Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6241–6246, June 1996
Evolution

A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov.

(Archaea/Crenarchaeota/evolution/symbiosis/Porifera)

CHRISTINA M. PRESTON*, KE YING WU*, TADEUSZ F. MOLINSKI†, AND EDWARD F. DELONG*‡

*Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, Santa Barbara, CA 93106; and †Department of Chemistry, University of California, Davis, Davis, CA 95616

Communicated by Carl R. Woese, University of Illinois, Urbana, IL, February 2, 1996

ABSTRACT **Archaea, one of the three major domains of extant life, was thought to comprise predominantly microorganisms that inhabit extreme environments, inhospitable to most Eucarya and Bacteria. However, molecular phylogenetic surveys of native microbial assemblages are beginning to indicate that the evolutionary and physiological diversity of Archaea is far greater than previously supposed. We report here the discovery and preliminary characterization of a marine archaeon that inhabits the tissues of a temperate water sponge. The association was specific, with a single crenarchaeal phylotype inhabiting a single sponge host species. To our knowledge, this partnership represents the first described symbiosis involving Crenarchaeota. The symbiotic archaeon grows well at temperatures of 10°C, over 60°C below the growth temperature optimum of any cultivated species of Crenarchaeota. Archaea have been generally characterized as microorganisms that inhabit relatively circumscribed niches, largely high-temperature anaerobic environments. In contrast, data from molecular phylogenetic surveys, including this report, suggest that some crenarchaeotes have diversified considerably and are found in a wide variety of lifestyles and habitats. We present here the identification and initial description of *Cenarchaeum symbiosum* gen. nov., sp. nov., a symbiotic archaeon closely related to other nonthermophilic crenarchaeotes that inhabit diverse marine and terrestrial environments.**

Phylogenetic comparisons of universal, highly conserved macromolecular features of diverse biota have resulted in fundamental conceptual changes in evolutionary biology. In particular, the recent elucidation of evolutionary relationships among disparate life forms has revealed that prokaryotes (defined as microorganisms lacking a membrane-bound nucleus) consist of two evolutionarily distinct lineages (1–4). Current data suggest that contemporary life on Earth has diverged into three major phylogenetic lineages (1–5), designated domains (5), two of which are prokaryotic (Bacteria and Archaea) and one of which is eukaryotic (Eucarya). Archaea, the most recently recognized of the domains, contains cultivated members that span a fairly limited range of phenotypes, represented by extreme halophiles, sulfur-metabolizing thermophiles, thermophilic sulfate-reducers, and methanogens (6). Although the number of new species of cultivated Archaea has increased dramatically in recent years (7), the variety of phenotypic motifs found within the Archaea has not. Virtually all new archaeal isolates can still be categorized into one of the four commonly encountered phenotypes listed above.

In contrast to those of Bacteria, the major physiological motifs of known and cultivated Archaea have appeared limited. Methanogenesis, aerobic or anaerobic heterotrophic oxidation of sugars and peptides, and chemolithoautotrophic growth on reduced sulfur compounds or hydrogen, represent three of the major catabolic themes (6). Limited capacity for phototrophic energy generation, nitrogen fixation, and denitrification has also been demonstrated in various archaeal groups. Of the two major branches of Archaea, cultivated Crenarchaeota, comprised predominantly of anaerobic thermophiles metabolizing elemental sulfur, have appeared the most limited phenotypically (7).

Recent molecular phylogenetic surveys of bacterial and archaeal diversity in cold oxygenated ocean waters (8–11) and terrestrial soils (12) have suggested the existence of additional crenarchaeal phylotypes. Unfortunately, these unusual archaea have to date resisted laboratory cultivation, and so their phenotypic and physiological properties remain unknown. A low-growth temperature range for marine planktonic archaea has been inferred from circumstantial evidence—e.g., their ecological distribution and the relatively low percent G+C content of their ribosomal RNA genes (8–11). Nevertheless, it has not been conclusively demonstrated that these microorganisms actually grow in cold habitats and do not instead emanate from allochthonous sources (e.g., hydrothermal environments).

We recently surveyed microbial populations associated with marine sponges by these new molecular methods (13). In the course of that study, we detected significant amounts of archaeal nucleic acids associated with the tissues of a single marine sponge species. We present here the identification and initial description of the sponge-associated archaeon and discuss the general implications of our observations for archaeal biology.

METHODS

Field Collection, Sponge Maintenance, and Identification. Sponges were collected at depths of 10–20 m by SCUBA diving at two different sites located just offshore of Santa Barbara, CA. The encrusting sponges were removed along with a small amount of their rock substrate, placed in collection bags, and transported to aquaria within 2 hr. Captive specimens were maintained in flowing seawater tanks. Criteria used in sponge specimen identification included gross morphology, color, oscule arrangement, encrusting shape and thickness, and the size and shape of siliceous spicules, which included both oxea and style types. No microscleres or ectosomal skeleton were present, and the choanosomal skeleton was halichondroid-like (Mary Kay Harper and Welton Lee, personal communication). The sponge harboring the archaeal symbionts represents a single species and was most similar to *Axinella mexicana*, based on the morphological criteria listed above and by comparison to type specimens (Welton Lee, personal communication). Because of small differences in spicule size and skeletal

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ssu, small subunit; DAPI, 4′,6-diamidino-2-phenylindole.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U51469).

‡To whom reprint requests should be addressed. e-mail: delong@marbtech.lscf.ucsb.edu.

arrangement, however, this sponge may represent a new species and will therefore be referred to as "*Axinella* sp." throughout this report.

Nucleic Acid Extraction and Hybridization. A vertical cross section of sponge (0.5 g) was mechanically dissociated in 0.22-μm filtered, autoclaved seawater using a tissue homogenizer. Cell lysis was accomplished by incubating the dissociated cells in 1 mg of lysozyme per ml for 30 min at 37°C followed by an incubation for 30 min at 55°C with 0.5 mg of proteinase K per ml and 1% SDS. The tubes were finally placed in a boiling water bath for 60 sec to complete lysis. The protein fraction was removed with two extractions with phenol:chloroform:isoamyl alcohol (50:49:1), pH 8.0, followed by a chloroform:isoamyl alcohol (24:1) extraction. Nucleic acids were ethanol-precipitated and resuspended in TE buffer (10 mM Tris·HCl/1 mM Na_2-EDTA, pH 8.0). Approximately 5 μg of DNA was purified by CsCl equilibrium density gradient ultracentrifugation on a Beckman Optima tabletop ultracentrifuge using a TLA100 rotor (9).

For RNA extractions, vertical cross sections of tissue (150 mg) were removed, and total rRNA was extracted in hot phenol:$CHCl_3$, pH 5.1 as described (14), with the following modifications. The sponge section was immersed in 0.7 ml of 50 mM sodium acetate buffer (pH 5.1), 0.7 ml of phenol (pH 5.1), and 0.5 ml of sterile glass beads (diameter, 0.1 mm). The mixture was mechanically disrupted for 4 min in a mini-beadbeater (Biospec Products, Bartlesville, OK), incubated at 60°C for 15 min, disrupted for an additional 2 min, and finally incubated at 60°C for 3 min. The sponge-derived nucleic acids or purified RNA standards were denatured, serially diluted, applied to nylon membranes (Hybond-N; Amersham), immobilized by UV crosslinking, and hybridized with domain-specific probes as described (9, 15). The amount of radioactive probe bound to immobilized rRNA was quantified using a radioanalytic gas proportional counting system (Scanalytics, Billerica, MA).

Cloning, Sequencing, and Phylogenetic Analysis. For direct sequencing of archaeal small subunit (ssu) rDNA, PCR mixtures contained one biotinylated and one unbiotinylated Archaea-targeted primer (1.0 ng/μl; ref. 9). Following amplification, the biotinylated strand was purified using avidin-coated magnetic beads (Dynal, Great Neck, NY; ref. 16) and sequenced directly using Sequenase 2.0 (United States Biochemical). To compare this sponge-associated archaeon sequence to those of other Archaea, bootstrap neighbor-joining analyses (Kimura two-parameter model for nucleotide substitution, 500 bootstrap iterations) were performed (403 nucleotide residues, positions 569–966, *Escherichia coli* numbering). Reference sequences, as well as sequence editing and phylogenetic analysis software, were obtained via anonymous ftp from the Ribosomal RNA Database Project (University of Illinois, Urbana, IL) (17).

To recover the full ssu rRNA gene and flanking regions, a "fosmid" (F-factor-based cosmid; ref. 18) library was prepared from prokaryotic cells retrieved from sponge tissue by differential centrifugation. Sponge tissue was incubated in calcium- and magnesium-free artificial seawater (460 mM NaCl/11 mM KCl/7 mM Na_2SO_4/2 mM $NaHCO_3$) containing 0.25 mg of Pronase per ml at room temperature for 1 hr. The sponge tissue was rinsed in artificial seawater and homogenized in a blender, and large particles and spicules were removed by low-speed centrifugation (4000 rpm, Sorvall GSA rotor, 4°C). The supernatant was next centrifuged at 5000 rpm for 5 min at 4°C to remove large sponge cells, and the resulting supernatant was centrifuged at 10,000 rpm in a GSA rotor at 4°C for 20 min to collect the remaining prokaryote-containing cell fraction. The resulting cell pellet was used to isolate DNA and construct a fosmid library, as described (18). A fosmid clone containing the complete archaeal ssu rRNA gene was identified using Archaea-biased ssu rRNA PCR primers (9). The ssu rRNA gene was subcloned into pBluescript vector (Stratagene), and the purified double-stranded plasmid was directly sequenced. Maximum likelihood analysis (19) was performed on a total of 1189 nucleotide positions, with the software FASTDNAML, version 1.0 (20) using empirical base frequency, global branch swapping, and bootstrapping options.

Whole Cell Hybridization. A vertical cross section of a captive sponge was fixed for 7 hr at 4°C in 3.7% formaldehyde diluted in 0.22 μm-filtered, autoclaved seawater. The section was subsequently rinsed in sterile seawater and macerated with a tissue grinder. Spicules and large aggregates of sponge tissue were removed by low-speed centrifugation before spotting onto gelatin-subbed slides. To reduce background, the slides were treated with acetic anhydride (21). Slides were dehydrated in an ethanol series (50%, 75%, and 95%, 2 min each) and air-dried (22). Hybridization conditions were as described (23, 24) but without the addition of bovine serum albumin. A mixture of four marine crenarchaeal-specific fluor-labeled oligonucleotide probes (5 ng/μl each) was added, and hybridizations were incubated at 40°C overnight in a moist chamber to prevent evaporation. After hybridizaton, slides were washed in 1× SET (150 mM NaCl/20 mM Tris·HCl, pH 7.8/1 mM Na_2-EDTA) at 45°C for 10 min, stained with 10 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml for 5 min at room temperature, washed for 10 min in 1× SET at room temperature, quickly rinsed in sterile water, and air-dried. After mounting in Citifluor AF1 (Citifluor, London), slides were examined under epifluorescence using a Zeiss Axioskop 20. Photographs were taken using Kodak Tmax 400 black and white film, using 0.5-sec exposures for DAPI-stained cells, and 120- to 240-sec exposures for cells visualized by fluor-labeled probe binding. A suite of the following four oligonucleotide probes, specific to "group I" marine Crenarchaeota (9) and labeled at their 5′-end with Texas Red (Molecular Probes), were employed simultaneously in hybridizations: GI-131 (TCC CGT CCA TAG GTT AGG), GI-538 (TCC TGA CCA CTT GAG GTG), GI-554 (TTA GGC CCA ATA ATC MTC CT), and GI-655 (GTA CCG TCT ACY TCT CCC ACT CC). Two negative controls were employed with each experiment, using identical cell preparations and hybridization conditions. One negative control consisted of hybridizations in which four unlabeled marine group I Archaea-specific probes were added in 50-fold excess (250 ng/μl) of the four Texas Red-labeled probes added at their standard concentration (5 ng/μl). An additional negative control consisted of hybridizations containing four, fluor-labeled Eucarya-specific probes (25).

RESULTS

Detection and Quantitation of Sponge-Associated Archaea. Initially, sponge-associated Archaea were detected by PCR amplification of total sponge DNA using Archaea-specific, ssu rRNA-targeted primers (9). Every individual of the *Axinella* sp. examined ($n = 23$), collected at different sampling sites and at different times of year, yielded positive results in PCR assays using primers that target archaeal rRNA in general (Table 1). In contrast, Archaea were not consistently detected in any other temperate or tropical sponges examined (Table 1; ref. 13) or in microbial assemblages of aquarium seawater surrounding captive sponges (data not shown).

These PCR results were subsequently verified by quantifying the relative amount of archaeal rRNA extracted from sponge tissue (Table 1; ref. 13) using domain-specific rRNA-targeted oligonucleotide probes (9, 14, 15). Quantitative oligonucleotide probe hybridization experiments indicated that from 0.5% to 5% of the total sponge tissue rRNA was derived from Archaea, in all sponge individuals of this species examined ($n = 13$, Table 1). Results with one individual *Axinella* sp., sampled over a period of 6 months, showed that the percentage of sponge-associated archaeal rRNA remained relatively constant (Fig. 1) over time. Other sponge individuals of this same

Table 1. Detection of Archaea in sponge tissue by PCR amplification and domain-specific rRNA-targeted oligonucleotide probe hybridization

Sponge species	Specimen No.	Collection date and site	Archaeal rDNA	Relative % Eucarya	Domain-specific Bacteria	rRNA Archaea
Axinella sp.	S1	2/94NR	+	97.4	2.0	0.5
	S2	2/94NR	+	95.9	2.7	1.4
	S3	3/94NR	+	97.4	2.2	1.3
	S4	3/94NR	+	98.0	ND	1.0
	S5	3/94NR	+	95.4	1.6	3.1
	S6	6/94NR	+	—	—	—
	S7	6/94NR	+	—	—	—
	S8	6/94NR	+	—	—	—
	S9	6/94NR	+	—	—	—
	S10	6/94NR	+	—	—	—
	S11	6/94NR	+	—	—	—
	S12	7/94NR	+	94.4	2.7	2.8
	S13	7/94NR	+	90.4	6.6	3.0
	S14	7/94NR	+	—	—	—
	S15	7/94NR	+	—	—	—
	S16	10/94NR	+	92.0	2.2	4.8
	S17	10/94NR	+	—	—	—
	S18	10/94NR	+	—	—	—
	HS1	10/94 HS	+	85.0	11	3.9
	HS2	10/94 HS	+	93.3	2.2	4.5
	HS3	10/94 HS	+	92.0	2.9	5.1
	HS4	10/94 HS	+	96.3	ND	3.6
	HS5	10/94 HS	+	94.7	1.4	3.9
Cliona celata	C1	1/94NR	−	96.0	4.0	ND
	C2	2/94NR	−	93.0	7.0	ND
	C3	2/94NR	−	86.0	14.0	ND
Tethya aurantia	T1	2/94NR	−	99	1.0	ND
Xestospongia sp.	1	Ref. 13	−	63	37	ND
	2	Ref. 13	−	44	56	ND
	3	Ref. 13	−	54	46	ND

NR and HS refer to different sample sites in the Santa Barbara Channel. + (−) indicate positive (negative) amplification of rDNA fragments of the predicted size, using Archaea-specific PCR primers and total sponge DNA as template; all sponge DNAs tested positive with Eucarya-specific rDNA PCR primers. Dashes indicate experiments not performed; ND, not detected.

species maintained for over 1 year in laboratory aquaria yielded similar results, demonstrating the stable maintentance of the sponge–archaeal association over time.

Phylogenetic Analysis of the Sponge-Associated Archaeon. We performed direct sequence analysis of PCR products amplified from sponge tissue extracts using the general archaeal rRNA-targeted oligonucleotide primers (9). Fifteen individuals of this *Axinella* species, collected at different sites and times, all yielded a single identical archaeal ssu rDNA sequence (data not shown). In contrast, when applied to marine planktonic populations, these same PCR primers consistently yield multiple rDNA phylotypes (9–11). The fact that only a single rDNA species was amplifed by the general, Archaea-targeted primers provides strong evidence that this *Axinella* sp. contained a single archaeal phylotype. These data also contrast strongly with results obtained using eubacterial rDNA primers, which indicated the presence of complex and heterogeneous sponge-associated eubacterial assemblages that vary in composition between different individuals of the same *Axinella* species (data not shown).

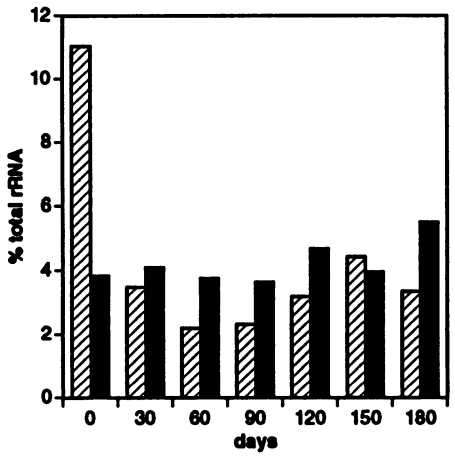

FIG. 1. Relative proportion of archaeal or bacterial rRNA within the tissue of an *Axinella* specimen maintained in a laboratory aquarium. The relative percentage of eucaryal rRNA was >85% at all time points. Hatched bars correspond to the relative percentage of eubacterial rRNA, and solid bars correspond to the relative percentage of archaeal rRNA.

Bootstrap neighbor-joining analysis of the PCR-amplified, directly sequenced ssu rDNA (403 nucleotide positions) consistently places the sponge-associated archaeon well within a previously described group of planktonic marine crenarchaeotes (Fig. 2*A*; ref. 9–12). The sponge-associated archaeal ssu rRNA does, however, contain particular diagnostic residues, which distinguished it from its planktonic relatives. Signature analysis revealed a total of 35 nucleotide residues that are diagnostic for and unique to the sponge-associated archaeon ssu rRNA, distinguishing it from all (twenty) other marine "Group I" crenarchaeote rRNA sequences available (Table 2).

The complete rRNA operon from the sponge-associated archaeon was identified in a fosmid clone library prepared from total sponge DNA, and the ssu rRNA was subsequently

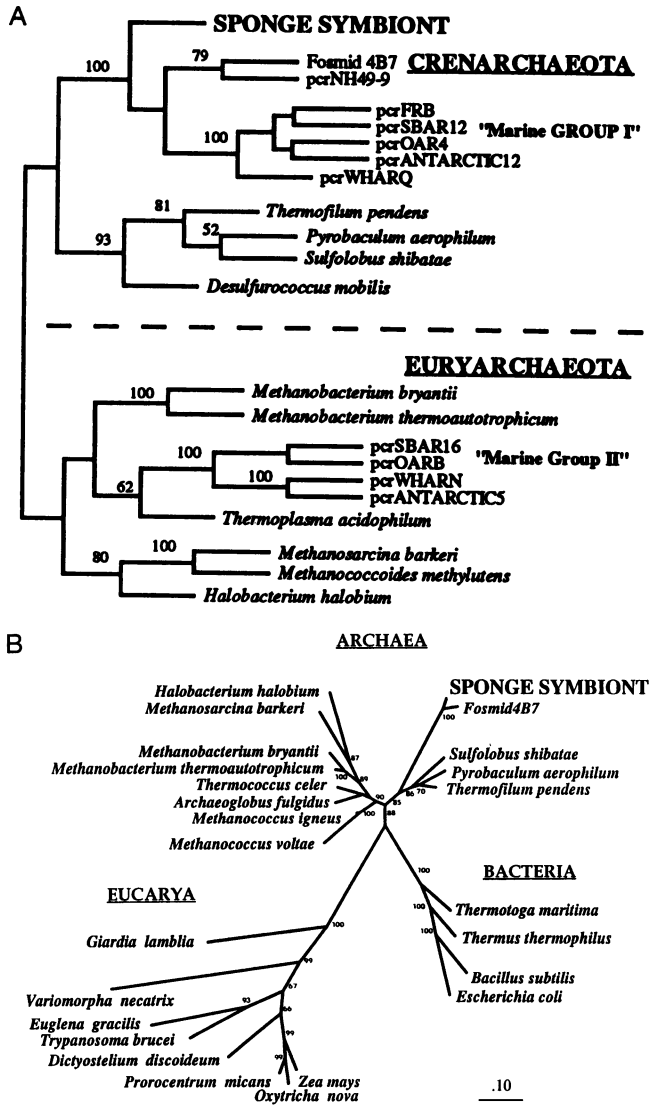

FIG. 2. Phylogenetic affiliation of the sponge-associated archaeon. (*A*) Neighbor-joining phylogenetic analysis of the sponge-associated archaeon rRNA. Reference sequences include rDNA genes of marine planktonic archaea amplified from mixed assemblages (indicated by the prefix "pcr"), as well as sequences of cultivated Archaea. "Fosmid 4B7" refers to a ssu rRNA sequence derived from a 40-kb genomic fragment of a free-living planktonic marine archaeon (18). Values represent the percentage of bootstrap replications >50% that support the branching pattern appearing to the right of the value. *Thermotoga maritima* and *Thermus thermophilus* were used as outgroups. (*B*) Phylogenetic relationship of the sponge archaeon determined by maximum likelihood analysis. Tree topology was inferred from 1189 residues, using FASTDNAML, version 1.0 (20). Values indicate the percentage of 250 bootstrap replications, which supported the branching pattern at various nodes in the majority-rule consensus tree. Scale bar corresponds to the expected number of changes per sequence position, for those positions changing at the median rate.

subcloned and sequenced. Maximum likelihood bootstrap analyses comparing this full sequence with representative rRNA sequences from the three domains supported the neighbor-joining analysis (Fig. 2*B*) and placed the sponge-associated archaeon well within the Crenarchaeota at a high bootstrap confidence level (85%; Fig. 2*B*). This affiliation is consistent with previous phylogenetic analyses of both ssu rRNA (9, 10, 18, 26) and the elongation factor 2 protein sequence (18) of the symbiont's planktonic archaeal relatives. This placement within the Crenarchaeota is also consistent with transversion signature analyses, in which the group shared 13 of 17 diagnostic

Table 2. Unique rRNA signature nucleotides distinguishing the sponge-associated symbiont from related planktonic crenarchaeotes

Nucleotide position	Group I consensus	Fosmid 4B7	Sponge symbiont
122	G	G	A
131	C	C	U
183.3	A	U	C
193.6	U	A	G
240	C	G	U
281	U	U	A
286	G	C	A
305	U	U	A
306	A	A	G
407	A	G	U
441	G	G	A
442	U	U	C
443	U	U	C
444	A	A	G
463.1	C	G	U
466	G	G	A
490	A/U	A	C
491	A	A	G
492	A	A	G
590	U	U	C
648	G/U	G	C
649	A	A	G
718	A	A	C
818	G/U	G	A
840.3	—	—	G
842	U	U	A
844	U/A	U	G
1044.10	A	A	U
1044.11	A	A	G
1139.4	U	U	C
1178	G	G	A
1185	G	G	A
1335	A	A	C
1356	U	U	C
1380	U	U	G

Nucleotide position in ssu rRNA is given in *E. coli* numbering. Positions having no counterpart in *E. coli* are designated by the nearest *E. coli* number followed by a decimal number. Group I consensus (9) corresponds to the consensus signature of marine planktonic crenarchaeal rDNA clones (8–11). "Fosmid 4B7" corresponds to a full-length rRNA gene recovered from a 40-kb genomic fragment of a free-living planktonic marine crenarchaeote (18).

crenarchaeal signature positions with all other cultivated crenarchaeotes, but only three residues in common with the corresponding euryarchaeal signature (data not shown).

Whole Cell Hybridization Analyses. Ribosomal RNA-targeted oligonucleotide probes were hybridized to formalin-fixed, homogenized sponge tissue to facilitate identification of sponge-associated Archaea by epifluorescence microscopy (22, 23). Hybridization with marine crenarchaeote-targeted (9) fluorescent oligonucleotide probes yielded a strong signal at opposite cellular poles of the sponge-associated archaeon. A central intracellular region in which little probe bound was also revealed in whole cell hybridizations (Fig. 3*A* and Fig. 4). This central area corresponded to regions of intense DAPI staining (Fig. 3*B* and Fig. 4), indicating the intracellular location of a DNA-containing nucleoid. The archaeal cells were rod-shaped, on average 0.8 μm long and 0.5 μm wide. No fluorescence of archaeal cells was observed when hybridizations were performed with a combination of four fluor-labeled Eucarya-specific negative control probes or in hybridization experiments in which Archaea-specific probes lacking a fluor were included simultaneously with the fluor-labeled probes, but at a 50-fold higher concentration.

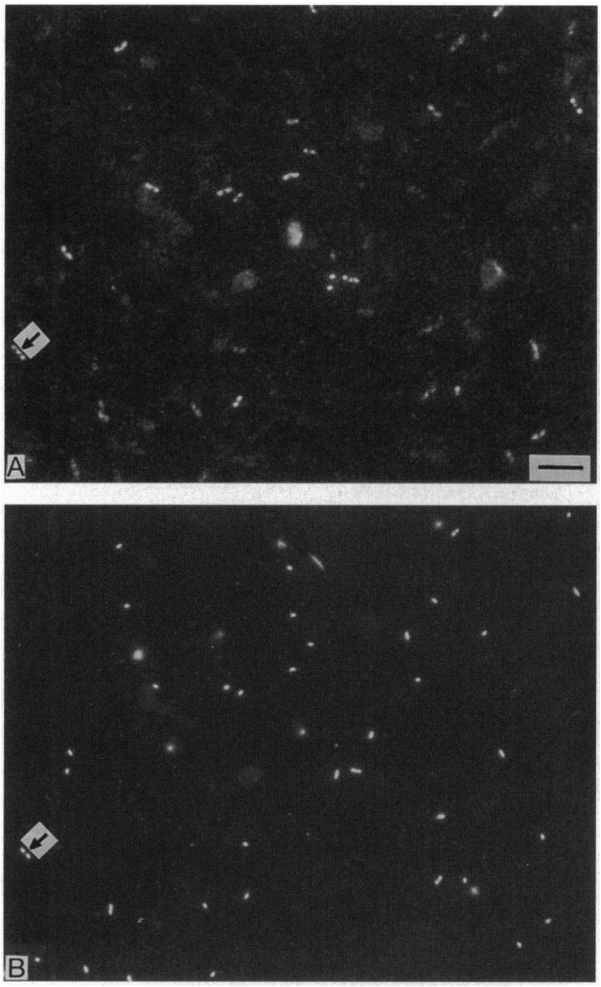

FIG. 3. Photomicrographs of sponge-associated archaeon hybridized with marine group I (9) Archaea-specific oligonucleotide probes labeled with Texas Red (*A*) and cells of the same field visualized with DAPI (*B*). Arrow indicates cell with an obviously visible dividing nucleoid, which revealed two distinct regions that partially excluded oligonucleotide probe binding (*A*). These intracelluar regions devoid of probe binding corresponded to central regions which are intensely stained with DAPI (*B*). Scale bar = 5 μm.

Of the total prokaryotic cells enumerated by DAPI staining (n = 1274), 65% were identified as Archaea by their strong binding to the marine group I-specific (9) oligonucleotide probes (Fig. 3 and Fig. 4). These results are consistent with rRNA abundances determined by oligonucleotide probe hybridization to bulk nucleic acids, which indicated that between 47% and 63% of the prokaryotic rRNA extracted from captive sponges was archaeal (Fig. 1). Actively dividing archaeal cells were recognized by the presence of two distinct regions in which no probe binding was evident (Fig. 3*A*, arrow, and Fig. 4, inset). The areas devoid of probe binding corresponded exactly to two distinct DNA-containing regions, readily visualized by intense DAPI-conferred fluorescence (Fig. 3*B*, arrow, and Fig. 4). Of the total sponge-associated Archaea detected by whole cell hybridization, ≈15% were in this apparent dividing state (Fig. 4, inset), indicating active growth of the archaeon within captive sponges maintained in aquaria for over 6 months.

DISCUSSION

Recent molecular phylogenetic surveys of naturally occurring prokaryote assemblages have suggested that standard cultivation approaches inadequately sample indigenous microbial

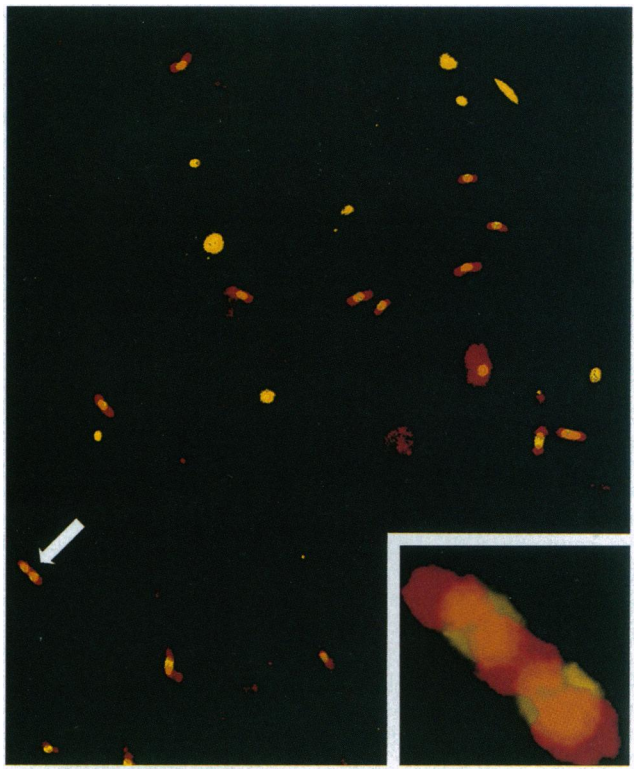

FIG. 4. Color image of sponge-associated archaeon simultaneously stained with DAPI and fluorescent oligonucleotide probes. The black and white photomicrographs of Figs. 3*A* and *B* were scanned, digitized, colorized, and superimposed to form a composite image of stained cells. Yellow corresponds to DAPI-stained regions (see Fig. 3*B*), and red corresponds to regions that bound the oligonucleotide probes (see Fig. 3*A*). The orange color results from the region of overlap between the two stains. The arrow points to the image of the same dividing cell that is indicated in Fig. 3. The inset is an enlargement of this image, showing the distribution of DAPI and rRNA-targeted stains.

communities. Nearly every ecological study that has employed a molecular phylogenetic approach to survey prokaryotes (8–12, 27–32) has revealed the existence of as yet uncultivated phylotypic groups. The extent and range of phylogenetic and phenotypic diversity that has escaped detection, however, remains to be quantitatively defined. The recent description of a large number of new phylotypes of thermophilic Archaea, all from a single Yellowstone hot spring, represents an outstanding example of such undocumented microbiological diversity (33).

In the marine environment, intimate associations between physiologically diverse Bacteria and a variety of eukaryotic hosts are common (34). Known symbioses involving Archaea, however, are limited to one archaeal group, the euryarchaeal methanogens (subgroups Methanomicrobiales and Methanobacteriales; ref. 35). Archaeal symbioses involving Crenarchaeota, or other nonmethanogenic Archaea, have not been described.

The consistent presence of a single, identical crenarchaeal phylotype in every specimen of the *Axinella* sponge examined, the archaeon's high abundance and apparent active cell division, and the persistence of the association over long time periods all strongly suggest that the sponge–archaeal partnership represents a true symbiosis. Although relatively close phylogenetic relatives of the sponge-associated archaeon have been found in plankton (8–10) and holothurian guts (11), these other phylogenetic surveys have consistently revealed multiple, highly related archaeal phylotypes, or "clusters" (8–11, 26). In contrast, only a single crenarchaeal phylotype was recovered from the many individuals of this single *Axinella* sponge host species examined, and this specific crenarchaeal

phylotype has not been observed in plankton or in seawater surrounding captive sponges.

The growth temperature of the sponge in its natural habitat ranges from ≈8°C to 18°C, and these Porifera (and their crenarchaeal symbionts) have remained healthy for years when maintained in laboratory aquaria at 10°C. This observation provides strong evidence that the marine crenarchaeotes, whose closest cultivated relatives are all thermophilic or hyperthermophilic, can thrive at low temperatures. Available phylogenetic and ecological data suggest that ancestral variants of hyperthermophilic crenarchaeotes, perhaps originally inhabiting marine hydrothermal systems, became well-adapted for growth in surrounding cold seawater. This colder environment may have been gradually exploited, initially by mesophilic crenarchaeal genetic variants, whose descendants eventually adapted to even lower temperatures of contemporary seas. Subsequently, mesophilic or psychrophilic crenarchaeotes apparently radiated into many diverse habitats, becoming widespread in marine plankton (8–10), entering into symbiotic associations with metazoa, and eventually invaded terrestrial environments (12). In analogy to other marine prokaryotic species (e.g., *Vibrio* spp., ref. 36), nonthermophilic marine Crenarchaeota occupy a wide variety of habitats, ranging from planktonic to symbiotic niches.

We here propose the specific name *Cenarchaeum symbiosum* DeLong and Preston gen. nov., sp. nov., for the sponge-associated archaeon. [Cen arch' ae um Gr. adj. *kainos* recent, and Gr. adj. *koinos* common; Gr. adj. *archaeo* ancient; M. L. neut. n. *Cenarchaeum* = genus of relatively recent (derived nonthermophilic phenotype) and common (non-"extremophilic") Archaea. sym bi o' sum M. L. neut. adj.; *symbiosum* = living together.] The following criteria (37, 38) were used for identification and description of the sponge symbiont: phylogenetic identity determined by ssu rRNA sequence; specific ssu rRNA signature nucleotides; cell identity, size and morphology determined by *in situ* hybridization; natural growth temperature; and the specificity of the symbiotic association within a specific host species. We also propose here the order Cenarchaeales (DeLong and Preston, order nov.), and the family name Cenarchaeaceae (DeLong and Preston, fam. nov.), for the clade containing the sponge-associated crenarchaeal symbiont, and its planktonic (8–10) and abyssal (11) marine relatives.

Available data (8–12), in combination with this report, suggest that mesophilic or psychrophilic Crenarchaeota are as ubiquitous as are the more readily cultured Bacteria. This group has simply gone unnoticed, despite its high abundance in some marine habitats, until appropriate methods for its detection and identification became available. It is now apparent that the crenarchaeal lineage includes not only thermophiles and hyperthermophiles, but nonthermophilic phenotypes as well. This kingdom contains members whose growth temperatures collectively span the full temperature range of liquid water, from its boiling point at hydrothermal vents (>100°C; ref. 7) to its freezing point in polar seas (<−1.5°C; ref. 10). Nonthermophilic crenarchaeotes have a wide ecological distribution and have radiated into diverse marine and terrestrial habitats (8–12). Archaea, in particular Crenarchaeota, were previously considered ecologically insignificant, being presumed to occupy mainly extreme and unusual environments. It is becoming increasingly evident that previously unrecognized members of the Archaea are abundant, globally distributed, and well-adapted to more pedestrian lifestyles and niches, including symbiotic partnership with eukaryotic hosts.

We thank Shane Andersen, Chris Gottschalk, and Jim McCullough for their expert diving assistance, Mary Kay Harper and Welton Lee for taxonomic identification of the sponge host, Kathy Foltz for use of aquarium facilities, and Bob Trench for advice and helpful discussions. This work was supported by a DuPont Young Investigator grant to E.F.D., National Science Foundation Grants OCE92-18523, and OPP94-18442 and the National Sea Grant College Program, National Oceanographic and Atmospheric Administration, U.S. Department of Commerce, Grant NA36RG0537, project R/MP57B through the California Sea Grant College Program.

1. Woese, C. R. & Fox, G. E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5088–5090.
2. Woese, C. R. (1987) *Microbiol. Rev.* **51,** 221–271.
3. Iwabe, N., Kuma, K., Hasegawa, M., Osawa, S. & Miyata, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9355–9359.
4. Gogarten, J. P., Kibak, H., Dittrich, P., Taiz, L., Bowman, E. J., Bowman, B. J., Manolson, M. F., Poole, R. J., Date, T., Oshima, T., Konishi, J., Denda, K. & Yoshida, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6661–6665.
5. Woese, C. R., Kandler, O. & Wheelis, M. L. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4576–4579.
6. Kates, M., Kushner, D. & Metheson, A. (1994) *Biochemistry of Archaea* (Elsevier, Amsterdam).
7. Stetter, K. O., Fiala, G., Huber, R. & Segerer, A. (1990) *FEMS Microbiol. Rev.* **75,** 117–124.
8. Fuhrman, J. A., McCallum, K. & Davis, A. A. (1992) *Nature (London)* **356,** 148–149.
9. DeLong, E. F. (1992) *Proc. Natl. Acad. Sci USA* **89,** 5685–5689.
10. DeLong, E. F., Wu, K. Y., Prezelin, B. B. & Jovine, R. V. M. (1994) *Nature (London)* **371,** 695–697.
11. McInerney, J. O., Wilkinson, M., Patching, J. W., Embley, T. M. & Powell, R. (1995) *Appl. Environ. Microbiol.* **61,** 1646–1648.
12. Ueda, T., Suga, Y. & Matsuguchi, T. (1995) *Eur. J. Soil Sci.* **46,** 415–421.
13. Brantley, S. E., Molinski, T. F., Preston, C. M. & DeLong, E. F. (1995) *Tetrahedron* **51,** 7667–7672.
14. Stahl, D. A., Flesher, B., Mansfield, H. R. & Montgomery, L. (1988) *Appl. Environ. Microbiol.* **54,** 1079–1084.
15. Raskin, L., Stomley, J. M., Rittman, B. E. & Stahl D. A. (1994) *Appl. Environ. Microbiol.* **60,** 1232–1240.
16. Hultman, T., Stahl, S., Hornes, E. & Uhlen, M. (1989) *Nucleic Acids Res.* **17,** 4937–4946.
17. Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, G. J., Fogel, K., Blandy, J. & Woese, C. R. (1994) *Nucleic Acids Res.* **22,** 3485–3487.
18. Stein, J. L., Marsh, T. L., Wu, K. Y., Shizuya, H. & DeLong, E. F. (1996) *J. Bacteriol.* **178,** 591–599.
19. Felsenstein, J. (1988) *Annu. Rev. Genet.* **22,** 521–565.
20. Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994) *Comput. Appl. Biosci.* **10,** 41–48.
21. Leitch, A. R., Schwarzacher, T., Jackson, D. & Leitch, I. J. (1994) *In Situ Hybridization* (Bios Scientific, Oxford, U.K.), p. 91.
22. Amann, R. I., Krumholz, L. & Stahl, D. A. (1990) *J. Bacteriol.* **172,** 762–770.
23. DeLong, E. F., Wickham, G. S. & Pace, N. R. (1989) *Science* **243,** 1360–1363.
24. Distel, D. L., DeLong, E. F. & Waterbury, J. B. (1991) *Appl. Environ. Microbiol.* **57,** 2376–2382.
25. Lim, E. L., Amaral, L. A., Caron, D. A. & DeLong, E. F. (1993) *Appl. Environ. Microbiol.* **59,** 1647–1655.
26. Olsen, G. J. (1994) *Nature (London)* **371,** 657–658.
27. Amann, R. I., Ludwig, W. & Schleifer, K. H. (1995) *Microbiol. Rev.* **59,** 143–169.
28. Giovannoni, S. J., Britschgi, T. B., Moyer, C. L. & Field, K. G. (1990) *Nature (London)* **345,** 60–63.
29. Ward, D. M., Weller, R. & Bateson, M. M. (1990) *Nature (London)* **344,** 63–65.
30. Schmidt, T. M., DeLong, E. F. & Pace, N. R. (1991) *J. Bacteriol.* **173,** 4371–4378.
31. Liesak, W. & Stackebrandt, E. (1992) *J. Bacteriol.* **174,** 5072–5078.
32. Spring, S. Amann, R., Ludwig, W., Schleifer, K. H. & Petersen, N. (1992) *Syst. Appl. Microbiol.* **15,** 116–122.
33. Barns, S. M., Fundyga, R. E., Jeffries, M. W. & Pace N. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1609–1613.
34. Smith, D. C. & Douglas, A. E. (1987) *The Biology of Symbiosis* (Edward Arnold, Baltimore).
35. Embley, T. M. & Finlay, B. J. (1994) *Microbiology* **140,** 225–235.
36. Baumann, P., Baumann, L., Woolkalis, M. J. & Bang, S. S. (1983) *Annu. Rev. Microbiol.* **37,** 369–398.
37. Murray, R. G. E. & Stackebrandt, E. (1995) *Int. J. Syst. Bacteriol.* **45,** 186–187.
38. Sneath, P. H. A. (1992) *International Code of Nomenclature of Bacteria.* (Am. Soc. Microbiol., Washington, DC).