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ORIGINAL ARTICLE

Use of Real-Time qPCR to Quantify Members of the Unculturable Heterotrophic Bacterial Community in a Deep Sea Marine Sponge, *Vetulina* sp

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Abstract In this report, real-time quantitative PCR (TaqMan® qPCR) of the small subunit (SSU) 16S-like rRNA molecule, a universal phylogenetic marker, was used to quantify the relative abundance of individual bacterial members of a diverse, yet mostly unculturable, microbial community from a marine sponge. Molecular phylogenetic analyses of bacterial communities derived from Caribbean Lithistid sponges have shown a wide diversity of microbes that included at least six major subdivisions; however, very little overlap was observed between the culturable and unculturable microbial communities. Based on sequence data of three culture-independent Lithistid-derived representative bacteria, we designed probe/primer sets for TaqMan® qPCR to quantitatively characterize selected microbial residents in a Lithistid sponge, *Vetulina*, metagenome. TaqMan® assays included specificity testing, DNA limit of detection analysis, and quantification of specific microbial rRNA sequences such as *Nitrospira*-like microbes and *Actinobacteria* up to 172 million copies per microgram per Lithistid sponge metagenome. By contrast, qPCR amplification with probes designed for common previously cultured sponge-associated bacteria in the genera *Rheinheimera* and *Marinomonas* and a representative of the CFB group resulted in only minimal detection of the *Rheiheimera* in total DNA extracted from the sponge. These data verify that a large portion of the microbial community within Lithistid sponges may consist of currently unculturable microorganisms.

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Introduction

"Environmental genomics" or "molecular microbial ecology" refers to the cultivation-independent characterization of unseen microbial diversity *in situ* using modern biotechnology methods such as sequencing of cloned small subunit (SSU) rDNA or larger genomic fragments in shotgun recombinant libraries [13, 19, 43, 66]. This approach has revealed a large discrepancy between microbes that can be cultured and those that are actually present in many different habitats and environmental gradients, sometimes referred to as "the great plate count anomaly" [7, 59].

In the sea, analysis of cloned SSU rDNA libraries of coastal and pelagic marine habitats reveal that only slightly more than half of prokaryotic phyla have been successfully cultured [46]. Conversely, genera such as *Vibrio*, *Oceanospirillum*, *Roseobacter*, and *Alteromonas*, which make up the bulk of commonly cultured marine bacteria [54], appear with minimal frequency in many culture-independent environmental libraries [8, 14, 19, 46].

Marine sponges often host a rich but relatively uncharacterized "metagenome" because of the presence of a diverse microbial community that can comprise >50% of the total sponge biomass [24, 50, 51, 68, 69, 71]. The associations between microorganisms and sponges appear

to be ancient [3], although the ecological roles of the participants remain mostly unknown. Many diverse studies [light and electron microscopy, fluorescence *in situ* hybridization (FISH), culture methods] confirm the finding of a wide taxonomic diversity of microbial consortia and their likely adaptations to specific sponge host environments [10, 12, 26, 40, 41]. In common with other recent molecular studies [11, 23, 53, 68], our molecular taxonomic survey of a subset of the 17,000 heterotrophic sponge-derived microbes in the Harbor Branch Marine Microbial Culture Collection (HBMMCC) applied 16S SSU rDNA sequence analysis to the identification of bacteria [50, 54]. Recent results indicate that sponges possess diverse and often non-overlapping culturable and unculturable cohorts of heterotrophic microorganisms [17, 26, 35, 37, 50, 54].

Studies of microbial diversity lead to many interesting questions with respect to microbial ecology, distribution patterns, and potential symbioses of sponge-associated microbes: Are commonly cultured marine microbes actually rare in their source habitats and why? How common are the culture-independent rRNA sequences in any specific sponge microbial community? Furthermore, what are the relative abundances of unculturable and culturable microbes in different habitats or multicellular hosts? They are rarely studied together, but likely to be variable and dependent on many environmental factors [8, 52]. Because we now have an extensive dataset of rDNA signature sequences from both cultured HBMMCC isolates and culture-independent clones derived from diverse marine invertebrate hosts [50, 54; http://www.hboi.edu/dbmr/dbmr_hbmmd.html], some of the most intriguing questions regarding potential multiple partner symbioses can now be addressed [24].

The present study uses SSU rRNA gene sequence data and quantitative real-time PCR (TaqMan® qPCR) to provide a quantitative analysis of marine sponge microbial communities. qPCR methods were originally developed to quantify the number of specific DNA locus copies in a sample [22, 32], and have also recently been used to characterize non-cultured microbial communities [25, 33, 57, 60]. In this study, TaqMan® qPCR is applied to quantify the numbers of uncultured sponge-associated microbes and heterotrophic isolates from the Lithistid sponge genus *Vetulina* using specific probe/primer sets designed to amplify cultured and uncultured bacterial SSU rDNA sequences. Microorganism-specific TaqMan® assays were then tested for target specificity and limits of detection (LOD). The resulting assays were applied to source sponge metagenomic DNA to demonstrate the efficacy of this sensitive technique on a unique organismal model and quantitatively characterize a subset of the associated culturable and unculturable microbes in this unique microcosm.

Materials and Methods

Marine Sponge Samples, Bacterial Cultures and DNA Isolation

All sponges used in this study are members of the polyphyletic order "Lithistida" [30], with a focus on a specimen of *Vetulina* sp. collected at a depth of 212 m off of the northwest coast of Curacao. For the purposes of this research, the sponge was designated "V-1"; however, this specimen has been deposited into the Harbor Branch Oceanographic Museum with the acquisition number 003:01011. Collections were made with the HBOI Johnson Sea Link II submersible [47]. After collection, a small piece of sponge tissue was removed for microbial isolation studies, a second piece was stored in 100% ethanol as a reference specimen for subsequent taxonomic identification using microscopy and spicule analyses, and the remainder was immediately frozen at −20°C.

To isolate heterotrophic bacterial cultures, a small section of sponge tissue including both the pinacoderm and mesohyl regions was gently rinsed with sterile artificial seawater (ASW, Kester–Sieburth formulation) [56], cut into smaller pieces and homogenized in 20 ml of sterile ASW using an ethanol sterilized Virtis grinder. Samples were serially diluted and plated onto either low nutrient LN, [Bacto Peptone (Difco), 0.5 g; yeast extract (Difco), 0.5 g; Bacto-agar (Difco), 16 g; 75% (*v*/*v*) ASW, 1 l] or Marine Agar 2216 (Difco) media and incubated at ambient temperature (approximately 22–25°C) for 2 to 4 weeks. After this time, discrete colonies were transferred to fresh plates of the isolation medium, incubated, and then repurified until the isolate was axenic. Isolates were then transferred to Marine Agar 2216 and maintained on this medium.

DNA extraction of microbial isolates was performed as previously described [54], whereas total sponge metagenomic DNA was isolated from 1–5 g of frozen sponge sample using a modified rigorous guanidine thiocyanate procedure, which included pulverizing sponge mesohyl in liquid nitrogen [35]. Integrity of high molecular weight DNA was verified by gel electrophoresis.

All culture-independent SSU rRNA gene clone names are designated with "V-1" (for *Vetulina*) representing the host sponge source that precedes a numeric designation (229 or 289) for the specific library, followed by a letter for each individual clone.

Sequence Analysis of SSU rRNA of Microbial Isolates

To allow the selection of bacterial 16S SSU sequences for the TaqMan® assays, molecular sequence and phylogenetic analyses were performed. As in previous studies [35, 54],

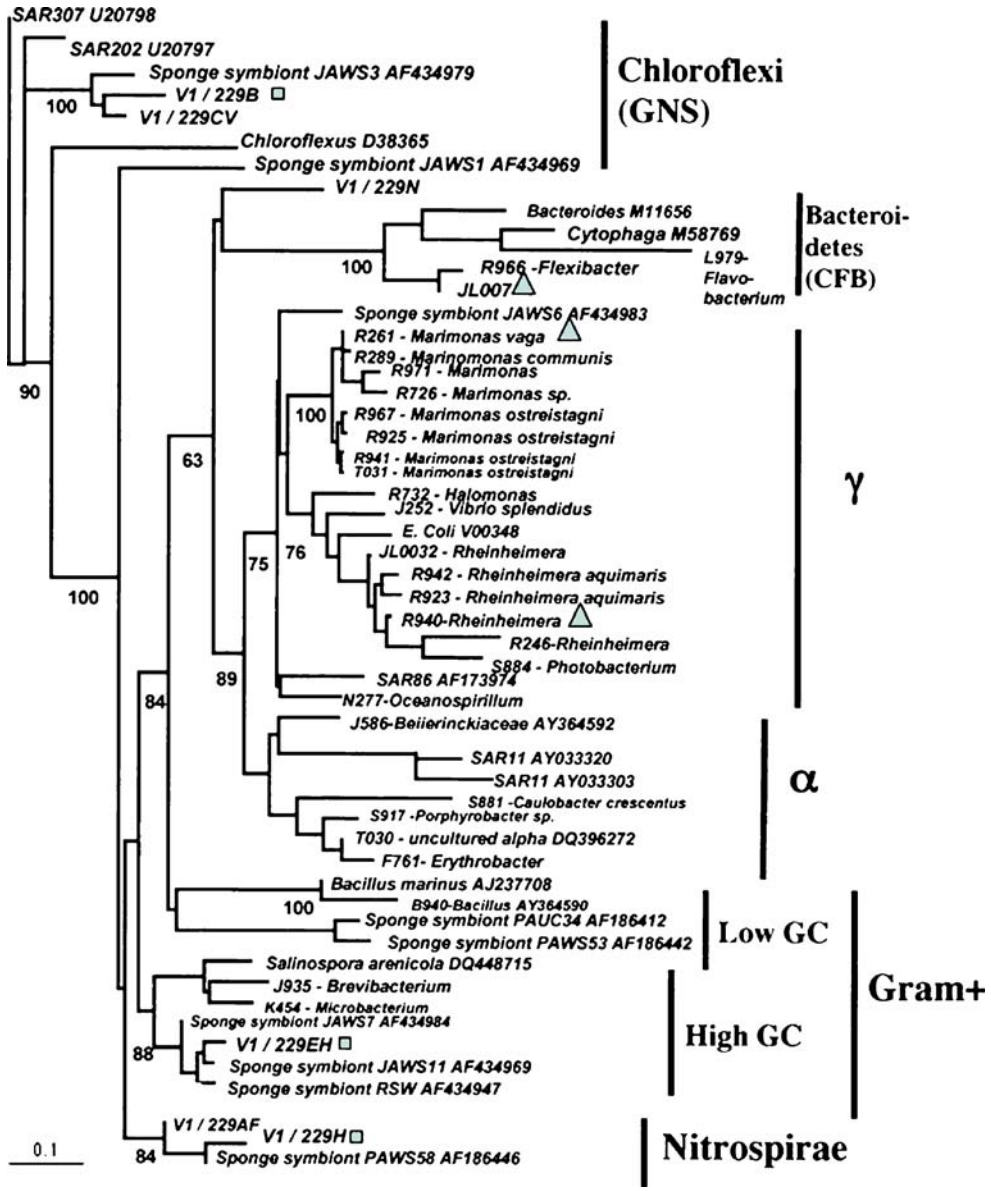

Figure 1 Neighbor joining phylogeny based on 16S SSU rRNA gene sequence sequences of sponge (mostly Lithistid)-derived eubacteria. Other marine HBMMCC microbes are included as references [17, 54]. Based on the given sequence data, MODELTEST chose the Tamura–Nei model [45] for correcting evolutionary distances, since the model takes into account excess transitions, unequal nucleotide frequencies, and variation of substitution rate among different sites, with the following parameters: Proportion of invariable sites (I) = 0.1050 and variable sites (G) Gamma distribution shape parameter = 0.6447. Bootstrap percentages for 500 replicates are shown below the appropriate nodes. Brackets indicate major eubacterial subdivisions. Greek α and γ symbols denote alpha- and gammaproteobacteria groups, respectively. "CFB" clade is synonymous with the Bacteroidetes, and the Chloroflexi are synonymous with GNS, which stands for "green non-sulfur" bacteria. Triangles and squares indicate culture isolate and culture-independent rDNA sequences, respectively, which were chosen for TaqMan® assays. Taxa followed by GenBank accession numbers were used as reference or outgroup sequences

the universal bacterial primers Ecoli9 [5′ GAG TTT GAT CAT GGC TCAG 3′] and Loop27rc [5′ GAC TAC CAG GGT ATC TAA TC 3′] were used to amplify about 800 bp of the 5′ end of the SSU rRNA gene. Standard PCR conditions of 94°C denaturation for 45 s, 53°C annealing for 60 s, and 72°C extension for 60 s repeated for 30 cycles were applied. Primer Eco9 is virtually equivalent to the 27F primer of Lane [34], as both sequences overlap slightly and have been shown to amplify a wide phylogenetic diversity of bacteria including *Cyanobacteria*, *Spirochetes*, *Deltaproteobacteria*, and *Planctomycetes* from other sponges in the Harbor Branch Collection [35, 50; Lopez et al., unpublished data].

Positive SSU PCR products from the metagenomic DNA template were cloned into the Topo TA cloning vector pCRII (Invitrogen) to isolate single SSU gene fragments for

Table 1 PCR primers and labeled probes used for specific TaqMan® assays

Target isolate or SSU clone	PCR primer pair (5′ - 3′)[a]	TaqMan® probe sequence	Expected amplicon size
V-1/229B	F- CGAAGGGGCTTCGGCC Rc- AACCTTTCCTCTCTACGTCGTAACTTAGA	TGAGTAACACGTAAGTGATCTGCC CCGG	139
V-1/229H	F- CGAGAGGCTCTTCGGAGTAGTACA GT Rc- CCGCATCCACGATGCAA	CCATACCCTTCCGGTCCTTCGGG	153
V-1/229 EH	F - TCTGGTCGAGTGGCGAACG Rc - CGGAGCGTCGGAGCCTT	TTGGCTAATACCGGATGCCCTCAG ATG	136
R261	F- AACGCGTAGGAATCTGCCTAGTAG Rc- AATCTCACGCAGGCTCATCTAATAG	AACGCATGCTAATACCGCATACGC CCTA	126
R940	F - TATAGGGAGCTGCCCGACAGA Rc -TAATCCCATATAGGTGCATCCGA	AGTTGGAAACGACTGTTAATACCG CATAATGTCTACG	123
JL007	F - CAAGTCGAACGGTAGACTACTTTCG Rc- TTGTACCGATAAATCTTTAATTAC CTTCAGA	ACGCGTATGCAACCTACCTTGTAC AGGG	164

[a] Forward (F) and Reverse complement (Rc) primers are shown

DNA sequencing. Using the above primers, PCR products derived from isolates were directly sequenced with an Applied Biosystems (ABI) "big-dye" cycle sequencing kit and automated ABI DNA sequencers at the Interdisciplinary Center for Biotechnology Research (University of Florida, Gainesville, FL).

New SSU rRNA gene sequences were queried against the Ribosomal Database Project (RDP) II or GenBank using BLASTN [1, 5; http://www.ncbi.nlm.nih.gov/blast/. Potential PCR chimera formation in clones was determined by both CHIMERA CHECK [5] and verifying the sequence similarity at the terminal ends in each clone. Novel Lithistid-derived microbe SSU gene sequences from HBMMCC isolates and clones have been deposited into GenBank with the following Accession numbers: DQ869279–DQ869304, and EF450317–EF450324.

Molecular Phylogenetic Analyses

Reference 16S rDNA sequences were retrieved and compared to data from GenBank release 154 (June 2006), which includes other common and previously determined "sponge symbiont" 16S rDNA sequences to identify major bacterial clades. These sequences with their accompanying accession numbers are included in phylogenetic reconstructions (Fig. 1). After using secondary structure models as a guide, highly gapped poorly aligned regions were removed before tree reconstructions. SSU rRNA sequence alignments were made with CLUSTAL X [64] and are available from the authors upon request. Phylogenetic reconstructions employed either distance, likelihood or parsimony algorithms using PAUP * version 4.0b10 [62]. However, because of the large genetic distances often involved in the SSU datasets, phenetic distances with the neighbor-joining algorithm were typically employed for phylogenetic reconstructions. MODELTEST was used to determine optimal DNA substitution models [45].

TaqMan® Assay Design and Specificity Testing

To develop specific probe/primer sets, 16S rRNA gene sequences were derived from three cultured (JL007/R966, R940, and R261) and three culture-independent Lithistid-derived microbial representatives (229B, 229EH, 229H), respectively. All were subsequently aligned with each other using Lasergene software. Isolate JL007 could not be revived after assays began, and so isolate R966 was used in its place as a closely related bacterium that was isolated from the same sponge sample. Primers and probes were manually designed to hyper-variable regions observed in alignments (Table 1). Resulting probe/primer sequences were BLAST-searched to verify specificity. To confirm specificity experimentally, each developed assay was tested against a panel of culturable, unculturable, and sponge metagenomic DNA. This panel included the three cultured targets (R940, R261, and R966) plus three randomly selected isolates from the HBMMCC (R246, R726, and R732). R246 was an additional isolate from *Vetulina* V-1; R726 and R732 were isolated from a *Corallistes* sp. collected at a depth of 280 m off of the south central coast of Curacao. Cultured organism DNA was assayed using TaqMan® templates consisting of purified genomic material, whereas for uncultured microbial DNA, templates consisted of quantified plasmids containing cloned 16S rRNA gene target sequences.

For TaqMan® PCR, duplicate 50 µl reactions consisted of 1× Universal Master Mix (ABI), 400 nM target-specific primers, 100 nM 5′ 6Fam and 3′ end Tamra-labeled target-specific probe (ABI), 2.5 units Platinum Taq polymerase (Invitrogen), and 10 µl of template DNA. This DNA was

present in either 10^6 copies for plasmids or 5 ng for genomic DNA. All DNA (plasmid and genomic) was quantified using PicoGreen and a spectrofluorimeter (Spectramax Gemini XS), and plasmid copies were determined based upon molecular weight. All DNA dilutions were carried out in molecular grade water. PCR reactions were spiked with a synthetic internal positive control (IPC) DNA with IPC-specific primers and a HEX-labeled probe for detection. Inhibition is often found in environmental samples; thus, the IPC is used as a positive control to monitor potential inhibition.

PCR was performed in a Bio-Rad iCycler capable of four-color fluorescence detection. The following cycling conditions were used: one cycle at 50°C for 2 min for UNG nuclease digestion; one cycle at 94°C for 2 min to activate hot-start polymerase; and 50 cycles consisting of 94°C for 15 s and 60°C for 1 min. UNG nuclease is present in the Universal Master Mix (ABI) and degrades previously amplified DNA (due to uracil incorporation), thus, preventing sample contamination from previous amplifications.

Amplification detection is expressed based upon threshold cycle (Ct) values [22]. A corresponding baseline ensures that all samples are compared equally with respect to the Ct values generated. Thus, lower Ct values represent faster entry into exponential amplification and a correspondingly higher level of template.

Table 2 Lithistid-derived microbial 16S rRNA sequences for TaqMan® analysis

SSU rRNA source	Nearest GenBank match/Accession No./ % identity to match	GenBank No.	Major Bacterial Subdivision or Phylum
Vetulina Cultured Isolates			
R246	*Rheinheimera* sp. 3006 / AM110966.1 / 97	AY36 8567	*Gammaproteobacteria*
R966	*Marinicola seohaensis / 95*	DQ86 9301	*Bacteroidetes*
R940[a]	*Rheinheimera baltica* / AJ441082 / 97	DQ86 9300	*Gammaproteobacteria*
R261[a]	*Marinomonas communis* / DQ011528.1 / 99	AY36 8567	*Gammaproteobacteria*
R289	*Marinomonas communis* strain LMG 2864 / DQ011528.1 / 99	EF45 0317	*Gammaproteobacteria*
R923	*Rheinheimera aquimaris* strain SW- 369 / EF076758.1 / 98	EF45 0318	*Gammaproteobacteria*
R925	*Marinomonas ostreistagni* / AB242868.1/ 97	EF45 0319	*Gammaproteobacteria*
R941	*Marinomonas ostreistagni* / AB242868.1 97	EF45 0320	*Gammaproteobacteria*
R942	*Rheinheimera aquimaris* strain SW-369 / EF076758.1 / 98	EF45 0321	*Gammaproteobacteria*
R965[c]	*Vibrio* sp. 1		*Gammaproteobacteria*
R966	*Marinicola seohaensis* / AY739663 / 100	AY37 1413	*Bacteriodetes*
R967	*Marinomonas ostreistagni* / AB242868.1/ 97	EF45 0322	*Gammaproteobacteria*
S711	*Marinomonas vaga*		*Gammaproteobacteria*
T030	Uncultured organism clone ctg-NISA106 / DQ396272.1/ 100	EF45 0323	*Alphaproteobacteria*
T031	*Marinomonas ostreistagni* / AB242868.1 97	EF45 0324	*Gammaproteobacteria*
JL007[a]	Marine bacterium HP11 / AY241557 / 95	DQ86 9296	*Bacteroidetes*
Cultured Isolates from other sources			
R726	*Marinomonas communis* / DQ011528.1 / 98	DQ86 9298	*Gammaproteobacteria*
R732	*Halomonas* sp. SW1.1A AY536541.1 / 99	DQ86 9299	*Gammaproteobacteria*
Uncultured clones			
V-1/229B[b]	Uncultured *Chloroflexi* clone Dd-spT- 92/ AY897076.1 / 96	DQ86 9283	*Chloroflexi*
V-1/229EH[b]	Uncultured *Actinobacterium* clone XA4A05/ AY954063.1 / 96	DQ86 9282	*Actinobacteria*
V-1/229H[b]	Uncultured *Nitrospirae* bacterium clone S.Ionian-H02/ AY534032 / 97	DQ86 9281	*Nitrospirae*
V-1/229N	Uncultured sponge symbiont JAWS10/AF434968.1 / 98	DQ86 9280	unknown
V-1/229AF	Uncultured *Nitrospira* sp. Clone/ AY711669.1 / 90	DQ86 9285	*Nitrospirae*

[a] SSU rDNA sequence from cultured isolate used for TaqMan probe/primer design as described in text
[b] culture-independent SSU rDNA sequence used for TaqMan probe/primer design as described in text (see Table 1)
[c] identity of this isolate was based on RFLP profiling (Sfanos et al, 2005) and not direct sequencing

Limit of Detection Studies

Template DNA samples were quantified by PicoGreen, and copy numbers were estimated based upon molecular weight of the template. Template DNA was diluted in molecular grade water to obtain 10^2 to 10^6 DNA copies per 50 μl PCR reaction. The PCR reactions were prepared as previously described. For all samples, TaqMan® PCR was conducted in duplicate and included appropriate no-template controls to monitor sample contamination.

Quantification of Target DNA Sequences in Sponge Metagenome

For quantification, a standard curve of target DNA was prepared from 10^2 to 10^6 copies per 50 μl PCR reaction. Five nanograms of sponge metagenome DNA was individually assayed with the 229B, 229EH, 229H, JL007, R940, and R261 probe/primer sets. PCR reactions were prepared as described previously. Standard curve samples were quantitatively defined in the software setup. TaqMan® PCR was conducted as described previously, including both duplicate experimental sponge metagenome samples and the comparative standard curve. Estimated copy numbers of target sequence were automatically calculated by the PCR software (iCycler Bio-Rad) based upon the resulting standard curve amplification.

Results

SSU rRNA Sequence Analysis

The HBMMCC microbial inventory [54] characterized many (n=359) culturable bacteria from Lithistid sponge specimens. The growth conditions selected for this study allowed the recovery of 16 isolates from *Vetulina* V-1, selected based on colony morphology and subsequently identified by 16S rRNA gene sequencing. Of these, seven have been identified as *Marinomonas* spp and four as *Rheinheimera* spp. (Table 2). Most of the culture-independent SSU rRNA gene sequences included in this paper remain part of a larger phylogenetic analysis (Lopez et al., unpublished data), and therefore, detailed treatment of the microbial systematics is not presented here. Nonetheless, phylogenetic analysis identified unique 16S SSU rRNA gene sequences that either (1) appeared in different microbial groups or clades or (2) were distantly related among all Lithistid-derived sequences. For example, despite being in the same green non-sulfur grouping (with 100% bootstrap support), V-1/229CV and V-1/229B clones shared only 89% rDNA sequence similarity with each other and about 84% similarity with their closest neighbor, sponge symbiont JAWS3. V-1/229N appeared similar to another previously identified sponge symbiont, JAWS10 [23]. Molecular phylogenetic analyses facilitated the selection of unique cultured isolates for TaqMan® targets and specificity testing. Bootstrap support for the major clades in the neighbor-joining tree of Fig. 1 was fairly high (~70%), whereas the branching patterns confirm the wide taxonomic spectrum of heterotrophic bacteria coexisting within these sponges, which echoes previous studies [24, 35]. The relatively long branch lengths of the Bacteroidetes (CFB) clade led us to choose isolate R966 as the basis for one TaqMan® probe, whereas both cultured *Gammaproteobacterium* sequences, R940 and R261, still had a high uncorrected genetic divergence between them of 14.7%. These isolates also represented a large proportion of the readily cultured bacteria from this sponge.

The *Vetulina* 16S rDNA clones selected as TaqMan® probe targets have large sequence divergences both to each other and to current cultured bacterial database entries, and have higher similarity (95–97%) only to "unclassified" or "uncultured" environmental sequences (Table 2). For example, after BLAST queries, clone 229EH had only 95% similarity to an uncultured gram-positive/high GC bacterium (*Actinobacteria*). Clone 229H had slightly higher identity (97%) to a *Nitrospirales* bacterium. Interestingly, no V-1 uncultured clones grouped within the commonly cultured *Alphaproteobacteria*. The majority of the culture-independent clones appear highly divergent and within basal clades such as green non-sulfur or uncultured *Actinobacteria* [18]. Some previously determined 16S rDNA sequences, such as SAR11 and SAR202, ubiquitous in the marine environment (e.g., water column, adjacent sediments, marine snow) were included as reference

Table 3 Specificity Testing of Six TaqMan Assays

TaqMan¨ Assay						
DNA Template	229B	229EH	229H	JL007	R940	R261
V-1/229B	**25.2**	N*	N	N	N	N
V-1/229EH	N	**25.9**	N	N	N	N
V-1/229H	N	N	**25.3**	N	N	N
JL007	N	N	N	**23.0**	N	N
R940	N	N	N	N	**29.9**	N
R261	N	N	N	N	N	**22.3**
R246	N	N	N	N	**26.8**	N
R726	N	N	N	N	N	**22.5**
R966	N	N	N	**21.5**	N	N
R732	N	N	N	N	N	N
Sponge V-1	**29.8**	**26.7**	**29.5**	N	**35.7**	N
Blank	N	N	N	N	N	N

Results are mean Ct values (n=2 reactions). Headers for each column denote the specific primer/probe used in the TaqMan® Assays as listed in Table 1, with the specific DNA template shown in each row
*N= No amplification

sequences [7, 15, 38, 46] and also appear distinct from both the unculturable and culturable Lithistid-derived bacteria. The low sequence similarity of the Lithistid 16S rDNA clones with previously characterized marine environmental sequences strongly suggested that many are sponge-specific microbial associates and are not native to other environments such as the water column (also see [10]). Overall, the phylogenetic reconstructions reveal a pattern where culture-independent 16S rDNA clones appear distinct from cultured HBMMCC isolate rRNA sequences [54].

Specificity Testing

To assess specificity, designed probe/primer sets were tested on the DNA for each cultured and uncultured microorganism. As shown in Table 3, each of the probe/primer sets reacted well with its intended target in the six primary assays. In addition, none of the six assays cross-reacted with each other's target. Because microbial DNA from the sponge metagenome contains a broad range of uncharacterized organisms, these assays may still recognize closely related organisms. The low Ct values (<30) for each of the tests suggest that the probe/primer sets worked well (e.g., with 10^6 copies plasmid DNA, 5 ng genomic DNA). Table 3 also shows that the three TaqMan® assays for uncultured organisms produced a positive signal with original source Lithistid sponge genomic DNA as the template. Conversely, only one of the assays for cultured organisms produced a positive signal (that for isolate R940 sequences) with a high Ct value when using total sponge genomic DNA as the template. Sequencing of each of the amplicons verified that they matched the original target sequence (data not shown).

There were three instances where the cultured isolate assays detected a signal from another DNA source. As expected, the JL007 assay produced a signal with the R966 because their rRNA sequences show close (97.4%) sequence similarity. Similarly, both R246 and R940 templates are detected by the R490 probe as both resembling *Rheinheimera* spp (Table 2), and R726 and R261 templates both have similarity to *Marinomonas communis* as detected by the R261 probe (Tables 1 and 2). Furthermore, in all three cases, assay cross-reactivities are supported by the close relationship of each pair in the phylogenetic tree (Fig. 1). Thus, in each case where sequence information is available, cross-reactivity was limited to instances where sequences are identical or highly similar to the target sequence.

Limits of Detection

Preliminary limits of detection studies of the six assays were performed using cloned plasmid sequences (uncultured) or 16S rDNA PCR amplicons (cultured organisms). DNA samples were quantified by picogreen and copy numbers estimated based on molecular weight. A lower limit of 100 copies was reliably detected for all assays, except 229H and 229EH sequences where the lower limit increased to 1,000 copies. As these levels represent appreciable detection sensitivity, the lack of detection of the cultured sequences in the sponge sample is unlikely to be the result of insensitivity.

Quantification Studies

Standard curves of starting quantity vs Ct were used to estimate starting target DNA quantities in unknown samples. Therefore, sponge metagenomic DNA (5 ng) was assayed using the three TaqMan® assays for uncultured organisms previously shown to be present in the metagenome DNA. Duplicate PCR reactions were performed as described above.

As can be seen in Table 4, each of the three unculturable target organism rDNA sequences was detected in *Vetulina* sponge metagenome DNA samples. The estimated quanti-

Table 4 Quantification of Uncultured Target Organisms in *Vetulina* Sponge Metagenome DNA

TaqMan® Assay

	229B		229H		229EH	
Sample	Mean Ct	Est Copy #	Mean Ct	Est Copy #	Mean Ct Est	Copy #
Blank	NA	0	NA	0	NA	0
Positive 1E6 copies	23.0	960,000	23.7	670,000	22.7	920,000
Positive 1E3 copies	34.1	910	37.6	500	33.0	870
Sponge DNA (5 ng)	26.0	150,000	24.5	454,000	22.8	860,000
Extrapolated copy # per µg Sponge DNA		30,000,000		90,800,000		172,000,000
*Correl. Coeff.**		*0.999*		*0.972*		*0.999*

**Standard curve consisted of 4 levels ranging from 1E3 to 1E6 copies per reaction*

ties range from 150,000 to 860,000 copies per sample. In contrast, of the three culturable organisms from the sponge samples, only R940 was detected (Ct=35.7) at a trace level, suggesting presence of very low levels of this microbe compared to the unculturable organisms.

Lower Ct values correlate with the increasing copy number of template DNA compared with the other assays. Within a specific assay, a three-cycle difference between Ct values is roughly equivalent to a tenfold difference in the quantity of DNA present. For each of the uncultured organisms, the number of genome copies ranged from 3×10^7 to 1.72×10^8 copies per microgram of sponge DNA, indicating a relatively large population of these uncultured microbes.

Discussion

Prokaryotes are extremely versatile and can colonize virtually any available habitat [2, 20, 65] For example, Delong [6] has described disparate taxonomic and functional differences between free-living and aggregate (marine snow) bacterial populations. Several other studies of 16S rRNA gene libraries indicate that bacterioplankton profiles vary with depth [8, 14, 54].

The mesohyl of marine sponges represents another unique niche to which microorganisms adapt [24, 26, 27, 69, 70], and may in fact be a key factor in determining diversity. Sponge-associated microbial community profiles are expected to vary according to pertinent abiotic (depth, latitude) and biotic (host sponge, secondary metabolites) conditions [16, 54] (Lopez et al., unpublished data). Microbial adaptation to specific sponge mesohyl microenvironments [28, 29, 31] may explain (1) the low sequence similarity to previously identified 16S rRNA gene clones from the surrounding environment and cultured isolates and (2) the general difficulty of culturing sponge-specific symbionts [21, 39–41]. However, the inability to culture the vast majority (~99.0%) of microbial diversity in nature has long been recognized [4, 15], and thus, is not unique to sponge-associated microbial communities.

The Lithistid-derived 16S rRNA gene clones used in this study probably represent unique or distantly related microbial taxa that span the diversity of the sponge-associated microbial community (Fig. 1). This is a small part of a larger study that is defining the microbial population of these Lithistid sponges that will be published separately. By focusing on a few diverse clones, we were able to show that qPCR is able to quantify the presence of three uncultivated microbes within the sponge mesohyl and revealed that these microbes are present as large populations with a 5.7-fold difference between the most common (229EH) and least common (229B) clone.

Specificity testing and limits of detection studies showed that positive qPCR amplification was specific to target DNA template and could detect as low as 100 to 1,000 copies of template; however, several isolates that had been cultured from these sponges could not be detected. Because sponge samples spiked with target DNA showed detection (as positive controls), the PCR was not inhibited by any specific factors in the samples. *M. communis* (R261, R726) represents about 0.48% of all isolates in our culture collection and was a common isolate from *Vetulina* V-1. In addition, *Marinomonas* spp. do appear to be relatively common as marine cultures [36, 72]. *Rheinheimera*-like bacteria (R246, R940) have also been isolated from several oceans, and both occur in other Lithistid sponges [54]. The poor amplification of a culturable subpopulation suggests that such organisms are present in very low numbers or are transient within the microbial community, possibly as a result of the copious volume of water filtered daily by sponges [9, 42, 48].

Our results add to the consensus from accumulating environmental genetics studies of various habitats that the majority of 16S SSU rRNA gene sequences from these environmental habitats do not match those of previously cultured species [8, 14, 19, 59]. It is also generally acknowledged that relative abundances of these "unculturable" taxa may not be adequately inferred from simple proportions within rDNA clone libraries as a result of inherent biases in DNA extraction or PCR methods [61]. However, because characterization of larger DNA fragments using shotgun metagenomics methods is still costly and labor-intensive [8, 67], the TaqMan® assay represents a rapid method for quantifying relative abundances of individual members of diverse microbial consortia [59]. Using this technology, we were able to support previous findings [23, 27, 34, 35] that the majority of DNA sequences identified in sponge metagenomes represent uncultured organisms, and we have extended this information by showing that large populations of these microorganisms are present within the community.

While this study demonstrated the versatility of quantitative PCR, TaqMan® assays can be further optimized to provide additional sensitivity through small changes in probe/primer concentration and annealing time. For example, some cross-reactivity was observed within the assays (Table 3), but is expected in complex microbial communities which may have several closely related strains. This result can be avoided in the future by probing alternative more divergent gene sequences such as ribosomal or heat shock proteins [73] that will result from wider sampling of genomic sequences

With the use of TaqMan® PCR for the analysis of sponge metagenome DNA, this study demonstrates the ability to use molecular data to extrapolate potential

population dynamics within the sponge community [49]. The quantitative nature of the analysis shows that the uncultured organisms tested by this method are present in marked levels within the sponge. In addition to qPCR methods, quantitative assessment of culturable and unculturable microbial communities has advanced with FISH surveys of sponges and other invertebrates. Recently, FISH has been used to demonstrate the localization of bacterial clades within sponge mesohyl and embryonic tissue [55] and to show differences in bacterial communities along a spatial gradient in Antarctic soft corals [69]. The FISH method is preferred for visualizing spatial location of specific microbes, whereas qPCR remains more precise in its quantification of target molecules.

This study also opens the door to applying transcriptome-based analyses to complex microbial communities. TaqMan® qPCR can be used to specifically target and quantify not only the presence of unculturable microorganisms but also specific gene expression within microorganisms and macroorganismal hosts. Characterization of specific gene expression within the unculturable population could demonstrate the interplay of microbes, their chemical signals [44, 63], and the role of unusual but potent secondary metabolites possibly used in microbe–microbe or microbe–sponge symbiotic relationships [53]. More studies beyond the scope of this paper are required to determine the answers pertaining to the precise classification, spatial distribution, and the ecological roles of bona fide microbial symbionts with their sponge hosts and how each adapts to the presence of other microbes within the mesohyl [23, 27].

After the microbial taxonomic inventory of our Lithistid sponges, TaqMan® PCR demonstrates a quantitative characterization of a subpopulation of unculturable microbes within a complex symbiotic microcosm, the marine sponge. The qPCR results also strongly suggest that uncultured bacteria, previously identified solely by SSU rDNA methods, comprise a dominant portion of the microbial community of these Lithistid sponges' mesohyl. Together with other emerging biotechnologies, such as more rapid DNA sequencing methods of genomes and metagenomes [58], and coordinated efforts to inventory marine life (e.g ICoMM—the International Census of Marine Microbes http://www.icomm.mbl.edu), qPCR assays will continue to contribute to the understanding of marine microbial diversity and biogeochemical processes in various environments.

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