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Phylogenetic Diversity of Bacteria Associated with the Marine Sponge *Rhopaloeides odorabile*†

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Molecular techniques were employed to document the microbial diversity associated with the marine sponge *Rhopaloeides odorabile*. The phylogenetic affiliation of sponge-associated bacteria was assessed by 16S rRNA sequencing of cloned DNA fragments. Fluorescence in situ hybridization (FISH) was used to confirm the presence of the predominant groups indicated by 16S rDNA analysis. The community structure was extremely diverse with representatives of the *Actinobacteria*, low-G+C gram-positive bacteria, the β- and γ-subdivisions of the *Proteobacteria*, *Cytophaga*/*Flavobacterium*, green sulfur bacteria, green nonsulfur bacteria, planctomycetes, and other sequence types with no known close relatives. FISH probes revealed the spatial location of these bacteria within the sponge tissue, in some cases suggesting possible symbiotic functions. The high proportion of 16S rRNA sequences derived from novel actinomycetes is good evidence for the presence of an indigenous marine actinomycete assemblage in *R. odorabile*. High microbial diversity was inferred from low duplication of clones in a library with 70 representatives. Determining the phylogenetic affiliation of sponge-associated microorganisms by 16S rRNA analysis facilitated the rational selection of culture media and isolation conditions to target specific groups of well-represented bacteria for laboratory culture. Novel media incorporating sponge extracts were used to isolate bacteria not previously recovered from this sponge.

The use of molecular approaches for describing microbial diversity has greatly enhanced the knowledge of population structure in natural microbial communities. It is widely accepted that culture-based techniques are inadequate for studying bacterial diversity from environmental samples, as many bacteria cannot be cultured using current and traditional techniques (20). Cloning and sequencing of 16S rRNA genes give data that can be used to describe complete microbial community composition and can indicate possible nutritional requirements and physiological niches of many microbes based on information already available for known phylogenetic relatives (11, 38). This may assist in the experimental manipulation of culture conditions to provide the correct growth environment for targeted bacteria. One of the limitations associated with the construction of 16S rDNA clone libraries from total environmental DNA is that it requires the use of PCR, which precludes quantitative estimates of abundance for each organism. This can be overcome to some degree by the use of fluorescence in situ hybridization (FISH) probing, which allows the cells to be visualized and semiquantified (30).

The biology of the bacterium-sponge relationship has elicited considerable interest among researchers investigating marine organisms as sources of natural products. Antimicrobial compounds have been isolated from sponge-associated bacteria on numerous occasions, and this has prompted the suggestion that microbial symbionts play a role in the defense of their host sponge (5, 21, 22, 44). Marine sponges produce a wide array of other natural products and bioactive secondary metabolites (4, 10, 18, 35; for a review of recent reports, see reference 14). In some instances, the origin of these compounds has also been shown to be bacteria associated with sponges. For example, *Vibrio* spp. associated with the sponge *Dysidea* sp. were shown to synthesize cytotoxic and antibacterial tetrabromodiphenyl ethers (13). The diketopiperazines associated with the sponge *Tedania ignis* were found to be produced by a *Micrococcus* sp. (43). Recently, the antifungal peptide theopalauamide, isolated from the marine sponge *Theonella swinhoei*, was shown to be contained in a novel δ-proteobacterial symbiont (42a).

Secondary metabolite production can be assigned to symbiotic microorganisms only when synthesis has been demonstrated in cultures isolated from the host species (15) and it is still possible that these compounds are simultaneously being produced by the host. In many instances, the limited availability of sponge material may preclude the commercial production of bioactive compounds of potential pharmaceutical importance (34). The isolation of bioactive compounds from symbiotic bacteria could overcome these limitations by providing a consistent yield using large-scale laboratory culture, eliminating the need to harvest sponges from the natural environment.

Rhopaloeides odorabile is a common Great Barrier Reef sponge. *R. odorabile* possesses an unusual group of C_{20} diterpenes which show variation in yield with changing environmental parameters, such as depth and light exposure (46). It has been hypothesized that these fluctuations combined with observed variability in external appearance (pigmentation and shape) may be due to variation in symbiotic microbial communities. A previous study investigated the culturable bacterial community associated with *R. odorabile* (48). This study found

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TABLE 1. Sequences of oligonucleotide probes used for FISH

Probe	Sequence	rRNA target site (position)[a]	Specificity	% Formamide[b]	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338–355	*Bacteria*	20	2
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	β-*Proteobacteria*	35	29
GAM42a	GCCTTCCCACATCGTTT	16S, 1027–1043	γ-*Proteobacteria*	35	29
HGC69a	TATAGTTACCACCGCCGT	23S, 1901–1918	*Actinobacteria*	25	39
LGC354a, -b, and -c	YSGAAGATTCCCTACTGC	16S, 354–371	Part of low-G+C gram-positive division	35	31
CF319a	TGGTCCGTGTCTCAGTAC	16S, 319–336	*Cytophaga-Flavobacterium*	35	29
PLA46	GACTTGCATGCCTAATCC	16S, 46–63	*Planctomycetes* division	30	33
GSB532	TGCCACCCCTGTATC	16S, 532–546	Part of green sulfur division	10 (45°C)	47
NonEUB338	ACTCCTACGGGAGGCAGC	16S, 338–355	*Bacteria* (negative control probe)	20	2

[a] *E. coli* numbering
[b] All hybridization temperatures were 46°C unless otherwise stated.

the culturable community to be dominated by a single bacterial strain, designated NW001, which is a member of the α subdivision of the *Proteobacteria*. Strain NW001 has a close association with *R. odorabile*, which is stable over space and time, and hence variations in the population of this bacterial strain are unlikely to account for the observed variation in diterpene production.

The present study aimed to investigate the diversity of the total bacterial community within the sponge *R. odorabile*. 16S rRNA gene sequence data and FISH with group-specific oligonucleotide probes were used to provide a culture-independent investigation into community composition. Phylogenetic data on microbial community composition in sponges are of biotechnological interest since these data will assist in the rational selection of culture conditions to increase the diversity of bacteria available for natural product screening.

MATERIALS AND METHODS

Sample collection. Specimens of the marine sponge *R. odorabile* (class Demospongiae; order Dictyoceratida; family Spongiidae) were collected by scuba diving at a depth of 13 m from Davies Reef (Great Barrier Reef, Australia; latitude, 18°49.53′S; longitude, 147°38.45′E). Sponges were transferred directly to plastic bags containing seawater to prevent contact of sponge tissue with air. In the laboratory, sponge tissue was immediately placed at −80°C for 3 days and lyophilized prior to molecular manipulation.

PCR and cloning. DNA was extracted from freeze-dried tissue of three sponges using a modified version of the method described by Pitcher et al. (36). Dried tissue (1.5 g) was ground in liquid nitrogen and suspended in 5 ml of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) containing 50 mg of lysozyme ml^{-1} and incubated at 30°C for 30 min. Guanidinium thiocyanate buffer (GES; 10 ml) was added, and the solution was vortexed for 5 min. GES was prepared by dissolving guanidinium thiocyanate (60 g) in 20 ml of 100 mM EDTA with heating to 65°C, cooling, and addition of 5 ml of 10% (wt/vol) Sarkosyl in a final volume of 100 ml. Samples were transferred to ice, and ammonium acetate was added to a final concentration of 2.5 M. Nucleic acids were recovered using a standard phenol-chloroform extraction followed by precipitation with isopropanol. DNA from all sponges was pooled and further purified by electrophoresis in a 1.2% (wt/vol) low-melting-point agarose gel. DNA fragments larger than 2 kb were excised and recovered from the agarose using Microcon 50 microconcentrators (Amicon Inc., Beverly, Mass.). DNA was quantitated using a spectrophotometer, and PCR was performed using 100 ng of DNA with primers designed to amplify 16S rRNA fragments from all members of the *Bacteria*: 519f, 5′-CAG CMG CCG CGG TAA TWC-3′, and 1406r, 5′-ACG GGC GGT GTG TRC-3′ (19). Prior to amplification, a high-fidelity *Taq* DNA polymerase-containing master mix (including primers) was digested with *Alu*I at 37°C for 60 min and 60°C for 30 min to digest any bacterial DNA contaminating the enzyme (*Taq* Hi-Fi; Gibco BRL, Life Technologies, Gaithersburg, Md.). Cycling conditions were as follows: initial denaturation at 94°C for 1.3 min, 30 cycles of 94°C for 1 min, 54°C for 1.3 min, and 72°C for 2 min, and a final extension of 5 min at 72°C in a Perkin-Elmer thermal cycler. PCR products were purified by electrophoresis in a 1% (wt/vol) agarose gel, and bands of approximately 950 bp were excised and recovered using a gel extraction kit (Qiagen, Inc., Chatsworth, Calif.). Purified PCR products were cloned with a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen, San Diego, Calif.). Plasmids were checked for inserts by digestion with *Eco*RI restriction endonuclease (Promega, Inc., Madison, Wis.). A negative-control cloning reaction was performed in which no sponge-extracted DNA was added.

Sequencing and phylogenetic analysis. Plasmid inserts from 70 clones were sequenced using the M13 forward and reverse primers, the PRISM Ready Reaction kit, and ABI 310 and 373 automated sequencers (PE Applied Biosystems, Foster City, Calif.). Sequences were compared to those in databases using the Basic Local Alignment Search Tool (BLAST) algorithm (1) to identify known sequences with a high degree of similarity. Sequences were examined for the formation of chimeras using the program CHECK_CHIMERA (28). Partial sequences were manually compiled and aligned using Phydit software (8). Evolutionary trees were generated using the neighbor-joining (41), Fitch-Margoliash (16), and maximum parsimony (26) algorithms in the PHYLIP package (version 3.5c; J. Felsenstein, University of Washington, Seattle). Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated as described by Jukes and Cantor (25). The robustness of inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values of >50% were shown on the trees.

FISH. Sections (10 μm) were prepared from three sponges collected at Davies Reef and three sponges from Lizard Island. All sample preparations and FISH reactions were performed as described elsewhere (48). All probes were labeled with the indocarbocyanine fluorochrome Cy3 and synthesized by MWG AG Biotech (Ebersberg, Germany). Due to the strong autofluorescent nature of the sponge tissue, it was necessary to photobleach the slides under strong halogen or fluorescent light for 60 s prior to hybridization. This decreased the autofluorescence to a level which enabled discrimination between probe-conferred signal and autofluorescence. Images were captured with a cooled charge-coupled device using Kontron software (KS2000) in the red and green channels using Zeiss filter sets 10 and 15. Final images were merged and viewed in Adobe Photoshop so that autofluorescence would appear yellow and the probe-conferred signal would appear red. Oligonucleotide probes used during this study are listed in Table 1. A negative control probe (NonEUB338) with the antisense sequence of the domain-level probe EUB338 was used to check for nonspecific hybridization.

Culture of sponge-associated bacteria. After examination of the clone library sequence results, specialized media were prepared to promote the growth of selected sponge-associated microorganisms. Media contained sponge components prepared as follows. (i) A water extract of sponge tissue was prepared by placing 100 g of freshly collected tissue in sterile distilled H_2O for 4 h. The water extract was filter sterilized prior to addition to media. (ii) An organic extract was prepared from the same sponge sample by consecutive extractions with hexane, dichloromethane, and methanol. (iii) The remaining extracted sponge tissue was ground with a mortar and pestle and incorporated directly into media. The media used were marine agar 2216 (Difco, Detroit, Mich.) for the isolation of heterotrophic bacteria, Emerson agar (Difco), M3+ Casamino Acids agar (40), glycerol-asparagine agar (49), actinomycete isolation agar (Difco), yeast malt extract agar (4 g of yeast extract $liter^{-1}$, 10 g of malt extract $liter^{-1}$, 4 g of dextrose $liter^{-1}$, 20 g of NaCl $liter^{-1}$), starch-casein agar (10 g of soluble starch $liter^{-1}$, 1 g of casein $liter^{-1}$, 0.5 g of K_2HPO_4 $liter^{-1}$, 20 g of NaCl $liter^{-1}$), and raffinose-histidine agar (10 g of raffinose $liter^{-1}$, 1 g of L-histidine $liter^{-1}$, 0.5 g of $MgSO_4$ $liter^{-1}$, 0.01 g of $FeSO_4$ $liter^{-1}$, 20 g of NaCl $liter^{-1}$). All media contained Difco Bacto agar (20 g $liter^{-1}$) to produce solid media. All media (with the exception of marine agar 2216) were supplemented with a final concentration of 10 μg of nalidixic acid ml^{-1}, 10 μg of cycloheximide ml^{-1}, and 25 μg of nystatin ml^{-1}. Cycloheximide and nystatin were added to the media to inhibit fungal growth,

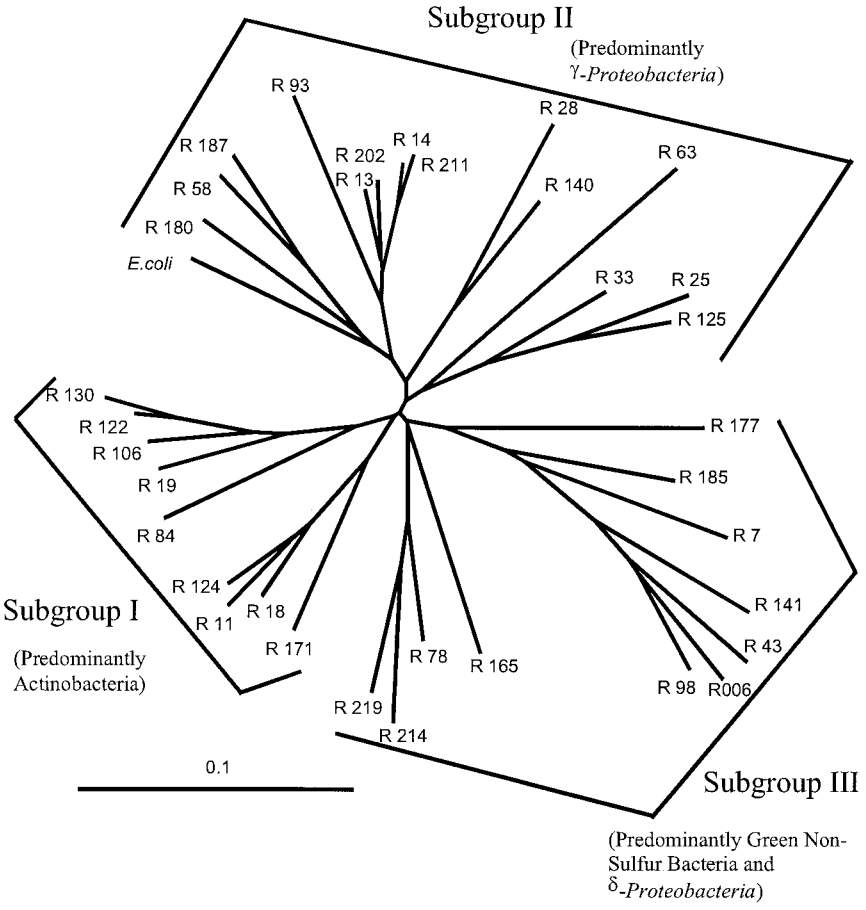

FIG. 1. Radial cladogram showing the diversity of bacterial clone sequences from *R. odorabile*. The neighbor-joining tree is based on 823 bp of 16S rRNA gene sequence and includes the 34 clones for which unique sequence was obtained. The scale bar represents 0.1 substitution per nucleotide position.

which could overgrow plates incubated for long times. Nalidixic acid inhibits many fast-growing gram-negative bacteria that would otherwise have overgrown plates and prevented isolation of slow-growing actinomycetes. Sponge extracts were included in each different medium to a final concentration of 0.1% in gradient plates. This was achieved by placing media containing sponge extract in a petri dish at a 15° inclination. After the agar had set, the petri dishes were set flat and medium containing no extract was poured over the top. This produced plates with a concentration gradient of sponge extract across the petri dish. All cultures were incubated at 28°C, with replicates being cultured aerobically and under microaerophilic conditions. All media were prepared at pH 7.0 (standard) and pH 5.0 (pH of bulk sponge tissue homogenate).

Three individual sponges were collected from Davies Reef and processed for microbial culture as described elsewhere (48). In addition, sponge tissue was pretreated at 50°C for 60 min or −130°C for 60 min prior to processing in an attempt to decrease the number of heterotrophic bacteria and allow the growth of actinomycete strains. All cultured bacteria were categorized using morphologic characteristics and their ability to grow on marine agar 2216, and actinomycete isolation agar was checked. Novel bacterial status was assigned to isolates after comparison of colony morphotype and microscopic appearance of Gram-stained preparations with previously obtained isolates. 16S rRNA sequence data were obtained for selected isolates which exhibited morphologic characteristics not observed in previous attempts to isolate bacteria from *R. odorabile* on standard media (6, 48).

RESULTS

Bacterial diversity indicated by 16S rRNA gene cloning analysis. PCR of total DNA isolated from *R. odorabile* tissue using 16S rRNA primers specific for bacteria yielded a band of the expected size of 950 bp. Combined PCR products were cloned into the vector pCR2.1-TOPO, yielding 240 independent clones. Seventy of these clones were sequenced and subjected to phylogenetic analysis. In total, 34 independent sequence profiles were obtained. The sequence results indicate that a high diversity of bacterial phylotypes was present within the sponge *R. odorabile* (Fig. 1). Overall, 30% of the clones clustered within the *Actinobacteria* and 41% within the γ-subdivision of the *Proteobacteria.* None of the sequences corresponded exactly to any known bacterial species, including strain NW001, which was previously isolated from *R. odorabile* (48).

Subgroup I (predominantly *Actinobacteria*). 16S rRNA analysis revealed that clone R 124, residing within subgroup I, had a close relationship to previously reported actinobacterial sponge symbionts and that clones R 11, R 18, R 122, R 84, and R 130 were more distantly related to these same symbionts (Fig. 2) (GenBank accession no. AF186415 and AF186411). Clones R 171, R 19, and R 106, clustering in the *Actinobacteria*, were only distantly related to their closest described relatives, which include some thermophilic (*Aerothermobacter marianis*) and acidophilic (*Ferromicrobium acidophilum*) actinomycete species.

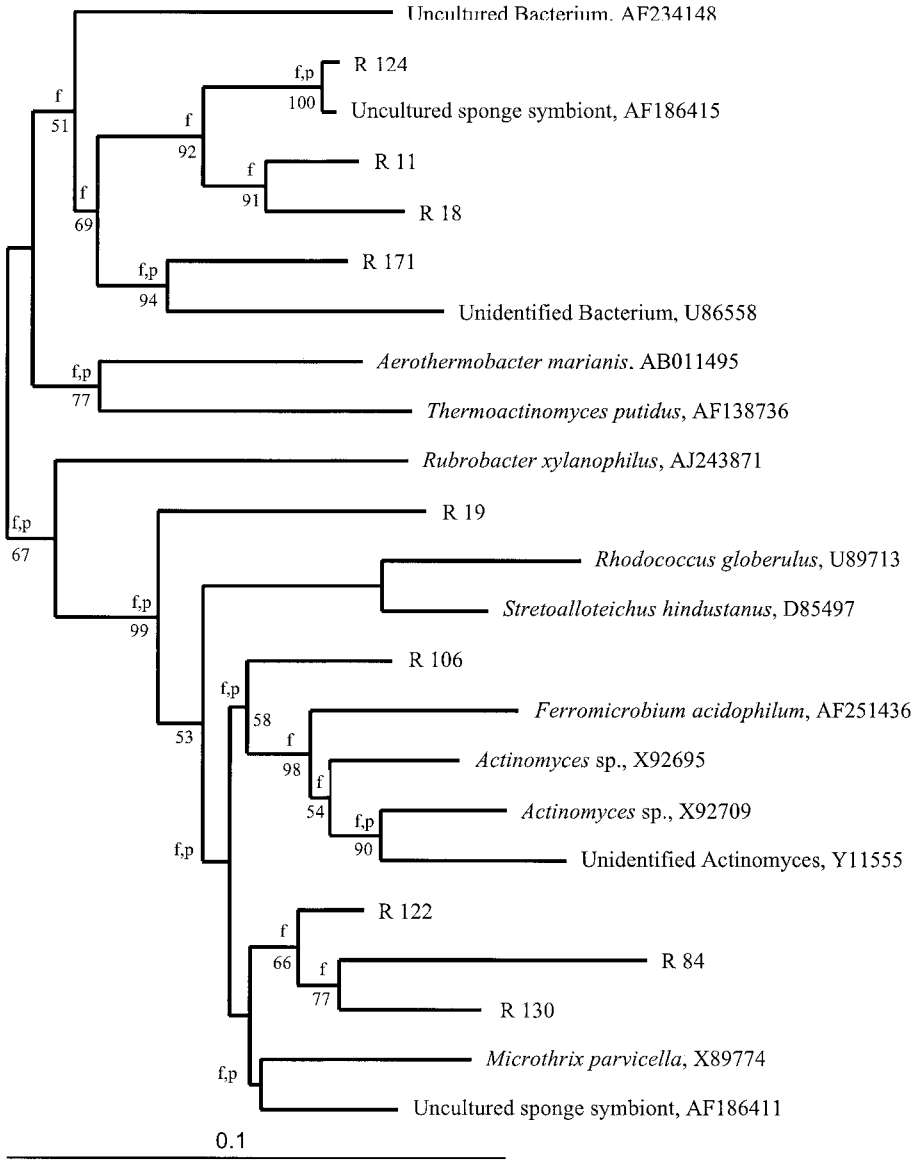

FIG. 2. Neighbor-joining phylogenetic tree from analysis of 709 bp of 16S rRNA gene sequence from clones clustering with the *Actinobacteria* (subgroup I in Fig. 1). "f" and "p" indicate branches that were also found using the Fitch-Margoliash and maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 resampled data sets. Only values of >50% are shown. The scale bar represents 0.1 substitution per nucleotide position.

Subgroup II (predominantly γ-*Proteobacteria*). Several of the bacteria in the γ-proteobacterial cluster (Fig. 3) are distantly related to previously described endosymbionts of marine tubeworms and bivalves, including the *Bathymodiolus thermophilus* symbiont, the *Riftia pachyptilla* symbiont, and an *Anodontia phillipiona* symbiont (GenBank accession no. M99445, U77478, and L25711). The majority of clones from this tree (R 202, R 140, R 93, R 33, R 25, and R 125) are affiliated with a number of uncultured or unidentified γ-*Proteobacteria*. Two small clusters of clones (R 13, R 14, and R 211; R 58 and R 187) have no previously described relatives. Subgroup II also contains a clone sequence closely related to *Cytophaga* sp., a member of the *Bacteroidaceae* (bootstrap value of 100%).

Subgroup III (predominantly green nonsulfur bacteria and δ-*Proteobacteria*). One distinct clade, supported by a bootstrap value of 89%, included five of the clones (R 141, R 43, R 6, R 98, and R 177) and the only other known members of this clade were other unidentified or uncultured bacteria cloned from environmental samples (Fig. 4). The closest relatives to these clones were photosynthetic flexibacteria, belonging to the *Chloroflexaceae* group in the green nonsulfur bacteria (GenBank accession no. AF005747, AF142799, and U20798). Clones R 28 and R 165 were distantly related to uncultured members of the δ-*Proteobacteria* (GenBank accession no. AJ237601 and AF154090). The closest known relative of clones R 78, R 214, and R 219 was an unidentified bacterium within the *Acido-*

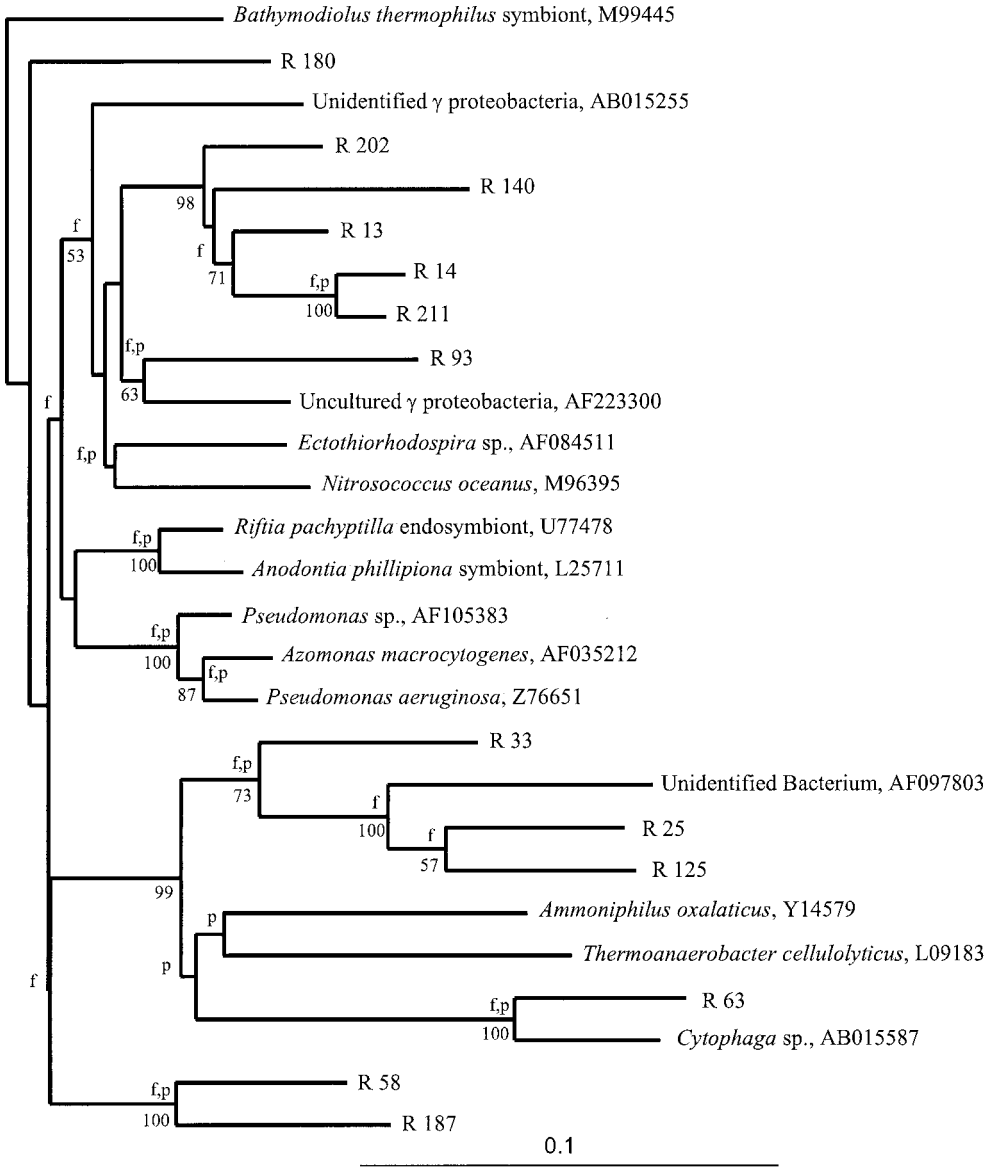

FIG. 3. Neighbor-joining phylogenetic tree from analysis of 804 bp of 16S rRNA gene sequence from clones clustering within the predominantly γ-*Proteobacteria* (subgroup II in Fig. 1). "f" and "p" indicate branches that were also found using the Fitch-Margoliash and maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 resampled data sets. Only values of >50% are shown. The scale bar represents 0.1 substitution per nucleotide position.

bacterium/*Holophaga* group (GenBank accession no. Z95729). Clone R 7 was highly unusual and had no known relatives.

FISH visualization. FISH with several group-specific probes confirmed the general community structure indicated by 16S rRNA gene analysis of the bacterial clone library. The EUB338 probe (specific for most members of the *Bacteria*) revealed that a high density of bacterial cells is present within the mesohyl region of *R. odorabile* (Fig. 5A). There were numerous γ-*Proteobacteria* (probe GAM42a) (Fig. 5B) and *Cytophaga*/*Flavobacterium* organisms (probe CF319a) (Fig. 5C) within the sponge tissue, particularly in the regions surrounding the choanocyte chambers. Planctomycetes were clearly evident within the mesohyl region and appeared to form clusters around spongin fibers and choanocyte chambers (probe PLA46) (Fig. 5D). The β-*Proteobacteria* were also prevalent throughout the sponge tissue and appeared to be intracellular (within sponge cells) in some instances (probe BET42a) (Fig. 5E). FISH also confirmed the presence of *Actinobacteria* (probe HGC69a) (Fig. 5F) and low-G+C gram-positive bacteria (probe LGC354a, -b, and -c) (Fig. 5G). A few cells hybridized with the green sulfur bacterium probe and were apparently both inter- and intracellular (probe GSB532) (Fig. 5H). No bacteria hybridizing to any group-specific probes were present in the aquiferous channels, associated with spongin or collagen fibers, or within the pinacoderm cells. No nonspecific binding of Cy3-labeled probe to sponge tissue was evident in negative control reactions. Results obtained with each group-specific probe were generally consistent for all sponges sampled from both Davies Reef and Lizard Island.

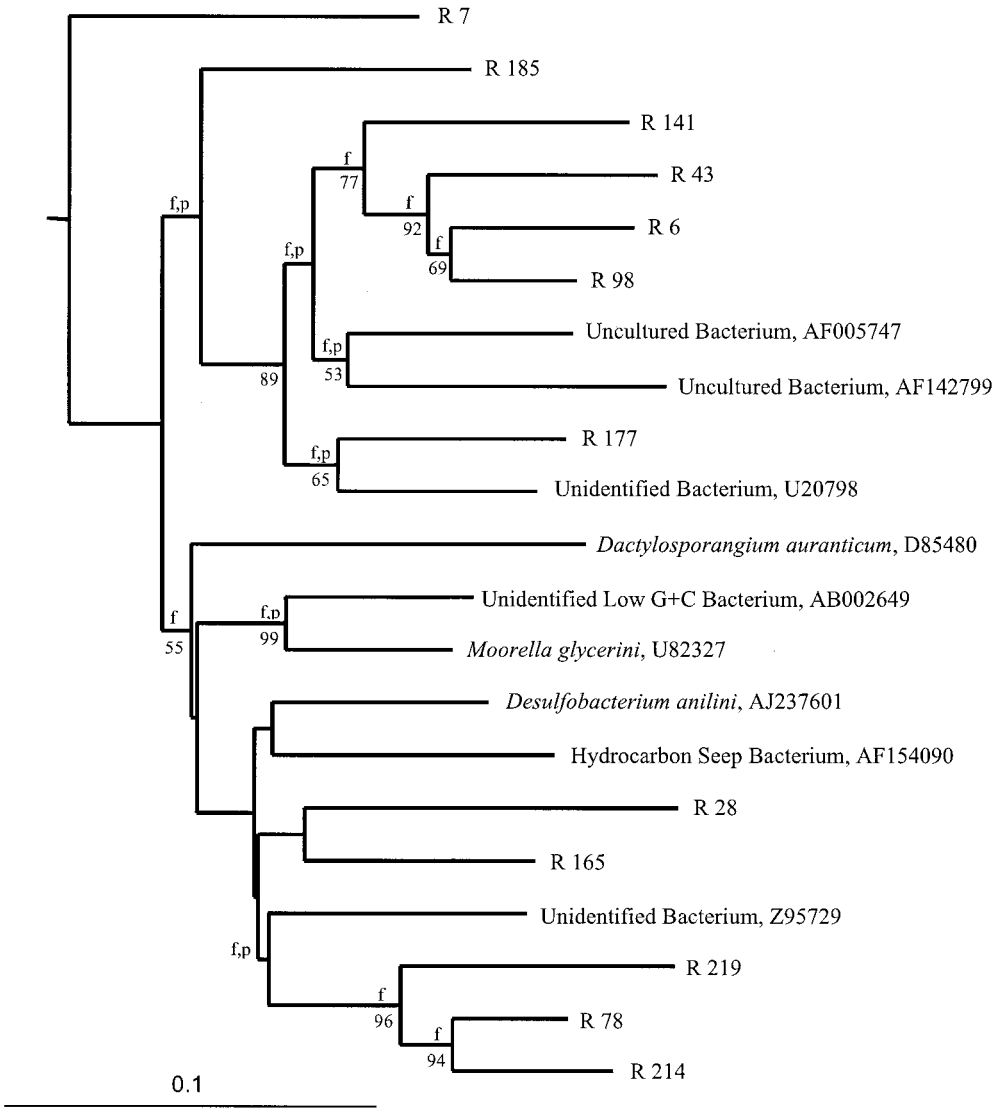

FIG. 4. Neighbor-joining phylogenetic tree from analysis of 811 bp of 16S rRNA gene sequence from clones clustering with green nonsulfur bacteria and δ-*Proteobacteria* (subgroup III in Fig. 1). "f" and "p" indicate branches that were also found using the Fitch-Margoliash and maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 resampled data sets. Only values of >50% are shown. The scale bar represents 0.1 substitution per nucleotide position. *E. coli* was used as an outgroup.

Culture-based studies. The use of standard culture media targeting *Actinobacteria* and media containing sponge extract resulted in the isolation of 42 bacterial isolates not previously cultured from *R. odorabile*. In all cases, morphotypes obtained in this study were regarded as novel if they had not been observed in previous culture-based studies, in which marine agar 2216 was the sole isolation medium and in which 223 bacterial strains were cultured from *R. odorabile* (6, 48). In general, media without added sponge extract produced the largest number of different colony morphotypes (Fig. 6A). The inclusion of an organic extract of sponge tissue in the culture media dramatically decreased the diversity of bacteria cultured (Fig. 6A). The largest diversity of bacterial morphotypes was observed on plates of marine agar 2216 and actinomycete isolation agar, while starch casein, raffinose-histidine, and yeast malt extract media yielded the lowest diversity (Fig. 6A). The addition of aqueous sponge extract to marine agar 2216, starch-casein agar, Emerson agar, and actinomycete isolation agar resulted in an increase in the number of novel cultivated morphotypes (Fig. 6B). The inclusion of sterile sponge tissue in the media did not result in the growth of many novel morphotypes (Fig. 6B). Pretreatment of sponge tissue at 50°C or −130°C did not produce any colony morphotypes that were not observed when pretreatment was omitted (data not shown). Additionally, culture media at pH 5.0 and incubation under microaerophilic conditions were unsuccessful for isolating novel morphotypes (data not shown). In general, the addition of sponge extracts to the media decreased the total number of morphotypes isolated. However, it did result in the appearance of several morphotypes not previously observed from

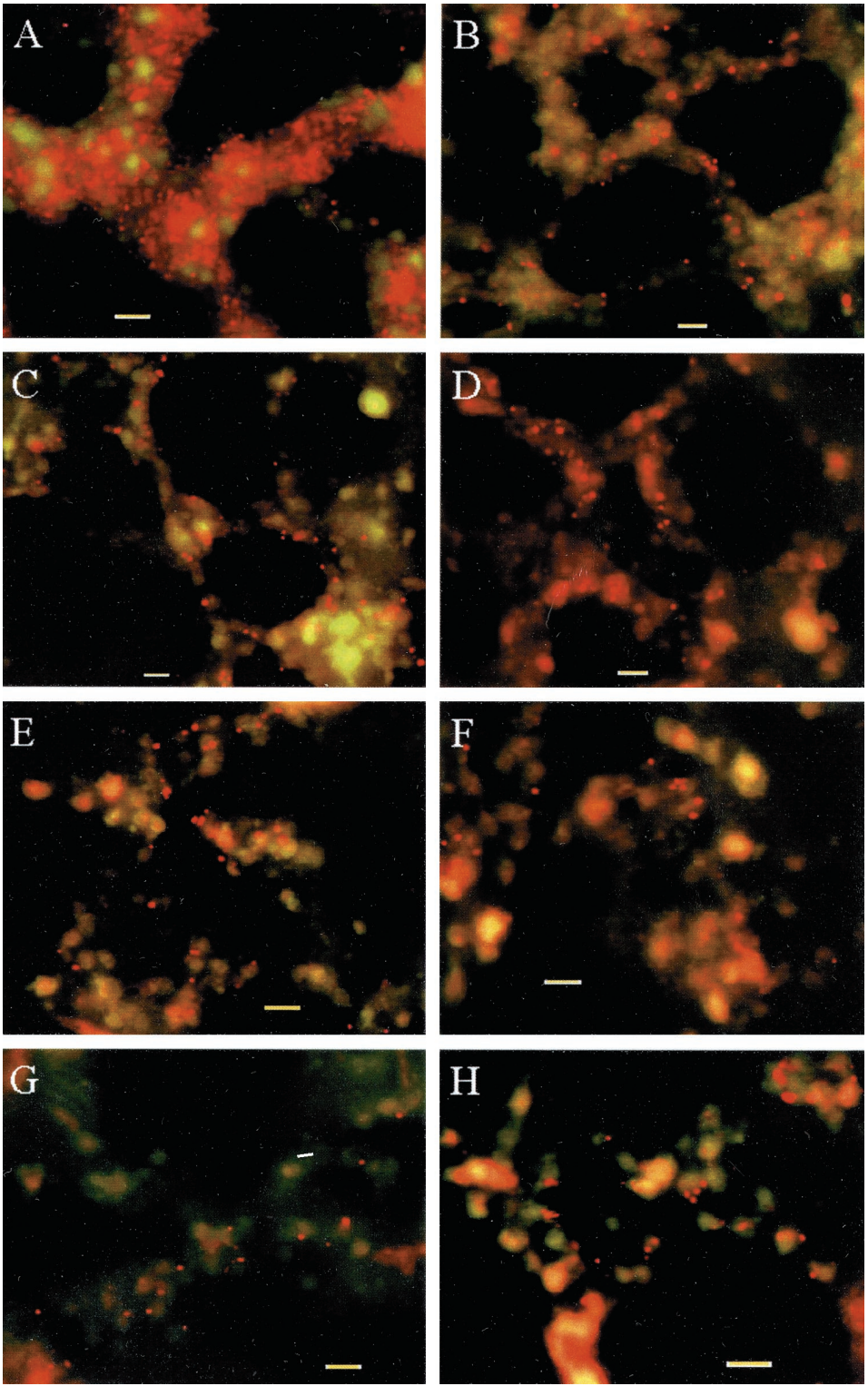

FIG. 5. Epifluorescence micrograph of cryosections of *R. odorabile* visualized by FISH. The mesohyl region was hybridized with Cy3-labeled EUB338 probe (A), Cy-3-labeled GAM42a (B), Cy-3-labeled CF319a (C), Cy-3-labeled PLA46 (D),Cy-3-labeled BET42a (E), Cy-3-labeled HGC69a (F), Cy-3-labeled LGC354 (G), and Cy-3-labeled GSB532 (H). Bar (all panels), 10 μm.

samples of *R. odorabile*. None of these novel morphotypes grew on marine agar 2216, and only two of the isolates obtained from Emerson agar and one isolate from yeast malt extract agar were capable of growth on actinomycete isolation agar. 16S rRNA gene sequencing of a selection of these morphotypes revealed bacteria with phylogenetic affiliations to *Pseudonocardia*, *Gordonia*, and *Bacillus* and other uncultured, unidentified organisms (Fig. 7).

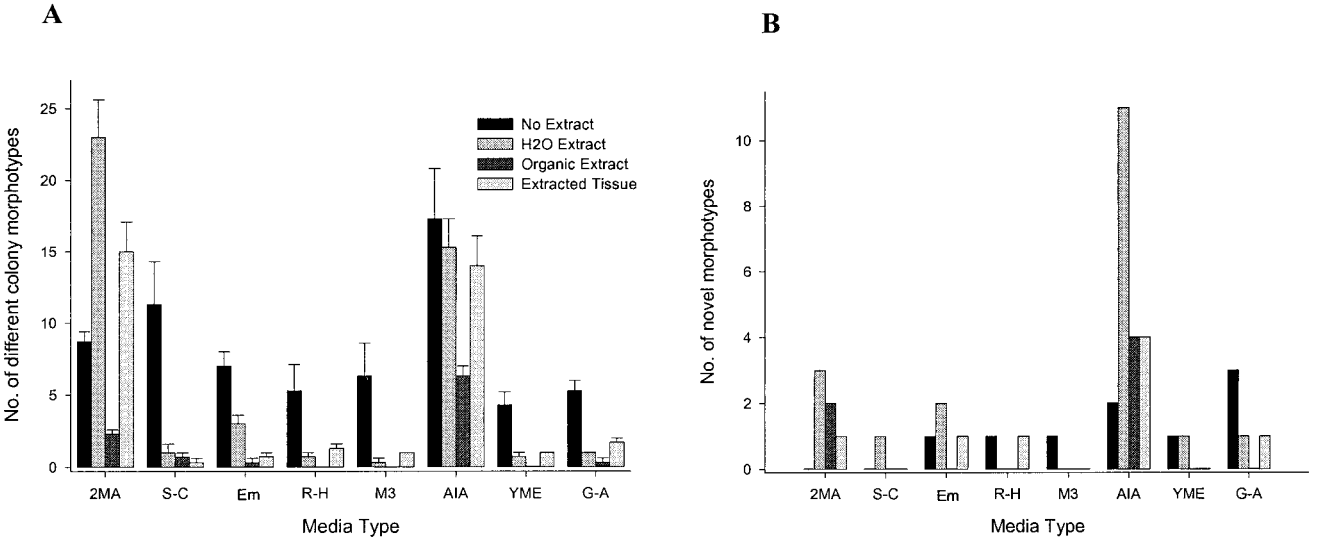

FIG. 6. (A) Effect of using actinomycete-selective media supplemented with sponge extract on total number of bacterial morphotypes isolated from *R. odorabile*. Each medium preparation contained either an H_2O extract, an organic extract, or dried sterile sponge tissue from freshly collected specimens of *R. odorabile*. The addition of no extract was tested to evaluate the potential of each medium for culturing *Actinobacteria*. Medium abbreviations: 2MA, marine agar 2216; S-C, starch-casein agar; Em, Emerson agar; R-H, raffinose-histidine agar; M3, M3+ Casamino Acids agar; AIA, actinomycete isolation agar; YME, yeast-malt extract agar; and G-A, glycerol-asparagine agar. (B) Effect of using actinomycete-selective media supplemented with sponge extract on the number of novel (not previously observed) bacterial morphotypes isolated from *R. odorabile*.

DISCUSSION

The α-proteobacterium strain NW001 has previously been reported as the predominant pure-cultured bacterium from *R. odorabile* using aerobic culture at 28°C (48). The relationship between this bacterium and *R. odorabile* was highly specific and stable over spatial and temporal gradients. Previous studies of culturable microbes associated with this sponge have also demonstrated the presence of symbionts related to *Pseudoalteromonas* spp. (6) and cyanobacteria, in particular, *Leptolyngbya* and *Plectonema* (48). However, only 0.1% of microbes from this sponge were amenable to culture using traditional techniques, and therefore the vast majority of microorganisms associated with *R. odorabile* could not be identified using a culture-based approach. The molecular taxonomic analysis of sponge-associated bacteria from *R. odorabile* indicates that there is a diverse assemblage of bacteria residing within this sponge; however, none of these previously cultured microorganisms were identified in the present study.

Sequences of cloned 16S rDNA fragments fell into three broad categories. Subgroup I (9 sequences) was predominated by actinobacterial clones, subgroup II (14 sequences) contained clones most closely affiliated with γ-*Proteobacteria*, and subgroup III (11 sequences) included clones distantly aligning with members of the green nonsulfur bacteria and δ-*Proteobacteria* groups. Duplicate sequences comprised 52% of the 70 clones analyzed in the present study, indicating that examination of the entire 240 clones comprising the clone library would further increase the known diversity of bacteria associated with *R. odorabile*. Strain NW001, the predominant culturable bacterium isolated from this sponge, was surprisingly absent in clones sequenced to date. This provides further evidence to suggest that additional molecular analysis would be required to fully document the microbial diversity from this sponge.

Many of the clones from *R. odorabile* were phylogenetically more closely related to gram-positive than gram-negative bacteria. In early culture-based studies of marine microbiology, approximately 95% of bacterial isolates were found to be gram negative (50). However, it has more recently become apparent that the proportion of gram-positive bacteria in most marine habitats has been underestimated (24). It is significant that nine of the clones were related to the *Actinobacteria*, since this group is of particular interest in screening for novel bioactive compounds. It is well established that a bias may occur in PCR amplification from mixtures of 16S rRNA templates; for example, Polz and Cavanaugh (37) found that template containing GC-rich permutations in priming sites may be overrepresented. In this study, the presence of *Actinobacteria* in tissue of *R. odorabile* was demonstrated by FISH and novel culture methods, confirming that the high representation of these bacteria in the 16S rDNA clone library was not artifactual. Cells hybridizing to the HGC69a probe were located throughout the mesohyl regions of the sponge tissue.

Our finding of a novel and abundant actinomycete assemblage associated with *R. odorabile* is the first indication that sponges may provide a prolific source of novel actinomycetes for natural product screening, although there are previous reports of the isolation of single strains of actinomycetes from marine sponges (for example, see references 5 and 21). It will be interesting to investigate whether *R. odorabile* is unusual in this regard or whether many marine sponges harbor novel actinomycetes. There is now considerable evidence for the presence of a diverse assemblage of actinomycetes in the marine environment generally (9, 23, 32, 45).

Subgroup II consisted of strains related to the γ-*Proteobacteria*. Many of the previously described marine endosymbionts fall within the γ-subdivision of the *Proteobacteria* (11, 12, 17,

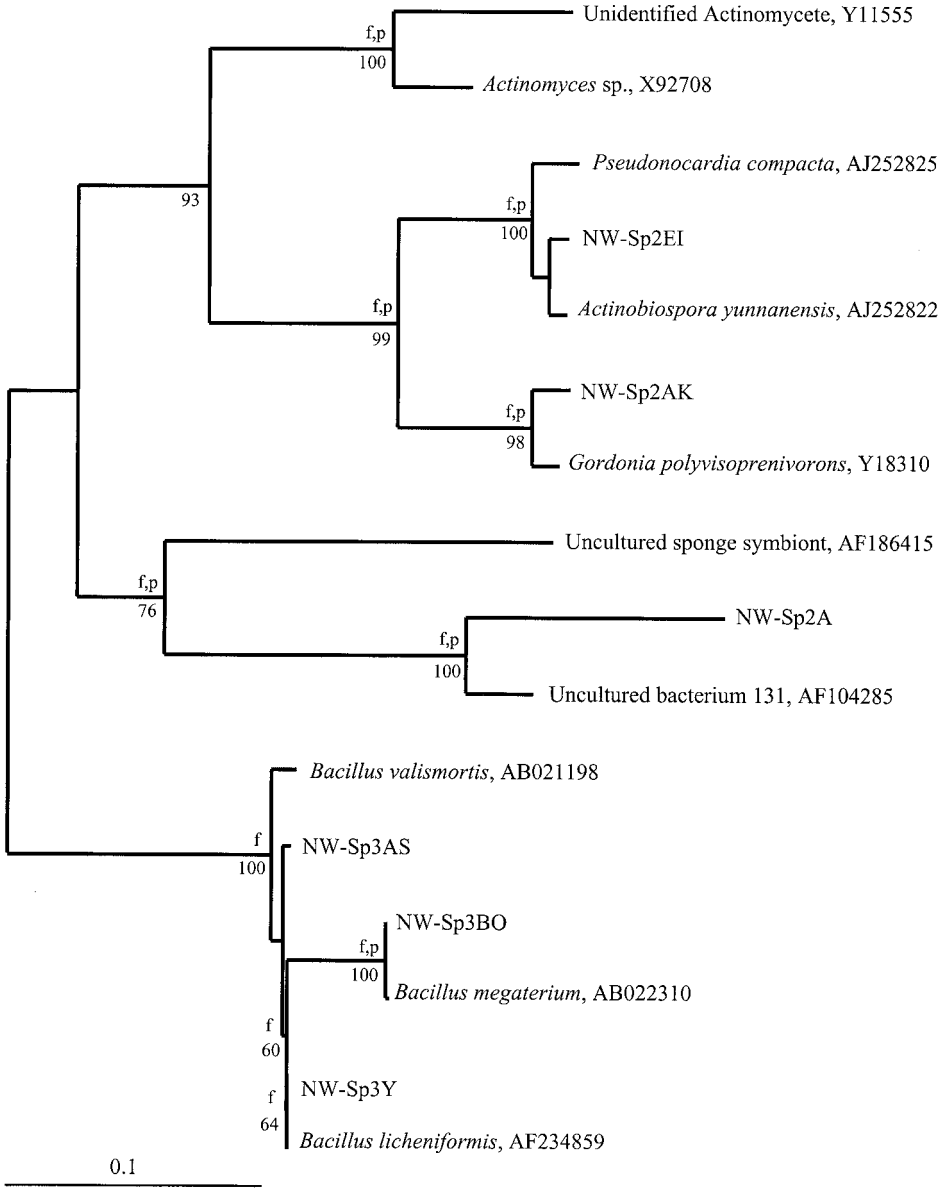

FIG. 7. Neighbor-joining phylogenetic tree from analysis of 305 bp of 16S rDNA sequence obtained from organisms cultured using sponge extract media. "f" and "p" indicate branches that were also found using the Fitch-Margoliash and maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 resampled data sets. Only values of >50% are shown. The scale bar represents 0.1 substitution per nucleotide position.

38). Lopez et al. (27) reported the presence of hitherto undescribed γ-*Proteobacteria* and novel uncultivated strains in the lithistid sponge *Discodermia.* An investigation of microbial diversity in *Aplysina cavernicola* using FISH revealed that δ-*Proteobacteria* were the predominant bacteria in this case, followed by the γ-*Proteobacteria* and representatives of the *Bacteroides* subclass (17). Biochemical characterization of culturable sponge-associated microorganisms from *Ceratoporella nicholsoni* revealed that at least 78% of these bacteria were members of the γ-*Proteobacteria* genera *Vibrio* and *Aeromonas* (42).

FISH analysis revealed that γ-*Proteobacteria* were prevalent throughout the mesohyl and were especially predominant in regions surrounding the choanocyte chambers of *R. odorabile*. These chambers are lined with choanocyte and archaeocyte cells, which are directly involved in nutrient uptake. It is therefore interesting to speculate that these γ-*Proteobacteria* may have a symbiotic role related to nutrition of *R. odorabile*.

The remaining 11 clones reside within subgroup III and have no previously described close relatives, confirming the presence of novel bacteria within this sponge. (Fig. 4). Distant relatives of clones R 141, R 43, R 6, R 98, and R 177 include green nonsulfur bacteria which were isolated from a deep subsurface paleosol (AF005747) (7) and an Antarctic maritime lake (AF142799).

The affiliation of clones from each subgroup with anaerobic

and/or microaerophilic relatives suggests that low-oxygen microniches may be present within the sponge mesohyl. Most sponges alternate between periods of high pumping velocity and periods where little or no water is processed (3). It is possible that oxygen becomes limiting during periods of low water circulation. Active respiration by the large number of bacteria present in the mesohyl coupled with low water circulation may result in anaerobic conditions. The results of the microbial community 16S rRNA gene analysis suggest that culture under low-oxygen conditions may be useful for obtaining additional bacterial isolates from sponge tissue. The use of microaerophilic conditions in the present study did not promote growth of any novel morphotypes.

The presence of significant numbers of bacteria related to the *Actinobacteria*, the γ-*Proteobacteria*, the *Cytophaga-Flavobacterium* group, the green nonsulfur bacteria, and the low-G+C gram-positive group, indicated by clone analysis, was confirmed by FISH. In addition, FISH revealed the presence of β-*Proteobacteria*, *Planctomyces*, green sulfur bacteria, and α-*Proteobacteria*, as predicted by previous culture-based studies (6, 48). These FISH results confirm that complete molecular analysis of the bacterial diversity would require an even larger number of clones to be analyzed. Combined use of 16S rRNA gene sequence analysis and FISH gave a more comprehensive overview of the bacterial community composition in *R. odorabile*. FISH results clearly show bacteria closely associated with cells of the sponge tissue. Bacteria that contain sufficient rRNA to generate probe-positive hybridization signals are likely to be metabolically active, suggesting that these bacteria have an intimate relationship with *R. odorabile* rather than being present within the sponge for consumption as a food source. Also, transient microbes being digested for food are more likely to be within the aquiferous system of the sponge.

Analysis of the phylogenetic affiliation of sponge-associated microorganisms facilitated a rational selection of additional culture media and isolation conditions for growth of a wider range of bacteria from *R. odorabile*. Media specific for the isolation of actinomycetes were included once the presence of these microorganisms was revealed by molecular analysis. The inclusion of these media in microbial cultivation studies from *R. odorabile* resulted in the isolation of novel actinomycete strains not previously observed. The use of specialized media containing extracts of sponge tissue was another useful approach for targeting novel bacteria not cultured using standard medium preparations. Using these approaches, additional cultured organisms related to members of the *Actinomycetales* (strains NW-Sp2EI and NW-Sp2AK), the low-G+C gram-positive bacteria (strains NW-Sp3AS, NW-Sp3BO, and NW-Sp3Y), and an uncultured bacterium related to *Geobacter* spp. (strain NW-Sp2A) were cultured. It is possible that some of these strains are significant components of the actinomycete assemblage associated with *R. odorabile*, and further FISH studies with probes specific for these strains could be used to elucidate this point. However, none of these strains corresponded to those obtained from the clone library.

The comprehensive 16S rRNA-based molecular approach to describing microbial community composition in *R. odorabile* was valuable in revealing the large diversity of bacteria associated with this sponge and enabling the rational design of culture methods for the isolation of additional sponge-associated microbes. *R. odorabile* hosts a diverse and complex assemblage of sponge-associated bacteria, many of which are only distantly related to previously described bacteria. These results illustrate just how challenging it may be to culture microbial symbionts from invertebrates which are responsible for production of novel pharmaceutically important compounds.

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