Vol. 67: 1–14, 2012
doi: 10.3354/ame01571

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online July 27

Characterization of bacterial epibionts on the cyanobacterium *Trichodesmium*

L. R. Hmelo[1,2], B. A. S. Van Mooy[1], T. J. Mincer[1,*]

[1]**Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA**

[2]***Present address:*** **Department of Microbiology, School of Medicine, University of Washington, Seattle, Washington 98195, USA**

ABSTRACT: Interest in *Trichodesmium,* a nitrogen-fixing genus of *Cyanobacteria*, has been fueled by its prominent role in the marine nitrogen cycle. However, it is often overlooked that *Trichodesmium* occur in the ocean as colonies and are only one member of a complex microbial consortium. In the present study, we used 16S rRNA gene sequences to phylogenetically classify the communities associated with 2 morphological types of *Trichodesmium* colonies, tufts and puffs, which were collected at the Bermuda Atlantic Time Series (BATS) site in the Sargasso Sea. Both *Trichodesmium* morphotypes were most closely related to *T. thiebautii* at ≥99% identity. Non-cyanobacterial sequences from both types of colonies were dominated by *Flavobacteria*, *Sphingobacteria*, and *Alphaproteobacteria*. However, the epibiotic communities possessed significantly lower diversity than bacterioplankton; major seawater planktonic taxa, such as the SAR11 clade and *Archaea*, were conspicuously absent. Moreover, several epibiotic taxa appeared to be novel. Among the tuft or puff 16S rRNA clone libraries constructed in the present study, epibionts sharing common operational taxonomic units at the 97 percent sequence identity (PSI) threshold or higher were not observed, presenting the possibility that these 2 morphologies could select for different epibiotic communities. While our data are representative of a single sample point in time and space, these data suggest that *Trichodesmium* possess an epibiotic microbial community of relatively low diversity, distinct from that observed in bacterial picoplankton.

KEY WORDS: *Trichodesmium* · Epibiont · *Cyanobacteria* · Cultivation-independent · Pelagic microbial community

INTRODUCTION

Scientific interest in the cyanobacterial genus *Trichodesmium* has largely been driven by the significant role of these microbes as N_2 fixers (Capone et al. 1997, Karl et al. 1997). They are capable of fixing at least 80 Gt N yr^{-1} in extremely low-nutrient, oligotrophic marine environments; as such, they are a major source of fixed carbon and reduced nitrogen to these environments and possibly fuel intense microbial activity in surface waters (Letelier & Karl 1996, Capone et al. 1997, Carpenter et al. 1997, Sohm et al. 2011).

Without exception, heterotrophic bacteria, and frequently filamentous *Cyanobacteria*, are observed attached to the surface of *Trichodesmium* spp. (Herbst & Overbeck 1978, Paerl et al. 1989, Siddiqui et al. 1992). Cell densities of bacterial epibionts of *Trichodesmium* are reported to range from 8.2×10^8 cells ml^{-1} (Sheridan et al. 2002) to 2.6×10^{11} cells ml^{-1} (Paerl 1982), which is ~3 to 5 orders of magnitude more concentrated than typical counts of planktonic bacteria in the euphotic zone of the open ocean. It is extraordinarily difficult to isolate *Trichodesmium* spp. in axenic culture, suggesting that they have evolved an essential relationship with their epibionts (Waterbury

*Corresponding author. Email: tmincer@whoi.edu

1991). Heterotrophic bacteria are known to colonize the surface of marine eukaryotic algae (Fisher et al. 1998, Sapp et al. 2007) and *Cyanobacteria* (Herbst & Overbeck 1978, Paerl 1982, Siddiqui et al. 1992, Nausch 1996, Sheridan et al. 2002, Stevenson & Waterbury 2006, Simmons et al. 2008) with some associations being quite specific (Paerl & Gallucci 1985, Fisher et al. 1998, Stevenson & Waterbury 2006, Tuomainen et al. 2006). For example, the cyanobacterium *Anabaena* sp. strain SSM-00 transfers fixed nitrogen to its epibiont *Rhizobium* sp. strain WH2K (Behrens et al. 2008). In many cases, associations between heterotrophic bacteria and phytoplankton are quite specific (Fisher et al. 1998, Paerl & Gallucci 1985, Stevenson & Waterbury 2006, Tuomainen et al. 2006). In the case of *Anabaena* sp. strain SSM-00 and *Rhizobium* sp. strain WH2K, transfer of fixed nitrogen from host to epibiont has been shown (Behrens et al. 2008).

The production of organic matter by phytoplankton and the concomitant release of fixed carbon and nitrogen into the upper ocean is a major geochemical process in the ocean. Viral lysis and programmed cell death are 2 mechanisms that are known to mediate the termination of *Trichodesmium* blooms and subsequent release of fixed nutrients into the environment (Hewson et al. 2004, Berman-Frank et al. 2007). Attached bacteria contribute to the transfer of fixed nutrients from *Trichodesmium* to the upper ocean by consuming *Trichodesmium*-produced organic exudates (Herbst & Overbeck 1978) and funneling this material into the microbial loop; bacteria thereby retain nitrogen fixed by *Trichodesmium* in the euphotic layer of the ocean (Nausch 1996).

Nitrogen fixed by *Trichodesmium* fuels new production, which is potentially a major driver for carbon export into the deep ocean. If degradation of these colonies is partially mediated by attached bacteria, these organisms could influence the residence time of fixed nitrogen and carbon in the upper ocean as well as the composition of the material exported into the deep ocean. The heterotrophic epibionts attached to nitrogen-fixing *Cyanobacteria* may also achieve broad ecological impacts by influencing the longevity of blooms by recycling organic matter and nutrients (Tuomainen et al. 2006). These epibionts are also suspected to aid the efficiency of *Trichodesmium* iron solubilization and uptake by contributing to the formation of anoxic microzones within *Trichodesmium* colonies (Roe et al. 2012).

Individual *Trichodesmium* cells typically form multi-cell trichomes, which can bundle together in multi-trichome colonies (Carpenter et al. 2004). These colonies tend to be dominated by 2 morphologic types, a spherical 'puff' type and a fusiform 'tuft' type. Bacteria on tuft-type colonies have been observed to occur in higher densities (Sheridan et al. 2002) and exhibit higher hydrolytic enzyme activity (Nausch 1996) relative to those on puff-type colonies, although the diversity of metazoa and microzooplankton associated with puff-type colonies appears to be greater than that associated with tufts (Sheridan et al. 2002). Considering the global environmental significance of this specific algal-bacterial association, the identity of ubiquitous heterotrophic epibionts associated with *Trichodesmium* spp. has been relatively understudied. Here, we report results of a culture-independent 16S rRNA gene sequence-based clone library survey of the microbial epibiont community associated with *Trichodesmium* colonies collected in the Sargasso Sea.

MATERIALS AND METHODS

Sample collection

Trichodesmium spp. colonies were collected under non-bloom conditions (i.e. no obvious bloom was observed during any time of collection) in September 2008 on the RV 'Atlantic Explorer' at the Bermuda Atlantic Time Series (BATS) station. Aggregates were collected from the near surface (the upper ~20 m) by a hand-held 130 µm plankton net, and individual colonies were gently picked using an inoculating loop. Colonies were sequentially washed 3 times with 0.2 µm filtered seawater. Individual colonies were preserved for DNA extraction. Colonies were preserved in SET buffer (0.75 M sucrose, 50 mM Tris pH 8, 40 mM EDTA pH 8) and frozen in liquid N_2 at sea and then transferred to a −80°C freezer in Woods Hole.

DNA extraction and clone library construction

Clone libraries of bacterial 16S rRNA gene sequences were constructed from 2 samples. One sample consisted of 10 puff-type colonies, and the other sample consisted of 10 tuft-type colonies. DNA was obtained according to a protocol adapted from DeLong et al. (2006). Aliquots of community *Trichodesmium* spp. DNA (25 to 100 ng) were added as template to polymerase chain reactions (PCR) for amplification of the SSU rRNA gene sequence. Each PCR consisted of the following (all amounts are final concentrations): 0.2 mM each dNTPs, 0.5 µM each

forward primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and reverse primer 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (primers were custom synthesized by Invitrogen), 2 units 'Easy A' thermostable proofreading polymerase (Stratagene), and 1× 'Easy A' reaction buffer containing 2 mM $MgCl_2$, for a 20 µl final reaction volume. PCR amplification conditions were as follows: an initial denaturation step of 2 min at 94°C, 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C. The final 3 steps were repeated for a total of 30 cycles. To avoid bias, the amplification products were monitored, and we determined that these conditions represented the minimum number of cycles needed to obtain sufficient product for cloning while avoiding saturation of the reaction (Suzuki & Giovannoni 1996). Reconditioning PCR was carried out to reduce heteroduplex formation (Thompson et al. 2002) as follows: initial reaction products were diluted 10-fold and re-amplified using parameters identical to above, except that only 3 thermal cycles were performed. Triplicate PCR amplifications were pooled and cloned into PCR 4-TOPO plasmid vectors using a TOPO TA cloning kit (Invitrogen). From each library, 384 clones were picked, and the clone insert DNA was amplified using PCR consisting of the following (all reagents reported at final concentrations): 0.2 mM each dNTPs, 0.5 µM each forward primer M13F (5′-GTA AAA CGA CGG CCA G-3′) and reverse primer M13R (5′-CAG GAA ACA GCT ATG AC-3′) (Invitrogen), and 1 U Paq5000 DNA polymerase (Stratagene) in 1× Paq5000 reaction buffer containing 2.25 mM $MgCl_2$ for a 20 µl final reaction volume. A total of 35 reaction cycles were completed using the same parameters as above. In total, 384 bacterial SSU rRNA clones were prepared for sequencing from each library.

Sequencing

Clone inserts were sequenced in the forward direction using the primer 27F. End-sequencing was performed offsite either by MWG-Operon or Agencourt Biosciences. A total of 339 raw sequences were generated from the tuft-colony library, and 331 raw sequences were generated from the puff-colony library. Sequences were typically between 700 and 750 base pairs in length.

Raw sequences were aligned via the web-based SINA aligner (www.arb-silva.de/aligner/). Aligned sequences were imported into ARB (Ludwig et al. 2004) (version 07.12.07). The sequences were de-replicated at 97, 99, and 100% identity thresholds using FastGroup II (Yu et al. 2006) (http://biome.sdsu.edu/fastgroup). Unique sequences were submitted to the Bellerophon server (Huber et al. 2004) (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) to identify putative chimeras. Sequences submitted to Bellerophon were internally aligned using ClustalW and evaluated with a Huber-Hugenholtz correction and a window size of 200. Sequences identified as chimeric by Bellerophon were confirmed or disregarded with information from the Ribosomal Database Project (RDP II) chimera detection software (Cole et al. 2003) (http://rdp.cme.msu.edu/). Chimeric sequences were excluded from further analyses (5 sequences were removed from the tuft-colony library, and 6 sequences were removed from the puff-colony library). An additional 22 sequences were excluded from the tuft-library and 20 additional sequences were excluded from the puff-library because the sequences were very short (<430 bp).

PCR with archaeal and eukaryotic primers

Trichodesmium total community DNA extracts were screened for the presence of archaeal 16S rRNA genes and eukaryotic 18S rRNA genes. The PCR was performed as described above, using Paq5000 DNA polymerase, with the following modifications: archaeal primers Ar20F (5′-TTC CGG TTG ATC CYG CCR G-3′) and Ar958R (5′-YCC GGC GTT GAM TCC AAT T-3′) were added to a final concentration of 0.5 µM with an annealing temperature of 60°C. The parameters for eukaryotic 18S rRNA gene amplification were as follows: primers EukF (*Escherichia coli* forward position 7, 5′-AAC CTG GTT GAT CCT GCC AGT-3′) and EukR (*E. coli* reverse position 1534, 5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) were added at a final concentration of 0.5 µM with an annealing temperature of 65°C. DNA from a fosmid template clone 4B7 (a member of the Group I *Crenarchaea*) (Stein et al. 1996) and a eukaryotic enrichment produced from seawater collected during HOTS cruise 179 (http://hahana.soest.hawaii.edu/hot/hot_jgofs.html) at Station ALOHA at 25 m were used as positive amplification controls for archaeal and eukaryote screening, respectively.

Taxonomic assignments and phylogenetic analyses

The Ribosomal Database Project II Classifier (Cole et al. 2003) was used to assign provisional taxonomic

affiliations to 97% similar operational taxonomic units (OTUs). BLASTn searches (Altschul et al. 1997) of the National Center for Biotechnology Information 16S rRNA gene sequence database were performed and showed taxonomic placements of many of our clones to be novel at or above the genus level, explaining their low bootstrap values in the automatic classifier. The proportions of major taxonomic groups were calculated, and the BLASTn results are available in Tables S1 & S2 in the supplement at www.int-res.com/articles/suppl/a067p001_supp.pdf. All BLASTn searches were performed with the GenBank database updated on 1 January 2010.

Rarefaction, coverage, and richness were all calculated at a 97 percent identity threshold (PSI) using the software resource Fast Group II (Yu et al. 2006). Confidence intervals of 95 and 98% were calculated for rarefaction results using variance data provided in the Fast Group II calculations. The coverage of our clone libraries (percentage of actual species richness detected) was calculated according to Good (1953) and Chao & Yang (1993). The former algorithm estimates coverage related to relatively rare OTUs, while the latter estimates coverage based on the abundance of dominant OTUs, providing a lower and upper bound on coverage, respectively. Species richness was estimated using the Chao1 nonparametric richness estimator (Chao 1987) (Table 1), yielding the probable number of OTUs in the sample on the basis of singletons and doubletons detected in the clone library (Chao 1987). The Shannon diversity index, which takes into account species richness and evenness of species distribution, was calculated according to the equations provided by Pielou (1966), as were approximations of the variance of resultant confidence intervals. All sequences were deposited in GenBank under accession nos. GU725472 to GU726134.

Table 1. Statistical description of puff-colony and tuft-colony libraries analyzed in the present study (excluding sequences related to *Trichodesmium*), and Sargasso Sea bacterioplankton clone libraries published by Carlson et al. (2009). OTUs and the Chao1 richness index were calculated using the FastGroup II web-based bioinformatics platform (described in 'Materials and methods'). The Shannon index and the approximation of variance therein were calculated at the 97, 99, and 100 percent sequence identity (PSI) threshold using equations presented by Pielou (1966). Variance in the Shannon index is presented for the 95% confidence intervals as absolute variance and the index range falling within the given confidence limits. The number of OTUs and the value of the Chao1 are calculated at the 97, 99, and 100 PSI threshold. The results of the 97 PSI analysis are described in detail in the 'Results' section

Percent similarity	No. of clones analyzed	OTUs	Richness index: Chao1	Diversity index: Shannon-Wiener	95% CI Shannon-Wiener diversity index
Puff-colony library					
100	162	49	151	2.7	± 0.22; 2.5–2.9
99	162	22	32	2.2	± 0.21; 2.0–2.4
97	162	18	23	1.8	± 0.19; 1.7–2.0
Tuft-colony library					
100	164	33	78	2.5	± 0.21; 2.2–2.6
99	164	18	24	1.8	± 0.19; 1.6–2.0
97	164	15	21	1.7	± 0.17; 1.7–2.1
Sargasso Sea library					
100	189	141	547	4.8	± 0.11; 4.6–4.9
99	189	98	268	4.1	± 0.15; 4.0–4.3
97	189	70	141	3.4	± 0.25; 3.1–3.7

RESULTS

Cyanobacteria

Although this study was focused on the epibiont communities associated with *Trichodesmium* spp., we chose a DNA extraction method that has been shown to lyse a wide range of cells (DeLong et al. 2006). As a result, high numbers of cyanobacterial 16S rRNA gene sequences were recovered (the puff library yielded 143 cyanobacterial clones from a total of 305 community clones, and 213 cyanobacterial clones were obtained from 312 total tuft library community clones). These data provided a valuable insight into the cyanobacterial 'base' of the colonies (Fig. 1).

In total, 100% of the cyanobacterial clones (143 total) in the puff-colony library were related to *Trichodesmium thiebautii* (Genbank accession no. AF013027) at the 99 PSI threshold. The tuft colonies contained a more diverse cyanobacterial community, although all of the tuft-colony derived cyanobacterial clones were determined to be members of the Order *Oscillatoriales* that includes the genus *Trichodesmium*. In total, 69% of clones (n = 148) were related to *T. thiebautii* at 99 PSI. However, 31% of the clones (n = 65) were most closely related (93 to 96 PSI) to an uncultured bacterium (EF630220) that shared 92 PSI with a cultured *Limnothrix*-like species (GenBank accession no. EF088338). The *Limnothrix*-like phylotypes were included in the cal-

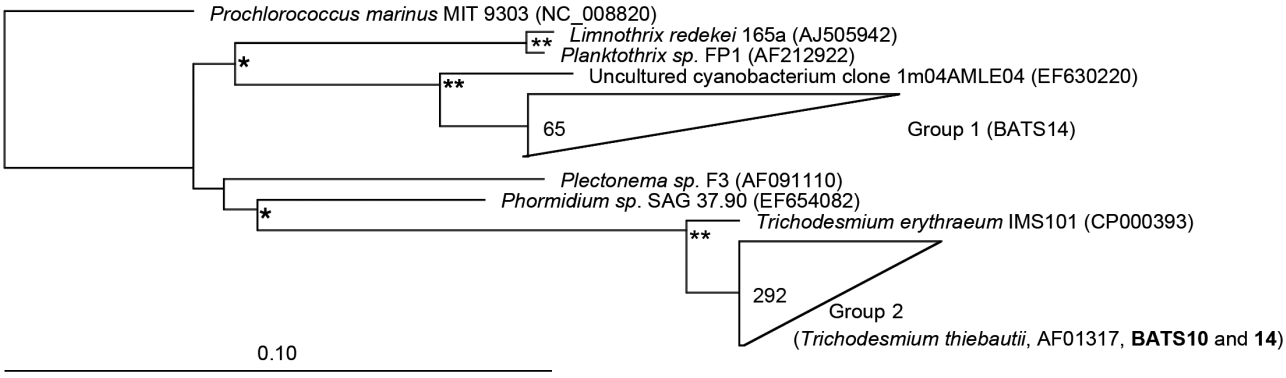

Fig. 1. Phylogenetic tree containing cyanobacterial 16S rRNA gene sequences recovered from *Trichodesmium* colonies in the present study. This tree was created using the neighbor-joining algorithm in ARB and bootstrapped 1000 times using Phylip (version 3.68). *bootstrap values of ≥60%; **bootstrap values of ≥90%. Sequences generated in the present study in **bold**. Accession numbers are in parentheses following all sequences. Sequences within Group 1 are at least 97% identical to the sequence BATS14_298, accession no. GU725542. Sequences within Group 2 are at least 97% identical to the sequence BATS14_001, accession no. GU725789. BATS10 identifies puff-colony library sequences, and BATS14 identifies tuft-colony sequences. The scale bar corresponds to 10 base substitutions per 100 nucleotide positions

culations of epibiotic bacterial richness, evenness, and rarefaction estimations of diversity.

Overview of epibiotic bacteria

The puff-colony and tuft-colony libraries displayed some similarities at the class level (Fig. 2a), although there was significant heterogeneity at more specific ranks. As is evident in Figs. 3 & 4, epibiotic OTUs recovered from the puff colony and the tuft colony are mutually exclusive. The

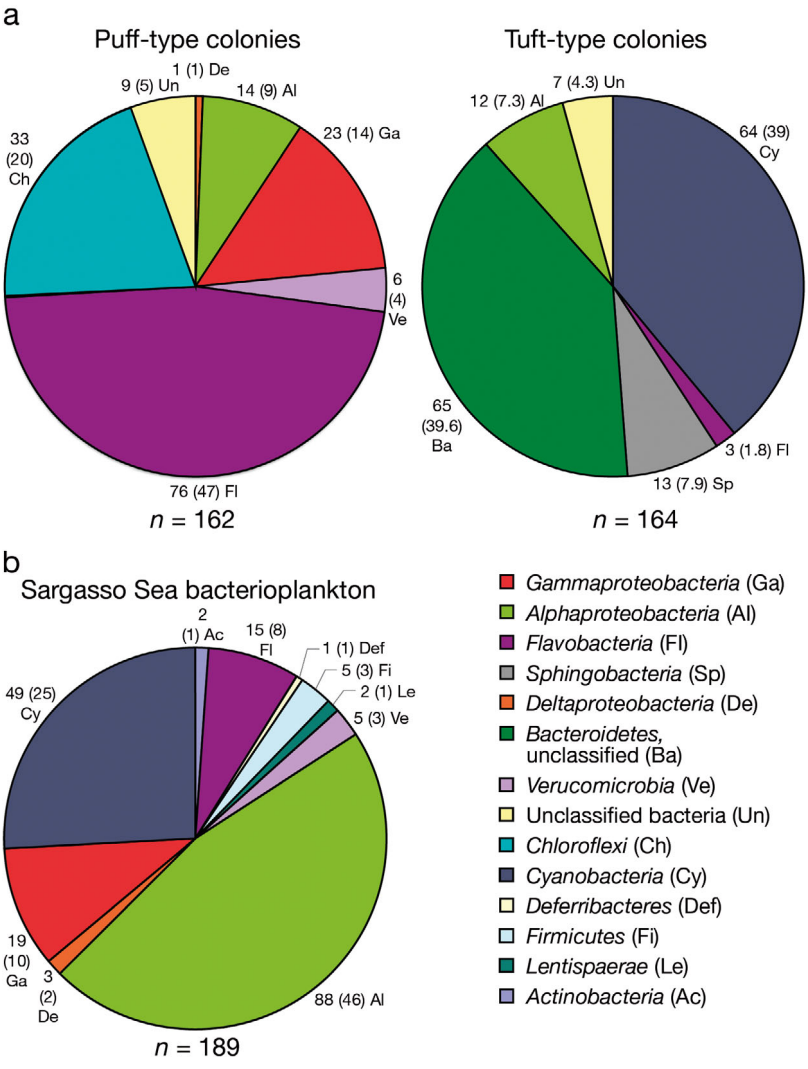

Fig. 2. Class-level 16S rRNA gene sequence-based phylogenetic composition of (a) puff-colony and tuft-colony epibiont communities and (b) Sargasso Sea bacterioplankton (for comparison, data from Carlson et al. 2009). Values listed adjacent to each 'wedge' of the pie chart provide the number of clones in that category followed by the percentage (in parentheses) of the total clones recovered (excluding clones related to *Trichodesmium*). The total number of clones recovered is listed underneath each chart. Classifications were made using the Ribosomal Database Project II automatic classifier. Sequences were assigned to the most specific taxonomic category that could be assigned with 80% bootstrap confidence. For more detailed phylogenetic analysis of 'unclassified' sequences, see Figs. 3 & 4

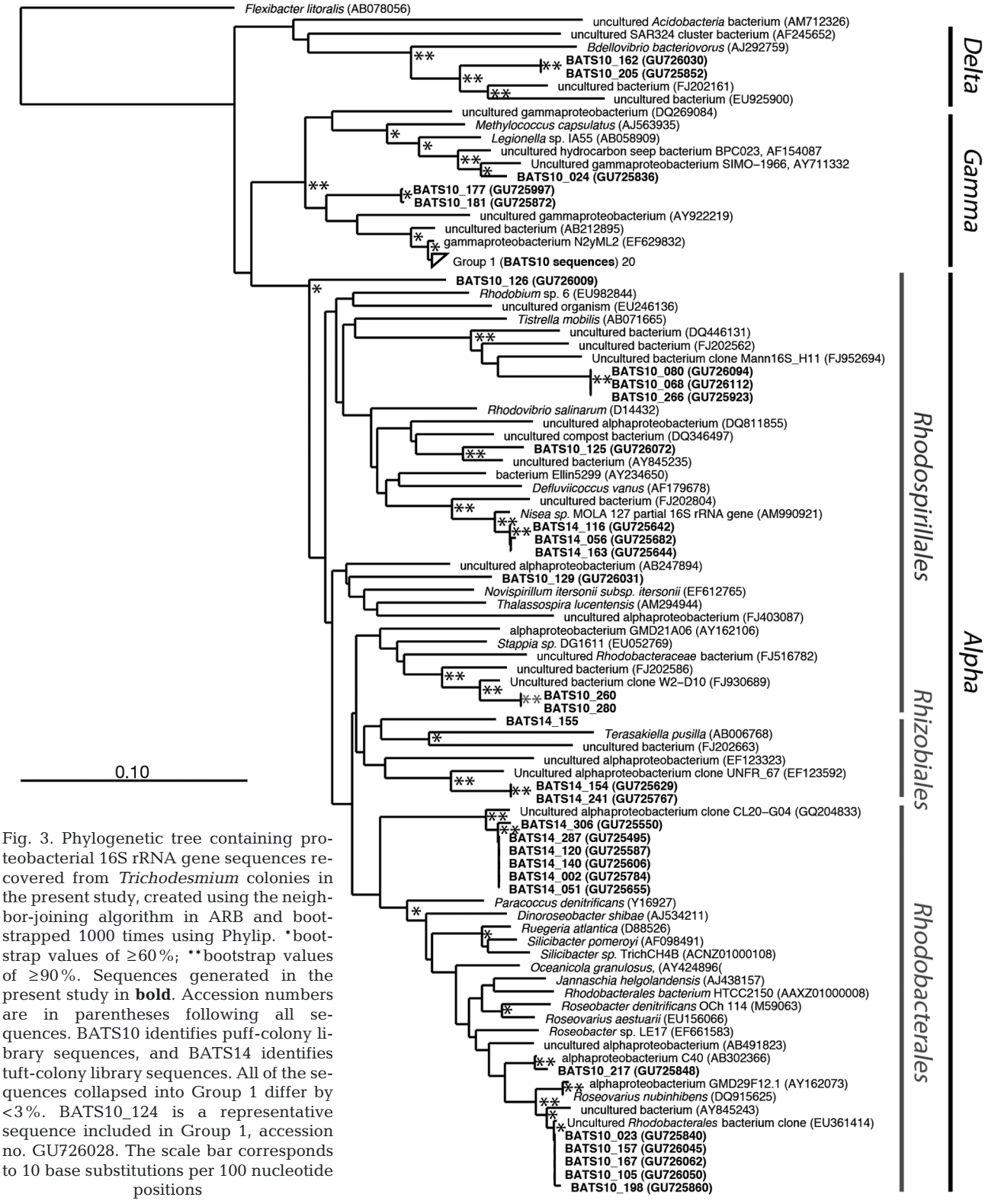

Fig. 3. Phylogenetic tree containing proteobacterial 16S rRNA gene sequences recovered from *Trichodesmium* colonies in the present study, created using the neighbor-joining algorithm in ARB and bootstrapped 1000 times using Phylip. *bootstrap values of ≥60%; **bootstrap values of ≥90%. Sequences generated in the present study in **bold**. Accession numbers are in parentheses following all sequences. BATS10 identifies puff-colony library sequences, and BATS14 identifies tuft-colony library sequences. All of the sequences collapsed into Group 1 differ by <3%. BATS10_124 is a representative sequence included in Group 1, accession no. GU726028. The scale bar corresponds to 10 base substitutions per 100 nucleotide positions

Ribosomal Database Project II Classifier (Cole et al. 2003) was used to assign provisional taxonomic assignments, which were refined using ARB. Using an 80% bootstrap confidence threshold within the RDP II classifier, we determined that *Flavobacteria* composed the greatest percentage (47%) of non-

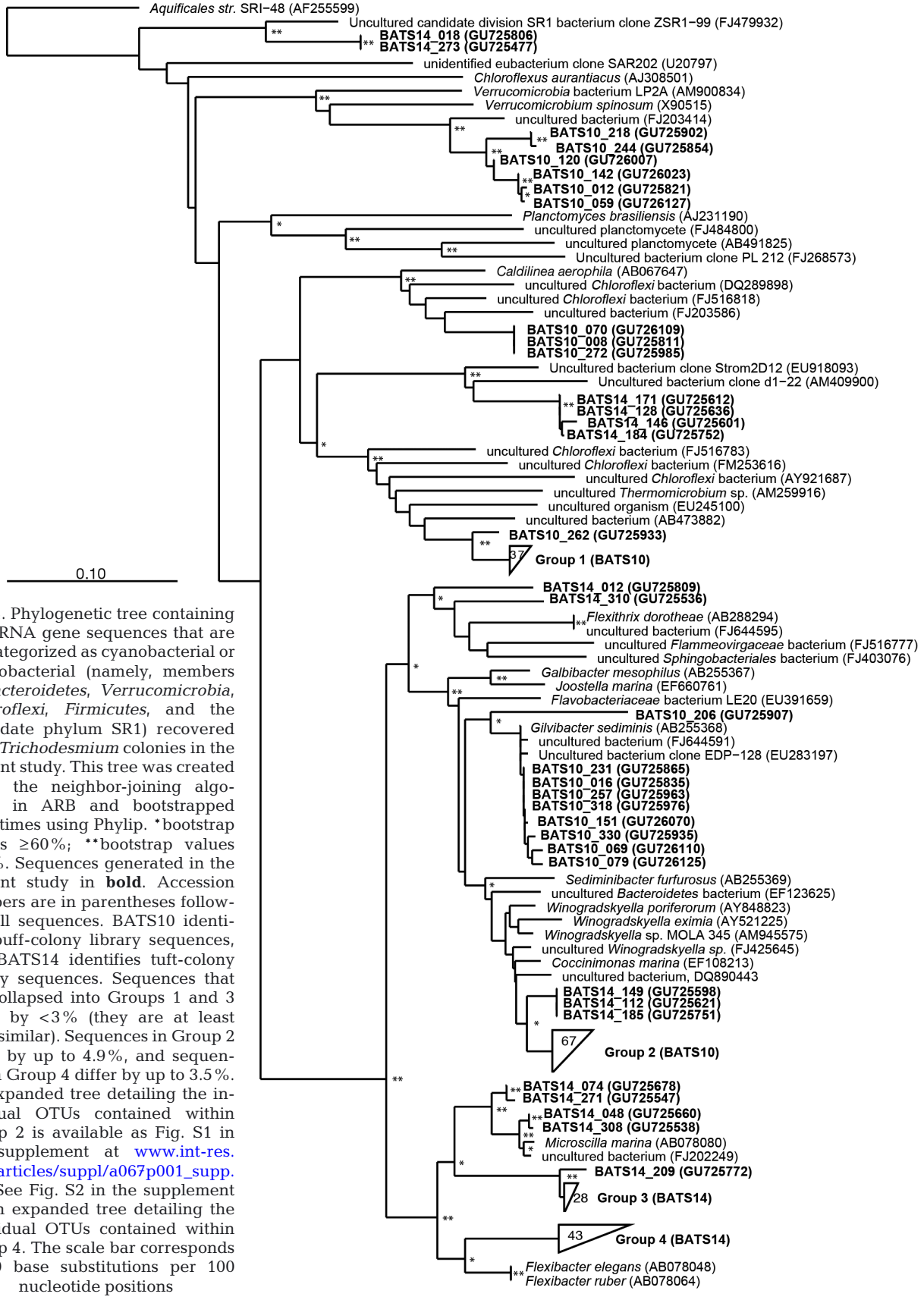

Fig. 4. Phylogenetic tree containing 16S rRNA gene sequences that are not categorized as cyanobacterial or proteobacterial (namely, members of *Bacteroidetes*, *Verrucomicrobia*, *Chloroflexi*, *Firmicutes*, and the candidate phylum SR1) recovered from *Trichodesmium* colonies in the present study. This tree was created using the neighbor-joining algorithm in ARB and bootstrapped 1000 times using Phylip. *bootstrap values ≥60%; **bootstrap values ≥90%. Sequences generated in the present study in **bold**. Accession numbers are in parentheses following all sequences. BATS10 identifies puff-colony library sequences, and BATS14 identifies tuft-colony library sequences. Sequences that are collapsed into Groups 1 and 3 differ by <3% (they are at least 97% similar). Sequences in Group 2 differ by up to 4.9%, and sequences in Group 4 differ by up to 3.5%. An expanded tree detailing the individual OTUs contained within Group 2 is available as Fig. S1 in the supplement at www.int-res.com/articles/suppl/a067p001_supp.pdf. See Fig. S2 in the supplement for an expanded tree detailing the individual OTUs contained within Group 4. The scale bar corresponds to 10 base substitutions per 100 nucleotide positions

cyanobacterial clones from the puff-colony library, with *Chloroflexi* (20%) second most abundant (Fig. 2). *Flavobacteria* were represented in the tuft-colony library (2%), while *Sphingobacteria*, which were entirely absent from the puff-colony library, were represented by 8% of the clone sequences. 'Unclassified' *Bacteroidetes* (unclassifiable at an 80% bootstrap threshold) composed a large percentage (40%) of the total tuft-colony epibiotic community and were most closely related to the class *Sphingobacteria* (see Fig. 4, Groups 3 and 4). *Alphaproteobacteria* composed 7% of tuft-colony clones, while 4% remained unclassified.

Epibiotic *Alphaproteobacteria*

Alphaproteobacteria are represented in both the puff-colony and tuft-colony clone libraries (Fig. 2). The alphaproteobacterial sequences observed in the puff-colony library were dominated by members of the order *Rhodobacterales* (79% of puff-colony associated alphaproteobacterial clones, 50% bootstrap threshold). The remainder of the puff-colony *Alphaproteobacteria* clones were determined to be members of the order *Rhodospirillales* (7%) or were unclassifiable within the *Alphaproteobacteria* with >50% bootstrap confidence (14% of puff-colony alphaproteobacterial clones could not be further classified). Alphaproteobacterial sequences associated with the tuft colonies examined in the present study were also associated with *Rhodobacterales* (50% of total tuft-colony alphaproteobacterial clones) and *Rhizobiales* (8%). In total, 42% of tuft-colony alphaproteobacterial clones were classified as *Rhodospiralles*. Finer scale placement of the *Alphaproteobacteria* clones is shown in Fig. 3.

Eukaryotes and *Archaea*

No gene sequences of archaeal 16S rRNA or eukaryotic 18S rRNA were amplified from the *Trichodesmium* puff-colony or tuft-colony total community DNA extracts, even though amplification of our positive controls was successful.

Clone library coverage, richness, and diversity

Clone libraries from aggregate samples of puff-type and tuft-type *Trichodesmium* colonies were statistically assessed for coverage, richness, and diversity. For comprehensive coverage and richness calculations, we binned sequences by PSI threshold. Richness and diversity data are provided for OTUs at 97, 99, and 100 PSI (Table 1). All sequences related to *T. thiebautii* at the 95 PSI level and higher were subtracted from analyses such that the richness, evenness, and rarefaction estimations of diversity we present pertain only to the epibiotic community.

The coverage of each library was estimated using 2 independent measures that rely on the occurrence of either rare (Good's coverage index) (Good 1953) or abundant (Chao's coverage index) (Chao 1987) OTUs. These estimates indicate that at 97 PSI, the puff-colony library captured 75 to 95% of actual species richness and the tuft-colony library captured a similar range of 68 to 94% of actual richness. These percentages of coverage decrease if calculations are considered at 99 or 100 PSI OTUs (data not shown). A complementary indicator of probable species richness (Chao1) (Chao & Yang 1993) was calculated, and at 97 PSI, the puff-colony library yielded a probable 23 OTUs and the tuft-colony library yielded 21. Due to our library coverage being <100%, the actual measured richness was lower: 18 OTUs were detected in the puff-colony library, and 15 OTUs were detected in the tuft-colony library. These richness values are likely to be low estimates because we chose to analyze our samples as 97 PSI OTUs, and species richness estimates increase if calculations are considered at 99 or 100 PSI OTUs (see Table 1).

Rarefaction curves were calculated for each *Trichodesmium* epibiont library to determine the likelihood that our sampling effort was sufficient to produce an unbiased estimate of species richness and diversity in the samples (Fig. 5). In the case of both libraries, the curves approach an asymptote, which indicates that our sampling effort was sufficient to yield reliable richness predictions. The termini of the rarefaction curves of the tuft-library and puff-library do not overlap (Fig. 5), indicating that the difference in richness between the 2 libraries is significant at the 95% confidence interval. This difference was found not to be significant at the 98% confidence interval (data not shown). The value of the Shannon index, which considers the evenness of OTU distribution in addition to the OTU richness, is slightly higher in the puff-colony library (1.8) compared to the tuft-colony library (1.7); the 95% confidence intervals for these richness estimates overlap, indicating that the difference is not significant.

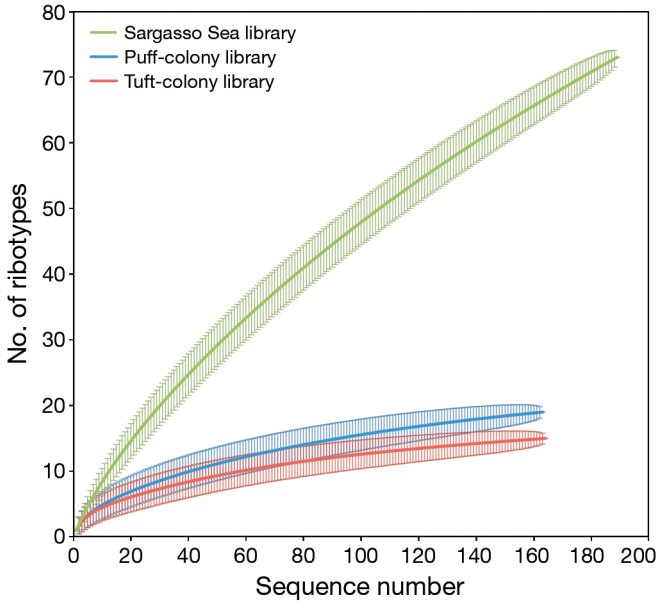

Fig. 5. Rarefaction curve of puff-colony and tuft-colony 16S rRNA gene sequence clone libraries generated in the present study as well as a Sargasso Sea clone library (BATS site, 40 m depth, Carlson et al. 2009). Error bars represent 95% confidence intervals. All *Trichodesmium* phylotypes of ≥95 percent sequence identity present in puff-colony and tuft-colony libraries were excluded from the rarefaction calculation

DISCUSSION

Trichodesmium spp. frequently exist not as individual trichomes but in colonies of trichomes that can occur in distinct morphological varieties and in close association with numerous types of other microbes (Sheridan et al. 2002, Carpenter et al. 2004). It is our observation as well as others (J. Waterbury pers. comm., E. Webb pers. comm.) that *Trichodesmium* spp. are difficult to obtain and maintain in axenic culture, which could imply an essential dependence of *Trichodesmium* spp. on its epibiotic bacteria. Relatively little work has been devoted to the *Trichodesmium* colony as a consortial community. In the present study, we set out to investigate the identity of the bacteria associated with puff-type or tuft-type colonies of wild *Trichodesmium* spp. and how these bacterial communities compared with that of ambient seawater.

It is well known that the Sargasso Sea and other oligotrophic marine biomes can harbor a high diversity of bacterioplankton. Since the foundational work at BATS by Giovannoni et al. (1990) and Chisholm et al. (1988), it has been recognized that the Sargasso Sea is consistently dominated by major lineages of bacteria such as SAR11, *Prochlorococcus*, and *Synechococcus* (Morris et al. 2005, Carlson et al. 2009). Using a dataset published by Carlson et al. (2009), we calculated the estimated richness of Sargasso seawater collected at the BATS site from 40 m and determined a richness estimate of 141 at the 97 PSI threshold (189 clones; Table 1). Similarly, Pommier et al. (2007) found that Sargasso seawater collected at 5 m had an estimated richness (Chao1) of 150 at the 97 PSI threshold (571 clones). Although values from both the Carlson et al. (2009) dataset and Pommier et al. (2007) were based on samples collected on different cruises than our samples of *Trichodesmium* colonies, these estimates of the number of OTUs in seawater exceed our estimates for the puff colonies or tuft colonies (23 and 21 OTUs, respectively) by several fold. Thus, we posit that the number of species (i.e. OTUs at 97 PSI) associated with *Trichodesmium* colonies is likely to be substantially lower than in the surrounding seawater. This suggests that compared to seawater, the phycosphere of *Trichodesmium* selects for a specific subgroup of organisms. We observed a slightly greater species richness in the puff-colony library (18 OTUs were observed at 97 PSI) relative to the tuft-colony library (15 OTUs were observed at 97 PSI), an observation which mirrors the richer community of microzooplankton and metazoa previously documented in puff-type colonies (Sheridan et al. 2002). Comparison of 95% confidence intervals of rarefaction calculations suggests that the observed difference in richness between the 2 libraries is significant (see 'Results' and Fig. 5). Although the observed difference is significant, these data are only representative of colonies sampled on one date at one location. More sampling will be necessary to determine whether higher bacterial community richness on puff colonies relative to tuft colonies is a reproducible characteristic of these communities.

The nutrient and redox conditions of seawater differ quite dramatically from those in the interior of a *Trichodesmium* colony (Paerl & Bebout 1988), and different metabolic strategies are likely to be required to thrive in either setting. In contrast to seawater, the interior of puff- and tuft-type colonies is more reducing (as measured by cellular tetrazolium salt reduction and O_2-microelectrode measurements) and becomes anoxic during the night (Paerl & Bebout 1988, Paerl et al. 1989). For this reason alone, it is not surprising that bacteria commonly observed in marine picoplankton clone library datasets are absent in our *Trichodesmium* clone libraries. Specifically, abundant Sargasso Sea picoplankter ecotypes SAR11, SAR 86, *Prochlorococcus*, or *Synechococcus*

identified by previous workers (Treusch et al. 2009, Carlson et al. 2009) were not detected in our libraries. The OTUs we recovered cluster more closely with clones and isolates from various benthic environments; in particular, we detected numerous clones that are most similar to those associated with sponges (e.g. uncultured *Cyanobacteria* and uncultured *Gammaproteobacteria* corresponding to GenBank accession nos. EF630220 and EF629832; Mohamed et al. 2008) and corals (e.g. uncultured *Gammaproteobacteria* and uncultured *Verrucomicrobia* corresponding to GenBank accession nos. FJ202161 and FJ203414; Sunagawa et al. 2009). We did not find close relationships between members of our library and common surface associated or algal associated organisms (e.g. specific members of the *Roseobacteria* clade [Mayali et al. 2008, Wagner-Döbler et al. 2009] or an epibiont isolated from the surface of *Trichodesmium* in the Caribbean Sea [GenBank accession no. ACNZ01000108]).

A quarter of the clones (excluding those related to *Trichodesmium*) recovered in the puff-colony library shared ≤90 PSI with nearest neighbors in GenBank. The community associated with the tuft colonies contained an even greater proportion of novel sequences; 81% of clone sequences share ≤90 PSI with their neighbors in GenBank. Only 21% and 9% of OTUs from the puff-colony and tuft-colony library, respectively, share 97 PSI or greater with their nearest GenBank relatives. These results are similar to those obtained during a study of the bacterial flora associated with another filamentous N_2-fixing cyanobacterium, *Nodularia*, in which the majority of epibiont clones associated with *Nodularia* represented novel taxa (Tuomainen et al. 2006).

Archaea were conspicuously absent from the *Trichodesmium* colonies. *Euryarchaea* may represent up to 30% of the microbial community in the upper 100 m of the open ocean water column (Frigaard et al. 2006), and *Crenarchaea* are one of the most abundant groups of organisms in the ocean when bathy- and mesopelagic communities are considered (Karner et al. 2001) and can be important nitrifiers (Nicol & Schleper 2006). *T. thiebautii* is known to migrate vertically in the water column over the diel cycle (Villareal & Carpenter 1990), and *Trichodesmium* spp. have been observed at depths of 100 m and greater (Letelier & Karl 1998, Davis & McGillicuddy 2006), the edge of the crenarchaeotal range in oligotrophic waters (Karner et al. 2001). It is interesting that such important nitrifying organisms as the *Crenarchaea* or other relatives to known nitrifying bacteria, such as the genera *Nitrosospira*, or *Nitrosococcus*, appear not to participate in nitrogen cycling in association with the ocean's most abundant nitrogen fixer. It has been posited that light could inhibit the nitrifying processes and could be a major reason why nitrifying *Bacteria* and *Archaea* are typically not found in the euphotic zone (Ward 1985, Mincer et al. 2007). In the case of *Trichodesmium,* which may have the ability to vertically migrate to higher light regions during daylight hours via gas vesicle buoyancy (Villareal & Carpenter 1990), this could be a particularly difficult environment for a nitrifier to inhabit. Our lack of archaeal 16S rRNA gene sequence signal in our libraries is echoed in our recent and ongoing metagenomic characterization of wild *Trichodesmium* spp., where *Archaea* were also found to be absent or exceedingly rare (T. Mincer, S. Dyhrman & B. Van Mooy unpubl. data).

There have been reports of eukaryotes associated with *Trichodesmium* colonies, but despite our best efforts, we were unable to amplify any eukaryotic SSU rRNA gene sequences from either the tuft or puff *Trichodesmium* community DNA (e.g. Sheridan et al. 2002). It is possible that eukaryotes may be less tightly associated with the colonies and more easily separated during the sample preparation process. It is important to consider that our samples were collected under non-bloom conditions, and the contribution of *Archaea* and eukaryotes might increase during a bloom event.

We detected several types of bacteria that are seemingly ubiquitous on living surfaces in the ocean, namely, *Alphaproteobacteria*, *Gammaproteobacteria*, and members of the *Bacteroidetes*. The presence of *Alphaproteobacteria* is not surprising as many members of this group are opportunistic colonizers and opportunitrophs (Polz et al. 2006) known to colonize surfaces and form biofilms (Buchan et al. 2005, Dang et al. 2008). Members of the order *Rhodobacterales*, which comprise a majority of the puff-colony-associated alphaproteobacterial sequences (79%) and half of the tuft-colony-associated alphaproteobacterial sequences (50%) detected in the present study, have been identified as ubiquitous early colonizers of surfaces in the oceans (Dang et al. 2008); it is striking that the *Rhodobacterales* contribute to only half of the alphaproteobacterial clones associated with the tuft-colony sample. Members of a specific clade within the *Rhodobacterales*, the *Roseobacter* clade, are of particular interest from the standpoint of microbial community development as they are known to produce antibacterial compounds and have been shown to communicate using acylated homoserine lactones,

activities which may considerably impact the development of a biofilm community (Gram et al. 2002, Dang et al. 2008, Hmelo 2010).

In addition to *Alphaproteobacteria*, we detected *Gammaproteobacteria* and *Deltaproteobacteria* in the puff-colony library. *Gammaproteobacteria*, belonging to the *Thiotrichales*, were detected only in the puff-colony library (no *Gammaproteobacteria* were detected in the tuft-colony library), which was surprising because *Gammaproteobacteria* are generally very common on marine surfaces, including particles and particularly algae (Fisher et al. 1998, Dang & Lovell 2000, Tuomainen et al. 2006, Sapp et al. 2007, Dang et al. 2008). Two deltaproteobacterial clones were detected within the puff-colony library displaying closest relatives in GenBank to uncultivated organisms cloned from benthic environments (e.g. sponges, sediments, and biofilms at hydrothermal vents). The nearest cultivars of these deltaproteobacterial clones were related to the bactivorous *Bdellovibrio* genus at 87%; however, based upon these data, it is unclear if bactivory occurs in the *Trichodesmium* phylosphere.

In our tuft-colony samples, we observed abundant cyanobacterial community members in addition to *Trichodesmium*, including a group distantly related to *Limnothrix* (92 PSI). Cyanobacterial filaments described as *Phormidium*-like or *Plectonema*-like have been reported in association with *Trichodesmium* colonies numerous times (Paerl et al. 1989, Siddiqui et al. 1992, Dyhrman et al. 2002, Hewson et al. 2009, Hynes et al. 2009), although to our knowledge, no absolute phylogenetic identification has been ascribed to this group. In contrast to our tuft-colony sample, we detected no cyanobacterial OTUs other than those related to *T. thiebautii* in the puff-colony sample. However, in the puff-colony sample, we did detect significant numbers of OTUs related to other possible phototrophic groups, such as the members of the *Roseobacter* clade of the *Alphaproteobacteria*, known to be aerobic anoxygenic phototrophs (Buchan et al. 2005). Within the tuft-library, we did not detect OTUs that clustered with *Roseobacter*-clade genera (Buchan et al. 2005).

In one published report within which puffs and tufts were examined independently, Sheridan et al. (2002) noted that the matrix of puff colonies contained bacteria and microflagellates, whereas the matrix of tuft colonies contained bacteria and cyanobacterial filaments. Siddiqui et al. (1992) noted that while contaminating cyanobacterial filaments were present within both colony types, they were significantly more abundant and more reliably associated with tuft colonies (Sheridan et al. 2002). Thus, both our observations and those from the literature indicate that it may be common for tuft colonies to host additional cyanobacterial species other than *Trichodesmium*, although the role of these additional *Cyanobacteria* remain unclear.

The 2 libraries constructed in the present study, representative of the 2 colony morphotypes collected at BATS, do not contain any common OTUs at the 97 PSI threshold. Previous research has concluded that bacterial density (Sheridan et al. 2002), enzyme activities (Nausch 1996, Stihl et al. 2001), and redox gradients from the outside to the inside of the colony (Paerl et al. 1989) differ substantially between puff and tuft colonies. These variations should lead to significant differences in the types of ecological niches provided by the 2 types of colonies. Our data are thus consistent with the hypothesis that puff colonies and tuft colonies can host very different environments on a microbial scale. However, our samples were aggregated from 10 morphologically identical colonies, and thus, we cannot speculate as to the extent of colony-to-colony variability within a given morphotype. An alternative albeit unlikely explanation that would also satisfy our data is that tuft and puff colonies are ecologically equivalent and that all 20 individual colonies collected in the present study host mutually exclusive bacterial flora.

A recent survey of the microbial diversity associated with the marine alga *Ulva australis* determined that 6 individuals selected from the same waters had nearly mutually exclusive flora associated with them (Burke et al. 2011). In turn, algal-associated species were mutually exclusive with seawater species (Burke et al. 2011). Burke et al. (2011) propose an explanation drawing on lottery competition theory (Sale 1976). They suggest that a guild of bacteria, a group that contains a high degree of ecological redundancy, exists whose members could all exploit the unique niche provided by the surface of *U. australis*. We suggest that this same mechanism may explain the differences we observed between our puff- and tuft-colony clone libraries if the 2 types of colonies provide equivalent niches. Perhaps the essential services required by *Trichodesmium* can be fulfilled by a variety of bacteria that all compete for settlement on its surface, consistent with lottery competition theory. This hypothesis remains to be tested by evaluating the bacterial communities associated with a number of individual colonies of both morphotypes. Unfortunately, we were unable to extract a sufficient amount of epibiont DNA from a single colony to perform this analysis.

The high species richness of seawater reflects a great number of low-abundance free-living bacteria, many of which may not actively participate in the biogeochemical cycling of elements in seawater (Pedrós-Alió 2006). The bacteria that are abundant on *Trichodesmium* may have at one time been 'seeded' from these low-abundance members of the seawater community (i.e. the ones least likely to be represented in a cultivation-independent analysis of seawater). From our data, it is impossible to determine where the seedbank for the unusual *Trichodesmium* associated sequences lie, whether within the *Trichodesmium* particulate community itself or the within the community of rare OTUs associated with the ambient seawater community.

In conclusion, the data we present here indicate that *Trichodesmium*-associated bacterial communities are distinct from other marine pelagic and benthic microbial communities. Both the lack of major seawater taxa and the high abundance of novel OTUs suggest that the bacterial epibionts of *Trichodesmium* spp. inhabit a unique niche which remains to be explored. These data provide a phylogenetic basis for investigations of these adaptive strategies.

*Acknowledgements. W*e gratefully acknowledge the assistance of M. Miller for microbial strain maintenance and media preparation. We also thank E. Webb and 2 anonymous reviewers, whose comments significantly improved this manuscript. We are also grateful to M. Lomas and the BATS team for affording us a berth on their cruise and for the assistance of the captain and crew of the RV 'Atlantic Explorer'. This work was funded by grants to T.J.M. and B.A.S.V.M. from the WHOI Ocean Life Institute and the National Science Foundation (OCE-0825407) and an award to T.J.M. from The Penzance Endowed Fund in Support of Assistant Scientists.

LITERATURE CITED

Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W, Lipman J (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Behrens S, Losekann T, Pett-Ridge J, Weber PK and others (2008) Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced element labeling-catalyzed reporter deposition fluorescence *in situ* hybridization (EL-FISH) and NanoSIMS. Appl Environ Microbiol 74:3143–3150

Berman-Frank I, Rosenberg G, Levitan O, Haramaty L, Mari X (2007) Coupling between autocatalytic cell death and transparent exopolymeric particle production in the marine cyanobacterium *Trichodesmium.* Environ Microbiol 9:1415–1422

Buchan A, Gonzalez JM, Moran MA (2005) Overview of the marine *Roseobacter* lineage. Appl Environ Microbiol 71: 5665–5677

Burke C, Thomas T, Lewis M, Steinberg P, Kjelleberg S (2011) Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis.* ISME J 5:590–600

Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ (1997) *Trichodesmium*, a globally significant marine cyanobacterium. Science 276:1221–1229

Carlson CA, Morris R, Parsons R, Treusch AH, Giovannoni SJ, Vergin K (2009) Seasonal dynamics of SAR11 populations in the euphotic and mesopelagic zones of the northwestern Sargasso Sea. ISME J 3:283–295

Carpenter EJ, Harvey HR, Fry B, Capone DG (1997) Biogeochemical tracers of the marine cyanobacterium *Trichodesmium.* Deep-Sea Res I 44:27–38

Carpenter EJ, Subramaniam A, Capone DG (2004) Biomass and primary productivity of the cyanobacterium *Trichodesmium* spp. in the tropical N Atlantic ocean. Deep-Sea Res I 51:173–203

Chao A (1987) Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43: 783–791

Chao A, Yang MCK (1993) Stopping rules and estimation for recapture debugging with unequal failure rates. Biometrika 80:193–201

Chisholm S, Olson R, Zettler E, Goericke R (1988) A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334:340–343

Cole JR, Chai B, Marsh TL, Farris RJ and others (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31: 442–443

Dang H, Lovell CR (2000) Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl Environ Microbiol 66:467–475

Dang H, Li T, Chen M, Huang G (2008) Cross-ocean distribution of *Rhodobacterales* bacteria as primary surface colonizers in temperate coastal marine waters. Appl Environ Microbiol 74:52–60

Davis CS, McGillicuddy DJ Jr (2006) Transatlantic abundance of the N_2-fixing colonial cyanobacterium *Trichodesmium.* Science 312:1517–1520

DeLong EF, Preston CM, Mincer T, Rich V and others (2006) Community genomics among stratified microbial assemblages in the ocean's interior. Science 311: 496–503

Dyhrman ST, Webb EA, Anderson DM, Moffett JW, Waterbury JB (2002) Cell-specific detection of phosphorus stress in *Trichodesmium* from the western North Atlantic. Limnol Oceanogr 47:1832–1836

Fisher MM, Wilcox LW, Graham LE (1998) Molecular characterization of epiphytic bacterial communities on charophycean green algae. Appl Environ Microbiol 64: 4384–4389

Frigaard NU, Martinez A, Mincer TJ, DeLong EF (2006) Proteorhodopsin lateral gene transfer between marine planktonic Bacteria and Archaea. Nature 439:847–850

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63

Good IJ (1953) The population frequencies of species and the estimation of population parameters. Biometrika 40: 237–264

Gram L, Grossart HP, Schlingloff A, Kiorboe T (2002) Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. Appl Environ Microbiol 68: 4111–4116

Herbst V, Overbeck J (1978) Metabolic coupling between the alga *Oscillatoria redekei* and accompanying bacteria. Naturwissenschaften 65:598–599

Hewson I, Govil SR, Capone DG, Carpenter EJ, Fuhrman JA (2004) Evidence of *Trichodesmium* viral lysis and potential significance for biogeochemical cycling in the oligotrophic ocean. Aquat Microb Ecol 36:1–8

Hewson I, Poretsky RS, Dyhrman ST, Zielinski B and others (2009) Microbial community gene expression within colonies of the diazotroph, *Trichodesmium*, from the Southwest Pacific Ocean. ISME J 3:1286–1300

Hmelo L (2010). Microbial interactions associated with biofilms attached to *Trichodesmium* spp. and detrital particles in the ocean. PhD thesis, Massachusetts Institute of Technology, Woods Hole, MA

Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317–2319

Hynes AM, Chappell PD, Dyhrman ST, Doney SC, Webb EA (2009) Cross-basin comparison of phosphorus stress and nitrogen fixation in *Trichodesmium.* Limnol Oceanogr 54:1438–1448

Karl D, Letelier R, Tupas L, Dore J, Christian J, Hebel D (1997) The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature 388:533–538

Karner MB, DeLong EF, Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409:507–510

Letelier R, Karl D (1996) Role of *Trichodesmium* spp. in the productivity of the subtropical North Pacific Ocean. Mar Ecol Prog Ser 133:263–273

Letelier RM, Karl DM (1998) *Trichodesmium* spp. physiology and nutrient fluxes in the North Pacific subtropical gyre. Aquat Microb Ecol 15:265–276

Ludwig W, Strunk O, Westram R, Richter L and others (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Mayali X, Franks PJS, Azam F (2008) Cultivation and ecosystem role of a marine *Roseobacter* clade-affiliated cluster bacterium. Appl Environ Microbiol 74: 2595–2603

Mincer TJ, Church MJ, Taylor LT, Preston C, Karl DM, DeLong EF (2007) Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. Environ Microbiol 9: 1162–1175

Mohamed NM, Enticknap JJ, Lohr JE, McIntosh SM, Hill RT (2008) Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. Appl Environ Microbiol 74:1209–1222

Morris R, Vergin K, Cho J, Rappé M (2005) Temporal and spatial response of bacterioplankton lineages to annual convective overturn at the Bermuda Atlantic Time-series study site. Limnol Oceanogr 50:1687–1696

Nausch M (1996) Microbial activities on *Trichodesmium* colonies. Mar Ecol Prog Ser 141:173–181

Nicol GW, Schleper C (2006) Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? Trends Microbiol 14:207–212

Paerl HW (1982). Interactions with bacteria. In: Carr NG, Whitton BA (eds) The biology of cyanobacteria. University of California Press, Los Angeles, CA, p 441–461

Paerl HW, Bebout BM (1988) Direct measurement of O_2-depleted microzones in marine *Oscillatoria*: relation to N_2 fixation. Science 241:442–445

Paerl HW, Gallucci KK (1985) Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial-bacterial association. Science 227:647–649

Paerl HW, Bebout BM, Prufert LE (1989) Bacterial associations with marine *Oscillatoria* sp. (*Trichodesmium* sp.) populations: ecophysiological implications. J Phycol 25: 773–784

Pedrós-Alió C (2006) Marine microbial diversity: Can it be determined? Trends Microbiol 14:257–263

Pielou EC (1966) The measurement of diversity in different types of biological collections. J Theor Biol 13:131–144

Polz MF, Hunt DE, Preheim SP, Weinreich DM (2006) Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. Philos Trans R Soc Lond B 361:2009–2021

Pommier T, Canback B, Riemann L, Bostrom K and others (2007) Global patterns of diversity and community structure in marine bacterioplankton. Mol Ecol 16:867–880

Roe KL, Barbeau K, Mann EL, Haygood MG (2012) Acquisition of iron by *Trichodesmium* and associated bacteria in culture. Environ Microbiol 14:1681–1695

Sale P (1976) Reef fish lottery. Nat Hist 85:60–65

Sapp M, Schwaderer AS, Wiltshire KH, Hoppe HG, Gerdts G, Wichels A (2007) Species-specific bacterial communities in the phycosphere of microalgae? Microb Ecol 53: 683–699

Sheridan C, Steinberg D, Kling G (2002) The microbial and metazoan community associated with colonies of *Trichodesmium* spp.: a quantitative survey. J Plankton Res 24:913–922

Siddiqui P, Bergman B, Carpenter E (1992) Filamentous cyanobacterial associates of the marine planktonic cyanobacterium *Trichodesmium*. Phycologia 31:326–337

Simmons TL, Coates RC, Clark BR, Engene N and others (2008) Biosynthetic origin of natural products isolated from marine microorganism-invertebrate assemblages. Proc Natl Acad Sci USA 105:4587–4594

Sohm JA, Webb EA, Capone DG (2011) Emerging patterns of marine nitrogen fixation. Nat Rev Microbiol 9:499–508

Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF (1996) Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. J Bacteriol 178: 591–599

Stevenson BS, Waterbury JB (2006) Isolation and identification of an epibiotic bacterium associated with heterocystous *Anabaena* cells. Biol Bull 210:73–77

Stihl A, Sommer U, Post AF (2001) Alkaline phosphatase activities among populations of the colony-forming diazotrophic cyanobacterium *Trichodesmium* spp. (Cyanobacteria) in the Red Sea. J Phycol 37:310–317

Sunagawa S, DeSantis TZ, Piceno YM, Brodie EL and others (2009) Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata.* ISME J 3:512–521

Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62: 625–630

Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res 30:2083–2088

Treusch AH, Vergin KL, Finlay LA, Donatz MG and others (2009) Seasonality and vertical structure of microbial communities in an ocean gyre. ISME J 3:1148–1163

Tuomainen J, Hietanen S, Kuparinen J, Martikainen PJ, Servomaa K (2006) Community structure of the bacteria associated with *Nodularia* sp. (Cyanobacteria) aggregates in the Baltic Sea. Microb Ecol 52:513–522

Villareal TA, Carpenter EJ (1990) Diel buoyancy regulation in the marine diazotrophic cyanobacterium *Trichodesmium thiebautii*. Limnol Oceanogr 35:1832–1837

Wagner-Döbler I, Ballhausen B, Berger M, Brinkhoff T and others (2009) The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea. ISME J 4:61–77

Ward BB (1985) Light and substrate concentration relationships with marine ammonium assimilation and oxidation rates. Mar Chem 16:301–316

Waterbury JB (1991). The cyanobacteria: isolation, purification, and identification. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The prokaryotes, 2nd edn. Springer-Verlag, New York, NY, p 2058–2078

Yu Y, Breitbart M, McNairnie P, Rohwer F (2006) FastGroupII: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. BMC Bioinformatics 7:57 doi:10.1186/1471-2105-7-57

Editorial responsibility: Douglas Capone, Los Angeles, California, USA

Submitted: August 1, 2011; Accepted: May 1, 2012
Proofs received from author(s): July 3, 2012