

Coral-associated Archaea

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ABSTRACT: The coral holobiont includes the coral, zooxanthellae, fungi, endolithic algae, and >30 species of Bacteria. Using culture-independent techniques, we now show that Archaea are also abundant and widespread on corals. Sequence analyses of Archaea on 3 species of Caribbean corals revealed that coral-associated Archaea are novel, diverse, and include representatives from both the Crenarchaeota and Euryarchaeota. Unlike zooxanthellae and Bacteria, the Archaea do not appear to form species-specific associations with reef-building corals. Fluorescent *in situ* hybridizations with peptide nucleic acid (PNA) probes showed that Archaea were present at >10⁷ cells cm⁻² on *Porites astreoides*, comprising nearly half of the prokaryotic community. This study and one by Kellogg (Mar Ecol Prog Ser 273:81–88) show that Archaea are abundant, diverse, and potentially important components of the coral holobiont.

KEY WORDS: Coral · Archaea · 16S rDNA · Fluorescent *in situ* hybridization · FISH · Peptide nucleic acid probe · PNA

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INTRODUCTION

Archaea are prokaryotes representing a third Domain of life and are genetically distinct from Bacteria and Eukaryotes (Woese et al. 1990). Three kingdoms have been assigned to Archaea: the Crenarchaeota, the Euryarchaeota (Woese et al. 1990), and the Korarchaeota (Brans et al. 1996). Initially Archaea were believed to be limited to anaerobic, hyperthermal, and highly saline habitats (DeLong 1998). However, culture-independent techniques based on 16S rDNA analyses have shown that Archaea are common in marine and other environments (DeLong 1992, Fuhrman et al. 1992, 1993, 1994, DeLong et al. 1994, Fuhrman & Davis 1997, Massana et al. 1997, 2000, Murray et al. 1998, 1999, Karner et al. 2001). Archaea have been shown to reside in coastal and offshore temperate waters worldwide (DeLong 1992, Fuhrman et al. 1992, 1993, Massana et al. 1997), Antarctic surface water (DeLong et al. 1994, Murray et al. 1998, 1999), and circumpolar deep ocean waters (DeLong et al.

1994, Murray et al. 1998, 1999). Pelagic Archaea may numerically dominate the marine plankton inhabiting meso- and bathypelagic zones of the ocean (Fuhrman et al. 1993, 1994, Karner et al. 2001). Marine Archaea are represented by 3 groups: Marine Group I (Crenarchaeotes) and Marine Group II (Euryarchaeotes; DeLong 1992), as well as Marine Group III, Euryarchaeotes, representing a third lineage of marine Archaea found in deep sea samples collected from both the Atlantic and Pacific Oceans (Fuhrman & Davis 1997).

Archaea form symbiotic relationships with macroorganisms, the best studied being euryarchaeal methanogens that live symbiotically in ruminants (Smith & Hungate 1958). The first characterization of crenarchaeal symbiosis was described when the Marine Group I related archaeon *Cenarchaeum symbiosum* was shown to have a highly specific relationship over space and time with the marine sponge *Axinella mexicana* (Preston et al. 1996). Subsequent studies revealed that other closely related Crenar-

chaetotes were associated with 3 other *Axinella* sponges (Margot et al. 2001). Each species of *Axinella* was inhabited by a specific archaeal phylotype, suggesting a consistent association of Crenarchaeotes with *Axinella* sponges (Margot et al. 2001). It has also been shown that both Euryarchaeotes and Crenarchaeotes are associated with specific tissues on the Great Barrier Reef sponge *Rhopaloeides odorabile*, indicating the possibility of a beneficial relationship between host and microbiota (Webster et al. 2001).

Corals form symbioses with algae and prokaryotes. They live symbiotically with zooxanthellae (unicellular dinoflagellates) and display patterns of specific associations that are often related to depth (Rowan & Knowlton 1995a,b, Rowan et al. 1997, Rowan 1998). On the Great Barrier Reef, for example, numerous species of coral host different taxa of algal symbionts in shallow versus deep water (Baker 1997). Symbiotic relationships and specific associations between corals and their bacterial communities have also been described (Peters 1984, Shashar et al. 1994, Ritchie & Smith 1995, 1997, Santavy 1995, Rohwer et al. 2001, 2002). Different species of nitrogen-fixing bacteria are associated with certain corals and or their skeletons (Williams et al. 1987, Shashar et al. 1994, Kuhl et al. 1995). Santavy (1995) demonstrated that a bacterial species forms ovoids inside *Porites astreoides* and appears to be a normal component of the coral's life. More recently, a γ -proteobacterium designated PA1 was detected on *Porites* spp. in both Panama and Bermuda, suggesting that coral-bacterial associations are maintained across distant locations (Rohwer et al. 2002). Also, *Montastrea franksi* appears to harbor a specific α -proteobacteria (Rohwer et al. 2001). Corals are also associated non-specifically with other organisms, including the protist Apicomplexa (Toller et al. 2002), several types of fungi (Le Campion-Alsumard et al. 1995a,b, Bentsis et al. 2000), and endolithic algae (Odum & Odum 1955, Shashar et al. 1997). Here we show that Archaea are diverse and abundant on reef building corals. A similar study by Kellogg (2004, this volume) reaches similar conclusions based on different coral species from the U.S. Virgin Islands.

MATERIALS AND METHODS

Sample collections and DNA preparations. Coral samples were collected at Whale Bone Bay, Bermuda (August 1999) and Bocas del Toro, Panama (April 1999 and June 2000). A punch and hammer was used to remove 1.3 cm diameter cores from *Colpophyllia natans*, *Porites furcata*, *Diploria strigosa*, *Montastrea franksi*, and *Porites astreoides*. The latter 3 species were sampled in both Bermuda and Panama, while *C.*

natans and *P. furcata* were only sampled in Panama. *Acropora cervicornis* and *Acropora prolifera* samples were collected using bone clippers from La Parguerra, Puerto Rico (January 2002). After harvesting, the samples were immediately placed in a Ziploc bag underwater. At the surface each sample was washed with 0.2 μ m filtered and autoclaved seawater to remove any loosely associated microbes, placed on ice, returned to the lab, and frozen. Samples of *P. astreoides* and *D. strigosa* from Panama were also placed in 2% paraformaldehyde and stored at 4°C for the fluorescent *in situ* hybridizations (FISH).

For the DNA extraction, each frozen coral sample was airbrushed (<2.7 bar) with 10 \times TE [100 mM Tris (hydroxymethyl)aminomethane hydrochloride (pH 8.0)/10 mM ethylenediaminetetraacetic acid (EDTA)] to remove the tissue and associated microbes. Two ml of the coral tissue/TE slurry was pelleted for 30 min at 10 000 $\times g$ at 4°C. Total DNA was extracted from the pellet using the Ultra Clean Soil DNA Kit (Mo Bio).

To check the DNA, partial bacterial 16S rDNA were PCR amplified using the Bacteria-specific 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') and the universal 1492R primer (5'-TACGGYTACCTTGTTACGACTT-3'; Amann, et al. 1995). The PCR reaction contained 1 \times REDTaq Buffer, 1 U REDTaq (Sigma), 200 μ M each dNTP, 1 μ M each primer, ~1 μ g target DNA (5 min at 94°C, 30 cycles of: 1 min 94°C, 1 min 65°C – 0.5°C per cycle, 3 min at 72°C, 10 min at 72°C).

Analyses of Archaea 16S rDNA. DNA from samples that were positive for bacterial DNA were then PCR amplified using the 21F (5'-TTCCGGTTGATCCYGC-CGGA-3'; DeLong 1992) and 915R (5'-GTGCTGCC-CGCCAATTCCT-3'; Stahl & Amann 1991) Archaea-specific primers. PCR products positive for archaeal DNA from both Bermuda and Panama were cloned into pGem Easy Vector as recommended by the manufacturer (Promega). To minimize the effect of overamplification of rare sequences in the latter cycles of PCR, 1/10 dilutions of the input DNA were made. The dilution that gave a detectable signal was used in the subsequence cloning and sequencing.

Transformants were selected for on Luria-Bertani (LB)/ampicillin plates with X-gal (5-bromo-4-chloro-indoly β -D-galacto-pyranoside). White colonies were picked into 96-well plates and subjected to PCR with M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGAC-3') to check for inserts. The PCR products that contained inserts were cleaned using the Ultra Clean PCR Clean-Up Kit (Mo Bio) and sequenced using the 21F primer.

Analysis of 16S rDNA sequences. FastGroup was used to dereplicate the libraries of 16S rDNA sequences (Seguritan & Rohwer 2001). FastGroup dereplicates 16S rDNA libraries by comparing all the

sequences in a data set to each other, grouping similar sequences together, and outputting a representative sequence from each group. For this study, ambiguous bases (N) were removed at the 5' end, sequences were trimmed to the 534 conserved site from the 3' end, and sequences >97% identical were placed in the same group (Seguritan & Rohwer 2001). Sequences containing many N's and those less than 300 bp in length were also removed by FastGroup. BLASTN (www.ncbi.nlm.nih.gov/blast/blast.cgi) was then used to characterize each sequence group. Groups identified as cloning vector sequences were removed from the data set. The identity of each sequence was also determined using ARB (www.arb-home.de/).

Richness and microbial diversity predictions.

Chao1 was used to estimate the number of species (i.e. species richness) of the archaeal communities associated with *Montastrea franksi*, *Diporia strigosa*, and *Porites astreoides* (Chao 1984, Colwell & Coddington 1994).

$$S_{\text{chao1}} = S_{\text{obs}} + n_1^2 / 2n_2$$

S_{chao1} is the species richness calculated using the occurrence of singlets (n_1) and doublets (n_2) and the number of different groups observed in the library (S_{obs}). Diversity of the microbial populations associated with these same corals was characterized using the Shannon-Wiener index (H' ; Shannon & Weaver 1949), where P_i is the percent of the total number of sequences for 1 group.

$$H' = -\sum [P_i(\ln P_i)]$$

Fluorescent *in situ* hybridizations (FISH) using peptide nucleic acid (PNA) probes. Coral samples preserved in 2% paraformaldehyde were airbrushed (<2.7 bar) into a Ziploc bag with filtered autoclaved seawater (FASW). The total volume of tissue slurry was recorded for subsequent calculations of number of cells per cm² coral surface. Tissue slurries were stored at 4°C. One hundred µl of coral tissue slurry was diluted into 5 ml of 0.2 µm filtered phosphate buffered saline (PBS) and vacuum filtered (<0.7 bar) onto a 0.2 µm Anodisc (Whatman) using a tower. The filters were washed with 5 ml PBS and then treated with 2 ml of 50% ethanol (-20°C) for 5 min. Filters were washed again with 5 ml PBS, placed into a sterile Petri dish, then 100 µl of hybridization buffer from the PNA Micro Dx FISH Reagent Kit (Applied Biosystems) containing the PNA probe (Applied Biosystems), 0.25 to 0.5 µM final concentration, was pipetted onto the center of the filter. Filters were incubated in the dark for 30 min at 45°C (Archaea-specific probe: 5'-TGC TCC CCC GCC AAT TCC-3'; DeLong 1992; fluorescein labeled) or 55°C (EuBacteria-specific probe: 5'-CTG CCT CCC GTA GGA-3'; Perry-O'Keefe et al. 2001; Cy3 or fluo-

rescein labeled). To ensure specificity of PNA probes, control hybridizations using the *Halobacterium* sp. and *Salmonella* sp. were run concurrently with the coral hybridizations. A no-probe hybridization was also run with each coral sample to compare fluorescence from the probe against the auto-fluorescent properties of the coral tissues. There was an easily discernible difference in the signal between the probe positive and no-probe hybridizations. Filters were washed 3 times at 45°C (Archaea) or 55°C (Bacteria) for 10 min with 5 ml pre-warmed wash buffer from the PNA Micro Dx FISH Reagent Kit. After the washing step, the filters were dried in a 37°C incubator and counterstained with DAPI (4',6'-diamidino-2-phenylindole; 5 µg ml⁻¹). Filters were mounted onto slides with 20 µl of anti-fade solution (50% glycerol, 50% PBS and 0.01% ascorbic acid).

Cells were visualized using a Leica DM RBE microscope equipped for epifluorescence with a mercury bulb (50 W) and filter set XF57-1 (Omega). Images were captured using a charged coupled device (CCD) camera (Olympus America) using the DAPI filters, as well as the fluorescein or Cy3 filter set for probe specific cell counts. Cells positive for both DAPI and probe were counted in 10 to 20 fields selected at random.

Calculation of coral surface area. To determine the surface area of coral samples a standard curve of measured aluminum foil size (mm²) and weight (mg) was constructed. Pieces of foil were cut at different sizes from 1 to 5 cm² and weighed to determine the relationship of surface area to g foil. Pieces of foil were then carefully cut to fit onto coral surface, weighed, and the surface area of coral samples was estimated by comparison to the standards.

RESULTS AND DISCUSSION

Occurrence of coral-associated Archaea

To determine if Archaea are associated with healthy corals, total DNA was harvested from 6 coral species and assayed using Archaea-specific PCR. As shown in Table 1, archaeal 16S rDNAs were found on 26% *Colpophyllia natans*, 100% *Diploria strigosa*, 39% *Montastraea* spp., and 62% *Porites* spp. collected from Bocas del Toro, Panama (BT). Archaea were also found on 50% of *Porites astreoides* samples collected from Whale Bone Bay, Bermuda (BM). Archaeal 16S rDNAs were not detected on *D. strigosa* and *M. franksi* samples from Bermuda or on *Acroporid* samples from Puerto Rico. To ensure that PCR quality DNA was present all samples were PCR amplified using Bacteria-specific primer 27F and uni-

Table 1. Detection of Archaea on corals by PCR amplification with 21F and 915R archaeal specific primers. Coral samples were collected from Whale Bone Bay, Bermuda (BM), Bocas del Toro, Panama (BT), and La Parguerra, Puerto Rico (PR)

Coral species	Location	Date	PCR positive for archaeal 16S rDNA	Total no. of samples screened	% positive
<i>Colpophyllia natans</i>	BT	Apr-99	5	19	26
<i>Diploria strigosa</i>	BM	Aug-99	0	6	0
	BT	Jun-00	4	4	100
<i>Montastraea annularis</i>	BT	Apr-99	2	3	67
<i>Montastraea franksi</i>	BM	Aug-99	0	5	0
	BT	Apr-99	0	2	0
	BT	Jun-00	3	8	38
<i>Porites astreoides</i>	BM	Aug-99	3	6	50
	BT	Jun-00	15	24	63
<i>Porites furcata</i>	BT	Apr-99	7	11	64
	BT	Jun-00	3	5	60
<i>Acropora cervicornis</i>	PR	Jan-02	0	8	0
<i>Acropora prolifera</i>	PR	Jan-02	0	3	0

versal primer 1492R. All of the samples used were positive with the Bacteria-specific PCR. This does not mean that Archaea were not present on the negative samples because our limit of detection was $\sim 10^7$ targets per μl of DNA.

16S rDNA analyses of coral-associated Archaea

A total of 353 archaeal 16S rDNA sequences were obtained from 6 coral colonies representing 3 coral species. Using a 97% identity cut off, these sequences contained 93 archaeal ribotypes. The most abundant ribotype was observed in 58 clones. Using the Chao1 estimator (Chao 1984, Colwell & Coddington 1994), it was calculated that this population of corals harbors ~ 213 different species of Archaea. The Shannon-Wiener diversity index (Shannon & Weaver 1949) for the community was 3.4, indicating that archaeal communities on corals are diverse, rivaling diversity estimates of coral-associated bacterial communities (*Acropora* spp., $H' = 2.2$; *Diploria strigosa*, $H' = 4.2$; *Montastraea franksi*, $H' = 3.4$; *Porites* spp., $H' = 2.5$; taken from Rohwer et al. 2002).

The archaeal community on corals consists of species from both the Crenarchaeota and the Euryarchaeota (Fig. 1), including some species from Marine Groups I and II. In another study on coral-associated Archaea (Kellogg 2004), archaeal species from Marine Groups I, II, and III were found. Seventy-three percent of the sequences were most closely related to Crenarchaeotes and 27% to Euryarchaeotes. The 2 most abundant sequences in the community were closely related to Crenarchaeotes (BT60MF7AA7, BT60MF7AF7) and represented 33% of all the clones. These

2 archaeal ribotypes were found on all 3 coral species, showing that coral-associated Archaea were not species-specific. The third most abundant ribotype was a Euryarchaeote (BT60PA9AC8) present on only 1 coral sample, *Porites astreoides* (PA9) from Panama. The archaeal community of PA10, a *P. astreoides* colony located next to PA9, contained mostly Crenarchaeotes (95%). This shows that individual coral colonies may be dominated by a particular archaeal ribotype. One archaeal ribotype (BT60PA10AD9) was found on all 3 coral types from both Panama and Bermuda collected in different years. This may be a generalist Archaea that can associate with several coral species. In a concurrent study by Kellogg (2004) another generalist coral-associated Archaea was found to make up 48% of all sequences on 3 different coral species. In contrast to zooxanthellae and Bacteria, the Archaea associated with corals appear to be non-specific.

Direct counts of Archaea and Bacteria on corals using PNA probes

FISH with Domain-specific PNA probes was used to directly count Archaea and Bacteria on corals. Control experiments using *Halobacterium* sp. (Archaea) and *Salmonella* sp. (Bacteria) were first performed to determine hybridization conditions for these probes. As shown in Fig. 2A, the Archaea-specific probe only annealed to the *Halobacterium* sp., and not to the *Salmonella* sp. in the mixture (Fig. 2B; *Salmonella* sp. cells are the smaller, brighter rods in the DAPI-stained field). Similarly, the Bacteria-specific probe only bound to the *Salmonella* sp. cells (Fig. 2C,D).

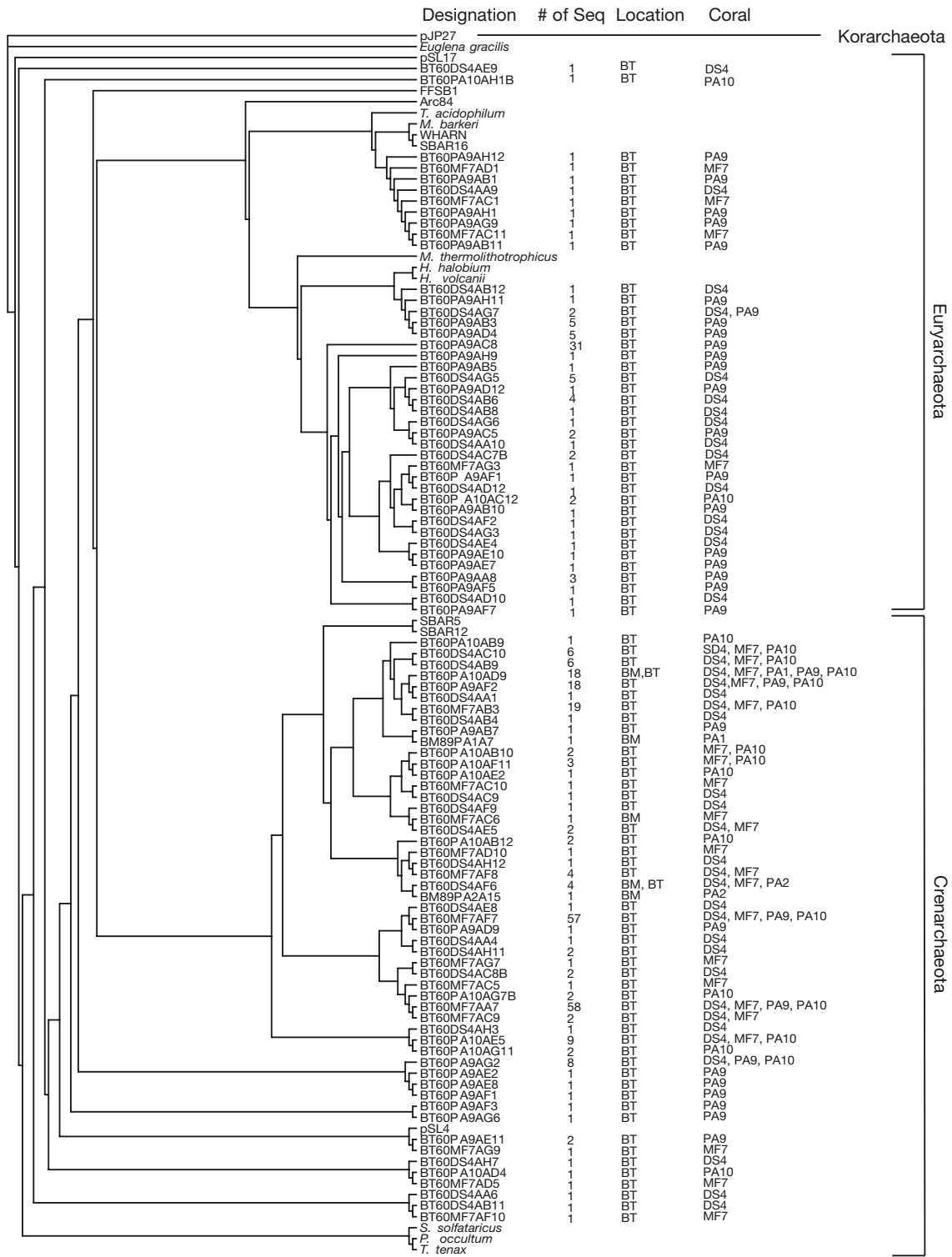


Fig. 1. Identity of archaeal groups from 16S sequencing. The neighbor-joining tree was built from an ARB-alignment. *Euglena gracilis* was used as an outgroup. The number of clones that each sequence represented is shown (# of Seq), as well as the location (BT = Bocas del Toro, Panama; BM = Whale Bone Bay, Bermuda), and coral species that the ribotype appeared on (Coral; DS = *Diploria strigosa*, MF = *Montastrea franksi*, PA = *Porites astreoides*). Designation labels are coded as follows: (1) the first 2 letters represent location (BM, BT), (2) the next 2 numbers represent the month and the last digit of the year, respectively, in which the sample was collected, (3) the next 2 letters followed by a numerical digit represent coral species and colony identity, and (4) the last numbers represent the clone designation in a micro-titer plate. The sample designated on the tree is 1 representative from each group. Full names of cultured microbes and GenBank accession numbers (in parentheses) for 16S rRNA genes used as guide sequences: *Halobacterium halobium* NCIMB 777 (AJ002949); *Haloferax volcanii* (AB074566); *Methanosarcina barkeri* strain Fusaro (AAAR02000001); *Methanococcus thermolithotrophicus* (M59128); *Sulfolobus solfataricus* P2 (NC002754); *Pyrodictium occultum* (M21087); *Thermoproteus tenax* (AY538162); *Thermoplasma acidophilum* DSM 1728 (NC002578). GenBank accession numbers for uncultured 16S rRNA genes used as guide sequences: Arc84 (AY029298); FFSB1 (X96688); pJP27 (L25852); pSL4 (U63341); pSL17 (U63339); WHARN (M88078); SBAR5 (M88075); SBAR12 (M88076); SBAR16 (M88077)

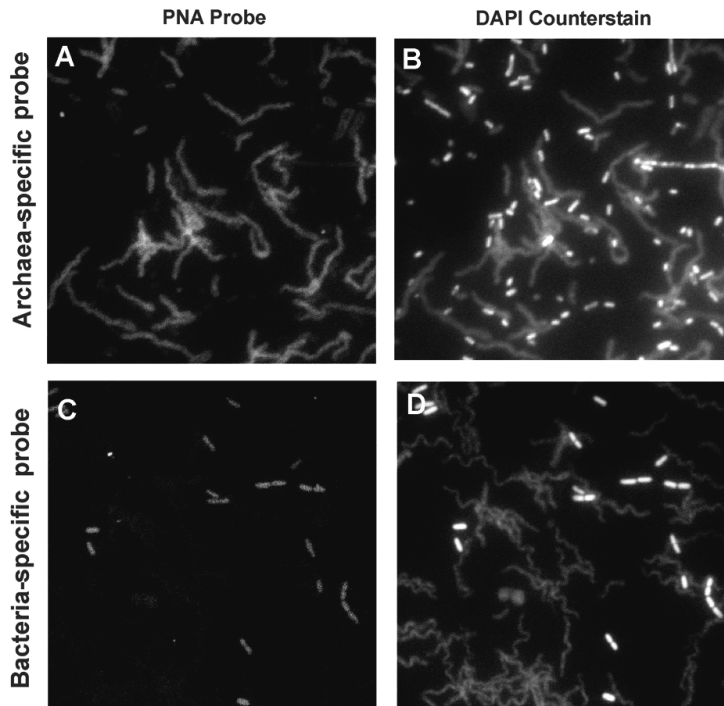
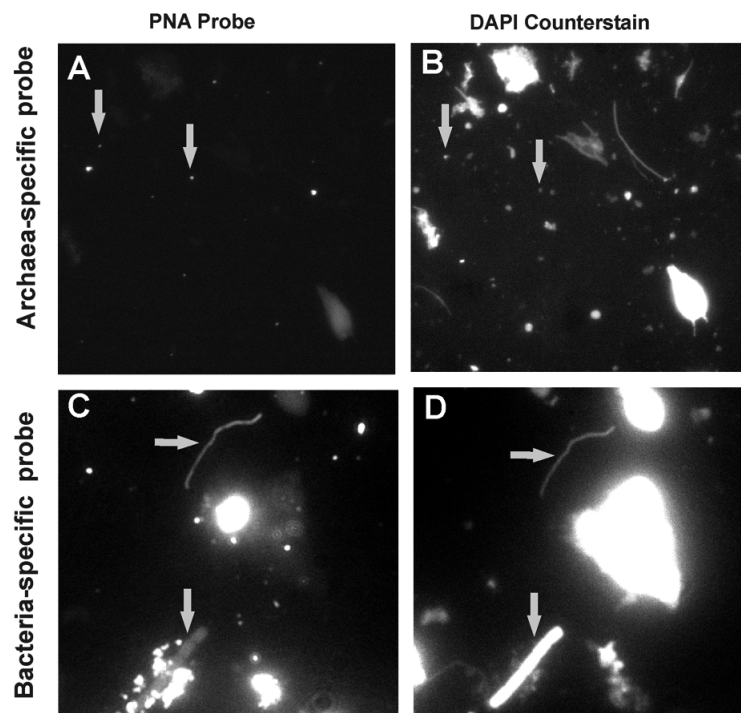


Fig. 2. Control experiments for the peptide nucleic acid (PNA) fluorescent *in situ* hybridizations (FISH) using *Halobacterium* sp. and *Salmonella* sp. to show that the probes were Domain specific. A mixture of *Halobacterium* sp. and *Salmonella* sp. were (A) hybridized with Archaea-specific DNA probe and (B) DAPI counterstained. On a separate filter, the same *Halobacterium* sp. and *Salmonella* sp. were (C) hybridized with a Bacteria-specific PNA probe and (D) counterstained with DAPI counter-stain. *Halobacterium* sp. are the long cells, while *Salmonella* sp. are the short rods

CONCLUSIONS

Cells positive for both the Archaea-specific probe and nucleic acid stain DAPI were counted as Archaea (arrows in Fig. 3). Likewise, cells positive for the Bacteria-specific probe and DAPI were counted as Bacteria. Total cell counts with a nucleic acid stain were not done because the coral lysates were extremely messy and prokaryotic cells could not be distinguished from other biological entities (e.g. mitochondria). There were an average of 8.5×10^7 Archaea cm^{-2} on *Porites* spp. (Table 2). More Archaea were observed on corals than Bacteria (average 4.9×10^7 Bacteria cm^{-2}). The number of Bacteria in the samples may have been underestimated in this study because we did not use a mixture of different probes (Bythell et al. 2002). Archaea-specific PNA hybridizations were also conducted on 4 Caribbean *Acropora* sp. samples, but no cells were detected.

Fig. 3. Example of peptide nucleic acid (PNA) fluorescent *in situ* hybridizations (FISH) to count Archaea and Bacteria on *Porites astreoides*. The Archaea-specific PNA probe was (A) hybridized to a paraformaldehyde fixed slurry from *P. astreoides* and (B) counterstained with DAPI. The Bacteria-specific PNA probe was (C) hybridized to the same coral sample and (D) counterstained with DAPI. Cells positive for both the PNA probe and DAPI were counted. Examples of positives are indicated with arrows



Archaea were commonly associated with corals, reaching concentrations of $>10^7 \text{ cm}^{-2}$. However, not all corals species (e.g. *Acroporids*) had detectable Archaea associates. In contrast to the coral-associated Bacteria, Archaea do not appear to form specific associations with corals. In fact, there may even be generalist Archaea associated with corals (Kellogg 2004, and present study). The coral-associated Archaea appear to be as abundant and diverse as the Bacteria (e.g. $H'_{\text{Bacteria}} = 2.2$ to 4.2 ; $H'_{\text{Archaea}} = 3.4$). Corals harbor diverse and abundant communities of Bacteria, as well as eukaryotic zooxanthellae, endolithic algae, fungi, and protists. The presence of Archaea on corals demonstrates that coral colonies

Table 2. Direct counts using fluorescent *in situ* hybridization (FISH) of Archaea and Bacteria on *Porites* spp. Both Archaea and Bacteria were present at $>10^7$ cm⁻². nd = not determined

Coral sample	No. of Bacteria cm ⁻² ($\times 10^7$)	No. of Archaea cm ⁻² ($\times 10^7$)	Bacteria: Archaea
<i>P. astreoides</i> 1	1.5	1.3	1.17
<i>P. astreoides</i> 2	1.5	1.9	0.78
<i>P. astreoides</i> 3	1.6	5.1	0.31
<i>P. astreoides</i> 4	2.9	nd	nd
<i>P. astreoides</i> 5	3.2	2.8	1.12
<i>P. astreoides</i> 6	4.9	12	0.40
<i>P. astreoides</i> 7	7.6	29	0.26
<i>P. astreoides</i> 8	7.6	8.5	0.90
<i>P. astreoides</i> 9	11	nd	nd
<i>P. astreoides</i> 10	7.7	17	0.44
<i>P. astreoides</i> 11	nd	8.6	nd
<i>P. furcata</i> 1	nd	4.1	nd
Average	4.9	8.5	0.67

harbor organisms from all 3 Domains of life. The main challenge in future studies is to determine what role the Archaea play in the coral holobiont.

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