponge-specific microbes were once a generalist marine species, thriving in all marine ecosystems, including sponges. Those strains that inhabited sponges have since evolved to become genetically dis-

tinct from their free-living counterparts. Support for these scenarios comes from another quarter, with various fatty acids of likely microbial origin occurring in a wide range of sponges irrespective of host phylogeny or geographic location (401, 403). The apparent absence of some of these biomarkers from marine sediments and seawater led to the suggestion that the compounds and their microbial producers have been present in the sponges since ancient times (403).

It is likely that any ancient sponge-microbe symbiosis would be obligate for one or both partners, potentially involving a reduction in microbial genome size if the symbiont has developed a nutritional dependence on its host. This has been demonstrated for many obligate insect endosymbionts (e.g., see references 252, 437, and 501), but it is unknown whether such tight host-symbiont coupling occurs in sponges. Integration of host and symbiont genomes was discussed in the sponge context by Sara and colleagues (337), while a recent paper offers evidence for lateral gene transfer from a fungus to the mitochondrion of its host sponge (327). Such gene transfer would not be without precedent among marine invertebrates, as it is believed that the ascidian *Ciona intestinalis* laterally acquired a cellulose synthase gene from a bacterium (253). Future genome sequencing of sponges and their microbial associates should offer valuable insights into the nature of these symbioses.

As noted earlier, not all sponge species harbor abundant microbial communities, and it is worthwhile to take a moment to consider these organisms. Freshwater sponges, for example, typically contain a paucity of microbial associates, and it has been suggested that this is due to an obligate requirement for sodium ions by the symbiotic bacteria (469). When freshwater sponges colonized their new habitat from the sea some 20 to 50 million years ago, it is presumed that existing symbionts were lost. Many marine sponges also harbor only relatively small numbers of microorganisms. These so-called low-microbial-abundance sponges (148) often cooccur with the high-microbial-abundance bacteriosponges, so habitat variation cannot be invoked as an explanation for these differences. Whether these sponges once contained, but later lost, large communities of microbial symbionts is unknown. It is also unknown whether the (comparatively few) microorganisms in low-microbial-abundance sponges are phylogenetically similar to those in their high-microbial-abundance counterparts.

Based on sequence information already at hand, the nearest we can come to addressing these and the following hypotheses is to consider estimated rates of 16S rRNA evolution for members of given sponge-specific clusters and to attempt to infer when the last common ancestor of sponge-specific microbes from different sponges might have occurred. If one assumes equal mutation rates in different bacterial lineages and asserts that a 1 to 2% 16S rRNA sequence difference corresponds to approximately 50 million years of evolution (259), then sequence differences of at least 10% would be required to place a common ancestor of these organisms back in the late Precambrian (~600 million years ago). Here we consider two examples, the cluster representing the cyanobacterium "*Candidatus* Synechococcus spongiarum" (426) and the "*Poribacteria*" (100). The "*Ca*. Synechococcus spongiarum" cluster is one of the largest of all sponge-specific sequence clusters, is well supported by all tree construction methods, and contains 52 sequences from 21 sponges located around the world (Fig. 5). We chose three of these sequences as an example, derived from the sponges *Theonella conica* (sampled from east Africa; GenBank accession number AY701309), *Aplysina aerophoba* (from the Mediterranean Sea; GenBank accession number AJ347056), and *Antho chartacea* (from southeastern Australia; GenBank accession number EF076240). The minimum pairwise 16S rRNA similarity among these sequences (after correcting for different sequence lengths) is 97.9%. This is a very minor difference when one considers the phylogenetically disparate hosts (the last two sponges are in different orders, while *T. conica* is in a different subclass) and their geographically distinct locations. Even if one assumes that cyanobacteria evolve very slowly, we argue that greater sequence divergence would be expected if these bacteria had indeed been living (separately) within their host sponges for 600 million years. This should be especially true for endosymbiotic microorganisms, which are believed to evolve more rapidly due to increased fixation of mutations within their small populations (259). These members of the "*Ca*. Synechococcus spongiarum" cluster may therefore have a much more recent common origin, reflecting a role of horizontal (i.e., environmental) transmission consistent with scenario 2 or 3 in Fig. 16. However, consideration of other sequences within the same cluster can yield a quite different result. The two least similar sequences within the "*Ca*. Synechococcus spongiarum" cluster are only ~93% similar, suggesting a much older separation of these particular strains. A comparable degree of similarity is seen in comparing sequences from the "*Ca*. Synechococcus spongiarum" cluster with those from free-living relatives. We suggest that a combination of vertical and horizontal symbiont transmission (scenario 2) could explain the observed data. Possible vectors responsible for horizontal symbiont transmission could include sponge-feeding animals, such as fishes and turtles (205, 274), analogous to the coral-feeding fireworm *Hermodice carunculata*, which acts as a vector for the coral pathogen *Vibrio shilonii* (386).

In our second example, we consider the "*Poribacteria*." At first glance, there appears to be a strong case for an evolutionarily ancient relationship between these bacteria and their sponge hosts. The members of this monophyletic, exclusively sponge-specific bacterial lineage differ in their 16S rRNA sequences by up to 15% and are some 20% dissimilar to their nearest nonsponge relative (derived from Antarctic sediment) (Fig. 11). Such high divergence within the cluster, together with the low similarity to the next most similar known organism, is suggestive of an ancient symbiosis with sponges. However, the two least similar "*Poribacteria*" sequences were taken from closely related (same family) sponges collected at the same Bahamas location, perhaps indicating horizontal symbiont transfer between these hosts. If the associations were ancient and involved strict coevolution of host and symbiont, then their respective phylogenies would be more congruent, with the least similar microbes being hosted by the least similar sponges. Furthermore, the long naked branch leading to the "*Poribacteria*" in the 16S rRNA tree could potentially be explained by faster rates of evolution in these bacteria. "*Poribacteria*" are a sister phylum to the *Planctomycetes* (446), which are sometimes believed to exhibit higher rates of evolution than other lineages (392). Like the case for "*Ca.* Synechococ-

cus spongiarum," a combination of vertical and horizontal symbiont transmission is thus the most likely scenario here, although the acquisition of these bacteria exclusively from the environment also cannot be ruled out.

Perhaps the most convincing evidence for a long-standing, symbiotic relationship between sponges and at least some microorganisms comes from demonstrations of coevolution. Despite difficulties in addressing this issue due to the phylogenetic complexity of sponge-associated microbial communities, several authors have now shown coevolution between sponges and microbes. In the first study, the mitochondrial cytochrome oxidase subunit 1 (*CO1*) gene and its bacterial homolog were amplified from several halichondrid sponges and their associated bacteria (98). A *CO1*-based phylogenetic tree of six putatively alphaproteobacterial symbionts was largely congruent with a tree containing sequences from the corresponding host sponges, suggesting that cospeciation had occurred (although there also appeared to have been a host switch event at one point). Subsequent studies of the filamentous cyanobacterium *Oscillatoria spongeliae* indicated a high degree of host specificity for various dictyoceratid sponges, with evidence of cospeciation as well as indications of some host switching (316, 396). Ongoing studies of this system by Thacker and coworkers (R. W. Thacker, personal communication) should further elucidate the complex evolutionary relationships among these tropical sponges and their cyanobacterial associates. Coevolution requires that the host and symbiont maintain close associations over evolutionary time, and as mentioned above, the mechanism by which this presumably occurs in sponges is vertical transmission of microorganisms in host eggs or larvae. An additional point to consider at this stage is that the phylogeny of sponges themselves is not fully resolved (40). Molecular data are often incongruent with traditional sponge taxonomy, which is based largely on morphological properties, such as growth form and spicule characteristics (8, 37, 190). Accordingly, our understanding of symbiont evolution in sponges will continue to develop only in parallel with improvements in our knowledge of host phylogeny. A recently initiated *CO1* sequencing project for taxonomically diverse sponges (www.spongebarcoding.org) is a step in the right direction for achieving the latter goal.

The final type of evidence for ancient, close associations between sponges and microorganisms comes from the fossil record (43, 261, 377). Reef mounds constructed by siliceous sponges and cyanobacterial mats, with the latter represented in part by stromatolites still found today, flourished in (sub)tropical marine waters as far back as the early Cambrian (43). The fact that sponges and microbes closely coexisted hundreds of millions of years ago is thus clear, but the nature of that interaction (e.g., whether microbes lived within sponge tissues) remains less certain.

Scenario 2: Parental and environmental symbiont transmission. Demonstrated vertical transmission is generally considered a strong indicator of symbiosis, yet this does not rule out the possibility of horizontal (e.g., environmental) transmission of the same microbe as an additional mechanism. Indeed, this phenomenon has already been shown for insect-bacterium symbioses (reviewed in reference 67), and here we borrow from the well-developed literature on this topic. In aphids, the primary (obligate) bacterial symbiont *Buchnera aphidicola* is vertically transmitted, whereas secondary (facultative) symbionts can be transferred either vertically or horizontally (329). Given that facultative symbionts can confer fitness advantages upon their hosts, maintenance of these populations—by whatever mechanism—is of clear benefit. Interestingly, it was recently shown that secondary symbionts in aphids can also be transmitted via the sperm, yielding a different infection pattern from that which would be expected based on strictly maternal transmission (237). As discussed in a subsequent section of this article, both maternal and paternal transmissions of cyanobacterial symbionts have been documented for a marine sponge (424). Another finding from the insect world which is relevant to our discussion is that certain bacteria can invade novel host species and form stable associations, perhaps using similar mechanisms for invasion to those found in pathogenic bacteria (67). Provided that host chemical and immune defenses can be evaded, it therefore seems entirely plausible that marine microbes could invade, and establish themselves within, sponges from which they were previously absent.

While phylogenetic trees of primary insect symbionts are congruent with those of their hosts, episodes of horizontal transfer in secondary symbionts obscure the coevolutionary signal for these microorganisms. As mentioned in the preceding section, in which the cases of the "*Poribacteria*" and "*Candidatus* Synechococcus spongiarum" were highlighted, the available molecular evidence for sponge-associated microorganisms supports a combination of vertical and horizontal transmission (not just overall, but for specific individual lineages). Another salient example is the alphaproteobacterial sponge associate represented in Fig. 14; this bacterium occurs widely in sponges (95, 453) and appears to be vertically transmitted (95) yet is closely related to bacteria isolated from seawater. Issues of 16S rRNA sequence resolution notwithstanding (i.e., minor rRNA differences may hide major ecological or even genomic differences) (178, 323), this example underscores the complexities involved with considerations of sponge-microbe evolution.

Scenario 3: Environmental acquisition. In the third scenario, putatively sponge-specific microorganisms are, in fact, also present in the surrounding seawater, but at such low abundance that standard methods fail to detect them. Several mechanisms exist by which the same microbes may then be detected upon contact with a sponge. Firstly, it is possible that sponges absorb specific microbial types from seawater, a process which would imply some degree of recognition of particular microorganisms (e.g., by the sponge's innate immune system) (244). Recognition of symbionts versus food bacteria has already been proven experimentally (see the following section), and if a given microbe encounters favorable conditions (e.g., high nutrients), it may multiply to the extent to which it can then be detected by the applied methods. Alternatively, a type of subtractive enrichment may occur, whereby those microbes which cannot resist phagocytosis by sponge cells are consumed and hardier bacteria (e.g., those with protective capsules) (482) survive and are physically enriched near the choanocyte chambers due to the sponge's filtering activities. If such resistant bacteria are capable of out-competing other potential colonizers, they may establish themselves within the sponge tissue. These possibilities can be placed under a banner of specific enrichment. Unspecific enrichment is another, at least theo-

retical, alternative; in this case, microorganisms would simply be concentrated by sponges during filtering to the extent to which they can then be detected by the applied methods. Although it is not easy to prove any of these hypotheses correct, it is, in principle, even more difficult to prove them wrong. Finding a sponge-specific microbe actively living in the ocean—independent from a host sponge—would lend support to the enrichment hypothesis. The converse is less convincing: if such cells are not detected outside sponges, it may simply reflect insufficient sampling.

Methodological considerations are of paramount importance in discussing the environmental acquisition scenario. Even after many studies of marine microbiology, it still cannot be discounted that many or most of the so-called sponge-specific microbes are in fact also present in seawater, but only at a low abundance which is not detected by standard methods. But how likely is it that sponge-specific microbes are actually present in other environments and that, due to methodological limitations, we simply fail to detect them? Given recent findings regarding the high level of diversity of uncommon microorganisms in the so-called rare biosphere (276, 376), the enrichment scenarios seem entirely plausible. Deep sequencing of seawater-derived 16S rRNA amplicons should provide further insights into the diversity of marine microorganisms, perhaps also yielding sequence types which are presently considered sponge specific. An interesting aside is that remarkably few of the >1,000 16S rRNA sequences obtained from the Sargasso Sea metagenome (440) are closely related to those sequences in sponge-specific clusters. That study employed a direct cloning approach and was thus free of PCR biases which might otherwise have resulted in the missing of certain sequence types. In this context, it will be very interesting to examine the upcoming results of the Sorcerer II expedition (www.venterinstitute.org), during which seawater samples are being collected from around the world. Irrespective of what such studies find, if the sponge specificity of any microbe is to be disproven, then it will be necessary to demonstrate activity of the said microbe outside a sponge host, as merely demonstrating its presence is not sufficient.

Highly relevant to this discussion is an interesting point made recently by R. Hill (154) regarding the abundance of microorganisms in seawater and the potential consequences for sponge microbiology. Central to this argument is the immense filtering capacity of sponges, i.e., up to 24,000 liters of seawater per day for a 1-kg sponge (443). Given that the typical cell density of bacteria in seawater is about 10^6 cells/ml, then such a sponge could take in a staggering 2.4×10^{13} bacterial cells per day. Thus, even if a specific bacterium is present in seawater at only 1 cell/ml (i.e., one-millionth of all cells present), then during a single day a sponge could still filter some 24 million cells of this organism from the water column (154). The implications are obvious: an organism which is perennially extremely rare in seawater (and therefore never detected by applied molecular methods) could conceivably be concentrated within a pumping sponge (e.g., in the choanocyte chambers, prior to phagocytotic ingestion) to an extent which is readily detectable by PCR or even hybridization-based methods. If it occurs widely (but is undetected) in seawater, then one can imagine a situation in which it is easily detected from different sponges at different locations and erroneously considered a sponge symbiont. Hunting for sponge-specific microbes in other prolific filter feeders, such as ascidians (rather understudied thus far, from a microbial perspective, but see references 239 and 413), may lead to the identification of these organisms from other, nonsponge sources. These arguments might seem to paint a grim picture for proponents of the sponge-specific microbe concept, but the following must also be considered. If it were really the case that such nonspecific enrichment of rare seawater microbes occurs in sponges, then surely sponge-derived 16S rRNA gene libraries would be dominated by other microbes which are known to be abundant in seawater. This does not happen. To illustrate the point, we use again the example of the cyanobacterium "*Candidatus* Synechococcus spongiarum": if "*Ca*. Synechococcus spongiarum"-type organisms are very rare in the ocean but are concentrated to sufficient levels to be detected in gene libraries from sponges, then the exceedingly abundant (and closely related) planktonic *Prochlorococcus* and *Synechococcus* strains should be much better represented. However, a cursory look at Fig. 5 reveals only a few such sequences from sponges. We thus feel there is a strong case for rejection of the unspecific enrichment hypothesis.

Regrettably, a lack of sufficient (appropriate) data prevents us from ending this section with firm conclusions regarding the origin of sponge-microbe associations. Even with almost 2,000 sponge-derived 16S rRNA sequences at our disposal, it is sobering how little can actually be inferred about the evolution of sponge-microbe associations. For almost every argument in favor of an ancient symbiosis, there exists a rational counterargument which invokes (recent) environmental acquisition as the probable driver of the association. For example, a sponge-specific cluster at the end of a long naked branch in a phylogenetic tree could reflect early divergence of this group from its relatives, or it may simply reflect insufficient sampling of closely related lineages and/or accelerated evolutionary rates of the sponge-specific organisms. Additionally, while low levels of sequence divergence within a sponge-specific cluster argue against an ancient association, the corollary does not necessarily hold: extensive intracluster sequence divergence could equally indicate a long, separate evolutionary history (e.g., among different hosts) or selective enrichment of diverse (but still monophyletic) organisms from seawater. So where do we stand at the moment? At this stage, there is no clear indication for scenario 1 (ancient symbioses), though this will be addressed more satisfactorily in the future once additional information on sponge phylogeny is available. The existing data point more towards scenario 2, whereby particular microbes may be passed vertically between generations, but with some horizontal exchange also occurring. Further consideration of potential vectors for the latter process would be worthwhile. Although we consider this scenario most likely, the supporting data are also consistent with scenario 3, i.e., environmental acquisition. This highlights a major current limitation from a methodological perspective, namely, our inability to distinguish between facultative sponge symbionts and specifically enriched microorganisms. Presumably, the former prefer to inhabit sponges but can tolerate conditions in seawater, while enriched microbes may simply tolerate sponges long enough to be detected by applied methods. Such apparently complex patterns of microbial transmission are not without precedent in

the animal kingdom, with insect-bacterium symbioses providing a valuable framework for future considerations of sponge-microbe symbioses. A well-studied marine system that has contributed greatly to our understanding of symbiont transmission—and, indeed, of host-microbe associations in general—is that of the squid *Euprymna scolopes* and its bioluminescent symbiont, *Vibrio fischeri* (195, 230, 258, 442). *V. fischeri* is acquired from the surrounding seawater by the juvenile squid, which, via a remarkable stepwise process coined "winnowing," prevents all other bacteria from being established, culminating in a monoculture of *V. fischeri* in its light organ (258). The phylogenetically less complex microbial communities encountered in hosts such as insects and the squid light organ (typically, in insects, there are one or two primary endosymbionts and one or two secondary endosymbionts) facilitate a depth of understanding of these processes thus far not achievable for the highly diverse microbial communities in sponges.

ECOLOGICAL ASPECTS: FROM SINGLE CELLS TO THE GLOBAL SCALE

Establishment and Maintenance of Sponge-Microbe Associations

The mechanisms by which associations between sponges and microorganisms are established are not well understood. As discussed at length in the previous section, the fundamental question of symbiont origin (i.e., whether symbionts were passed down from an ancestral sponge or obtained contemporaneously from seawater) remains unresolved, as do many of the mechanisms of sponge-microbe interactions and the regulation of microbial communities in these hosts. Extensive studies by Müller and colleagues have attempted to address the underlying bases of sponge-microbe interactions at the molecular level (36, 241, 243, 249, 399, 400, 444, 465). Sponges are often regarded as primitive animals, yet their morphological simplicity belies the possession of a surprisingly complex immune system (244). Indeed, the refinement of such a system has undoubtedly contributed to the success of sponges throughout their long evolutionary history, especially when one considers the immense numbers of (potentially pathogenic) microorganisms to which they are exposed due to their filter-feeding activities. Detailed studies of the Adriatic sponge *Suberites domuncula* have revealed immune responses against both gram-negative (36, 243, 464) and gram-positive (400) bacteria, illuminating one means by which sponges may select for and against certain microbes from the surrounding environment. In the case of the former, exposure of *S. domuncula* to the bacterial endotoxin lipopolysaccharide (LPS)—derived from gram-negative cell walls—elicited an increase in synthesis of two compounds with pronounced antibacterial activity (243). Confirmation that the compounds were indeed synthesized by the sponge was obtained by cloning of the gene encoding a key enzyme in the relevant biosynthetic pathway. A receptor for LPS on the sponge cell surface was later identified, as was a signal transduction pathway which is upregulated upon exposure to increased LPS levels (464). The immune response of *S. domuncula* to gram-positive bacteria takes a quite different form: upon exposure to peptidoglycan in the bacterial outer cell wall, the sponge responds with a rapid activation of endocytosis, followed by the release of lysozyme (400). Receptors for fungi (277) and viruses (466) also occur in sponges. For more detailed discussions of the various immune responses and signal transduction pathways in sponges, the reader is referred to recent reviews dealing with this topic (e.g., see references 244 and 246).

In another recent study from the Müller group, using *S. domuncula* and its alphaproteobacterial symbiont SB2 as a model system, the importance of oxygenation of sponge tissue in mediating the relationship was demonstrated (241). Specifically, it was shown that strain SB2 grew preferentially on minimal media with the aromatic compound protocatechuate, rather than glucose, as the carbon source. The bacterium can obtain protocatechuate in situ from the sponge, which produces this and other diphenols via the activities of the enzyme tyrosinase. Interestingly, tyrosinase activity and expression of the tyrosinase-encoding gene in *S. domuncula*, as well as the number of *pcaDC* genes in strain SB2 (responsible for bacterial utilization of protocatechuate and used here as a proxy for SB2 abundance on the surface [exopinacoderm] of the sponge), were all maximal under aerated conditions (241). Coupled with the observed loss of SB2 cells from the sponge surface under low-oxygen conditions, it was asserted that the oxygen level is responsible for regulating the bacterial fauna in sponges. Whether this type of mechanism is important in other sponge-microbe systems remains to be determined.

The coexistence of microbial symbionts with bacterium-digesting archaeocytes in the sponge mesohyl has long interested sponge biologists. In a series of landmark experiments, Wilkinson and coworkers fed tritium-labeled sponge- and seawater-derived bacteria back to host sponges and found that sponge symbionts passed through uneaten, whereas seawater bacteria were largely consumed (482). Two different mechanisms were proposed to account for this: either (i) symbionts are specifically recognized by the sponge and deliberately not ingested or (ii) bacteria use extracellular masking capsules to avoid detection by sponge cells (482, 483). While neither theory has been tested explicitly (but see the preceding discussion on sponge immune responses), the latter explanation is in favor today, with several studies reporting the existence of slime layers and sheaths on symbiotic bacteria (106, 427, 473). The results of recent experiments by the Hentschel group were consistent with earlier findings: seawater-derived bacteria were consumed by *Aplysina aerophoba* some 2 orders of magnitude faster than was a consortium of sponge-derived bacteria (459). In addition, when a green fluorescent protein-labeled food bacterium was fed to the sponge, it was rapidly digested within the sponge tissues. All of these findings carry interesting implications for the evolution of sponge-microbe associations (also see the previous section). If presumed symbionts are not taken from the seawater (i.e., either as colonizers or as a food source), then this suggests vertical transmission as the mechanism by which these associations are maintained.

It is now established beyond doubt that many sponges (at least among those in marine environments) harbor diverse and abundant microbial communities. What is far from established is how, if at all, the composition and density of these communities are regulated. The potential role of phages and protozoa in regulating microbial communities within sponges is of interest, but virtually nothing is known about this to date (but see

reference 211). Predatory bacteria, perhaps related to the deltaproteobacterial genus *Bdellovibrio*, could also be involved in structuring microbial communities in sponges (468). At least for cyanobacteria, it has been suggested that their abundance is directly proportional to the number of sponge cells, implying some degree of influence by the sponge over symbiont growth and reproduction (477). The high photosynthetic rates of cyanobacteria in sponges (see also the following section) should, with all things being equal, result in cyanobacterial growth to the extent that host tissues would be overwhelmed. It is thus likely that the sponge exerts some control over its symbiont populations, with several mechanisms being proposed, including the following: sponges consume excess symbionts, sponges eject symbionts when stressed, the host sponge steals photosynthate from the cyanobacteria, and sponges starve the symbionts (477). With some debate over the extent of sponge consumption of symbionts and no evidence for expulsion of excess symbionts, Wilkinson argued that the last two scenarios (steal and starve) are most likely. There is strong evidence for stealing of photosynthate from symbionts in other systems (e.g., coral-zooxanthella [128] and freshwater *Hydra-Chlorella* [232] symbioses), and it seems plausible that sponges may also produce some sort of host release factor to induce the release of large quantities of fixed carbon from the cyanobacteria. Along these lines, a host release factor was recently described for the symbiosis between the sponge *Haliclona cymaeformis* and its macroalgal symbiont *Ceratodictyon spongiosum* (129). In contrast, the starve hypothesis holds that if a sponge can somehow restrict the symbiont's access to essential nutrients, such as nitrogen and phosphorus, then symbiont protein synthesis and cell division would be restricted. Consequently, an excess supply of carbon-rich photosynthate would be excreted from the symbiont (156, 477). To the best of our knowledge, neither scenario has been proven unequivocally or disproven for any sponge-microorganism association. More generally, remarkably little is known about communication, or chemical cross talk, between sponges and their microbial associates. Marine sponges produce a wide variety of secondary metabolites, some of which could potentially enable them to select for or against particular types of microorganisms (185) (although the nonspecific nature of many antimicrobial compounds suggests that such selection may generally be limited to broader classes of microbes, e.g., gram-positive versus gram-negative bacteria). Conserved bacterial signaling systems, as exemplified by the acyl homoserine lactone (AHL) regulatory systems of many gram-negative bacteria (111, 369), often mediate colonization-related traits (e.g., biofilm formation, swarming, and virulence) and offer one means by which sponges could interact or interfere with bacteria. Bacteria capable of AHL production have already been reported from marine sponges (361, 389), as have other putative signaling molecules, such as diketopiperazines (1, 162, 182). It is highly likely that sponges produce metabolites which allow them to disrupt AHL-regulated phenotypes, as shown for the macroalga *Delisea pulchra* (126, 127, 223, 224) and various terrestrial plants (302, 393). Indeed, inhibition of bacterial swarming by chemical extracts from sponges has recently been shown, though it has yet to be clarified whether this is an AHL-specific effect (184).

Associations between sponges and microorganisms can be maintained over different generations in either of the following two ways: (i) microbes can be recruited from the surrounding water by filter feeding (i.e., horizontal or environmental transmission) or (ii) microbial symbionts can be passed on from the parent sponge via reproductive stages (i.e., vertical transmission) (Fig. 17). Although horizontal transmission of symbionts has been demonstrated convincingly for several marine symbioses (e.g., squid-*Vibrio fischeri* [258] and hydrothermal vent tubeworm-chemoautotrophic bacterium [257] symbioses), it is the latter mechanism which has received most attention for sponges. Bacteria have now been found in embryos or larvae from all three classes of Porifera (see reference 97 and references therein), including species with highly varied reproductive strategies. Sexual reproduction in sponges involves either vivipary (where larvae are brooded within the animal) or ovipary (whereby eggs, generally fertilized externally, develop outside the sponge). Evidence for vertical transmission of bacteria has been reported for both types (97, 116, 118, 181, 362, 419, 422, 431), while asexual reproduction, i.e., budding, could also contribute to symbiont transfer in some species (146). Indeed, gemmules, the asexual buds of freshwater sponges, contain symbiotic zoochlorellae in at least some species (372), while a bud protruding from the surface of the marine sponge *Tethya orphei* contained a symbiotic cyanobacterium (117).

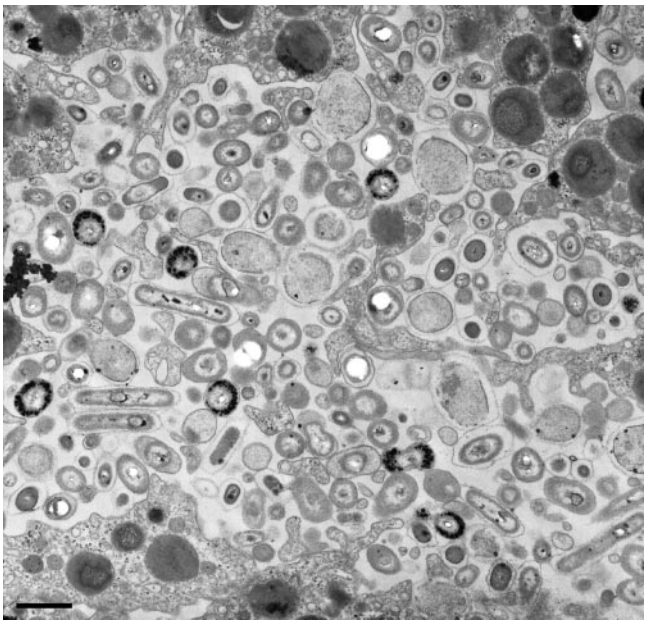

FIG. 17. Vertical transmission of microbial symbionts by a marine sponge. A transmission electron micrograph of a *Chondrilla australiensis* larva is shown, indicating a diverse range of bacterial morphotypes. Bar = 1 μm. (Modified from reference 420 with permission of the publisher.)

The vast majority of reports dealing with vertical transmission in sponges have been based on transmission electron microscopy (TEM) observations. Such studies have contributed greatly to our understanding of this phenomenon, with the identification of several mechanisms by which symbiotic microbes can be incorporated from maternal mesohyl tissue into eggs or embryos (reviewed in reference 97). These include phagocytosis of microbial cells by the oocyte directly from the adult mesohyl as well as transfer of microbes from parent

sponge to embryo along an "umbilical cord." Intriguingly, in the Australian sponge *Chondrilla australiensis*, eggs containing a cyanobacterial symbiont (of the "*Candidatus* Synechococcus spongiarum" type [426]) were distributed throughout the sponge mesohyl, whereas cyanobacteria are normally confined to the better-illuminated periphery, or cortex, of the sponge (422). Nurse cells, probably derived from choanocytes (425), have been invoked as a possible mechanism by which cyanobacteria are transported to the eggs (422, 424). These cells, which fuse with eggs and release their contents (including cyanobacteria) into the egg cytoplasm prior to spawning, presumably phagocytose the symbionts in the cortex before moving to the developing eggs deeper within the sponge. Remarkably, Usher and coworkers were also able to demonstrate the presence of cyanobacteria in sperm cells, indicating that both parents are capable of transferring symbionts to offspring (424). Sponges of the genus *Chondrilla* were also the subject of another recent TEM study (which additionally employed immunocytochemical techniques), in which vertical transmission of an endosymbiotic yeast was shown (221).

The drawback of the TEM approach is that, with some exceptions (e.g., cyanobacteria [423, 424]), even phylum-level identification of the relevant microorganisms is not possible due to an insufficient number of distinguishing morphological characters. Multiple bacterial morphotypes have been observed in sponge larvae (suggesting transmittance of a complex assemblage) (e.g., see reference 424), yet little or nothing is known of their phylogenetic affiliations. The recent application of molecular techniques in this area (95, 266) thus offers the potential for exciting new insights into the phylogenetic (and, in principle, metabolic) complexity of transmitted microbial assemblages. A 16S rRNA gene library constructed using cyanobacterium-specific PCR primers confirmed the presence (as indicated by TEM) of a single cyanobacterial type in both larvae and adults of the Red Sea sponge *Diacarnus erythraenus* (266). The transmitted cyanobacterium is highly similar to the aforementioned "*Ca.* Synechococcus spongiarum"-type symbiont of *Chondrilla australiensis*. A range of molecular techniques were used to examine the bacterial community in larvae of the Caribbean sponge *Mycale laxissima*, revealing a much more diverse population than that recovered by cultivation efforts (95). A single alphaproteobacterium, related to *Pseudovibrio denitrificans* and previously reported from many sponges (Fig. 14), was the only bacterium from the larvae that could be grown on a standard marine medium. In contrast, sequences representing a diverse assemblage comprising *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, and *Proteobacteria* (including the isolated alphaproteobacterium) were recovered from a 16S rRNA gene library based on DNAs isolated from the larvae (95). Similarly diverse microbial communities were found in larvae of the sponges *Corticium* sp. (366) and *Ircinia felix* (352), using 16S rRNA-based approaches, such as gene libraries, FISH, and DGGE. Broad congruence between larva- and adult-associated microbial communities in these studies indicated that a significant subset of the resident microbes is transferred in this way. Future molecular studies could provide information on the metabolic properties of the transferred symbionts (e.g., via analysis of functional genes or FISH-microautoradiography), which should improve our understanding of the mechanistic bases of these symbioses.

Whatever the underlying mechanisms for the establishment and maintenance of sponge-microbe associations, it is apparent that in many cases such associations are highly stable and resistant to external disturbance (reviewed in reference 148). In at least some other instances (e.g., see reference 457), this is not the case. Briefly, neither starvation, exposure to antibiotics, nor transplantation to different depths could elicit major changes in bacterial community composition in *Aplysina aerophoba* (105) and *Aplysina cavernicola* (407). Similarly, even translocation of *Aplysina fistularis* from its natural depth of 4 m to a new depth of 100 m was not enough to significantly affect cyanobacterial abundance in this sponge (although the sponge did die at depths of >100 m) (222). In contrast, this change resulted in a loss of cyanobacterial symbionts from the cooccurring sponge *Ircinia felix*. The importance of cyanobacterial symbionts—at least to some sponges—was recently demonstrated by Thacker in a series of elegant field experiments (394). To test the hypothesis that a greater benefit to the host is derived from more specialized symbionts, shading experiments were conducted with the tropical sponges *Lamellodysidea chlorea* (containing the host-specific cyanobacterium *Oscillatoria spongeliae*) and *Xestospongia exigua* (which contains the generalist symbiont "*Ca*. Synechococcus spongiarum"). Shaded individuals of the encrusting sponge *L. chlorea* lost >40% of their initial area, but the chlorophyll *a* concentration in the remaining sponge tissue (a proxy of cyanobacterial abundance) did not change, implying a close, putatively mutualistic relationship between the sponge and *O. spongeliae*. In contrast, *X. exigua* lost relatively little mass but did lose many of its "*Ca*. Synechococcus spongiarum" symbionts, suggesting that this relationship is less tight-knit than the other (394). While the mechanism by which symbionts are lost from *X. exigua* is unclear, the existing data do suggest that the specialist *O. spongeliae* provides a greater benefit for its host sponge than does the generalist "*Ca*. Synechococcus spongiarum" for its host. Although the necessary data are currently lacking, one could speculate that the degree of host specificity (of individual symbionts or even entire communities) may explain some of the different results obtained from previous perturbation experiments (148). More generally, the extent of host specificity among marine eukaryote-associated microbes may have substantial implications for microbial diversity on a wider scale (390). If most symbionts are highly host specific, then their overall diversity will be much higher than if the same hosts harbor mostly generalist species. Finally, Roberts and coworkers recently reported the results of experimental manipulations with the photosynthetic symbiont-containing Australian sponge *Cymbastela concentrica* (322). Shade and, to a lesser extent, silt treatments (both designed to mimic the physical effects of sewage effluent discharge) led to lowered chlorophyll *a* concentrations within the sponge, while increased salinity and nutrient loads had negligible effects.

Physiology of Sponge-Associated Microorganisms

A lack of pure cultures for most sponge-associated microorganisms has contributed to a paucity of knowledge about their physiological characteristics. What we do know has arisen

from a combination of the existing pure-culture studies, process data, and inferred metabolic properties from analysis of 16S rRNA, functional genes, and metagenome data. Collectively, microbes in sponges are capable of, among other processes, photosynthesis, methane oxidation, nitrification, nitrogen fixation, sulfate reduction, and dehalogenation. Here we summarize the current knowledge by first examining, in turn, the major nutrient cycles within sponges.

Carbon. Heterotrophy is a common form of carbon metabolism in sponges, either via consumption of microbes from seawater or via microbial uptake of dissolved organic carbon (495). However, for many sponges, particularly those in tropical regions, carbon metabolism centers around the activities of photosynthetic microorganisms, such as cyanobacteria (11, 58, 59, 381, 470, 474, 477, 486). Many tropical sponges contain substantial populations of these oxygenic autotrophs, and nowhere is the contribution of microbial symbionts to the host sponge more evident than in this case (see also the next section). Translocation of photosynthates (mostly as glycerol) from cyanobacteria to the host has been shown for marine sponges (476), while glucose produced by a chlorella-like green alga was passed to its freshwater sponge host, *Ephydatia fluviatilis* (475). Phototrophic sponges—those whose carbon nutrition depends heavily on cyanobacterial symbionts—receive >50% of their energy requirements from cyanobacteria (474), allowing these species to thrive in the low-nutrient, high-light areas commonly found on tropical reefs. On the Great Barrier Reef, phototrophic sponges comprise approximately half of the total sponge biomass on outer reefs, where the water is cleaner, but are much less common inshore, where terrestrial runoff and turbidity are greater (470, 485). Similarly, phototrophic sponges are largely absent from Caribbean reefs, where only small numbers of sponge-associated cyanobacteria are present (470). Phototrophy has also been demonstrated for at least one temperate sponge (57), while numerous others are known to contain photosynthetic symbionts (321, 336, 390, 430). The sponge *Cymbastela* sp. from temperate South Australia was capable of compensating photosynthetically (i.e., the rate of photosynthesis equals its rate of respiration) at a 4- to 5-m depth in winter, while it was a net producer at the same depth in summer (57). In contrast, Great Barrier Reef sponges may derive much of their nutrition from photosynthetic symbionts as deep as 15 to 30 m, due to the clearer water and, therefore, decreased light attenuation (58). Some sponges are apparently obligate phototrophs, with their lower depth limits determined by the availability of light for photosynthesis (58). Others function as mixotrophs, combining symbiont-derived nutrition with filter feeding, while still others contain no photoautotrophic symbionts and derive all of their carbon nutrition from filter feeding (477).

The extent to which other photosynthetic associates (e.g., diatoms, dinoflagellates, and phototrophic sulfur bacteria) contribute to carbon cycling within sponges is less clear. The Mediterranean sponges *Cliona viridis* and *Cliona nigricans* both contain symbiotic dinoflagellates (zooxanthellae), and for *C. viridis*, at least, it appears that sponge metabolism depends on the photosynthetic activity of these symbionts (353). Indeed, the growth of *C. viridis* was greater in individuals maintained under natural light conditions than in those maintained in constant darkness, reflecting the contribution of photosynthetic symbionts to host metabolism (324). Conversely, in at least one case, it appears that diatoms in Antarctica may parasitize the sponge host, using its metabolic products as an energy source (16). The sponge *Cymbastela concentrica* in southeastern Australia contains high densities of diatom-like cells in the illuminated periphery (M. W. Taylor, unpublished data), but further work is required to elucidate whether this association is phototrophic in nature. The occurrence in this sponge of at least some cyanobacteria in addition to the diatoms (Longford et al., unpublished data) will complicate efforts in this direction. Some freshwater sponges (e.g., *Spongilla lacustris*) contain zoochlorellae, and although the symbiosis is not obligate (aposymbiotic individuals occur in areas of deep shade), it appears that algal photosynthesis can contribute to host metabolism and growth (109, 333, 475).

An unusual form of nutritional symbiosis is that between methanotrophic bacteria and deep-sea cladorhizid sponges (428, 429, 431). These remarkable sponges, which possess no aquiferous system but instead prey on tiny swimming organisms, are believed to obtain a significant portion of their nutrition from the consumption of methanotrophs. Methane serves as a carbon source and substrate for energy production in methanotrophs, and in this particular system it is derived from a deep-sea mud volcano (429). In other sponges, the presence of methanogenic archaea may lead to methane production within anoxic zones. A 16S rRNA gene sequence affiliated with the methanogens of the phylum *Euryarchaeota* (456) is the sole piece of evidence for this at present, but the documented existence of anoxic microhabitats within sponges (159) suggests that these associations could be more widespread. Other chemoautotrophic microbial processes that have been observed in sponges and may also contribute to sponge nutrition are nitrification and sulfur oxidation. These are dealt with in the following sections.

Nitrogen. After carbon, nitrogen is the most important nutrient for life, as it is required for the synthesis of amino acids and, subsequently, proteins. In oligotrophic waters where nitrogen levels are low (e.g., coral reefs), symbiotic microorganisms may contribute to the nitrogen budget of sponges via fixation of atmospheric nitrogen, N_2 (479, 484). The first evidence for this came from measurements of nitrogenase activity in three Red Sea sponges (479). The activity of this enzyme, the catalyst for microbial N fixation, was estimated using an acetylene reduction test (for caveats, see reference 484) and could be measured only in *Siphonochalina tabernacula* and *Theonella swinhoei*, both of which contained cyanobacteria. In contrast, *Inodes erecta*, which contained only noncyanobacterial microorganisms, showed no evidence of N fixation. Additionally, nitrogenase activity was higher in illuminated tissue than in that maintained in the dark and did not correlate with the abundance of the heterotrophic bacterial communities in *S. tabernacula* and *T. swinhoei*. Taken together, these data suggested that nitrogenase activity was due mainly to the presence of cyanobacteria, many of which are capable of N fixation (479). A subsequent study provided more concrete proof of N fixation in sponges by demonstrating incorporation of the stable isotope $^{15}N_2$ into various amino acids in *Callyspongia muricina* (484). Whether microbial N fixation is of major ecological significance for sponges remains uncertain, but it does appear that its occurrence in sponges is not limited to cyanobacteria.

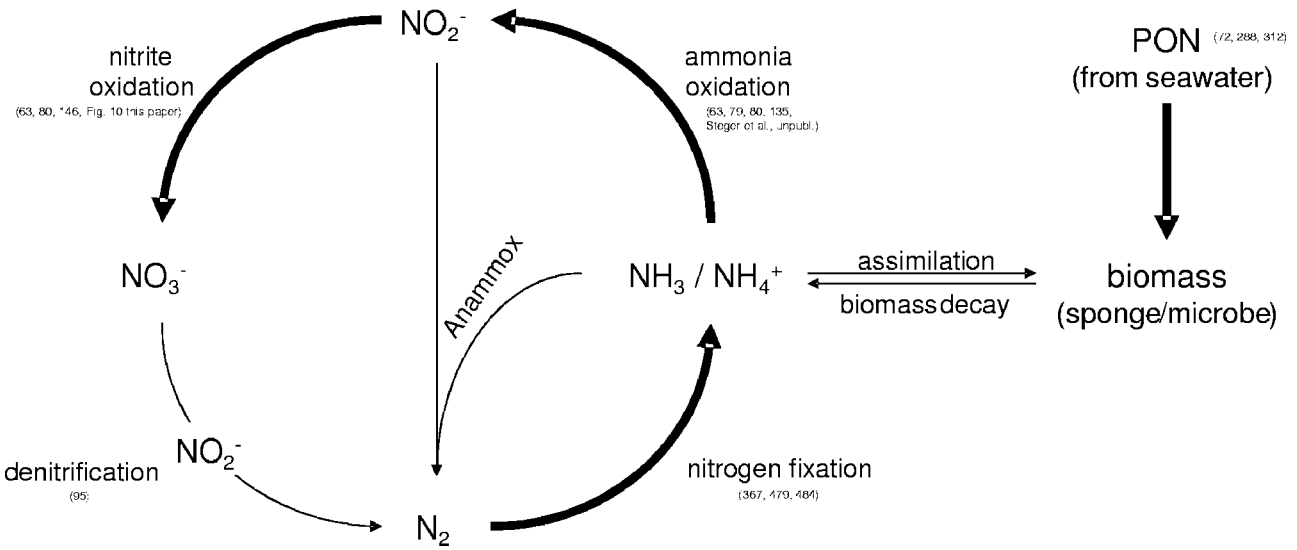

FIG. 18. Current state of knowledge about the nitrogen cycle in sponges. Thick arrows signify those processes which have been demonstrated in sponges; references (given in parentheses) pertain to either the process or the implicated microorganisms. PON, particulate organic nitrogen.

Heterotrophic nitrogen-fixing bacteria were reported from a *Halichondria* sp. (367), and the *nifH* gene (encoding a subunit of the nitrogenase reductase enzyme) has been amplified from both alpha- and gammaproteobacteria inhabiting several Caribbean sponges (N. M. Mohamed, Y. Tal, and R. T. Hill, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006).

Nitrification in sponges has also received attention. The two steps of nitrification, i.e., the biological conversion of ammonia to nitrite and then to nitrate, are catalyzed, in turn, by ammonia-oxidizing and nitrite-oxidizing microorganisms (33, 194). Ammonia, which can be toxic to eukaryotes, is a metabolic waste product and could accumulate within sponge tissues, particularly during periods of low pumping activity. The release of nitrate (and in some cases nitrite) from incubated sponges provided the first indication of nitrification within these organisms, with estimated rates often far exceeding those for other benthic substrata (63, 80). These results suggested the presence of nitrifying microorganisms, and indeed, 16S rRNA sequences from both ammonia-oxidizing betaproteobacteria (79; Taylor et al., unpublished data) and nitrite-oxidizing bacteria of the genus *Nitrospira* (Fig. 10) (146; Longford et al., unpublished data) have been recovered in molecular surveys of sponges. The widespread presence of *Nitrospira* in sponges may indicate low nitrite availability in these hosts, as members of the *Nitrospira* typically favor low-nitrite habitats (356, 448). Nitrifying microorganisms are among the few for which metabolic capabilities can generally be inferred from 16S rRNA data, and sequences representing several types of ammonia-oxidizing bacteria–in the genera *Nitrosospira* and *Nitrosomonas*–-were identified from the Australian sponge *Cymbastela concentrica* (Taylor et al., unpublished data). The finding of only *Nitrosomonas eutropha/europaea*-affiliated ammonia oxidizers in a previous study of six tropical sponges (79) may have been due to the use of overly specific PCR primers (both of the primers used have mismatches to all *Nitrosospira*-affiliated ammonia oxidizers and also to many *Nitrosomonas* spp. [193, 300]). Nitrite oxidizers belonging to the *Nitrospira* genus are frequently recovered in 16S rRNA gene surveys of sponges, yet in at least one case (80), extensive release of nitrite indicated that oxidation of ammonia and nitrite could be uncoupled. Interestingly, ammonia-oxidizing archaea, whose existence was just proven in 2005 (192), also exist in sponges. Metagenomic reconstruction of "*Candidatus* Cenarchaeum symbiosum," the abundant and yet uncultivated crenarchaeote in the Californian sponge *Axinella mexicana* (294), revealed the existence of ammonia monooxygenase (*amoA*) genes (required for ammonia oxidation) in this organism (135), while PCR-based surveys for *amoA* indicated that archaeal ammonia oxidizers are widespread in marine sponges (D. Steger et al., unpublished data). Whether archaea or bacteria are the key ammonia oxidizers in sponges remains to be determined, but in at least one system (soils), ammonia-oxidizing archaea appear to greatly outnumber their bacterial counterparts (204).

Numerous gaps remain in our knowledge of nitrogen cycling in sponges (Fig. 18). For example, the existence of anoxic zones in at least some sponges (159) suggests the potential for both denitrification and anaerobic ammonium oxidation (anammox). Neither process has been reported for sponges thus far, and our own efforts to amplify 16S rRNA genes from known anammox bacteria from several sponges have yielded no results. Denitrification is catalyzed by a phylogenetically diverse range of microorganisms, and it is risky to infer the ability to denitrify from 16S rRNA sequence data alone. Nonetheless, it is worth noting that a common alphaproteobacterial associate of marine sponges (95, 147, 453) (Fig. 14) is very closely related to the marine denitrifier *Pseudovibrio denitrificans* (368), and at least some of the sponge-derived strains have also tested positive for denitrification (95). The role of sponge filter feeding in providing particulate organic nitrogen is also of interest (312), with evidence that uptake of ultraplankton can yield sufficient nitrogen to sustain both the tropical sponge *Haliclona cymaeformis* and its macroalgal symbiont (72, 288).

Sulfur. Several lines of evidence point to the widespread occurrence of sulfur-metabolizing microorganisms in sponges. For starters, two of the key microbial players in the sulfur cycle, namely, sulfate reducers and sulfur oxidizers, have been found in multiple sponges. Sulfur-oxidizing bacteria from the families *Rhodospirillaceae* and *Chromatiaceae* (*Alpha-* and *Gammaproteobacteria*, respectively) were isolated from *Ircinia* sp. and *Euspongia officinalis* in the 1970s (173). In that paper, the bacteria were referred to as phototrophic sulfur bacteria, and additional, earlier isolations of green sulfur bacteria (phylum *Chlorobi*) were also discussed (see reference 173 and the Eimhjellen [1967] citation within). FISH signals for *Chlorobi* were later found in the Great Barrier Reef sponge *Rhopaloeides odorabile* (458). The above-mentioned sulfur bacteria oxidize reduced sulfur compounds such as hydrogen sulfide. This substrate is presumably derived from the activities of sulfate-reducing bacteria, which have also been obtained from sponges (159, 160, 173, 225, 358). An endosymbiotic sulfur cycle comprising sulfate-reducing and sulfide-oxidizing bacteria has already been demonstrated for a marine oligochaete (83), and the above data suggest that a similar process takes place in at least some sponges.

The most extensive work on sulfur metabolism within sponges has been conducted by Hoffmann, Reitner, and colleagues (159, 160, 225, 310, 358). They detected sulfate-reducing bacteria by FISH in the Mediterranean sponges *Chondrosia reniformis* and *Petrosia ficiformis* (225, 358), as well as in the cold-water sponge *Geodia barretti* (159, 160). In *G. barretti*, FISH detection of sulfate reducers belonging to members of the genera *Desulfoarculus*, *Desulfomonile*, and/or *Syntrophus* (estimated abundance, 1.8% of the total bacterial community) was complemented by isotopic measurements of sulfate reduction rates and analysis of oxygen profiles within the sponge (159). Sulfate reduction is an anaerobic process, and through the use of microelectrodes (354), these authors were able to demonstrate the presence of anoxic zones within the sponge, particularly during periods of pumping inactivity (159). The estimated sulfate reduction rates in *G. barretti*, of up to 1,200 nmol SO_4^{2-} cm^3 sponge day^{-1}, are among the highest recorded in natural systems. Intriguingly, analysis of lipid biomarkers suggested that bacterially derived carboxylic acids (perhaps from sulfate reducers) may be transferred to the host for subsequent synthesis into other compounds (159). The accumulation of toxic hydrogen sulfide was also addressed by Hoffmann and coworkers, who calculated that the activities of sulfide-oxidizing bacteria could, together with chemical reoxidation processes and the use of oxidized iron from seawater as an electron acceptor, be sufficient to balance microbial sulfide production. Although it is pure speculation at this stage, it is also possible that sulfur-oxidizing symbionts enable sponges to occupy sulfide-rich environments. The base of the Micronesian sponge *Oceanapia* sp., for example, can be buried up to 20 cm deep in the sediment (360), where anoxic conditions with high sulfide concentrations may prevail.

Interestingly, 16S rRNA gene sequences which are highly similar to those from known sulfate reducers (e.g., *Desulfobacterium* or *Desulfomicrobium* spp.) (Fig. 8) have only rarely been recovered from sponges. Ahn and colleagues did find *Desulfovibrio*-related organisms in enrichment cultures grown on *Aplysina aerophoba*-derived brominated phenolic compounds (3), but these sequences do not appear to have been deposited in GenBank and were therefore not included in our analyses. Analysis of functional genes [e.g., *dsrAB*, encoding the dissimilatory (bi)sulfite reductases] (447, 502) would be one way to gain further insights into the composition of sulfur-metabolizing microbial guilds within sponges.

A final aspect of sulfur metabolism that has received much attention in marine systems is that of dimethylsulfoniopropionate (DMSP) and its cleavage product, dimethylsulfide (DMS). These compounds are thought to play a role in global climate regulation (497), while DMSP may also protect marine algae and invertebrates from herbivores/predators and oxidative damage (384, 436). In a recent survey of DMSP content in a variety of coral reef invertebrates, high levels in corals were attributed to symbiotic zooxanthellae, while the much lower DMSP concentrations typical of sponges were presumed to be diet derived (435). It is currently unknown whether the levels in sponges are sufficient to play a role in predator deterrence or whether those sponges with symbiotic zooxanthellae have higher DMSP contents.

Other aspects of microbial metabolism in sponges. In contrast to the case for several major chemical elements (namely, carbon, nitrogen, and sulfur), to our knowledge virtually nothing is known about phosphorus cycling within marine sponges. We assume that sufficient phosphorus is obtained from the sponge's diet of microorganisms.

The degradation of halogenated chemicals within the Mediterranean sponge *Aplysina aerophoba* was the subject of an interesting recent study (3). This and other sponges are rich sources of brominated compounds such as bromophenols and bromoindoles, and it was predicted that microorganisms within such sponges may be capable of dehalogenation. Indeed, by establishing enrichment cultures from sponge tissue, in the presence or absence of various electron acceptors, the authors of that study were able to demonstrate reductive debromination under methanogenic and sulfidogenic, but not denitrifying, conditions (3). Antibiotic inhibition of dehalogenation activity indicated that it was the microbes, not the sponge, which were responsible.

The production of a wide range of secondary metabolites by sponge-associated microorganisms is well known. We provide examples of some pharmacologically relevant metabolites in a later section (see "Biologically Active Chemicals from Marine Sponge-Microbe Consortia and Their Commercial-Scale Supply") and, immediately below, outline the potential benefit(s) of these metabolites to the sponge-microbe association.

The Varied Nature of Sponge-Microbe Interactions

Sponges and the microorganisms living within and around them display the full gamut of interactions, from microbial pathogenesis and parasitism (sometimes resulting in sponge death) to microbes as the major food source for heterotrophic sponges and to mutualistic (or at the very least commensalistic) associations in which both partners appear to benefit. We first consider the putative benefits of symbiotic microorganisms to the host sponge.

Mutualism/commensalism. It is clear that sponges benefit greatly from the diverse metabolic properties of their associated microorganisms (see the preceding section). The provi-

sion of photosynthates and (perhaps to a lesser extent) fixed nitrogen from cyanobacteria (11, 474, 476, 477, 479, 486) is presumably a key factor in the ecological success of many sponges on nutrient-poor tropical reefs. Cyanobacterial symbionts may be equally important to juvenile and adult sponges. Sponge larvae are generally thought to be lecithotrophic (i.e., nourished from finite stored nutrients) (219), with no capacity for filter feeding (though some may assimilate dissolved organic matter from seawater [175]), so the energy gained from photosynthetic cyanobacteria should contribute to (i) gamete and larval longevity in the water column (424) and (ii) (once sponges are settled) the rapid growth required to outcompete algae and other photosynthetic organisms for substratum in illuminated areas (477, 478). Larval mortality may consequently be lower for those harboring cyanobacterial symbionts. The importance of photosynthetic symbionts to their hosts is evident in the typically flattened morphologies of phototrophic sponges, with the thinner species containing dense accumulations of cyanobacteria throughout the tissue. In contrast, mixotrophic sponges—those which utilize both filter feeding and photosynthetic symbionts for nutrition—may reduce their reliance on symbionts with age. Juveniles possess a high proportion of symbiont-containing tissue, which reduces as the sponge grows thicker and increases the amount of filter-feeding tissue (474, 478). Interestingly, endosymbiont photosynthesis can also bring with it certain costs for the sponge, such as the following: (i) morphological adaptation for improved light capture may occur at the expense of filter-feeding capacity (477), and (ii) oxidative stress may result from the presence of high levels of photosynthetically produced molecular oxygen, necessitating an enhancement of antioxidant defenses compared with those of asymbiotic specimens (303, 304). Another role for cyanobacteria and their pigments has also been proposed, namely, protection of sponges from excessive illumination (336). Although this role has not been proven experimentally, one expects that this could be particularly important for intertidal species, where radiation (UV and photosynthetically active radiation) is especially high (381). The documented occurrence of UV-absorbing mycosporine-like amino acids in sponges harboring cyanobacteria (e.g., *Dysidea herbacea* [13]) also supports the hypothesis of shading by the symbionts.

Microbial metabolism may benefit the host sponge in other ways. As mentioned earlier, Hoffmann and colleagues (159) described the likely transfer of carboxylic acids from anaerobic bacteria to the sponge *Geodia barretti*. Methanotrophic bacteria may supplement the nutrition of non-filter-feeding, carnivorous sponges in methane-rich deep-sea habitats (429, 431), while symbiotic zooxanthellae (dinoflagellates) enhance boring and growth rates in clionid sponges (153). Elimination of toxic metabolic by-products is another possible role played by sponge-associated microbes. The sulfur-oxidizing bacteria mentioned above oxidize reduced sulfur compounds, such as highly toxic hydrogen sulfide, to less harmful forms. Sulfide may accumulate in anoxic zones due to the activities of sulfate reducers, particularly during periods of low pumping activity (159). Similarly, ammonia and nitrite, which can be toxic to eukaryotes, are products of sponge and microbial metabolism but may be oxidized to harmless forms via the activities of nitrifying microorganisms. While negative effects of nitrite on the development of some juvenile freshwater sponges have been demonstrated in laboratory experiments (179), it is less clear whether ammonia or nitrite ever accumulates to sponge-harming levels in nature. Microelectrode studies to address such questions (354) would be of great interest and should further our understanding of the role of nitrifying microorganisms in sponges. Also of interest will be the data derived in the future from sponge genome projects. These should help to identify possible absent metabolic pathways in the host, whose functions may instead be filled by symbiont-derived factors.

Further putative benefits for sponges from their microbial partners include increased structural rigidity (due to mucous production by bacteria) (472), direct incorporation of dissolved organic matter from seawater (480, 495), digestion and recycling of insoluble sponge collagen (481), and microbial production of secondary metabolites that are of use to the host. In several cases, production of bioactive metabolites has tentatively been ascribed to bacterial symbionts, and these may serve to protect the sponge from pathogens, predators, and foulers (e.g., see references 147, 351, and 417). In some other cases, molecules produced by microbial symbionts could potentially be used as precursors for the biosynthesis of defense metabolites by sponges. Whatever the exact mechanism, it is likely that the chemical defenses of many sponges include both host- and symbiont-produced metabolites (see also "Harming the host: pathogenesis, parasitism, and fouling" and Biotechnology of Sponge-Microbe Associations: Potential and Limitations).

The observant reader may have noticed that the preceding discussion deals almost exclusively with putative benefits for the host, with little mention of advantages for the symbiont(s). Even when the benefits to the host sponge are obvious (e.g., in phototrophic sponges), it is not necessarily clear what benefit the microbial partner derives from the association. It is thus difficult, and often impossible, to confidently assign a mutualistic rather than just a commensalistic label to a sponge-microbe association. Presumed benefits for microbial symbionts of sponges include a generous supply of nutrients, as well as shelter—from predators or high light levels—in the sponge tissue (336).

Microorganisms as a food source for sponges. With the primary exception of phototrophic sponges (described above), most sponges are thought to obtain the bulk of their carbon nutrition via the consumption of microorganisms from the water column (though uptake of dissolved organic carbon may also be significant [e.g., see reference 495]). Bacteria (including both cyanobacteria and presumed heterotrophs) as well as eukaryotic microalgae can satisfy the entire food requirements of sponges (307), with the potential for dense sponge communities to significantly deplete the surrounding water of microbial cells (289, 309). Early studies of particle feeding in sponges indicated that as much as 96% of bacterial cells were removed from the inhalant seawater by the filtering activities of the sponge (308). These results were supported by the later application of more sophisticated techniques, in particular flow cytometry (23, 289, 290). Pile and colleagues reported grazing of the Atlantic sponge *Mycale lingua* on various types of plankton (<10 μm in size), with retention efficiencies ranging from 93% for *Prochlorococcus*-type cyanobacteria down to 72% for the smallest photosynthetic eukaryotes (290). Similar methodologies applied to the encrusting New Zealand sponge *Polymastia*

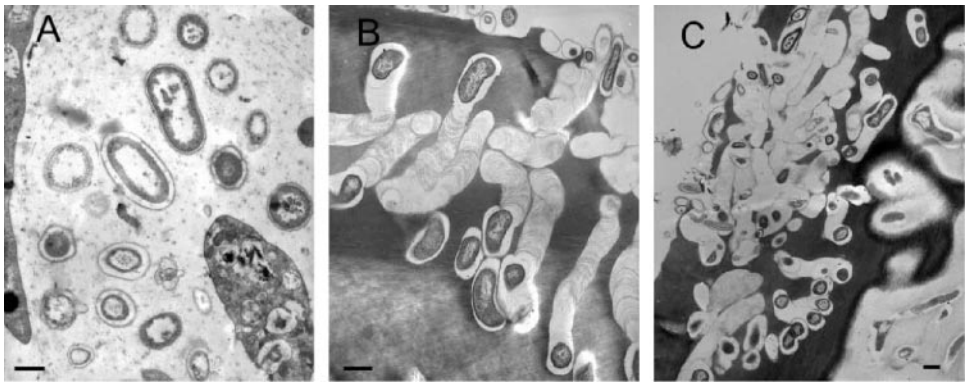

FIG. 19. Effect of a bacterial pathogen on a marine sponge. Transmission electron micrographs of *Rhopaloeides odorabile* tissue are shown, displaying (A) the diversity of bacterial morphotypes in healthy tissue, (B) a sponge experimentally infected with the alphaproteobacterial pathogen strain NW4327, and (C) consequent necrosis of the sponge tissue. Bar = 500 nm. (Reprinted from reference 455 with permission of the publisher.)

croceus showed the best retention of *Synechococcus*-type cyanobacteria (94%) and picoeukaryotes (88%), with somewhat poorer retention of *Prochlorococcus*-type cyanobacteria and other (noncyano-) bacteria (74 and 46%, respectively) (23). The lower retention of some cell types suggested that *P. croceus* was selective in its feeding. Laboratory experiments involving the feeding of symbiotic versus seawater bacteria to other sponges lend strong support to the notion of selective feeding (459, 482).

The generally highly efficient removal of particles from seawater is due largely to the extraordinarily large number of choanocyte chambers ($\sim 1 \times 10^7$ per cm^3) in sponge tissues (306). With each chamber containing as many as 150 choanocytes (371), coupled with the ability of pinacocytes (epithelial cells) to capture larger particles (308, 412), any food particle passing through the intricate aquiferous system of a sponge is subjected to intense grazing pressure. Interestingly, it now appears that even viruses can be retained by sponges, with some 23% of viral particles being removed from seawater by the Red Sea sponge *Negombata magnifica* (131). Considering the enormous abundance of viruses in seawater (1 million to 100 million per ml) (387), this could represent a significant flux of nutrients in ecosystems containing large sponge populations. While most studies of sponge feeding have been conducted with demosponges (e.g., see references 89, 196, 307, 313, and 379), microbial retention efficiencies of 90% or more have also been reported for hexactinellids (494, 496). The deep-sea hexactinellid *Sericolophus hawaiicus* was somewhat less efficient, with microbial retention efficiencies ranging from 47% for bacteria to 54% for photosynthetic eukaryotes of <3 μm (291).

Consumption of symbiotic microorganisms has also been raised as a possible food source for sponges (336, 337). The first report of apparent widespread disintegration, both intra- and extracellularly, of cyanobacterial symbionts was from Sara in the early 1970s (336). His TEM observations suggested that the Mediterranean sponge *Ircinia variabilis* actively degraded *Aphanocapsa*-type cyanobacteria (now considered *Synechococcus* spp.) (421) both in the sponge mesohyl and within certain sponge cells, providing an important source of photosynthetically fixed carbon to the host sponge (336). These results have since been questioned, with the suggestion that the observed lysis of symbionts was in fact an artifact of the histology procedure (477). Wilkinson argued that while some cyanobacterial cells may be digested intracellularly (e.g., see reference 473), this is the exception rather than the rule. However, several other reports of bacterial (including cyanobacterial) consumption by sponges have since emerged (172, 222, 266, 422), lending weight to the notion that certain sponges may "farm" bacteria as a food source (172). Indeed, phagocytosis and subsequent intracellular digestion of bacteria are the presumed mechanisms of nutrient transfer between a carnivorous *Cladorhiza* sponge and its methanotrophic symbionts (429, 431).

Harming the host: pathogenesis, parasitism, and fouling. Deleterious effects of microbes on sponges may be direct (i.e., pathogenesis or parasitism) or indirect (e.g., microbial films promoting surface fouling). The various reported instances of sponge disease have generally been attributed to bacteria or fungi (199), yet in most cases the responsible microbe(s) has not been identified unequivocally. A notable exception is a 2002 study by Webster and colleagues (455) in which they isolated a pathogenic alphaproteobacterium (designated strain NW4327) from an infected individual of the Great Barrier Reef sponge *Rhopaloeides odorabile*. Strain NW4327, which is related to the tumor-forming symbionts of *Prionitis* sp. macroalgae (12) and to the causative agent of juvenile oyster disease (34, 35), was shown to infect and kill healthy sponge tissue (455). The mechanism by which this occurred was via degradation of the collagenous spongin fibers, with almost the entire sponge surface subject to tissue necrosis following experimental inoculation with strain NW4327 (Fig. 19). Similarly infected tissue, with documented bacterial attack of spongin fibers, was evident during a devastating outbreak of disease in commercially important Mediterranean sponges during the late 1980s (433). Mass mortalities of commercially important sponges (e.g., *Spongia* spp.) have occurred several times in both the Mediterranean (199, 298) and Caribbean (119, 199, 375), virtually eliminating commercial sponge fisheries in some areas. Not only sponges, but also corals and other epibenthic organisms, experienced extensive mortality during a 1999 episode in the northwestern Mediterranean (52). This outbreak coincided with a sudden increase in seawater temperature, with subsequent laboratory studies suggesting the additional involvement of both protozoans and fungi. Increased microbial virulence

and/or compromised host resistance linked to global warming has already been postulated as a cause of many mass mortalities of marine organisms (139, 140), and it will be of great interest (and concern) to see how marine sponges are affected by predicted rises in seawater temperature in the future. Other reports of diseases in sponges include the so-called *Aplysina* red band syndrome, afflicting aplysinid sponges on Bahaman reefs (262), cyanobacterial overgrowth of *Geodia papyracea* (330), and repeated observations of diseased sponges on a Panamanian coral reef over a 14-year period (492). Bleaching of *Xestospongia muta* and other Caribbean sponges has also been reported (64, 91, 251, 441), but it remains to be established whether, as is the case for some corals (325, 326) and an Australian macroalga (R. J. Case, A. Low, W. C. Chen, S. Longford, G. R. Crocetti, N. A. Tujula, P. Steinberg, and S. Kjelleberg, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006), bacterial pathogens are to blame. Bacteria of two genera (*Bacillus* and *Pseudomonas*) were identified as possible agents of disease in the Papua New Guinean sponge *Ianthella basta* (54). This fan-shaped sponge has undergone significant mortality at a number of inshore sites, leading to speculation that the putative pathogen(s) may be of terrestrial origin. A simple model was recently developed to describe the role of sponge morphology in recovery from disease, with branching sponges being the most likely to recover (493). To our knowledge, nothing is known about diseases of freshwater sponges.

Parasitism of sponges by diatoms has been reported for several Antarctic species (16, 51). Bavestrello and coworkers found a negative correlation between chlorophyll *a* (used as a proxy for diatom abundance) and sponge carbohydrate levels (16), while in a parallel study of the hexactinellid sponge *Scolymastra joubini*, they described a degradation of sponge internal tissue in areas of dense diatom aggregations (51). The diatoms in *S. joubini* were of the genus *Melosira* and appeared to enter the host either through the ostia (inhalant openings) (Fig. 2) or via active incorporation by the sponge pinacoderm (dermal membrane). Why sponges should actively incorporate potentially harmful diatoms is not clear, although consumption of diatoms as a food source is one possibility (113, 114). Alternatively, the silica-encased diatoms may "trick" the sponge cells into taking them up (51), a plausible explanation given the tendency of some sponges to incorporate siliceous particles from the surrounding environment (15, 17, 18, 107). Currently, nothing is known about the nature of the interactions between sponges and diatoms in tropical and temperate systems (65, 390).

Microbes may also harm sponges in a less direct manner, for example, by promoting the fouling of sponge surfaces. Any surface in the marine environment, biotic or abiotic, is subject to intense fouling pressure. During the colonization process, a new surface will first develop a biochemical conditioning film, followed by microbial fouling (e.g., colonization by bacteria and diatoms). This biofilm then acts as a precursor to attachment by macrofouling organisms, such as invertebrates and macroalgae (71, 450), which in the worst cases can negatively affect sponge nutrition by blocking feeding channels or can increase hydrodynamic drag, resulting in sponge dislodgment from the substratum.

It is important that sponges not be considered mere helpless targets for potentially harmful microorganisms. Compounds with antibacterial, antifungal, or antifouling properties are produced by many sponges (19, 32, 112), and those chemicals with more specific effects may allow the host sponge to select for harmless or even beneficial microbes while deterring deleterious types. Interestingly, the resident microbial community may also participate in host defense, and there are numerous examples of the antimicrobial potential of apparently indigenous microbes (56, 147, 203, 397, 402). In addition, at least one sponge, *Halichondria panicea*, prevents fouling and sedimentary clogging of its ostia by sloughing off its outer tissue layer every few weeks (14), while the innate immune systems of sponges (see "Establishment and Maintenance of Sponge-Microbe Associations") are also believed to play a role in the prevention of microbial invasion.

The Big Picture: Temporal and Biogeographic Variability in Microbial Communities of Sponges

Variability in sponge-associated microbial communities has been examined at a number of levels, such as over time (days to months) (105, 201, 390, 462), within and among individuals of the same sponge species (mm to thousands of km) (7, 146, 201, 365, 390, 391, 404, 454, 462), and among different host species (146, 151, 208, 390). Other studies have investigated the spatial and/or temporal distributions of specific microbial taxa within sponges (100, 226, 294, 421, 453). If an emergent theme is to be identified from these studies, it is that, with some exceptions, sponge-associated microbial communities appear to be relatively stable, with little variation in time and space (148). The main caveat to this statement is that the methods employed may not always detect the variability which is present. We review the main published findings on these topics and, where appropriate, use our own phylogenetic analyses (see Evolution and Diversity of Sponge-Associated Microorganisms) to aid our discussion of symbiont biogeography.

Considering first those studies in which the whole microbial community was targeted, only a few papers deal with temporal variability. The first examined such variation in aquarium-maintained *Aplysina aerophoba* (105). The authors used several methods to characterize the resident microbial community, including cell counts (both 4′,6′-diamidino-2-phenylindole [DAPI]- and cultivation-based), TEM, FISH, and DGGE. What they revealed was an extremely abundant bacterial community (6.4×10^8 cells per g sponge tissue) which varied little during an 11-day incubation period, even under starvation conditions or upon exposure to antibiotics. Although DGGE banding patterns changed slightly during the antibiotic treatment, relative levels of abundance of the major bacterial groups, as assessed by FISH, stayed fairly constant irrespective of treatment (105). The lack of observed temporal variability as well as the apparent resistance of the community to disturbance suggests that *A. aerophoba* harbors a highly stable microbial community. Similarly, cultivation for up to 8 months did not seem to greatly alter the bacterial community in *Geodia barretti*, at least at the broad phylogenetic levels targeted by the applied FISH probes (160). Temporal variability has also been examined among sponges in the field. A 16S rRNA gene-DGGE study found bacterial communities in the temperate Australian sponges *Callyspongia* sp., *Stylinos* sp., and *Cymbas-*

tela concentrica to be highly stable over the course of a year, while additional sampling of the last species revealed a similar lack of variation on a shorter (days to weeks) time scale (390). Another DNA fingerprinting method, terminal RFLP analysis, also identified only relatively minor temporal changes in bacterial community composition on the surface of the sponge *Mycale adhaerens* from Hong Kong (201). In contrast to these studies, the bacterial community profile of the North Sea sponge *Halichondria panicea*, as assessed by 16S rRNA gene-DGGE, varied considerably over a 10-month period (462). The archaeal community, also assessed by DGGE, varied little. Another study by the same group, on the North Sea sponge *Pachymatisma johnstonia*, demonstrated stable bacterial communities in specimens sampled at different times (2 years apart) from two Orkney Isles collection sites (A. Wichels, S. Kuppardt, and G. Gerdts, presented at the 10th International Symposium on Microbial Ecology, Cancun, Mexico, 2004).

Spatial variability in sponge-associated microbial communities has been studied from the millimeter to the interocean scale. Taylor and coworkers examined spatial variability within and among individuals of three cooccurring Australian sponges (390). In all cases, the variation in 16S rRNA gene-DGGE banding patterns (and inferred community compositions) was minor, with even the least similar samples for a species sharing >70% of bands. For the 30% or less of bands which did vary, most of the variation could be ascribed to differences among rather than within individual sponges. Considerable differences were seen among different host species, with one sponge in particular harboring a distinct bacterial community compared to those in the other two species (390). In another study, Wichels et al. found differences between mesohyl-inhabiting microorganisms and transient microbes present in the sponge aquiferous system (462). The latter fraction of microbes was targeted by gently compressing *Halichondria panicea* tissue within a syringe and collecting the outflowing water. Although incomplete separation of aquiferous system and tissue fractions may have sometimes disguised differences (462), in general there did seem to be distinct communities between the two sample types. Marked differences were also evident between outer (cortex) and inner (endosome) tissues in the Mediterranean sponge *Tethya aurantium* (404). Cell separation techniques used in natural product research on sponges have also identified patterns of microbial distribution within sponge tissues. For example, cyanobacterial symbionts in the ectosome (outer tissues) of *Theonella swinhoei* were readily separated from a filamentous bacterium (later identified as the deltaproteobacterium "*Candidatus* Entotheonella palauensis" [351]) which occurs exclusively in the inner endosome (27, 28). It is typical for phototrophic symbionts, such as cyanobacteria, to be prevalent in the outer, well-illuminated surfaces of host sponges, while other microorganisms may dominate the inner core (148).

Moving up to the next spatial scale, we consider geographic patterns of variability. A 16S rRNA gene-DGGE study of the Antarctic sponges *Homaxinella balfourensis*, *Kirkpatrickia variolosa*, *Latrunculia apicalis*, *Mycale acerata*, and *Sphaerotylus antarcticus* revealed that associated bacterial communities were highly consistent, both among individual sponges at the same sampling site and also among three different sampling sites separated by some 10 km (454). The first molecular study of large-scale biogeographic variability in sponges was the 2002 study by Hentschel and colleagues (146), which we discussed at length in previous sections. The sponges *Rhopaloeides odorabile* (458), *Aplysina aerophoba*, and *Theonella swinhoei* contained substantially overlapping microbial communities whose sequences often fell in monophyletic, sponge-specific clusters, despite wide (host) phylogenetic and geographic separation (146). A 2005 study employed 16S rRNA gene-DGGE to investigate the bacterial community in the sponge *Cymbastela concentrica* along the eastern Australian coast (391). At eight sampling sites spanning 500 km of coastline within the temperate range of the sponge, bacterial community composition varied little. However, *C. concentrica* sponges from a tropical location >1,000 km away had a seemingly very different resident bacterial community. Seawater collected during sponge sampling varied comparatively little between the tropical and temperate locations. Allopatric speciation (resulting from adaptation to geographically separated hosts) is one possible explanation for the different communities, although latitudinal changes in environmental factors (e.g., temperature and light) could also be responsible. A more prosaic explanation is that the *C. concentrica* individuals from the two locations could simply be distinct (sub)species, although molecular taxonomy studies would be needed to confirm this (391). Cultivation efforts by Sfanos and colleagues in which 17,000 bacterial isolates were obtained and more than 2,000 were screened by 16S rRNA-based RFLP fingerprinting and/or sequencing often yielded the same bacterium from many sponge hosts from multiple locations (365). The most extreme case was that of an alphaproteobacterium (GenBank accession no. AY362009), which was recovered from 18 sponge species (plus several nonsponge sources, such as a coral and a sea cucumber) spread among various Caribbean and eastern Atlantic locations (365).

Several papers have examined the temporal and spatial variability of particular sponge-associated microorganisms, often providing valuable clues to the nature of the sponge-microbe association (i.e., true symbionts would be expected to maintain a long and consistent relationship). For example, "*Candidatus* Cenarchaeum symbiosum," the sole archaeon present in the marine sponge *Axinella mexicana*, was recorded from all 23 individuals of this sponge collected from the Californian coast over a 9-month period (294). Furthermore, archaeal rRNA levels stayed relatively constant (and high) for more than a year in aquarium-maintained *A. mexicana*, indicating a highly stable relationship between the sponge and its archaeal inhabitant. A subsequent study demonstrated temporally and spatially stable associations between three Mediterranean axinellid sponges (*Axinella damicornis*, *Axinella verrucosa*, and *Axinella* sp.) and their single, host-specific crenarchaeal associates (which, in all cases, were related to "*Ca.* Cenarchaeum symbiosum") (226). The cultivable fraction of the microbial community in *Rhopaloeides odorabile* was always dominated, irrespective of sampling time or location, by a specific alphaproteobacterium (453). This bacterium, which is closely related to *Pseudovibrio denitrificans* (Fig. 14), comprised >80% of the total heterotrophic bacterial colony count in samples collected over a 460-km portion of the Great Barrier Reef as well as in those collected during four consecutive seasons at one reef. Another alphaproteobacterium, affiliated with the genus *Rhodobacter*, was found in all samples of the sponge *Halichon-*

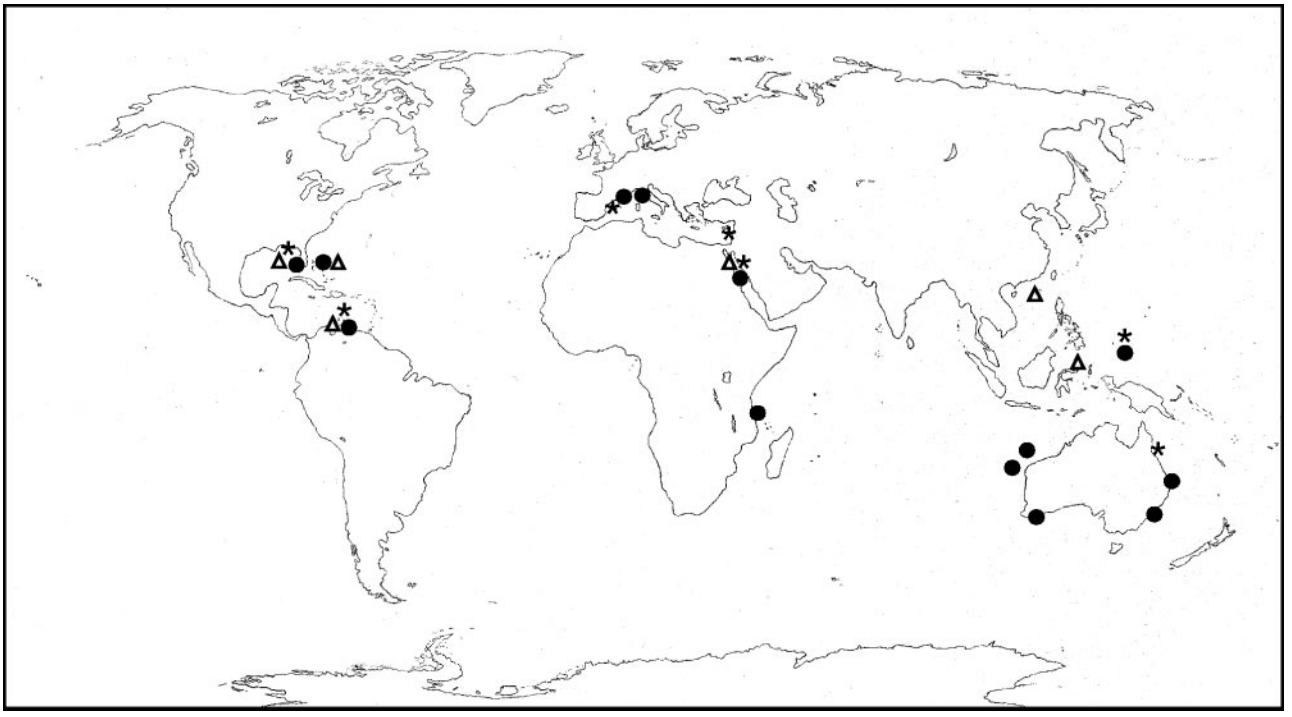

FIG. 20. Global distributions of selected monophyletic, sponge-specific clusters. Symbols refer to collection locations for representatives of the "*Ca.* Synechococcus spongiarum" (*Cyanobacteria*) (circles), *Actinobacteria* (triangles), and *Acidobacteria* (stars) clusters. In the last cluster, coral-derived sequences from the Mediterranean are also present.

dria panicea from the Adriatic, Baltic, and North seas (7). In a much earlier study, Wilkinson et al. isolated a particular bacterium (or at least a highly similar one, as molecular data were not feasible at that time) from several sponges in the Mediterranean and the Great Barrier Reef (483).

The biogeography of sponge-associated cyanobacteria has recently come under close scrutiny. Usher and colleagues performed an extensive survey of cyanobacterial symbionts, sampling nine sponge species (from six genera) in the Mediterranean Sea and the Pacific, Southern, and Indian Oceans (421). In addition, one of these sponges, *Chondrilla australiensis*, was sampled from eight Australian locations spanning several thousand kilometers and a wide temperature range. 16S rRNA gene sequences representing at least four closely related lineages of *Synechococcus* spp. were recovered from the various host sponges and included, most notably, "*Candidatus* Synechococcus spongiarum" (426), which was present in four of the sampled sponges, including all sampled individuals of *C. australiensis* (421). Interestingly, the "*Ca.* Synechococcus spongiarum" sequences from the Usher et al. study comprise, together with other sequences obtained independently from the Caribbean (78, 151, 342, 383), Red Sea (383), east Africa (383), Micronesia (146, 394), Mediterranean (146), and southeastern Australia (this study), one of the largest documented monophyletic, sponge-specific clusters (Fig. 5). In total, "*Ca.* Synechococcus spongiarum"-like sequences have been recovered from 21 sponge species from around the world, making this organism similarly widely distributed as its free-living *Synechococcus*/*Prochlorococcus* counterparts (273, 340).

Many of the sponge-specific clusters from other phyla are also widely distributed. The "*Poribacteria*," for example, have so far been identified from sponges in the Mediterranean, Caribbean, and eastern Pacific (100; this study), while a large sponge-specific cluster within the *Actinobacteria* (Fig. 13) contains sequences from the Red Sea (146), South China Sea (data not shown), Indonesia (235), and various Caribbean locations (235, 342; this study). Numerous other such examples exist, indicating an apparently global distribution of many sponge-specific microbes (Fig. 20). Hill and colleagues (151) attempted to relate the occurrence of major (sponge-associated) bacterial taxa to the geographic location where the host sponge was collected. They suggested that some patterns could be discerned whereby specific taxa might be better represented in, e.g., tropical but not cold-temperate sponges. However, we argue that such statements are premature and that there are insufficient data to draw firm conclusions at present. For example, many (or most) of the existing 16S rRNA gene libraries from sponges have not been sampled exhaustively, and missing taxa may well be represented in a library of clones but not yet identified due to low sequence coverage. Furthermore, different geographic areas (and different habitat types within an area) have not been studied equally well, so there could be biases towards apparently more diversity in some areas which have received more attention. The construction of 16S rRNA gene libraries from sponges in underrepresented locations (e.g., Africa, South America, and western North America) would go a long way towards improving our understanding of sponge symbiont biogeography.

Throughout this review, we have attempted to carefully evaluate published data in the context of the methods and ap-

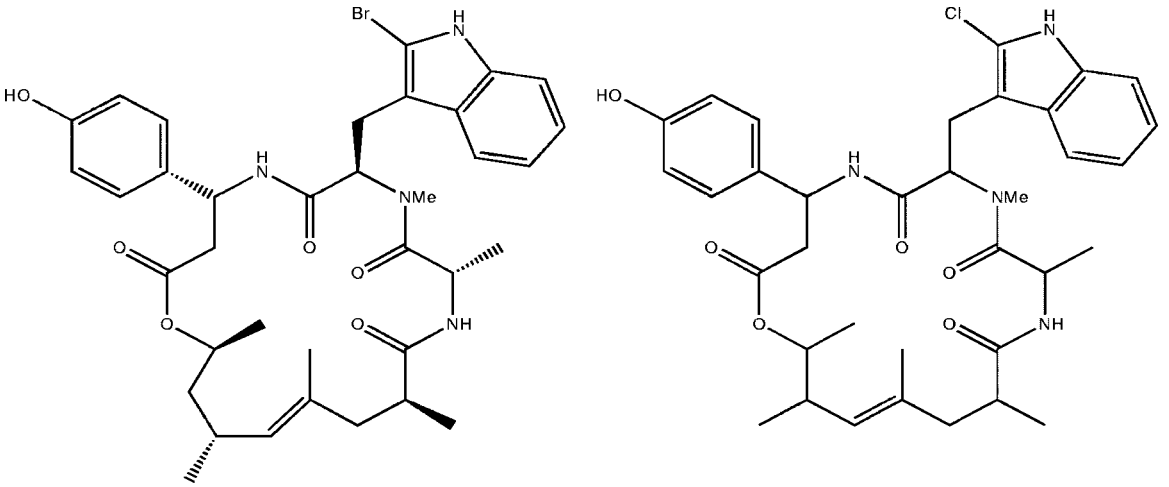

FIG. 21. Chemical structures of jaspamide (left), from *Jaspis* sp. sponges, and chondramide D (right), from the deltaproteobacterium *Chondromyces crocatus*. Note the remarkable structural similarities between the compounds.

proaches used for a particular study. This is never more critical than when considering variability in complex microbial communities. Appropriate sampling designs are important for investigations of any ecological system, yet in the past this has often been overlooked in microbial ecology circles (238, 390). The fact that one should analyze sufficient replicate samples to encompass biological variability is beyond question, yet many of the available methods for characterizing microbial communities are seemingly incompatible with this goal. DNA fingerprinting techniques such as DGGE, terminal RFLP analysis, and automated ribosomal intergenic spacer analysis offer high-throughput analyses of large numbers of samples, but together they suffer from one major drawback: without considerable efforts (e.g., sequencing of excised DGGE bands), the bands or peaks representing particular microorganisms have no identity. If banding patterns alone are compared as proxies of community composition, one must acknowledge that observed changes in a community could equally likely be due to changes among two strains of the same microbial species or to a much more significant shift, such as from a member of one bacterial phylum to another. Conversely, FISH and 16S rRNA gene library analyses can provide detailed quantitative and phylogenetic information, respectively, yet neither approach is well suited to analyzing large numbers of samples. Based on our own experience as well as reports in the literature (e.g., see reference 458), autofluorescence in sponges can also create difficulties for FISH analyses. A need therefore exists for a phylogenetically informative yet rapid means of assessing microbial community structure. Microarrays offer particular promise in this regard, with a range of 16S rRNA- and functional gene-based microarrays already available (reviewed in references 122 and 215). The highly parallel nature of microarrays provides the potential, for example, to survey the presence of multiple sponge-specific clusters in a single assay, something which is not possible with other existing techniques. Importantly, symbiont function could also be addressed via the so-called isotope array approach (2).

BIOTECHNOLOGY OF SPONGE-MICROBE ASSOCIATIONS: POTENTIAL AND LIMITATIONS

Biologically Active Chemicals from Marine Sponge-Microbe Consortia and Their Commercial-Scale Supply

An enormous number of biologically active compounds have been isolated from marine sponges and their associated microorganisms. Indeed, sponges are the most prolific marine producers of novel compounds, with more than 200 new metabolites reported each year (see reference 32 and preceding reviews in that series). Furthermore, more sponge-derived compounds are in clinical and preclinical trials (e.g., as anticancer or anti-inflammatory agents) than compounds from any other marine phylum (31). The occurrence in unrelated sponges of structurally similar compounds, particularly those which were otherwise known exclusively from microorganisms, led to speculation that such compounds (including some already in drug trials) were of microbial origin (27, 143, 280, 427) (Fig. 21). Since chemical synthesis of natural products can be problematic and expensive due to their structural complexity (4, 48, 373), the realization that at least some compounds may be produced by microbes raised hopes of obtaining a sustainable, essentially unlimited supply of compounds for testing and subsequent drug production (e.g., via cultivation of the relevant bacteria) (280, 297). Today, the true origin of most sponge compounds has still not been proven unambiguously and remains a key issue among marine natural product chemists. The possibility of convergent evolution of biosynthetic pathways among different sponges has also been raised (332). It is not our intention to comprehensively review sponge-derived natural products; such reviews are the subject of chemistry rather than microbiology per se, and many excellent reviews dedicated to this topic already exist (31, 32, 191, 236, 279, 280). Rather, we focus our attention on selected important examples and highlight some of the difficulties involved with obtaining a consumer-ready end product.

Sponge (or microbe)-derived compounds span a wide range of chemical classes (e.g., terpenoids, alkaloids, peptides, and

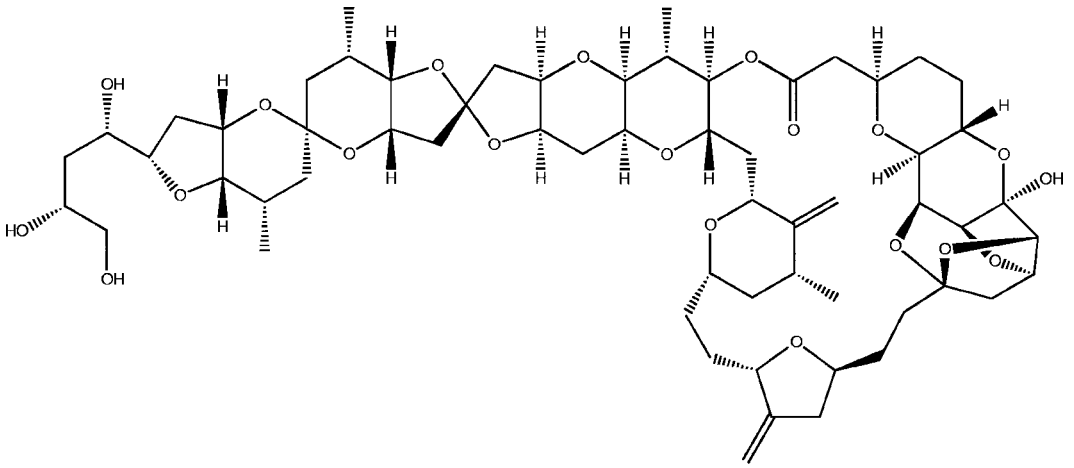

FIG. 22. Chemical structure of halichondrin B.

polyketides) with an equally wide range of biotechnologically relevant properties (e.g., anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, and antifouling) (31, 32, 112, 186, 229, 236, 279, 280). The attention of natural product chemists and pharmaceutical companies, at present, is focused firmly on anticancer drugs, with several promising sponge-derived compounds in clinical and preclinical cancer trials (31, 255, 370). The large number of novel, active metabolites being reported from sponges every year begs the question of why such chemicals have not yet made it to pharmacy shelves. To date, and to the best of our knowledge, not a single compound obtained from a sponge has been approved as a drug, with a major brake on progress being the so-called supply problem (138, 297, 408). (The nucleoside analogs Ara-A and Ara-C, commercialized as antiviral and anticancer agents, respectively, could arguably be considered the sole exceptions. They were not isolated directly from sponges but are synthetic derivatives based on compounds from the Caribbean sponge *Cryptotethia crypta* [24, 25].) Biologically active natural products are often produced in relatively small amounts, and often by rare animals whose natural populations cannot sustain the extensive collections required for clinical trials. Alternative means for producing large amounts of metabolites are therefore required. We illustrate this issue by using two examples, the anticancer compounds halichondrin B and peloruside A.

The halichondrins are a group of polyether macrolides that exhibit potent antitumor activities (158, 415). First isolated from the Japanese sponge *Halichondria okadai* in the mid-1980s (158), they were subsequently found in several other sponges from diverse geographic locations, including *Axinella* spp., *Phakiella carteri*, *Raspailia agminata*, and *Lissodendoryx* sp. (138). Halichondrin B (Fig. 22) was particularly sought after due to its high cytotoxicity, and its total synthesis was reported as early as 1992 (4). However, due to the structural complexity of the compound, many steps were required for its synthesis, rendering total synthesis impractical for industrial-scale production. While the occurrence of halichondrins in many unrelated sponges suggested a microbial origin, little was known about the microbiology of the relevant sponges, and thus alternative avenues were investigated (to our knowledge, the precise [i.e., sponge versus microbial] origin of the halichondrins has never been determined unambiguously). *Lissodendoryx* sp., collected from the coast of southern New Zealand, yielded the largest amounts of halichondrins and therefore became a focus of drug supply efforts (138, 250). Based on the potency of halichondrin B and its projected demand if approved for human use, the requirement for clinical trials was estimated to be ~10 g, with annual requirements as a commercial drug of 1 to 5 kg (138). Given that 1 metric ton of *Lissodendoryx* sp. sponges yielded only 300 mg of halichondrin B and that the entire natural biomass of the sponge was estimated to be only 289 metric tons, collection from the wild was quickly ruled out. Aquaculture of *Lissodendoryx* sp. was then investigated, with promising initial results (250). However, scale-up of the operation to the levels necessary for commercial production was not achieved due to a lack of funding (M. J. Page, personal communication), and the small amounts of compound present in the sponge tissue may render the aquaculture option economically untenable in this case anyway (373). Nevertheless, halichondrin B may yet prove to be a success story, with a synthetic analog, E7389, in phase I clinical trials as an anticancer compound (370). This simplified version of halichondrin B is more amenable to chemical synthesis but retains the biological activities of the original compound (60).

Our second example concerns the macrocyclic lactone peloruside A (460) (Fig. 23). Isolated from the New Zealand demosponge *Mycale hentscheli*, peloruside A shows promising

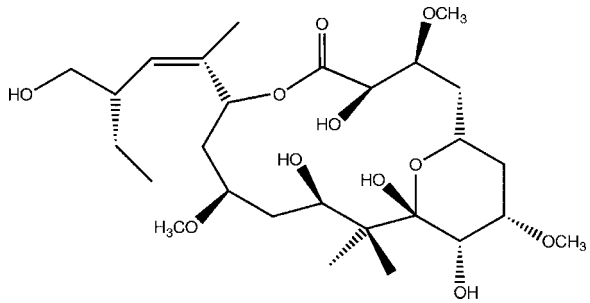

FIG. 23. Chemical structure of peloruside A.

anticancer properties, acting in a similar manner and potency to the widely used cancer drug paclitaxel (Taxol) (166). With the compound currently in preclinical trials, two avenues are being pursued in parallel to ensure a sufficient supply of the compound for subsequent clinical trials. Chemical synthesis is one approach, with several groups recently reporting partial or total synthesis (e.g., see reference 177). A New Zealand consortium, working together with a U.S. pharmaceutical company, is currently investigating whether cost-effective, industrial-scale synthesis is achievable (137). An alternative supply option for peloruside A is being explored by the same group, with aquaculture of *M. hentscheli* looking highly encouraging (137, 271). With 200 kg of sponge yielding a mere 2 g of pure peloruside A, scaling-up is a priority, with the goal of growing >500 kg of sponge over the coming year (137). Other compounds of pharmaceutical interest are also produced by *M. hentscheli*, namely, the cytotoxic polyketide mycalamide A and the macrolide pateamine (165, 256, 270, 278). Concentrations of these metabolites in natural sponge populations vary significantly in time and/or space (270), suggesting that complex ecological and physical factors may be involved in their production. An improved understanding of the ecological roles of these and other compounds could greatly benefit metabolite harvesting programs, and indeed, ecological observations are often used to guide the initial stages of drug discovery in marine environments (295, 359). Ongoing microbiological investigations with *M. hentscheli* (452; S. A. Anderson, unpublished data) should also benefit future drug development efforts with this sponge.

Supply issues notwithstanding, the pharmacological potential of marine sponges and other sessile invertebrates (e.g., corals, bryozoans, and ascidians) is enormous. Although progress toward the commercial product stage has been slow, it is highly likely that at least one of the several compounds now in clinical trials (or a synthetic analog) will be commercialized within the next few years. A combination of improved chemical synthesis methods with the various approaches outlined in the following section should ensure a bright future for this field, with sponge-derived natural products being utilized either in their natural form or as inspiration for new, laboratory-generated compounds (e.g., via chemical proteomics) (287). As a footnote to this discussion, the freshwater sponges should also be mentioned. Their chemistry has received much less attention than that of their marine counterparts, and while various lipids and a compound with antipredator activity have been reported (77, 311), it is unclear whether these sponges produce many, if any, compounds of pharmaceutical interest.

Methods for Accessing the Hidden Chemistry of Marine Sponges

A number of (nonsynthesis) approaches are available for accessing biologically active natural products from sponges and the microorganisms within them (Fig. 24). For convenience, we split these into the following three main themes: cultivation of metabolite-producing microbes, sponge culture, and molecular biological methods, such as metagenomics. In addition, we highlight the importance of metabolite localization studies for improving our knowledge of which partner (sponge or microorganism) is responsible for metabolite production.

Cultivation of metabolite-producing microorganisms. Cultivation of sponge-associated microorganisms that produce bioactive compounds is the most direct method for large-scale production of these chemicals (154), and cultivation approaches are widely practiced among those targeting bioactive compounds (46, 47, 81, 130, 147, 154, 163, 176, 189, 235, 364, 365, 378, 453). The potential payoffs from the cultivation approach are obvious and substantial: if metabolite producers can be isolated on artificial media and grown to significant cell numbers (while continuing to produce the relevant metabolite), then this obviates the need for large-scale harvesting of natural sponge populations, with its environmentally and financially negative implications.

Two broad strategies for isolating microbial producers of bioactive compounds were outlined by Hill in a recent review (154). The first is to use a wide range of media in an effort to grow as many different sponge-associated microbes as possible. Since growth under different culture conditions may influence which metabolites are produced, the use of many different media and conditions should help to maximize the chemical diversity from a given microorganism (154). Bacteria associated with deep-sea benthic invertebrates have been the subject of extensive cultivation efforts by the Harbor Branch Oceanographic Institution, with a range of nutrient-poor to nutrient-rich media being utilized (130, 264, 365). Approximately 17,000 isolated microbes, most from deep water and mostly from sponges, are present in the Harbor Branch Oceanographic Marine Microbial Culture Collection (365). These include representatives of the *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* and are the subject of natural product screening. An alternative, more targeted approach is to go after specific microbial groups with proven track records in the production of bioactive compounds. Many such groups, including cyanobacteria, fungi, and actinomycetes, are well known from sponges (148, 163), with actinomycetes being the subject of a particularly interesting success story. Sponge-derived actinomycetes of the genus *Micromonospora* produce manzamines, alkaloids with, among other things, potent antimalarial properties (10, 93, 155, 301). The first hint that manzamines were of microbial origin came from the finding of these compounds in many distantly related, geographically disparate sponge species. Subsequent cultivation-dependent and -independent characterization of the microbial communities in two Indonesian manzamine-producing sponges, 01IND 35 and 01IND 52, revealed highly diverse assemblages, with the recovery of actinomycetes provoking intensive culturing efforts in their direction (154). Growth of the sponge-derived *Micromonospora* sp. has since been achieved on a large scale in 20-liter fermentations, with maintenance of manzamine production (R. T. Hill, personal communication). Improvements in this process should greatly facilitate passage of these compounds through the various stages of the drug-testing process. Actinomycete-selective media were also used successfully with the sponges *Pseudoceratina clavata* (188), *Xestospongia* spp. (235), *Hymeniacidon perlevis* (498), and *Craniella australiensis* (209). In the last study, many of the cultivated actinomycetes displayed broad-spectrum antimicrobial activities.

There are numerous other examples of the production of biologically active compounds by sponge-derived microbial isolates. An antibacterial peptide was isolated from both the

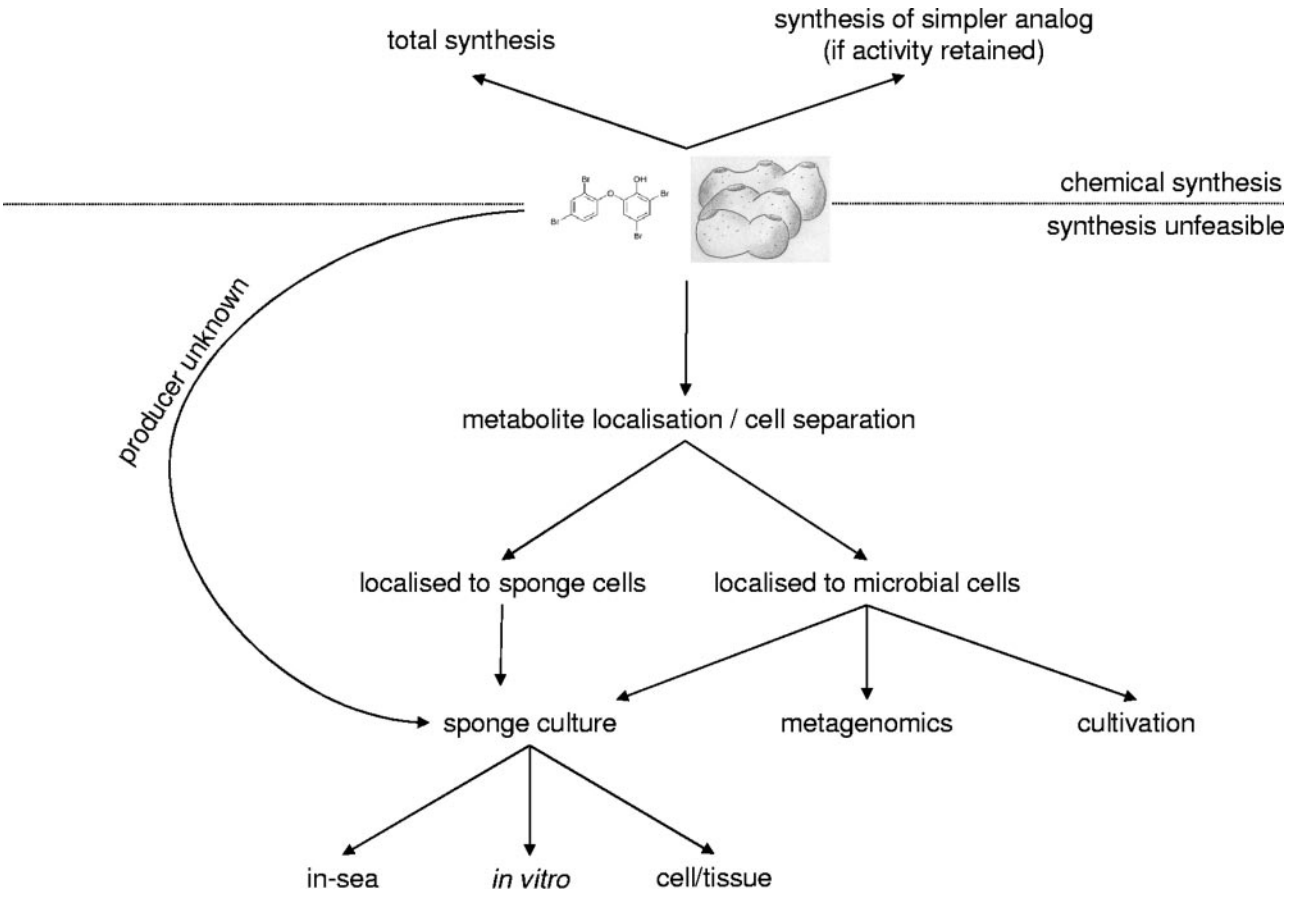

FIG. 24. Approaches for obtaining bioactive metabolites from marine sponges.

sponge *Hyatella* sp. and an associated *Vibrio* sp. (260), while a glycoglycerolipid produced by a *Halichondria panicea*-derived *Microbacterium* sp. had antitumor properties (463). In another study, several quinolones, including one with both antimicrobial and cytotoxic activities, were isolated from a pseudomonad from the Pacific sponge *Homophymia* sp. (45). Although the mechanistic basis was not identified, 27 bacteria isolated from the Mediterranean sponges *Aplysina aerophoba* and *A. cavernicola* exhibited antimicrobial activities in a series of assays (147). Given the activity of some of these isolates against clinically important multiresistant *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, assay-guided fractionation and subsequent chemical characterization of any active components could prove particularly profitable. Terrestrial fungi have a long-standing reputation as prolific producers of bioactive natural products (44), and it is hardly surprising that sponge-associated fungi also show promise in this regard. Many examples exist of sponge-derived fungi that produce bioactive compounds (29, 42, 44, 163, 174, 176, 295, 296, 451). Although many of the isolated fungi are of suspected terrestrial origin (i.e., they are closely related to typical terrestrial species), to some extent this does not matter for drug discovery purposes; even if sponges act only as mere accumulators of contaminant fungi, these microorganisms can still be targeted, and once they are isolated, there may be no need to attempt reisolation from the original sponge hosts. Interestingly, it appears that unlike bacteria, fungi are not the source of any natural products previously ascribed to marine sponges (191).

The success of efforts to isolate sponge-associated microorganisms that produce bioactive compounds is dependent upon a number of factors. Most significantly, the majority of environmental microorganisms, including those in sponges, have proven resistant to cultivation by standard techniques (105, 380). Although various authors have reported improved culturability of sponge-associated bacteria via, for example, supplementing the media with sponge tissue extracts (458) or catalase and sodium pyruvate (264), the proportion of total sponge bacteria that can be isolated has remained low. Only 0.06, 0.1, 0.15, and 0.7% of total bacteria could be cultured from the sponges *Candidaspongia flabellata* (47), *Rhopaloeides odorabile* (47, 453), *Aplysina aerophoba* (105), and 01IND 35/01IND 52 (154), respectively. Santavy and colleagues were able to achieve somewhat better recovery (3 to 11%) from the Caribbean sclerosponge *Ceratoporella nicholsoni*, although obviously, even in this case, some 90% of the resident bacteria were not captured by cultivation attempts (335). While cultivation difficulties are hardly confined to sponges, it is nevertheless likely that many of the sponge associates are obligate symbionts which may have evolved with the sponge over hundreds of millions of years (see Evolution and Diversity of

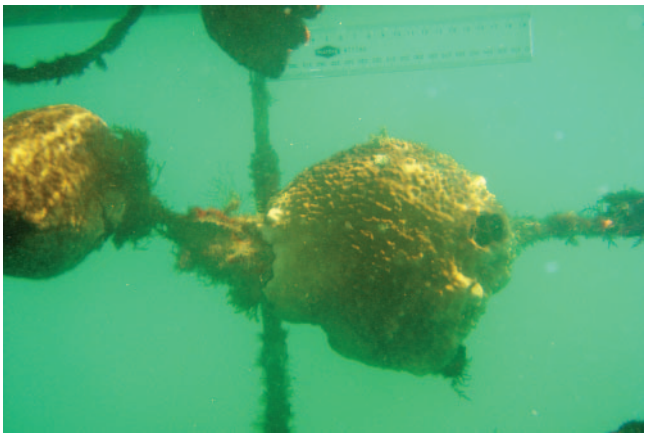

FIG. 25. In-sea aquaculture of the Great Barrier Reef sponge *Rhopaloeides odorabile*. (Image courtesy of Rocky de Nys [James Cook University, Australia], reproduced with permission.)

Sponge-Associated Microorganisms) and will, due to nutritional or other dependencies, be extremely difficult to obtain in pure culture. Furthermore, those that can be isolated may not necessarily produce the compound anymore, as they may require some as yet unknown cue or metabolic intermediates from the host sponge. Additionally, and for unknown reasons, some bacteria simply stop producing the compound of interest after a certain time on artificial media (145). This could be due to any of a number of genetic reasons relating to a lack of selective pressure in pure culture, e.g., point mutation in a key gene or loss of a mobile genetic element carrying the biosynthesis genes.

Sponge culture. Culturing sponges is another way to address the supply problem, irrespective of whether compound production is due to the sponge or the symbiont. Methods employed for the cultivation of sponges for drug production vary widely in scale and sophistication, from sea-based aquaculture to in vitro cultivation (in closed or semiclosed systems) to cell and tissue cultures (22). The technical and economic potential of each of these was reviewed recently (373).

In-sea sponge aquaculture has received considerable attention for its potential to cost-effectively address the metabolite supply issue (Fig. 25) (84–88, 132, 137, 231, 248, 271, 299, 439). Of all approaches, it has the advantage of most closely simulating the conditions encountered by sponges in nature, and practitioners have been able to draw upon more than a century of experience in farming bath sponges. On the negative side, the inherent unpredictability of marine environments can create problems (e.g., due to atypical climatic conditions or storm damage) (439) which are easily avoided in controlled in vitro systems. The outcomes of sponge aquaculture trials have varied widely, with success dependent upon a number of factors, including the type of farming structure (84, 85, 132), sponge growth form (86), farming location (271), and season of transplantation (86, 87). If sponge survival can be ensured, then growth increases of up to 5000% per year (relative to the starting size) are achievable, depending on the sponge species examined (271). Crucially, bioactive metabolites are typically retained in farmed sponges (84, 87, 132, 248, 250, 271).

The consequences of environmental variability can be sidestepped by cultivating sponges under semienclosed or even fully closed conditions (22, 90, 141, 161, 267–269). Although this is generally more expensive than sea-based aquaculture, an obvious advantage is the ability to control environmental parameters, such as growth temperature, water movement, and food supply, as well as to eliminate biomass loss due to storms or disease outbreaks (see "The Varied Nature of Sponge-Microbe Interactions"). Potential problems in recirculating systems include a buildup of toxic secondary metabolites and metabolic wastes, such as ammonia (22). Considerable growth (>200% in 1 to 2 months) of *Pseudosuberites andrewsi* explants was achieved in a bioreactor (268), with even more growth (>1,000% in 45 days) observed for the sponge *Crambe crambe* in a closed system (21). Like the case for sea-grown sponges, metabolite production has been observed for sponges subjected to in vitro cultivation (e.g., see references 75 and 90). However, one must remember that despite its promise, this technology remains in its infancy, and to our knowledge, there are no examples of industrial-scale in vitro cultivation of sponges.

Sponge cell culture for the production of biologically active metabolites represents the other extreme of the scale continuum from sea-based aquaculture. Although sponge cells can be dissociated readily and even induced to divide in suspension for several cycles, it has so far proven impossible to establish continuous cell lines (reviewed in references 293, 319, and 320). Primmorphs, which are three-dimensional aggregates comprising proliferating and differentiating sponge cells, have therefore generated much recent interest since they can be maintained for long periods (66, 240, 242, 247, 293). Particularly exciting was the finding by Müller and coworkers that primmorphs from *Dysidea avara* grown in a bioreactor produced the secondary metabolite avarol, which is both characteristic of this sponge and of great pharmacological interest due to its strong biological activities (e.g., antitumor, antibacterial, and antiviral activities) (242). In contrast, single *D. avara* cells did not produce avarol. Another interesting aspect of primmorphs, especially within the context of this review, is that symbiotic microorganisms can be retained within them (247, 398), potentially allowing for primmorph production of both sponge- and microbe-derived compounds. Since their initial demonstration in *Suberites domuncula* (66, 247) and then *D. avara*, primmorphs have been generated from a wide range of sponges, including *Axinella polypoides*, *Cliona celata*, *Halichondria panicea*, *Petrosia ficiformis*, and *Stylotella agminata* (374, 434, 500). In the coming years, it should become clear whether primmorphs can be scaled up sufficiently to overcome the supply problem for many promising drug leads.

Surprisingly little information exists on the microbiology of cultured sponges (in any system), yet this could be of vital importance if metabolites are produced by microbial associates. For example, if sponges are cultured away from their natural environment, then metabolite-producing symbionts may conceivably be lost, or if metabolites are diet derived (as suspected for okadaic acid) (297), then a change in diet would presumably result in a loss of compounds. Moreover, even if the desired metabolite is produced by the sponge itself, microbial symbionts may still be of direct (e.g., by providing metabolic precursors) or indirect (e.g., by affecting general sponge health) significance (154).

Metagenomics. One of the most exciting developments in molecular biology from a drug discovery perspective has been the advent of environmental genomics, or metagenomics (92, 104, 136, 207, 346, 438). Metagenomics refers to the analysis of genome fragments from a complex microbial community and offers the potential for large-scale, sustainable production of bioactive metabolites, including those produced by uncultivated microorganisms. If biosynthesis genes can successfully be cloned and expressed in another (cultivated) microorganism, such as *E. coli*, then this could ensure an unlimited supply of a specific metabolite (48, 143). Different approaches for the cloning and heterologous expression of biosynthesis genes from marine invertebrate symbionts were recently the subject of a comprehensive review by Hildebrand and colleagues (149). The successful application of many of these methods is exemplified by studies by Haygood and coworkers on the symbiosis between the bryozoan *Bugula neritina* and its bryostatin-producing gammaproteobacterial symbiont, "*Candidatus* Endobugula seritina" (69, 70, 142, 143, 149, 150). Here we focus our attention on the results of recent metagenomic studies involving sponges.

Two main types of analysis have been used to extract biotechnologically relevant information from metagenome libraries: one is based on function, whereby libraries are screened for the expression of specific traits, and the other is based on screening for sequences themselves (346). While screening for functional traits (e.g., antibiotic production or quorum-sensing inhibitors) has been successful (to various degrees) in other environments (68, 318, 489), studies of sponge metagenomics, to our knowledge, have been exclusively sequence based. To date, the major foci of such studies have been the polyketide synthase (PKS) and nonribosomal peptide synthetase genes (187, 284, 285, 342). The PKSs are responsible for the synthesis of bacterial polyketides, a diverse group of pharmacologically important natural products which include the antibiotics erythromycin and tetracycline as well as antitumor, immunosuppressive, and cholesterol-lowering agents (197). Type I PKSs are organized in a modular fashion, lending themselves to the combinatorial biosynthesis of novel polyketides with potentially useful pharmaceutical properties (197). A number of marine drug candidates, including the bryostatins, discodermolide, and the aforementioned peloruside A, belong to this class of compounds (104, 150). The modular nature of the polyketides suggests that environmentally retrieved PKS fragments, which may not produce intact bioactive compounds, could still be useful by providing modules for combinatorial polyketide synthesis (187, 197). A large part of the sponge-PKS story comes from work by Piel and colleagues (279–286). Their study of the polyketide onnamide in sponges was greatly facilitated by prior metagenomic investigations into the production of a structurally highly similar antitumor compound, pederin, in beetles of the genus *Paederus* (281, 283, 286). The microbial community within these beetles is much less complex than that of sponges, allowing easier access to the genome of the bacterial pederin producer (349). Ultimately, pederin production was linked to a beetle symbiont closely related to *Pseudomonas aeruginosa*, although evidence for lateral gene transfer of the pederin-type genes suggests that compound production may not necessarily correlate with rRNA-based bacterial phylogeny (283, 285). Armed with a sound understanding of the genetic bases of pederin biosynthesis, Piel et al. investigated the production of onnamide in the sponge *Theonella swinhoei* from Japan (285). Ketosynthase (KS) fragments were PCR amplified directly from the sponge metagenome, revealing a diverse range of sequence types. More importantly, PCR-based screening of a 60,000-clone cosmid library with the same primers yielded a single KS-positive clone, which was fully sequenced. Strong indications existed for a bacterial origin of this genome fragment (e.g., a lack of introns and small intergenic distances), which should correspond to almost the entire region of the polyketide structure needed to obtain an antitumor compound (285). In addition to the obvious biotechnological importance of this study, as heterologous expression of such gene clusters could lead to an inexhaustible supply of target metabolites for clinical trials, interesting ecological and evolutionary questions were also raised. For example, how did such similar biosynthetic pathways come to be present in symbionts of such dissimilar hosts, i.e., marine sponges and terrestrial beetles?

PKSs have also been studied in other sponges by using metagenomic approaches. In an attempt to isolate genes encoding the promising antitumor compound discodermolide from the Caribbean sponge *Discodermia dissoluta*, Schirmer and colleagues (342) employed a two-step approach. First, degenerate KS primers were used to amplify 256 sequences (85 different KS sequences), including several from *trans*-AT-type PKS domains of the pederin and onnamide types. A selection of the derived sequences was then used to create a probe pool for the screening of 155,000 macroarrayed fosmid and cosmid clones; given the proportion of bacterial inserts in the studied libraries (>90%) and the average insert size (35 kb), >4 Gb (some 1,000 bacterial genome equivalents) were screened (342). In total, 1,025 PKS-positive clones (0.7% of all analyzed clones) were identified. Interestingly, sequencing of selected fosmid and cosmid clones revealed suprisingly little overlap between these KS domains and those derived from the same sample by the direct PCR approach. A PKS consistent with the biosynthesis of discodermolide was also not found (342). Direct amplification of KS domains was also combined with the construction of fosmid libraries to study PKSs in another sponge, the Great Barrier Reef species *Pseudoceratina clavata* (187, 188). Each approach led to the retrieval of five KS domains, all of which fell into an apparently sponge-specific KS cluster (together with sequences obtained from *Discodermia dissoluta*, *Theonella swinhoei*, and an unidentified sponge) following phylogenetic analysis (187). Cultivated bacteria from *P. clavata* were also screened by PCR for KS domains, with 10 such domains detected in representatives of the *Actinobacteria*, *Alphaproteobacteria*, and *Firmicutes*. None of the KS domains from isolates clustered with the metagenome-derived sequences, highlighting the importance of a polyphasic approach to encompass as much of the PKS diversity as possible (187).

Although not part of a drug isolation strategy per se, no discussion of sponge metagenomics would be complete without considering the seminal work of DeLong and colleagues (134, 135, 294, 343–345). Over the past decade, the symbiosis between the Californian sponge *Axinella mexicana* and the psychrophilic crenarchaeote "*Candidatus* Cenarchaeum symbiosum" (294) has been a model system for environmental

genomics. "*Ca.* Cenarchaeum symbiosum" was the first known symbiotic crenarchaeote, and despite its as yet uncultivated status, several factors have made it a suitable target for genomic studies: it is the only archaeon in the sponge and dominates the microbiota, consistently comprising some 65% of all prokaryotic cells; it is always associated with the sponge; and the symbiosis is maintained for a long time in aquaria (294). Relatively large amounts of biomass and DNA have therefore been available, with physical enrichment for the archaeal cells greatly facilitating the construction of large-insert genomic libraries for this organism. The first published results from the genomic analyses outlined the characterization of a DNA polymerase which was homologous to those of cultivated archaeal hyperthermophiles and yet, as revealed by heterologous expression in *E. coli*, was inactivated at temperatures above 40°C, reflecting the symbiont's low-temperature lifestyle (345). Initial studies based on the 16S rRNA gene indicated the presence of a single archaeal phylotype in *A. mexicana* (294), so subsequent genome-derived indications of the presence of two closely related variants were unexpected (343). Although the two variants differed <0.8% in their 16S and 23S rRNA genes and had an identical gene order for a studied 28-kb region, variations in DNA identity of up to 20% were observed for protein coding regions, with up to 30% variation for intergenic regions (343). These findings thus highlighted the difficulties created by genomic microheterogeneity in assembling environmentally retrieved genome fragments, and these difficulties remain today (76, 414, 440). Despite such obstacles, the DeLong group was able to assemble a closed genome for "*Ca.* Cenarchaeum symbiosum" (134), which has already yielded important insights into the metabolism of the sponge symbiont in particular and of marine *Crenarchaeota* in general (135). For example, genome reconstruction revealed the potential of "*Ca.* Cenarchaeum symbiosum" to function either autotrophically (as an ammonia oxidizer) or mixotrophically. In an extension of the environmental genomics approach, homologues of genes involved in carbon and nitrogen metabolism were also found in metagenome libraries from ocean waters worldwide, demonstrating the ubiquity of these metabolic pathways among marine crenarchaeotes (135). Conversely, certain genes encoding cell surface, regulatory, or defense mechanisms were not recovered from the free-living relatives of "*Ca.* Cenarchaerum symbiosum," suggesting that these could be involved specifically with the establishment and maintenance of the symbiosis (134).

The organism-oriented approach taken for "*Ca.* Cenarchaeum symbiosum" was also employed in a recent study of the sponge-specific candidate phylum "*Poribacteria*" (100, 101). Virtually nothing is known about the physiology and genetic makeup of these bacteria, and since there are no cultured representatives, metagenomics offered a promising approach. The sole entry point into the "*Poribacteria*" genome was the 16S rRNA gene sequence, and a single fosmid clone among 29,000 clones (corresponding to 1.1 Gb of DNA in total) was positive in an initial 16S rRNA PCR-based screening (101). Analysis of this 39-kb insert revealed 27 open reading frames, including one encoding a new kind of molybdenum-containing oxidoreductase and several encoding unusual transmembrane proteins (101).

The potential of metagenomics and other cloning approaches to revolutionize natural product research with sponges is undeniable, yet there remain considerable technical challenges. For example, if the microbial communities under study are highly complex, then the genomes of target organisms (e.g., "*Poribacteria*") may remain largely hidden against a background of genomes from other symbionts (but see Conclusions and Future Directions for possible means of enriching specific genomes). Other potential problems include the use of inappropriate host organisms for expression studies (some hosts may not express the genes of interest) (104, 136) and the large sizes of many gene clusters, which can prohibit their successful cloning (although there exists the theoretical possibility of reconstructing biosynthetic pathways via the assembly of many smaller, overlapping sequence reads). In still other cases, nonclustering of biosynthesis genes may be a problem, with the genes of interest spread across different parts of the symbiont's genome (281). On the bright side, the relevant technology is developing quickly. Massive sequencing efforts, such as the recent Sargasso Sea study (440), may provide one means of accessing rare genomes and the biotechnologically relevant information within them. Indeed, an ambitious small- and large-insert sequencing study along these lines was recently initiated for a temperate Australian sponge (S. Kjelleberg, P. Steinberg, and T. Thomas, personal communication). Similarly, the new pyrosequencing technology from 454 Life Sciences (227) has already been applied successfully to complex environmental samples (204) and may prove valuable in future sponge metagenomics projects. Furthermore, high-throughput screening techniques have developed to the extent that a single worker can now screen, from start to finish, a library of 400,000 clones for a particular gene in a mere 4 days (169).

Cell separation and metabolite localization. The aforementioned techniques each offer the potential to generate large quantities of biologically active metabolites from sponge-microbe associations. All three approaches (microbial cultivation, sponge culture, and metagenomics) can, in principle, be undertaken without prior knowledge of which partner (sponge or microorganism) produces a given compound. However, the most rational approach is to first establish which cell type(s) is responsible for metabolite production, as this can determine a logical strategy for future efforts (Fig. 24). Many researchers have employed cell separation techniques for this purpose (27, 28, 99, 103, 120, 314, 315, 332, 416, 417). A major breakthrough in this area came with the realization that chemically fixed sponge and microbial cells retain their natural products in a form amenable to chemical analyses, such as high-performance liquid chromatography and nuclear magnetic resonance spectroscopy (416). Coupled with the relative ease with which one can dissociate sponge tissue, this opened up a number of possibilities for natural product research. In the 1990s, researchers in Faulkner's laboratory thus took advantage of cyanobacterial autofluorescence to separate, via flow cytometry, the filamentous cyanobacterium *Oscillatoria spongeliae* from sponge and other microbial cells in dissociated tissue of *Dysidea herbacea* (416, 417). Of three major types of metabolites known previously from this sponge, two—polychlorinated peptides (416) and polybrominated biphenyl ethers (417)—were found exclusively inside *O. spongeliae* cells. In contrast, sesquiterpenoids were confined to the sponge cells. While autofluorescing cells

(e.g., cyanobacteria) have been separated from other cell types (e.g., sponge and noncyanobacterial microbes) by flow cytometry, other characteristics, such as cell size and, provided the natural products are retained, FISH probe-conferred fluorescence, could also be used to differentiate sponge and symbiont cells by this technique.

A more common approach has been to separate different cell types in dissociated sponge tissue via centrifugation (27, 28, 314). Again, the Faulkner group was instrumental in establishing these approaches for sponge natural product study. In the chemically rich lithistid sponge *Theonella swinhoei*, Bewley and colleagues (28) identified four distinct cell types by electron microscopy (sponge cells, unicellular cyanobacteria, unicellular heterotrophic bacteria, and filamentous heterotrophic bacteria [the term "heterotrophic bacteria" is used here in reference to noncyanobacterial microorganisms; these cells could, in principle, be from chemolithoautotrophic bacteria]). By following gross dissection of the sponge (into the endosome [inner tissues] and ectosome [outer tissues]) with dissociation and centrifugation, they were able to obtain relatively clean fractions comprising each cell type. The macrocyclic polyketide cytotoxin swinholide A was found only in the fraction containing the unicellular heterotrophic bacteria, while the potent antifungal glycopeptide theopalauamide (350) was associated with a filamentous bacterium (later identified as a deltaproteobacterium, "*Candidatus* Entotheonella palauensis" [351]) (28). Density gradient centrifugation provides yet another opportunity for separating sponge and microbial cells. In this case, dissociated sponge tissue is placed above a density gradient (consisting of, e.g., Ficoll or Percoll), and following centrifugation, various cell fractions are formed based on differing densities (103, 120, 121, 315). Individual fractions can then be examined chemically and microscopically to correlate the occurrence of compounds with specific cell types. For *Haliclona* sp., Garson and coworkers used Percoll gradients to demonstrate localization of the cytotoxic alkaloids haliclonacyclamine A and B within the sponge cells rather than in associated dinoflagellates (120).

Depending on the properties of and prior knowledge about the compounds under study, localization may be achieved without the need for prior cell separation. For example, brominated compounds, which are often biologically active and can be present in large amounts in sponges, may be visualized in situ by X-ray energy-dispersive microanalysis (406, 410). Specific antibodies for the toxin latrunculin B were used to prove its localization in sponge but not symbiont cells within the Red Sea demosponge *Negombata magnifica* (125). In a novel approach for sponges, catalyzed reporter deposition-FISH was recently used to demonstrate mRNA expression from the *dysB1* genes (responsible for the biosynthesis of polychlorinated peptides) in cells of the cyanobacterium *Oscillatoria spongeliae* in *Dysidea* (*Lamellodysidea*) *herbacea* (102). The biological result was consistent with earlier work by others (103, 416), but more significantly, this study proved the utility of the method for the investigation of target chemicals in sponges. The main limitation of the method is that a certain level of genetic information must first be available for the studied, or a related, biosynthetic pathway.

Although strongly suggestive, even the localization of a metabolite to a particular cell type is not unequivocal proof of its production in that cell or, indeed, even in that organism at all. A compound may diffuse or be exported away from its site of synthesis, especially if it is toxic to the producer (149). The recent demonstration of swinholide A production by free-living marine cyanobacteria (9) is a case in point: while Bewley and coworkers (28) found this compound only in heterotrophic bacteria (outlined above), it now seems plausible that cyanobacterial symbionts in *Theonella swinhoei* may produce the compound but excrete it to be stored elsewhere. Alternatively, both the heterotrophic bacteria and the cyanobacteria may share the capacity for swinholide A biosynthesis, perhaps due to lateral gene transfer (9). Interphylum and even interdomain transfer of natural product pathways has been reported a number of times (e.g., the β-lactam biosynthesis genes, responsible for the production of antibiotics, including penicillins and cephalosporins, are found in both fungi and bacteria [41]). Yet another potential explanation is that a given metabolite could be derived from the sponge's diet. There is evidence suggesting that this may be the case for okadaic acid, which has been isolated from various sponges but is known to be produced by free-living marine dinoflagellates (297, 467). Interestingly, while localization studies have very often implicated the host sponge as the source of bioactive metabolites, in at least some cases it appears likely that intracellular or cell surface-associated bacteria may have been present but overlooked (149). In such cases, microbial production of metabolites cannot be ruled out completely.

It is important to note that while any of the aforementioned localization techniques may give an indication of which is the metabolite-producing organism, none of them directly allows the harvesting of large amounts of the desired compounds (in contrast to cultivation of the relevant microorganism, sponge culture, or heterologous expression of biosynthesis genes). The chief benefit of such studies is therefore to improve our understanding of metabolite production within the sponge-microbe association, identifying the putative producer(s) and thus providing the basis for targeted cultivation and/or molecular approaches.

Other Biotechnologically Relevant Aspects of Sponges

In addition to the by now well-known pharmacological potential of marine sponges and their associated microbiota, several other aspects are also worth highlighting. For example, the farming of certain sponges, particularly those in the genera *Spongia* and *Hippospongia*, for their use as bath sponges has been going on for more than a century. The industry, based mainly in the Caribbean and Mediterranean, has been hit numerous times by mass outbreaks of sponge disease (see "Harming the host: pathogenesis, parasitism, and fouling"). A better understanding of sponge microbiology in general, and disease ecology in particular, should contribute to the future success of these endeavors. The enormous filtering capacity of sponges has led to the suggestion that they be farmed in a bioremediation context, e.g., to reduce the high bacterial loads resulting from sewage discharges and marine fish farms (110, 233, 379). Such an approach could be optimized financially by farming sponges that, in addition to their role as a biofilter, are useful as bath sponges or produce bioactive metabolites which can subsequently be harvested (233).

Another aspect relates to the remarkable structural properties of the silica skeletons of demosponges and hexactinellid (glass) sponges. A series of recent studies examined in detail the siliceous spicules of *Euplectella aspergillum*, a deep-sea hexactinellid from the western Pacific (5, 6, 385). Individual spicules exhibit, in addition to their role in providing structural support, fiber-optic properties similar to those of fibers utilized in the telecommunications industry (5, 385). Importantly from a technical point of view, spicules are formed at ambient temperatures, and the study of this process may allow some of the inherent problems associated with the commercial (high-temperature) manufacture of optical fibers to be overcome (385). Further insights into how organisms synthesize such sophisticated biological materials were gained from electron microscopy observations of spicule organization in the same sponge (6). The spicules of *E. aspergillum* provide great structural stability due to their intricate arrangement at several spatial levels, ranging from nanometers to centimeters, perhaps inspiring the future development of new materials by humans (6). As mentioned earlier, sponges (e.g., the hexactinellid sponge *Scolymastra joubini*) (51) may derive at least some of their silica from diatoms, while in turn, spicules may extend the range of photosynthetic symbionts within sponge tissue via their conduction of light (115). Ongoing work on sponge skeletogenesis (e.g., see references 245, 357, 418, and 490) should provide fascinating insights into its biological and, potentially, commercial implications.

CONCLUSIONS AND FUTURE DIRECTIONS

As indicated at the beginning of this article, even the significant recent advances in our understanding of sponge-microorganism associations have not closed numerous gaping holes in our knowledge of these systems. It is startling how little is known about many fundamental aspects of sponge symbiont biology, particularly in the areas of symbiont metabolism and evolution. On the other hand, the ever-increasing research interest in this topic (Fig. 1) promises a bright future for the field. To close, we offer our thoughts on where some of this research attention could best be directed.

Detailed studies of symbiont transmission and sponge-microbe coevolution will greatly facilitate our understanding of the evolution of these systems. Improved host phylogenies, from species to class levels, will be critical in this regard, and recent efforts in this direction are highly encouraging. "Hunting" for apparently sponge-specific microorganisms in other, nonsponge habitats (e.g., seawater and other filter-feeding invertebrates) will also be important. However, merely proving the presence of a sponge-specific microbe outside a sponge host is not sufficient to disprove its sponge-specific nature. For example, a sponge damaged by a predator or storm may disintegrate and spread its microbial inhabitants into the water column. It is thus necessary to prove the activity of such microorganisms outside the sponge host, using methods which link microbial identity with function (e.g., FISH-microautoradiography or stable isotope probing). This will be no small feat if the organism is extremely rare in the ocean, as will almost certainly be the case.

The function and physiology of sponge-associated microbes are increasingly important research topics, reflecting our current paucity of knowledge about many of the microbial associates of sponges. For many, or even most, symbionts in sponges, all that is known so far is their 16S rRNA gene sequence: their metabolism remains a black box. Despite various caveats, the 16S rRNA data assembled here, together with analyses of so-called functional genes, will provide a solid framework for the application of recently developed molecular tools for ecophysiological analyses of uncultivated microbes (2, 445, 449). Moreover, future combinations of (hypothesis-generating) metagenomic data with such tools should be particularly profitable, especially for enigmatic microbes such as the "*Poribacteria*." Another area warranting further attention is that of the molecular and biochemical bases of sponge-microbe interactions. Recent work on the innate immune responses of sponges has already provided many important insights, but much remains unknown about host-symbiont signaling and regulation.

Although many aspects of sponge-microbe associations are interesting and important from a basic research perspective, we acknowledge that it is largely biotechnological interest that will sustain this field into the future. It is thus fitting to end our review with this topic. The biotechnological potential of sponge-microbe associations has been widely (and justifiably) lauded, yet the transition from initial compound discovery to large-scale commercial production remains difficult. Chemical synthesis of sponge-derived compounds or their simpler derivatives offers the most reliable option for sustainable, long-term drug supply if the said compounds can be produced cost-effectively. Emerging technologies such as metagenomics and high-throughput microbial cultivation approaches offer exciting potential for accessing those compounds which are produced by microorganisms. Current problems for metagenomics due to the complexity of microbial communities in sponges may be overcome in the future by single-cell genomic approaches (such as multiple displacement amplification) applied to specific cell types which have been separated by, e.g., FISH and flow cytometry (e.g., see reference 183). The use of sponge larvae as starting material for metagenomic studies of drug-producing microbes has also been suggested, as these are most likely to represent the true symbionts and, perhaps, may be simpler communities (279). The demonstrated complexity of vertically transmitted communities (reviewed in this article) suggests, however, that even this will be challenging. Combinatorial biosynthesis, as exemplified by an elegant recent study of *Prochloron* sp. symbionts of ascidians (82), provides further potential for exploiting the chemical novelties present in sponge-microbe associations. Looking to the future, it is clear that even greater integration among microbiologists, chemists, geneticists, zoologists, and aquaculture experts will be crucial in order to wring the most (data) out of sponges and their microbial partners.

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