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Multiple Approaches To Enhance the Cultivability of Bacteria Associated with the Marine Sponge *Haliclona* (*gellius*) sp.▽†

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Three methods were examined to cultivate bacteria associated with the marine sponge *Haliclona* (*gellius*) sp.: agar plate cultures, liquid cultures, and floating filter cultures. A variety of oligotrophic media were employed, including media with aqueous and organic sponge extracts, bacterial signal molecules, and siderophores. More than 3,900 isolates were analyzed, and 205 operational taxonomic units (OTUs) were identified. Media containing low concentrations of mucin or a mixture of peptone and starch were most successful for the isolation of diversity, while the commonly used marine broth did not result in a high diversity among isolates. The addition of antibiotics generally led to a reduced diversity on plates but yielded different bacteria than other media. In addition, diversity patterns of isolates from agar plates, liquid cultures, and floating filters were significantly different. Almost 89% of all isolates were *Alphaproteobacteria*; however, members of phyla that are less commonly encountered in cultivation studies, such as *Planctomycetes*, *Verrucomicrobia*, and *Deltaproteobacteria*, were isolated as well. The sponge-associated bacteria were categorized into three different groups. The first group represented OTUs that were also obtained in a clone library from previously analyzed sponge tissue (group 1). Furthermore, we distinguished OTUs that were obtained from sponge tissue (in a previous study) but not from sponge isolates (group 2), and there were also OTUs that were not obtained from sponge tissue but were obtained from sponge isolates (group 3). The 17 OTUs categorized into group 1 represented 10 to 14% of all bacterial OTUs that were present in a large clone library previously generated from *Haliclona* (*gellius*) sp. sponge tissue, which is higher than previously reported cultivability scores for sponge-associated bacteria. Six of these 17 OTUs were not obtained from agar plates, which underlines that the use of multiple cultivation methods is worthwhile to increase the diversity of the cultivable microorganisms from sponges.

Bacteria have been called the unseen majority on Earth, with an estimated number of 4×10^{30} to 6×10^{30} cells (47). In addition, they might as well be termed the uncultivable majority, because no matter whether they are derived from soil, the aquatic environment, or human gut, only a minority can be readily cultured (8, 10, 11). The bacteria associated with marine sponges are no exception. Despite the efforts of a number of research groups, cultivability values from only 0.1 to 11% have been reported (16, 36, 40, 45). Cultivation of sponge-associated microbes has received considerably more attention, since a number of publications have shown that bacteria were the actual producers of some bioactive metabolites that had been previously ascribed to their hosts (1, 2, 15, 29). In addition, cultivation of microbes will remain an important tool in the genomics era, to make complete genomes available for sequencing and analysis.

The general approach for cultivation is to provide nutrients and an environment similar to the natural environment to grow the target species. The microenvironment to which bacteria are exposed may be significantly different from their apparent natural environment. This is especially significant for strains that reside inside the mesohyl of marine sponges, where the microenvironment has little similarity to the macroenvironment (seawater) that is generally used in most cultivation experiments. For example, inside the sponge mesohyl, anoxic conditions frequently occur when the sponge temporarily ceases to pump water (21, 22). In addition, iron may be available at higher concentrations in the mesohyl than in the surrounding seawater due to the presence of siderophores (18). Moreover, many sponges have been found to host cyanobacteria that are producers of numerous bioactive compounds (reviewed in reference 12) and require light for growth. Another factor that might be of importance for the cultivation of obligate symbionts are sponge-specific lectins, which are one of the core elements of the mesohyl (33). Furthermore, patience may prove to be of utmost importance to cultivate these bacteria that have adapted to oligotrophic conditions (6). The doubling time of many microorganisms in nature is on the order of 100 days (47), which is much longer than most reported cultivation experiments.

In the present work, we studied the cultivability of *Haliclona* (*gellius*) sp.-associated bacteria by examining a matrix of different low-nutrient media and environmental conditions in order to mimic aspects of microenvironments that are found in sponges. In addition to aerobic and anaerobic cultures on low-nutrient agar plates, we used low-nutrient liquid cultures, following the dilution-to-extinction principle (3), to isolate species that are not able to grow on the agar-air interface. Fur-

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thermore, we applied floating polycarbonate filters, mimicking the inner structures of the filter-feeding sponge, to culture bacteria on top of low-nutrient media. To assess cultivation success, we compared the isolates with previously obtained 16S rRNA gene sequences from a clone library from *Haliclona* (*gellius*) sp. (42).

MATERIALS AND METHODS

Sample collection. Specimens of *Haliclona* (*gellius*) sp. (identified by R. W. M. van Soest and W. L. Lee) were collected near the Monterey, CA, harbor (coordinates, 36.60N, 121.89W) at a depth of approximately 12 m on 24 May 2006 (Hg5) and 23 January 2007 (Hg6). The sponges were rinsed three times with sterile artificial seawater (natural sea salt mix; Oceanic Systems, Dallas, TX) before grinding the tissue with a sterilized mortar and pestle. Two tissue volumes of sterile artificial seawater (ASW) were added to obtain a homogeneous cell suspension. For specimen Hg5, the cell suspension was divided in aliquots of 1.2 ml and mixed with 0.6 ml 50% sterile glycerol in ASW. The samples were frozen to −20°C before they were stored at −80°C. The cell suspension of Hg6 was immediately used for a cultivation experiment.

Chemicals. Natural seawater was collected approximately 50 km offshore from San Francisco and kept at the UC Berkeley animal care facility. After collection at the animal care facility, it was immediately autoclaved and stored at 4°C until further use.

A previously frozen *Haliclona* (*gellius*) sp. specimen was used to prepare the aqueous, organic, and spicule extracts. The specimen was ground with a sterilized mortar and pestle, and 2 ml of the ground tissue was extracted overnight with 40 ml of double-distilled water (ddH_2O) or 40 ml methanol for the aqueous or the organic extract, respectively. Subsequently, the extracts were filter sterilized using a 0.22-μm filter and stored at −20°C. The spicule extract was prepared by dissolving the cake that remained on the filter of the aqueous extract in 50 ml of 5 mM EDTA in ddH_2O. After incubation for 24 h, the suspension was centrifuged at 138 × *g* for 10 min. The pellet was rinsed twice with ddH_2O before it was stored in 20 ml of ddH_2O at −20°C.

A fluorescein diacetate (FDA) stock solution was prepared by dissolving 5 g FDA (Sigma) in 100 ml acetone, and it was stored at −20°C. A working solution was prepared for each experiment by diluting 10 μl stock solution in 1 ml acetone. Subsequently, the FDA working solution was mixed at a 1:1 ratio with a 3% NaCl solution.

Cultivation conditions. *Haliclona* (*gellius*) sp. isolates were obtained from (i) agar plates, (ii) liquid media, and (iii) floating filters.

(i) Agar plate cultures. Nineteen different agar media were used for the cultivation of sponge-associated bacteria, and they were assigned numbers as follows: medium 1, basic agar (1 liter natural seawater [NSW]); 2, marine agar 2216 (Difco); 3, actinomycete isolation agar (1 liter NSW, 1 g peptone from casein, 0.1 g asparagine, 4 g sodium propionate, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 1 mg $FeSO_4 \cdot 7H_2O$, 1 ml glycerol); 4, raffinose-histidine agar (1 liter NSW, 2 g raffinose, 0.2 g histidine) (modified from reference 46); 5, 60:40 agar (600 ml NSW, 400 ml ddH_2O); 6, glycerol-arginine agar (1 liter NSW, 2 ml glycerol, 0.3 g arginine) (35); 7, chitosan agar (1 liter NSW, 2 g chitosan); 8, peptone-starch agar (1 liter NSW, 2 g starch, 0.2 g peptone from casein) (modified from reference 32); 9, fluid thioglycolate agar (1 liter NSW, 1 g peptone from casein, 0.3 g yeast extract, 0.3 g D-glucose, 0.05 g L-cystine, 0.5 g sodium thioglycolate); 10, mucin agar (1 liter NSW, 1 g mucin) (36); 11, Delicious agar (1 liter NSW, 0.3 g peptone from casein, 0.1 g yeast extract, 0.01 g D-glucose); 12, Mueller-Hinton agar (1 liter NSW, 1 g yeast extract, 0.5 g starch, 2 g peptone from casein); 13, Delicious antibiotic agar (1 liter NSW, 0.3 g peptone from casein, 0.1 g yeast extract, 0.01 g D-glucose, 0.5 g penicillin, 1 g streptomycin); 14, synechococcus agar (1 liter NSW, 0.21 g $NaNO_3$, 0.0053 g NH_4Cl, 0.25 g Na_2SO_3, 0.0124 g $Na_2CO_3 \cdot 2H_2O$, 0.5 g cycloheximide); 15, charcoal agar (1 liter NSW, 1 g yeast extract, 2 g activated charcoal, 0.4 g L-cysteine · H_2O) (modified from reference 7); 16, aqueous sponge extract agar (per liter, 880 or 960 ml NSW, 0.01 g D-glucose, 120 or 40 ml aqueous sponge extract); 17, organic sponge extract agar (per liter, 880 or 960 ml NSW, 0.01 g D-glucose, 120 or 40 ml organic sponge extract); 18, sponge spicule agar (per liter, 960 or 992 ml NSW, 0.01 g D-glucose, 40 or 8 ml sponge spicule extract); 19, crenarchaeote agar (1 liter NSW, 0.124 g $Na_2CO_3 \cdot 2H_2O$, 0.053 g NH_4Cl, 1 ml tungsten-selenite solution) (48; modified from reference 27). In addition to the basic version (a), several variations of the latter medium were used: the addition of 0.2 g streptomycin (b), 0.1 g penicillin and 0.2 g streptomycin (c), 0.5 g penicillin and 1 g streptomycin (d), 0.2 g streptomycin and 40 ml aqueous sponge extract (e), 0.2 g streptomycin and 40 ml organic sponge extract (f), and 0.2 g streptomycin and 8 ml spicule extract (g). All media contained 15 g Noble agar to produce solid medium, and all media except basic agar and marine agar 2216 were supplemented with 1 ml trace metal solution (36), 1 ml phosphate solution (36), and 1 ml vitamin solution (BME vitamins [diluted 10-fold]; Sigma). Filter-sterilized carbonate, tungsten-selenite, L-cysteine, vitamin, and antibiotic solutions were added after autoclaving the media to prevent evaporation, precipitation, or inactivation. The pH of all media was 7.5, except for synechococcus agar (pH 8.0) and crenarchaeote agar (pH 7.0). Media 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 16, 17, and 18 were also used for anaerobic cultivation experiments. For those experiments the media were additionally supplemented with 0.42 g/liter $NaNO_3$, 0.96 g/liter sodium propionate, and 0.001 g resazurin. For the actinomycete isolation agar (medium 3) and the fluid thioglycolate agar (medium 9), only the resazurin was added. For media 1 to 11, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 dilutions of the cryopreserved cell suspension of Hg5 (see above) were distributed on the agar surface with a few sterilized glass beads. In addition, one plate was included as a negative control. For media 12 to 19, only 10^1, 10^2, and 10^3 dilutions and the negative control were used for the inoculation, as the experiment with media 1 to 11 had shown that these dilutions were most successful. Plates were incubated in the dark at 13°C, except medium 12 (in the dark at room temperature [RT]) and medium 14 (in the light at RT). Growth was monitored by weekly counts of the colonies on the plates for a period of 3 months.

TABLE 1. Setup of the large-number liquid cultures

No. of cells/well	Culture label, based on indicated no. of cells/well and additive(s)[a]						
	No additive	AHSL	cAMP	AHSL + cAMP	dfaB	Carbon source	AHSL + cAMP + dfaB + carbon source
2	A	C	E	G	I	K	M
4	B	D	F	H	J	L	N

[a] AHSL, acyl homoserine lactones; dfaB, desferrioxamine B.

(ii) Liquid medium cultures. Three liquid media experiments were performed. In the first experiment, an exploratory liquid cultivation was done in two sets of 78 plastic tubes with a cultivation volume of 10 ml NSW. Five approximate inoculum densities were used: 2 (18 tubes), 4 (18 tubes), 10 (18 tubes), 20 (10 tubes), and 200 cells/tube (10 tubes). The cell concentration in each inoculum was determined by a microscopic cell count of a 4′,6-diamidino-2-phenylindole-stained sample. In addition, four tubes were included as a negative control. In half of the tubes, only NSW was used, while for the other half the NSW was supplemented with 1 mg/liter peptone from casein, 1 ml trace metal solution (36), 1 ml phosphate solution (36), and 1 ml vitamin solution (BME vitamins [diluted 10-fold]; Sigma). For the first set of 78 tubes, a cryopreserved sponge homogenate was used (Hg5), while for the second set a fresh homogenate from a different specimen (Hg6) was used. The tubes were incubated in a shaker at 13°C for 4 months.

In the second experiment, a "large-number experiment" was performed to test the effect of certain potentially growth-stimulating compounds. Fourteen deep 96-well plates (assigned letters A to N) were inoculated with approximately 2 or 4 cells per well containing 1 ml of NSW (Table 1). The acyl homoserine lactones that were tested were a mixture of *n*-butyryl-DL-homoserine lactone, *n*-hexanoyl-DL-homoserine lactone, *n*-heptanoyl-DL-homoserine lactone, and *n*-octanoyl- DL-homoserine lactone (Sigma) and were applied at 1, 10, 100, and 1,000 nM each. Cyclic AMP (cAMP; Sigma) was tested at 10 μM, desferrioxamine B at was tested at 1, 10, 100, and 1,000 nM, and a mixture of yeast extract, peptone from casein, glucose, and sodium citrate was tested with each component at 1 mg/liter. The plates were incubated on a shaker at 13°C for 3 months.

For the third set of experiments, 14 96-well plates were incubated via flow cytometric cell sorting. A sample of the inoculum was applied to the flow cytometer, and each well in the 96-well plates was inoculated with one "event," i.e., a bacterium, a sponge cell, or a piece of cell debris. Eleven plates contained 100 μl medium supplemented with the four different homoserine lactones at 1,000 nM each, cAMP at 10 μM, desferrioxamine B at 1,000 nM, and a mixture of yeast extract, peptone from casein, glucose, and sodium citrate at 1 mg/liter each. Three 96-well plates contained 100 μl medium supplemented with a "high" carbon load: a mixture of yeast extract, peptone from casein, glucose, and sodium citrate at 100 mg/liter each. The plates were incubated on a shaker at 13°C for 3 months.

(iii) Floating filter cultures. Fourteen different media were applied for the floating filter cultivation experiment: medium 1, basic medium (1 liter NSW); 2, aqueous sponge extract medium (0.8 liter NSW, 0.2 liter aqueous sponge extract); 3, organic sponge extract medium (0.9 liter NSW, 0.1 liter organic sponge extract); 4, mucin medium (1 liter NSW, 100 mg mucin); 5, raffinose medium (1 liter NSW, 100 mg raffinose); 6, Delicious medium (1 liter NSW, 33 mg yeast extract, 33 mg peptone, 33 mg glucose); 7, Delicious antibiotic medium (1 liter NSW, 33 mg yeast extract, 33 mg peptone, 33 mg glucose, 10 mg penicillin, 20 mg streptomycin). Media 8 to 14 were identical to media 1 to 7, respectively, but were supplemented with a mixture of acyl homoserine lactones (100 nM each [details are provided above for the liquid medium cultures]), cAMP (10 μM), desferrioxamine B (100 nM), and amphotericin B (2.5 mg/liter). All media were supplemented with 1 ml trace metal solution (36) 1 ml phosphate solution (36), and 1 ml vitamin solution (BME vitamins [10-fold diluted]; Sigma). Filter-sterilized vitamin, acyl homoserine lactone, cAMP, desferrioxamine B, and antibiotic solutions were added after autoclaving the media to prevent evaporation, precipitation, or inactivation. The pH of all media was 7.5. Black polycarbonate filters (GE Osmonics, Minnetonka, MN) with a diameter of 47 mm and a pore size of 0.1 μm were autoclaved and mounted on a sterile glass filter holder. Each filter was first rinsed with 40 ml ddH_2O before 5 ml diluted cell suspension was filtered. A total of 98 filters were prepared: three different inoculum concentrations in duplicate for all 14 media and for each medium a blank filter was included as a negative control. The three inoculum concentrations were chosen so that they were comparable to the 10^1, 10^2, and 10^3 dilutions that were used for the agar plate cultivation experiment (the same number of cells per unit of surface area). The filters were placed on top of 13 ml medium in a petri dish by using sterile tweezers. High petri dishes were used (height, ~1.5 cm) to establish a large headspace to facilitate handling of the petri dishes and to have more oxygen available to the cells. The petri dishes were sealed and incubated in the dark at 13°C for 39 to 80 days.

Viability analysis. All liquid cultivations were checked for growth by staining the samples with FDA prior to performing PCR. The cell suspensions from tubes and deep-well plates were concentrated in 105 μl 3% NaCl. A volume of 100 μl was used for the viability analysis, and 5 μl FDA working solution in NaCl was added to these samples. A 5-μl aliquot was taken from all wells from the third liquid culture experiment, where the cultivation volume was only 100 μl. The 5 μl FDA working stock was added immediately to the remaining 95 μl of these samples. All samples to which the FDA working stock was added were incubated for 24 h (a long incubation time was used because of the extremely low cell concentrations [17]) in the dark before absorption was measured at 490 nm. A 3% NaCl solution and 1×10^6 E. coli cells/ml were used as negative and positive controls, respectively. Samples with an absorption of >0.02 were selected for direct PCR.

Colony PCR. Colonies from agar plates and floating filters were picked using sterilized tooth picks and dissolved in 10 μl ddH_2O. For microcolonies (which were picked from the floating filters), sterilized metal pins were used and they were dissolved in 5 μl ddH_2O. Subsequently, all samples (including the 5-μl aliquots from the liquid culture samples) were stored at −20°C for at least one night. Aliquots (1.5 μl) of these samples was used to amplify the 16S rRNA gene(s) in a 25-μl PCR mixture with 0.4 μM universal bacterial primers 8F (AGA GTT TGA TCC TGG CTC AG [13]) and 1492R (GGT TAC CTT GTT ACG ACT T [28]), 0.4 mM each deoxynucleoside triphosphate, 1.25 U AmpliTaq Gold (Applied Biosystems), 1× PCR buffer, and 0.125 μl dimethyl sulfoxide. For the microcolonies from the floating filters, 5 μl of DNA template was used for the 25-μl PCR mixtures. The PCR program was as follows: initial denaturation for 12 min at 94°C; 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C; a final extension step for 10 min at 72°C. The PCR products were visualized on a 0.8% agarose gel. Products from successful PCRs were purified (MultiScreen $PCR_{\mu 96}$; Millipore, Billerica, MA) and sequenced with the 8F primer.

Sequence analysis. Sequence chromatograms were manually inspected and assembled using the Vector NTI software (Invitrogen, Carlsbad, CA). Sequences with similarities of >97% were considered 1 OTU. The species boundary has recently been revised to 98.7 to 99% identity for sequences to be considered to belong to the same OTU (43). However, this is only true for sequences with no ambiguous nucleotides, which is not the case for our PCR products, which were directly sequenced. All sequences were checked for possible chimeric origins by using Bellerophon (version 3) from the greengenes website (23). Sequences were deposited at the NCBI GenBank. Nearest neighbors were determined by comparison to the NCBI GenBank database using BLAST searches (6 March 2009).

Phylogenetic analysis. 16S rRNA gene sequences of the OTUs of *Haliclona* (*gellius*) sp. isolates, nearest neighbors of *Haliclona* (*gellius*) sp. isolates, and *Haliclona* (*gellius*) sp. clones from a previous study (42) were imported in the ARB software package (31). DNA sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB program and refined manually. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (4). The default program parameters were used, except we allowed a minimum block length of 5 and gaps in 50% of positions. Phylogenetic trees were created by Bayesian analysis, using MrBayes v3.0b4 (24) at the freely available Bioportal server (www.bioportal.uio.no). All parameters were treated as unknown variables with uniform prior probability densities at the beginning of each run, and their values were estimated from the data during the analysis (39). All Bayesian analyses were initiated with random starting trees and were run for 1×10^7 generations. The nucleotide identities of *Haliclona* (*gellius*) sp. isolate and *Haliclona* (*gellius*) sp. clone sequences that grouped together in the tree were established by using the BLAST2 tool from NCBI. Identities of 97% and higher between *Haliclona* (*gellius*) sp. isolates and *Haliclona* (*gellius*) sp. clones were regarded as successfully isolated sponge-associated bacteria.

MDS. Different treatments (cultivation methods and media) were compared for the distribution of isolates among OTUs observed in this study by using the PRIMER 6 software (5). The data were pretreated by using the square root of the abundance of the isolates per OTU. Bray-Curtis similarity was used to calculate resemblance between treatments, and nonmetric multidimensional scaling (MDS) was used to plot the analysis.

Nucleotide sequence accession numbers. Sequences were deposited with NCBI GenBank under accession numbers EU346387 to EU346643, EU642556, and EU642557.

RESULTS AND DISCUSSION

Cultivation methods and media. A total of 3,903 growing cultures were identified. These cultures included 2,278 colonies picked from agar plates, 528 liquid cultures (from 2,836 seeded cultures), and 1,097 colonies picked from floating filters. A total of 205 different OTUs were obtained from all 16S rRNA gene PCR products. Sixty-eight OTUs represented more than one colony, while 137 OTUs represented a single colony.

No significant differences based on 16S rRNA gene sequence were found between the anaerobic and aerobic agar plate cultures (data not shown), and therefore colonies obtained with the same medium under aerobic and anaerobic conditions were combined. The number of PCR products obtained for each medium (Table 2) reflects the number of colonies that grew on the plates. In general, fewer PCR products (<100) were obtained from the extremely nutrient-poor media, such as basic agar, 60:40 agar, synechococcus agar, and crenarchaeote agar. For the relatively recalcitrant carbon source chitosan, the number of PCR products was also low. An equally poor result with the latter carbon source was obtained with the cultivation of associated bacteria from 10 Mediterranean sponge species (34). The organic sponge extract agar also turned out to be relatively unsuccessful with respect to the number of PCR products obtained. Because no methanol control was included, it is unclear whether this was because of the low availability of nutrients or because of the presence of toxic compounds in the organic extract. No colonies were formed on Delicious antibiotic agar or charcoal agar, most likely because of the presence of high penicillin and streptomycin concentrations and charcoal concentrations, respectively. Despite the high nutrient concentrations and the absence of antibiotics in marine agar and Mueller-Hinton agar, a low number of PCR products was obtained. For marine agar, the colony number remained low because some fast-growing colonies quickly covered the surface of the agar plates, leaving little space for slower growers. From the oligotrophic media, such as actino-

TABLE 2. Summary of the number of PCR products obtained and diversity (number of OTUs) for all media and cultivation methods

Agar plates			Liquid media			Floating filters		
Medium[a]	No. of PCR products	No. of OTUs	Medium[a]	No. of PCR products	No. of OTUs	Medium[a]	No. of PCR products	No. of OTUs
1, basic	92	18	Tubes	37	20	1, basic	0	0
2, marine	54	15	dw A	0	0	2, Aq sp ex	8	5
3, Actino	165	28	dw B	2	1	3, Or sp ex	8	6
4, Rafhis	118	19	dw C	0	0	4, mucin	99	29
5, 60:40	41	13	dw D	0	0	5, Raf	6	3
6, Glyarg	135	26	dw E	0	0	6, Delici	38	17
7, chitosan	68	22	dw F	0	0	7, Delici + ps	0	0
8, Pepsta	151	30	dw G	0	0	8, basic+	14	6
9, Fluthi	131	20	dw H	5	4	9, Aq sp ex+	5	4
10, mucin	109	29	dw I	0	0	10, Or sp ex+	4	2
11, Delici	114	26	dw J	1	1	11, mucin+	47	23
12, Mulhin	62	17	dw K	0	0	12, Raf+	26	9
13, Delici + ps	0	0	dw L	5	5	13, Delici+	52	18
14, Synech	47	13	dw M	0	0	14, Delici + ps+	20	10
15, charcoal	0	0	dw N	3	3			
16, Aq sp ex	120	18	nw 1–11	0	0			
17, Or sp ex	22	13	nw 12–14	86	5			
18, Sp sp ex	164	32						
19, Crenarch	24	8						

[a] See Materials and Methods for explanations of the different media used (names for some media have been abbreviated here; the full names (and additional components) of the media, by number, can be found in the text). Media used for liquid cultures were not assigned numbers.

mycete agar, raffinose-histidine agar, glycerol-arginine agar, peptone-starch agar, fluid thioglycolate agar, mucin agar, Delicious agar, aqueous sponge extract agar, and sponge spicule agar, higher numbers of PCR products and OTUs were obtained. In total, 151 OTUs were obtained from agar plate cultures.

Only 139 clean PCR products were obtained from the liquid cultures because, first of all, growth occurred in only approximately 20% of the inoculated cultures, and second, in many cultures in which growth occurred, different 16S rRNA gene templates were present, which resulted in multiple signals when they were sequenced. The lower number of clean PCR products resulted in a considerably lower species diversity obtained from liquid cultures (36 OTUs) compared to the agar plates, which corresponds to the observations of Schoenborn and colleagues (41), who compared the success of agar and liquid cultures for the isolation of soil bacteria. However, 11 OTUs were exclusively obtained from liquid cultures and not from agar plates or floating filters (see Fig. S1 in the supplemental material).

Two types of colonies appeared on the floating filters: (i) macrocolonies (Fig. 1), which were comparable to the colonies that were picked from the agar plates, and (ii) whitish to transparent microcolonies that were barely visible with the naked eye. The microcolonies were much more abundant than the macrocolonies and represented 80% of all floating filter colonies that were picked. Colony formation occurred on floating filters on almost all media. However, on Delicious antibiotic medium no colonies formed, similar to the result on Delicious antibiotic agar in the plate experiment. No PCR products were obtained from colonies that appeared on basic medium. Mucin medium and Delicious medium were most successful for obtaining colonies and OTUs from *Haliclona* (*gellius*) sp. tissue compared to the other media used for the floating filter cultures. The addition of the mixture of acyl homoserine lactones, cAMP, desferrioxamine B, and amphotericin B to the media had no observable effects, except that their additions led to colony formation on Delicious antibiotic medium. In total, 60 OTUs were obtained from floating filters. As for the cultivation on agar plates and in liquid media, 23 floating filter-specific OTUs were obtained (see Fig. S1 in the supplemental material).

When profiles of isolates obtained with different media and cultivation techniques were compared (Fig. 2), we found that the addition of antibiotics to agar plates (medium 19 and derivatives) yielded a different profile of OTUs than with the other agar media, but the cultivation technique used had the strongest effect on the OTU profiles obtained. The OTU profiles obtained from agar plates showed little resemblance with the OTU profiles obtained from liquid media. Also, OTU profiles obtained from floating filter media containing aqueous (media 2 and 9) or organic (media 3 and 10) sponge extract or raffinose (5) were different from other treatments. Previous studies using the dilution-to-extinction method have led to the

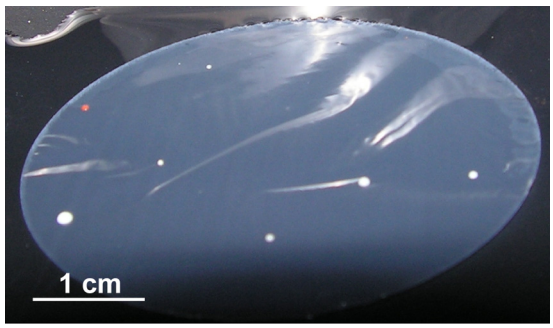

FIG. 1. Example of a floating filter taken from a petri dish. The white macrocolonies and one red macrocolony are visible on top of the filter surface.

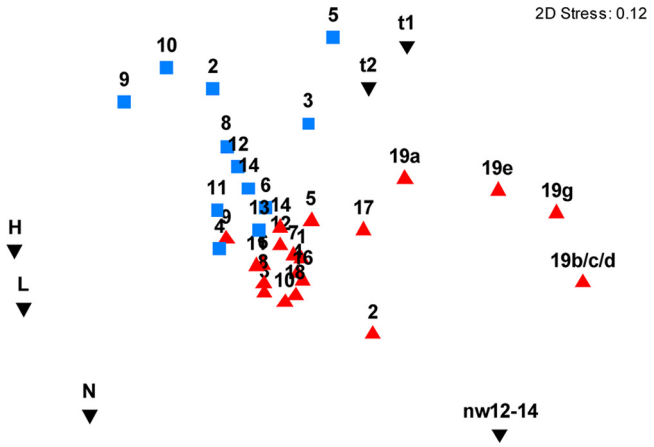

FIG. 2. MDS plot of similarity between OTUs obtained with different treatments. Red triangles correspond to agar plate cultures, black triangles to liquid cultures, and blue squares to floating filter cultures. The numbers and letters depicted above the triangles and squares refer to the media numbers (see Materials and Methods). Media 19b, -c, and -d were pooled, as they yielded the same isolates. t1 refers to the liquid cultivation experiment using a cryopreserved extract of Hg5, while t2 refers to the liquid cultivation experiment using a freshly prepared extract from Hg6. Only treatments that resulted in more than 1 OTU are depicted. The distance between data points in the figure is a measure for the relative similarity between the OTU profiles obtained for the different treatments, i.e., data points that are closer to each other indicate a higher similarity.

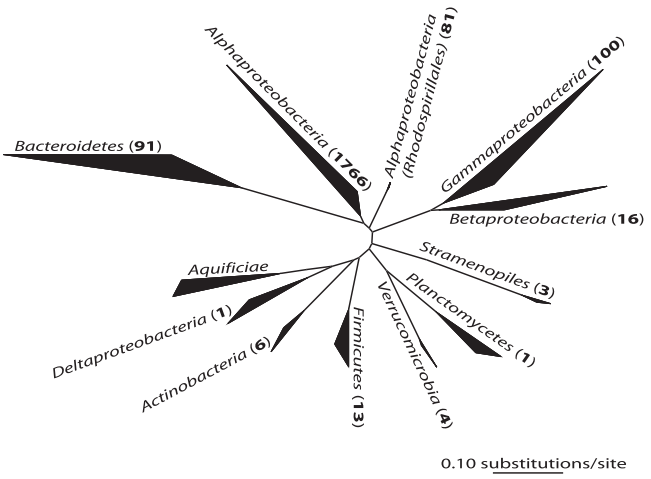

FIG. 3. Collapsed Bayesian phylogram of isolates from *Haliclona* (*gellius*) sp. and nearest neighbors (BLAST search, 6 March 2009). The numbers in parentheses indicate the number of isolates for each phylum/class.

isolation of the ubiquitous, but previously uncultivable, representatives of the SAR11 clade (38). The floating filter technique has been scarcely applied but has resulted in the isolation of the moderately thermophilic bacterium *Thiobacillus ferrooxidans*, while use of conventional solid media was unsuccessful for growing this species (9). Moreover, this technique was used to isolate a novel *Verrucomicrobium* sp. with no cultured near relatives (37). A similar trend could be seen for the *Haliclona* (*gellius*) sp. isolates; while only 28% of the agar plate isolates had nearest neighbors (BLAST search) that were derived from clones, this number rose to 36% and 52%, respectively, for liquid culture isolates and floating filter isolates.

Identification of cultivated bacteria. *Alphaproteobacteria* represented the large majority of the isolates, at almost 89% of all sequences obtained (Fig. 3). This is in sharp contrast to the distribution of 16S rRNA gene sequences previously obtained from an environmental sample of *Haliclona* (*gellius*) sp. tissue, where *Gammaproteobacteria*-derived sequences dominated the library, at 55% of the PCR products, while *Alphaproteobacteria* represented only 7% (42). This is not an unusual discrepancy, as *Alphaproteobacteria* dominate isolates from many marine sponges (34, 45), while 16S rRNA gene libraries from environmental tissue samples show a different distribution (44, 46). The *Gammaproteobacteria* and *Bacteroidetes* represented 5 and 4% of the cultured isolates from *Haliclona* (*gellius*) sp., respectively, and the *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Stramenopiles*, *Verrucomicrobia*, and *Betaproteobacteria* and *Deltaproteobacteria* together represented approximately 2% (Fig. 3). The difference in cultivability between *Alphaproteobacteria* and *Betaproteobacteria* and *Gammaproteobacteria* is also reflected in the origin of the 16S rRNA gene sequence of the nearest neighbors: 67 and 55% of the *Betaproteobacteria* and *Gammaproteobacteria* nearest neighbors, respectively, were derived from clones (and not isolates), while for the *Alphaproteobacteria*, nearest-neighbor clones only accounted for 28% of the OTUs.

The nearest neighbors of the isolates can mostly be divided in three groups: (i) seawater isolates or clones, (ii) marine sediment isolates or clones, and (iii) marine invertebrate-derived isolates or clones. Nine *Haliclona* (*gellius*) sp. isolates from the latter group are present in marine invertebrate-specific clades (Fig. 4; the complete phylogenetic tree can be found in Fig. S1 of the supplemental material). All nearest beta- and gammaproteobacterial sponge neighbors were derived from clone sequences, indicating that we have cultivated previously uncultivated sponge-specific microbes. In contrast to the beta- and gammaproteobacterial sponge neighbors, only 33% of the alphaproteobacterial sponge neighbors were obtained from clone sequences.

In order to assess the cultivability of *Haliclona* (*gellius*) sp.-associated bacteria, 16S rRNA gene sequences of the isolates were compared to a previously prepared 16S rRNA gene library from an environmental sample from the same sponge specimen (42). Three categories of *Haliclona* (*gellius*) sp.-associated bacteria are defined to discuss the (un)cultivability:

(i) OTUs that were obtained from sponge tissue and from sponge isolates. Seventeen OTUs that were previously found in the clone library from the sponge were also obtained by cultivation (Table 3). Eight of them belong to the *Alphaproteobacteria*, six to the *Gammaproteobacteria*, two to the *Bacteroidetes*, and one to the *Firmicutes*. Six of these 17 OTUs were not obtained from agar plates, which indicates that the use of multiple cultivation methods is valuable for increasing the diversity of cultivable microorganisms from sponges. Three OTUs (OTUHg15, OTUHg19, and OTUHg23), which were detected in clone libraries of several *Haliclona* (*gellius*) sp. specimens, were also recovered from the cultivation experiment (Fig. 5). In total, 5 of the 36 OTUs that were present in the clone library of the tissue sample of *Haliclona* (*gellius*) sp. specimen 5 (Hg5, the specimen that was also used for the

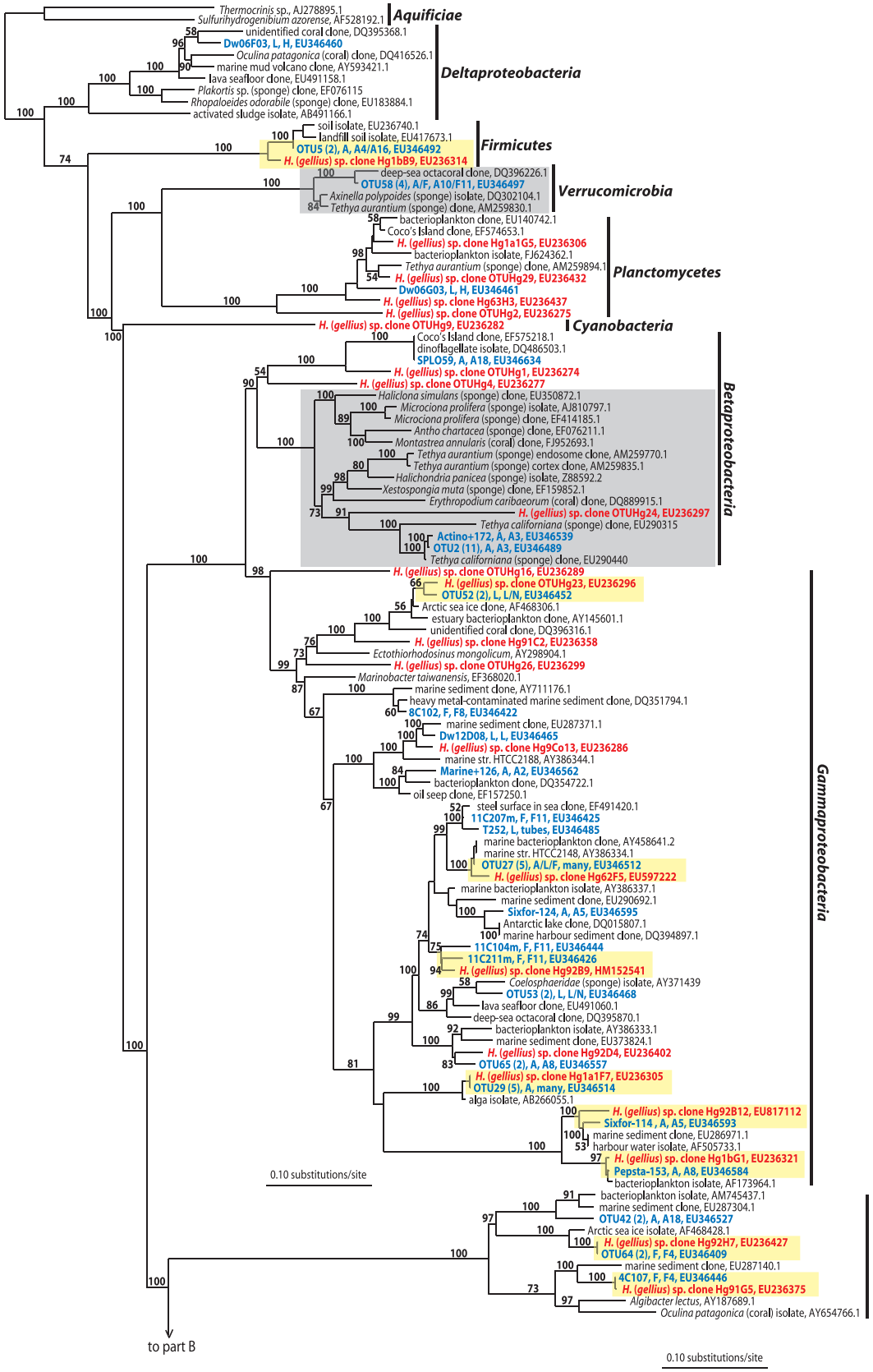

FIG. 4–*Continued on following page*

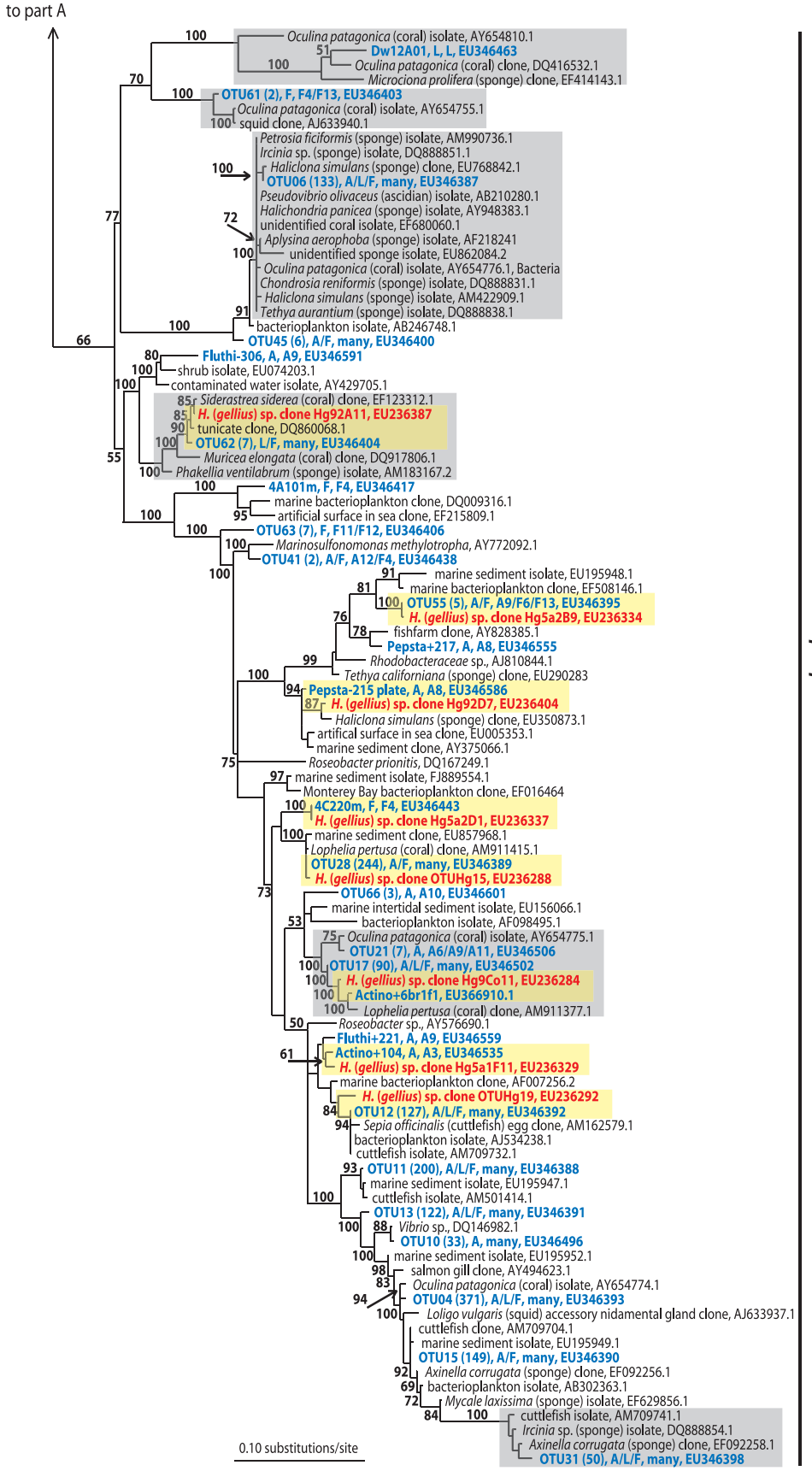
to part A
Oculina patagonica (coral) isolate, AY654810.1
Dw12A01, L, L, EU346463
Oculina patagonica (coral) clone, DQ416532.1
Microciona prolifera (sponge) clone, EF414143.1
OTU61 (2), F, F4/F13, EU346403
Oculina patagonica (coral) isolate, AY654755.1
squid clone, AJ633940.1
Petrosia ficiformis (sponge) isolate, AM990736.1
Ircinia sp. (sponge) isolate, DQ888851.1
Haliclona simulans (sponge) clone, EU768842.1
OTU06 (133), A/L/F, many, EU346387
Pseudovibrio olivaceus (ascidian) isolate, AB210280.1
Halichondria panicea (sponge) isolate, AY948383.1
unidentified coral isolate, EF680060.1
Aplysina aerophoba (sponge) isolate, AF218241
unidentified sponge isolate, EU862084.2
Oculina patagonica (coral) isolate, AY654776.1, Bacteria
Chondrosia reniformis (sponge) isolate, DQ888831.1
Haliclona simulans (sponge) isolate, AM422909.1
Tethya aurantium (sponge) isolate, DQ888838.1
bacterioplankton isolate, AB246748.1
OTU45 (6), A/F, many, EU346400
Fluthi-306, A, A9, EU346591
shrub isolate, EU074203.1
contaminated water isolate, AY429705.1
Siderastrea siderea (coral) clone, EF123312.1
H. (gellius) sp. clone Hg92A11, EU236387
tunicate clone, DQ860068.1
OTU62 (7), L/F, many, EU346404
Muricea elongata (coral) clone, DQ917806.1
Phakellia ventilabrum (sponge) isolate, AM183167.2
4A101m, F, F4, EU346417
marine bacterioplankton clone, DQ009316.1
artificial surface in sea clone, EF215809.1
OTU63 (7), F, F11/F12, EU346406
Marinosulfonomonas methylotropha, AY772092.1
OTU41 (2), A/F, A12/F4, EU346438
marine sediment isolate, EU195948.1
marine bacterioplankton clone, EF508146.1
OTU55 (5), A/F, A9/F6/F13, EU346395
H. (gellius) sp. clone Hg5a2B9, EU236334
fishfarm clone, AY828385.1
Pepsta+217, A, A8, EU346555
Rhodobacteraceae sp., AJ810844.1
Tethya californiana (sponge) clone, EU290283
Pepsta-215 plate, A, A8, EU346586
H. (gellius) sp. clone Hg92D7, EU236404
Haliclona simulans (sponge) clone, EU350873.1
artifical surface in sea clone, EU005353.1
marine sediment clone, AY375066.1
Roseobacter prionitis, DQ167249.1
marine sediment isolate, FJ889554.1
Monterey Bay bacterioplankton clone, EF016464
4C220m, F, F4, EU346443
H. (gellius) sp. clone Hg5a2D1, EU236337
marine sediment clone, EU857968.1
Lophelia pertusa (coral) clone, AM911415.1
OTU28 (244), A/F, many, EU346389
H. (gellius) sp. clone OTUHg15, EU236288
OTU66 (3), A, A10, EU346601
marine intertidal sediment isolate, EU156066.1
bacterioplankton isolate, AF098495.1
Oculina patagonica (coral) isolate, AY654775.1
OTU21 (7), A, A6/A9/A11, EU346506
OTU17 (90), A/L/F, many, EU346502
H. (gellius) sp. clone Hg9Co11, EU236284
Actino+6br1f1, EU366910.1
Lophelia pertusa (coral) clone, AM911377.1
Roseobacter sp., AY576690.1
Fluthi+221, A, A9, EU346559
Actino+104, A, A3, EU346535
H. (gellius) sp. clone Hg5a1F11, EU236329
marine bacterioplankton clone, AF007256.2
H. (gellius) sp. clone OTUHg19, EU236292
OTU12 (127), A/L/F, many, EU346392
Sepia officinalis (cuttlefish) egg clone, AM162579.1
bacterioplankton isolate, AJ534238.1
cuttlefish isolate, AM709732.1
OTU11 (200), A/L/F, many, EU346388
marine sediment isolate, EU195947.1
cuttlefish isolate, AM501414.1
OTU13 (122), A/L/F, many, EU346391
Vibrio sp., DQ146982.1
OTU10 (33), A, many, EU346496
marine sediment isolate, EU195952.1
salmon gill clone, AY494623.1
Oculina patagonica (coral) isolate, AY654774.1
OTU04 (371), A/L/F, many, EU346393
Loligo vulgaris (squid) accessory nidamental gland clone, AJ633937.1
cuttlefish clone, AM709704.1
marine sediment isolate, EU195949.1
OTU15 (149), A/F, many, EU346390
Axinella corrugata (sponge) clone, EF092256.1
bacterioplankton isolate, AB302363.1
Mycale laxissima (sponge) isolate, EF629856.1
cuttlefish isolate, AM709741.1
Ircinia sp. (sponge) isolate, DQ888854.1
Axinella corrugata (sponge) clone, EF092258.1
OTU31 (50), A/L/F, many, EU346398
0.10 substitutions/site
Alphaproteobacteria

TABLE 3. OTUs that were discovered both in a 16S rRNA gene clone library of *H.* (*gellius*) sp. (based on Sipkema et al. [42]) and among isolates of the same sponge species[a]

Sponge clones		Sponge isolates		Method(s)	% identity	Phylum or class
Clone name (specimen no.)	Accession no.	Isolate name (specimen no.)	Accession no.			
Hg1bB9 (1)	EU236314	OTU5 (5)	EU346492	A	98	*Firmicutes*
OTUHg23 (1, 6)	EU236296	OTU52 (5)	EU346452	L	97	*Gammaproteobacteria*
Hg1a1F7 (1)	EU236305	OTU29 (5)	EU346514	A	100	*Gammaproteobacteria*
Hg1bG1 (1)	EU236321	pepsta-153 (5)	EU346584	A	98	*Gammaproteobacteria*
Hg62F5 (6)	EU597222	OTU27 (5/6)	EU346512	A/L/F	98	*Gammaproteobacteria*
Hg92B9 (9)	HM152541	11C211 (5)	EU346426	F	97	*Gammaproteobacteria*
Hg92B12 (9)	EU817112	sixfor-114 (5)	EU346593	A	98	*Gammaproteobacteria*
OTUHg15 (5, 9)	EU236288	OTU28 (5)	EU346389	A/F	99	*Alphaproteobacteria*
OTUHg19 (5, 9)	EU236292	OTU12 (5/6)	EU346392	A/L/F	98	*Alphaproteobacteria*
Hg5a1F11 (5)	EU236329	actino + 104 (5)	EU346535	A	97	*Alphaproteobacteria*
Hg5a2B9 (5)	EU236334	OTU55 (5)	EU346395	A/F	99	*Alphaproteobacteria*
Hg5a2D1 (5)	EU236337	4C220 m (5)	EU346443	F	100	*Alphaproteobacteria*
Hg92A11 (9)	EU236387	OTU62 (5)	EU346404	L/F	98	*Alphaproteobacteria*
Hg92D7 (9)	EU236404	pepsta-215 (5)	EU346586	A	98	*Alphaproteobacteria*
Hg9Co11 (9)	EU236284	actino + 6br1f1 (5)	EU366910	A	98	*Alphaproteobacteria*
Hg91G5 (9)	EU236375	4C107 (5)	EU346446	F	99	*Bacteroidetes*
Hg92H7 (9)	EU236427	OTU64 (5)	EU346409	F	99	*Bacteroidetes*

[a] The specimen number is included in parentheses after the clone or isolate name. Four specimens (1, 5, 6, and 9) were used to make the clone library, and two of these specimens (5 and 6) were used for the cultivation experiment (specimen 6 was used only for a small part of the liquid cultures). Sponge clones shown in boldface were obtained from multiple *Haliclona* (*gellius*) sp. specimens. The identity between the clone and matching isolate (based on BLAST2 analysis) is included. The letters A, F, and L in the method column refer to agar plates, floating filter cultures, and liquid cultures, respectively.

cultivation experiment) were also obtained from cultures, thus representing 14% of the bacterial diversity.

Twelve OTUs were cultivated from the extract of Hg5 and were not obtained in a clone library from the same individual sample but were found in clone libraries from other individual samples (Fig. 4). This implies that these OTUs were actually present in the extract of Hg5 but not included in the clone library, despite the considerable size of the clone library of each individual (approximately 200 clones/individual). The expected presence of "extra OTUs" was confirmed by rarefaction analysis of these clone libraries (42). If the percentage of cultivated OTUs (17 OTUs) that were also present in the total clone library of the sponge (Hg1 + Hg5 + Hg6 + Hg9 = 170 OTUs) is assessed, a cultivation score of 10% is obtained, which is a good result compared to values that have been previously reported for sponges. The large range of cultivability numbers in the literature (0.1 to 11%) is partly explained by the use of different definitions of the "cultivable fraction." Friedrich et al. (16) and Webster and Hill (45) used epifluorescence microscopy to estimate the bacterial cell concentration in sponge tissue of *Aplysina aerophoba* and *Rhopaloeides odorabile*, respectively, and defined the cultivability as the number of CFU divided by the total number of bacteria in the sponge. Both groups obtained a very similar cultivability: 0.15 and 0.1 to 0.23%, respectively, with similar cultivation setups. Santavy et al. (40) employed the same definition as the above-mentioned authors but estimated the bacterial cell concentration in the sponge by conversion of bacteria counted on transmission electron microscopy images to a three-dimensional model and obtained a cultivability of 3 to 11%. Olson and McCarthy (36) defined cultivability as the number of different bacterial species that could be cultured divided by the total bacterial diversity, based on denaturing gradient gel electrophoresis (DGGE), and obtained a value of 5%. We have used the same definition as Olson and McCarthy and express cultivability as the percentage of the OTUs present in sponge tissue that were also obtained by cultivation.

A number of the above-mentioned isolates from *Haliclona* (*gellius*) sp. have only a low identity to previously isolated bacteria (Table 4). For example, isolates OTU52, 4C107, Actino + 104, and 11C211 have only 90%, 94%, 95%, and 95% identity, respectively, to the nearest isolate, which is on or below the genus boundary (30).

(ii) OTUs that were obtained from sponge tissue but not from sponge isolates. Despite the fact that the recoverability of 10 to 14% of the bacterial species associated with an environmental sample of *Haliclona* (*gellius*) sp. is a relatively good score, it must be noted that the clone library OTUs that were regarded as the most stable associates of the sponge microflora (*Planctomycete* OTUHg2, *Betaproteobacteria* OTUHg1 and OTUHg24, *Gammaproteobacteria* OTUHg26 [Fig. 4], and *Crenarchaea* OTUHgAr2 [42]) were not among the cultivated OTUs. This

FIG. 4. Bayesian phylogram of 16S rRNA gene sequences of a selection of the isolates from *Haliclona* (*gellius*) sp. and nearest neighbors (the complete phylogenetic analysis with all OTUs is presented in Fig. S1 of the supplemental material). *Haliclona* (*gellius*) sp. isolates are shown in blue and boldface, and *Haliclona* (*gellius*) sp. clones are in red and bold. Yellow boxes contain *Haliclona* (*gellius*) sp. isolates and clones that are ≥97% identical. Gray boxes represent marine invertebrate-specific clusters. The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. Nodes with PP values of <50 are not indicated. *Thermocrinis* sp. and *Sulfurihydrogenibium azorense* were used as outgroups.

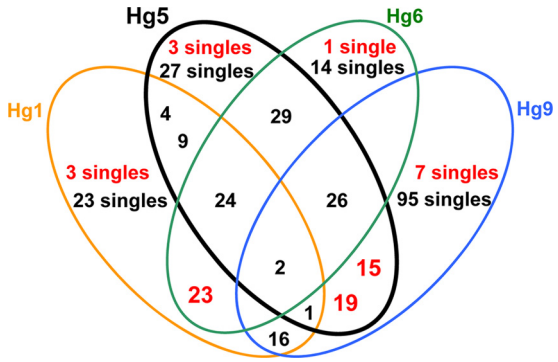

FIG. 5. Venn diagram of the OTUs in the clone library of the sponge that were also obtained by cultivation. Four sponge individual samples (each represented by one oval) were made to prepare the clone library (Hg1, Hg5, Hg6, and Hg9; see Sipkema et al. [42]). Each number (black or red) refers to an OTU that was found in the clone library of more than one sponge individual. The positions of the numbers in the Venn diagram show in which individuals each of the clone library OTUs were detected. OTU numbers in red were found in the clone library and were also obtained by cultivation. OTU numbers in black were found in the clone library but were not obtained by cultivation. The number of singles in black represents the number of OTUs that were present in the clone library of only one of the individuals. The number of singles in red represents the number of these singles that were also obtained by cultivation. The extract of Hg5 was used to inoculate the cultures (Hg6 was used only for one small liquid culture experiment).

confirms the current state of the art, that numerically abundant environmental bacteria are rarely isolated in cultivation experiments (26). We obtained one *Planctomycete* isolate from a liquid culture, but it is only distantly related to clone library OTUHg2 (BLAST2 identity, 84%). Sponge-specific OTUHg24 and OTUHg26 from the clone library have no cultured near relatives among the *Haliclona* (*gellius*) isolates or any other isolates (Fig. 4A) and may require highly specific yet unknown conditions for cultivation. To date, there has been only one report about the cultivation of a marine group 1 crenarchaeote (27). A variety of media (agar plate cultivation medium 19 and its derivatives) were based on the work of Könneke et al. (27). For all cultures that did not yield a bacterial 16S rRNA gene PCR product, PCR was repeated with archaeal 16S primers (methods not described), but crenarchaeal PCR products were never obtained. A study of the genomes of the most abundant, but uncultivable, microbial sponge symbionts, similar to the study by Hallam and colleagues (19, 20), who studied the genome of *Cenarchaeum symbiosum*, may hold the key to their cultivability and provide more information about the role of these microorganisms in the sponge-microbe consortium. High-throughput cultivation methods that have been applied for the isolation of previously uncultured microorganisms (6, 25, 49) are less likely to overcome the uncultivability of the most prominent sponge symbionts, as a low abundance of the cells in the inoculum is not the problem.

(iii) OTUs that were not obtained from sponge tissue but were obtained from sponge isolates. From the 205 OTUs that were obtained from isolates, only 17 matched with OTUs that were also obtained in a clone library from a tissue sample of the sponge. The question then arises: where did all the other OTUs from the isolates come from? A first possibility would be that they are laboratory-derived contaminations. However, controls that were included in all experiments were, with a few exceptions, negative, and it is therefore unlikely that most of the "extra" OTUs were derived from laboratory contamination. A large group of *Haliclona* (*gellius*) sp. isolates had nearest neighbors isolated or cloned from seawater, and although the sponges were rinsed three times with sterile artificial seawater before the inoculum was prepared, this rinsing does not remove bacteria that are present in the canal system

TABLE 4. OTUs from isolates of *Haliclona* (*gellius*) sp. that share ≤95% identity with the nearest isolate[a]

Sponge isolates				Nearest isolate		Phylum
Isolate name	Accession no.	Method(s)	% identity	Source	Accession no.	
Dw06F03	EU346460	L	86	Activated sludge	AB491166	*Deltaproteobacteria*
OTU58	EU346497	A/F	95	Sponge	DQ302104	*Verrucomicrobia*
Dw06G03	EU346461	L	94	Bacterioplankton	FJ624362	*Planctomycetes*
OTU2	EU346489	A	87	Sponge	Z88592.2	*Betaproteobacteria*
OTU52	EU346452	L	90	Soda lake	AY298904	*Gammaproteobacteria*
OTU53	EU346468	L	95	Sponge	AY371439	*Gammaproteobacteria*
OTU65	EU346557	A	90	Bacterioplankton	AY386333	*Gammaproteobacteria*
Marine + 126	EU346562	A	92	Bacterioplankton	AY386344	*Gammaproteobacteria*
Sixfor-124	EU346595	A	94	Bacterioplankton	AY386337	*Gammaproteobacteria*
T252	EU346485	L	94	Bacterioplankton	AY386337	*Gammaproteobacteria*
Dw12D08	EU346465	L	94	Bacterioplankton	AY386344	*Gammaproteobacteria*
8C102	EU346422	F	91	*Marinobacter taiwanensis*	EF368020	*Gammaproteobacteria*
11C211	EU346426	F	95	Bacterioplankton	AY386337	*Gammaproteobacteria*
OTU63	EU346406	F	95	Intertidal flat	EU156066	*Alphaproteobacteria*
OTU66	EU346601	A	90	Marine sediment	FJ889554	*Alphaproteobacteria*
Actino + 104	EU346535	A	95	Bacterioplankton	AF007256	*Alphaproteobacteria*
Pepsta + 217	EU346555	A	92	*Rhodobacteraceae*	AJ810844	*Alphaproteobacteria*
Dw12A01	EU346463	L	91	Coral	AY654810	*Alphaproteobacteria*
4A101	EU346417	F	91	Bacterioplankton	AB330821	*Alphaproteobacteria*
OTU42	EU346527	A	94	Bacterioplankton	AM745437	*Bacteroidetes*
4C107	EU346446	F	94	Coral	AY654766	*Bacteroidetes*

[a] Results are based on a BLAST search. The letters A, F, and L in the Method(s) column refer to agar plates, floating filter cultures, and liquid cultures, respectively.

and choanocyte chambers at the moment that the sponge is being processed. These bacterial species are likely to be present at low concentrations, but if they are easily cultivable they can skew the distribution of isolated bacteria. Other bacteria that are present in extremely low numbers in the sponge tissue may also be cultivated, although they may not be detected in a 16S rRNA gene clone library. This hypothesis is supported by rarefaction analysis of the diversity present in the clone library from the environmental sample. It was shown that 48 to 77% of diversity was covered (42), and a number of the isolates may correspond to these "hidden" OTUs in the clone library. Similar discrepancies between clone libraries or DGGE patterns on one hand and isolates on the other hand were found in studies with *Suberites zeteki* and two *Scleritoderma* sp. sponges (36, 50).

Isolates that were most prominent in the cultivation experiment (>100 colonies and cultures) were all *Alphaproteobacteria*. The most frequently obtained sequence was culture OTU4 (371 times), which is a member of the *Rhodobacteraceae* family and has been frequently isolated from marine sediments and seawater, coral mucus, squid nidamental glands, and sponges. The isolation of this family from a variety of sources suggests that it is an opportunistic cosmopolitan species. The same can be said for OTU11 (found 200 times), OTU13 (122), and OTU15 (149), which are related to OTU4. Despite the fact that culture OTU6 (133) was not discovered in the clone library of *Haliclona* (*gellius*) sp., its nearest neighbors are isolates from the marine sponges *Haliclona simulans*, *Chondrosia reniformis*, and other sponge- and marine invertebrate-derived *Alphaproteobacteria*. Only one of the sequences in the sponge-specific clade was obtained from a clone, while all others were derived from isolates. This suggests that the bacterium may be present in many marine invertebrates at low numbers and is therefore generally not detected in clone libraries.

In addition to the *Haliclona* (*gellius*) sp. isolates from category 1 that share only low identity with previously cultivated bacteria, more isolates with low identity were obtained (Table 4). For example, the isolates that represent the *Betaproteobacteria* OTU2 belong to a diverse marine invertebrate-specific clade (Fig. 4A) and share only 87% identity with the nearest isolate. Also, the isolated *Deltaproteobacteria* DW06F03 shares only 86% identity with the nearest isolate.

Conclusions. Diversification of cultivation methods led to an improved cultivability of targeted bacteria (previously detected in a clone library of the sponge). A matrix of cultivation methods and mainly oligotrophic media resulted in the isolation of 10 to 14% of the bacterial diversity found in *Haliclona* (*gellius*) sp. The use of alternative cultivation methods to agar plate cultivation was particularly rewarding for the isolation of previously uncultivated species or uncultivated genera (Table 4), which underlines the importance of marine sponges for the discovery of new microorganisms. Despite the cultivation of a variety of bacterial species and genera that had not been amenable to cultivation before, the associated microorganisms that are thought to be the most important partners for the sponge were not obtained in culture. (Meta)genome sequencing of these microorganisms could overcome the knowledge gap and provide indications of the putative role of the microorganism in the sponge-microbe network and present new leads for the rational design of highly specific cultivation media and conditions.

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REFERENCES

1. **Bewley, C. A., N. D. Holland, and D. J. Faulkner.** 1996. Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. Experientia **52:**716–722.
2. **Brantley, S. E., T. F. Molinski, C. M. Preston, and E. F. DeLong.** 1995. Brominated acetylenic fatty-acids from *Xestospongia* sp., a marine sponge-bacteria association. Tetrahedron **51:**7667–7672.
3. **Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Robertson.** 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl. Environ. Microbiol. **59:**881–891.
4. **Castresana, J.** 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. **17:**540–552.
5. **Clarke, K. R., and R. M. Warwick.** 2001. Changes in marine communities: an approach to statistical analysis and interpretation. PRIMER-E, Plymouth, United Kingdom.
6. **Connon, S. A., and S. J. Giovannoni.** 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. **68:**3878–3885.
7. **Daly, J. G., and R. M. W. Stevenson.** 1985. Charcoal agar, a new growth medium for the fish disease bacterium *Renibacterium salmoninarum*. Appl. Environ. Microbiol. **50:**868–871.
8. **Davis, K. E. R., S. J. Joseph, and P. H. Janssen.** 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. **71:**826–834.
9. **De Bruyn, J. C., F. C. Boogerd, P. Bos, and J. G. Kuenen.** 1990. Floating filters, a novel technique for isolation and enumeration of fastidious, acidophilic, iron-oxidizing, autotrophic bacteria. Appl. Environ. Microbiol. **56:** 2891–2894.
10. **Eckburg, P. B., et al.** 2005. Diversity of the human intestinal microbial flora. Science **308:**1635–1638.
11. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. I. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.
12. **Faulkner, D. J., et al.** 2000. Symbiotic bacteria in sponges: sources of bioactive substances, p. 107–119. *In* N. Fusetani (ed.), Drugs from the sea. Karger, Basel, Switzerland.
13. **Felske, A., H. Rheims, A. Wolterink, E. Stackebrandt, and A. D. L. Akkermans.** 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class *Actinobacteria* in grassland soils. Microbiology **143:** 2983–2989.
14. Reference deleted.
15. **Flatt, P., et al.** 2005. Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge *Dysidea* (*Lamellodysidea*) *herbacea* and symbiotic cyanobacterium *Oscillatoria spongeliae* by CARD-FISH analysis. Mar. Biol. **147:**761–774.
16. **Friedrich, A. B., I. Fischer, P. Proksch, J. Hacker, and U. Hentschel.** 2001. Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. FEMS Microbiol. Ecol. **38:**105–113.
17. **Green, V. S., D. E. Stott, and M. Diack.** 2006. Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. Soil Biol. Biochem. **38:**693–701.
18. **Guan, L. L., H. Onuki, and K. Kamino.** 2000. Bacterial growth stimulation with exogenous siderophore and synthetic *N*-acyl homoserine lactone autoinducers under iron-limited and low-nutrient conditions. Appl. Environ. Microbiol. **66:**2797–2803.
19. **Hallam, S. J., et al.** 2006. Genomic analysis of the uncultivated marine crenarchaeote *Cenarchaeum symbiosum*. Proc. Natl. Acad. Sci. U. S. A. **103:**18296–18301.
20. **Hallam, S. J., et al.** 2006. Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analysis of marine Crenarchaeota. PLoS Biol. **4:**520–536.
21. **Hoffmann, F., et al.** 2005. An anaerobic world in sponges. Geomicrobiol. J. **22:**1–10.
22. **Hoffmann, F., et al.** 2008. Oxygen dynamics and transport in the Mediterranean sponge *Aplysina aerophoba*. Mar. Biol. **153:**1257–1264.
23. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317–2319.
24. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics **17:**754–755.

25. **Ingham, C. J., et al.** 2007. The micro-petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proc. Natl. Acad. Sci. U. S. A. **104:**18217–18222.
26. **Joseph, S. J., P. Hugenholtz, P. Sangwan, C. A. Osborne, and P. H. Janssen.** 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl. Environ. Microbiol. **69:**7210–7215.
27. **Könneke, M., et al.** 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature **437:**543–546.
28. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellows (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY.
29. **Lee, K. L., J. H. Lee, and H. K. Lee.** 2001. Microbial symbiosis in marine sponges. J. Microbiol. **39:**254–264.
30. **Ludwig, W., et al.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19:**554–568.
31. **Ludwig, W., et al.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
32. **Mincer, T. J., P. R. Jensen, C. A. Kauffman, and W. Fenical.** 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. Appl. Environ. Microbiol. **68:**5005–5011.
33. **Müller, W. E. G., et al.** 1981. Lectin, a possible basis for symbiosis between bacteria and sponges. J. Bacteriol. **145:**548–558.
34. **Muscholl-Silberhorn, A., V. Thiel, and J. F. Imhoff.** 2008. Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. Microb. Ecol. **55:**94–106.
35. **Olson, J. B., C. C. Lord, and P. J. McCarthy.** 2000. Improved recoverability of microbial colonies from marine sponge samples. Microb. Ecol. **40:**139–142.
36. **Olson, J. B. and P. J. McCarthy.** 2005. Associated bacterial communities of two deep-water sponges. Aquat. Microb. Ecol. **39:**47–55.
37. **Pol, A., et al.** 2007. Methanotrophy below pH 1 by a new Verrucomicrobia species. Nature **450:**874–878.
38. **Rappe, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni.** 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature **418:**630–633.
39. **Ronquist, F., J. P. Huelsenbeck, and O. Van der Mark.** 2005. MrBayes 3.1 manual. School of Computational Science, Florida State University, Tallahassee, FL.
40. **Santavy, D. L., P. Willenz, and R. R. Colwell.** 1990. Phenotypic study of bacteria associated with the Caribbean sclerosponge, *Ceratoporella nicholsoni*. Appl. Environ. Microbiol. **56:**1750–1762.
41. **Schoenborn, L., P. S. Yates, B. E. Grinton, P. Hugenholtz, and P. H. Janssen.** 2004. Liquid serial dilution is inferior to solid media for isolation of cultures representative of the phylum-level diversity of soil bacteria. Appl. Environ. Microbiol. **70:**4363–4366.
42. **Sipkema, D., B. Holmes, S. A. Nichols, and H. W. Blanch.** 2009. Biological characterisation of *Haliclona* (?*gellius*) sp.: sponge and associated microorganisms. Microb. Ecol. **58:**903–920.
43. **Stackebrandt, E., and J. Ebers.** 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today **33:**152–155.
44. **Thiel, V., S. C. Neulinger, T. Staufenberger, R. Schmaljohann, and J. F. Imhoff.** 2007. Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. FEMS Microbiol. Ecol. **59:**47–63.
45. **Webster, N. S., and R. T. Hill.** 2001. The culturable microbial community of the Great Barrier Reef sponge *Rhodopaloeides odorabile* is dominated by an α-proteobacterium. Mar. Biol. **138:**843–851.
46. **Webster, N. S., K. J. Wilson, L. L. Blackall, and R. T. Hill.** 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. Appl. Environ. Microbiol. **67:**434–444.
47. **Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U. S. A. **95:**6578–6583.
48. **Widdel, F., and F. Bak.** 1992. Gram negative mesophilic sulfate reducing bacteria, p. 3352–3378. *In* A. Ballows et al. (ed.), The prokaryotes. A handbook on the biology of bacteria. Springer, New York, NY.
49. **Zengler, K., et al.** 2005. High-throughput cultivation of microorganisms using microcapsules. Methods Enzymol. **397:**124–130.
50. **Zhu, P., Q. Li, and G. Wang.** 2008. Unique microbial signatures of the alien Hawaiian marine sponge *Suberites zeteki*. Microb. Ecol. **55:**406–414.