RESEARCH ARTICLE

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Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida)

Abstract Marine sponges harbor dense and highly diverse bacterial communities, and some percentage of the microflora appears to be specialized for the sponge habitat. Bacterial diversity was examined in *Chondrilla nucula* Schmidt to test the hypothesis that some subset of sponge symbiont communities is highly similar regardless of the species of host or habitat requirements of the host. *C. nucula* was collected from a mangrove channel on Lower Matcumbe Key in the Florida Keys (25°53′N; 80°42′W) in August 1999. Domain-specific universal bacterial primers were used to amplify the 16S rDNA gene from genomic DNA that had been extracted from sponges and the surrounding water. An RFLP technique was used to assess diversity of sponge-associated and environmental bacterial communities. The clone library from *C. nucula* contained 21 operational taxonomic units (OTUs). None of the 53 OTUs from adjacent water samples were found in the *C. nucula* library indicating that a distinct community was present in the sponge. Sequence analysis indicated that *C. nucula* harbors a microbial community as diverse as the microbes from other sponges in different habitats around the world. Phylogenetic analysis placed several *C. nucula* clones in clades dominated by bacteria that appear to be sponge specialists (e.g., Acidobacteria, Bacteroidetes, and Cyanobacteria). Proportional representation of major bacterial taxonomic groups represented in symbiont communities was compared as a function of geographic location of sponge hosts. This study supports the hypothesis that sponges from different oceans existing in dissimilar habitats harbor closely related bacteria that are distinct from other bacterial lineages and appear specialized for residing within sponges.

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Introduction

Understanding the natural world depends in part on being able to identify the species present on the planet. During attempts to survey species diversity, it is important to examine microbial communities associated with host organisms because the symbiotic habitat may contain a major portion of prokaryotic diversity (e.g., Bull et al. 1992; Amann et al. 1995). Here, we use 'symbiosis' in a strict ecological sense whereby a close relationship between individuals of two or more species exists, but assume nothing regarding the nature of the relationship (e.g., mutualism, commensalism). Among eukaryotes harboring bacterial symbionts, many marine sponges have extraordinarily diverse microbial communities in their tissues. This diversity may in part be explained by the varying physical, chemical, and biological conditions within the sponge host, which may affect microbial ecology (e.g., number of species supported in the system; relative abundance) and evolution (e.g., specialization through niche partitioning). Despite the evolutionary and ecological significance of these associations, we know relatively little about how they are structured or how they function. Determining how these associations originated and are maintained will broaden our understanding of microbial–host interactions.

Prokaryotic sponge symbionts occur both intra- and extracellularly (e.g., Vacelet 1975; Wilkinson 1978). Early work explored symbiont communities using traditional microbiological techniques on culturable symbionts (e.g., phenotypic assays) and/or microscopic examination of sponge tissue. These studies found that microbes can occupy up to 50% of the sponge volume (Vacelet 1975; Wilkinson 1978; Santavy et al. 1990). Furthermore, the microbial community appears to be

sponge-specific and distinct from the surrounding bacterioplankton (e.g., Wilkinson et al. 1981; Santavy and Colwell 1990; Santavy et al. 1990). However, culture-based techniques uncovered only 3–11% (or less) of the total number of species occupying sponge hosts (Santavy et al. 1990).

Within the last 5 years, many studies have explored sponge bacterial symbiont diversity using culture-independent approaches afforded by molecular techniques (e.g., Lee et al. 2001; Hentschel et al. 2003; Hill 2004). Detailed symbiont surveys have been conducted in a number of ecosystems including: Antarctica; Great Barrier Reef and Botany Bay, Australia; Santa Barbara, California; Mediterranean; Western Caroline Islands, Palau; and deep-sea habitats (Hentschel et al. 2003; Hill 2004; Webster et al. 2004; Olson and McCarthy 2005). Several studies have found that the sponge bacterial symbioses are stable across spatial and temporal scales (e.g., Friedrich et al. 2001; Hentschel et al. 2002; Webster et al. 2004) but others have challenged this hypothesis. For example, although *Cymbastela concentrica* exhibited limited, short-term changes in microbial communities over small geographic distances, the microbial community present in temperate *Cymbastela concentrica* was distinct from the community existing in *Cymbastela concentrica* from tropical waters of Australia (Taylor et al. 2004, 2005). These differences highlight the need for additional work in the analysis of sponge-associated microbial diversity.

To date, surveys of sponge microbial symbionts have provided surprising results and generated a number of important questions. For instance, several sponge species from the Mediterranean and Caribbean appear to harbor a previously unknown, sponge-associated microbial lineage, tentatively defined as the Phylum Poribacteria (Fieseler et al. 2004). It appears likely that host-symbiont specificity in these relationships is high. Another intriguing hypothesis proposed by Hentschel et al. (2002) is that sponges harbor uniform microbial communities (but see Taylor et al. 2004). Support for this hypothesis has come from a number of studies. Most recently, lithistid sponges living in deep-sea habitats were found to harbor symbionts also found in shallow water lithistid and verongid sponges from the Pacific and Mediterranean (Olson and McCarthy 2005). Thus, sponges belonging to different orders, occupying highly dissimilar habitats, harbor similar members in their microbial communities.

Microbial communities harbored by Caribbean sponges remain poorly studied (but see Fieseler et al. 2004). Our goal was to begin to describe some of the symbionts found in the common sponge *C. nucula* from the Florida Keys, Florida. Specifically, we were interested in testing the hypothesis that sponges harbor a uniform microbial community (sensu Hentschel et al. 2002) by determining whether a sponge common in the tropical western Atlantic harbored symbionts found in sponges in other oceans (e.g., the Indo-Pacific and

Mediterranean). It is important to note that *C. nucula* represents a species complex (Klautau et al. 1999), and the species designations for the various populations are still under consideration. The precise phylogenetic or taxonomic position of *C. nucula* populations is not currently known (Klautau et al. 1999), and given that species identification of the sponge host was not carried out in this study, it is likely that the *C. nucula* from the Caribbean region will be assigned to a new species. In the Florida Keys, this population of *C. nucula* is an important competitor for space on coral reefs (Hill 1998) and releases nitrogen into reef ecosystems (Corredor et al. 1988). Identifying members of the bacterial symbiont community may provide insights into microbial contributions to sponge growth and metabolic capabilities, which may have broader ecological consequences for coral reef structure.

Materials and methods

Sample collection

Chondrilla nucula Schmidt was collected in August 1999 at a depth of < 3 m from a mangrove channel in Lower Matcumbe Key in the Florida Keys (25°53′N; 80°42′W). Sponges that were > 5 m apart were collected and were shipped overnight to Fairfield University. On the same date, a single seawater sample (1 l) was collected adjacent to the sponges and shipped (overnight) to Fairfield. Upon arrival, sponges were rinsed twice in sterile artificial seawater, and 50 mm^3 sections were taken from just below the pinacoderm into the choanoderm with a sterile scalpel. Seawater was also vacuum filtered (0.2 μm Nalgene$^®$ filter) at this time. Material was stored at −80°C prior to DNA extraction.

Construction of 16S rDNA libraries and sequence acquisition

Total DNA was extracted from sponge tissue, and the filtered biomass from the seawater sample, using a modified cell-lysis method using lysozyme, SDS, and phenol-chloroform extraction as previously described (Tsai and Olson 1991). Primers fD1 (5′-ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-cccgggatccaagcttAAGGAGGTGATCCAGCC-3′) for the domain Bacteria (Weisburg et al. 1991) were used to amplify near full-length (≈1,500 bp) 16S rDNA sequences from *C. nucula* community DNA. Lower case primer sequences represent restriction sites, which were not used for cloning in this study. The amplified products from three individual sponges were pooled and then cloned into the pCR$^®$2.1 TOPO vector (Invitrogen). The seawater library was constructed in the same manner, but was based on a single sample of water. One hundred colonies were picked for each library.

Clone inserts were purified using the QIAquick Gel Extraction kit (Qiagen) and analyzed using amplified ribosomal DNA restriction analysis (ARDRA). Clone inserts were cut with *Hpa* II and *Hha* I and digestion products were run on molecular screening grade agarose (Roche™). Agarose gels were analyzed visually. Of the 100 clones that were picked, we were able to analyze 78 and 91 clones by ARDRA for the *C. nucula* and ambient seawater libraries, respectively. Twenty-one of the *C. nucula* clones that had ARDRA profiles that were distinct from clones isolated from the water column were sequenced using the Beckman Coulter CEQ™ DTCS kit with the CEQ8000 DNA Analysis System, or the ABI Big Dye™ Terminator Cycle Sequencing kit and ABI DNA sequencer (Perkin Elmer™). The resulting 21 sequences ranged in length from 323 to 634 bp and were submitted to GenBank® (Accession numbers: DQ079030–DQ079050). In cases ($n = 4$) where sequences from multiple clones with identical ARDRA patterns were compared, pairwise analysis revealed no nucleotide differences over the region that had been sequenced (ranging from 250 to 495 bp).

We used the BLAST algorithm to identify related sequences from the GenBank® database (Altschul et al. 1990). BLAST searches were conducted with all sequenced clones, and preliminary taxonomic assignments were based on results from GenBank®. Sequences were checked for possible chimeric origins using the CHECK_CHIMERA software available through the Ribosomal Database Project.

Rarefaction and phylogenetic analysis

The extent of diversity in the clone libraries was estimated by rarefaction analysis of ARDRA profiles using the algorithm of Hurlbert (1971) and Heck et al. (1975) as implemented in the program Analytic Rarefaction 1.3. Resulting curves allowed for comparison of taxon richness between seawater and the *C. nucula*-associated microbial community. We focused our phylogenetic examination on unique sequences that consistently retrieved sponge-specific symbionts during BLAST searches. Phylogenetic relationships were inferred for symbiont sequences using distance and maximum likelihood (ML) methods. Sequences were aligned using ClustalW employing default parameters (Thompson et al. 1994). PAUP* 4.0b8 (Swofford 1999) was used to estimate bootstrap support for distance trees using a full heuristic search method and TBR branch swapping algorithm. Initial trees were obtained using neighbor joining. For ML analysis, models of sequence evolution that best fit the data were chosen based on results from Modeltest (v3.06, Posada and Crandall 1998). Tree-Finder (Jobb 2004) was used to estimate ML trees and their corresponding bootstrap support. Bootstrap analyses for both the distance and ML trees involved 1,000 replicates. Sponge symbionts from distant taxonomic groups were chosen as outgroups for all analyses.

Geographic analysis

We compiled data from a number of studies that had examined sponge symbiont diversity to compare trends in taxonomic composition of sponge microbial diversity across large geographic distances. Criteria for inclusion in this study were that authors attempted extensive community surveys through the construction of 16S rDNA libraries. Sequences were obtained from published reports from multiple regions of the world's oceans (including cold-temperate and subtropical North Atlantic, Mediterranean, Red Sea, subtropical and tropical Pacific, and the Southern Ocean). Taxonomic groupings were assigned based on published results or were estimated from consensus BLAST surveys. The relative percentage of a particular taxon within the library was estimated by dividing the number of taxa belonging to a particular bacterial phylum by the total number of unique clones obtained in that study.

Results

Taxon richness

ARDRA profiles indicated that a diverse community of bacterial species exists in *C. nucula* collected from a mangrove channel in Lower Matecumbe Key, Florida Keys, Florida. The clone library contained 21 operational taxonomic units (OTUs) (i.e., distinct ARDRA patterns) and the seawater library contained 53 OTUs. These OTUs were used in subsequent rarefaction analysis. The sponge microbial community was distinct from the bacterial community sampled from the ambient seawater given that none of the 21 OTUs from *C. nucula* were found in the seawater. Rarefaction analysis indicated that the sponge community had lower taxon richness than ambient seawater bacterial communities (Fig. 1). Although the *C. nucula* curve lay below the seawater curve, there was substantial overlap of the 95% confidence intervals (Fig. 1). Because extrapolation based on rarefaction analysis is an unreliable and inappropriate method for estimating total species richness (Gotelli and Colwell 2001); we could not estimate the total number of taxa that might be expected in the sponge or water column. However, it appears that > 20 taxa of bacteria were present in the *C. nucula* tissues and > 50 taxa in the water column at the time of sampling in 1999 (Fig. 1).

Phylogenetic analysis

Partial 16S rDNA sequences were obtained for clones with unique ARDRA patterns. Of the 21 sequences obtained, five *C. nucula* clones consistently retrieved other sponge microbial symbionts during BLAST searches, and we focused our analysis on these sequences. The most commonly encountered sequences came from

Fig. 1 *Chondrilla nucula.* Rarefaction analysis of diversity in sponge symbiont community compared to the bacterial community in the water column. *Curves* overlap substantially but begin to diverge after 20 sampled clones. At least 21 taxa of bacteria were associated with *C. nucula* and > 50 taxa were present in the water column. *Dashed lines* represent 95% confidence intervals
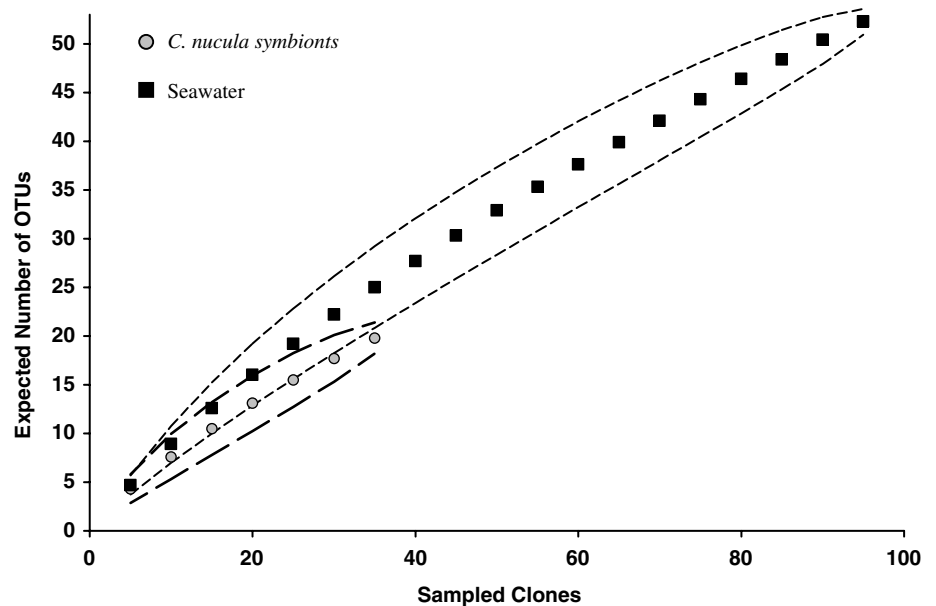
microbes harbored by sponges in the Indo-Pacific, Red Sea, and Mediterranean (Hentschel et al. 2002). The data presented here include representatives from the Acidobacteria, Bacteroidetes, Cyanobacteria, and the γ-Proteobacteria.

The Acidobacteria clones in this survey (*Cnuc8* and *Cnuc56*) fell within strongly supported clades entirely occupied by other sponge symbionts (Fig. 2a, b). *Cnuc8* and *Cnuc56* sequences aligned with different regions of the 16S gene, so it was not possible to compare both in the same phylogeny. Our analysis provided robust support for the Acidobacteria clusters (Acido-I, Acido-II, and Acido-III) of Hentschel et al. (2002). The monophyletic clade that included *Cnuc8* (Fig. 2a) was comprised of sponges from tropical and subtropical regions as well as sponges from shallow- and deep-water habitats (e.g., *Scleritoderma* sp.). This well-supported clade includes many of the acidobacterial symbionts belonging to the Acido-I group. The monophyletic clade that included *Cnuc56* included sponges from the Mediterranean and tropical Pacific, which belong to the Acido-III group (Fig. 2b). The sponge-specific Acido-II group was placed in a clade that included novel, geographically widespread soil microbiota (Kuske et al. 1997), but no *C. nucula* microbes isolated in this study belonged to this group.

One of the Bacteroidetes identified in our surveys (*Cnuc9*) was placed in a strongly supported monophyletic clade that included other sponge-specific bacteroids (Fig. 3). These bacterial representatives were collected from hosts living in the Red Sea, Mediterranean, and Pacific Oceans. This clade also included *R. marinus*, which is associated with the dinoflagellate *Peridinium* sp. (according to GenBank® data). Thus, Bacteroidetes that appear to be associated with a host organism (sponge or perhaps protistan) clustered in our analysis while free-living bacteroids fell outside of the symbiont-specific clade (Fig. 3).

The cyanobacterial symbiont isolated in our survey (*Cnuc6*) was placed near cyanobacterial species isolated from *Theonella swinhoei* of the tropical Indo-Pacific (Fig. 4). Bootstrap support for this placement was ≈80%. Several other cyanobacterial sponge symbionts fell outside of this clade, and did not cluster together in our analysis.

There were few sponge-specific clusters in the phylogenetic analysis of γ-Proteobacteria sequences (Fig. 5). *Cnuc2* retrieved bacterial symbionts from two sponge species from Woods Hole, Massachusetts in the northwest Atlantic during BLAST searches. Phylogenetic analysis based on the distance optimality criterion indicated a potential grouping of *Cnuc2* with two microbes isolated from *Microciona prolifera* and *Suberites domuncula* (Fig. 5). A free-living microbe found in a Georgia salt marsh of the southeastern US was included in this analysis. The ML analysis separated each of these sequences creating a polytomy. Another sponge-specific lineage was identified that included sponges from Palau, the Mediterranean, and Red Sea.

Geographic distribution of major sponge symbiont lineages

The diversity of microbes harbored by sponges living in different oceans is impressive (Table 1). Although limited to the examination of single sponge samples (due to the high cost associated with these survey efforts), some interesting trends were observed when the relative proportion of different taxonomic groups were compared. Only the polar sponges were associated with Firmicutes and Planctomyces, and it is important to note that up to one-third of the bacterial community for two of the Antarctic sponges were of uncertain taxonomic affiliation (Table 1). Spirochaetes were only found in *Aplysina*
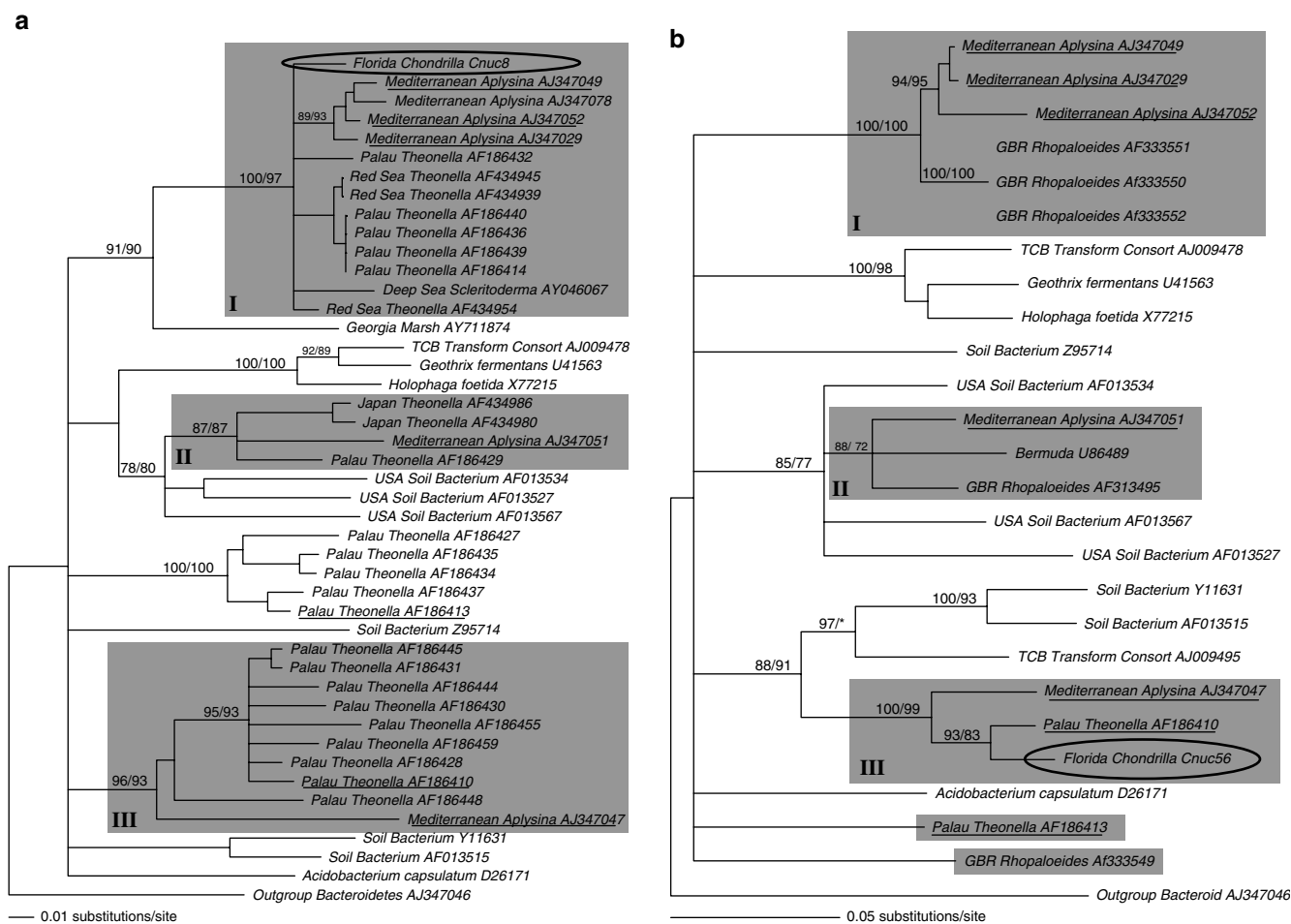
a

I
Florida Chondrilla Cnuc8
Mediterranean Aplysina AJ347049
Mediterranean Aplysina AJ347078
Mediterranean Aplysina AJ347052
Mediterranean Aplysina AJ347029
Palau Theonella AF186432
Red Sea Theonella AF434945
Red Sea Theonella AF434939
Palau Theonella AF186440
Palau Theonella AF186436
Palau Theonella AF186439
Palau Theonella AF186414
Deep Sea Scleritoderma AY046067
Red Sea Theonella AF434954
Georgia Marsh AY711874
TCB Transform Consort AJ009478
Geothrix fermentans U41563
Holophaga foetida X77215

II
Japan Theonella AF434986
Japan Theonella AF434980
Mediterranean Aplysina AJ347051
Palau Theonella AF186429
USA Soil Bacterium AF013534
USA Soil Bacterium AF013527
USA Soil Bacterium AF013567
Palau Theonella AF186427
Palau Theonella AF186435
Palau Theonella AF186434
Palau Theonella AF186437
Palau Theonella AF186413
Soil Bacterium Z95714

III
Palau Theonella AF186445
Palau Theonella AF186431
Palau Theonella AF186444
Palau Theonella AF186430
Palau Theonella AF186455
Palau Theonella AF186459
Palau Theonella AF186428
Palau Theonella AF186410
Palau Theonella AF186448
Mediterranean Aplysina AJ347047
Soil Bacterium Y11631
Soil Bacterium AF013515
Acidobacterium capsulatum D26171
Outgroup Bacteroidetes AJ347046

—— 0.01 substitutions/site

b

I
Mediterranean Aplysina AJ347049
Mediterranean Aplysina AJ347029
Mediterranean Aplysina AJ347052
GBR Rhopaloeides AF333551
GBR Rhopaloeides Af333550
GBR Rhopaloeides Af333552
TCB Transform Consort AJ009478
Geothrix fermentans U41563
Holophaga foetida X77215
Soil Bacterium Z95714
USA Soil Bacterium AF013534

II
Mediterranean Aplysina AJ347051
Bermuda U86489
GBR Rhopaloeides AF313495
USA Soil Bacterium AF013567
USA Soil Bacterium AF013527
Soil Bacterium Y11631
Soil Bacterium AF013515
TCB Transform Consort AJ009495

III
Mediterranean Aplysina AJ347047
Palau Theonella AF186410
Florida Chondrilla Cnuc56
Acidobacterium capsulatum D26171
Palau Theonella AF186413
GBR Rhopaloeides Af333549
Outgroup Bacteroid AJ347046

—— 0.05 substitutions/site

Fig. 2 Acidobacteria. Bootstrap support values given for groupings common to distance and maximum likelihood (*ML*) analyses (*numerator* and *denominator* respectively). *Asterisk* indicates node was not supported by that method. *Underlined sequences* are common to phylogenies in both (**a**) and (**b**). Sponge sequences are *highlighted in shaded boxes*. Name of each sponge clone includes its geographic location, genus of the host from which it was derived, and accession number. **a** *C. nucula* clone *Cnuc8* (designated by *oval*) had high similarity with other sponge-specific acidobacterial symbionts. *Cnuc8* clone fell within a well-supported clade that included other sponge symbionts from the Acido-I group. Tree based on analysis of 377 characters. **b** *C. nucula* clone *Cnuc56* (*oval*) also had high similarity with other sponge-specific symbionts, falling within a clade with high bootstrap support that included sponge-specific symbionts from the Acido-III group. Tree based on analysis of 302 characters

aerophoba and *C. nucula* of the Mediterranean and subtropical Atlantic, respectively. The γ-Proteobacteria were found in all sponges examined and represented from 2 to 86% of the clone library (Table 1). The Bacteroidetes and α-Proteobacteria were also major representatives of the libraries examined (92 and 83% respectively). These three groups represented from 56 to 100% of the microbial community in polar (Southern Ocean) and cold-temperate (*Halichondria panicea*) sponges but only 2–62% for subtropical and tropical sponges. This difference was due in part to the fact that the polar and cold-temperate sponges did not harbor several groups that were found in warm-temperate and tropical sponges (Table 1). Indeed, the Acidobacteria, δ-Proteobacteria, Chloroflexi, Cyanobacteria, and Nitrospira were only detected in libraries constructed for warm water sponges.

Discussion

We uncovered substantial diversity in the bacteria associated with *C. nucula* collected in the Florida Keys, Florida that was comparable to the diversity found in other marine sponges. Our data, and those of other studies, provide a framework for future studies of the role these diverse microbes play in the ecology, evolution, and development of marine sponges. Our data also provide support for the hypothesis that several species of marine sponges (separated by large geographic distances and habitat differences) harbor closely related microbial symbionts, which may be specialized for exploiting the intra-sponge habitat.

A greater number of OTUs were identified in Florida water samples than in the symbiont community associ-

Fig. 3 Bacteroidetes.
Phylogenetic analysis of
sequence obtained from *C.
nucula*. *Cnuc9* (*oval*) was in a
well-supported clade including
symbionts from Mediterranean,
Red Sea, and Pacific Ocean
sponges. *Cnuc9* and symbiont
from *Aplysina aerophoba* were
placed in clade with strong
bootstrap support. A non-free-
living *Rhodothermus marinus*
specimen associated with the
dinoflagellate *Peridinium* sp.
was also in that clade. Tree
based on analysis of 409
characters
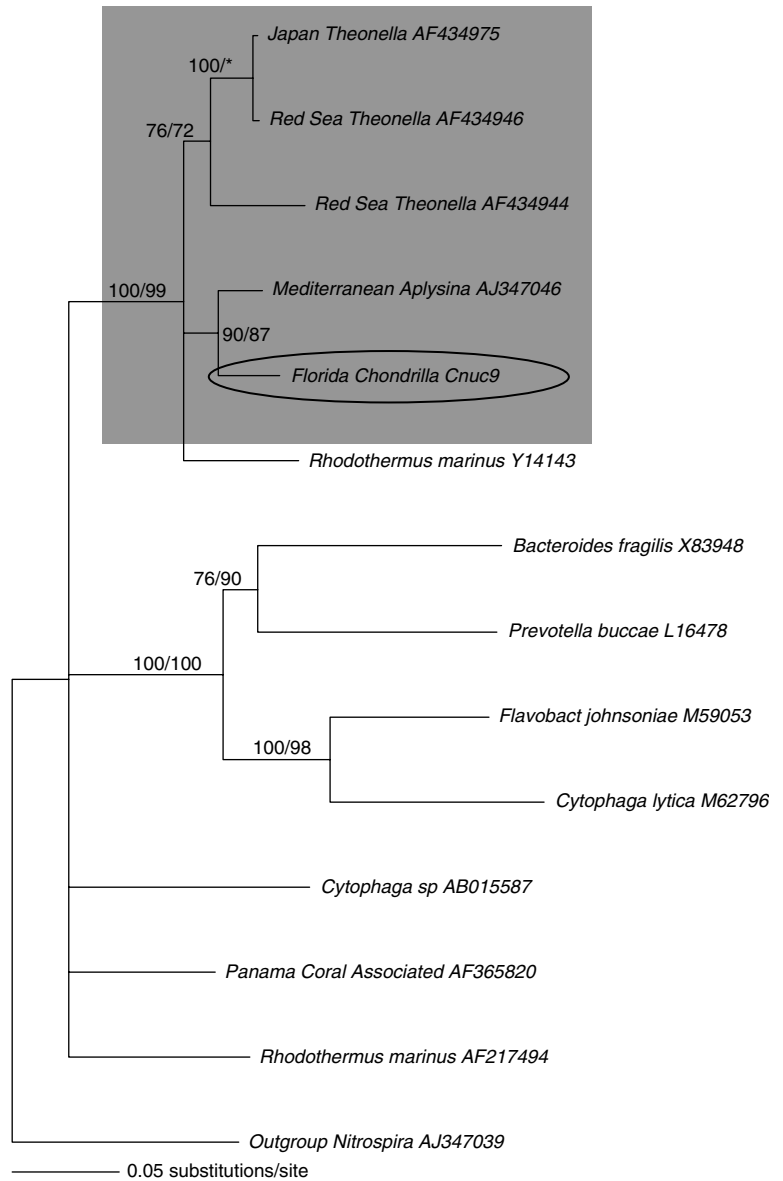
ated with *C. nucula*. Neither curve approached a plateau indicating that the full bacterial diversity has yet to be determined [see caveats for rarefaction analysis in Gotelli and Colwell (2001)]. The intra-sponge habitat and ambient seawater may contain taxonomically distinct species assemblages that would be sampled with different thoroughness, given the PCR-based strategy employed in this survey. An important future objective in assessing the microbial species richness present in *C. nucula* is to examine the temporal and spatial stability of the association using a combination of bacterial primers. Evidence from *Cymbastela concentrica*, which ranges through temperate and tropical waters of Australia, indicates that changes in microbial symbiont communities can occur (Taylor et al. 2004, 2005).

Two *C. nucula* sequences (*Cnuc8* and *Cnuc56*) clearly belong to well-supported, sponge-specific lineages within the Acidobacteria. These putative sponge specialists

have been found in hosts living in Indo-Pacific, Mediterranean, Red Sea, and Caribbean waters. These sponge specialists are not restricted to shallow water hosts since the deep-sea *Scleritoderma* spp. harbor Acido-I symbionts (Olson and McCarthy 2005). It is interesting to note that the sister taxa to the Acido-II group include ecologically important soil microbes with a wide geographic distribution. The physiological characterics of this group deserve greater attention to determine what properties allow for successful invasion of such apparently dissimilar habitats.

A potential sponge-specific lineage, belonging to the Bacteroidetes, was also identified for one *C. nucula* clone (*Cnuc9*) but the clade was not entirely sponge-specific. *R. marinus*, which is typically associated with a dinoflagellate, was also included in the clade. Whether this represents a Bacteroidetes lineage specialized for occupying host tissues (poriferan or protistan) deserves

Fig. 4 Cyanobacteria. *C. nucula Cnuc6* clone (*oval*) was placed in a clade that included sponge cyanobacterial symbionts from Mediterranean and Indo-Pacific host sponges. Other sponge cyanobacterial symbionts are shown in *shaded boxes*. Tree based on analysis of 333 characters
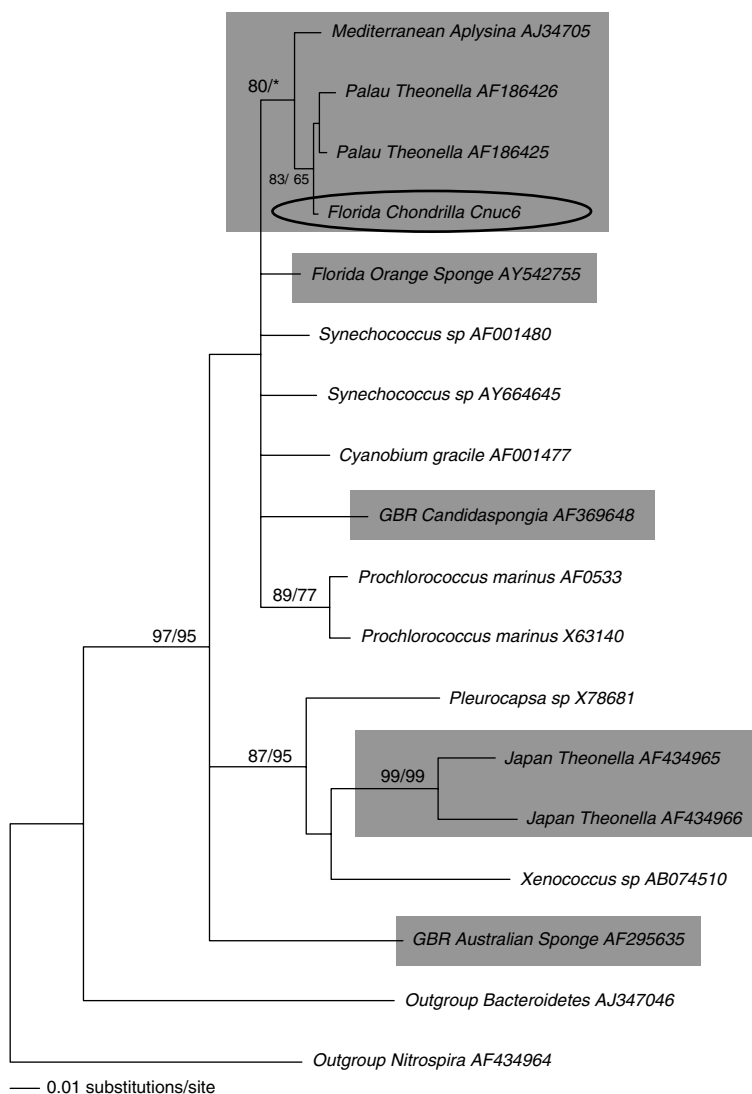

additional attention, but there appears to be a bias in this lineage for existing in sponge tissues. The Bacteroidetes contain species that range from pathogens to commensals and they often have unusual physiological capabilities (e.g., lipid metabolism). Of particular note is the recent finding that Bacteroidetes are involved in generating reproductive (i.e., cytoplasmic) incompatibilities in a parasitoid wasp (Hunter et al. 2003). Whether these microbes play a role in the evolution of their poriferan hosts by erecting species boundaries is worthy of future research.

Recent work on the cyanobacterial symbionts of marine sponges has yielded interesting findings (Usher et al. 2004a, b; Steindler et al. 2005). It appears that sponges host at least four distinct, but closely related, species of *Synechococcus* (but see Steindler et al. 2005). It is not clear from our analysis to which group the *Cnuc6* sequence might belong. *C. nucula* has been assumed to harbor *Aphanocapsa feldmannii* (e.g., Wilkinson 1980), but the true species status of the cyanobacterium associated with *C. nucula* at our site remains unknown. Our

sequences did not cover the same region of 16S rDNA as many of the sequences available in GenBank®, so we could only focus on the sequences shown in Fig. 4. However, our sequence aligns most closely aligned with representatives of the group 6 *Synechococcus* reported in Honda et al. (1999). A number of cyanobacterial sequences from the Caribbean have recently been published in GenBank®, and the analysis of these sequences will answer several questions about the relationships between and among the Caribbean, Pacific, Red Sea, and Mediterranean cyanobacteria that have been identified thus far (Steindler et al. 2005).

Our data resolve little regarding the specificity of γ-Proteobacteria associated with sponges. *Cnuc2* was similar to microbes isolated from temperate sponges of the western North Atlantic. Support for this clade was relatively low, and a free-living bacterium isolated from salt marshes of Georgia was included in this branch. None of the other γ-Proteobacteria isolated from sponges created a robust grouping, but symbionts from Palau, the Mediterranean, and Red Sea did cluster

Fig. 5 γ-Proteobacteria. Distance methods placed *C. nucula* Cnuc2 clone (*shaded oval*) in a clade that included a free-living bacterium and microbial symbionts from *Microciona* and *Suberites*. There was relatively weak support for a sponge-specific grouping in this analysis. Deep-sea vestimentiferans and shallow-water mollusk endosymboints were placed in a well-supported clade (*arrow*). Tree based on analysis of 385 characters
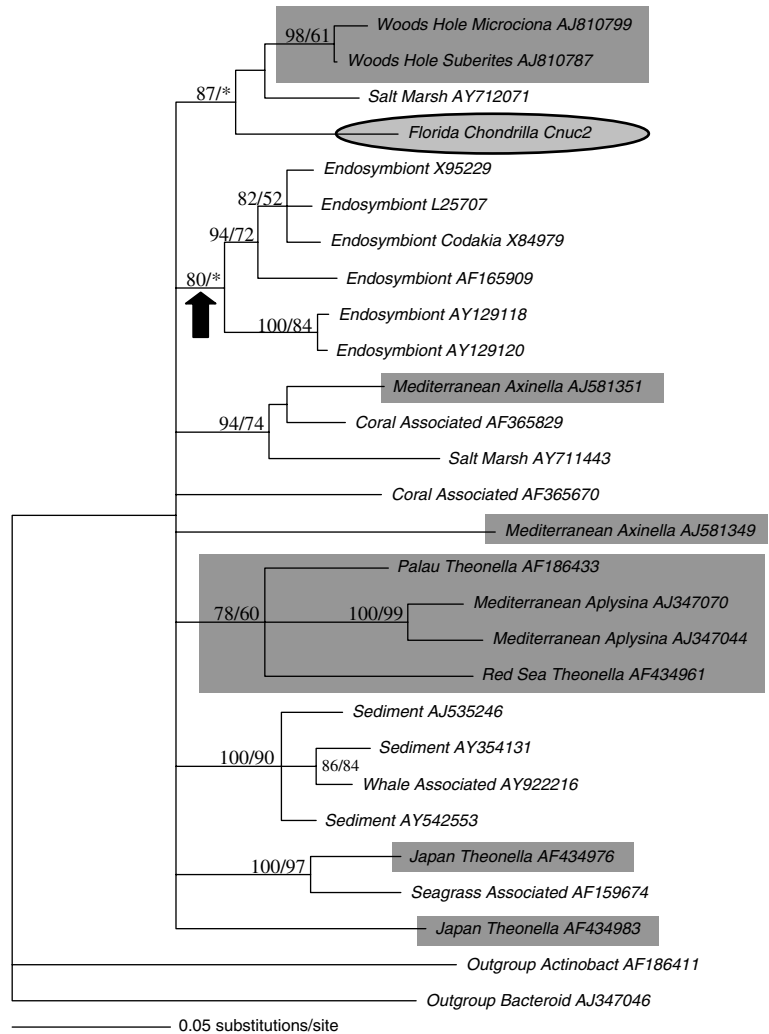
together. A relatively robust clade with endosymbionts from shallow water mollusks and deep-sea vestimentiferans was identified (Fig. 5). The γ-Proteobacteria may represent an opportunistic and generalist group of bacteria with limited specificity for a particular host (e.g., whale-, coral-, and seagrass-associated microbes). Several of the γ-Proteobacteria, however, have interesting physiological capabilities (e.g., sulfur metabolism) that may benefit their hosts.

It is important to keep in mind that the data shown in Table 1 are relative frequencies of bacterial types isolated from clone libraries. Whether the PCR-based strategy employed here accurately reflects species richness remains to be seen, but implementation of hybridization techniques (e.g., Friedrich et al. 1999; Webster et al. 2001b; Fieseler et al. 2004) has already begun to clarify our understanding of the relative abundance and spatial location of some of the sponge-specific symbionts. However, several interesting trends were observed in this study. Bacterial communities harbored by polar, and to some extent cold-temperate, sponges appear to be distinct from those harbored by subtropical or tropical sponges. Webster et al. (2004), however, found one

α-Proteobacterial symbiont from tropical *Rhopaloides odorabile* that clustered with symbionts from Antarctic sponges, and in this regard, the placement of *Cnuc2* next to temperate North Atlantic sponges is intriguing. Taylor et al. (2004) found similar symbionts residing in temperate sponges of Australia and warmer water sponges such as *T. swinhoei*.

It is somewhat surprising that δ-Proteobacteria, Chloroflexi, Cyanobacteria, and Nitrospira were not detected in the *T. swinhoei* collected in the Red Sea, despite their relative commonness in *T. swinhoei* from Japan and Palau. It would be worthwhile exploring why Acidobacteria, δ-Proteobacteria, Chloroflexi, Cyanobacteria, and Nitrospira have not been detected in polar and cold-temperate sponges, and why Firmicutes and Planctomyces have not been found in tropical and subtropical sponges (but see Pimentel-Elardo et al. 2003). It is important to determine whether the distribution of sponge specialist bacteria is more strongly influenced by properties of the host or environmental conditions. *C. nucula* is a perfect case study for this question because Atlantic populations of this sponge are considered to consist of multiple cryptic species (Klautau et al. 1999;

Table 1 Relative frequencies of major bacterial taxonomic groups isolated from clone libraries of multiple sponge species

Taxonomic group	Southern Ocean					Pacific Ocean			Red Sea	Mediterranean	Atlantic Ocean	
	Sa[a]	Kv[a]	Hb[a]	La[a]	Ma[a]	Ro[b]	TsP[c]	TsJ[c]	TsI[c]	Aa[c]	Cn[d]	Hp[e]
γ-Proteobacteria	85.7	28.6	33.3	31.3	55.6	32.3	2.1	11.1	23.5	3.3	23.7	28.6
Bacteroidetes	14.3	57.1		6.3	11.1	3.3		5.6	11.8	1.6	4.8	25
α-Proteobacteria			14.3	60	18.8	6.5		11.1	11.8	13.1	33.3	25
Actinobacteria						13	4.2	11.1	35.3	13.1		21.4
Acidobacteria						16	43.7	11.1	17.6	9.9	14.3	
δ-Proteobacteria							16.7	11.1		5	4.8	
Chloroflexi						16	10.4	11.1		41		
Cyanobacteria							4.2	11.1		4.9	4.8	
Nitrospira							10.4	5.6		1.6		
Spirochaetes										1.6	4.8	
Firmicutes			6.7	6.3								
Planctomyces				6.3								
Uncertain affiliation				31.3	33.3	12.9	8.3	11.1		4.9	9.5	

Host sponges are listed based on the major body of water where they reside along an approximate south–north continuum. Percentages calculated by determining the frequency of unique clones compared to all clones uncovered in each survey. Taxonomic assignments of bacteria were based on percent identities obtained during BLAST searches or designations from the literature

[a] Webster et al. (2004): *Sa Sphaerotylus antarcticus*; *Kv Kirkpatrickia variolosa*; *Hb Homaxinella balfourensis*; *La Latrunculia apicalis*; *Ma Mycale acerata*

[b] Webster et al. (2001a): *Ro Rhopaloides odorabile*

[c] Hentschel et al. (2002): *Ts Theonella swinhoei* from Palau, Japan, or Israel; *Aa Aplysina aerophoba*

[d] *Cn Chondrilla nucula* from Florida Keys

[e] Obtained from GenBank®; *Hp Halichondria panicea* from northeastern Atlantic

see Usher et al. 2004a, b for Australian species of *Chondrilla*). Any differences or similarities between and among the bacterial symbionts harbored by these sponges would provide insights into forces that structure these communities.

Our data support the hypothesis that some subset of the bacterial community harbored by marine sponges is uniform (sensu Hentschel et al. 2002) across large geographic distances, which indicates these are ancient symbioses. The interaction between the host sponge and members of its symbiont community is likely to have important ecological and evolutionary ramifications (e.g., Wilkinson 1987; Margot et al. 2002; Piel et al. 2004). Many sponges studied to date are broadcast spawners (e.g., *C. nucula*) and may require some level of reinfection each generation. However, several studies have demonstrated vertical transmission of microbes into gametes and larvae regardless of sponge reproductive mode or type of bacterial symbiont (e.g., Kaye 1991; Gaino and Sara 1994; Sciscioli et al. 1994; Usher et al. 2001). Indeed, the host sponge may control the composition of the microbial community in their offspring through a series of complicated interactions between the oocytes and nurse cells that attach to the developing eggs (Usher et al. 2001). If this is so, the high degree of similarity among different individuals of the same species might be explained by a surprising degree of control by the host sponge over the types of microbes that persist in its tissues. The majority of sponges are bactivorous so the presence of substantial microbial populations in their tissues indicates some degree of selectivity and regulation by the host. Complicated cell recognition pathways must exist, given the high degree of diversity within the sponge (e.g., Pimentel-Elardo et al. 2003). Identifying and describing the mechanisms by which this discernment is achieved is an important goal. The diversity uncovered in this and other studies provides an opportunity to address these and other important questions.

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