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UNIVERSITY OF CALIFORNIA

Santa Barbara

Prokaryotic Diversity in Marine Sponges: A Description of a Specific Association between the Marine Archaeon, *Cenarchaeum symbiosum*, and the Marine Sponge, *Axinella mexicana*

A Dissertation submitted in partial satisfaction

of the requirements for the degree of

Doctor of Philosophy

in

Ecology, Evolution, and Marine Biology

by

Christina Marie Preston

Committee in charge:

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December 1998

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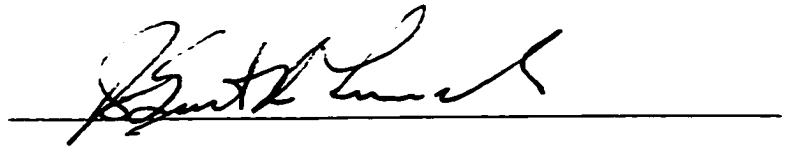

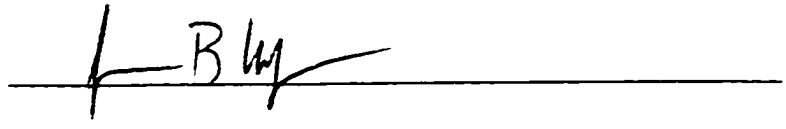

Committee Chairperson

December 1998

This work is dedicated to
C. R. "Bud" Fowkes & Richard and Mary Preston
for they are the people who have taught me the most about life.

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ABSTRACT

Prokaryotic Diversity in Marine Sponges: A Description of a Specific Association between the Marine Archaeon, *Cenarchaeum symbiosum* and the Marine Sponge *Axinella Mexicana*.

by
Christina Marie Preston

Many marine sponges possess dense microbial populations, but little is known regarding specific members or interactions of the microbial community within sponges. This study utilized molecular phylogenetic techniques, including quantitative ribosomal RNA (rRNA) hybridization with domain-specific oligonucleotide probes and phylogenetic analysis of isolated 16S rRNA gene sequences, to assay the diversity of prokaryotes within marine sponges (*Xestospongia* sp, *Cliona celata*, and *Axinella mexicana*). All the sponges harbored diverse prokaryotic assemblages. The majority of rRNA phylotypes were similar to those commonly found in the marine environment. However, the dominance of *Prochlorococcus*-like rRNA gene clones within *Xestospongia* sp., and the presence of archaeal rRNA and rDNA within *A. mexicana* might suggest that these prokaryotes represent sponge-specific microflora.

The association between the marine archaea and *A. mexicana* was further investigated to determine if the relationship was specific and persistent. rRNA hybridization studies showed that archaea inhabited all specimens of *A. mexicana* from the Santa Barbara Channel and Monterey, CA, and that the association could be maintained over 4 years in laboratory aquaria. Phylogenetic analysis of the 16S rDNA showed that the archaeal population of a single individual consisted of highly-related strains of an archaeon named

Cenarchaeum symbiosum that were affiliated with, but distinct from uncultivated marine planktonic crenarchaeotes. The presence of the strains in natural populations of *A. mexicana* was investigated using strain-specific 23S rRNA oligonucleotide probes and 16S rRNA signature sequence analysis. Several, highly related rRNA phylotypes (>99.2% sequence similarity) were detected in single specimens of *A. mexicana*. Sequence analysis of the 16S rRNA gene also indicated minor differences in the Monterey and Santa Barbara Channel strains of *C. symbiosum*. Using *in situ* hybridization with fluorescently-labeled 16S rRNA oligonucleotide and 16S/23S ribosomal polynucleotide probes that target marine crenarchaea, the sponge-associated archaea were identified and found to inhabit extracellular regions of the sponge matrix. The results of the above study indicated that the association between *A. mexicana* and *Cenarchaeum symbiosum*, is a symbiosis. The association represents the first description of *Archaea* inhabiting a sponge, and the first specific association between crenarchaea and a metazoan.

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INTRODUCTION

Anton de Bary (1879) defines symbiosis as the living together of unrelated organisms. Descriptions of symbiotic systems in the literature also imply that the associations are both persistent and specific (Dubos and Kessler, 1963). Specificity occurs when two interacting components show selectivity for one another (Weiss, 1954). When the two components are organisms involved in a symbiotic association, specificity is further defined as the interactions between the "two components of a symbiotic pair and the expression of the complementariness of all their dynamically interacting attributes" (Dubos and Kessler, 1963). Schoenberg and Trench (1980) proposed that interactions between the host and symbiont which lead to selection were a reflection of specificity, for example, the acquisition of a free-living symbiont from a mixed population by each generation of a host. One important aspect in showing specificity of an association is determining the identity of its members, and its persistence and stability. In this study, symbiosis is defined as a consistent, persistent, and specific association between two or more phenotypically distinct organisms. The outcome of the association, be it mutualistic, commensal, or parasitic is not addressed here.

Prokaryotes commonly inhabit plants, vertebrates, invertebrates, and protista in a wide variety of symbiotic associations. Often, the metabolic capability of the eucaryote host increases due to the presence of a symbiont. For example, metazoans (e.g. *Cnidaria* and *Porifera*) "photosynthesize" via their autotrophic symbionts (Douglas, 1994). Symbioses within invertebrates are

widespread; prokaryotes are specifically associated with members of all invertebrate phyla including the *Porifera* (Saffo, 1992). Marine sponges contain dense populations of microorganisms; in some sponges up to 60% of their tissue volume is attributable to prokaryote biomass (Santavy *et al.*, 1990; Vacelet and Donadey, 1977; Wilkinson, 1978). A mixed consortia of prokaryotes, mainly cyanobacteria and heterotrophic bacteria, are found free-living within the mesohyl or intracellularly within specialized cells called bacteriocytes or cyanocytes (Levi and Porte, 1962; Vacelet and Donadey, 1977; Wilkinson 1978; Ibid. 1987; Vacelet, 1981; Rutzler, 1990). It has been proposed that prokaryotes (including cyanobacteria or blue-green algae) within the sponge provide the sponge host with nutrients (Wilkinson, 1987), are involved in nitrogen cycling (Diaz and Ward, 1997; Shieh and Lin, 1994; Wilkinson and Fay, 1979), and have been implicated in the production of secondary metabolites that deter predation on the sponge or encroachment of other sedentary organisms (Unson *et al.*, 1994; Molinski, 1993; Kobayashi and Ishibashi, 1993; Faulkner *et al.*, 1993; Unson and Falkner, 1993).

Evidence that the prokaryotic photosynthentic symbiont marine sponge *Dysidea herbacea* was the source of secondary metabolites was shown by Unson and Faulkner (1993) and Unson *et al.*, (1994). Two features of the cyanobacterial symbiont, thylakoid membranes and autofluorescent pigments, allowed its identification in all individuals of *D. herbacea* producing the metabolite and its separation from non-autofluorescent sponge cells using flow cytometry. The source of the secondary metabolite was then shown to be present only in the fraction containing the cyanobacterial symbionts (Unson and

Faulkner, 1993; Unson *et al.*, 1994). Morphological characters of the cyanobacterial symbiont were thus used in defining its specificity, location, and role within the sponge (Berthold *et al*, 1982; Unson *et al.*, 1994; Unson and Faulkner, 1993).

The majority of prokaryotes, however, do not have defining morphological characteristics. Thus, morphological studies provide little information regarding the identity of the prokaryote, especially in its localization within host tissue. Traditionally, the classification of prokaryotes has relied on biochemical and phenotypic analysis (Austin, 1988). This requires that the organism must first be isolated, and thus, the microbe is not defined in its natural environment. The identification of bacterial symbionts of sponges has focused on the comparison of isolates obtained from the sponge and those from the surrounding seawater (Wilkinson, 1978; Santavy and Colwell, 1990). Those isolates obtained only from sponge-derived inoculum were interpreted as symbionts. However, the specificity of such associations has not been established. It is not clear whether cultures obtained were chance isolates, or specific inhabitants of particular sponge species. Experiments re-infecting sponges with bacteria isolated from them were not performed (Wilkinson, 1978; Santavy and Colwell, 1990), thus Koch's postulates were not satisfied. Additionally, culturing techniques fail in definitively determining if the prokaryote is a true symbiont, because the physical association between the host and symbiont is lost upon isolation.

Cultivation-based techniques often do not recover the majority of the population. Comparisons of total prokaryotic counts estimated from direct

enumeration and from the number of colonies on culture media indicate that cultivation techniques retrieve only a small percentage of the total microbial population. Santavy *et al.* (1990) recovered only eleven percent of the bacterial population in a Caribbean sclerosponge using cultivation-based techniques. Similar studies in bacterioplankton have shown that less than one percent were cultivated (Ferguson *et al.*, 1984; Hobie *et al.*, 1977; Kogure *et al.*, 1979). Cultivation studies of the sponge microbial community have mainly utilized media that select for aerobic or facultative anaerobic heterotrophic bacteria (Santavy and Colwell, 1990; Wilkinson *et al.*, 1981; Santavy *et al.*, 1990), although enrichment media for gram positive bacteria (Santavy *et al.*, 1990), cyanobacteria (Santavy *et al.*, 1990), anaerobic phototrophic bacteria (Imhoff and Truper, 1976), and nitrogen fixing bacteria (Shieh and Lin, 1994) have also been used. Those microorganisms that are readily cultivated from sponges may not represent dominant members of the prokaryotic association, but rather those capable of growing on the enrichment media. In addition, many symbionts have not been cultivated outside their host (see Baumann, 1998; Baumann and Moran, 1997), and thus in these cases, cultivation based techniques cannot be used to identify or characterize the symbiont.

Phylogenetic studies based on universally conserved macromolecules, [e.g.. ribosomal RNA (rRNA), Lane *et al.*, 1987; Olsen, 1988; Woese, 1987], have been used to determine the phylogenetic relationships of all organisms and in doing so have altered the classification of life into three domains, the *Eucarya, Bacteria*, and *Archaea* (Woese *et al.*, 1990). They have also laid the groundwork for the development of molecular techniques to directly access the

diversity of mixed populations (see reviews by Ward *et al.*, 1992; Pace, 1996; Pace, 1997) without the requirement that the microorganism first be in pure culture. The first report directly sequenced 5S rRNA to determine the phylogenetic affiliation of unculturable symbionts (Stahl *et al.*, 1984). Other studies have utilized shotgun cloning to isolate individual rRNA genes whereby fragments of genomic DNA from environmental samples were cloned then screened for rRNA genes (DeLong *et al.*, 1989; Schmidt *et al.*, 1991). Alternatively reverse transcription of rRNA, and amplification of ribosomal DNA (rDNA) using the polymerase chain reaction (PCR) followed by cloning, have been used to isolate individual rRNA genes (see reviews by Pace, 1997; Pace, 1996). The rRNA gene sequence is then used to infer the phylogeny of microorganisms using reference rRNA sequences of cultivated and environmental rRNA in databases such as the Ribosomal Database Project (RDP, Maidak *et al.*, 1997). Molecular phylogenetic approaches have led to the discovery of many previously undetected and thus far uncultivated prokaryotic lineages including the SAR11 cluster (Giovannoni *et al.*, 1990), marine "Group 1" archaea (DeLong, 1992; Fuhrman *et al.*, 1992), and marine "Group 2" archaea (DeLong, 1992). The marine "Group 1" archaea and the SAR11 cluster have been shown to have a worldwide distribution, and account for significant proportions of the prokaryotic biomass in plankton (DeLong *et al.*, 1994; Massana *et al.*, 1997; Murray *et al.*, 1998; Massana *et al.*, 1998; Moyer *et al.*, 1998; Fuhrman and Davis, 1997; McInerney *et al.*, 1997; Giovannoni *et al.*, 1990; Mullins *et al.*, 1995). The discovery of previously undetected lineages

using molecular phylogenetic methods exemplifies the fact that culture-based methods have not fully described prokaryotic diversity within the environment.

The identification of microorganisms in mixed assemblages by rRNA gene phylogeny is one application of rRNA gene sequence data. Different regions within the rRNA exhibit different mutation rates creating different levels of sequence conservation along the length of the molecule ranging from universally conserved to more variable domains (Woese, 1987). Thus, oligonucleotide probes can be designed to target different phylogenetic groups of organisms. The more variable regions within the rRNA molecule are ideal for designing probes that target specific strains or lineages while more conserved regions are domain-specific or are universally found in all organisms (Amann *et al.*, 1995). The probes can then be used to quantify (rRNA hybridization studies) and identify (*in situ* hybridization studies) the presence of specific groups of microorganisms in their natural environment, and in cases when the only the rRNA phylotype has been recovered, evidence that the organism exists.

Phylogenetic analyses using rRNA gene sequence have been successfully applied to study host-symbiont associations, specifically, in cases where the symbiont cannot be cultured (Stahl *et al.*, 1984; Distel *et al.*, 1988; Amann *et al.*, 1991). In addition, they have been used to confirm (Distel *et al.*, 1991) or refute (Jannasch and Nelson, 1994; Felbeck and Distel, 1992; Distel and Wood, 1992) the authenticity of the putative symbiont isolate. rRNA gene sequence has also been used to investigate the diversity, specificity, and evolution of symbioses in aphids (Unterman *et al.*, 1989; Munson *et al.*, 1991), clams and mussels (Distel *et al.*, 1988; Durand and Gros, 1996; Krueger and

Cavanaugh, 1997), bioluminescent fish (Haygood and Distel, 1992), and cnideria (Rowan and Powers, 1991; Rowan and Knowlton, 1995; McNally *et al.*, 1994; Gast and Caron, 1996). Symbiont-specific oligonucleotide probes developed from rRNA sequence data were successfully used in *in situ* hybridization studies to localize the putative symbiont within its host (Distel *et al.*, 1991; Cary *et al.*, 1993; Cary *et al.*, 1997), or to investigate how the symbiont is transmitted to each generation of host (Cary and Giovannoni, 1993; Gros *et al.*, 1996). Only one study has utilized 16S rRNA gene phylogeny to investigate the bacterial diversity within marine sponges (Althoff *et al.*, 1998).

The research presented here utilizes molecular phylogenetic techniques to access the diversity of prokaryotes within several species of marine sponges, *Cliona celata*, *Tethya aurantia*, and *Axinella mexicana* found off of the coast of California, and the Australian sponge, *Xesotspongia* sp. One goal was to determine whether specific associations exist between the marine sponges and prokaryotes. Sponges are filter feeders, and collect bacteria as food (Bergquist, 1978). Prokaryotic rRNA phylotypes recovered from the sponge could represent sponge-specific microflora or transient microorganisms captured during filtration. It is therefore necessary to determine if the rRNA phylotypes recovered from the sponge are similar to those commonly found in seawater. The abundance of archaeal and bacterial rRNA within the sponges was estimated using 16S rRNA hybridization probes. Individual prokaryotic 16S rRNA genes were amplified from the sponges *C. cliona* and *Xestospongia* sp. using PCR, cloned, and sequenced to determine their phylogenetic affiliation.

During the initial investigation of prokaryotic diversity within these sponges, archaeal rRNA and rDNA were detected in several individuals of *A. mexicana* (chapter 1). Prior studies utilizing culturing techniques had only identified bacterial populations in sponges; no members of the Archaea had yet been detected. The remainder of the chapters focus on the investigation of the archaeal population within *A. mexicana* and indicate that these organisms constitute a symbiosis (as defined above). The archaea were found to be related to a uncultivated clade of planktonic archaea (chapter 2). This particular lineage of archaea has resisted cultivation attempts, and the physiology and phenotypic properties of the group are currently unknown. Thus, studies of the archaea and the archaeal-sponge association must utilize techniques that do not require the isolation of the archaea in pure culture. Quantitative rRNA hybridization studies were used to assay the contribution of archaeal rRNA to total rRNA from natural populations of *A. mexicana*, and to monitor the percentage of archaeal rRNA in sponges held in aquaria (chapter 2). Information obtained from sequence data was used to determine the phylogenetic position of the archaea and its presence in natural populations of *A. mexicana* from the Santa Barbara Channel and Monterey, CA (chapter 2). The diversity of the archaeal population within *A. mexicana* was characterized (chapter 3), and fluorescent probes were developed (both oligonucleotide and polynucleotide) to elucidate the morphology, location, and density of the archaea in *A. mexicana* (chapters 2 & 4). The distribution of the archaea of *A. mexicana* in other environments (chapters 2 & 3), as well as the presence of archaea in other species of marine sponges (chapter 1, chapter 3, & appendix 2) was also investigated.

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CHAPTER 1

Prokaryotic diversity within marine sponges as determined by 16S ribosomal RNA abundance and 16S RNA Gene Sequence.

Introduction

Many marine sponges harbor a dense bacterial community which can comprise up to 60% of their mesohyl volume (Santavy *et al.*, 1990; Vacelet and Donadey, 1977; Wilkinson, 1978). The sponge-associated bacteria have been implicated in the production of secondary metabolites (Unson and Faulkner, 1993; Faulkner *et al.*, 1994), in nitrogen cycling (Diaz and Ward, 1997; Shieh and Lin, 1994; Wilkinson and Fay, 1979), and in the production of nutrients for the sponge host (Wilkinson, 1987; Rutzler, 1990; Reiswig, 1981). The majority of studies describing sponge-associated bacterial populations have relied solely on culturing techniques that often retrieve only a small percentage of the total microbial population, or electron microscopic (EM) techniques, which provide little information on microbial identity expect for a few morphologically distinct prokaryotes (e. g. cyanobacteria). Santavy *et al.* (1990) estimated that in a Caribbean sclerosponge, 89% of the invertebrate-associated bacteria were "unculturable", and so the majority of the prokaryotic population remained unidentified in their study.

The cultivable and morphologically distinct bacterial types that have been isolated or observed in sponges include cyanobacteria (Berthold *et al.*, 1982; Vacelet, 1981; Wilkinson, 1978; Wilkinson, 1981; Hinde *et al.*, 1994), anaerobic photoheterotrophic bacteria (Imhoff and Truper, 1976), methane-oxidizing bacteria (Vacelet *et al.*, 1995; Vacelet *et al.*, 1996; Harrison *et al.*,

1994), gram positive bacteria (Lee and Choi, 1998), and aerobic and facultative anaerobic heterotrophic bacteria (Zimmerman *et al.*, 1990; Shieh and Lin, 1994; Wilkinson, 1978; Santavy *et al.*, 1990; Vacelet, 1975; Burlando *et al.*, 1988). In all cases, more than one bacterial species has been isolated or more than one bacterial morphology was observed using EM, suggesting that a mixed consortia of bacteria inhabit sponges. Additionally, many of the sponge-bacterial isolates were not recovered from the surrounding seawater. These were interpreted as sponge-specific microorganisms (Wilkinson, 1978; Santavy *et al.*, 1990; Wilkinson *et al.*, 1981; Santavy, 1985; Santavy and Colwell, 1990). In contrast, three studies found bacterial isolates from the sponge and seawater were similar (Madri *et al.*, 1971; Bertrand and Vacelet, 1971; Wilkinson, 1978).

Since cultivation-based techniques recovered a low percentage of the prokaryotic population (11% see above ref. Santavy *et al.* 1990; <1% of bacterioplankton, Hobbie *et al.*, 1977), the majority of the diversity within the community remained undescribed. Molecular phylogenetic techniques, on the other hand, do not require that the organisms of the community first be cultivated and so they can be used to directly access the diversity of microbial communities (Pace, 1996). The technique involves the isolation of nucleic acids from the community and the subsequent recovery of rRNA genes. Individual rRNA genes are isolated using PCR with rRNA-specific primers and propagated in clone libraries. The individual rRNA genes can then be sequenced and compared to the rRNA genes of known organisms to determine its phylogenetic affiliation. Initial studies of bacterial diversity utilizing these techniques showed that rRNA phylotypes isolated in environmental gene libraries are not similar to

those of cultivated microbes (Giovannoni *et al.*, 1990; DeLong, 1992; Fuhrman *et al.*, 1992; DeLong *et al*, 1994; Liesak and Stackebrandt, 1992). A comparison of bacterial 16S rRNA genes recovered from the same environmental sample (bacterioplankton) using molecular phylogenetic and standard cultivation techniques, showed little similarity, indicating that microorganisms that are the most abundant are not readily cultivated (Suzuki *et al.*, 1997). One study, however, suggested that many readily cultivated bacteria represented those common in bacterioplankton (Rehnstam *et al.*, 1993). Nonetheless, the analyses of the prokaryotic communities via 16S rRNA gene sequence allows a more direct assessment of diversity, and avoids many of the selection biases introduced with cultivation based techniques.

Only a single study has employed this approach to investigate the bacterial flora found within sponges. Althoff *et al.*, (1998) found that the sponge, *Halichondria panicea*, harbored a mixed consortia of bacterial rRNA genes which was dominated by a rDNA clone most closely affiliated with the Alpha Proteobacterium, *Rhodobacter*. The *Rhodobacter*-like clone consistently colonized sponges collected from different locations suggesting a possible symbiotic association between the bacterium and sponge.

A similar approach was used here to determine the phylogenetic identity of sponge-associated microflora. In addition, the relative prokaryotic biomass in marine sponges was also estimated. Both archaeal and bacterial-specific rDNA primers and rRNA oligonucleotide probes were employed to determine the presence of the two prokaryotic Domains within marine sponges. Prior to this study, the presence of archaea in marine sponges had not yet been investigated.

Our ultimate goal is to identify those bacterial species which are consistently and specifically associated with particular species of *Porifera*. Here I present a general survey of the diversity of prokaryotes within marine sponges collected from California and Australia.

Methods

All marine sponges, except *Xestospongia* sp., were collected with SCUBA from Naples Reef located off the coast of Santa Barbara, CA. The Australian sponge *Xestospongia* sp. was obtained from T. F. Molinski. All sponges from Santa Barbara were identified by their gross morphology, color, and size and shape of the spicules (see appendix 1 for descriptions of individual sponges and chapter 2 for the identification of *Axinella mexicana*). A portion of sponge was removed, rinsed in filter sterilized seawater, and frozen within two hours of collection. The sponges were stored frozen at -80°C until nucleic acids were extracted.

In order to determine the prokaryotic diversity within marine sponges without using cultivation techniques, bacterial rDNA genes were isolated, cloned and sequenced. In addition, the relative percentage of prokaryotic rRNA was quantified by hybridizing domain-specific oligonucleotide probes to total nucleic acids extracted from marine sponges.

Nucleic acids were extracted as follows: A subsample of sponge (0.5 g) was mechanically dissociated in lysis buffer (40 mM EDTA, 50 mM Tris-HCl pH 8.3, and 0.75 M sucrose) using a tissue homogenizer. Cells were incubated in 1 mg/mL lysozyme for 30 minutes at 37°C, followed by an incubation in 0.5 mg/mL proteinase K and 1% SDS for 30 minutes at 55°C, and finally immersed

in a boiling water bath for 60 seconds. Nucleic acids were purified using phenol:chloroform:isoamyl alcohol (50:49:1, vol:vol:vol) pH 8.0 and chloroform:isoamyl alcohol (24:1, vol:vol) extraction, precipitated with two volumes of 100% ethanol and 1/10 volume of 4 M ammonium acetate, rinsed in 80% ethanol, air dried, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0). DNA was quantified using a Hoest dye fluorescence assay in a fluorometer (Hoefer Scientific Instruments). Crude nucleic acid preparations were used in the rRNA hybridization experiments (see below). No amplification products were obtained when the crude nucleic acid preparations were used in PCR, therefore, total DNA extracted from sponges was purified by cesium chloride equilibrium density gradient ultracentrifugation.

To purify the DNA, approximately 5 μg of the crude nucleic acids, 0.5 mM Cesium Chloride, and 0.5 mg/mL ethidium bromide were mixed and centrifuged overnight on a Beckman Optima tabletop ultracentrifuge using a TLA100 rotor at 100,000 rpm (Weeks *et al.*, 1986). The band of DNA was visualized under UV light, collected using a syringe, extracted three times with an equal volume of water saturated butanol to remove the ethidium bromide, and washed three times with 1 mL TE buffer in a Centricon 100 microconcentrator (Amicon) to remove the cesium chloride. The purified DNA was then used in PCR reactions (see below).

Eucaryl and archaeal small subunit ribosomal RNA genes (ssu rRNA or rDNA) were amplified in PCR reaction mixtures using domain-specific primer pairs (table 1-1). Bacterial rRNA genes were amplified using a bacterial-specific forward primer and a universal reverse primer (table 1-1). Each 100 μl

Table 1-1. PCR primer pairs used to amplify the ssu rRNA genes from purified nucleic acids of marine sponges.

Target Organisims	Primer	Primer Sequence (5' to 3')[a]	ODP designation[b]	Reference
Eucarya	EukF	AACCTGGTTGATCCTGCCAGT	---	Medlin et al., 1988
	EukR	TGATCCTTCTGCAGGTTCACCTAC	---	Medlin et al., 1988
Bacteria	Eubac27F	AGAGTTTGATCCTGGCTCAG	D-Bact-0009-a-S-20	Lane, 1991
	1492R	GGTTACCTTGTTACGACTT	S-*-Univ-1492-a-A-19	Lane, 1991
Archaea	Arch21F	TTCCGGTTGATCCYGCCGGA	S-D-Arch-0002-a-S-20	DeLong, 1992
	Arch958R	YCCGGCGTTGAMTCCAATT	S-D-Arch-0940-a-A-20	DeLong, 1992

[a]Y= U/C; M=A/C

[b]named according to nomenclature of the Oligonucleotide Probe Database (Alm *et al.*, 1996)

PCR reaction contained 1X PCR buffer (10 X PCR buffer was 500mM KCl, 100 mM Tris-HCl pH 8.3, 20 mM $MgCl_2$, 0.01% gelatin and 0.5% NP40), 200 μM dATP, dCTP, dGTP, and dTTP (Promega), 0.150 μM of each primer, 2.5 U Taq DNA polymerase (Fisherbiotech). Ten nanograms of DNA isolated from dinoflagellates, *Shewanella* sp. strain SC2A, or *Haloferax volcanii* were used as the positive control for the eucaryl, bacterial, or archaeal primer pairs, respectively. The negative control was just the PCR reaction mixture; no DNA was added. In general, 1 μL and 1μL of a 1 to 10 dilution of the purified DNA (approximately 5 to 50 ng) was used as the template in PCR reactions. The conditions for PCR were as follows: an initial denaturation step at 92°C for 3 minutes, 30 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and followed with a final extension at 72°C for 5 minutes using a Perkin Elmer 2400 (DeLong, 1992). Amplification products were visualized by electrophoresis on 1% agarose gels (wt/vol) in 0.5X TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA) containing 0.25 μg/mL ethidium bromide.

The diversity of bacteria associated with *C. celata* (specimen c2) and *Xestospongia* sp. (specimen x2) was assessed by cloning and sequencing the amplified bacterial rDNA. Multiple bacterial PCR reactions from each individual were pooled, and concentrated by ethanol precipitation. Two volumes of 100% ethanol and 1/10 volume 4 M sodium acetate pH 5.1 were added to the pooled PCR products, held for at least 2 hours at -20°C, centrifuged for 30 minutes at 12,000 xg, and washed in 80% ethanol. The resulting pellet was resuspended to 1/10 the original pooled PCR volume with nanopure water. rRNA genes were inserted into β-galactosidase gene of the pTA cloning vector

(version 2.0, Invitrogen) and transformed into *E. coli* INVαF' according to the manufacture's protocol. Transformation mixtures were plated onto LB agar plates containing 50 μg/mL kanamycin and 50 μl X-Gal (2% stock solution, wt/vol), incubated overnight at 37°C, and stored at 4°C until complete color development of the colonies. After blue/white colonies appeared, transformants that contained inserts, as determined by their white colony color, were inoculated into 2 mL of LB media containing 50 μg/mL kanamycin and incubated overnight with shaking at 37°C. Bacterial cells were collected by centrifugation at 12,000 xg for 30 seconds, and the plasmids were extracted as follows. Each cell pellet was resuspended in 100 μl of ice cold solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA). Two-hundred microliters of freshly made solution 2 (0.2 N NaOH, 1% SDS) was then added. One-hundred and fifty microliters of 3 M K+, 5M acetate pH 5.5 was then added, and the tubes vortexed gently. The tubes were spun for 10 minutes at 12,000 xg, and the supernatant transferred into a tube containing 720 μL 100% ethanol. The plasmid DNA was precipitated, washed in 80% ethanol, and resupended in 50 μl TE buffer containing 0.1 mg/mL RNAse A.

To determine if plasmids contained inserts of the correct size, plasmid minipreps were digested overnight with 1.2 U/μL EcoR1 in 1X restriction buffer H (Promega, WI), and the fragments separated on a 1% agarose gel. Clones that contained the bacterial rDNA inserts were subsequently amplified with M13F and M13R primers using the same conditions as described above for the amplification of 16S rRNA genes. One microliter of a 1 to 10 dilution of the plasmid miniprep was used as the template in PCR reactions. Amplified

products were checked on a 1% agarose gel. The amplified cloned rDNA gene were digested with 1 U/μL of HaeIII in 1X restriction buffer C (Promega, WI), overnight at 37°C. The restriction fragment length polymorphism (RFLP) pattern for each cloned rRNA gene was determined after electrophoresis on a 2.5% low melt agarose gel (wt/vol). Clones were grouped on the basis of the RFLP patterns of both the EcoRI and HaeIII digests.

A representative of the major RFLP patterns was sequenced using Sequenase (version 2.0; US Biochemical) according to the manufacture's recommendations. Ten microliters of isolated plasmid DNA was denatured with 0.2 M NaOH for 30 minutes at 37°C and then concentrated using 2 volumes of ethanol and 60 mM ammonium acetate pH 4.5. Denatured plasmids were resuspended in 2 μL sequenase reaction buffer and 8 μL of 519r primer (5 ng/μl stock; 5' GWATTACCGCGGCKGCTG; Lane, 1991), and incubated at 37°C for 10 minutes. During the 10 minute incubation, the premix [for 6 reactions: 6μL DTT, 6 μL diluted labeling mix (6 μL dGTP labeling mix in 24 μL ddH_2O), 3 μL [α-^{35}S]-dATP (Dupont/NEN), 12 μL diluted sequenase (2 μL sequenase in 10 μL sequenase dilution buffer), and 1 μL pyrophosphate] was prepared on ice and 2.5 μL of each ddNTPs was aliquoted to individual wells of a multi-well tray held at 37°C. To each annealing tube, 5 μL of premix was added, and incubated for one minute at room temperature. Then, 3.5 μL were aliquoted into each ddNTP, and incubated for 10 minutes at 37°C. Reactions were terminated by the addition of 6 μL of stop mix. Before loading onto 6 % Long Ranger sequencing gel (J. T. Baker), reactions were placed in boiling water for 5 minutes to denature the strands, and placed on ice. Short gels were run for 4

hours and long gels were run for 6.5 hours at 1,000 volts. Gels were dried on a gel dryer (Fisher) and exposed to X-ray film (Fuji Medical X-ray Film, Japan). Gels were read by hand. The sequences from the long and short gels for each clone were entered into GDE and aligned. The consensus rDNA sequence for each clone was used in phylogenetic analysis. The phylogenetic affiliation using distance matrix analysis (DeSoete, 1983) of the rDNA clones was determined by comparing them to rRNA gene sequences of known microorganisms in databases such as the Ribosomal Database Project (Maidak *et al.*, 1997).

The proportion of archaeal, bacterial and eucaryl rRNA in marine sponges was determined by quantifying the relative binding of radioactively-labeled, domain-specific oligonucleotide probes (Giovanonni *et al.*, 1988; Stahl *et al.*, 1988; Raskin *et al*, 1994) to crude nucleic acids from sponges. rRNA hybridization experiments were performed as previously described (DeLong, 1992; Massana *et al.*, 1997) with the following modifications. The sponge-derived crude nucleic acids and RNA standards (Eucarya: *Saccharomyces cerevisiae;* Bacteria: *Cytophaga johnsanii;* Archaea: *Haloferax volcanii*) were denatured with 0.5% gluteraldhyde in 50 mM sodium phosphate buffer pH 7.2 for 10 minutes. A four-fold serial dilution was made for the RNA standards and an eight-fold dilution series were made for the sponge-derived nucleic acids. The nucleic acids were then applied to nylon membranes (Hybond-N; Amersham) with vacuum using a slot blot apparatus, and immobilized by UV crosslinking (Stratalinker, Stratagene). Four replicate blots were made for each set of samples. Each replicate blot was hybridized to one of the domain-specific oligonucleotide probes. The blots were prehybridized for 30 minutes at 45°C in

hybridization buffer (0.9 M NaCl, 50 mM Na_2HPO_4, 5 mM EDTA, 0.5% SDS, 10X Denhart's solution, 0.5 mg/mL polyadenosine; Massana *et al.*, 1997) before the addition of $2X10^7$ cpm of the respective 16S rRNA oligonucleotide probe labeled at the 5' end with [γ-$d^{32}P$]-ATP (Dupont/NEN) using T4 polynucleotide kinase (Stahl and Amann, 1991). Oligonucleotide probes used in the study are listed in table 1-2. Blots were hybridized overnight at 45°C. Blots were then washed at room temperature for 30 minutes in wash buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris and 1% SDS; Massana *et al.*, 1997), and then placed in pre-equilibrated wash buffer at the high stringency temperature for 30 minutes. High stringency wash temperatures were 37°C for Univ1392 and Euk1209, 45°C for Bact338, and 56°C for Arch915 (DeLong, 1992; Massana *et al.*, 1997). The blots were subsequently air dried and the amount of radioactive probe bound to the serially diluted, immobilized rRNA was quantified using a radioanalytic gas proportional counting system (Scanalytics, Billerica, MA).

To quantify the relative amount of rRNA belonging to each domain, the slope of the line representing the amount of nucleic acids blotted versus the total counts per minute was determined for each sponge sample probed with each domain-specific probe. To account for differences in the binding efficiencies of the oligonucleotide probes, the slope of the signal (cpm bound per unit rRNA) of each domain-specific probe was normalized to the Univ1392 probe signal for each control template. The correction factor for each domain-specific probe was determined by dividing the slope of the Univ1392 probe binding to the control rRNA by the slope of the domain-specific probe binding to the same control

Table 1-2. Domain-specific and universal oligonucleotide probes used in rRNA hybridization studies.

Oligonucleotide Probe	Sequence (5' to 3)	ODP designation[b]	Reference
Univ1392	ACGGGCGGTGTGTRC	S-*-Univ-1392-a-A-15	Stahl *et al.*, 1988
Euk1200	GGGCATCACAGACCTG	S-D-Euca-1209-a-A-16	Giovannoni *et al.*, 1988
Bact338	GCTGCCTCCCGTAGGAGT	S-D-Bact-0338-a-A-18	Amann *et al.*, 1990
Arch915	GTGCTCCCCCGCCAATTCCT	S-D-Arch-0915-a-A-20	Amann *et al.*, 1990

[a]R=A/G;

[b]named according to nomenclature of the Oligonucleotide Probe Database (Alm *et al.*, 1996)

rRNA. The domain-specific correction factor and the slope of the sponge samples probed with the respective domain-specific oligonucleotide probe were multiplied to express the probe-derived signal in terms of the Univ1392 probe. The relative percentage of rRNA of each domain was determined by dividing the normalized domain specific slope by the sum of the normalized domain specific slopes (Eucarya+Bacteria+Archaea). The percentage of the Univ1392 hybridization signal (HS) was also calculated for each probe and is defined as the corrected domain-specific slope/slope of the Univ1392 bound to the same sample X 100.

Results

Detection of prokaryotic rRNA genes in marine sponge

Purified nucleic acids were used as templates in PCR reactions to determine if Archaeal and/or Bacterial rDNA were present within marine sponges. Eucaryal-targeted PCR amplifications were also performed to ensure that there was no inhibitory compounds that would prevent amplification of prokaryotic rRNA genes and give a false negative result. In all cases, eucaryl rRNA genes were amplified from purified nucleic acids from *Cliona celata*, *Tethya aurantia*, *Axinella mexicana*, an unknown species (m1), and *Xestospongia* sp. (see figure 1-1 and table 1-3). Amplification products of the correct size were observed in all PCR reactions using the bacterial primer pair, indicating all sponges contained bacterial rRNA genes (table 1-3; figure 1-1). Archaeal rDNA genes were amplified from *A. mexicana* (all 5 specimens). One individual of *C. celata* (c4) produced a weak amplification signal with the archaeal-specific rDNA primers.

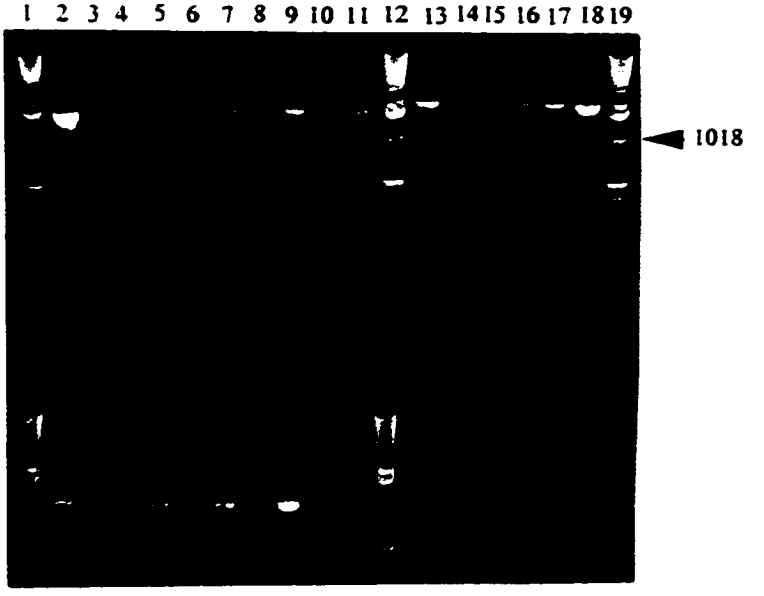


Figure 1-1. Amplification products from PCR indicating the presence or absence of eucarya (lanes 15-18, top) , bacterial (lanes 4-11, top), and archaeal (4-11, bottom) rRNA genes within *A. mexicana* and *C. celata*. Templates used in the reactions are as follows: *A. mexicana* DNA from individuals s3, s4 and s5 was used in reactions present in lanes 4 to 9 (top and bottom) and lanes 15 to 17 (top). Amplifications using *C. celata* (specimen c4) DNA are in lanes 9, 10 (top and bottom) and 18 (top). Each sponge sample was amplified twice using either 1µL of a 1/10 dilution (lanes 4,6,8 & 10) or 1 µL (lanes 5,7,9, &11) of CsCl purified sponge DNA as the template in PCR reactions containing the bacterial and archaeal primers. All eucaryl amplifications contained 1µl of purified sponge DNA. The predicted sizes of the products were 1.8 kb for Eucarya, 1.5 kb for Bacteria, and 0.95 kb for Archaea. Lanes 2 and 3 (top) contain the Bacterial positive control, *Shewanella* sp. SC2A and the negative control, respectively. The positive and negative controls for the Eucaryl PCR are located in lanes 13 and 14 respectively, and the positive and negative controls for the archaeal amplification are in lanes 2 and 3 (bottom). Lanes 1 (top and bottom), 12 (top and bottom), and 19 (top) contain the 1 KB ladder (BRL, Gibco; NY).

Table 1-3. PCR amplification of Eucaryal, Bacterial, and Archaeal rDNA genes from marine sponges.

Sponge Species	Specimen No.	Detection of Domain-Specific ssu rRNA Genes[a]		
		Eucarya	Bacteria	Archaea
Cliona celata	c1	+	+	-
	c3	+	+	-
	c4	+	+	weak+
Tethya aurantia	t4	+	+	-
Axinella mexicana	s1	+	+	+
	s2	+	+	+
	s3	+	+	+
	s4	+	+	+
	s5	+	+	+
unidentified	m1	+	+	-
Xestospongia sp.	x1	+	+	-
	x2	+	+	-
	x3	+	+	-

Presence (+) or absence (-) of an amplification product of the predicted size with the primer pairs employed to detect the presence of Eucaryl, Bacterial, and Archaeal ssu rRNA genes. Predicted amplification sizes were approximately 1.8 kilobases (kb), 1.5 kb, and 0.95 kb for Eucarya, Bacteria, and Archaea.

Relative percentages of domain-specific rRNA in different marine sponges.

The detection of prokaryotes in different marine sponges using PCR was confirmed with rRNA hybridization experiments. In addition, the percentage of prokaryotic rRNA within the sponge should indicate if a biologically active/abundant prokaryotic population is present. The relative amount of rRNA contributed by each domain was determined from slot blots of serially-diluted crude nucleic acids immobilized on nylon filters hybridized to radioactively labeled domain-specific oligonucleotide probes. The relative proportion of prokaryotic rRNA (Archaeal & Bacterial) in sponges varied between different species, but was comparable within individuals of the same species (table 1-4). Individuals of *Xestospongia* had the highest bacterial signal. Almost one half of the total sponge rRNA signal was bacterial. The sponge species examined from Santa Barbara contained a lower percentage of prokaryotic rRNA (less than 15% of the total rRNA) compared to the Australian sponge. *C. celata*, *T. aurantia*, and the unidentified species of sponge only contained bacterial rRNA. Approximately 4% of the total rRNA of the *A. mexicana* was prokaryotic, which was comparable to other sponges collected from Santa Barbara. However, one half of the prokaryotic signal was attributable to the presence of archaeal rRNA (see chapters 2 and 4). Although archaeal rRNA was not detected in any other sponge species from the Santa Barbara Channel, archaeal rDNA was weakly amplified from one specimen (c4) of *C. celata* (see table 1-3).

In most cases, the sum of the corrected domain-specific probe slopes accounted for a relatively low percentage of the Univ1392 probe slope (table 1-4, the values in parentheses). Hybridization conditions including the quality of

Table 1-4. Relative percentage of the total rRNA isolated from various sponges as determined by rRNA hybridization to domain-specific oligonucleotide probes.

Species[a]	Specimen No.	Relative % of Domain-specific rRNA[b] (HS)		
		Eucarya	Bacteria	Archaea
Cliona celata	c1	87 (44.0)	13(6.3)	ND
	c3	93 (33.9)	7 (2.9)	ND
	c4	93 (47.6)	7 (3.3)	ND
Tethya aurantia	t4	99 (50.3)	1.(1.5)	ND
?	m1	99 (52.4)	1 (.7)	ND
Axinella mexicana	s1	89 (43.1)	10 (4.6)	1.3 (0.6)
	s2	90 (68.2)	3 (2.4)	6.3 (4.7)
Xestospongia sp.	x1	63 (55.7)	37 (46.5)	ND
	x2	44 (50.6)	56 (44.1)	ND
	x3	54 (63.9)	46 (33.2)	ND

[a]*Xestospongia* sp. collected from Bennett Shoal, Exmouth Gulf, Western Australia. All other sponges collected from Naples Reef, located off the coast of Santa Barbara, CA.

[b] ND not detected

The numbers in parentheses represent hybridization signal (HS) which was calculated as the normalized domain-specific slope/slope of the Univ1392 probe bound to the same sample X 100.

the rRNA, wash temperature, differences in the specific activity and binding efficiencies of the oligonucleotide probes, degeneracy in the oligonucleotide probe sequence, and the secondary structure of the target region in the rRNA (Raskin *et al.*, 1994; Zheng *et al.*, 1997) could account for these differences. Another potential complication is that the domain-specific probe's target sequence may not be present in all the members of the Domain. If a portion of the population within the sponge did not contain the target sequence of one of the domain-specific probes, then that rRNA was undetected.

Analysis of Bacterial Population by rRNA Gene Cloning and Sequencing.

The use of domain-specific rRNA primers and oligonucleotide probes indicated the presence of bacterial rRNA and rDNA, but in utilizing the domain-specific probes, individual members of the prokaryotic community were not identified. Thus, in order to access the diversity of the bacterial population, the PCR amplified rRNA genes from one individual of *C. celata* and *Xestospongia* sp. were cloned and sequenced. RFLP analysis was performed to identify unique groups of clones. A total of 38 rRNA gene clones of *C. celata*, and 65 from *Xestospongia* sp. were analyzed by RFLP analysis. EcoRI digests of the clones showed four and nine different banding patterns for *Xestospongia* sp. and *C. celata*, respectively. A second enzyme, HaeIII, which cuts more frequently, was used to further identify groups of clones. Nine RFLP patterns were observed more than once in the bacterial 16S rRNA clone library obtained from *Xestospongia* (table 1-5). However, the majority of the clones from *Xestospongia* sp. (n= 31) had unique HaeIII banding patterns. The cloned bacterial 16S rRNA from *C. celata* exhibited nine different EcoRI banding

Table 1-5. Identification of the major RFLP banding patterns of Bacterial 16S rRNA gene clones from *Xestospongia* sp.

RFLP pattern[a]	Number of Clones	Most Similar to (Sab value)[b]
I-1	10	*Prochlorococcus marinus* str. SSW5 (.759)
I-2	4	*Prochlorococcus marinus* str. SSW5 (.741)
I-3	2	*Vibrio splendidus* (.899)
I-4	4	*Desulfovibrio desulfuricans* & Clone RB25(.435)
II-1	4	Endosymbiont of *Inanidrilus leukodermatus* (.627)
II-2	2	*Solemya Redi* Symbiont & Clone OM252 (.564)
III-1	2	*Stomatococcus mucilaginocus* (.454)
III-2	2	*Streptomyces thermodiasticus* (.482)
IV-1	3	Octopus spring microbial mat DNA type K (.536)

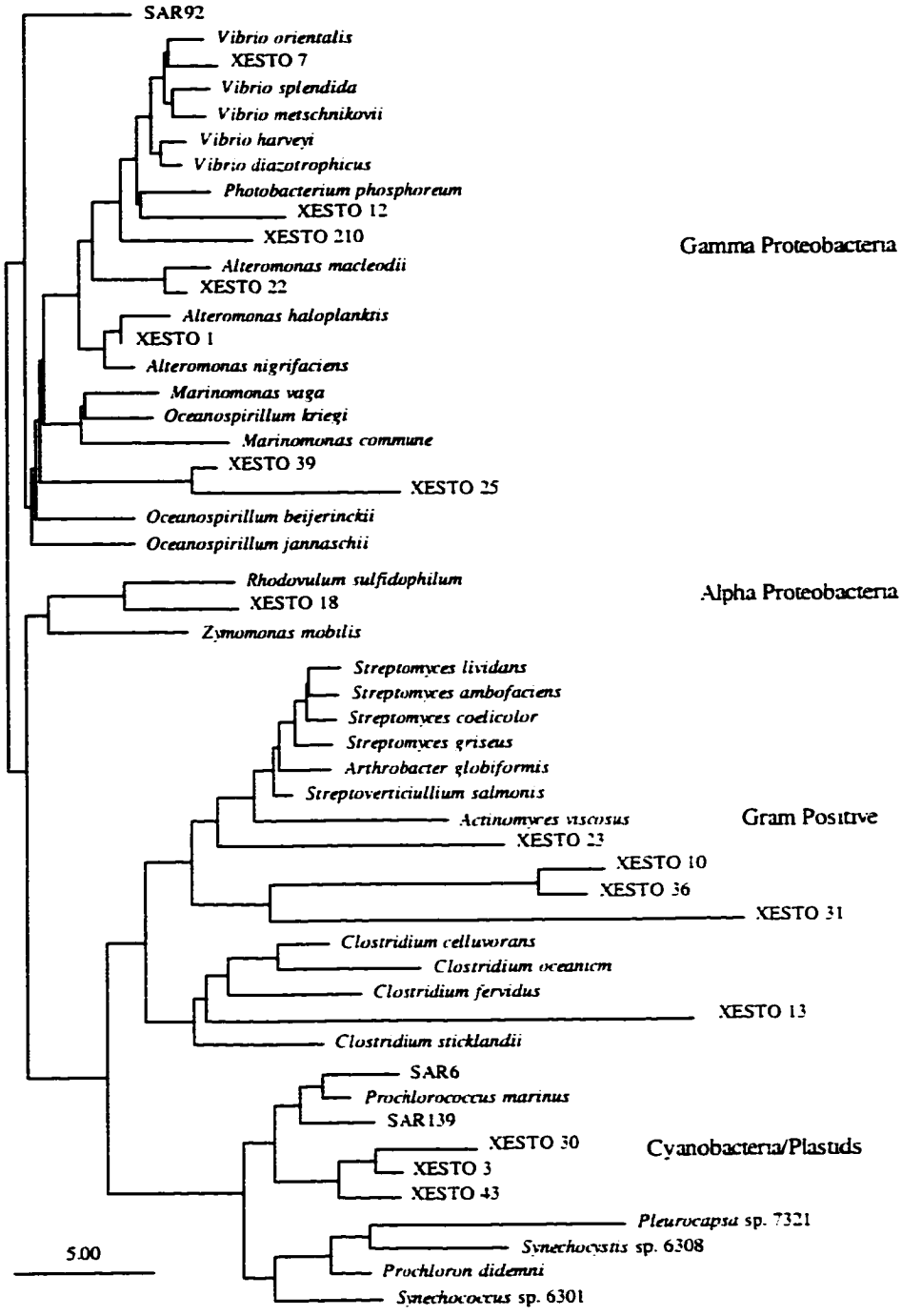
[a]RFLP patterns determined by digesting PCR amplified-cloned bacterial 16S rRNA genes with EcoR1 and HaeIII. Roman numerals indicate identical RFLP pattern after digestion with EcoR1.

[b]Similarity (Sab values) determined using Similarity rank available at the Ribosomal Database Project (Maidak *et al.*, 1997)

patterns, and only one HaeIII RFLP banding pattern was observed more than once. The diversity in the RFLP patterns of the rDNA clones suggests a diverse bacterial community within these two marine sponge, which may either represent sponge-specific microflora or planktonic microorganisms obtained during filter feeding.

The phylogenetic affiliation of the clones was determined by comparing the rRNA gene sequence of the clones to the rRNA genes of known bacteria using Similarity Rank available at the Ribosomal Database Project (RDP; Maidek *et al.*, 1997) and distance matrix analysis (DeSeote, 1983). The phylogenetic analysis of rDNA clones indicated that *C. celata* and *Xestospongia* contained relatively high bacterial species diversity. The majority of bacterial types within *Xestospongia* were from Gamma Proteobacteria, Cyanobacteria/Chloroplast, and the Gram Positive Bacteria (figure 1-2). Bacterial 16S rRNA clones belonging to the Cyanobacteria/Chloroplast lineage accounted for 21.5 % of the clones and were most similar to *Prochlorococcus marinus* (Sab = 0.759 and 0.741, table 1-5). In contrast, representative phylotypes of the gamma Proteobacteria and Gram Positive Bacteria were diverse. Most of the gamma Proteobacteria were highly related to the genera *Vibrio* (Sab .741-.891), *Alteromonas* (Sab .952 and .935), or *Oceanospirillum* (.603 and .658) which are commonly isolated from seawater. The Gram Positive bacterial rDNA clones were loosely affiliated with genera of the order Actinomycetales, which included *Stomatococcus, Streptomyces, Cellulomonas,* and *Microsporidia.* The presence of a dominant *Prochlorococcus*-like phylotype along with high bacterial rRNA, may suggest an active bacterial

Figure 1-2. Phylogenetic affiliation of bacterial rDNA clones obtained from *Xestospongia* sp. Partial sequences (region 240 to 446, *Escherichia coli* numbering) of the rDNA clones were compared to known rRNA sequences of microorganisms archived at the Ribosomal Database Project (Maidak *et al*, 1994) using distance analysis (DeSoete, 1983). The prefix XESTO designates the *Xestospongia* bacterial rDNA clones.



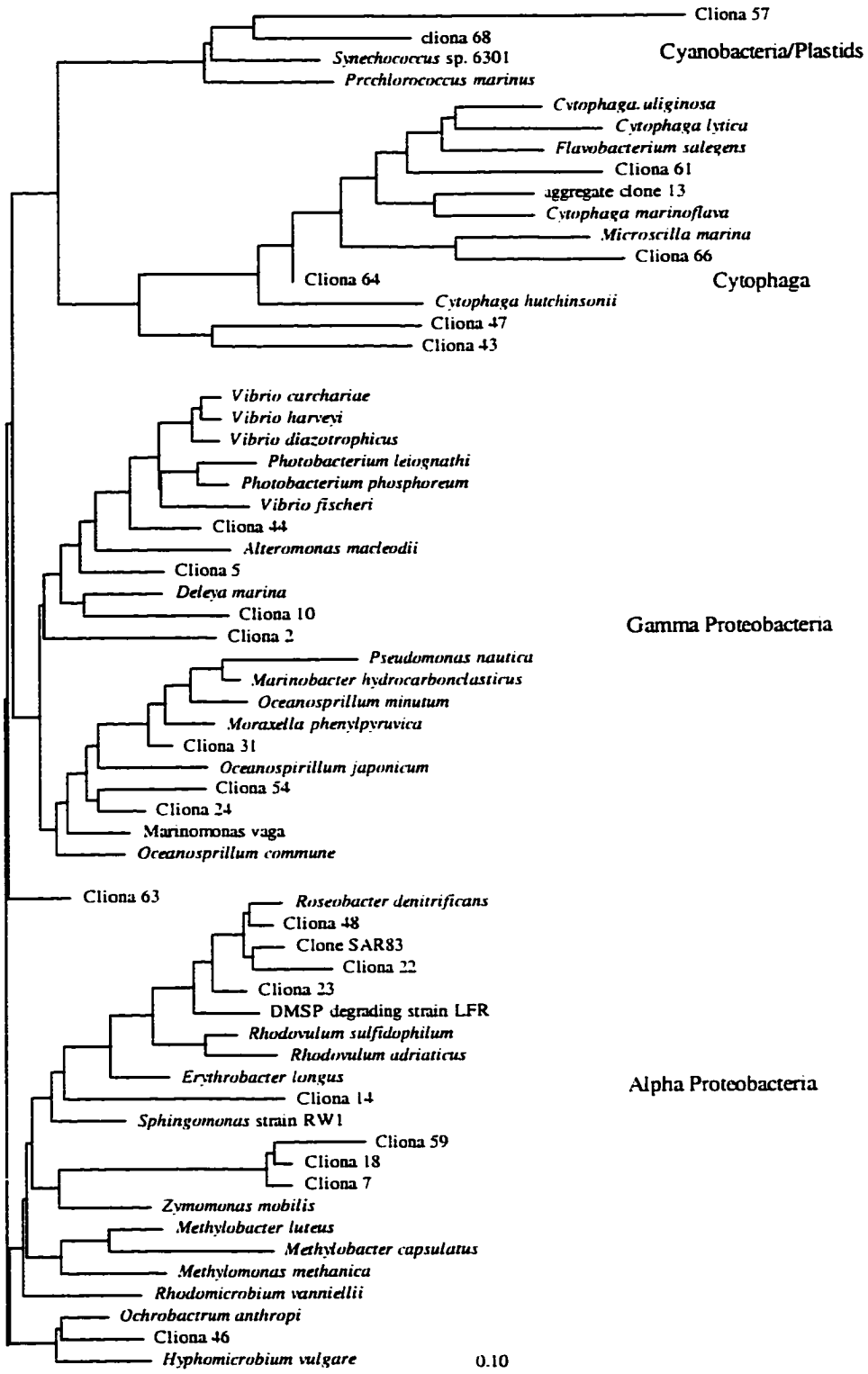
population within this individual of *Xestospongia*. Further analysis of other specimens are necessary to determine if photoautotrophs are common members of the prokaryotic community within this species of marine sponge.

Unlike the bacterial clone library of *Xestospongia*, no dominant rDNA phylotype was observed in the 16S clone library of the temperate marine sponge, *C. cliona*. Most of the rDNA clones were affiliated with the Alpha and Gamma Subdivisions of the *Proteobacteria*, or to the *Cytophaga* Lineage (figure 1-3). The majority of the Gamma Proteobacteria were closely affiliated (Sab values between .650 and .807) with bacteria commonly isolated from seawater including *Vibrio*, *Marinobacter*, *Altermonas*, and *Oceanosprillum* (Figure 1-3). Bacterial clones affiliated with the alpha Proteobacteria were most similar to environmental clones, marine Adriatic90 (Sab .496 and .519), ANG Clone 1A (Sab .932), OM42 (Sab .897), and the gill symbiont of *Prionitis lanceolata* (Sab .779). Results from the RFLP and phylogenetic analyses suggest a diverse bacterial community, rather than a dominating species type. These findings suggest that the bacterial population recovered in clonal libraries from *C. celata* most likely represent planktonic bacteria captured during filter feeding rather than sponge-specific microorganisms.

Discussion

Molecular phylogenetic analysis using conserved macromolecules (rRNA genes) provided a means to assay the prokaryotic community within sponges. In the California coast sponges investigated here, prokaryotic rRNA was present in relatively low amounts, and in the majority of sponges investigated, composed strictly of bacteria. The Australian sponge,

Figure 1-3. Phylogenetic affiliation of bacterial rDNA clones obtained from *C. celata*. Partial sequences (region 208 to 440, *E. coli* numbering) of the rDNA clones were compared to known bacterial rRNA sequences obtained from the Ribosomal Database Project (Maidak *et al*, 1994) using distance analysis (DeSoete, 1983).



Xestospongia sp. contained the highest amounts of bacterial rRNA relative to total sponge rRNA. The presence of high bacterial numbers is consistent with previous electron microscopic studies which documented high bacterial abundance within sponges (Santavy and Colwell, 1990; Vacelet and Donadey, 1977; Wilkinson, 1978).

Molecular cloning and sequencing of bacterial rRNA genes isolated from marine sponges allowed the identification of specific bacterial rRNA genes within the consortia. If the rRNA sequence of the clone is closely affiliated with rRNA of phenotypically described microorganisms, the functional/physiological role of the microorganisms can sometimes be inferred from its phylogenetic placement (Head *et al.* 1998; Pace, 1996). However, it is only a prediction; the functional/physiological role of the organism must still be verified. The majority of rDNA clones recovered from *Xestospongia* were either gram positive, members of the gamma Proteobacteria or highly related to the pelagic marine prochlorophyte, *Prochlorococcus* sp. Bacterial rDNA clones from *C. celata* were affiliated with the gamma Proteobacteria, alpha Proteobacteria and Cytophaga. If these microbial species represent specific members of the two sponge associations, then the potential bacterial metabolic strategies could include phototrophy (*Prochlorcoccus*-like clones; Chisholm *et al.*, 1992; *Roseobacter*; Shiba, 1992), facultative anaerobes and aerobic chemoorganotrophy (Gamma proteobacteria, *Vibrio* and *Altermonas;* Gauthier and Breittmayer, 1992; Farmer III and Hickman-Brenner, 1992), and aerobic degradation of complex polysaccharides (*Cytophaga* and *Streptomyces*; Coughlan and Mayer, 1992).

The prevalence of *Prochlorococcus*-like rDNA clones in the *Xestospongia* bacterial clone library may suggest a potential symbiont. However, although *Prochlorococcus*-like rRNA was dominant in the rRNA gene library of *Xestospongia* sp., its abundance in the sponge cannot be inferred due to inherent biases in PCR amplification and cloning methods. The number of copies of the rRNA gene and genome size (Farrelly *et al.*, 1995), the preferential amplification of the dominant members of the association (Ward *et al.*, 1992), preferential denaturation of the GC low templates (Reysenbach *et al.*, 1992), the use of degenerate primers (Polz and Cavanaugh, 1998), and template saturation kinetics (Suzuki and Giovannoni, 1996; Suzuki *et al.*, 1998) can all influence the ratio of the different rRNA phylotypes in clone libraries. The number of amplicons cloned of a particular ribotype that result after PCR amplification or rDNA phylotype ratios in clonal libraries may not represent the true ratios of the microorganisms in the natural sample (Suzuki *et al.*, 1998). It remains to be determined if the high abundance of bacterial rRNA in *Xestospongia* sp. was due to *Prochlorococcus* rRNA, but if so, it could be further evidence for a sponge-Prochlorophyte association. Associations between photoautotrops and sponges are common (Wilkinson, 1981), and thus would not be a surprising finding. However, the abundance of *Proclorococcus* in seawater where *Xestospongia* was collected, was not determined. The prevalence of *Prochlorococcus* clones in the 16S rRNA gene library of *Xestospongia* could reflect their abundance in the surrounding seawater and not a specific association with the sponge.

Sponges are filter feeders and have been shown to capture particles up to 50 μm. The major site of bacterial-sized particle capture (0.1 to 1.5 μm) occurs in the choanocyte chambers (Bergquist, 1978). The average bacterial density at 20m in the Santa Barbara Channel is about 1.0 $X10^6$ per mL (Murray, personal communication). A 50 g sponge (wet weight) can filter up to 950 L d^{-1} (for pumping rates see Bergquist, 1978) and have a filter efficiency of approximately 96% (Reiswig, 1971). Sponges, therefore, have the potential to concentrate up to $8.8X10^{11}$ bacterial cells d^{-1}. Bacteria detected within marine sponges could represent either bacteria that consistently inhabit the sponge matrix, or they could simply be collected food. Thus, not only is the detection of similar rRNA phylotypes in different individuals important, but so is the identification of the cellular location (mesohyl versus aquiferous system) within the host.

Relatively high bacterial diversity was found in microbial populations associated with *Xestospongia* and *Cliona* species. This result is similar to previous cultivation (Santavy and Colwell, 1990; Wilkinson, 1978; Santavy *et al.*, 1990), EM studies (Vacelet and Donadey, 1977; Bigliardi *et al.*, 1993), and molecular phylogenetic (Althoff *et al.*, 1998) studies which showed that sponges are inhabited by a diverse community of bacteria. Specifically, they found bacterial isolates of the genera *Cytophaga*, *Vibrio*, and *Aeromonas*, within sponges (Wilkinson, 1978; Santavy *et al.*, 1990; Wilkinson *et al.*, 1981; Santavy, 1985; Santavy and Colwell, 1990). Although these genera are commonly found in and isolated from seawater, the bacterial strains isolated from the seawater and sponge differed phenotypically (Santavy and Colwell, 1990; Santavy *et al.*, 1990; Wilkinson, 1978). In this study, bacterial rRNA

phylotypes from a single individual of two species of sponge were most similar to bacteria commonly found in marine environments. This result is similar to the finding of Madri *et al.* (1971), Bertrand and Vacelet (1971), and Wilkinson (1978; for *Ircinia wistarii*). It is not clear whether the bacteria detected in this study were consistently associated with *C. celata* and *Xestospongia* sp., or if they differed phenotypically from those which commonly inhabit seawater. A more detailed analysis of the bacterial population within each sponge is required to determine if a true specific association exists. This includes showing consistency and persistence of the association as well as determining the life histories of the host and its potential symbionts. The localization of specific prokaryotic types within the sponge using *in situ* hybridization with rRNA-targeted oligonucleotide probes should aid in determining if a specific association exists.

The detection of both archaeal rRNA and rDNA within the sponge *A. mexicana* is the most interesting result of this study. Prior to this study, archaea had not been found in association with marine sponges. Known symbioses involving archaea are limited to methanogens (Embley and Finlay, 1994). The absence of archaeal rRNA, despite the amplification of archaeal rDNA in a single specimen of *C. celata*, suggests that archaea are not a biologically active or abundant member of the prokaryotic community within this species of marine sponge. The identity and description of archaea within *A. mexicana* and its affiliation to the recently discovered planktonic marine archaea (DeLong, 1992, Fuhrman *et al.*, 1992) are presented in the subsequent chapters.

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CHAPTER 2

Detection and description of an association between a psychrophilic crenarchaeon, *Cenarchaeum symbiosum*, gen. nov., sp. nov, and the marine sponge, *Axinella mexicana*.

Introduction

Re-classification of organisms into three Domains of life, the Eucarya, Bacteria, and Archaea, using molecular phylogenetic analysis of conserved macromolecules, has most profoundly affected microbiology (Woese *et al.*, 1990). The prokaryotes are now recognized as two distinct lineages, Archaea and Bacteria. The relationship of the three Domains depends on which pair of homologous genes are used to construct the phylogeny (Forrtere, 1997), although the majority of trees constructed from genes involved in transcription and translation, support Archaea and Eucarya as being sister taxa (Iwabe *et al.*, 1989; Brown and Doolittle, 1995; Forrtere, 1997). Due to the differences in branching order of gene trees, Woese (1998) has proposed that the universal ancestor could have been a collection of cells in which genes were exchanged freely rather than a single organism or lineage that gave rise to the extant Domains. Thus, utilizing genes other than transcriptional and ribosomal rRNA or protein genes to root the tree would not resolve the branching order at the base of the tree due to lateral gene transfer of modular genes (Woese, 1998).

Of the prokaryotic lineages, Archaea are the least diverse phenotypically. Cultivated archaea have mainly been recovered from extreme environments and their major phenotypes include extreme halophiles, anaerobic and aerobic sulfur-metabolizing thermophiles, thermophilic sulfate reducers, and methanogens (Kates *et al.*, 1993; Fuchs *et al.*, 1991). The number of cultivated

archaea and unique environmentally isolated ribosomal RNA (rRNA) sequences have increased, yet no new phenotypic motifs have been described (Pace, 1996). The major physiological types of the archaea include methanogenesis, aerobic or anaerobic heterotrophic oxidation of sugars and peptides, and chemolithoautotrophic growth on reduced sulfur compounds or hydrogen. Limited phototrophic energy generation, nitrogen fixation, denitrification, iron oxidation, and iron reduction have also been demonstrated in several of the archaeal groups (Vargas *et al.*, 1998; Hafenbradl *et al.*, 1996; Kates *et al.*, 1993). Of the two major archaeal lineages, the Crenarchaea are the most limited phenotypically and physiologically (Stetter *et al.*, 1990).

The introduction of molecular phylogenetic methods has allowed the identification of microorganisms in the context of their environment without the necessity of cultivation. Thus, many previously undetected, and as yet uncultivated prokaryotic lineages have been described (planktonic archaea: DeLong, 1992 and Fuhrman *et al.*, 1992 : SAR 11 cluster: Giovannoni *et al.*, 1990; magnetococci: Amann *et al.*, 1995; MC clusters I, II, and III: Liesak and Stakebrandt, 1992). Novel archaeal rRNA phylotypes have been recovered from 16S rRNA gene libraries made from nucleic acids extracted from marine plankton (DeLong, 1992; Fuhrman *et al.*, 1992). The detection of the archaeal sequences in cold oxygenated seawater contradicts the paradigm that archaea inhabit only extreme environments. In fact, rRNA phylotypes related to those first detected in marine plankton, have also been described in soils (Kudo *et al.*, 1997; Bintrim *et al.*, 1997; Jurgens *et al.*, 1997), freshwater sediments (Schleper *et al.*, 1997a; MacGregor *et al.*, 1997), coastal marine sediments (Munson *et al.*,

1997), deep sea sediments (Kato *et al.*, 1997), subsurface environments (Chandler *et al.*, 1998), and the guts of holothurians (McInerney *et al.*, 1995) and marine fish (van der Maarel *et al.*, 1998) indicating the existence of both mesophilic and psycrophilic crenarchaea. The marine planktonic crenarchaea are distributed worldwide (DeLong, 1992; DeLong *et al.*, 1994; Fuhrman *et al.*, 1992; Fuhrman *et al.*, 1997; Massana *et al.*, 1997; Massana *et al.*, 1998; McInerney *et al.*, 1997; Moyer *et al.*, 1998; Murray *et al.*, 1998) and were shown to constitute a significant proportion of the biomass of bacterioplankton (DeLong *et al.*, 1994; Massana *et al.*, 1998; Murray *et al.*, 1998). The detection of the additional archaeal phylotypes has thus altered the perception of limited phylotypic and environmental diversity of archaea, and hints at the possibility of additional archaeal phenotypes.

The closest relatives of the mesophilic and psychrophilic crenarchaea all inhabit thermophilic and hyperthermophilic environments. However, their ecological distribution and relatively low G+C % of their 16S rRNA genes is consistent with a low temperature existence for these new crenarchaeal phylotypes. Prior to this study, no evidence directly supported the hypothesis that these organisms could grow at cold temperatures. In addition, it has been suggested that the same marine archaea found in plankton also emanate hydrothermal environments (Moyer *et al.*, 1998).

In chapter 1, I surveyed the prokaryotic diversity of marine sponges, and in the study detected the presence of archaea associated with a single species of marine sponge. Prior to this investigation, associations involving prokaryotes and *Porifera* only were known to occur with *Bacteria* (including *Cyanobacteria*

or blue-green algae). Here, I describe the identification, initial description, and specificity of the sponge-archaeal association in *Axinella mexicana*, as well as the general implications of the results to the biology of the Archaea.

Methods

Field Collection, sponge maintenance, and identification

Encrusting red sponges were collected between 10-20 m by SCUBA at two different sites off the coast of Santa Barbara, CA, Haskel Reef and Naples Reef, at Hermit Hole, North Santa Cruz Island in the Santa Barbara Channel, and at Coral Street, Monterey, CA. The sponges were removed along with a small amount of their rock substrate, placed in collection bags, and transported to aquaria within two hours of collection. A subsample of each sponge was frozen for ribosomal DNA (rDNA) or RNA (rRNA) analysis. Specimens were also maintained in flowing seawater tanks at either 10°C or ambient seawater temperature. Criteria used in sponge identification included gross morphology, color, oscule arrangement, encrusting shape and thickness, and the size and shape of siliceous spicules. To ensure that different individuals were of the same species, spicule analysis was performed on each sponge collected.

Nucleic acid extraction

Nucleic acids were extracted from sponges using two different techniques, one that recovered total nucleic acids and the other that specifically isolated RNA. Total crude nucleic acids were extracted and purified by cesium chloride density gradient as described in the methods of chapter 1. The purified DNA was used in PCR reactions to amplify archaeal rDNA (see below).

To extract RNA from the sponge, vertical cross sections of sponge (approximately 150 mg) were removed and total rRNA extracted in hot phenol:$CHCl_3$, pH=5.1, as previously described (Stahl *et al.*, 1988) with these modifications. The sponge section was immersed in 0.7 ml of 50 mM sodium acetate buffer, pH= 5.1 containing 0.7% SDS, 0.7 ml of phenol, pH=5.1, and 0.5 ml of sterile glass beads (diameter 0.1 mm, Sigma). The mixture was mechanically disrupted for 4 minutes in a mini-beadbeater (BioSpec Products), incubated at 60°C for 15 minutes, disrupted for an additional 2 minutes, and finally incubated at 60°C for 3 minutes. The aqueous phase was collected after centrifugation, extracted a second time using phenol pH 5.1:chloroform:isoamyl alcohol (50:49:1), precipitated with 100% ethanol, washed in 80% ethanol, and resuspended in TE buffer pH 8.0. The RNA was quantified spectrophotometrically. Crude nucleic acid and RNA extractions were used in rRNA hybridization experiments to quantify the relative percentage of prokaryotic rRNA in the sponge.

rRNA Hybridization Experiments

The proportions of archaeal, bacterial and eucaryal rRNA in freshly collected sponges and those maintained in aquaria were determined by quantifying the relative binding of radioactively-labeled, domain-specific oligonucleotide probes (Giovannoni *et al.*, 1988; Stahl *et al.*, 1988; Raskin et al, 1994) to crude nucleic acids or rRNA extracted from each individual sponge. The sponge-derived crude nucleic acids and purified RNA standards (Eucarya: *Saccharomyces cerevisiae*; Bacteria: *Shewanella putrifaciens, Pseudomonas nautica, Cytopha johnsanii*, or *Escherichsia coli*; Archaea: *Haloferax volcanii,*

Thermoplasma acidophilum, or *Sulfobus solfataricus*) were denatured, blotted, and hybridized to one of the domain-specific oligonucleotide probes or the universal oligonucleotide probe as described in chapter 1. The percentage of rRNA of each Domain within the different specimens of sponge was calculated as previously described (chapter 1).

Normalization to Univ1392 was subsequently found to overestimate the percentage of archaeal rRNA (Zheng *et al.*, 1996), and thus for several individuals a second universal probe was used. Blots were prepared as previously described (chapter 1), except that 5 replicate blots were made and one was hybridized to S-*-Univ-1390-a-A-18 (Univ1390; Zheng *et al.*, 1996). The high stringency wash for Univ1390 was performed at 45°C.

To determine the standard deviation of the hybridization signal (relative % rRNA), the sponge individual used in the time series experiment (hs1 10/94. see below) was serially diluted 8-fold and blotted five times on four replicate blots. Control rRNAs were replicated four times on each blot. The blots were hybridized to the domain specific probes, washed, and the relative percentage of each domain was calculated as previously described (see methods in chapter 1).

PCR amplification and Phylogenetic Analysis

To compare the archaeal population of different individuals of *A. mexicana*, the small subunit ribosomal DNA (ssu rDNA) genes were amplified with PCR and directly sequenced. The archaeal ssu rDNA was amplified in PCR reaction mixtures using one biotinylated [(S-D-Arch-0002-a-S-20 (Arch21F); DeLong, 1992] and one unbiotinylated archaeal-targeted primer [S-D-Arch-0940-A-20 (Arch 958R); DeLong, 1992]. Each PCR reaction contained

1X PCR buffer (10 X PCR buffer was 500mM KCl, 100 mM Tris-HCl pH 8.3, 20 mM $MgCl_2$, 0.01% gelatin and 0.5% NP40), 200 µM dATP, dCTP, dGTP, and dTTP (Promega), 10 ng/µl each primer, 0.025 U/µl Taq DNA polymerase (Fisherbiotech). DNA isolated from *Haloferax volcanii* (10ng) was used as the positive control. The negative control tube contained only the PCR reaction mixture. Between 5ng to 50ng of purified DNA from different individuals of *A. mexicana* were used as the template. The conditions for PCR were as follows: an initial denaturation step at 92°C for 3 minutes, 30 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and followed with a final extension at 72°C for 5 minutes using a Perkin Elmer 2400 (DeLong, 1992). Amplification products were visualized by electrophoresis on 1% agarose gels (wt/vol) in 0.5X TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA) containing 0.25 µg/ml ethidium bromide.

Following amplification, the biotinylated strand was purified using avidin-coated, magnetic beads (Dynal Inc; Hultman *et al.*, 1989) and sequenced directly according to the methods of Sequenase (version 2.0 , US Biochemical; chapter 1). 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, *E. coli* numbering). Reference sequences, as well as sequence editing and phylogenetic analysis software were obtained via anonymous ftp from the Ribosomal RNA Database Project (Maidak *et al.*, 1997).

In addition to directly sequencing PCR amplified archaeal 16S rRNA genes, the full ssu rRNA genes and flanking regions were recovered from a fosmid (F-factor based cosmid) library prepared directly from nucleic acids recovered from *A. mexicana* (Preston *et al.*, 1996). Fosmid clones containing the complete archaeal ssu rRNA gene were identified using archaeal-biased ssu rRNA PCR primers (DeLong, 1992). The ssu rRNA genes were subcloned into Bluescript vector (Stratagene, La Jolla), and the purified double-stranded plasmids directly sequenced (Preston *et al.*, 1996). Maximum-likelihood analysis (Felsenstein, 1988) was performed on a total of 1189 nucleotide positions, with the software "fastDNAml", v 1.0 (Olsen *et al.*, 1994) using empirical base frequency, global branch swapping, and bootstrapping options.

The bacterial 16S rDNA genes were amplified using the primer pair S-D-Bact-0009-a-S-20 (Bact27F, Lane, 1991) and S-*-Univ-1492-a-A-19 (Univ1492R, Lane, 1991) using the same conditions as described above for the amplification of archaeal small subunit genes. Multiple reactions were pulled and concentrated using a Microcon-100. Approximately 0.65 pmoles of amplified bacterial 16S rRNA genes were digested overnight at 37°C with 0.8 U/μL HaeIII and 1X buffer. Digests were visualized by electrophoresis on a 2.5% agarose gel (Nueseive 3:1, FMC) after staining with 0.5 μg/mL of ethidium bromide. The minimal number of bacterial rDNA genes recovered from each sponge was determined by calculating the total sum of the restriction fragments.

Maintenance and manipulation of the association in captivity

To determine if the archaeal -sponge association could be maintained in laboratory aquaria, sponge individual hs1 collected 10/94 was subsampled for rRNA analysis. The remainder of the specimen was placed in flowing seawater tanks at 10°C. Subsamples were taken monthly and the relative percentage of eucaryal, bacterial, and archaeal rRNA was determined by rRNA hybridization.

The effect of antibiotics on the proportion of total rRNA of each member of the association was determined using specimen hs1. The specimen had been maintained for 120 days in an aquarium prior to the start of the experiment. The sponge was divided into five equal portions. One section remained in the flowing seawater tank, another was placed in a 500 mL beaker containing 300 mL of raw seawater, and the other three were placed in 500 mL beakers containing 300 mL of raw seawater and 200 µg/mL antibiotics. The following antibiotic treatments were tested: cycloheximide only, gentamicin, streptomycin, and penicillin, and chloramphenicol only. The first week of the chloramphenicol treatment also contained 200 µg/mL tetracycline. Antibiotics and seawater were changed on a weekly basis. Samples were frozen for rRNA extraction and the relative change in percentage rRNA was determined by rRNA hybridization using domain-specific oligonucleotide probes as previously described (chapter 1). In addition, microorganisms in the seawater of each treatment were collected on 0.22 µm filters (Supor, Gelman Sciences) and stored dry at -20°C. Cells collected on filters were removed by scraping into sodium acetate buffer, and the RNA was extracted as described above. RNA (300 ng) from the surrounding seawater was blotted and hybridized to Univ1392 and

Arch915. The binding of each probe to its target rRNA sequence was determined by autoradiography.

Another *A. mexicana* individual kept in the flowing seawater tank, was subsampled and then cut into 5 pieces. One remained in the flowing seawater tank, and the others were placed in beakers containing 150 mL seawater to which 0.1% potassium nitrate, 0.1% ammonium chloride, 0.5 g/L sodium bicarbonate, or nothing was added. After 7 days, samples for nucleic acids extraction were taken. The change in the relative percentage of rRNA of archaea, bacteria, and eucarya was determined by rRNA hybridization.

Probe specificity

Specificity of the rRNA oligonucleotide probes designed to target marine crenarchaea (DeLong, unpublished data) in *in situ* hybridization studies was determined by slot blot hybridization. The specificity of the following probes were tested: S-O-Cenar-0131-a-A-18 (TCC CGT TCA TAG GTT AGG), S-O-Cenar-0538-a-A-19 (TCC TGA CCA CTT GAG GTC T), S-O-Cenar-0554-a-A-20 (TTA GGC CCA ATA ATC MTC CT), S-O-Cenar-0655-a-A-23 (GTA CCG TCT ACY TCT CCC ACT CC). rRNAs from cultivated archaea, eucarya, and bacteria, and crude nucleic acids from *A. mexicana* were denatured with 0.5% gluteraldhyde in 50 mM sodium phosphate buffer, pH 7.2. Cloned 16S rRNA genes from marine "Group I" and "Group 2" archaea (DeLong, 1992) were denatured with 0.5 N NaOH in 1.5 M NaCl. Slots contained between 50 and 100 ng of the cloned 16S rRNA genes or between 50 and 300 ng of crude nucleic acids from bacteria, eucarya, cultivated archaea, or *A. mexicana*. Replicate membranes were prepared, prehybridized for an hour at 45°C in

hybridization buffer before the addition of 2 X 10^7 cpm of one of the oligonucleotide probes radioactively labeled at the 5' end with ^{32}P. In addition to the marine "Group 1" crenarchaeal probes tested, two of the replicate blots were hybridized to either S-*-Univ-1392-a-A-15 and S-A-0915-a-A-20 to show that rRNA or rDNA was accessible to the oligonucleotide probes. The blots were then washed for 30 minutes at room temperature in 100 mL of wash buffer (chapter 1) followed by a high stringency wash for 30 minutes. The stringency washes were performed at 40 and 45°C for GI 538 and GI 131, 30, 35 and 40°C for GI 554 probe, and 40, 45, and 50°C for GI655. Blots were then air dried and exposed to x-ray film (Fuji Medical X-ray Film, Japan) to determine in the probes hybridized to the rRNA.

Fluor labeling of Oligonucleotide probes

The "Group 1"-specific oligonucleotide probes were synthesized by Operon (Operon Technologies Inc.) with a 5' NH_3 group for subsequent labeling with fluorescent molecules. Texas Red (Molecular Probes) or FITC fluorochromes were covalently linked to the oligonucleotide via the 5' NH_3 group as described below. One hundred micrograms of the amino-linked oligonucleotide and 100 mM carbonate buffer pH 9.2 were combined and the pH was adjusted to 9.0 with 0.1 N NaOH before the addition of 1.6 mg/mL fluorescein-5-isothiocyanate or Texas Red-X, succimimidyl ester (stock solution 10 mg/mL FITC or Texas Red in di-methyl formamide: Molecular Probes). The reaction mixture was incubated overnight at room temperature in the dark. Unincorporated fluors were removed using Sephadex G-25 spin column chromatography. Sephadex G-25 (Sigma) was hydrated overnight in an excess

of 10 mM Tris pH 7.5-8.0. Columns were made from 5 mL syringes plugged with glass wool, placed in a 15 mL falcon tube, and filled with approximately 5 mL of the hydrated sephadex. The column was then spun at low speed and rinsed with 10 mM Tris. The effluent was removed and the tip of the syringe placed in an epitube. The labeling mixture was then added to the top of the sephadex bed and spun for 1 minute. A half a milliliter of 10 mM Tris was added to the top of the bed and spun for an additional 15 minutes to drive the oligonucleotide probes into the de-capped epitube. The fraction was then dried down, resuspended in 25% sucrose, and purified on a 20% non-denaturing acrylamide gel to separate the unlabeled and labeled oligonucleotide probes. The labeled and unlabeled oligonucleotide probes were visualized on a TLC plate covered in saran wrap using a hand held UV lamp. The labeled oligonucleotide probes were cut from the gel, placed in plastic scintillation vials containing 1 mL of nanopure water, and eluted overnight at room temperature with constant shaking. The elute was filtered through a 0.22 μM syringe filter (Milex-GV, Milipore), and the concentration of the probes were determined spectrophotometrically. The probes were dried down in 250 μg aliquots and stored at -20°C until use. The probes were resuspended in nanopure water for a final working concentration of 50 μg/mL for use in whole cell hybridization studies.

Whole Cell hybridization

A vertical-cross section of a sponge held in an aquarium was fixed for seven hours at 4°C in 3.7% formaldehyde diluted in 0.22 μm filtered, autoclaved seawater or magnesium and calcium free artificial seawater (CMF-ASW; 460

mM NaCl, 11 mM KCl, 7 mM Na_2SO_4, 2 mM $NaHCO_3$). The section was subsequently rinsed in sterile seawater or CMF-ASW and macerated with a tissue grinder. Spicules and large aggregates of sponge cells were removed by low speed centrifugation. Archaeal enrichments (Schleper *et al.*, 1998) were also used in the whole cell hybridization experiments. Five microliters of the cell preparation was spotted onto gelatin-coated [0.1% gelatin, 0.01% $KCr(SO_4)_2$] teflon slides with 5 mm wells (Cel Line Associates). To reduce background the slides were treated with acetic anhydride (Leitch *et al.*, 1994) and washed in 2X SSC (0.3 M NaCl, 30 mM trisodium citrate, pH 7.0). Slides were then dehydrated in an ethanol series (50%, 75%, 95%, 2 minutes each) and air dried. Hybridization buffer contained 5X SET (0.75 M NaCl, 5 mM EDTA, and 0.1 M Tris-HCl pH 7.8), 0.1% dextran sulfate, 0.1% SDS, and 0.5 mg/ml polyadenosine (DeLong *et al.*, 1989; Distel *et al.*, 1991). A mixture of four marine crenarchaeal specific fluor-labeled oligonucleotide probes (0.8 nM each) labeled at their 5' end with Texas Red were added and hybridizations were incubated at 40°C overnight in a moist chamber to prevent evaporation. After hybridization, slides were washed in 1X SET at 45°C for 10 minutes, stained with 10 μg/ml DAPI for 5 minutes at room temperature, washed for 10 minutes in 1X SET at room temperature, quickly rinsed in sterile water, and air dried. After mounting in Citifluor AF1 (Citifluor Ltd., 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 second exposures for DAPI stained cells, and 120-240 second exposures for cells visualized by fluor-labeled probe binding. Three 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" archaeal-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). Two additional negative controls consisted of hybridizations containing three, fluorescein-labeled Eucaryal-specific probes (S-D-Euca-0309-a-A-17, Sogin and Gunderson, 1987; S-D-Euca-0502-a-A-16, Amann *et al.* 1990; Euk1209) or two, fluorescein-labeled Bacterial-specific probes (Bact338 and S-D-Bact-927-a-A-17; Giovannoni *et al.*, 1988) in addition to the Texas Red labeled marine crenarchaeal-specific probes.

Results

Identification of the sponge host

The encrusting red sponges were of a single species which was identified as *Axinella mexicana*, based on morphological criteria (see methods) and by comparison to type specimens (Welton Lee, personal communication). The red encrusting sponge contained both oxea and style type siliceous spicules (see appendix 1). No microscleres or ectosomal skeleton were present. The choanosomal skeleton was halichondroid-like (Mary Kay Harper and Welton Lee, unpublished data). Small differences in spicule size and skeletal arrangement were apparent between the type specimens and the sponges collected in this study. These characters were not significant enough to suggest a new species of sponge (Welton Lee, personal communication).

Verification of a sponge associated archaeon in *A. mexicana*

Initially, sponge-associated archaea were detected using archaeal-specific rDNA-targeted PCR amplification and rRNA-targeted oligonucleotide probes (chapter 1). Every individual of *A. mexicana*, collected at different sampling sites and at different times of year which was examined using the PCR assay (n=32), yielded positive results with archaeal-specific primers (table 2-1). Other sponge species including *Tethya aurantia* collected from Santa Barbara and *Xestospongia* sp. collected off the coast of Australia did not contain archaeal rDNA (chapter 1; Brantley *et al.*, 1995). However, archaeal rDNA was amplified from several tropical and Antarctic sponges (see appendix 2), and weakly amplified from one individual of *Cliona celata* collected off the coast of Santa Barbara (Chapter 1). No archaeal rRNA was detected in *C. celata* (Chapter 1).

Results from PCR analyses of purified nucleic acids from *A. mexicana* were subsequently verified by quantifying the relative amount of archaeal rRNA extracted from sponge tissue (table 2-1) using Domain-specific rRNA targeted oligonucleotide probes. These quantitative oligonucleotide probe hybridization experiments indicated that between 1.3 to 10.8% of the total rRNA extracted from *A. mexicana* was archaeal (n=35, table 2-1). The standard deviation of the correction factors and relative percentage of each domain within one blotting experiment normalized to Univ1392 was less than 0.1 and 0.01% respectively (table 2-2). A second universal probe (Univ1390) was used, as archaeal rRNA is overestimated when the domain specific probes are normalized to Univ1392 (Zheng *et al.*, 1996). The archaeal rRNA percentage calculated using Univ1392 was on average 2.4±0.88 times (n=7) higher than the same data normalized to

Table 2-1: Detection of Archaea in sponge tissue by PCR amplification with archaeal specific primers and domain-specific rRNA-targeted oligonucleotide probe hybridization.

Sponge Species	Specimen No.[a]	Collection date and site	Archaeal rDNA	Relative % of Domain-specific rRNA[b]		
				Eucarya	Bacteria	Archaea
A. mexicana	s1	2/94	+	89.2 (43.1)	9.6 (4.6)	1.3 (0.6)
	s2	2/94	+	90.1 (68.2)	3.5 (2.5)	1.7 (1.2)
	s3	3/94	+	94.4 (69.2)	3.5 (2.5)	1.7 (1.2)
	s4	3/94	+	96.3 (80.8)	ND	3.5 (1.3)
	s5	3/94	+	91.3 (65.4)	1.8 (2.9)	7.0 (5.0)
	s6	6/94	+	---	---	---
	s7	6/94	+	---	---	---
	s8	6/94	+	90.3 (100.3)	3.1 (3.5)	6.6 (7.3)
	s9	6/94	+	91.2 (113.3)	3.3 (4.0)	5.6 (6.9)
	s10	6/94	+	90.3 (99.4)	2.5 (2.8)	7.2 (7.9)
	s11	6/94	+	---	---	---
	s12	7/94	+	96.0 (223)	1.8 (4.3)	2.2 (5.1)
	s13	7/94	+	95.3 (151.7)	2.6 (4.2)	2.1 (3.3)
	s14	7/94	+	---	---	---
	s15	7/94	+	---	---	---
	s16	10/94	+	92 (83.7)	2.2 (2.9)	4.8 (4.3)
	s17	10/94	+	---	---	---
	s18	10/94	+	---	---	---
	s19	12/95	---	96.4 (183)	1.2 (2.2)	2.4 (4.6)
	s20	12/95	---	96	1.5	2.5
	s21	12/95	---	86.6 (172)	11.4 (22)	1.9 (3.8)
	s22	12/95	---	92.8 (141)	2.8 (2.3)	4.4 (3.6)
	s23	12/95	---	93.3 (84.7)	2.5 (2.3)	4.2 (3.8)
	s24	4/96	---	90.4 (81.9)	1.5 (1.4)	8.1 (7.3)
	s25	4/97	---	93 (126.0)	2.5 (3.3)	4.3 (6.1)

Table 2-1. (continued)

Sponge Species	Specimen No.[a]	Collection date and site	Archaeal rDNA	Relative % of Domain-specific rRNA[b]		
				Eucarya	Bacteria	Archaea
A. mexicana	s26	4/97	+	93 (89.6)	2.7 (2.5)	4.2 (4.1)
	s27	9/97	---	93.6 (107.5)	1.6 (0.7)	4.8 (5.7)
	s28	9/97	---	91.6 (99.5)	3.9 (3.3)	4.5 (5.1)
	s29	9/97	+	95.8 (131.2)	0.9 (0)	5.3 (4.7)
	s30	9/97	---	94.7 (120.9)	2.4 (4.7)	3.3 (4.3)
	hs1	10/94	+	85 (73.1)	11 (9.5)	3.9 (3.3)
	hs2	10/94	+	93.3 (127)	2.2 (3.0)	4.5 (6.0)
	hs3	10/94	+	92	2.9	5.1
	hs4	10/94	+	96.3	nd	3.6
	hs5	10/94	+	94.7	1.4	3.9
	hh1	2/97	+	77.8 (68.9)	12.0 (10.6)	10.3 (9.1)
	hh2	2/97	+	86.8(102.0)	2.4 (2.8)	10.8 (12.7)
	hh3	2/97	+	91.4 (105.7)	3.4 (3.9)	5.3 (6.1)
	ms1	6/98	+	95.8 (97.2)	2.0 (2.0)	2.2 (2.3)
	ms2	6/98	+	91.5 (104.0)	4.0 (4.5)	4.5 (5.1)
	ms3	6/98	+	94.3 (93.7)	2.3 (2.3)	3.4 (3.4)
	ms4	6/98	+	94.2 (99.3)	2.4 (2.5)	3.4 (3.6)

[a] Individuals of A. mexicana were collected at two different locations off the coast of Santa Barbara, CA (prefixes s and hs correspond to Naples Reef and Haskel Reef, respectively), and off of North Anacapa Island, CA (prefix hh).
Domain specific probes were normalized to the Univ1392 probe.

[b]Values outside parentheses represent the percentage of each normalized domain-specific signal/ sum of the normalized domain-specific signal and the values in parentheses indicate the percentage of the normalized domain-specific hybridization signal/Univ1392 hybridization signal. See chapter 1 for discussion of the differences in these calculations.
--- indicates experiments not performed. nd = not detected

Table 2-2. Standard deviation of the controls and relative percentage of each domain for one sponge individual as determined by rRNA oligonucleotide hybridization.

Correction factors (n=4)			
Eucaryal Bacterial Archaeal	1.10±0.06 0.32±0.06 0.46±0.04		
A. mexicana individual	Oligonucleotide Probe	Slope (n=5)[a]	% rRNA[b]
HS1 (10/94)	Universal	3.56±0.26	
	Eucarya	3.80±0.19	87.5±0.006 (114.3 ±8.72)
	Bacteria	0.63±0.03	4.3±0.002 (4.63±2.29)
	Archaea	0.85±0.02	8.2±0.004 (10.7±0.47)

[a] not normalized

[b]The average correction factor was used to normalize the slopes of each domain specific probe to Univ1392 in the calculation of the percentage rRNA contributed by each domain. The percent of the Universal hybridization signal for each domain-specific probe is given in parentheses.

Univ1390. The combination of the PCR amplification and rRNA hybridization results indicated that all individuals of *A. mexicana* collected to date from four different locations contained archaea (n=42).

A consistent percentage of archaeal rRNA, as determined from rRNA hybridization experiments, was detected in one individual of *A. mexicana* (hs1) maintained in flowing seawater tanks and sampled over a period of 18 months (figure 2-1). Other individuals of *A. mexicana* kept for over one year in laboratory aquaria yielded similar results (table 2-3), indicating the stability of the sponge-archaeal association in culture. Thus, studies involving the long term maintenance of the sponge-archaeon association in captivity are possible.

Cloning, sequencing and phylogenetic analyses

Two independent lines of evidence indicated that archaea inhabited all individuals of the marine sponge, *A. mexicana*. To determine the phylogenetic position of sponge-associated archaea and whether the archaeal population consists of either a single or multiple rDNA phylotypes, the rRNA genes were recovered, sequenced and compared to rRNA genes of planktonic archaea, cultivated archaea, bacteria, and eucarya. Direct sequence analysis of PCR amplified rDNA from a total of 23 different *A. mexicana* specimens (280 bases: only data from 15 are shown, figure 2-2) from the Santa Barbara Channel yielded nearly identical rDNA genes. Only at four nucleotide positions (175,183.7,194.7, and 361, *E. coli* numbering) were ambiguities consistently observed (figure 2-2). Cloned 16S rDNA genes recovered from a sponge fosmid library constructed from a single individual of *A. mexicana* (Preston *et al.*, 1996; Schleper *et al.*, 1998) contained two rRNA gene sequences which differed from

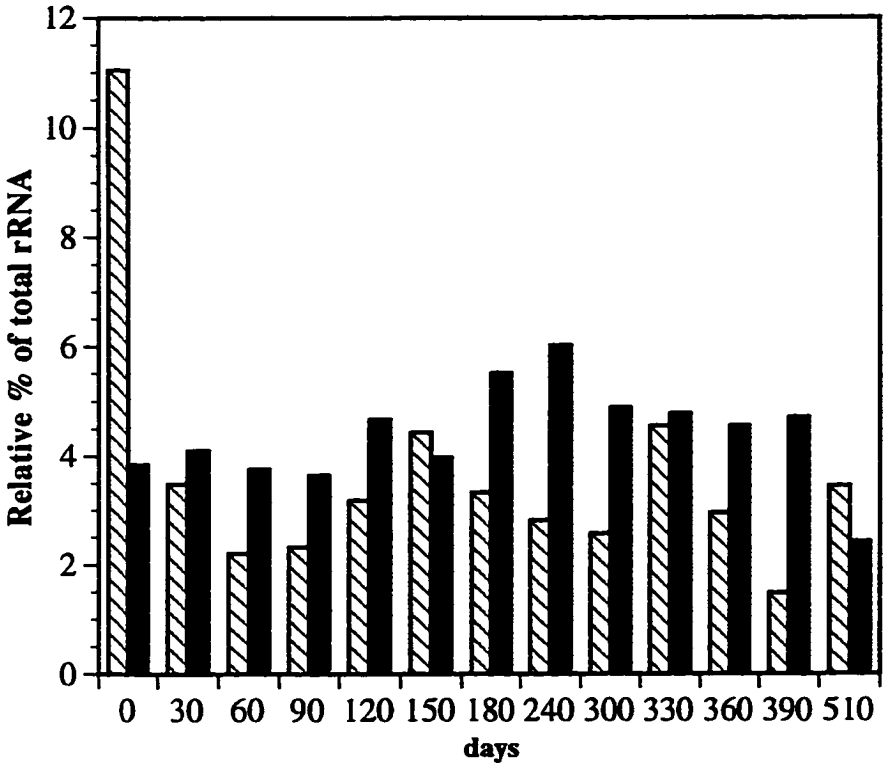


Figure 2-1. Maintenance of the sponge-archaeal association in the laboratory. The relative proportion of archaeal and bacterial rRNA as determined by rRNA hybridization with domain-specific oligonucleotide probes within a single sponge host (hs1) maintained at 10°C in a flowing seawater aquarium over a period of 18 months. The relative percentage of eucaryal rRNA was greater than 85% at all time points. Hatched bars correspond to the relative percentage bacterial rRNA and solid bars to the relative percentage archaeal rRNA.

Table 2-3. Detection of Archaeal rRNA in sponges held in flowing sea water aquaria using rRNA hybridization with domain-specific oligonucleotide probes.

Speciman[a]	length in captivity	Temperature	Relative % of Domain-Specific rRNA[a]		
			% Eucarya	% Bacteria	% Archaea
Aq1	>1 years	10°C	93.9 (73.0)	0.9 (0.7)	5.2 (4.1)
	>3 years	10°C	93.2 (103.4)	1.3 (1.4)	5.6 (6.2)
	>4 years	10°C	93.7 (88.5)	1.1 (1.0)	5.2 (4.9)
S20	0	---	93.3 (84.7)	2.5 (2.3)	4.2 (3.8)
	4 months	10°C	94.7 (109.6)	1.6 (1.9)	3.7 (4.3)
Aq4	≈2 years	Ambient	95.8 (98.6)	1.6 (1.6)	2.6 (2.6)
Aq5	≈2 years	Ambient	95.9 (77.6)	0.9 (0.8)	3.2 (2.6)
Aq6	≈2 years	Ambient	93.6 (94.6)	2.1 (2.2)	4.3 (4.3)

[a] Initial collection date of sponges Aq1 and Aq4-6 unknown.

[b]Values outside parentheses represent the percentage of each normalized domain-specific signal/ sum of the normalized omain-specific signal and the values in parentheses indicate the percentage of the normalized domain-specific hybridization signal/Univ1392 hybridization signal.

Figure 2-2. Comparison of archaeal ssu rRNA genes isolated from different specimens of *A. mexicana*. 16S archaeal rRNA gene sequences (region 131 to 406 *E. coli* numbering; 285 bases) were obtained by directly sequencing PCR amplification products from DNA of different *A. mexicana* individuals. In addition, s12a and s12b represent cloned 16S rRNA gene sequences recovered in a fosmid library from sponge individual s12. "Group 1" and "Group 2" refer to rRNA gene sequences from two planktonic groups of archaea recovered from the Santa Barbara Channel. Periods indicate the same nucleotide base as the reference sequence (s12a). Positions 175 and 183.7 were the only two nucleotide positions within this region of the archaeal 16S rRNA genes isolated from *A. mexicana* in which ambiguities were consistently observed. E. C. # refers to the corresponding base in *E. coli*.

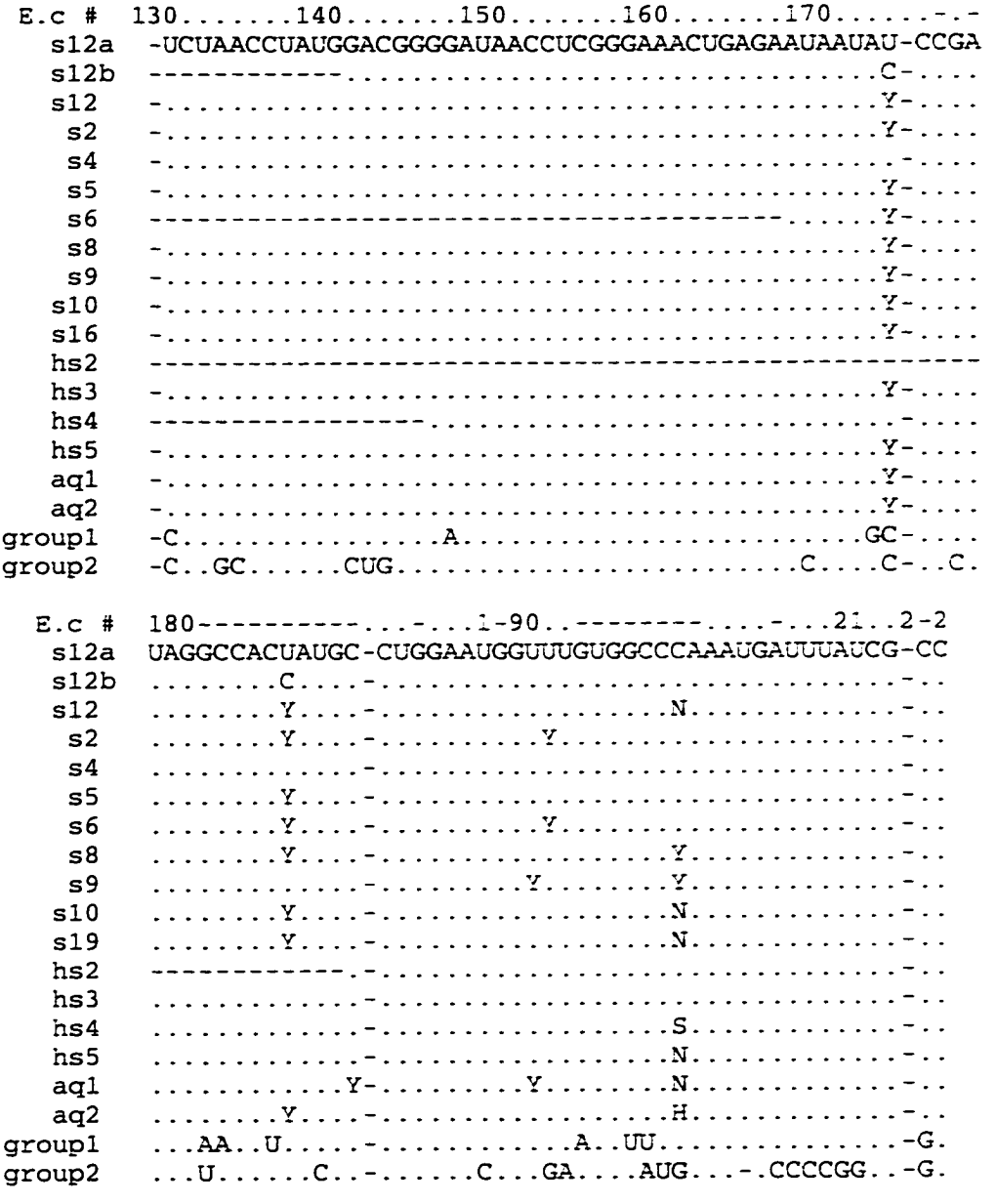


figure 2-2 (continued)

```
E.c #    0.......230.......240.......250.......260.......27
   s12a  GUAGGAUGGGACUGCGGUCUAUCAGCUUGUUGGUGAGGUAAUGGCCCACC
   s12b  ....................................................
   s12   ....................................................
    s2   ....................................................
    s4   ....................................................
    s5   ....................................................
    s6   ....................................................
    s8   ....................................................
    s9   ....................................................
   s10   ....................................................
   s19   ....................................................
   hs2   ....................................................
   hs3   ....................................................
   hs4   ....................................................
   hs5   ....................................................
   aq1   ....................................................
   aq2   ....................................................
group1   ....-...........C.......U.........................
group2   A.........C......CG......G....A..G.GU......UA..CU.

 E.c #   0.......280......290.......300........310.......32
   s12a  AAGGCUAUAACAGAUACGGGCUCUGAGAGGAGAGGCCCGGAGAUGGGUAC
   s12b  ....................................................
   s12   ....................................................
    s2   ....................................................
    s4   ....................................................
    s5   ....................................................
    s6   ....................................................
    s8   ....................................................
    s9   ....................................................
   s10   ....................................................
   s19   .......G............................................
   hs2   ....................................................
   hs3   ....................................................
   hs4   ....................................................
   hs5   ....................................................
   aq1   ....................................................
   aq2   ....................................................
group1   ...A....U....G..................UA..................
group2   U..C..UCG..GCG......U.G......C.AUA............A.U.
```

figure 2-2 (continued)

```
E.c #   0.......330.......340.......350.......360.......37
   s12a  UGAGACACGGACCCA-GGCCUAUGGGGCGCAGCAGGCGAGAAAACUUUGC
   s12b  ...............-..................................
   s12   ...............-.....................N............
    s2   ...............-..................................
    s4   ...............-..................................
    s5   ...............-..................................
    s6   ...............-..................................
    s8   ...............-..................................
    s9   ...............-..................................
   s10   ...............-.....................S............
   s19   ...............-..................................
   hs2   ........-S......-..................................
   hs3   ...............-.....................S............
   hs4   ...............-..................................
   hs5   ...............-.....................N............
   aq1   ...............-..................................
   aq2   ...............-..................................
group1   ...............G..................................
group2   .......A.A.U...G.U..C.C...A........U..C....C...GA.

  E.c #  0.......380.......390.......400....
   s12a  AAUGUGCGAAAGCACGACAAGGUUAAUCCGAGUGU
   s12b  ...................................
   s12   ...................................
    S2   ...................................
    s4   ........-..........................
    s5   ...................................
    s6   ........-..........................
    s8   ...................................
    s9   ...................................
   s10   ...................................
   s19   ..........................
   hs2   ...................................
   hs3   ..........................
   hs4   ...-K.......
   hs5   ...................................
   aq1   ...................................
   aq2   ............................
group1   ................................A..
group2   ....C.G....C.GU..UC...GG.C...A....C
```

one another at two of the ambiguous positions (positions 175 and 183.7 *E. coli* numbering). The combination of these nucleotides (C and U) would yield a Y after direct sequencing if both rRNA genes were present. These positions were consistently ambiguous (Y nucleotides) in different individuals of *A. mexicana* indicating the variation represents real differences in 16S rRNA genes either between rRNA operons within a single organism or between highly related but distinct co-occurring strains. All Crenarchaea characterized to date possess a single rRNA operon (Garrett *et al.*. 1991). The two rRNA phylotypes have been subsequently shown to belong to two distinct, highly-related strains of the same archaeal species (Schleper *et al.*, 1998). The variation at positions 194.7 and 361 could represent PCR or sequencing errors, or real archaeal rRNA phylotype differences in individuals of *A. mexicana*. The former is more likely, since ambiguities at these positions were observed with direct sequencing (s12) but not detected in the clone library (s12a and s12b) of this individual.

The archaeal 16S rRNA genes isolated from *A. mexicana* collected from Monterey, CA were most similar to the archaea that inhabited the Santa Barbara sponges (>98% similar, over 807 nucleotide positions). The Monterey archaea however, showed additional variation in the ribosomal gene sequence indicating additional rRNA variants and the possibility of Monterey-specific strains of the sponge-associated archaea (see chapter 3).

The phylogenetic placement as determined by the neighbor-joining method with bootstrap analysis of PCR amplified, directly sequenced archaeal ssu rDNA (403 nucleotide positions; figure 2-3) consistently placed the sponge associated archaea within a previously described lineage of as-yet uncultivated

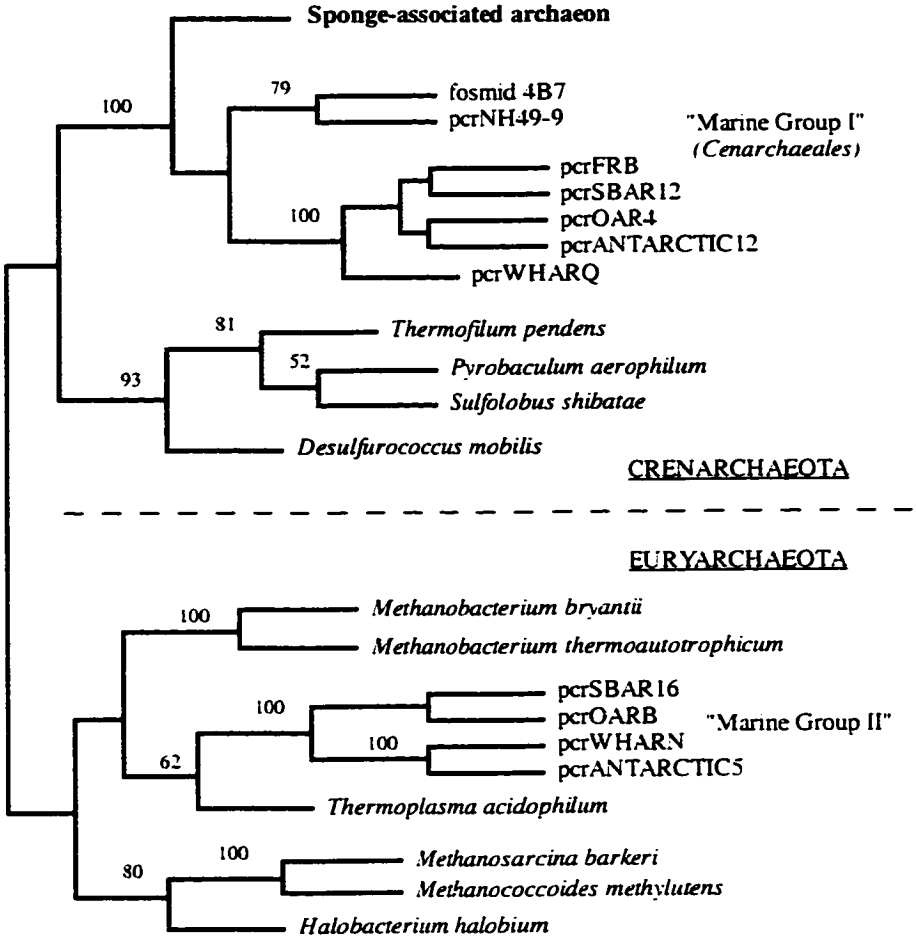


Figure 2-3. Phylogenetic affiliation of the sponge-associated archaeon as determined by neighbor-joining phylogenetic analysis of the sponge-associated archaeon rRNA (403 nucleotide positions). Reference sequences include individual rDNA genes of marine planktonic archaea amplified from mixed assemblages and cloned (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 (Stein *et al.*, 1996). Values represent the percentage of bootstrap replications greater than 50% that support the branching pattern appearing to the right of the value. *Thermotoga maritima* and *Thermus thermophilus* were used as outgroups.

planktonic marine Crenarchaeota (DeLong *et al.*, 1994; DeLong 1992; Fuhrman *et al.*, 1992). The highest unrestricted sequence similarity between the planktonic clones and archaeal symbionts was 95% over 523 nucleotide positions. Despite the high similarity, the archaeal 16S rRNA gene sequence (1,474 nucleotides) isolated from *A. mexicana* contains 35 unique or diagnostic nucleotide residues which distinguish it from all of the other marine "Group 1" Crenarchaeotal rRNA sequences available (table 2-4).

Maximum-likelihood bootstrap analyses comparing the full 16S rRNA gene sequence, recovered from a fosmid library (Preston *et al.*, 1996), to representative rRNA sequences from the three Domains supported the neighbor-joining analysis (figure 2-3) in the placement of the sponge-associated archaeon within the Crenarchaeote subdivision of the Archaea at a high bootstrap confidence level (85%, figure 2-4). The sponge associated archaeon shared 49 out of 53 of the interdomain signature nucleotides with Archaea, two with Bacteria, and one with Eucarya (table 2-5), confirming the phylogenetic positioning of the sponge-associated archaea within the Archaea. Positioning of the sponge associated archaea within the crenarchaeota branch was verified with transversion sequence analysis in which the sponge-associated archaea shared 13 of the 17 diagnostic signatures with other cultivated crenarchaeotes, but only 3 nucleotide positions with the euryarchaeota (data not shown). The placement of the sponge-associated archaea within the Crenarchaeal branch was also supported in phylogenetic analyses of other conserved macromolecules including the 23S rRNA gene sequence, DNA polymerase, GSAT protein, and the TATA binding protein (DeLong, unpublished data; Schleper *et al.*, 1997b).

Table 2-4. Unique rRNA signature nucleotides distinguishing the sponge-associated symbiont from related planktonic crenarchaeotes.

Nucleotide position[a]	"Group I" Consensus[b]	Fosmid 4B7[c]	sponge-associated archaea
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
840.4	-	-	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

[a]Nucleotide position in ssu rRNA is given in *E. coli* numbering. Positions having no counterpart in *E. coli* are designated by the nearest nucleotide in *E. coli* followed by a decimal number.

[b]"Group I" consensus (DeLong, 1992) corresponds to the consensus signature of marine planktonic crenarchaeal rDNA clones (DeLong, 1992; Furhman *et al.*, 1992; DeLong *et al.*, 1994; McInerney et al., 1995).

[c]"Fosmid 4B7" corresponds to a full length rRNA gene recovered from a 40 kb genomic fragment of a free-living planktonic marine crenarchaeote (Stein *et al.*, 1996).

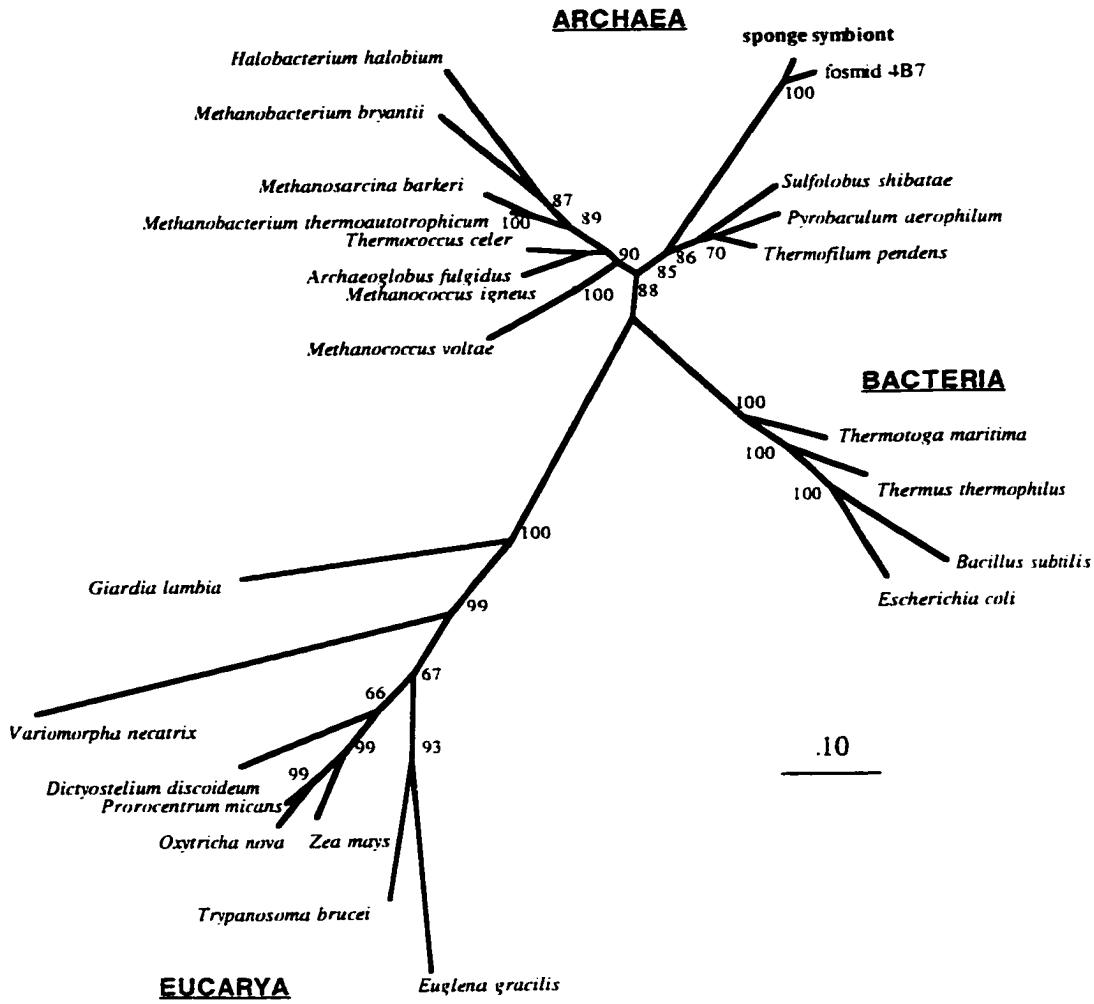


Figure 2-4. Phylogenetic relationship of the sponge archaeon determined by maximum-likelihood analysis. Tree topology was inferred from 1189 residues, using fastDNAml, version 1.0 (Olsen *et al.*, 1994). Values indicate the percentageof 250 bootstrap replications which supported the branching pattern at various nodes, of 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.

Table 2-5: Phylogenetic affiliation of the sponge symbiont using interdomain signature analysis[a].

Nucleotide Postition	Nucleotide				
	Eucarya	Eubacteria	Archaea	Group 1	Symbiont
33:551	A:U	A:U	Y:R	C:G	C:G
44.1:397	-:A	-:A	U:A	U:A	U:A
47.1 extra base?	Yes	No	Yes	Yes	Yes
52:359	G:C	Y:R	G:C	G:C	G:C
53:358	C:G	A:U	C:G	C:G	C:G
113:314	C:G	G:C	C:G	C:G	C:G
121	A	Y	C	C	C
292:308	R:U	G:C	G:C	G:C	G:C
307	Y	Y	G	G	G
335	A	C	C	C	C
338	A	A	G	G	G
339:350	C:G	C:G	G:Y	G:C	G:C
341:348	U:A	C:G	C:G	C:G	C:G
361	C	R	C	A	A
365	A	U	A	A	A
367	U	U	C	C	C
377:386	Y:R	R:Y	Y:G	U:A	U:A
393	A	A	G	G	G
500:545	U:A	G:C	G:C	G:C	G:C
514::537	G:C	Y:R	G:C	G:C	U:C
549	C	C	U	U	U
558	A	G	Y	U	G
569:881	G:C	Y:R	Y:R	C:G	C:G
585:756	U:A	R:Y	C:G	C:G	C:G
674:716	R:Y	G:A	G:C	G:C	G:C
675:715	U:A	A:A	U:A	U:A	U:A
684:706	G:Y	U:A	G:Y	G:C	G:C
716	Y	A	C	C	C
867	Y	R	Y	C	C
880	U	C	C	C	C
884	G	U	U	U	U
923	A	A	G	G	G
928	A	G	G	G	G
930	G	Y	A	A	A
931	G	C	G	G	G
933	A	G	A	A	A

Nucleotides that define the three domains Eucarya, Eubacteria, and Archaea from Winkler and Woese (1991) and Group 1 from DeLong (1992).

The bacterial population in the sponge was diverse. Restriction fragment length polymorphism (RFLP) analysis of the bacterial 16S rRNA genes (region 27 to 1492, *E. coli* numbering) amplified from *A. mexicana* showed variation in restriction patterns of different individuals, even those collected on the same date (lanes 3 and 4 or 8 and 9; figure 2-5). At least 4 different rRNA gene sequences were present in *A. mexicana* as determined by the sum of the size of the restriction fragments (figure 2-5). This differed from what was observed for the archaeal population which produced a single RFLP pattern (data not shown) belonging to a single species.

Physiological response to the addition of antibiotics and nutrients

In order to determine if the sponge-associated archaea have similar patterns of antibiotic resistance as their cultivated relatives and if the sponge-archaeal association could be manipulated to enrich for archaea, sections of *A. mexicana* were incubated in seawater containing antibiotics which specifically inhibit bacterial or eucaryal protein synthesis or bacterial cell wall formation. Resistance to a particular antibiotic can often be conferred by a single nucleotide change at a particular residue (Ramirez *et al.*, 1993; Amils *et al.*, 1993). Thus, the susceptibility of the archaea to several of the antibiotics could be predicted after analysis of nucleotide residues in the rRNA gene sequence which confer antibiotic susceptibility/resistance. A change at position 912 from a C in the 16S rRNA gene sequence causes resistance to streptomycin (Amils et al., 1993; Ramirez *et al.*, 1993) and from a G at position 2057 in the 23S rRNA causes resistance to chloramphenicol (Ramirez *et al.*, 1993). In gentamicin resistance, the secondary structure of the stem found around position 1410 in the 16S rRNA

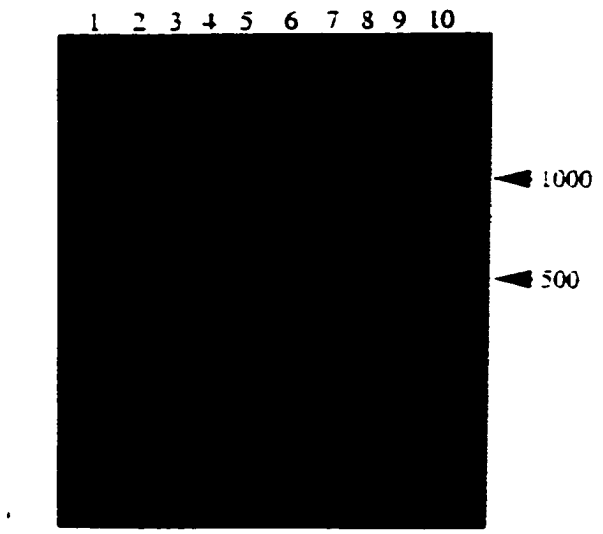


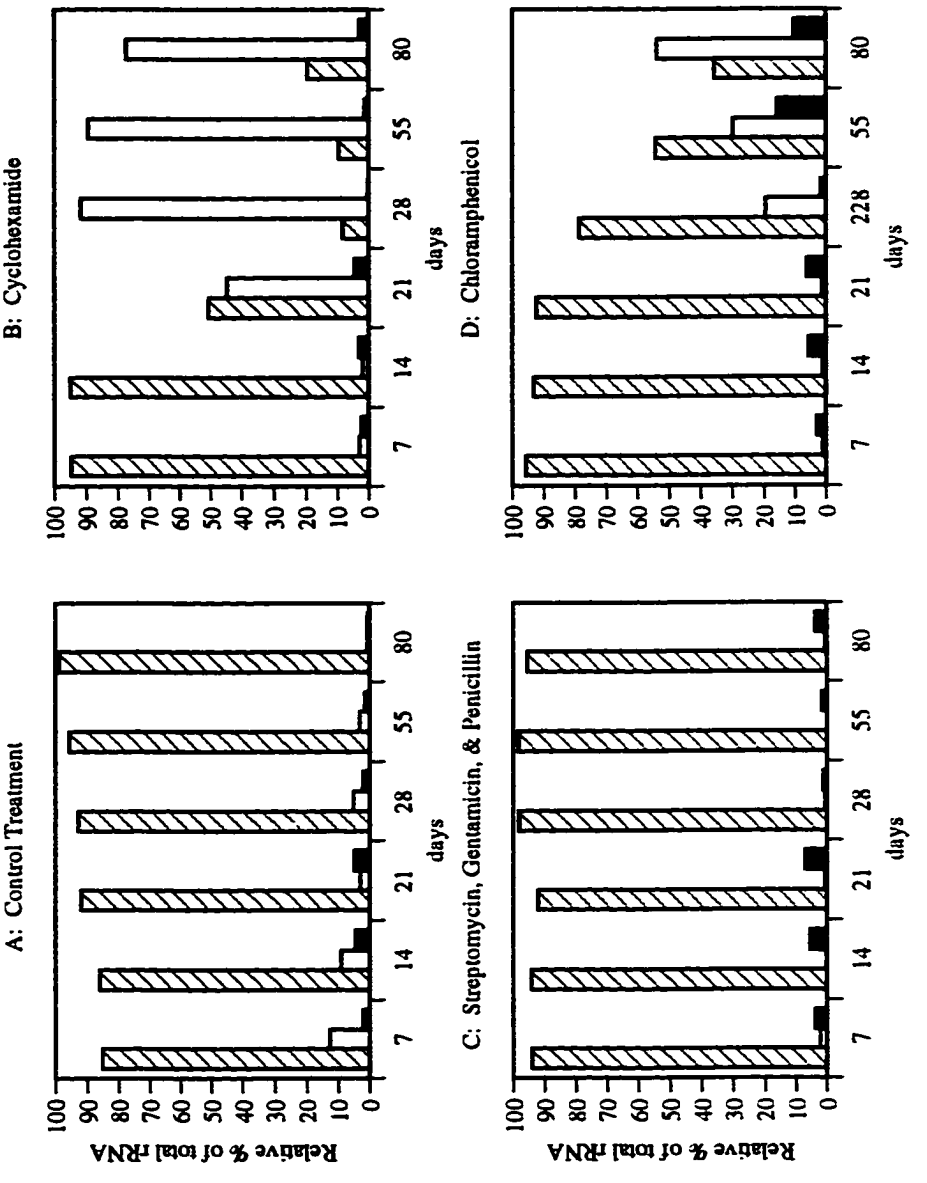
Figure 2-5. RFLP analysis of the 16S rRNA bacterial genes within different specimens of *A. mexicana*. PCR amplified bacterial 16S rDNA genes were digested with HaeIII and separated by electrophoresis on a 2.5% agarose gel. Lanes 1 and 10 contain a 100 base pair ladder (NEB). The specimen number and the sum of the band sizes for each lane follows: s4 (lane 2: 6,700 bp), s8 (lane 3: 5,590 bp), s9 (lane 4: 7,510 bp), s23 (lane 5: 11,260 bp), s26 (lane 6: 9,540 bp), hs4 (lane 7: 10,840 bp), hh1 (lane 8: 9,020 bp), and hh2 (lane 9: 9,880 bp), respectively. For dates and sites of collection see table 2-1. Arrows on the right point to the 1000 and 500 base pair fragments. Lanes 2 through 9 contain restriction digests from different individuals of *A. mexicana* .

is disrupted (Amils *et al.*, 1993). The marine archaea possess the nucleotide residue U at position 912 in 16S rRNA, an A at position 2057 in the 23S rRNA, and do not possess the G-C base pair between positions 1412-1489 and 1409-1491 in the 16S rRNA, thus should be resistant to streptomycin, chloramphenicol, and gentamicin, respectively. Erythromycin resistance is also conferred by a nucleotide change at position 2058 in the 23S rRNA (Amils et al., 1993; Ramirez *et al.*, 1993); the sponge-associated archaea do not have an A at this position, and therefore should be resistant. Erythromycin was not used in this study. In addition, the symbiont should also be resistant to penicillin because archaeal cell walls contain no peptidoglycan (Kandler and Konig, 1993).

Archaea were detected in all treatments except for day 28 of the cycloheximide treatment and day 80 of the control (figure 2-6). Antibiotic studies indicated that the bacterial antibiotics (chloramphenicol, streptomycin, gentamicin, and penicillin) did not adversely effect the sponge archaeal population (figure 2-6), which is consistent with the antibiotic data available on the archaea (Cammarano *et al.*, 1985). However, chloramphenicol resistant bacteria proliferated after 3 weeks, and thus, chloramphenicol was not as useful as the gentamicin, streptomycin, and penicillin treatment in eliminating the bacterial population. The cycloheximide treatment had little effect on the relative percentage of each domain until week 3. At that point there was a dramatic increase in the relative percentage of bacterial rRNA.

Between day 7 and day 28, nucleic acids were extracted from the seawater surrounding the sponge and probed to determine whether the archaea

Figure 2-6. The effect of eucaryal- and bacterial-targeted antibiotics on the archaeal that inhabit *A. mexicana*. Portions of hs1 maintained for 120 days in flowing seawater tanks were incubated in seawater with no additions (a), cyclohexamide (b), streptomycin, gentamicin, and penicillin (c), and chloramphenicol (d). The relative percentage of eucaryal (hatched bars), bacterial (white bars), and archaeal (black bars) was determined by rRNA hybridization with domain-specific oligonucleotide probes.



were being expelled from the sponge into the surrounding seawater. Oligonucleotide probe Arch915 did not bind to the nucleic acids extracted from the surrounding seawater indicating the absence of archaeal rRNA.

Other experiments were conducted to test the effects of nutrient supplements on the archaeal population. The addition of ammonium, nitrate, or bicarbonate had no effect on the relative percentage of archaeal rRNA of *A. mexicana* (data not shown).

Specificity of the rRNA oligonucleotide probes

Preliminary rRNA sequence analysis of the 16S rRNA gene indicated that the probes initially designed to specifically target the 16S rRNA of marine "Group 1" planktonic archaea (DeLong, unpublished data) were also complimentary to the rRNA gene sequence of the sponge archaeal symbiont. Check Probe at the Ribosomal Database Project (Maidak *et al.*, 1997) verified that these probes targeted only the marine "Group 1" archaea. However, secondary structure of rRNA and binding efficiency of the probes can influence probe signal. Thus, the specificity of the oligonucleotide probes was also tested experimentally by hybridizing radioactively labeled oligonucleotide probes to 16S rRNA genes cloned from marine planktonic crenarchaea and euryarchaea and the crenarchaea detected in *A. mexicana*.

Hybridization of all plasmid and rRNAs to either the universal or Arch915 probe was observed (data not shown), indicating that rRNA was present and accessible to the probes. No cross reactivity to the cloned rRNA genes of marine "Group 2" euryarchaea, or to native rRNA from cultivated archaea, bacteria, or eucarya was observed (figure 2-7). The probes hybridized

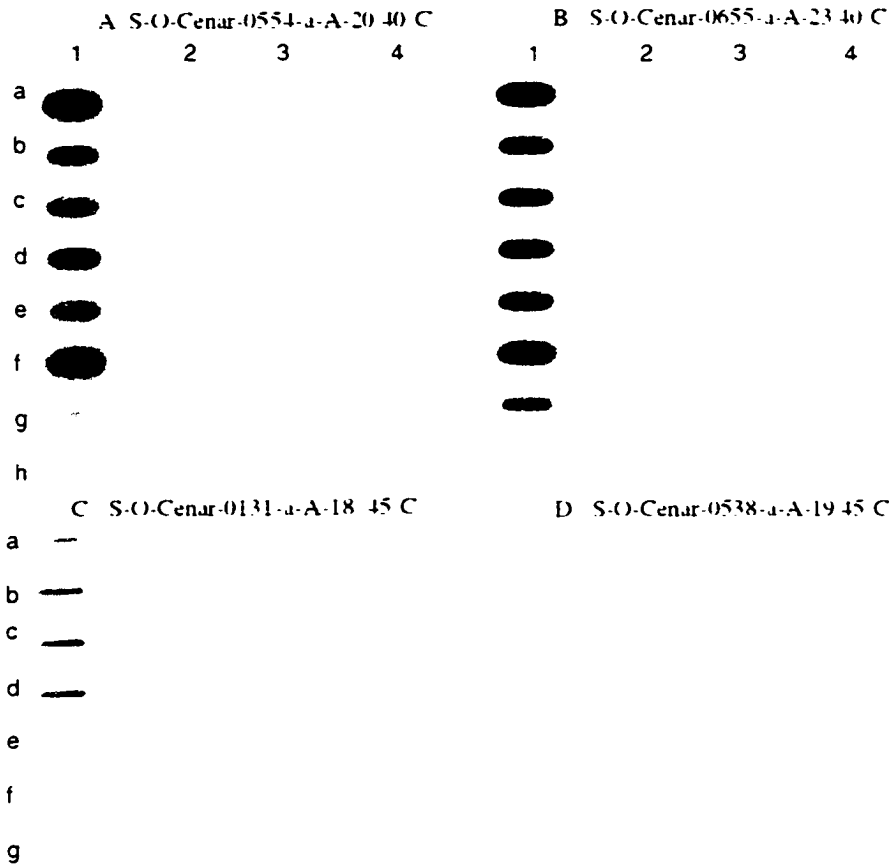


Figure 2-7. Slot blot autoradiographs showing the specificity experiments of the 4 marine crenarchaeal oligonucleotide probes [S-O-Cenar-0554-a-A-20 (a), S-O-Cenar-0655-a-A-23 (b), S-O-Cenar-0131-a-A-18(c), S-O-Cenar-0538-a-A-19 (d)]. The lowest temperature tested which provided the correct specificity is shown. Column 1 contains the following cloned 16S rRNA genes from marine "Group 1" archaea: SBAR5, WHARQ, OAR4, OAR7, fosmid 4B7 (DeLong, 1992; Stein *et al.*, 1996), and the archaeal 16S rRNA gene isolated from *A. mexicana*. Slot 1G contains crude nucleic acids extracted from *A. mexicana*. Column 2 contains the following 16S rRNA genes clones from marine Group 2 archaea: SBAR1A, WHARN, OAR22, and OAR B for panels a and b and OAR22 and OARB for panels c and d. Column 3 contains rRNA extracted from cultured Archaea. Representatives of the Archaea in panels a and b were *Haloferax volcanii*, *Thermoplasma acidophilum*, *Methanobacterium thermoautrophicum*, *Methanococcus jannaschii*, *Pyrococcus* strain GBD, *Desulfurococcus* strain SY, *Sulfolobus solfataricus* and in panels c and d: *H. volcanii*, *T. acidophilum*, *M. jannaschii*, *Desulfurococcus* strain SY, *Pyrococcus* strain GBD, and *S. solfataricus*. 3H in panels a and b and 3G in panels c and d contain rRNA extracted from *Saccharomyces cerevisiae*. Column 4 contains rRNA extracted from the following cultured bacteria: *Vibrio fischeri*, *Psuedomonas nautica*, *Rhodopseudomonas palustris*, *Paracoccus denitrificans*, *Commamonas testosteroni*, *Bacillus megaterium*, and *Syenechococcus* strain 6301. Slot 4H of panels a and b contain rRNA from *Saprospira grandi*.

to the cloned 16S rRNA genes from marine planktonic archaea, to the cloned 16S rRNA gene of the archaeal symbiont of *A. mexicana*, and to crude nucleic acids from the sponge. Thus, the target rRNA sequence of each of the oligonucleotide probes was present in the archaeal symbiont and the probes hybridized specifically to the marine "Group 1" archaea.

Whole cell hybridization analyses

To visualize the sponge-associated archaea, fluorescently-labeled, rRNA-targeted oligonucleotide probes were hybridized to formalin-fixed whole cell suspensions of homogenized sponge. Fluorescent whole cell hybridization with marine Crenarchaeotal-specific oligonucleotide probes yielded a strong hybridization signal at opposite cellular poles of the sponge-associated Archaea, and revealed a central area to which no rRNA-targeted probe bound (figure 2-8b; figure 2-9). This central area corresponded to a region of intense DAPI staining (figure 2-8a, figure 2-9), indicating a central intracellular location of the nucleoid. The archaeal cells identified using both the rRNA and DAPI stains, were rod-shaped, and on average 0.8 μm in length and 0.5 μm in width. In control experiments in which a fifty fold excess on unlabeled "Group 1" four mix was used in conjunction with the fluor labeled oligonucleotide probes at their standard concentration, no probe-derived fluorescence signal was observed (figure 2-10). This result indicated that the unlabeled probe out-competed the fluor-labeled probe for the target rRNA sequence. Thus, the fluorescence signal when only the fluor-labeled probes were used in hybridization experiments was due to binding of the oligonucleotide probes to their complimentary sequence in rRNA, and not due to nonspecific binding of the fluor molecule. Simultaneous

Figure 2-8. Photomicrographs of the sponge-associated archaea. Dissociated sponge cells were hybridized with marine "Group I" (DeLong, 1992) archaeal-specific oligonucleotide probes labeled with Texas Red (a), and cells of the same field visualized with DAPI (b). The arrows point to a cell that was undergoing division when the specimen was fixed. The nucleoid corresponds to two distinct regions which stained intensely with DAPI (b). Oligonucleotide probe binding was excluded in this area (a). The oligonucleotide probe bound to three intracellular regions of the dividing cell (a). Scale: 5 μm = 6 mm.

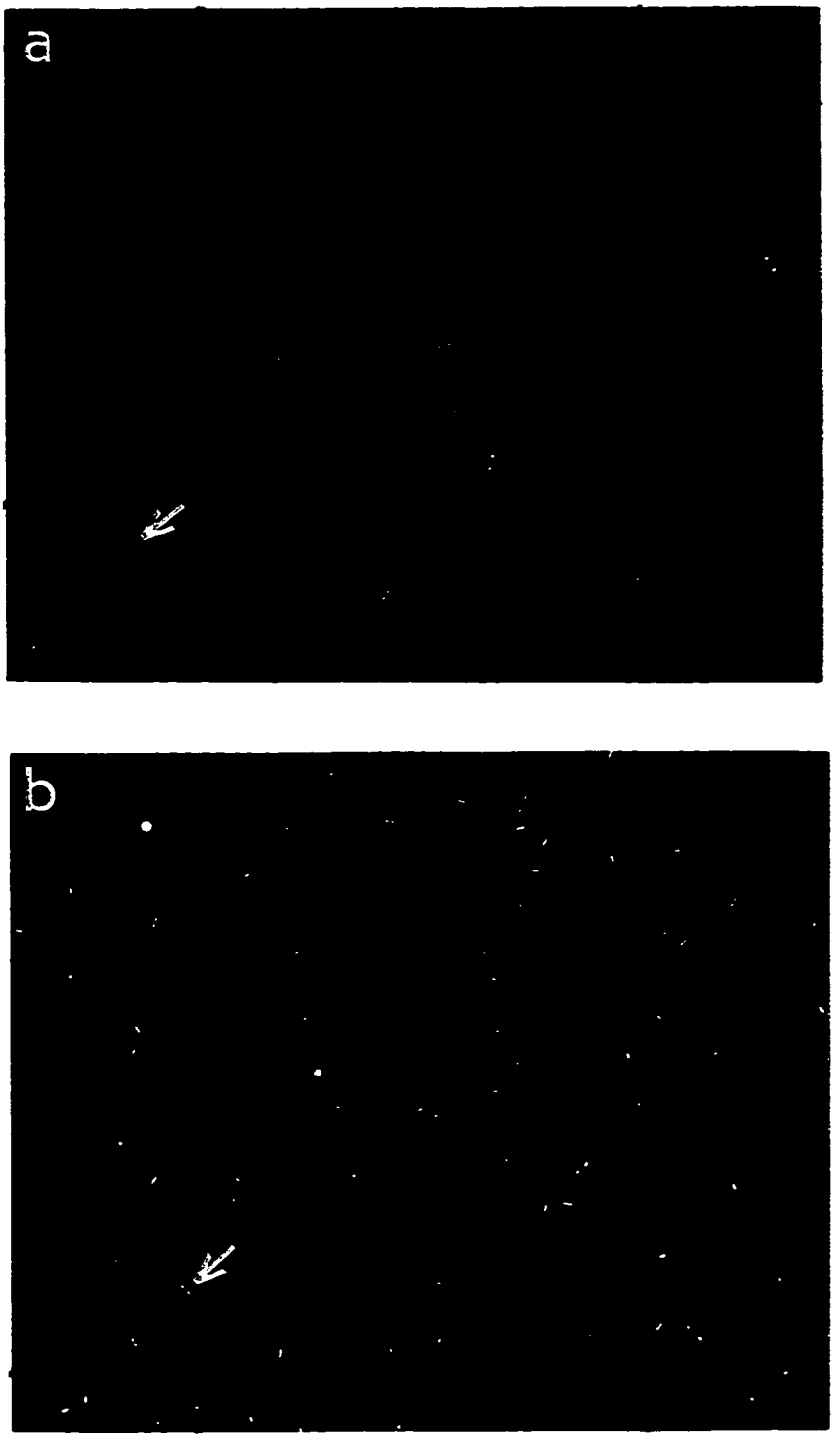


Figure 2-9. Color overlay showing the staining pattern of the sponge-associated archaea with DAPI and fluorescently labeled oligonucleotide probes. The black and white photomicrographs of figures 2a and 2b were scanned, digitized, colorized, and superimposed to form a composite image of stained cells. Yellow coloration corresponds to DAPI stained regions (see figure 2-8b), and red to regions which bound the rRNA-targeted oligonucleotide probes (see figure 2-8a). The orange color results from overlap between the two cell stains. The arrow points to the image of the same dividing cell that is indicated in figure 2-8. The inset is an enlargement of this image, showing the distribution of DAPI and rRNA targeted stains.

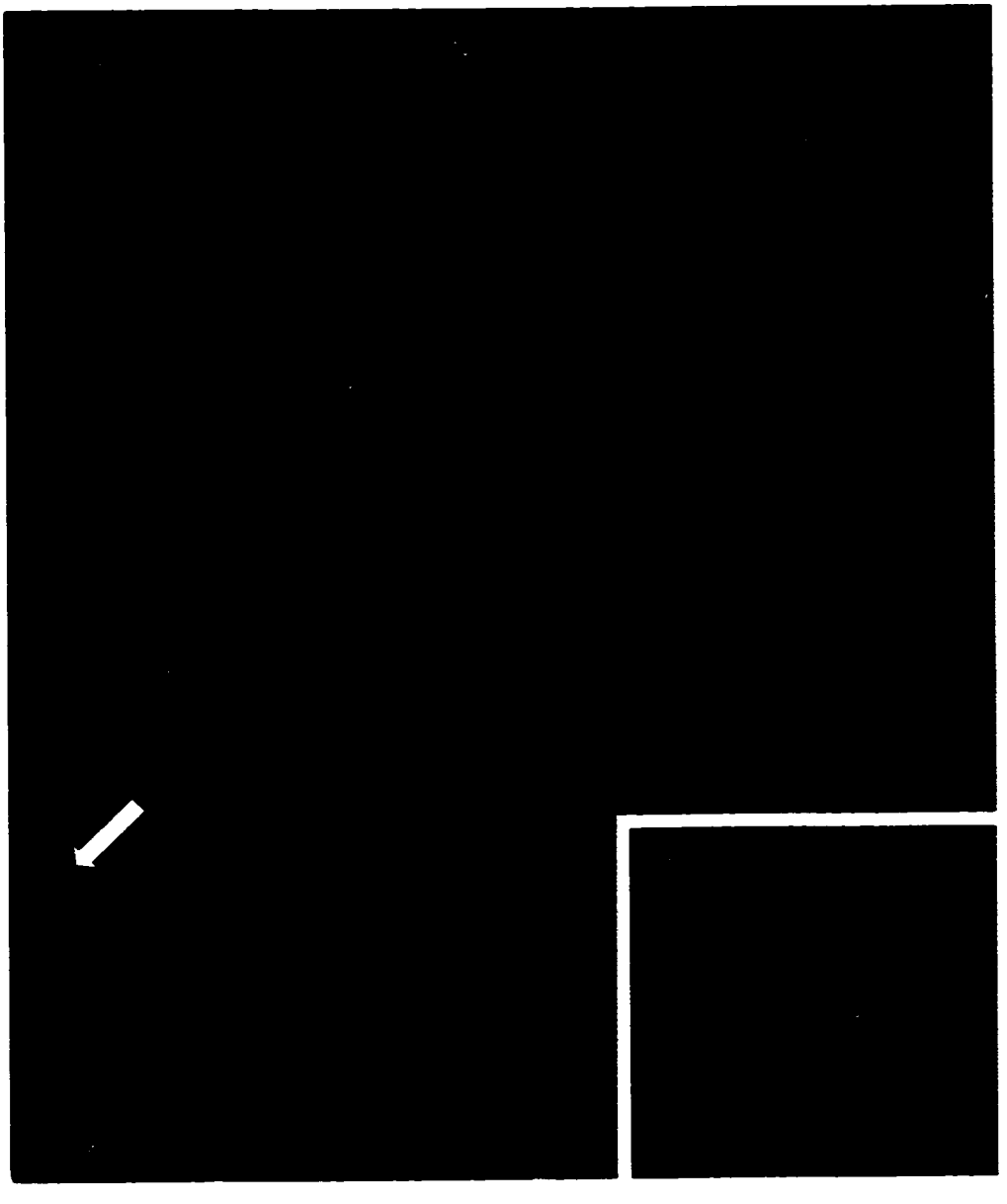
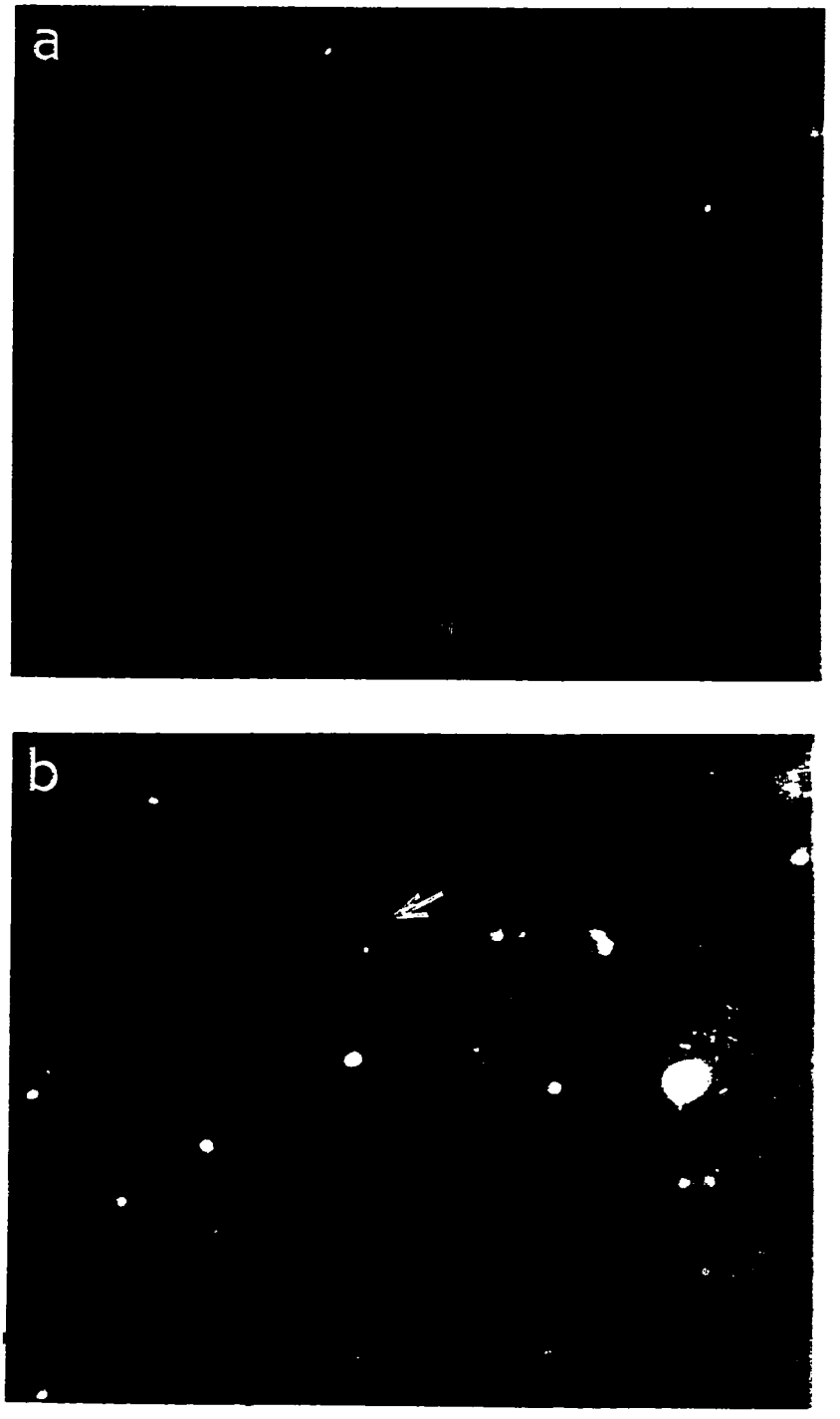


Figure 2-10. Decrease in the hybridization signal with the addition of an excess of unlabeled oligonucleotide probes that target the marine archaea. Photographs of dissociated sponge cells simultaneously hybridized to the Texas Red "Group 1" oligonucleotide probes at their normal concentration and a fifty fold excess of the same four mix unlabeled (a). The corresponding DAPI image is also shown (b). The arrow points to several archaeal cells (b; see figure 2-8 for the staining pattern of the archaea with DAPI) which did not stain with the Texas Red "Group 1"-specific oligonucleotide probes when a fifty fold excess of unlabeled probe was added. Scale: 5 μm= 6 mm

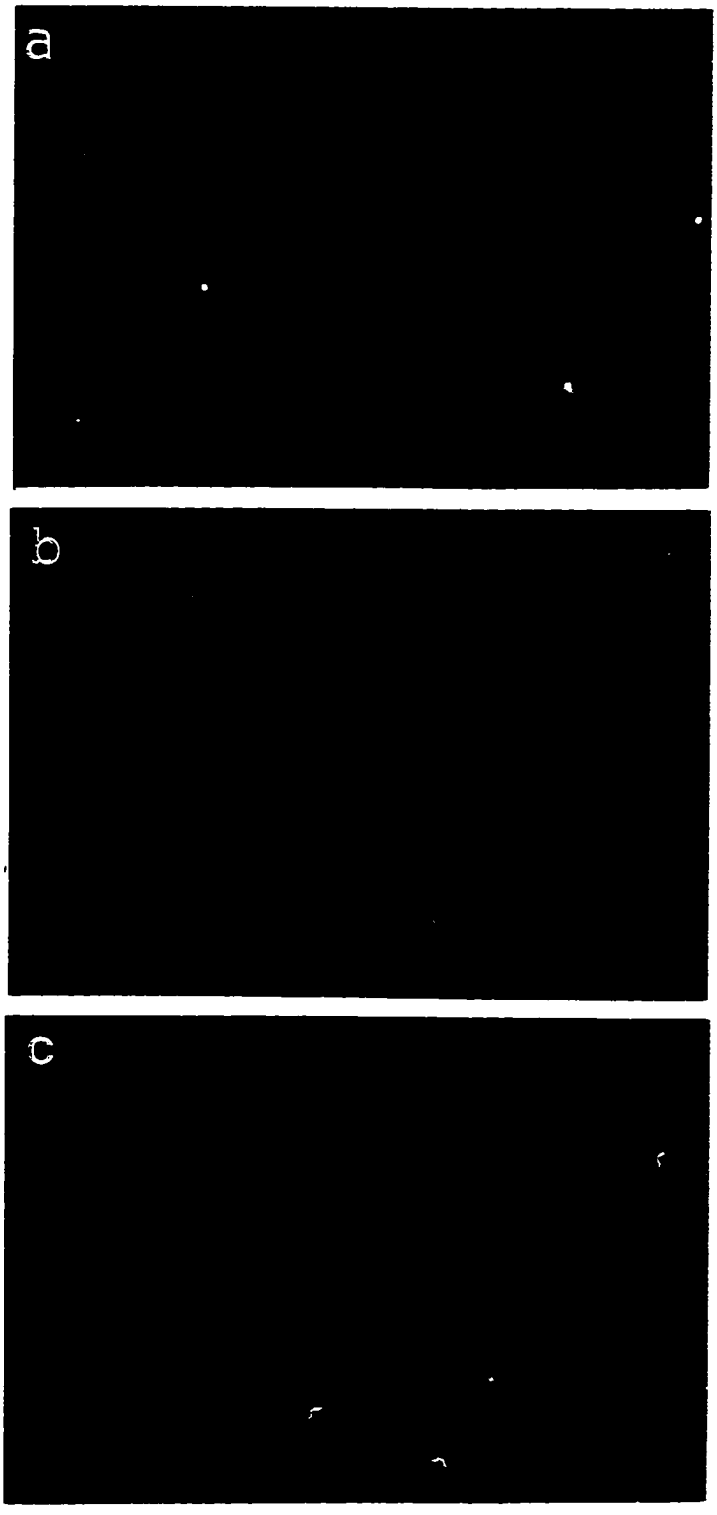


staining with the four "Group 1" probes and either Bacteria-specific probes (figure 2-11) or Eucarya-specific probes (figure 2-12) showed that cells that hybridized with the marine crenarchaeotal-specific probes did not hybridize with probes specific for Eucarya or Bacteria. In addition, the Bacterial-specific probes hybridized to diverse cell morphologies (data not shown), confirming the result of RFLP analysis of the bacterial 16S rRNA genes which indicated a diverse bacterial population in *A. mexicana*.

Of the total prokaryotic cells enumerated by DAPI staining from two specimens of *A. mexicana*, 50% and 65% (table 2-6) were identified as archaea by their strong binding to the marine Crenarchaeotal-specific oligonucleotide probes (figure 2-8). These results are consistent with previously determined rRNA abundances, which indicated that between 47 and 70% of the prokaryotic rRNA extracted from sponges held in aquaria was archaeal in origin (figure 2-1 and table 2-6).

Archaeal cells fixed during division were recognized by the presence of two regions showing no probe binding (arrow, Figure 2-8a; figure 2-9, inset), which corresponded exactly to two distinct, nucleoid regions visualized by their intense DAPI staining, and three regions to which the rRNA probe bound (arrow, figure 2-8b; figure 2-9, inset). Thirteen to 15% of the total sponge-associated archaeal cells detected by whole cell hybridization for the two individuals of *A. mexicana*, exhibited this staining pattern. This result indicates cell division of the archaeal symbionts within sponges kept in flowing seawater tanks at 10°C for up nine months (table 2-6).

Figure 2-11. Simultaneous staining of archaea and bacteria associated with *A. mexicana*. Archaea and bacteria from enrichment preparations were stained simultaneously with Texas Red labeled "Group 1"- (a) and FITC labeled bacterial-(b) specific oligonucleotide probes. Each probe bound to different cells. The same field visualized with DAPI is shown in panel c. Arrow heads in c point to several bacteria identified by their hybridization to bacterial-specific probes (b). Scale: 5 μm = 5 mm



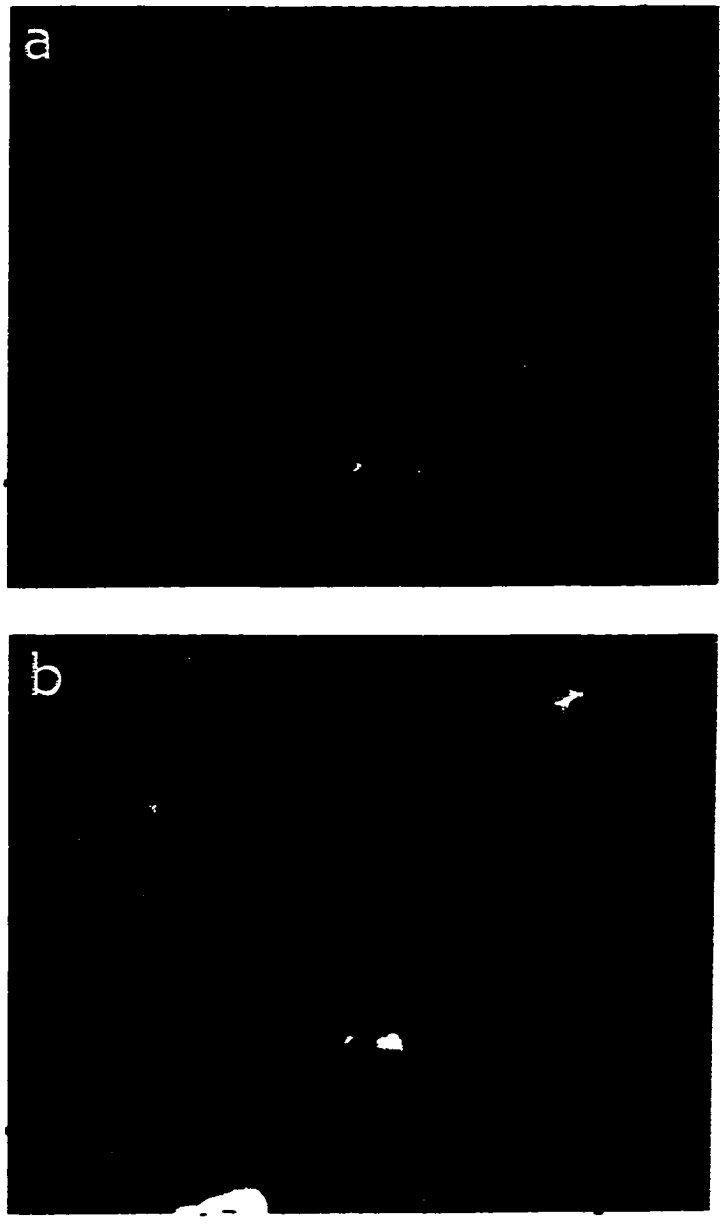


Figure 2-12. Fluorescent staining of archaea and sponge cells with oligonucleotide probes. Dissociated sponge cells were simultaneously hybridized to Texas Red-labeled "Group I" (a) and FITC-labeled eucaryl (b) oligonucleotide probes. Scale: 5 μm = 5 mm

Table 2-6 . Percentage of the total prokaryotic community that bound to the four fluor-labeled oligonucleotide probes.

Sponge Individual	Length in Captivity	Total Dapi Count[a]	Percent Archaea	Percent Archaea Actively Dividing	Relative Abundance of Archaeal rRNA[b]
s20	4 months	576	50%	13%	70%
hs1	9 months	1274	65%	15%	63%

[a]Prokaryotes only.

[b] of the total Prokaryotic rRNA as determined by quantitative rRNA hybridization determined using rRNA oligonucleotide probes.

Discussion

DeBary (1879) defines symbiosis as an association between distinct organisms. In the marine environment, intimate associations between physiologically diverse bacteria and a variety of eucaryotic hosts are common (Smith and Douglas, 1987; Cavanaugh, 1994). Well known examples include highly specific associations in which a multicellular, eucaryotic host derives benefit from the unique physiological capabilities of their prokaryotic symbionts. Known symbioses involving Archaea, however, are limited to one archaeal group, the euryarchaeal methanogens (subgroups *Methanomicrobiales* and *Methanobacteriales*; Embley and Finlay, 1994). The presence of methanogens, which serve as an electron or hydrogen sink, thermodynamically favors more complete oxidation and utilization of available carbon substrates in the host. Archaeal symbioses involving Crenarchaeota, or other nonmethanogenic archaea, have not previously been described.

Symbioses within the phylum *Porifera* are common and involve bacteria, dinoflagellates, and *Chlorella* (see Bergquist, 1978), but associations with archaea had not been reported prior to this study (chapter 1). Archaeal and bacterial rDNA and rRNA were initially detected in several specimens of *A. mexicana* (chapter 1). Here, I focused on determining the identity of the archaea and the specificity of the interaction between the archaea and *A. mexicana*. The archaea found within *A. mexicana* are most closely related to, but distinct from (table 2-4; figures 2-2 & 3) the crenarchaeal planktonic lineage (DeLong, 1992; Fuhrman *et al.*, 1992; Massana *et al.*, 1997; McInerney *et al.*, 1995; DeLong *et al.*, 1994; van der Maarel *et al.*, 1998). The archaeal rRNA phylotypes

recovered from different host individuals, were nearly identical. None of the planktonic rRNA phylotypes were found within *A. mexicana*. The consistent presence of archaeal rRNA, the detection of similar crenarchaeal rRNA phylotypes in every specimen of *Axinella mexicana* examined from the Santa Barbara Channel and Monterey, CA, their high cellular abundance, evidence of cell division, and the persistence of the association over long time periods, all strongly suggest that the sponge-archaeal partnership is specific and represents a symbiosis (see definition by DeBary above).

Analyses of picoplankton diversity at a mooring in the Santa Barbara Channel by rRNA gene cloning and sequencing, and denaturing gradient gel electrophoresis (Murray, Ph.D. dissertation, Massana *et al.*, 1997) did not detect the archaeal symbiont. However, the same rDNA sequence (region 172 to 468, *E. coli* numbering) belonging to the archaea that inhabits *A. mexicana* was detected in two Antarctic sponges (appendix 2). This result indicates that the archaeal symbiont (rRNA phylotype sequence s12a (101G10), figure 2-2 & Appendix 2) or its close relatives are not found exclusively in *A. mexicana*. In at least some other marine symbioses, the same symbiont as determined by rRNA gene sequence has been found to inhabit multiple hosts (Durand and Gros, 1996; Gast and Caron, 1996; Rowan and Powers, 1991). Despite the detection of the same archaeal rRNA phylotype in other marine sponges, the relationship between the archaea and *A. mexicana* still represents a specific association.

In most cases, only one individual from each of the different Antarctic and tropical sponges was analyzed. Thus, it remains to be determined if archaea consistently inhabit these species of sponge. Since sponges are filter feeders, it

will be necessary to rule out the possibility that archaea are simply food derived from the plankton. In cases where more than one non-*Axinella* sponge specimen was assayed for archaeal rDNA, they often gave conflicting results (appendix 2), indicating that the archaea may have been transient members of the prokaryotic population obtained from filter feeding or represent contaminants from the seawater. The detection of the planktonic crenarchaeal clone ANTARCTIC12 in three of the Antarctic sponges may have been due to its high relative abundance in bacterioplankton (Murray et al., 1998) at the time the sponges were collected.

In marine environments multiple, highly related archaeal phylotypes, or "clusters" have been consistently recovered in plankton (DeLong, 1992, Fuhrman *et al.*, 1992, DeLong 1994, Massana *et al.*, 1997; McInerney *et al.*, 1997; Fuhrman and Davis, 1997), holothurian guts (McInerney *et al.*, 1995), and marine fish (van der Maarel *et al.*, 1998). It remains to be determined if the marine "Group 1" archaea (DeLong, 1992) detected within the guts of holothurians and marine fish are permanent or transient members of the gut microflora. The archaeal phylotypes recovered from *A. mexicana* were more closely related to each other (>98% similarity), than to any of the planktonic archaea (highest similarity 95%). Additionally, all host sponges contained archaeal rRNA indicating that the association was very specific.

The techniques used to analyze the archaeal population in the sponge can be applied to study the marine planktonic archaea. For example, the specificity of the oligonucleotide probes for "Group 1" archaea was determined utilizing dissociated sponge cells. These probes have been subsequently used to quantity

the percentage of archaeal cells in plankton collected off the coast of Palmer Station, Avers Island, Antarctica (Murray *et al.*, 1998).

The fluorescently-labeled rRNA oligonucleotide probes and DAPI stain bound to different regions of the cell indicating that there is partitioning of the DNA and the rRNA within the cell. Nucleoid regions that exclusively contain DNA have been reported in Archaea and Bacteria (Robinow and Kellenberger, 1994; Rieger *et al.*, 1995; Poplawsik and Bernander, 1997). The structure of the nucleoid was shown to change during the cell cycle; it was most densely packed during exponential growth (Poplawsik and Bernander, 1997). A recent electron microscopic by study by Fuerst *et al.*, (1998) found sponges contained prokaryotic-like cells with a membrane bound nucleoid region. Based on the membrane and septum structure, and a partitioning of RNA and DNA similar to the archaeal symbiont of *A. mexicana*, Fuerst *et al.*, (1998) suggested that these cells may be archaeal. The detection and identification of archaea within these sponge species has yet to be confirmed with either molecular phylogenetic techniques or lipid analysis. If true, it would be an intriguing result, since no Archaea to date have been shown to have a membrane-bound nucleoid.

The growth temperature of the sponge in its natural habitat ranges from about 8 °C to 18 °C. These porifera (and their crenarchaeal symbionts) have remained healthy for years when maintained in laboratory aquaria at 10 °C or at ambient seawater temperature. This observation provides strong evidence that the marine crenarchaeotes, whose closest cultivated relatives are all thermophilic or hyperthermophilic, can thrive at low temperatures. A study of the

thermostability of the DNA polymerase of the archaeal symbiont also supports a non-thermophilic phenotype (Schleper *et al.*, 1997b).

The ecological distribution of marine archaea reported in the literature (DeLong, 1992, Fuhrman *et al.*, 1992, DeLong 1994, Massana *et al.*, 1997; Massana *et al.*, 1998; Murray *et al.*, 1998; McInerney *et al.*, 1995; van der Maarel *et al.*, 1998; Moyer *et al.*, 1998; McInerney *et al.*, 1997) and this report, suggest that mesophilic or psychrophilic crenarchaeota are ubiquitous. This lineage had 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 hyperthermophilic, thermophilic, and non-thermophilic phenotypes whose growth temperatures collectively span a wide temperature range, from >100°C (Stetter *et al.*, 1990; Blochel et al., 1997) to <-1.5 °C (DeLong *et al.*, 1994; Massana *et al.*, 1998; Murray *et al.*, 1998). Non-thermophilic crenarchaeotes have a wide ecological distribution, and have radiated into diverse marine and terrestrial habitats (Kudo *et al.*, 1997; Bintrim *et al.*, 1997; Jurgens *et al.*, 1997; Schleper *et al.*, 1997a; MacGregor *et al.*, 1997; Munson *et al.*, 1997; Kato *et al.*, 1997; Chandler *et al.*, 1998; McInerney *et al.*, 1995; van der Maarel et. al, 1998; Fuhrman *et al.*, 1992; Fuhrman *et al.*, 1997; DeLong *et al.*, 1994; Moyer *et al.*, 1998; Murray *et al.*, 1998; Massana *et al.*, 1997; Massana *et al.*, 1998; DeLong, 1992; Ueda et al., 1995). Archaea, in particular Crenarchaeota, were previously considered ecologically insignificant, and were presumed to occupy primarily extreme and unusual environments. It is becoming increasingly evident that the mesophilic and psycrophilic Archaea are abundant, globally distributed, and

well-adapted to non-extreme lifestyles and niches which includes a symbiotic relationship with a eucaryotic host. This situation is reminiscent of more common and easily cultivated marine prokaryotes (e.g. Vibrionaceae; Baumann *et al.*, 1983) which inhabit planktonic, epiphytic, and symbiotic niches.

The specific binomial name *Cenarchaeum symbiosum* gen. nov., sp. nov. {Preston *et al.*, 1996; [Cen arch' ae um Gr. adj. *kainos* recent, and Gr. adj. *koinos* common; Gr. adj. *archaeo* ancient; M. L. neut. n. *Cenarchaeum* the genus of relatively recent (derived nonthermophilic phenotype) and common (non-"extremophilic") archaea. sym bi o' sum M. L. neut. adj. *symbiosum* living together]} was proposed for the sponge-associated archaeon. The following criteria were used for identification and description of the sponge symbiont: phylogenetic placement as determined by ssu rRNA sequence, high similarity of 16S rRNA genes from different host individuals, specific ssu rRNA signature nucleotides, cell identity, cell size and morphology (determined by whole cell hybridization), natural growth temperature, and the specificity of the symbiotic association with *A. mexicana*.

Nearly every ecological study which has utilized molecular approaches to describe microbial diversity has led to the discovery of unique rRNA phylotypes belonging to organisms that have yet to be cultivated (DeLong, 1992; Fuhrman *et al.*, 1992; Amann *et al.*, 1995; Giovannoni *et al.*, 1990; Ward *et al.*, 1990; Schmidt *et al.*, 1991; Liesak and Stackebrandt 1992; Spring *et al.*, 1992). These studies revealed organisms isolated using standard cultivation techniques often inadequately represent the prokaryotic population (Suzuki *et al.*, 1997). The recent description of a large number of new phylotypes of

thermophilic Archaea and Bacteria, from a single Yellowstone hot spring, represents an example of high microbiological diversity revealed only after molecular phylogenetic analyses (Barns *et al.*, 1994; Hugenholtz *et al.*, 1998). The association between the psychrophilic crenarchaea and their metazoan host would have remained undetected without the aid of molecular phylogenetic tools. The physical distribution of the archaeal symbionts within host tissues, be it extra- or intracellular will be addressed in chapter 4. The trophic relationship between the host and symbionts remains to be investigated. The ability to maintain and manipulate the association in laboratory aquaria indefinitely should greatly aid future investigations.

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CHAPTER 3

Distribution of the genetic variation in *Cenarchaeum symbiosum* within its host *Axinella mexicana* and in other environmental samples

Introduction

Molecular phylogenetic surveys, using rRNA gene sequences, have become a standard method to identify members of prokaryotic communities. Their use has led to the discovery of many novel and previously undetected lineages (DeLong, 1997; Pace, 1997) including the SAR 11 cluster (Giovannoni *et al.*, 1990) and the group 1 crenarchaea (DeLong, 1992; Fuhrman *et al.*, 1992). One re-occurring result of these studies is the recovery of highly-related, but unique rRNA gene sequences which form clusters upon phylogenetic analysis (DeLong, 1992; Giovannoni *et al.*, 1990; Field *et al.*, 1997; Moore *et al.*, 1998; Amann *et al.*, 1996). The explanations put forth to account for the variation include artifacts generated during PCR amplification (Taq polymerase errors and chimeric genes), sequencing errors, sequence variation between rRNA operons of a single organism, and the presence of highly-related strains (Amann *et al.*, 1996; Field *et al.*, 1997; Moore *et al.*, 1998). The ecological distribution of the rRNA gene sequences (Moore *et al.*, 1998; Field *et al.*, 1997) and the *in situ* visualization of the variation of rRNA gene expression in naturally occurring bacterial populations (Amann *et al.*, 1996), indicates that the highly-related rRNA genes probably represent real organismal diversity .

Symbiotic organisms form phylogenetically distinct lineages, but often the members of the lineage occupy various hosts, rather than representing a

single symbiont population from one species host (Durand and Gros, 1996; McNally *et al.*, 1994; Stouthamer *et al.*, 1993). However, morphological variation within the symbiont population of a single host has been detected microscopically in some symbioses (Fisher, 1990; Finlay and Fenchel, 1991; McLean and Houk, 1973; Trench and Winsor, 1987), indicating the possibility of more than one symbiont type. Molecular evidence has shown that the morpholgically variable symbiont population was either due to morphological plasticity of a single symbiont (Distel *et al.*, 1988; Eisen *et al.*, 1992; Distel *et al.*, 1994; Kim *et al.*, 1995; Embley *et al.*, 1992), or the presence of phylogenetically distinct symbionts (Distel *et al.*, 1995; Unterman *et al.*, 1989; Rowan *et al.*, 1997). The majority of described natural symbiotic associations, however, consist of a monospecific culture of the symbiont within a particular invertebrate or protozoan host species (Distel *et al.*, 1988; Distel *et al.*, 1991; Durand and Gros, 1996; Distel and Cavanaugh, 1994; Krueger and Cavanaugh, 1997; Haygood and Distel, 1992; Embley and Finlay, 1993; Rowan and Powers, 1991). This however does not imply that a particular symbiont is found exclusively in a single species of host (Trench, 1997; Durand and Gros, 1996; Gast and Caron, 1996). In addition, Gast and Caron (1996) found that different individuals of the host, *Orbulina universa*, can harbor highly-related symbiont "strains" (>99.4% similar in rRNA gene sequence over 1,799 bases), but more than one strain (multiple rRNA gene sequences) was not detected within a single specimen. In fact, none of the above studies reported any microheterogenity (>97.5% similarity in rRNA gene sequence; Stackebrandt and Goebel, 1994) within a single symbiont population.

The methods used to identify the dominant members of the symbiont population may not have been rigorous enough to detect variation within each population (Haygood and Distel, 1992) and when variation was detected, it was explained by errors introduced by the methods used (Distel *et al.*, 1995; O'Neil *et al*, 1992). In addition, rRNA analysis may not be a good indicator of population level microheterogenity because small differences are likely not involved in selection (exception: antibiotic resistance, see Amils *et al.*, 1993 and Ramirez *et al.*, 1993) which defines the ecological distribution of strains (Cohan, 1996). Strains with identical rRNA gene sequence have been shown to differ physiologically and in protein coding and ITS gene sequence (Palys *et al.*, 1997; Trench, 1997; Cillia *et al.*, 1996; Stackebrandt and Goebel, 1994; Fox *et al.*, 1992). Many different strains of *Rhizobium etli* biovar *phaseoli* colonized the root nodules of a single bean plant as determined by multilocus enzyme electrophoresis (Souza *et al.*, 1994). In these cases, the microheterogenity would have remained undetected if only the small subunit rRNA gene was analyzed.

One study detected heterogeneity in rRNA genes within the symbiont population of an individual host and investigated the distribution of those variants throughout naturally occurring host populations. Rowan and Knowlton (1995) and Rowan *et al.*, (1997) found up to three phylogenetically distinct dinoflagellates (6 to 7% dissimilarity over 474 nucleotides of the ssu rRNA gene sequence) inhabited the cnidarian host, *Montastraea annularis*. The distribution of the symbionts within a host individual was correlated with depth and led to

hypotheses regarding the light adapted abilities of the dinoflagellate clades (Rowan and Knowlton, 1995; Rowan *et al.*, 1997).

The marine sponge, *Axinella mexicana*, was found to have a consistent and specific association with the archaeon, *Cenarchaeum symbiosum* (chapter 2). In preliminary sequencing of the archaeal rRNA genes of different individuals of *Axinella mexicana* and sequence data from cloned rRNA gene variants, two ambiguous positions were consistently observed within the 5' end of the 16S rRNA gene. This indicated either the presence of multiple copies of the rRNA gene or the presence of highly-related strains (chapter 2) in sponges from the Santa Barbara Channel. Evidence from chromosomal libraries showed that the ambiguities were due to the presence of two different, but highly-related genetic variants (variant A and B) of *C. symbiosum* (Schleper *et al.*, 1998). Complete sequence analysis of an overlapping 28 kilobase (kb) genomic fragment containing the rRNA genes of *C. symbiosum*, showed the two major variant types were 92.9% similar in 16S and 23S rRNA gene sequence, 87.8% similar in overall DNA similarity, 91.6% similar in amino acid sequence of open reading frames (ORF), and exhibited colinearity in their protein encoding genes (Schleper *et al.*, 1998). The level of similarity and the organization of the chromosomal contigs indicated that the two rRNA variants belong to two different, but highly-related strains of *C. symbiosum*, rather than rRNA operons of a single organism (Schleper *et al.*, 1998). The detection of the heterogeneity in the chromosomal libraries led to the present study to determine whether the major *C. symbiosum* strains occur consistently in natural archaeal populations that colonize *A. mexicana*. Two approaches were used in this investigation.

First, the nucleic acid sequence derived from PCR amplified archaeal 16S rRNA genes from individuals of *A. mexicana* was re-evaluated with respect to the two major variant types found in fosmid libraries. Secondly, oligonucleotide probes designed to specifically target the 23S rRNA molecule of each strain were hybridized to crude nucleic acid preparations from *A. mexicana* collected at various sites as well as surrounding environmental samples, in order to determine the spatial distribution of the *C. symbiosum* variants.

Methods

Nucleic Acid Extraction

To assay the distribution of the *C. symbiosum* variants within its host and surrounding environments, samples of *A. mexicana*, seawater, sediments, and other species of sponge from the collection site, as well as, seawater supplied to aquaria holding *A. mexicana* was collected. Nucleic acid extraction from sponges was previously described (see methods in chapter 1). Seawater and sediment samples from Naples Reef, Santa Barbara, CA, were collected on SCUBA at depth in collapsible 20 L cube containers and in sterile 50 mL falcon tubes, respectively. Microorganisms were collected from seawater supplied to the aquaria in which the sponges were held. In addition, a 10 gallon aquarium containing 15 sponge individuals was filled with seawater and stood for 48 hours with aeration before filtration. Bacterioplankton from the reef water and aquaria was concentrated on 0.22 μm Stervix filters after GF/A (Whatman, England) prefiltration. Nucleic acids were extracted from Stervix filters as previously described (Somerville *et al.*, 1989; Massana *et al.*, 1997). rRNA was

extracted from 0.4 g of sediment using the hot phenol bead beating technique (Stahl *et al.*, 1988; see methods in chapter 2).

PCR amplification and *in vitro* transcription of 23S rRNA Genes

The 23S rRNA genes of the two variants of *C. symbiosum* were amplified from fosmid DNA (fosmids 101G10 and 60A5) using the PCR primers L-O-g1-215-a-S-25 (AGAGAAATCAATAGAGATTTCCCAA) and a L-S-Csym-2733-a-A-23 (AGATGCTTTCAGCACTTAGCCTA). The conditions of the PCR were as follows: an initial denaturation at 92°C for 3 minutes, followed by 30 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes in a PE 2400 Thermocycler (Perkin Elmer). The amplified product was then ligated into the PCR vector 2.1 (Invitrogen) and transformed into *E. coli* strain INVaF' as previously described (chapter 1). Clones containing the correct insert size were identified after restriction enzyme digestion with EcoRI by gel electrophoresis. Clones with the correct orientation of the 23S gene and the T7 promoter were identified by PCR using either M13F or M13R and L-Csym-2734-a-A-23 as the primers. The plasmid containing the insert in the correct orientation was then amplified using the M13 forward and reverse primers. Amplified products of several reactions were pooled, treated with 0.1 mg/mL proteinase K for 1 hour at 37°C, phenol:chloroform extracted, and precipitated with ethanol. The 2.5 kb LSU amplicon was *in vitro* transcribed as described by the AmpliScribe™ T7 Transcription Kit (Epicentre Technologies). The transcript was then washed with DEPC treated water in a microconcentrator (Centricon 100, Amicon). The RNA concentration was determined spectrophotometrically. The RNA

transcripts were used to experimentally determine the Td (temperature at which 50% of the probe bound dissociates) of each 23S rRNA oligonucleotide probe and as controls in the rRNA hybridization studies.

rRNA hybridization using oligonucleotide probes

Two oligonucleotide probes, each specific for a different strain, were designed to target the 23S rRNA molecule. The sequences of the 23S rRNA for the two *C. symbiosum* variants were obtained from fosmid clones 101G10 (variant A) and 60A5 (variant B) and aligned to other known crenarchaeotal 23 S rRNA gene sequences. The variant specific probes differ at 3 nucleotide positions (L-St-CsymA-283-a-A-19; variant A: ACACTTCAACTATTTCCTG and L-St-CsymB-283-a-A-19; variant B ACACTTTGACTATTTCGTG). In addition, a third oligonucleotide probe, L-O-g1-264-a-A-17 (23S gI probe: CAGATTCAGTTTGGGCT), was designed to target the 23S rRNA of marine group 1 crenarchaea. Probes were labeled with [γ-^{32}P]ATP (Dupont/NEN) at the 5' end using polynucleotide kinase as previously described (Stahl *et al.*, 1988; chapter 1).

To experimentally determine the Td of the oligonucleotide probes, 200 ng of the LSU RNA transcripts of the two variants were denatured (see below), blotted onto nylon membranes (Hybond-N, Amersham) in duplicate, immobilized by UV crosslinking (Stratalinker, Stratagene), and hybridized to the *C. symbiosum* variant probes or the GI marine archaeal probe. Membranes were hybridized overnight at 45°C in hybridization buffer (0.9 M NaCl, 50 mM Na_2HPO_4, 5 mM EDTA, 0.5% SDS, 10X Denhart's solution, 0.5 mg/ml polyadenosine; Massana *et al.* 1997). The blots were then washed for 30

minutes at room temperature in 100 mL of wash buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris and 1% SDS; Massana *et al.*, 1997), then cut into individual slots, and washed in 500 µl of wash buffer for 30 minutes at increasing temperatures (Zheng *et al.*, 1996). The radioactivity in the wash buffer and on the membrane was determined using liquid scintillation counting. For the variant specific probes the following temperatures (°C) were used: 29, 34, 37, 40, 43, 46, 49, 51, 54, 62, 70, and 78 and for the 23S gI probe: 29, 34, 37, 42, 43, 45, 46°C, 48, 49, 51, 54, 62, and 70. The Td was calculated as the point at which 50% of the total cpm was washed from the membrane.

The specificity of the 23S oligonucleotide probes and the presence of the two *C. symbiosum* variants in naturally occurring populations of *A. mexicana* were determined as follows. Nucleic acids from archaea, eubacteria, eucarya, marine sponges, seawater, and sediment, and RNA transcripts were denatured using 2% gluteraldehyde in 20 mM sodium phosphate pH 7.2. Fosmid DNA controls containing the rRNA operon of the *C. symbiosum* variants or a planktonic crenarchaeon were denatured by boiling for 10 minutes and then placed on ice. Between 50 ng and 300 ng of the denatured nucleic acids were applied and UV crosslinked to nylon membranes before hybridization to the radioactively-labeled probes. Three replicate blots were prepared, and each was hybridized to a different oligonucleotide probe. The probes were hybridized overnight at 45°C, and then washed at room temperature for 30 minutes. A high stringency wash was then performed at 5°C below the experimentally determined Td for 30 minutes. Blots were air dried and exposed to x-ray film. The binding of each probe to its target rRNA was determined by

autoradiography and the total cpm quantified by radioanalytic gas proportional counting system (Scanalytics). The variant specific probes were normalized to the 23S gI probe to correct for differences in their binding efficiencies. The correction factor was determined by dividing the probe signal of 23S gI probe binding to the control rRNA transcript by the probe signal of the variant specific probe binding to the same rRNA transcript. The normalized variant specific probe signals were used to calculate the ratio of variant A to variant B in each sponge.

Quantitative rRNA hybridization with domain specific probes (S-*-Univ-1392-a-A-15, S-D-Arch-0915-a-A-20, S-D-Bact-a-A-18, and S-D-Euk-1209-a-A-16) was also performed on nucleic acids from seawater, sediment samples, and non-*A. mexicana* species from Monterey as previously described (Massana *et al.*, 1997; see methods in chapter 1).

Phylogenetic analysis of 16S rRNA genes recovered from A. mexicana collected in Monterey

To compare the 16S rRNA genes recovered from *A. mexicana* from Monterey and the Santa Barbara Channel, as well as those of other Archaea, bootstrap neighbor joining analyses (Kimura two parameter model for nucleotide substitution, 1000 bootstrap iterations) were performed (643 nucleotide residues, positions 111 to 844, *E. coli* numbering). The rRNA gene sequences were obtained as previously described (see chapter 2). Reference sequences, as well as sequence editing and phylogenetic analysis software were obtained via anonymous ftp from the Ribosomal RNA Database Project (Maidak *et al.*, 1997). In addition, the overall similarity of the 16S rRNA archaeal genes

(region 34-895, *E. coli* numbering), without correction, was also determined for the sponge-associated archaeal and several planktonic archaea.

Results

Determination of the Td and Specificity of the 23S rRNA Oligonucleotide Probes

The Td of the 23S rRNA oligonucleotide probes as determined by the elution method (Zheng *et al.*, 1996) using RNA transcripts (Poltz and Cavanaugh, 1997), was 41°C for the 23S gI probe and 46.5°C for the variant A and B probes (figure 3-1). The high stringency wash was thus performed at 36°C for 23S gI probe and 41.5°C for the variant A and B probes. The specificity of the 23S rRNA probes was shown by hybridizing the variant probes to rRNA from cultivated archaea, eubacteria, and eucarya. Probe specificity was further evaluated by hybridization to chromosomal contigs containing the rRNA operon of variant A, variant B, or a planktonic archaeon (figure 3-2). Fosmids C1H5, C4H1, C4H9, C7D4, C15A3, C20B5, 101G10, 60H6, 44F6, 69H2, and 69H4 hybridized to the variant A probe and fosmids C8B8, C17D6, and 60A5 hybridized to the variant B probe. These results confirmed analyses using RFLP analysis and rDNA sequencing which grouped the fosmids into the two major classes of variants (Schleper *et al.*, 1998). Thus, the variant A probe bound to the variant A-like, but not to the variant B-like chromosomal contigs and RNA transcripts of *C. symbiosum*. Likewise, the variant B probe bound only to variant B-like chromosomal contigs and RNA transcripts. Both variant probes bound to total nucleic acids from individuals of *A. mexicana* (figure 3-2, slots 5C and 5D). No cross hybridization of the variant specific probes to the 23S

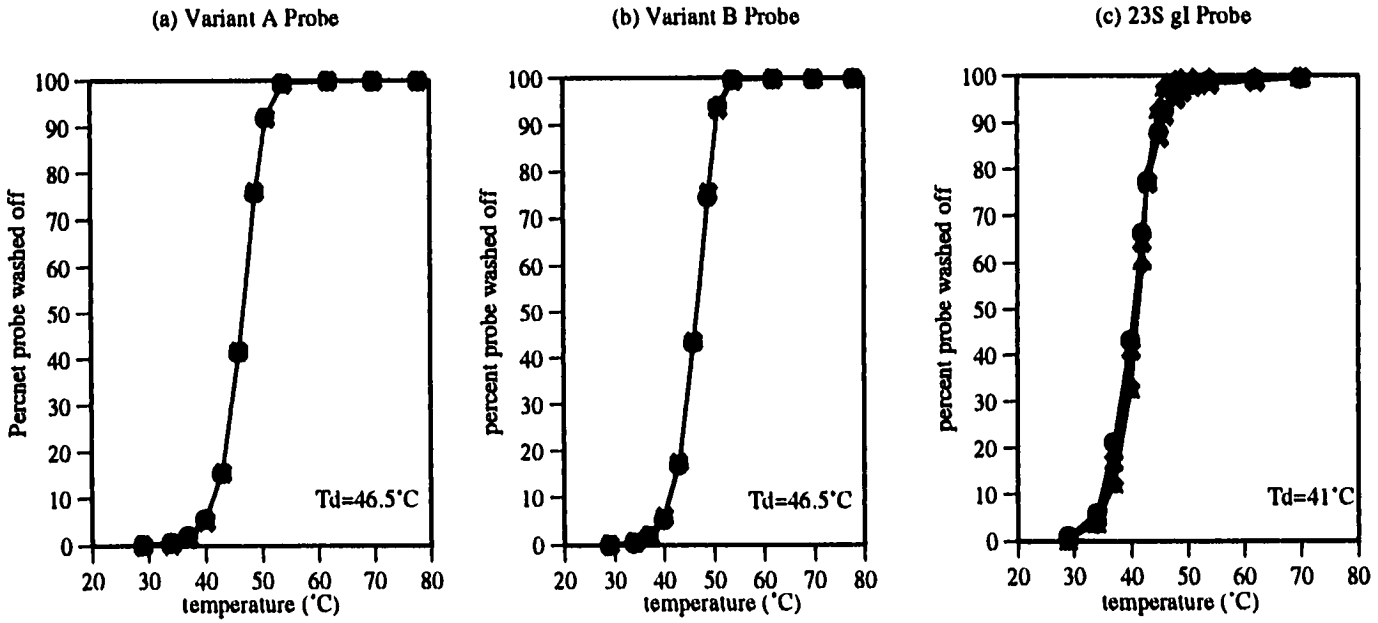

Figure 3-1: Td curves of the 23S rRNA variant (a and b) and g1 (c) specific oligonucleotide probes as determined by the elution method (Zheng *et al.*, 1996). In the Td study for the variant A and B probes, duplicate slots of rRNA transcripts from 101G10 and 60A5 were used, respectively. For the 23S gI probe, duplicate slots of each rRNA transcript were used. The Td for each of the probes was calculated at the temperature at which 50% of the probe was washed from the membrane.

Figure 3-2: Slot blot autoradiographs showing the specificity experiments of the 23S gl probe and the variant A and B probes. Columns 1 and 2 contain fosmids with rRNA operons of *C. symbiosium* (fosmids C1H5, C4H1, C4H9, C17D6, C8B8, C15A3, C7A6, C20B5, 101G10, 60H6, 44F6, 69H2, 69H4, and 60A5). 2G contains the rRNA operon of a planktonic crenarchaeon (fosmid 4B7; Stein *et al.*, 1996). Column 3 contains rRNA extracted from the following cultured Archaea: *Haloferax volcanii*, *Thermoplasma acidophilum*, *Methanococcus jannaschii*, *Desulfurococcus* strain SY, *Pyrococcus* strain GBD, and *Sulfolobus solfataricus*. 3G contains rRNA extracted from *Saccharomyces cerevisiae*. Column 4 contains rRNA extracted from the following cultured Eubacteria: *Shewanella* strain SC2A, *Pseudomonas nautica*, *Paracoccus denitrificans*, *Commamonas testosteroni*, *Bacillus megaterium*, *Rhodopseudomonas palustris*, *Syenechococcus* strain 6301. Column 5 contains rRNA transcripts of the two *C. symbiosium* variants (101G10 and 60A5), and nucleic acids extracted from *A. mexicana* (specimans HS4 and S28). Cells 2H-5H and 5E-5G contain no nuleic acids.

rDNA gene of a planktonic archaeon (Fosmid 4B7; Stein *et al.*, 1996) or to cultivated archaea, eucarya, or eubacteria was observed. The 23S gI oligonucleotide probe bound to all chromosomal contigs and rRNA transcripts from *C. symbiosum*, to the crude nucleic acids from *A. mexicana*, and to the chromosomal contig 4B7 (Stein *et al.*, 1996) containing the 23S rRNA gene of a planktonic archaeon. It did not bind to thermophilic crenarchaea, euryarchaeota, eucarya, or eubacteria used in this specificity study.

Distribution of the *C. symbiosum* variants

Sequence analysis of 590 bases at the 5' end of the 16S RNA genes of the two fosmids revealed two nucleotide positions (175 and 183.7, *E. coli* numbering, table 3-1 and 3-2) that distinguish the *C. symbiosum* variants. These signature sequences corresponded to ambiguities detected when PCR amplifications of the 16S archaeal rDNA genes from *A. mexicana* collected from the Santa Barbara Channel were directly sequenced (chapter 2). Additional sites of variation between the two 16S rRNA genes found in chromosomal libraries (Schleper *et al.*, 1998; Preston *et al.*, 1996) are shown in table 3-1. The two variable nucleotide positions located at the 5' end of the 16S rRNA gene, were used to determine the presence of the two archaeal variants in natural populations of *A. mexicana*. Variant A had a U at both signature positions and variant B had a C at both positions. The presence of both C and U at the signature positions indicated the presence of both variants. Of 21 *A. mexicana* individuals collected from the Santa Barbara Channel, 16 showed U/C (or Y) sequence ambiguities at the signature positions, thus indicating the presence of both strains. Five sponges (s4, s14, s26, s29, and hh1) yielded a U at the

Table 3-1. Variable nucleotide positions between variants of *C. symbiosum*.

Nucleotide Position[a]	Nucleotide[b]				
	101G10-variant A	60A5-variant B	ms1	ms2	ms4
26	A	A	T	T	---
175	T	C	C	C	C
183.7	T	C	T	C	T
185	C	C	T	C	Y
191.3	T	T	C	T	C
381	A	A	C	A	A
388	G	G	A	A	G
487	C	C	M	M	C
680	G	A	G	A	G
701	C	T	C	T	C
710	C	T	C	T	C
805	C	C	T	C	C
829	A	G	G	G	G
840.2	C	C	G	C	C
863	G	G	A	A	G
908	T	A	A	A	W
974	A	T	---	---	---
1044.31	C	T	---	---	---
1137	C	A	---	---	---

[a]Nucleotide positions numbered according to the 16S rRNA gene sequence of *E. coli*. Nucleotides without a corresponding position in *E. coli* are numbered using the nearest nucleotide in *E. coli* followed by a decimal number.

[b]W=A/T; --- = base at nucleotide position not determined. Nucleotide positions 6 to 1541 (*E. coli* numbering) compared for 101G10 and 60A5. For Monterey sponges, nucleotide positions for ms1, ms2 and ms4 used were 12 to 918, 20 to 950, and 34 to 950 (*E. coli* numbering), respectively.

Table 3-2 : Detection of *C. symbiosium* Variants in Natural Populations of *A. mexicana*

A. mexicana individual or isolated DNA source[a]	Variation in 16S rDNA at Positions[b]		Detection of Variants with 23S rRNA specific probes			
	175	183.7	Variant type A	Variant type B	Marine GI Archaea	ratio of variant A to B
101G10 RNA transcript	---	---	+	-	+	
60A5 RNA transcript	---	---	-	+	-	
fosmid 101G10 from s12	U	U	+	-	+	
fosmid 60A5 from s12	C	C	-	+	+	
s12	Y	Y	+	+	+	2.0
s1	---	---	+	+	+	1.9
s2	---	---	+	+	+	0.7
s3	Y	Y	+	+	+	1.5
s4	U	U	+	w	+	14.6
s5	Y	Y	---	---	---	---
s6	Y	Y	+	+	+	1.4
s7	---	---	+	w	+	5.7
s8	Y	Y	+	+	+	1.4
s9	Y	Y	+	w	+	5.3
s10	---	---	+	+	+	1.3
s11	Y	Y	+	+	+	1.3
s13	---	---	+	+	+	2.3
s14	U	U	-	w	+	B only
s16	---	---	+	+	+	1.6
s17	Y	Y	-	w	w	B only
s18	Y	Y	-	w	w	B only
s19	---	---	+	+	+	1.4
s20	---	---	+	+	+	1.1
s21	---	---	+	+	+	1.3
s22	---	---	+	+	+	3.6
s23	---	---	+	+	+	1.6
s24	---	---	+	+	+	0.4

Table 3-2 (continued)

s25	---	---	+	+	+	8.1
s26	U	U	+	+	+	10.4
s27	---	---	+	+	+	2.0
s28	---	---	+	+	+	2.7
s29	U	U	+	-	+	A only
s30	---	---	+	+	+	3.1
hs1	---	---	+	+	+	0.3
hs2	---	---	+	+	+	17.3
hs3	Y	Y	+	w	+	14.2
hs4	Y	Y	+	w	+	12.6
hs5	Y	Y	+	+	+	5.9
hh1	U	U	w	w	+	1.5
hh2	Y	Y	+	+	+	0.4
hh3	Y	Y	+	+	+	0.2
ms1	---	---	w	w	w	0.8
ms2	---	---	-	+	w	B only
ms3	---	---	+	+	+	2.0
ms4	---	---	+	-	+	A only

[a]Sponges collected at Naples Reef (s) and Haskel Reef (hs) in the Santa Barbara Channel, Hermit Hole (hh), N. Santa Cruz Island, and Monterey (ms), CA;

[b]Y= direct sequence of PCR product reveals C and U at the same position; --- = experiments not performed;

+ , w and - indicate positive, weak positive, and no binding of the 23S rRNA oligonucleotide probe to its target sequence as determined by autoradiography

signature position suggesting dominance of variant A. In no sequencing reaction was a C detected at both signature positions.

In a second approach, oligonucleotide probes specific for the 23S rRNA of either variant A or B were used to specifically detect the two major *C. symbiosum* variants in the total rRNA extracted from naturally occurring populations of *A. mexicana* from the Santa Barbara Channel. All sponge individuals from three different locations contained archaeal rRNA and at least one of the variant types (table 3-1). For 32 of the 36 sponges assayed from Santa Barbara, detectable signals were observed after hybridization with both the A and B variant probes, indicating the presence of both *C. symbiosum* variants. In three individuals (s14, s17, & s18), a hybridization signal was detected from only the variant B probe. The ratios of the normalized hybridization signal of variant A to variant B ranged from 0.2 to 17.3. In the majority of *A. mexicana*, variant A appeared to be the dominant strain (table 3-2).

Individuals of *A. mexicana* maintained in flowing seawater tanks from one to four years hybridized to both rRNA variant probes (table 3-3). *A. mexicana* individual hs1 collected 10/94 and maintained in an aquarium for 16 months harbored both symbiont strains throughout the time series experiment. Two additional sponges, aq1 and aq2, both held for less than a year in an aquarium, had ambiguities at the signature positions for the variants also indicating the presence of both variants (data not shown). Aq1 was sampled again four years later, and bound both the 23S rRNA variant specific probes (table 3-3). These results indicated that both symbiont strains still inhabited

Table 3-3: Detection of *C. symbiosium* Variants in *A. mexicana* held in flowing seawater tanks

A. mexicana individual or isolated DNA source*	Detection of Variants with 23S rRNA specific probes		
	Variant type A	Variant type B	Marine GI Archaea
Aq1 (>4 years)	+	+	+
Aq3 (1 year)	+	+	+
Aq4 (2 years)	+	+	+
Aq5 (2 years)	+	+	+
Aq6(2 years)	+	+	+
hs1 (field)	+	+	+
Aq-hs1 (3 months)	+	+	+
Aq-hs1 (5 months)	+	+	+
Aq-hs1 (8 months)	+	+	+
Aq-hs1 (1 year)	+	+	+
Aq-hs1 (16 months)	+	+	+
flowing seawater 3-10-95	-	-	-
flowing seawater 10-2-97	-	-	w
flowing seawater 6-10-98	-	-	+
Aquarium-seawater 6-12-98	-	-	-

Prefix Aq represents sponges held in aquaria.
* time in parentheses indicates the length of *A. mexicana* was maintained in captivity

sponges maintained in aquaria, and thus, the association involving both variants can be maintained in the laboratory.

Quantitative 16S rRNA hybridization studies indicated the presence of archaeal rRNA in seawater supplied to aquarium tanks, seawater surrounding captive sponges, and in seawater and sediments located at Naples Reef where the sponges were collected (table 3-4). Despite the detection of archaeal 16S rRNA and the hybridization of the g1 LSU probe to several of the samples, no probe signal was observed when the variant specific 23S rRNA gene probes were hybridized to nucleic acids extracted from seawater supplied to the tanks, seawater surrounding captive *A. mexicana* (table 3-3), or in seawater or sediments collected at depth at Naples Reef (table 3-5).

The presence of the variants of *C. symbiosum* in *A. mexicana* collected off the coast of Monterey, CA, was also investigated. A hybridization signal was detected between the 23S GI and both the variant probes to nucleic acids from ms1 and ms3, indicating the presence of both variants (table 3-2). In the other two individuals assayed, a detectable hybridization signal was observed with only one of the variant probes (table 3-2). The phylogenetic analysis of PCR amplified, directly sequenced archaeal ssu rDNA from *A. mexicana* from Monterey and the cloned 16S rDNA genes from *C. symbiosum* from the Santa Barbara Channel (643 nucleotide positions: figure 3-3) using the neighbor joining method with bootstrap analysis, consistently grouped the Monterey and Santa Barbara Channel archaeal sequences to the exclusion of the planktonic crenarchaea at a high bootstrap confidence level (100%). Unrestricted similarity analysis also showed that the archaeal 16S rRNA genes of *A. mexicana* collected

Table 3-4. Relative percentage of Eucarya, Eubacteria, and Archaea in seawater, sediments, and non-Axinellid sponges collected from Monterey as determined by 16S rRNA hybridization using domain-specific oligonucleotide probes.

rRNA source	Relative % of 16S rRNA using Domain-specific probes[a]			Amplification of Archaeal rDNA[b]
	Eucarya	Bacteria	Archaea	
Naples Seawater 4-22-97	10.5 (12.7)	80.9 (98.1)	8.6 (10.4)	---
Naples Seawater 9-22-97	3.6 (3.2)	94.9 (85.0)	1.5 (1.3)	---
Naples Sediment 4-22-97	50.5 (50.5)	47.7 (47.5)	2.0 (2.0)	---
flowing seawater (6-10-98)	11.6 (12.8)	75.6 (83.8)	12.8 (14.2)	---
aquarium seawater (6-12-98)	5.8 (6.9)	92.5 (110.4)	1.7 (2.0)	---
Unidentified sponge (m3)	97.5 (87.8)	1.8 (1.7)	ND (0)	-
Unidentified sponge (m4)	97.5 (75.2)	2.37 (1.8)	ND (0)	-
Unidentified sponge (cs3)	96.9 (116.8)	2.6 (3.1)	ND (0)	+
Unidentified sponge (cs5)	94.8 (106.2)	3.7 (4.1)	1.5 (1.7)	+

[a]Values outside parentheses represent the percentage of each normalized domain-specific signal/ sum of the normalized domain-specific signal and the values in parentheses indicate the percentage of the normalized domain-specific hybridization signal/Univ1392 hybridization signal. ND = not detected.

[b]Positive (+) or No (-) amplification of the predicted size (approximately 950 bp) using archaeal specific primers for the 16S rRNA gene. ---experiments not performed

Table 3-5: Detection of *C. symbiosum* variants in seawater, sediments, and other species of sponge

rRNA source	Detection of Variants with 23S rRNA Specific Probes		
	Variant type A	Variant type B	Marine GI
Naples Seawater 4-22-97	-	-	+
Naples Seawater 5-31-97	-	-	+
Naples Seawater 9-22-97	-	-	w
Naples Sediment 4-22-97	-	-	w
Naples-*Cliona celata*	-	-	-
Naples-*Tethya aurantia*	-	-	-
Monterey-*Microciona* sp.	-	-	-
Monterey-*cs3*	-	+	+
Monterey-*cs5*	-	+	+

+, w, and - indicate positive, weak and no binding of the 23S rRNA oligonucleotide probe to its target sequence as determined by autoradiography.

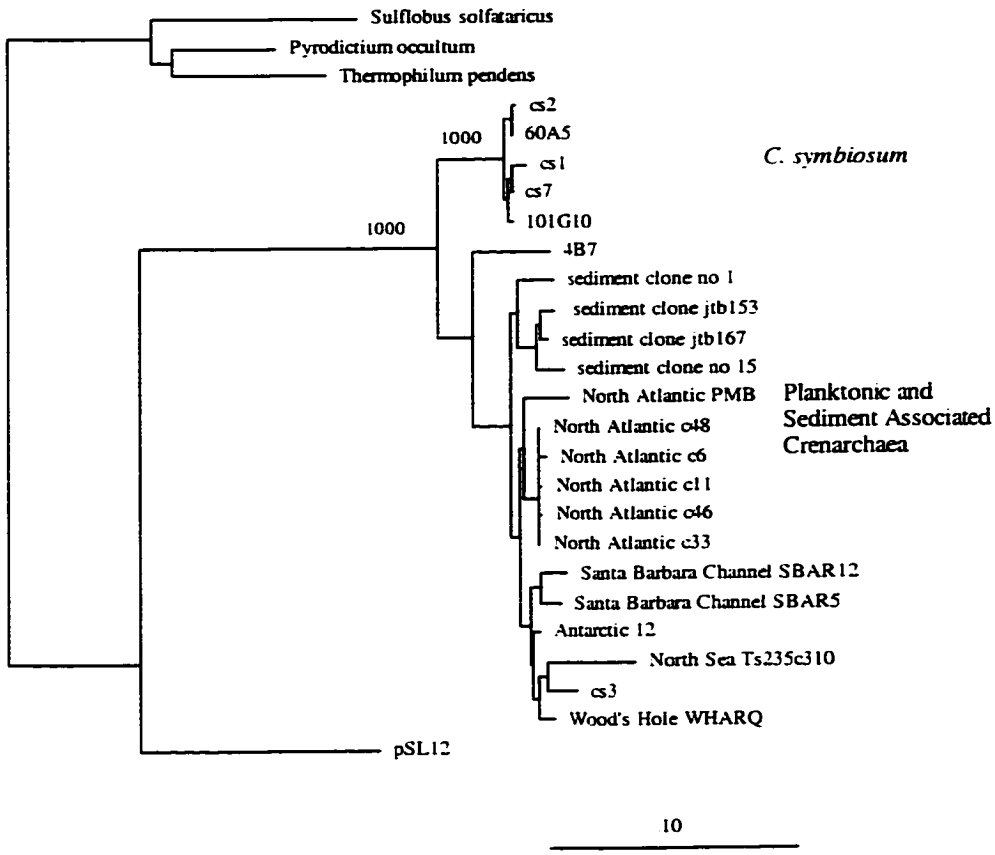

Figure 3-3. Phylogenetic affiliation of the archaea found in sponges collected from Monterey, CA. Archaeal 16S rRNA genes were amplified from *A. mexicana* (specimens ms1, ms2, and ms4) and an unknown sponge (cs3) and directly sequenced. The composite ssu RNA gene sequences (111 to 844, *E. coli* numbering, 643 nucleotide positions) were compared to the *C. symbiosum* variants detected in *A. mexicana* (101G10 and 60A5) from the Santa Barbara Channel and other planktonic and sediment-associated marine crenarchaea using the neighbor joining method (Kimura two parameter model for nucleotide substitution with bootstrap analysis 1000 iterations). Only bootstrap values at the nodes that separate the marine planktonic and sediment crenarchaea from *C. symbiosum*, and the two *C. symbiosum* variants from the Santa Barbara Channel are shown.

from Monterey were most similar to the *C. symbiosum* variants (> 98.6%, table 3-6). However, additional variation of the 16S rRNA gene sequence was detected in the sponges collected from Monterey when compared to archaea recovered from sponges collected in the Santa Barbara Channel (table 3-1) indicating the possibility of Monterey-specific strains.

Nucleic acids from other species of sponges, *Tethya aurantia* and *Cliona celata* collected from Naples Reef and *Microciona* sp. collected from Monterey, CA, did not contain archaea as determined previously by hybridization with archaeal specific 16S rRNA oligonucleotide hybridization (chapter 1, table 3-4). In addition, the total sponge nucleic acids did not bind the g1 23S rRNA oligonucleotide probes or either of the variant probes, indicating the absence of g1 archaeal and more specifically *C. symbiosum* rRNA (table 3-4). Sponge individuals cs3 and cs5 from Monterey, however, bound the 16S archaeal, 23S marine g1 and the variant B 23S rRNA oligonucleotide probes (table 3-4 & 3-5). Sequence analysis of the archaeal 16S rRNA gene sequence (region 13 to 895, *E. coli* numbering) from individual cs3 indicated that the archaea were more closely related to uncultivated planktonic archaea than to *C. symbiosum* (figure 3-3; table 3-6).

Discussion

The identification of highly-related rRNA variants of *Cenarchaeum symbiosum* in chromosomal libraries led to the investigation of whether these strains co-exist in naturally occurring populations of *Axinella mexicana*. *A. mexicana* inhabits the Eastern Coastline of the Pacific Ocean from Baja California, Mexico to Monterey, CA (Sim and Bakas, 1986; C. Diaz, personal

Table 3-6. Similarity matrix comparing the archaeal 16S rRNA genes from sponges collected from Monterey compared to those found in the Santa Barbara Channel.

	ms1	ms2	ms4	101G10	60A5	cs3
ms1						
ms2	98.3					
ms4	99.2	98.5				
101G10	98.9	98.6	99.3			
60A5	98.6	99.4	99.4	99.2		
cs3	91.2	91.5	91.2	91.5	91.5	
4b7	92.3	92.5	92.5	93.2	93.0	92.3
Ts235c310	89.0	89.1	89.1	89.8	89.6	95.0
sbar5	90.8	90.9	90.9	91.6	91.4	96.1
sbar12	90.8	90.8	90.8	91.6	91.4	96.0
WHARQ	91.2	91.6	91.6	92.0	92.0	97.5

Line delineates the sponge-associated and planktonic archaeal 16S rRNA gene sequences. Archaeal rRNA genes from *A. mexicana* collected from Monterey, CA (prefix ms) and an unknown species of sponge (prefix cs) collected at the same site. 101G10 and 60A5 represent the two archaeal 16S rRNA genes of *C. symbiosum* from the Santa Barbara Channel. 4B7, TS235C310, sbar5, sbar12, and WHARQ are crenarchaeal 16S rRNA phylotypes recovered from seawater (van der Maarel *et al.*, 1998; DeLong, 1992; Stein *et al.*, 1996). The region compared corresponds to nucleotide positons 34 to 895 in the 16S rRNA gene of *E. coli*.

communication). In this study, variation within symbiont populations of *A. mexicana* from Santa Barbara, the Channel Islands, and Monterey were investigated. Previously, 16S rRNA oligonucleotide probes specific for Archaea were used to estimate the relative abundance of the archaeal symbionts within their host. However, these probes target not only *C. symbiosum*, but also marine planktonic archaea commonly found in seawater. Thus, 23S rRNA oligonucleotide probes and signature positions in the 16S rRNA gene specific for each of the *C. symbiosum* strains were used to detect their presence within their host and other environments.

When the oligonucleotide probes specific to the 23S rRNA of marine archaea and the *C. symbiosum* variants were designed, only three 23S rRNA gene sequences were available, two of which represented the archaeal variants from *A. mexicana* and the other a planktonic crenarchaeon (fosmid 4B7; Stein *et al.*, 1996). The hybridization of the variant B probe to the unknown sponge (specimens cs3 and cs5), in spite of the fact that the archaeal 16S rRNA gene sequence of cs3 was more similar to planktonic archaea, suggests that the target sequence is not unique to *C. symbiosum*. However, no additional archaeal 16S rRNA phylotypes were detected in *A. mexicana* (chapter 2), thus the positive hybridization of the variant-specific probes to the nucleic acids from the sponge indicates the presence of one or both of the *C. symbiosum* variants. No probe signal was detected when the variant specific probes were hybridized to nucleic acids from seawater or sediment. Thus, archaeal 23S rRNA genes with these two target sequences, which includes the *C. symbiosum* variants of *A. mexicana*, were not detectable in these samples.

rRNA hybridization using 23S variant-specific oligonucleotide probes and direct sequencing of the archaeal 16S rRNA genes from sponges collected in the Santa Barbara Channel, did not always give identical results in the determination of which variants were present in a specimen. Discrepancies between the rRNA gene sequence and the hybridization data could be due to the ratio of the variants in the sponge or to a greater sensitivity of PCR when the archaeal rRNA hybridization signal was low (sponges s17 and s18). rRNA from variant A in sponges s4 and s26 was a much greater than the rRNA of variant B. Because both strains are amplified and sequenced collectively, having an overabundance of one variant may obscure the detection of the rarer variant. The use 16S rRNA gene sequence and rRNA hybridization studies that target LSU rRNA of the variants, each provided an independent assay of the diversity of the archaeal population within *A. mexicana*. The combined results of 16S rRNA sequence and 23S hybridization studies indicated the presence of both symbiont strains in all but one (s29, table 3-2) individual of *A. mexicana*. This could suggest that the two strains can exist independently, and that the association between *A. mexicana* and *C. symbiosum* involves either one or both of the symbiont strains. In the majority of *A. mexicana* investigated (table 5-1) and in chromosomal libraries constructed from sponges s12 and s23 (Schleper *et al.*, 1998; Preston *et al.*, 1996), variant A dominated over variant B. In some symbiont systems, the distribution of which symbiont inhabits its host at a particular location was determined by the physiological limitations of the symbiont (Secord, 1995; Rowan and Knowlton, 1995; Trench, 1997). The presence or dominance of one *C. symbiosum* strains could not be attributed to

the date or location of collection indicating that there was no geographical gradient or temporal control which determined which variant dominated (table 3-2).

The archaeal 16S rRNA gene sequences recovered from *A. mexicana* collected from Monterey, CA were most similar to *C. symbiosum* recovered from Santa Barbara Channel sponges. However, the archaeal rRNA gene sequences within sponges collected from the coast of Monterey differed from those collected in the Santa Barbara Channel, suggesting the presence of additional strains of *C. symbiosum*. The detection of ambiguities in the 16S rRNA gene sequence and the hybridization of both of the 23S rRNA variant-specific probes indicates that there is also microheterogenity in the symbiont population within a single specimen.

The data presented here could indicate that *C. symbiosum* may not have a free-living state. Alternatively, its abundance in seawater could be below the limits of detection with the methods used. To date, *C. symbiosum* has not been detected in seawater samples collected from Naples Reef, in studies investigating the archaeal diversity, utilizing cloning and sequencing as well as DGGE analysis of 16S rRNA genes, from the surface to 150 m at the Santa Barbara Mooring site, or in coastal seawater from Anvers Island, Antarctica even though planktonic archaea are abundant (Massana *et al.*, 1997; Murray, Ph. D. dissertation). The presence of the *C. symbiosum* in seawater collected from Monterey, CA, has yet to be investigated. However, *C. symbiosum* phylotype (variant A) was detected in Antarctic sponges (appendix 1), which could suggest

that the archaeon may be involved in symbiotic associations with sponges other than *A. mexicana.*

The archaeal population of *A. mexicana* was shown to maintain a constant rRNA level during a time course study (chapter 3) and observed to be actively dividing (chapter 2 and 4). The presence of a constant and active archaeal population and evidence that the symbiont strains were not being introduced or expelled from the host, suggests that the sponge may be digesting the symbionts. In addition, the detection of both strains throughout the time course study also indicates that the association involving both strains can be kept in captivity.

The persistent co-occurance of both rRNA variants shows that the two strains are stably maintained within *A. mexicana* collected from the Santa Barbara Channel. Genetic theory suggests that for highly-related cellular lineages to co-occur, they must exhibit ecological differences (Palys *et al.*, 1997). Highly-related sympatric strains even those with indistinguishable rRNA gene sequences, can vary at protein loci and form separate similarity clusters, each distinct cluster representing a separate ecological population (Palys *et al.*, 1997). Clusters could be ecologically identified, for example, by host range, virulence or symbiotic factors, distribution, or physiology (Palys *et al.*, 1997; Field *et al.*, 1997; Moore *et al.*, 1998). Although the physiological differences of the *C. symbiosum* variants is unknown, evidence from chromosomal libraries showed sequence differences between the two variants in protein coding regions (Schleper *et al.*, 1998). Competition experiments involving the initiation and maintenance of different symbiotic strains, showed that native strains could

outcompete other symbiotically-competent strains (McFall-Ngai and Ruby, 1998; Provasoli *et al.*, 1969). In cases where multiple symbiont types were stably maintained in a single host, the symbionts were either spatially separated (Unterman *et al.*, 1989; Fenchel and Ramsing, 1992) or physiologically distinct (Distel *et al.*, 1995), indicating that they were not competing for the same resources.

Likewise the co-occurring strains of *C. symbiosum* could occupy different regions of the host or differ physiologically such that the two are metabolically interdependent. Binding of both the 23S rRNA variant probes to nucleic acids extracted from cross sections of *A. mexicana* did not indicate vertical stratification of the symbiont strains (data not shown). Sponges have different cell types, but the cells are not organized into tissues (Bergquist, 1978). The cross sections of sponge analyzed in this hybridization study probably included all cell types. The determination of the distribution of the variants is beyond the resolution of rRNA hybridization studies, if each strain was associated with a particular sponge cell type. This would require the localization of the variant strains via *in situ* hybridization using probes that specifically target each strain.

To ensure that the symbiosis persists in subsequent generations of the host, the transmission of the symbiont strains probably involves a population of cells that maintains the level of archaeal diversity currently observed in *A. mexicana*. The mode of transmission and the distribution of the *C. symbiosum* strains within the sponge host is currently unknown. Investigations of symbiont transmission and distribution would certainly be interesting in light of the

detection of microheterogenity of the archaeal symbiont population of *Axinella mexicana*.

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CHAPTER 4

In situ localization of *Cenarchaeum symbiosum* in its sponge host, *Axinella mexicana*, utilizing polynucleotide probes that target 16S and 23S rRNA.

Introduction

Prokaryotes inhabit all extant marine *Porifera* (Wilkinson, 1984). The distribution of prokaryotes throughout the cellular matrix of different species of sponge varies, but generally sponges with a well developed canal system and low matrix density possess few bacteria while those with high matrix density harbor large bacterial populations (Vacelet and Donadey, 1977). Electron microscopic studies have shown that prokaryotes are mainly dispersed intercellularly throughout the mesohyl, often in contact with collagen fibrils, and between choanocyte chambers and archaeocytes (Vacelet and Donadey, 1977; Bertrand and Vacelet, 1971; Vacelet, 1975; Wilkinson 1978; Unson and Faulkner, 1993; Vacelet *et al.*, 1996). Bacteria have also been found intracellularly within specialized cells (bacteriocytes or cyanocytes) and vacuoles (Vacelet and Donadey, 1977; Wilkinson, 1978; Bigliardi *et al.*, 1993), and extracellularly within the canal system (Burlando *et al.*, 1988). Only a few electron microscopic studies have been able to positively identify and determine the distribution of specific members of the microbial population within sponges (Wilkinson, 1978; Vacelet *et al.*, 1996). This is mainly due to the lack of distinguishing morphological characteristics among the majority of prokaryotes.

Phylogenetically based oligonucleotide probes complimentary to regions within the rRNA and labeled with fluorescent molecules offer a means to identify morphologically indistinct prokaryotic microorganisms in their natural

environment. The specificity of oligonucleotide probes from universal to strain specific is determined by the region of the rRNA that the probe compliments. *In situ* hybridization using rRNA oligonucleotide probes has been successfully used to localize symbionts within host tissue (Amann *et al.*, 1991; Distel *et al.*, 1991; Springer *et al.*, 1992; Lloyd *et al.*, 1996; Dyal *et al.*, 1995; Embley *et al.*, 1992; Cary *et al.*, 1997), to reveal the spatial organization of prokaryotic cells in biofilms and activated-sludge (Amann *et al.*, 1992, Manz *et al.*, 1993, Wagner *et al.*, 1993; Wagner *et al.*, 1994), to identify uncultivated prokaryotes (chapter 2; Murray *et al.*, 1998), and to determine the distribution and abundance of microorganisms (Murray *et al.*, 1998; Spring *et al.*, 1992). However, many hybridization studies reported that less than 50% of the total prokaryotic cells were detected using oligonucleotide probes labeled with a single fluorochrome (Hahn *et al.*, 1992; Hicks *et al.*, 1992; Lee and Kemp, 1994; Spring *et al.*, 1998; DeLong *et al.*, 1989). Poor permeability or low growth rate of the cell could cause the fluorescence signal to be below the limit of detection (DeLong *et al.*, 1989). Alternatively, the probe's target sequence may not be present in a subset of the population. Different approaches to increase probe-derived signal include use of multiple oligonucleotide probes with similar specificity that target different regions of the rRNA (Lee *et al.*, 1993; Amann *et al.*, 1990), probes with multiple fluors (Amann *et al.*, 1990; DeLong, 1989; Zarda *et al.*, 1991; Ludwig *et al.*, 1994; Trebesius *et al.*, 1994), and indirect detection techniques (DeLong, 1990; Zarda *et al.*, 1990). However, oligonucleotide probes with multiple fluors were found to increase background fluorescence, but not the fluorescence signal (Wallner *et al.*, 1993; Lee *et al.*, 1993). In contrast, multiple

oligonucleotide probes used simultaneously did increase the fluorescence signal, but the number of sites in the rRNA that target the same group organisms limits the increase to two to three fold (Trebesius *et al.*, 1994). Indirect detection methods have also been successful, but the method involves large enzyme/substrate complexes, and thus additional permeablization steps must be performed to allow entry of the secondary molecule into cells. Polynucleotide probes (up to 300 nucleotides in length) differ from oligonucleotide probes (approximately 20 nucleotides in length) because they span larger regions of the target molecule. Thus, polynucleotide probes can incorporate a larger number of fluors (Trebesius *et al.*, 1994; Ludwig *et al.*, 1994). The longer rRNA probes do not have the resolution of strain-specific oligonucleotide probes. However, they were useful in the detection of specific groups under well-defined hybridization conditions (Ludwig *et al.*, 1994), and have been successful when oligonucleotide probes have failed (Spring *et al.*, 1998).

Multiple 16S rRNA oligonucleotide probes that target marine "group 1" archaea, were successfully used to determine the morphology and cellular abundance of the archaeal symbiont, *Cenarchaeum symbiosum* in disassociated sponge (chapter 2; Preston *et al.*, 1996). However, because of the low signal intensity of the oligonucleotide probes and the high background of sponge matrix, the location of the archaeal symbiont within its sponge host, *Axinella mexicana*, could not be readily determined in the intact sponge. Thus, in order to boost signal, RNA polynucleotide probes with multiple fluor residues were used in this study in combination with confocal scanning laser microscopy (CSLM) to investigate the location and distribution of *C. symbiosum* within its

host. Polynucleotide probes complementary to the rRNA of the two *C. symbiosum* variants and to the mixed bacterial population within the sponge were synthesized using *in vitro* transcription. The RNA polynucleotide probes span nearly the entire length of the 16S and 23S rRNA genes, and thus incorporated a greater number of target sites and fluor molecules than the four singly-labeled oligonucleotide probes used in initial studies (chapter 2; Preston *et al.*, 1996). The presence of highly conserved regions within the rRNA among disparate taxa requires that the specificity of the polynucleotide probes be rigorously tested prior to their use in CSLM studies.

Methods

Design of rRNA Polynucleotide Probes

Polynucleotide probes were designed to target the rRNA of either archaeal or bacterial populations found within *A. mexicana*. In order to isolate the rRNA operons, primer pairs S-D-Arch-21-a-S-20 and RNAOPT7GI or S-D-Bact-8-a-S-20 and RNAOPT7EUB (Table 4-1) were used to amplify the 16S/23S gene operons of the *C. symbiosum* variants contained in fosmids 60A5 and 101G10 or bacterial 16/23S rRNA gene operons from purified nucleic acids of *Axinella mexicana*, respectively. PCR reactions contained 1X low salt buffer (Stratagene), 0.025 units/μL TAQ plus (Stratagene), 200 μM dATP, dTTP, dCTP, and dGTP (Promega), 0.150 μM of each primer (table 4-1), and 0.1 ng/μl of template DNA. The conditions of PCR were an initial denaturation at 92°C for 3 minutes, 30 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3.5 minutes, and a final extension at 72°C for 5 minutes (Perkin Elmer 2400). PCR reactions were pooled, precipitated, and used as templates in *in*

Table 4-1. List of PCR primers used to amplify the 16S-23S rRNA genes for *in vitro* transcription.

PCR Primer[a]	Sequence (rDNA primer in bold)	rDNA primer Reference
S-D-Arch-21-a-S-20	**TTCCGGTTGATCCYGCCGGA**	DeLong, 1992
RNAOPT7GI (T7 promoter + L-O-Cenar-2610-a-A-18)	GCCAGTGAATTGTAATACGACTCACTATAGGG**CGCTTCCATCAGG CAGAG**	this study
S-D-Bact-8-a-S-20	**AGAGTTTGATCCTGGCTCAG**	Lane, 1991
RNAOPT7EUB (T7 promoter + L-D-Bact-1933-a-A-19)	GCCAGTGAATTGTAATACGACTCACTATAGGG**ACCCGACAAGGAA TTTCGC**	Amann *et al.*, 1995

[a] primers named using the nomenclature of the Oligonucleotide Probe Database (Alm *et al.*, 1996)

vitro T7 transcription reactions.

Fluorescein-labeled nucleotides were directly incorporated into RNA transcripts. One-hundred microliter transcription reactions contained 1X Ampliscribe buffer (Epicenter), 30 µL PCR amplified rDNA operons from either *C. symbiosum* or bacteria from the sponge, 500 µM ATP and GTP, 260 µM CTP and UTP, 140 µm fluorescein-12-CTP (DuPont/NEN) and fluorescein-12-UTP (Boehringer-Manheim), 10 mM DTT, and 1X T7 Ampliscribe enzyme solution (Epicenter). Transcripts were also labeled with N6 Aminohexyl ATP (Gibco, BRL) for later incorporation of Texas Red. A 10µL transcription reaction contained 6 mM UTP, CTP, GTP, and ATP, 4 mM N6 aminohexyl ATP, 1X ampliscribe buffer, 10mM DTT, 5 µl amplified bacterial DNA and 1X T7 Ampliscribe enzyme solution. All transcription reactions were incubated at 37°C for 2 hours and were subsequently washed with DEPC treated water in a Microcon 100 (Amicon).

The FITC and N6 aminohexyl labeled transcripts were then hydrolyzed to approximately 100 nucleotide fragments by adding 1/10 volume of 30 mM $MgCl_2$ and incubating for 15 minutes at 94°C (modification of Chee *et al.*, 1996). The reaction was terminated with the addition of 0.5M EDTA. The size of the fragments was determined by comparison to known RNA standards after agarose gel electrophoresis. Hydrolyzed transcripts containing N6 aminohexyl ATP were subsequently labeled with Texas Red. The hydrolyzed transcript was incubated in 250 mM $NaHCO_3$, pH 8.2 and 2.5 mg/ml Texas Red-X, succimimidyl ester (Molecular Probes) in dimethylformamide (DMF), for 2 hours at room temperature. Unincorporated dye was removed by washing with

DEPC treated water in a Microcon 100. The fluorescently labeled hydrolyzed probes were then used in *in situ* hybridization studies (see below).

Cell fixation

To prepare sponges for *in situ* hybridization, sections of sponge were fixed overnight with 3.7% formaldehyde in Calcium-Magnesium free artificial seawater (CMF-ASW; 460 mM NaCl, 11 mM KCl, 7 mM Na_2SO_4, 2 mM $NaHCO_3$) at 4°C, rinsed once in CMF-ASW, and stored at -20°C in CMF-ASW: ethanol (1:1 vol:vol) until hybridization. Sponge cells were prepared three different ways. A section of sponge was mechanically homogenized in CMF-ASW or 1X PBS (145 mM NaCl, 100 mM Na_2HPO_4, pH 7.2), spotted onto gelatin coated [0.1% gelatin, 0.01% $KCr(SO_4)_2$] teflon slides (Cel-Line Associates), air dried, and then serially dehydrated in ethanol (50, 75, and 100% for 2 minutes each). One millimeter cubed blocks of sponge were cut with razor blades, rinsed in hybridization buffer (see below), and directly used in hybridization studies. Lastly, a block of sponge (1 cm^3) was dehydrated in an alcohol series and embedded in paraffin wax (Johansen, 1940). Ten micron longitudinal sections were cut on a sliding microtome and mounted onto glass slides. Sections were de-paraffinized in xylene for 15 minutes, and subsequently washed in 100% and 95% ethanol for 10 minutes each prior to hybridization with the fluorescently-labeled probes.

In addition to the sponge, cultivated bacteria and archaea were also used in *in situ* hybridizations as negative controls in the specificity tests. *Vibrio (Photobacterium) angustum* (ATCC 33977) was grown to log phase in 2216 marine broth (Difco). A *Thermococcales* isolate (Kingdom Euryarchaeota) from

a high temperature petroleum reservoir (V. Orphan, unpublished data) was grown overnight to 48 hours at 80°C in 2216 marine broth: artificial seawater (1:1 vol:vol) with the addition of 5g/L S° (Jannasch *et al.*, 1995). Both cultures were fixed overnight by the addition of formalin (final concentration 3.7%), removed from formalin, and stored at -20°C in 4% NaCl:ethanol (1:1 vol:vol). Mixtures of dissociated sponge and either the cultivated archaea or bacteria were then used to test the specificity of the polynucleotide probes.

Specificity Tests and In Situ Hybridization Conditions

The optimal condition for each polynucleotide probe was determined experimentally using dissociated sponge tissue by altering the formamide concentration and temperature of the hybridization and wash buffers. All *in situ* hybridizations were performed in 5X SET buffer (0.75 M NaCl, 5 mM EDTA, and 0.1 M Tris-HCl pH 7.8), 0.1% dextran sulfate, 0.1% SDS, 0.5 mg/ml polyadenosine, and either 50% formamide at 55°C or 60% formamide at 60°C. The polynucleotide probes were added at a final concentration of 0.091 μM. Slides were sealed in a humid chamber containing hybridization buffer and hybridized overnight. Post-hybridization washes were conducted at 45, 50, or 55°C in 0.2X SET containing 50% formamide for 10 minutes, and followed by a room temperature wash in 0.2X SET. Cells were then stained with 1 μg/mL DAPI for 5 minutes at room temperature, dipped in nanopure water, air dried and mounted in Citifluor AF1 (Citifluor) before viewing using epifluorescence microscopy (Zeiss Axioskop). Color photographs were taken using Kodak 400 ASA or Fuji Sensia 400 ASA film. The exposure times were either 60 or 120 seconds for the FITC and Texas Red stained cells and 15 or 30 seconds for

DAPI stained cells. Slides were scanned (Nikon Scanner), and all color images were prepared as dye-sublimation prints.

To determine if the polynucleotide probes bind to bacteria and other archaea, dissociated sponge cells were mixed with either *V. angustum* or the *Thermococcales* isolate or prior to spotting onto gelatin coated slides. Slides were subsequently hybridized overnight at 55°C to the sponge bacterial or *C. symbiosum* polynucleotide probe in hybridization buffer (5X SET, 0.1% dextran sulfate, 0.1% SDS, 0.5 mg/ml polyadenosine, and 50% formamide). Cells hybridized to the bacterial polynucleotide probe were washed with 0.2X SET and 50% formamide at 45°C and 55°C. Those hybridized to the *C. symbiosum* polynucleotide probe were washed in the same buffer at only 55°C. In addition to the above experiments, dissociated sponge cells from two unidentified sponges from Monterey, CA were also used in *in situ* hybridization studies.

Pretreatment of RNAse

Cells were treated with RNAse to show that the polynucleotide probes were binding to rRNA and not to rDNA. Dissociated sponge cells spotted onto gelatin coated slides were rehydrated in 2X SSC (0.3 M NaCl, 30 mM trisodium citrate, pH 7.0) and then incubated in 10 µg/mL RNAse A (Sigma) at 37°C for 30 minutes. The cells were then washed in 2X SSC before treatment with 1% di-ethyl-pyrocarbonate (DEPC, Sigma) in 1X PBS for 30 minutes at 37°C (Bauman and Detvelzen, 1988; Bertin *et al.*, 1990). Slides were subsequently rinsed in 1X PBS, ethanol dehydrated, and air dried before hybridization with the polynucleotide probes as described above.

Staining for Confocal Microscopy

The three dimensional distribution of the archaea within the sponge with respect to the bacteria flora and individual sponge cells was determined by using the *C. symbiosum* polynucleotide probe in conjunction with one of the following: the bacterial polynucleotide probe, propidium iodide, or eucaryal-specific oligonucleotide probes. Either blocks or de-paraffinized sections of sponge were used. A $1mm^3$ block of sponge was placed in 10 μL of hybridization buffer containing the *C. symbiosum* and bacterial polynucleotide probe and washed using conditions described above. In a second set of experiments, only the FITC labeled *C. symbiosum* polynucleotide probe was used in the hybridization, and then the block of sponge was counterstained with 2.5 μg/ml of propidium iodide. Lastly, de-paraffinized sections were first hybridized to the FITC labeled *C. symbiosum* polynucleotide probe, washed, and subsequently hybridized to a suite of three Texas Red-labeled oligonucleotide probes that target Eucarya (S-D-Euca-0309-a-A-17, S-D-Euca-0502-a-A-16, S-D-Euca-1209-a-A-16; Amann *et al.* 1990; Lim *et al.* 1993). Hybridization conditions for counterstaining with Eucarya-specific oligonucleotide probes were as follows: the FITC stained sections were incubated overnight at 40°C in hybridization buffer containing 5X SET buffer, 0.1% SDS, 0.5 mg/ml polyadenosine and 1.9 μM of each oligonucleotide probe. After the hybridization buffer containing either the polynucleotide or oligonucleotide probe was added to the de-paraffinized sections, a coverslip was placed over the sections to prevent dehydration. Sections were then washed at 37°C and at room temperature in 1X SET for ten minutes each. Slides were then dipped in nanopure water and air dried. Blocks and sections of sponge were mounted in

Citifluor AF1 and viewed using a confocal microscope (Diaphot 200, Nikon). For CSLM, each channel in the z series was captured sequentially with a Biorad MRC 1024 with Lasersharp software.

The percentage of prokaryotes that hybridized to each of the probes was determined for dissociated sponge cell preparations. In addition, the number of archaea per volume of sponge was determined from confocal images of de-paraffinized sponge sections hybridized to both the Eucaryal oligonucleotide probes and *C. symbiosum* polynucleotide probe. The area of each optical section was determined using NIH Image (Version 1.61, by Wayne Rasband). The depth of each section was 0.7μm. Total *C. symbiosum* counts were determined for five z series images representing 90 optical sections. Consecutive Z series images were viewed simultaneously to insure that the *C. symbiosum* cells were counted only once through the depth series.

Results

The archaeal symbiont could not be identified or localized in TEM micrographs in either enrichment preparations of *C. symbiosum* or in thin sections of sponge (Vacelet, personal communication). Thus, a different approach was used to localize the archaeal symbionts within the host. PCR-amplified rRNA operons were used in transcription reactions to generate rRNA probes that target the 16S and 23S rRNA of either the bacteria or archaea found within the sponge, *A. mexicana*. The *C. symbiosum* polynucleotide probe was a mixture of the 16S and 23S rRNA operons of both variant A and variant B (Schleper *et al.*, 1998). The bacterial polynucleotide probe was a mixed

population probe complimentary to the 16S and 23S rRNA genes of the bacterial assemblage present in a single specimen of *A. mexicana*.

The conditions of specificity of the rRNA polynucleotide probes required to distinguish the different prokaryotic populations within *A. mexicana* were determined experimentally by altering the temperature and concentration of formamide in the hybridization and wash buffers. In hybridization studies using the bacterial and *C. symbiosum* polynucleotide probes simultaneously, each probe bound to different prokaryotic cell populations indicating that there was no cross hybridization of the bacterial rRNA polynucleotide probe to the archaea, or vice versa, in any of the hybridization and wash conditions tested (figure 4-1). The staining pattern that was previously observed for the archaeal cells using four "group 1"-specific oligonucleotide probes (see chapter 2) was also seen when the *C. symbiosum* polynucleotide probe was used. The *C. symbiosum* polynucleotide probe bound to opposite cellular poles of the target cell leaving an unstained center region which corresponded to a region that bound DAPI. At lower stringency washes, sponge cells stained yellow (panel A, figure 4-1) indicating that both the bacterial and *C. symbiosum* polynucleotide probes were binding nonspecifically. By increasing the wash temperature to 55°C, the background binding was minimized and both prokaryote populations remained distinguishable. However, the signal intensity of the bacterial rRNA polynucleotide probe decreased with the increase in specificity (figure 4-1, see also figure 4-3). Thus, to lower the background binding of the probes, subsequent experiments containing both polynucleotide probes were washed in 0.2X SET and 50% formamide at 55°C.

Figure 4-1 Double exposure photographs show the result of increasing stringency conditions on the specificity of the C. symbiosum and bacterial polynucleotide probes. Dissociated sponge cells were simultaneously hybridized to the Texas Red-labeled bacterial and FITC-labeled *C. symbiosum* polynuclotide probes at 55°C in hybridization buffer containing 50% formamide and washed with 0.2 X SET in 50% formamide at 45°C (A), 50°C (C) or 55°C (E) or at 60 °C in hybridization buffer containing 60% formamide and washed at 55°C in 50% formamide (D) . The corresponding DAPI stained field is also shown in the adjacent photograph (B, D, F and H, respectively). Scale bar = 10μm

The specificity of the rRNA targeted stain for *C. symbiosum* was tested by mixing cultivated archaea or bacteria with dissociated sponge tissue, hybridizing with one of polynucleotide probes, and counterstaining with DAPI. The morphology of the *Thermococcales* isolate (figure 4-2A arrow) and *V. angustum* (figure 4-2B arrow) differ from *C. symbiosum* and thus could be distinguished under phase contrast or after staining with DAPI. Two different hybridization conditions, 50% formamide at 55°C or 60% formamide at 60°C were tested for the *C. symbiosum* polynucleotide probe using a mixture of dissociated sponge cells and the *Thermococcales* isolate. The *C. symbiosum* polynucleotide probe bound weakly to the cultivated archaea at low stringency (55°C and 50% formamide hybridization, see arrows in figure 4-2 C). Although some cross reactivity was observed, the signal intensity of the probe binding to *C. symbiosum* was much greater compared to its binding to the cultivated archaeon. At the higher hybridization stringency, no cross reactivity between the cultivated archaeon and the *C. symbiosum* polynucleotide probe was observed (data not shown). Dissociated sponge cells were also mixed with the bacterium, *V. angustum* (figure 4-2B) and hybridized at 55°C with 50% formamide to the *C. symbiosum* polynucleotide probe. No fluorescence signal was observed from *V. angustum* after hybridization with the *C. symbiosum* polynucleotide probe (figure 4-2D), which indicated that hybridization and wash conditions could distinguish archaeal cells from cultivated bacteria. When *V. angustum* and disassociated sponge cell mixtures were probed with the sponge bacterial polynucleotide probe, the bacteria were fluorescent (figure 4-3 C & D). The result indicated that the negative result obtained when *V. angustum* was

Figure 4-2 Specificity of *C. symbiosum* polynucleotide probe to the archaeal symbiont in the presence of cultivated archaea and bacteria. Disassociated sponge cells spiked with either the *Thermococcales* isolate (A &C) or *V. angustum* (B &D) were hybridized to FITC labeled *C. symbiosum* polynucleotide probe. Arrows point to the location of either the *Thermococcales* isolate or *V. angustum* in the DAPI (A & B) and FITC (C & D) stained fields. Scale bar = 10μm

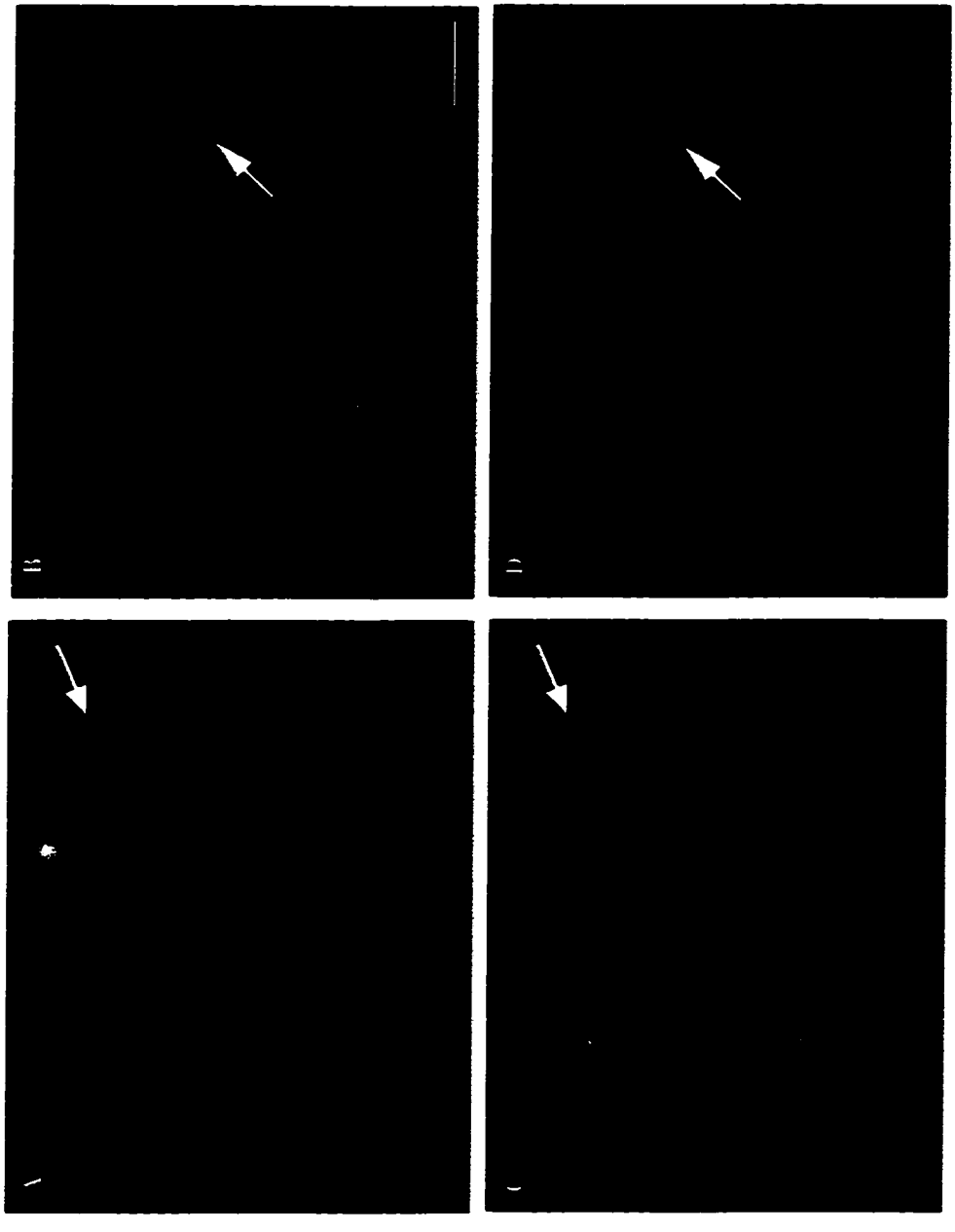
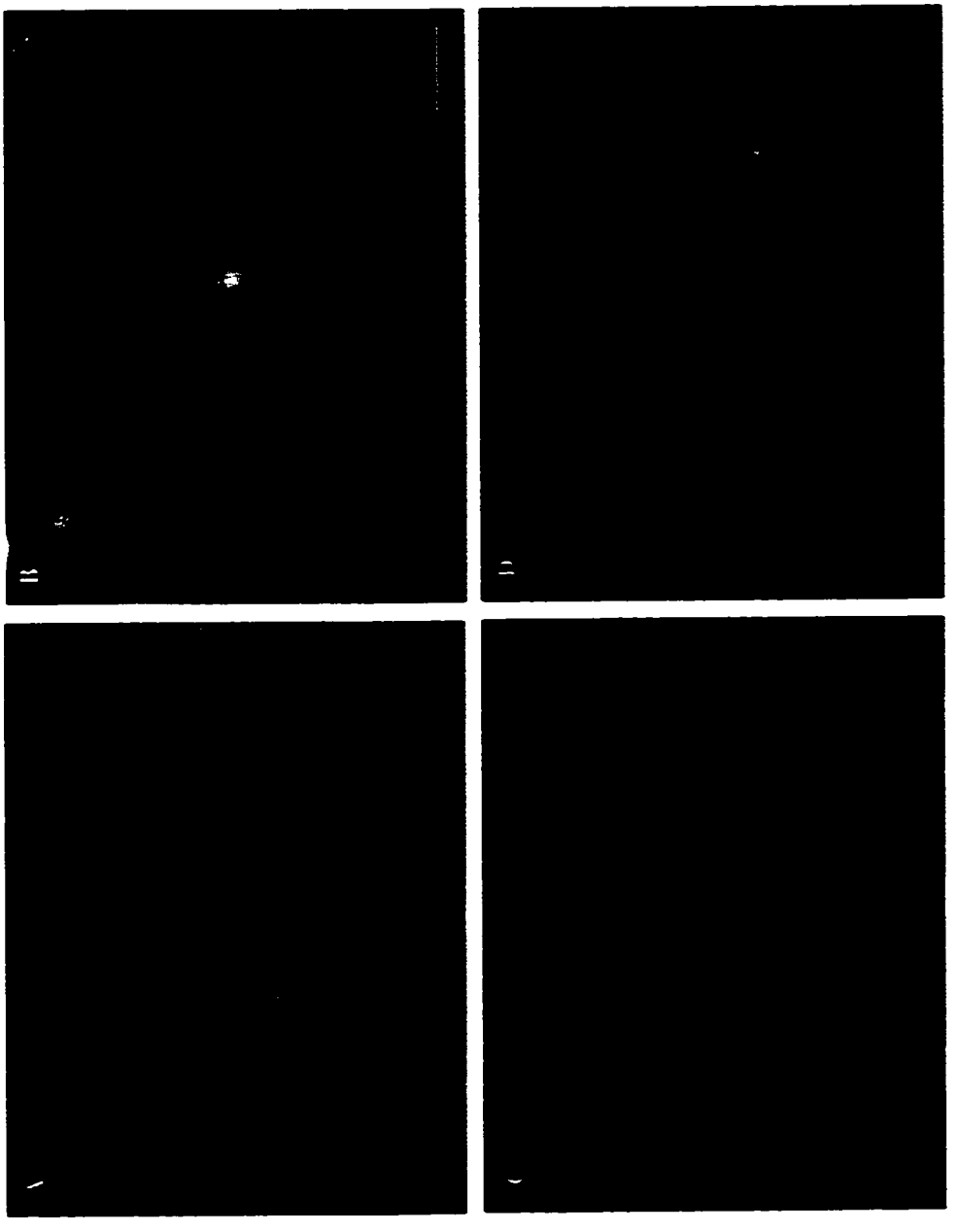


Figure 4-3: Specificity of the bacterial polynucleotide probe. Dissociated sponge tissue was spiked with *V. angustum*, hybridized to the bacterial polynucleotide probe, washed at either 45°C or 55°C, and counterstained with DAPI. The *V. angustum* were visible in the DAPI stained field (A and B) and after staining with the FITC labeled bacterial polynucleotide probe at both stringenies [45°C (A) and 55°C (B)]. Scale bar = 10μm



probed with *C. symbiosum* polynucleotide probe, was due to sequence specificity and not due to poor permeability of the bacterial cells. No *C. symbiosum* cells were visibly fluorescent, as determined by their staining pattern (chapter 2), when the sponge cells and *V. angustum* mixture was hybridized to the sponge bacterial polynucleotide probe (figure 4-3C & D). These experiments show that at 55°C the hybridization buffer containing 50% formamide could distinguish *C. symbiosum* cells from cultivated euryarchaea and bacteria.

When the dissociated sponge cells were treated with RNAse prior to hybridization with either the *C. symbiosum* or sponge bacterial polynucleotide probe, no probe signal was observed (figure 4-4A & B). Untreated cells were fluorescent when probed with either the *C. symbiosum* (figure 4-4C) or sponge bacterial (figure 4-4D) polynucleotide probe. The result indicated that the probes were binding to rRNA and not to rDNA or other cellular molecules. Similar studies by Bertin *et al.* (1989) and Bauman and Bentvelzen (1988) also indicated that their rRNA-targeted polynucleotide probes bound to RNA.

For optimal staining and specificity, hybridizations using *C. symbiosum* and bacterial rRNA polynucleotide probes were performed at 55°C in 50% formamide, followed by a post-hybridization wash at 55°C containing 50% formamide in hybridization studies. When the sponge bacterial rRNA polynucleotide probe was used alone, the hybridization conditions were the same, but the post hybridization wash was reduced to 45°C. The initial study to determine the conditions for specificity also indicated that the target cells were

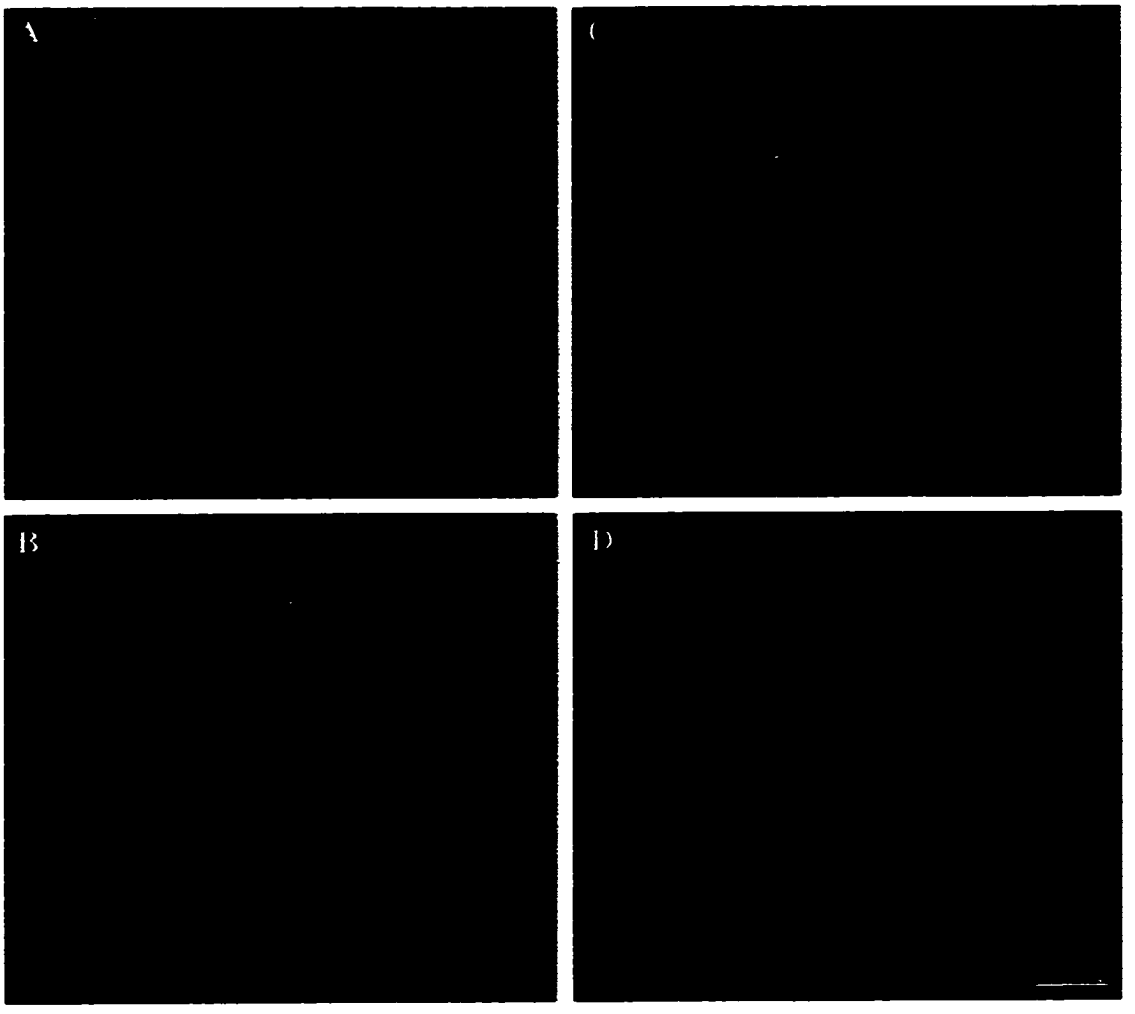


Figure 4-4: Effect of RNAse on probe-derived fluorescence signal. Disassociated sponge cells were treated with RNAse (A and B) or not (C and D) and then probed with either the *C. symbiosum* (A and C) or sponge bacterial (B and D) polynucleotide probe. Positive hybridization was only observed on untreated slides. Scale bar = 10μm

permeable to the hydrolyzed polynucleotide probes with normal fixation methods.

Once the correct hybridization and wash conditions for specificity were determined the polynucleotide probes were then used to determine the percentage of archaeal and bacterial cells out of the total prokaryotic cells. Prokaryotic cell counts were performed on dissociated sponge cells simultaneously hybridized with the *C. symbiosum* and bacterial polynucleotide probes. An average of 86.9 ±5.4% of the prokaryotic cells of *A. mexicana* were visible with one of the polynucleotide probes (Table 4-2). *C. symbiosum* accounted for between 45 to 68% of the prokaryotic cells in *A. mexicana* collected from both Santa Barbara and Monterey, CA. Between 16.7 to 28.2% of the *C. symbiosum* had a morphology indicative of cell division (chapter 2). Thirteen to 44.9% of the prokaryotic cells in *A. mexicana* were bacterial.

Less than 50% of the prokaryotic cells were detectable with the bacterial and *C. symbiosum* polynucleotide probes in dissociated cells from the other species of sponge investigated (Table 4-2). This could indicate a lower rRNA content in the prokaryotes of these species. Alternatively, part of the prokaryotic population within the other species of sponge could be distantly related to the prokaryotic population of *A. mexicana* such that the amount of probe binding was below detection. The relative intensity of probe signal of the bacterial population between different individuals of *A. mexicana* also varied, indicating the possibility of a variable bacterial population, either in the concentration of rRNA per cell or species composition. Restriction fragment length polymorphism analysis of the bacterial 16S rRNA genes from different

Table 4-2: Percentage of fluorescent cells after simultaneously probing with the *C. symbiosum* and sponge bacterial polynucleotide probes.

Sponge species[a]	Specimen No.	% fluorescent cells using the *C. symbiosum* probe	% dividing[b]	% fluorescent cells using the Bacterial probe	% hybridized	total cells[c]
A. mexicana	s27	51.2	16.7	37.8	89.0	445
	s28	68.3	19.0	13.0	81.3	571
	s29	61.1	28.2	19.4	80.6	180
	s30	61.0	24.7	32.7	93.7	318
A. mexicana.	ms1	61.8	18.5	23.2	84.9	544
	ms2	47.5	23.6	44.9	92.4	526
	ms3	45.9	20.4	35.7	81.6	512
	ms4	63.4	16.7	28.0	91.6	509
unidentified	cs5	2.8	0	46.2	49.5	521
unidentified	m4	0.8	0	33.2	36.9	485

[a] *A. mexicana* individuals collected from either Santa Barbara (prefix s) or Monterey (prefix ms), CA The two unidentified sponges were collected in Monterey.

[b] Percentage of *C. symbiosum* with morphology indicative of cell division (chapter 2, Preston *et al.*, 1996)

[c] Total prokaryotic cells were determined by DAPI counts.

individuals of *A. mexicana* indicated that the bacterial population was variable (chapter 2). Archaea accounted for 2.8% and 0.8% of the prokaryotic cells in the two unidentified species of sponge (Table 4-2). None of the archaeal cells had a morphology indicative of cell division (chapter 2, Preston *et al.*, 1996).

Confocal studies also confirmed that the *C. symbiosum* and the bacterial polynucleotide probes bound to different prokaryotic cell populations within the sponge matrix (figure 4-5a). The FITC labeled archaeal symbionts formed tracks around unstained regions. The bacteria were not interspersed within the tracks of archaea. Similar patterns in the distribution of the archaeal cells were observed when the nuclei of the sponge cells were stained (Figure 4-5b). The archaeal symbionts appeared in tracks that both surround individual sponge cells and lie between adjacent sponge nuclei. An unstained region most likely delineating the cytoplasm of the sponge cell, separated the sponge's nucleus from the archaeal symbionts (figure 4-5b, arrow).

The use of FITC labeled *C. symbiosum* probe in conjunction with the Texas Red labeled eucaryotic oligonucleotide probes allowed the identification of the archaeal symbionts in relation to the cytoplasm of individual sponge cells. Figure 4-6 shows consecutive sections through one confocal depth series. Most archaeal cells occupied regions of the sections where no eucaryotic probe bound. This could indicate either the archaea are intracellular and exclude the Texas red labeled eucaryl probes from binding, or extracellular occupying the matrix of the sponge. As the depth of the z series increased, the biovolume of the sponge cells increased (see boxed region in f and the corresponding area in sections g through l, figure 4-6) and the skeletal matrix decreased. The archaea were not present

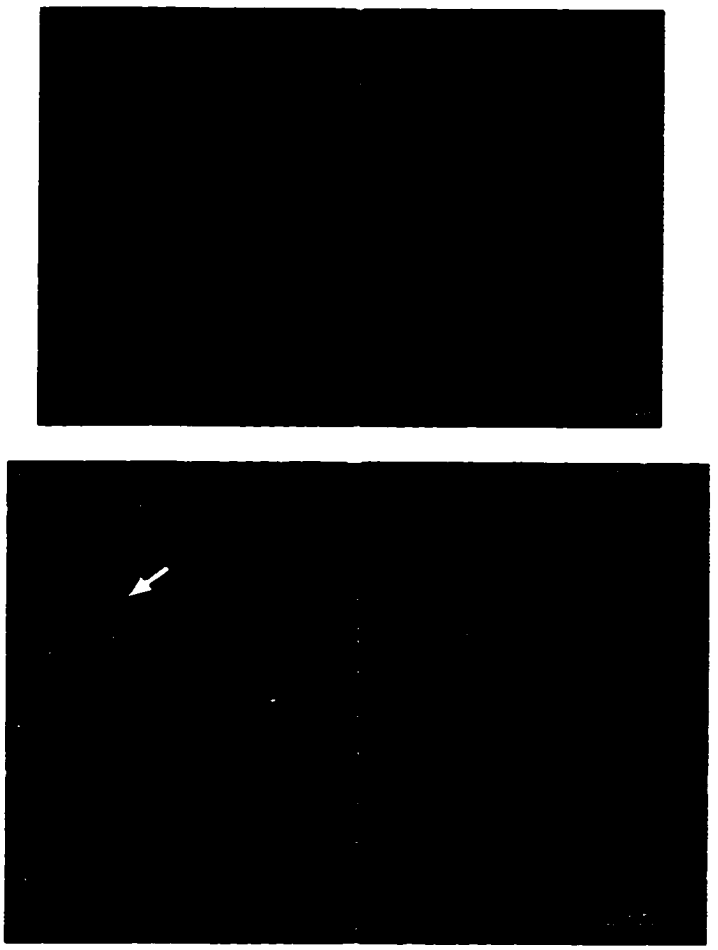
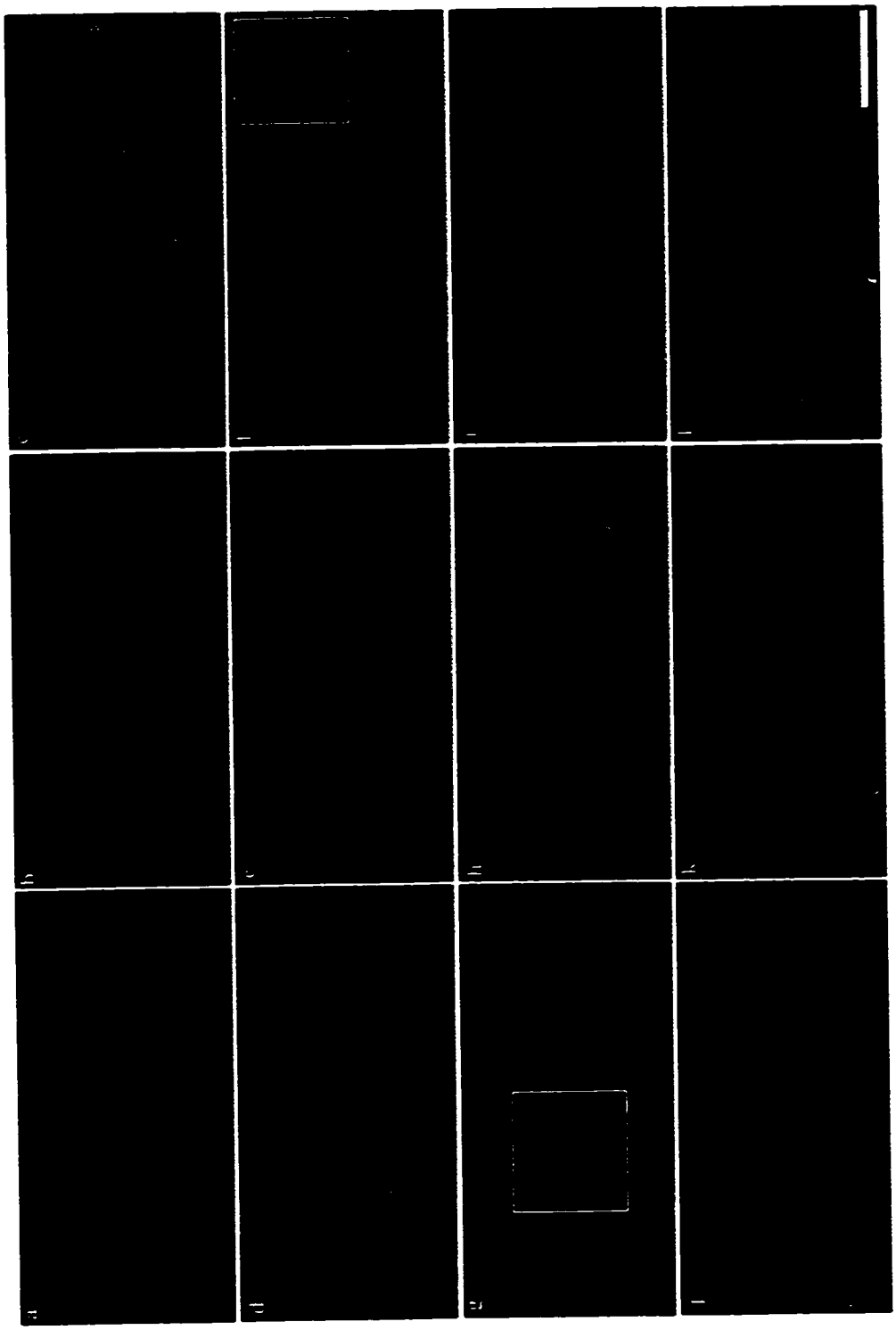


Figure 4-5 Stereo-pair micrographs showing the distribution of *C. symbiosum* within the sponge matrix. A 1 mm^3 block of sponge was either simultaneously hybridized with FITC labeled *C. symbiosum* and Texas Red labeled bacterial polynucleotide probes (a), or hybridized to the FITC labeled *C. symbiosum* polynucleotide probe and subsequently stained with 2.5 μg/ml of propidium iodide (b). The arrow in b points to the cytoplasm of a sponge cell. The stereo pairs represent 13 xy optical sections at 0.6 μm increments for A, and 21 xy sections at 0.5 μm increments for B. Scale bar = 5 μm.

Figure 4-6 Z series images through paraffin sections of sponge showing the distribution of the archaeal symbionts (green) in relation to individual sponge cells (red). De-paraffinized sections were stained with the FITC labeled *C. symbiosum* operon probes and subsequently stained with three Texas Red labeled oligonucleotide probes specific for Eucarya. Boxed regions in panel f and g and the corresponding area in other sections show regions where the boundary of individual sponge cells could be determined and show that at least most *C. symbiosum* cells occupied extracellular regions along the periphery of sponge cells. Panels a to i and i to l were taken at 1.0 μm and 0.5 μm increments, respectively. Scale bar = 5 μm



when the extracellular space was limited (panels i-l, figure 4-6), suggesting that the archaeal symbionts inhabit the skeletal matrix of the sponge. The boxes in panels f and g (figure 4-6) enclose regions where the cellular boundary of individual sponge cells could be determined and where archaeal cells were detected. The *C. symbiosum* polynucleotide probe bound to prokaryotic cells on the periphery of individual sponge cells, indicating an extracellular location of the archaeal symbiont. *C. symbiosum* cells were not distributed regularly throughout sections of *A. mexicana.* In regions where archaea were detected, their average density was $1.0 \times 10^7 \pm 7.9 \times 10^5$ cells/mm^3 of sponge. Approximately 8.3 ±2.9 % of the *C. symbiosum* had a morphology which indicated cell division (chapter 2, Preston *et al.*, 1996).

Discussion

Polynucleotide probes complimentary to the rRNA of the archaeal and bacterial populations found within *Axinella mexicana* were synthesized in order to determine the distribution of prokaryotes within the matrix of the sponge. The specificity and utility of polynucleotide probes depends on the composition and complexity of the system being examined, the region that the probes target, and the hybridization and post-hybridization conditions. Short polynucleotide probes up to 300 nucleotides designed to target variable regions of the rRNA molecule, were found to have group-level specificity under certain hybridization conditions (Trebesius *et al.*, 1994). Polynucleotide probes which targeted longer regions of the rRNA exhibited lower specificity mainly due to highly conserved regions in rRNA (Bertin *et al.*, 1990). Thus, the RNA targeted polynucleotide probes do not provide the resolution necessary for determining the spatial

distribution of very closely-related members of the microbial community. However, for group-level discrimination, polynucleotide probes can potentially be useful.

The association between *A. mexicana* and its microbial community includes representatives from all three domains of life. The archaeal population in the sponge consists of a single species, *C. symbiosum*, which is distantly related to the other inhabitants of the sponge (chapter 2, Schleper *et al.*, 1998; Preston *et al.*, 1996; Schleper *et al.*, 1997). Comparison of the rRNA polynucleotide sequence of *C. symbiosum* to that of other prokaryotes showed that the 16S/23S rRNA genes were less then 65% similar to the 16S/23S rRNA genes from bacteria and less than 75.2% similar to rRNA genes of cultivated archaea (table 4-3). In this study, I used polynucleotide probes that target nearly the entire small and large subunit rRNAs. The disadvantage of using rRNA targeted polynucleotide probes is the presence of highly conserved segments within these genes. Comparisons of 100 consecutive nucleotides from the 16S and 23S rRNA genes of *C. symbiosum* to those of other prokaryotes indicated that even in conserved regions of rRNA genes, the highest similarity to *C. symbiosum* did not exceed 80% for *E. coli* and 90% for either the cultivated archaea (figure 4-7). The majority of the forty-three consecutive seqments of 100 nucleotide from the rRNA genes of *C. symbiosum* were greater than 80% similar to the planktonic archaeon (32 out of 43; 74.4%). The percentage of consecutive nucleotide blocks of *C. symbiosum* with greater than 80% similarity to cultivated crenarchaea and euryarchaea was much lower (13.9% and 9.3%, respectively).

Table 4-3: Similarity of the rRNA polynucleotide of *C. symbiosum* to other prokaryotes[a]

	16S rDNA of *C. symbiosum*	23S rDNA of *C.symbiosum*
Planktonic "group 1" Crenarchaeon (4B7)	.932	.872
Sulfulobus shibatae	.738	---
S. acidocaldaris	.734	---
S. solfataricus	.735	.665
Thermofilum pendens	.746	.673
Desulfurococcus mobilis	.744	.666
Methanococcus vannielii	.714	.655
Methanobacterium thermoautotrophicum	.717	.639
Archaeoglobus fulgidus	.718	.654
Thermoplasma acidophilum	.713	.644
Thermococcus celer	.720	.668
Halobacterium halobium	.701	.622
Haloferax volcanii	.690	.619
Pyrococcus sp. [b]	.712	---
Photobacterium angustum	.604	---
Pseudomonas aeruginosa	.631	.585
Escherichia coli	.622	.584
Roseobacter denitrificans	.633	---
Oceanosprillum kriegii	.620	---

Full 16S and 23S rRNA gene sequences were obtained from the Ribosomal Database Project (Maidak *et al.*, 1994). Similarity values were calculated without correction.

[b] Comparison of 1292 nucleotides (18 to 1358 *E. coli* numbering) of *C. symbiosum* to the high temperature petroleum reservoir isolate (V. Orphan, unpublished data)

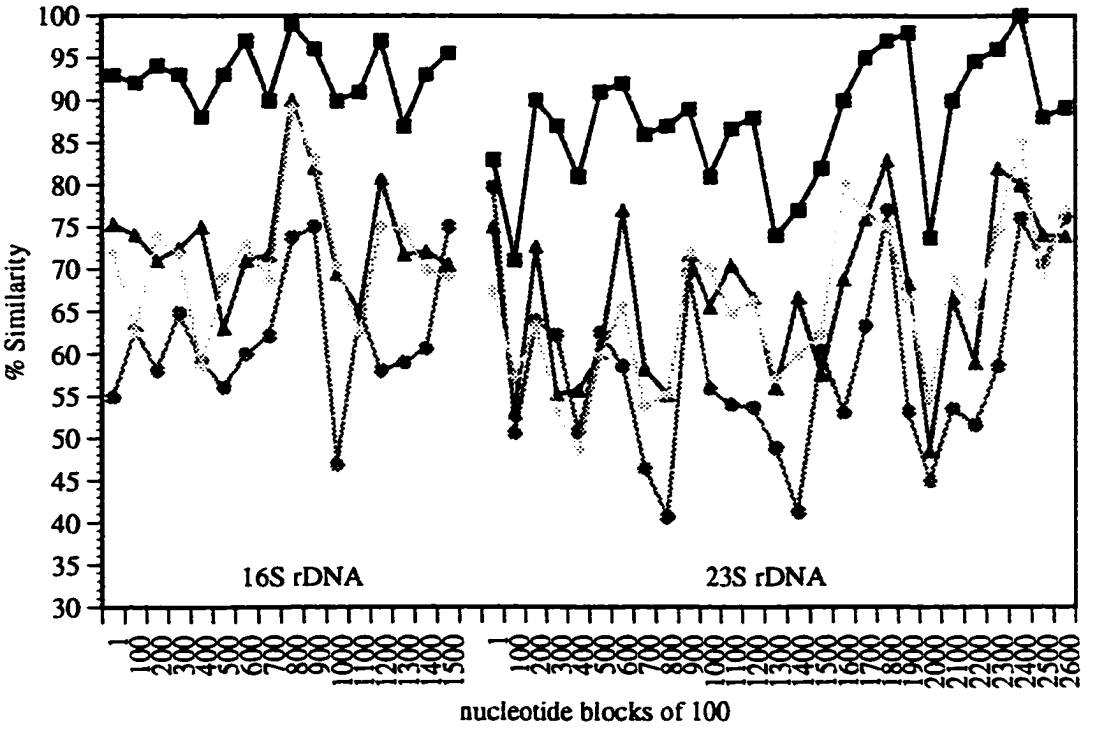


Figure 4-7 Percent similarity of seqments of 100 consecutive nucleotides through the 16S and 23S rRNA gene sequence of *C. symbiosum* (variant A) compared to the planktonic marine group I crenarchaeote clone 4B7 (squares, Stein *et al.*, 1996), *S. sulfataricus* (triangles), *A. fulgidus* (diamonds), and *E. coli* (circles). The 16S and 23S rRNA genes were obtained from the Ribosomal Database Project (Maidek *et al.*, 1994) and the unrestricted similarities were calculated.

The specificity of the polynucleotide probes was determined experimentally using dissociated sponge cells containing *C. symbiosum* and cultivated bacteria and archaea. Results of the specificity tests indicated that the *C. symbiosum* and sponge bacterial polynucleotide probes could distinguish the archaeal and bacterial populations, respectively, found within the sponge. Similarities of the 16S rRNA genes of the cultivated archaea and bacteria used in the specificity tests indicated that under the hybridization conditions, the probe could distinguish *C. symbiosum* from organisms that were 70% similar in 16S rRNA gene sequence (table 4-3).

The *C. symbiosum* polynucleotide probe do, bind to other species of planktonic marine crenarchaeota (DeLong and Taylor, unpublished data). When prokaryotes from seawater collected from Naples Reef were probed with the *C. symbiosum* polynucleotide probe, a portion of the prokaryotic cells were fluorescent (unpublished data) indicating the presence of archaea. However, *C. symbiosum* rRNA was not detected in these samples (chapter 3). The result is not surprising given the similarity of the rRNA gene of C. *symbiosum* and other marine g1 crenarchaea (82.6-92.3% similar in 16S rDNA gene sequence, also see planktonic clone 4B7 in table 4-3 and figure 4-7). Exhaustive surveys of chromosomal DNA libraries and rDNA analysis of different individuals of *A. mexicana* indicate that the only archaea present are strains of *C. symbiosum* (Preston *et al.*, 1996; Schleper *et al.*, 1998; chapter 2 & 3). Thus, the archaeal cells detected in *A. mexicana* with the *C. symbiosum* polynucleotide probe represent the archaeal symbionts (variants A and B), and not planktonic marine "group 1" crenarchaea.

The percentage of archaea in disassociated sponge material of captive sponges as determined using oligonucleotide probes (chapter 2) was similar to field collected sponges probed with the polynucleotide probe. However, the percentage of *C. symbiosum* observed dividing was much higher in the field collected sponges than those maintained in captivity. This difference suggests that although the *C. symbiosum/A. mexicana* association can be maintained in aquaria over long periods of time, the archaeal symbionts were less active than those in the field.

The majority of the archaeal symbionts were dispersed extracellularly throughout the mesohyl and in close association with sponge cells. *C. symbiosum* cells surrounded individual cells and occupied regions between adjacent sponge cells. The distribution of the archaea in *A. mexicana* was similar to the distribution of prokaryotes found in other species of sponge (Vacelet and Donadey, 1977; Bertrand and Vacelet, 1971; Vacelet 1975; Wilkinson 1978; Unson and Faulkner, 1993; Vacelet *et al.*, 1996), and the density of *C. symbiosum* within the sponge was similar to the prokaryotic density in the mesohyl of a marine sclerosponge (Santavy *et al.*, 1990).

The detection and localization of *C. symbiosum* within the sponge matrix utilizing polynucleotide probes, corroborates genetic evidence of a specific association between the archaeon and the sponge, *Axinella mexicana*. *In situ* hybridization studies utilizing polynucleotide probes identified an extracellular population of archaeal symbionts. As was observed in previous studies (Trebesius *et al.*, 1994, Spring *et al.*, 1998), the rRNA polynucleotide probes offer a significant advantage over oligonucleotide probes with respect to

sensitivity, although they do not offer as high a resolution in taxonomic discrimination as oligonucleotide probes. The distribution of each of individual *C. symbiosum* variants (Schleper *et al.*, 1998) could not be determined in this study, because the polynucleotide probes generated from each variant are 99.2 % similar over the entire 16S and 23S rRNA (Schleper *et al.*, 1998). Determining whether the variants co-localize, or alternatively inhabit different regions within the sponge host, will require the development of *in situ* probes and protocols with higher sensitivity, capable of distinguishing these two variants that are related at the species level.

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Concluding Remarks

The identification of sponge-specific prokaryotic associations is complicated because sponges concentrate microorganisms during filter feeding from the seawater. The microorganisms detected in sponges could be transient, and the types detected could be dependent on which prokaryotic species are present within the seawater. In this study, the majority of the rRNA phylotypes recovered from the marine sponges *Xestospongia* sp. and *Cliona celata* were closely related to those commonly found in the marine environment. The detection of archaeal rRNA and rDNA in the marine sponge, *Axinella mexicana*, was not enough evidence to support a sponge-specific association, because archaea were shown to constitute significant proportions of the prokaryotic biomass in the plankton (Murray et al, 1998, Massana et al, 1998; Massana et al., 1997; DeLong et al., 1994; DeLong, 1992). Thus, it was necessary to identify the archaeal symbiont using molecular phylogenetic techniques, to show that the same archaeal phylotype was present throughout different populations of *A. mexicana*, and to localize the archaea within the sponge.

Phylogenetic analysis of the archaeal population indicated that the archaea were affiliated with, but distinct from, an uncultivated group of marine planktonic crenarchaea (highest unrestricted sequence similarity 95%). The archaeal 16S rRNA phylotypes recovered from *A. mexicana* inhabiting the Santa Barbara Channel and Monterey, CA were more similar to each other than to planktonic archaea. However, Santa Barbara Channel and Monterey collected sponges appeared to have their own archaeal-specific strains which are very highly related to one another in their 16S rRNA gene sequence. Marine archaea

have a worldwide distribution, and many new planktonic and marine sediment 16S rRNA gene sequences have been reported, but none of those studies has recovered the rRNA phylotypes of any of the strains of *C. symbiosum* (DeLong, 1992; DeLong *et al.*, 1994; Fuhrman *et al.*, 1992; Fuhrman *et al.*, 1997; Massana *et al.*, 1997; Massana *et al.*, 1998; McInerney *et al.*, 1997; Moyer *et al.*, 1998; Murray *et al.*, 1998). This could indicate that *C. symbiosum* does not have a free-living component to its life history. Alternatively, *C. symbiosum* could be present in the seawater, but at such a low abundance that it was simply not detected with the methods used. The observation that *C. symbiosum* strains have a specific and consistent association with the marine sponge, *A. mexicana*, is consistent with a functional symbiosis between these two organisms.

Most symbioses involve a single symbiont inhabiting a particular host species (Distel *et al.*, 1988; Distel *et al.*, 1991; Durand and Gros, 1996; Distel and Cavanaugh, 1994; Krueger and Cavanaugh, 1997; Haygood and Distel, 1992; Embley and Finlay, 1993; Rowan and Powers, 1991; McNally et al., 1994; Gast and Caron, 1996). If rRNA variation was detected in prokaryotic populations of one individual, then the rRNA gene sequences were at least 6 to 7% different (Rowan and Knowlton, 1995). However, multiple highly-related rRNA phylotypes (16S rRNA genes 99.2% similar) were detected in each individual of *A. mexicana*, indicating variation within the archaeal population of a single host. The maintenance of this variation within a single generation suggests that either the strains are not competing, or that they are spatially separated. In addition, to maintain the variation in subsequent generations, the infection of the host must involve an inoculum that includes both strains.

In situ hybridization studies using the 16S rRNA oligonucleotide probes and the rRNA polynucleotide probes provided the first morphological description of a member of this marine crenarchaeotal lineage. In conjunction with rRNA hybridization studies of sponges maintained in the laboratory, these data provide evidence that these organisms, whose closest members are thermophiles and hyperthermophiles, grow at low temperatures. In most of the sponges, at least half of the prokaryotic cells in *A. mexicana* were archaeal. The archaea inhabited skeletal matrix of the sponge and were observed around the periphery, surrounding individual, and between adjacent sponge cells.

The known habitats of mesophilic and psychrophilic crenarchaea include bacterioplankton (DeLong, 1992; DeLong *et al.*, 1994; Fuhrman *et al.*, 1992; Fuhrman *et al.*, 1997; Massana *et al.*, 1997; Massana *et al.*, 1998; McInerney *et al.*, 1997; Moyer *et al.*, 1998; Murray *et al.*, 1998), marine and freshwater sediments (Schleper *et al.*, 1997; MacGregor *et al.*, 1997; Munson *et al.*, 1997; Kato *et al.*, 1997), soil (Kudo *et al.*, 1997; Bintrim *et al.*, 1997; Jurgens *et al.*, 1997), the guts of holothurians (McInerney *et al.*, 1995) and marine fish (van der Maarel *et al.*, 1998), and now a symbiosis with a marine sponge. The phenotypic and biochemical traits of the sponge-specific archaea as well as the mesophilic and psychrophilic crenarchaea still remain largely unknown. However, the association was maintained in the dark in laboratory aquaria indicating that the archaea are not obligate phototrophs. The type of symbiotic system (mutualistic, commensal, or parasitic) as well as the role of the archaea within *A. mexicana* also remain unknown.

Archaea were detected in other marine sponges, suggesting the possibility of additional archaeal-sponge associations. However, as stated above, marine archaea can constitute significant proportion of the prokaryotic biomass and the origin of the archaeal rRNA genes be they sponge-specific or planktonic must be determined. The tropical sponges and several Antarctic sponges contained archaeal rRNA genes highly similar to those found in the plankton. Preliminary evidence indicates that the same rRNA phylotype of *C. symbiosum* from the Santa Barbara Channel inhabits sponges found in Antarctica. The detection of the *C. symbiosum* phylotype in two unidentified Antarctic sponges is extremely interesting given that rRNA phylotype has not been detected in planktonic environments despite high archaeal biomass, and thus could represent additional sponge-archaeal associations.

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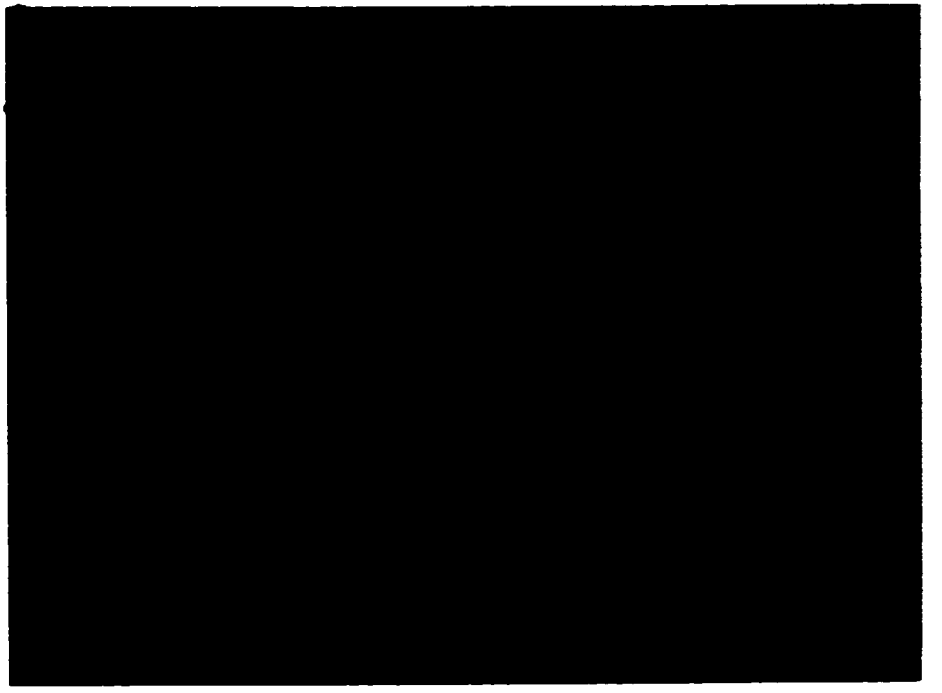
Appendix 1

Spicule preparations and gross morphology of marine sponges used in this study.

It is beyond the scope of this thesis to present a rigorous taxonomic identification of all of the marine sponges examined here. However, attempts were made to describe sponges using standard criteria (spicule shape and size, color, habitat, and morphology) commonly used to identify *Porifera*. The positive identification of *Axinella mexicana* was made by Dr. W. Lee and was based on spicule size and shape, color, habitat, skeletal structure, and comparison to type specimens (W. Lee and M. K. Harper, personal communication). The identification of additional specimens of *A. mexicana* were made by comparing the morphological characteristics (spicule size and shape) of each new specimen with the one originally identified by W. Lee as *A. mexicana*. The following figures describe some of the morphological characters of the marine sponges used in this thesis.

Figure A1-1. Morphological characteristics of *Axinella mexicana* collected from the Santa Barbara Channel.

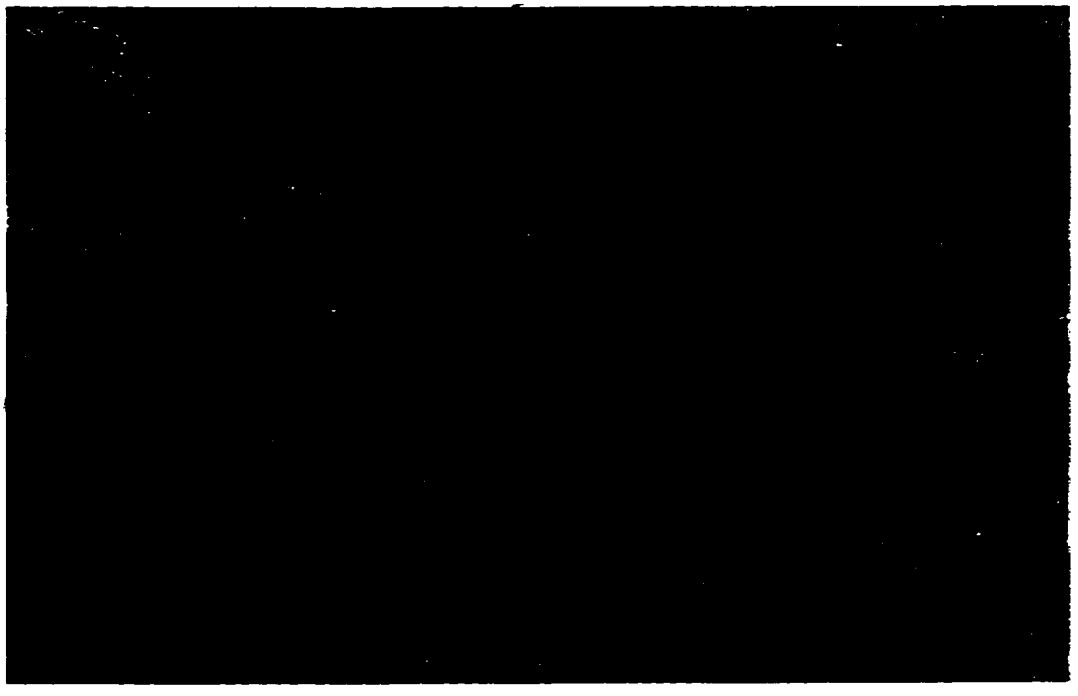


Specimens: s1-s30, hs1-hs4, hh1-hh3
Color: red to orange color
Shape: encrusting sponge from 1 to 3 cm thick
Depth and Location Collected: 9 to 15 m, Santa Barbara Channel- Naples Reef, Haskle Reef, and Hermit Hole (N. Anacapa Island)
Spicules: Megascleres only: Oxea and Styles. The size range was 200μm to 400μm for both types, with oxeas being the larger of the two. The oxea were bent near their midpoint and the style near the head. (see photo above; Scale 10mm=50μm)
Skeleton: plumo-reticulate, and a condensed spicular area at the attachment surface

Identification of the sponge was made by Dr. Welton Lee and Mary Kay Harper.

Monterey sponges (specimens ms1-ms4) had a similar type and size of the spicules, color and shape as those collected from the Santa Barbara Channel.

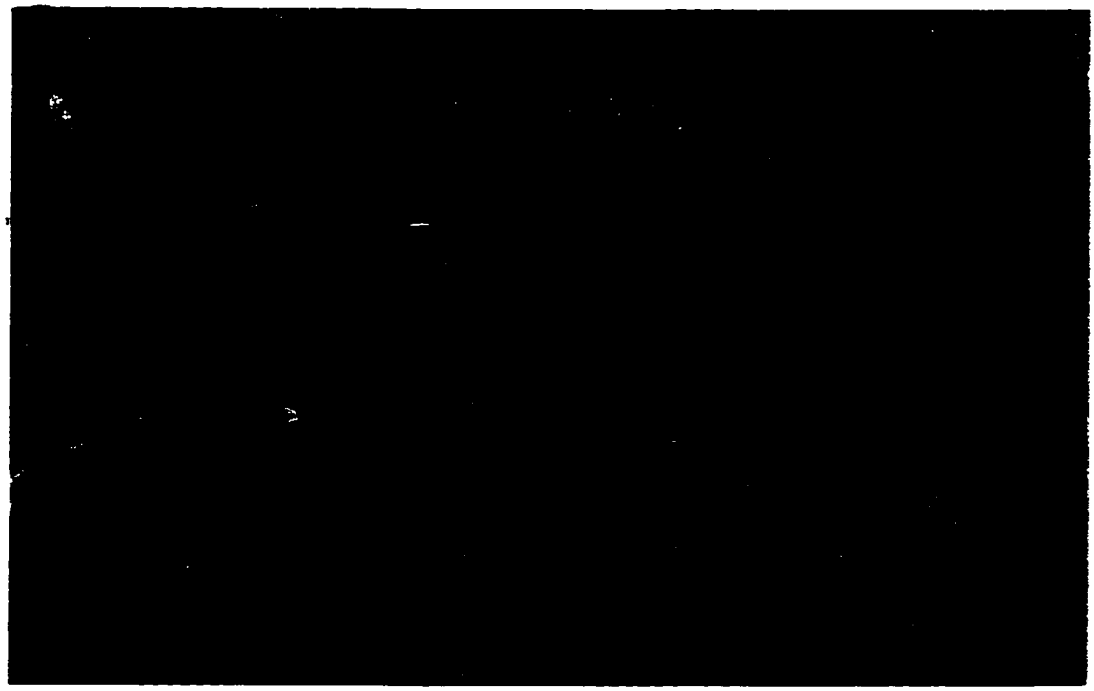
Figure A2-2. Spicule preparation of from *Cliona celata* and morpholgoical description of *Tethya aurantia.*



Sponge: *Cliona celata*
Color: Yellow
Shape: Boring sponge. Attached to calcareous material (scallop shell).
Depth and Location Collected: 9 to 15 m, Santa Barbara Channel, Naples Reef
Spicules: Megascleres: Tylosyles only. (see above photograph. Scale 10mm=50μm)

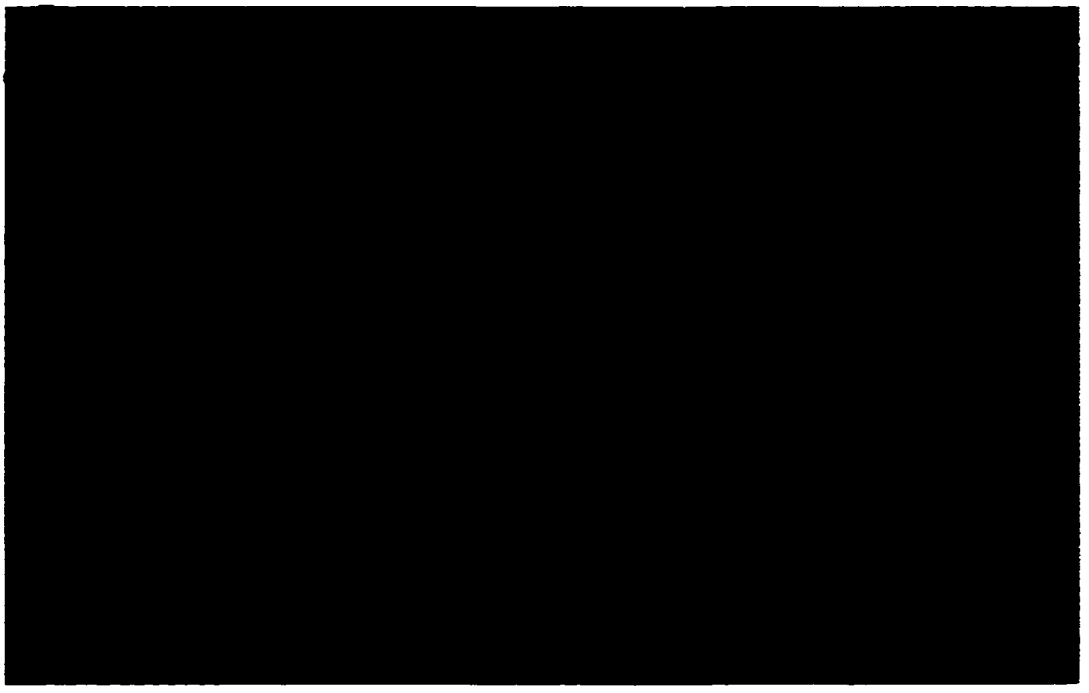
Sponge: *Tethya aurantia*
Color: mainly orange and yellow
Shape: globular, spherical, very fibrous
Depth and Location Collected: 9 to 15m, Santa Barbara Channel, Naples Reef.
Spicules: Megascleres: styles, tylostyles, simple triaxonMicroscleres: sphaeraster (no photograph shown)

Figure A1-3. Morphological characteristics of an unidentified sponge from the Santa Barbara Channel.



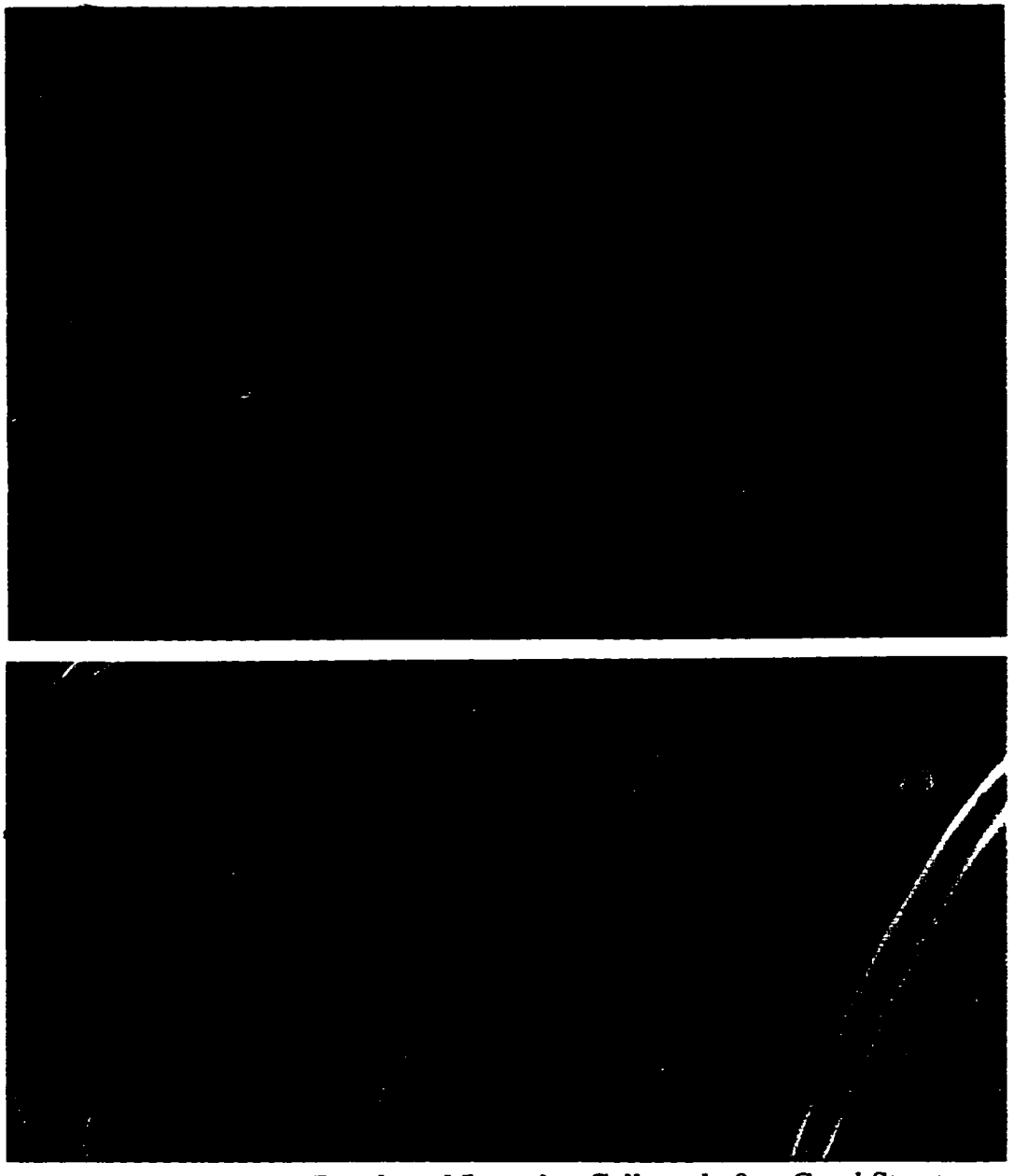
Specimen number: m1
Color: Red
Depth and Location Collected: 9 to 15 m, Santa Barbara Channel- Naples Reef
Shape: Massive, oscules raised
Spicules: Megascleres and Miroscleres present. (see above photograph, Scale: 10 mm= 50 μm)

Figure A1-4. Unidentified marine sponges, (specimens cs3 and cs5) from Monterey, CA.



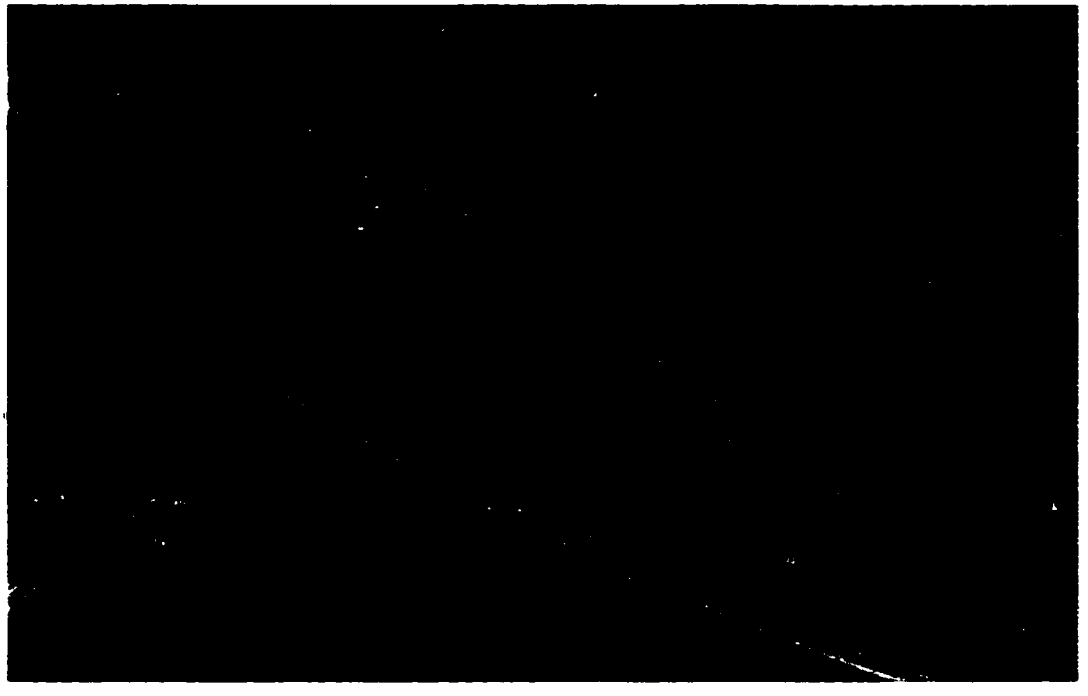
Specimens: cs3 and cs5
Color: red to yellow
Shape: encrusting
Depth and Location Collected: 9 m; Coral Street, Monterey, CA.
Spicules: Megascleres. (see above photograph, scale 10mm = 50μm).

Figure A1-5. Unidentified sponge (specimen m3 & m4) from Monterey, CA.



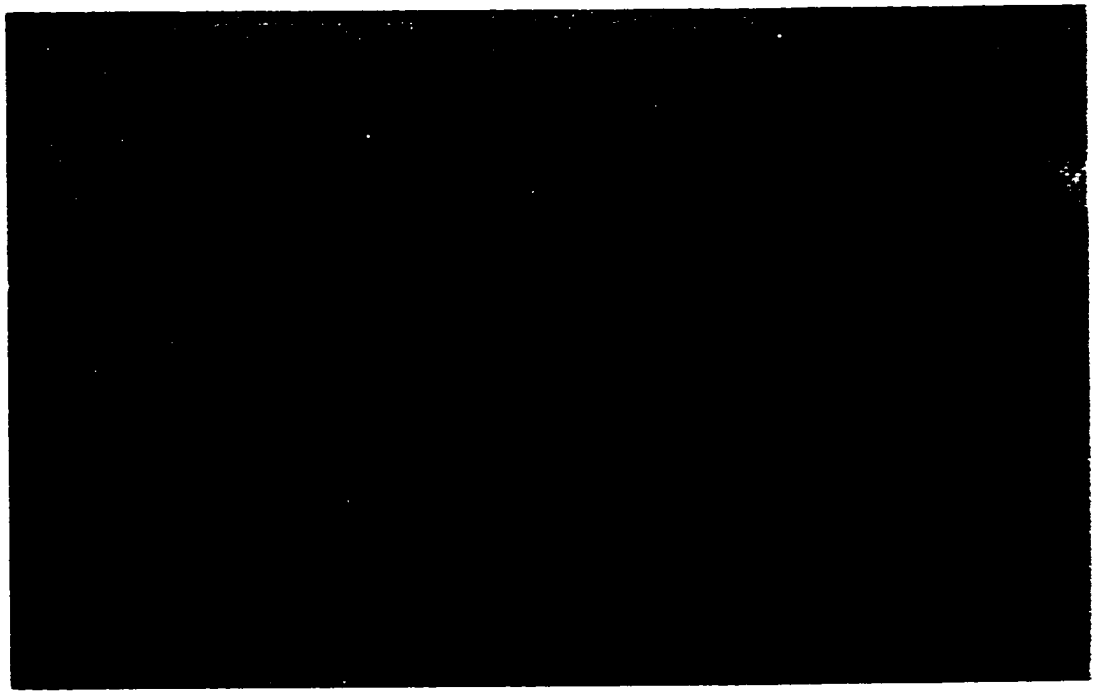
Specimens: m3 and m4 **Depth and Location Collected**: 9m; Coral Street, Monterey CA. **Spicules**: Megascleres and Miroscleres present. (see above photographs, Scales: top photo 10 mm= 50 µm and bottom photo 11mm = 30 µm)

Figure A1-6. Spicule preparations of several unidentified Antarctic sponges. collected off the coast of Avers Island, Antarctica.



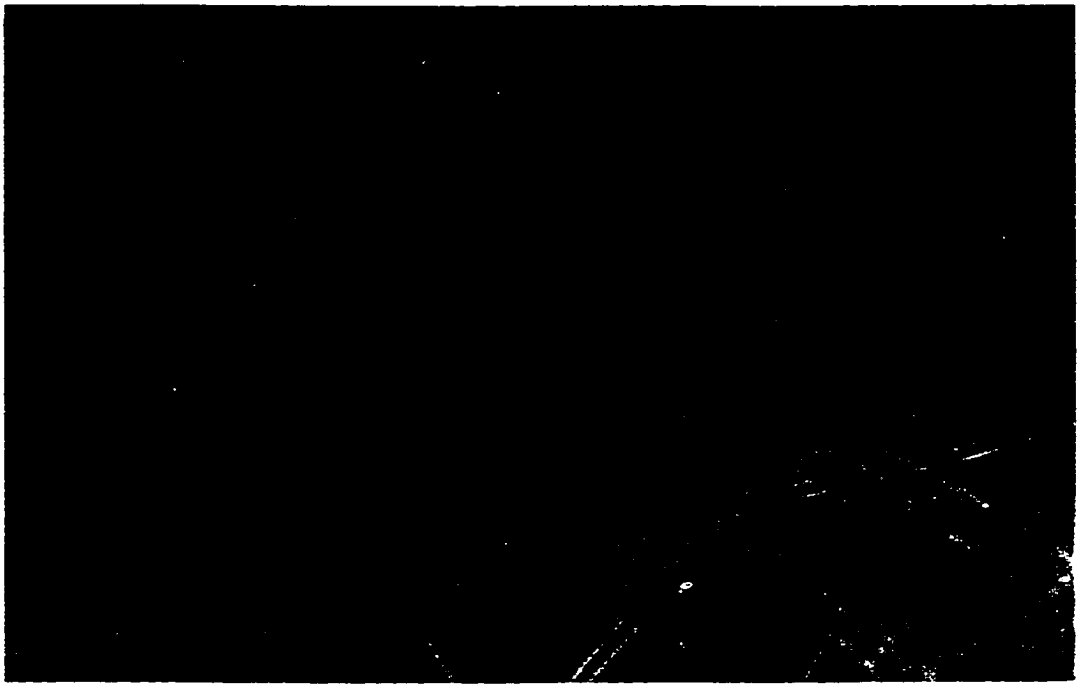
Specimens: Ant1 and Ant2, color orange. Spicules pictured above (Scale: 10mm = 50μm)

Figure A1-6 (continued)



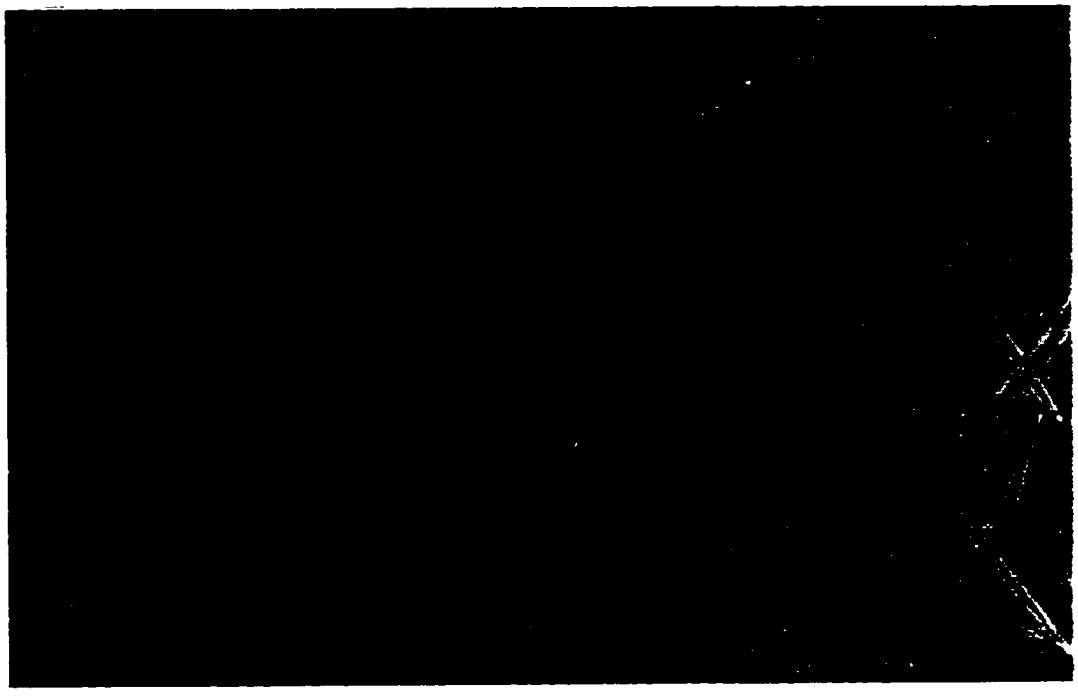
Specimen: Ant3, color light brown to tan, massive, finger sponge, large oscules at top. Spicules pictured above (Scale: 10mm = 50μm)

Figure A2-6 (continued)



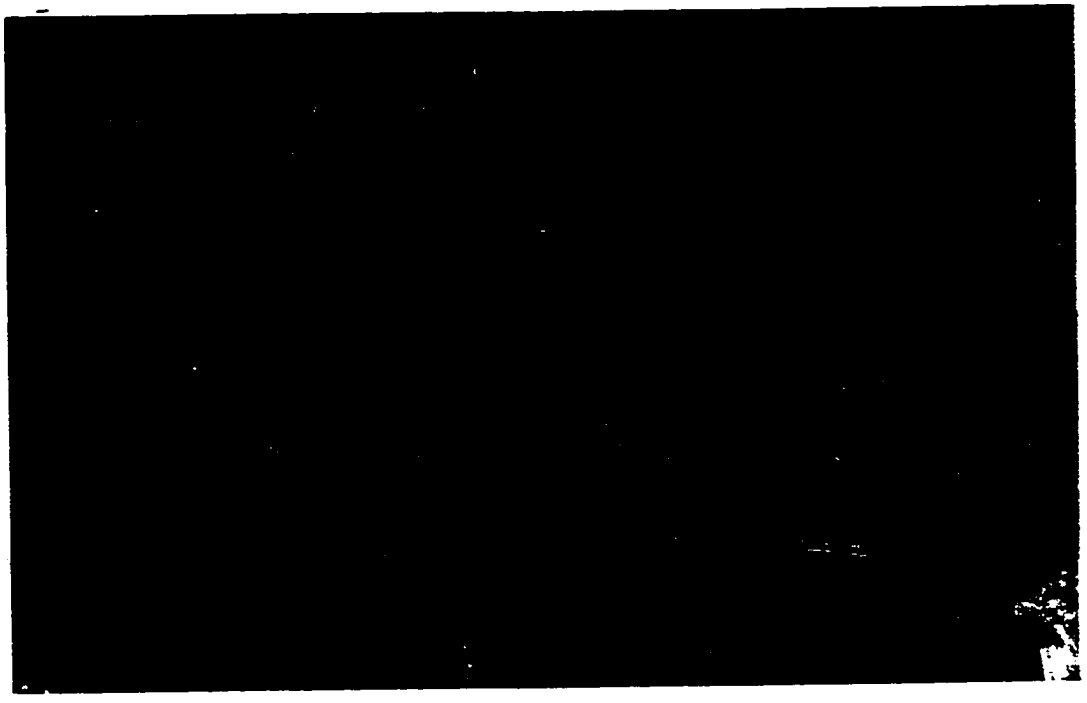
Specimen: Ant4, color light brown to tan. Spicules pictured above (Scale: 10mm = 50μm)

Figure A2-6 (continued)



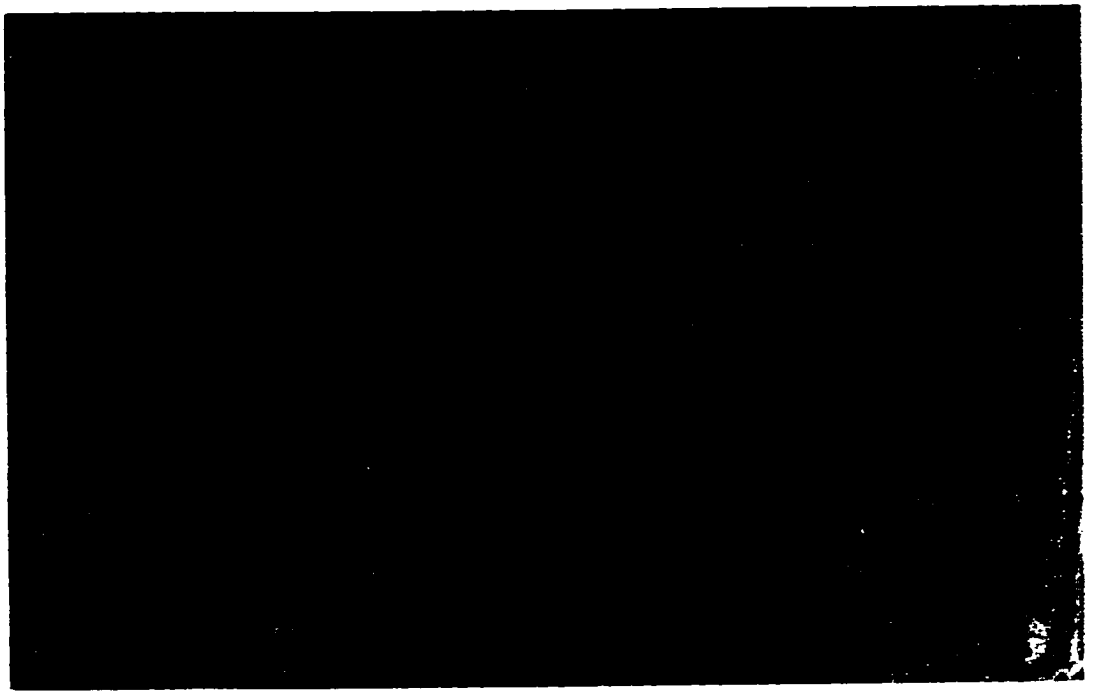
Specimen: Ant9: color tan. Spicules pictured above (Scale: 10mm = 50μm)

Figure A2-6: (continued).



Specimen: Ant11, color reddish-brown, fibrous. Spicules pictured above (Scale: 10mm = 50μm)

Figure A2-6: (continued).



Specimen: Ant12 color yellow to tan,
Spicules pictured above (Scale: 10mm = 50μm)

Other Sponges: No spicule photographs shown.
Ant5: color light brown, many ostia, single osculum
Ant6 and 14: mustard yellow, elastic skeleton, lots of projections from the surface
Ant7: light brown to tan branching sponge.
Ant8: color yellow to brown, thin finger sponge
Ant10: color white to light tan
Ant13: rust colored, fibrous skeleton
Ant15: color tan, tubular shape, large single osculum at top.

Appendix 2-1.

Table A2-1 Detection of Archaea in tropical and Antarctic sponges

Sponge Species or Specimen[a]	Location Collected[b]	Archaeal rDNA[c]	Sponge Archaea Most Similar to[d]	% Archaeal rRNA[e]
Xestospongia testudinaria (1)	Indonesia	-	---	---
X. wiedenmayeri (1)	Belize	weak +	---	---
Neofibularia nohlangere (2)	Belize	+ (1)	planktonic archaea clones (c6, c11, c33, c46, c48; 97.8)	---
Chondrilla nucula (1)	Fiji	weak +	planktonic archaea clones (c6, c11, c33, c46, c48; 95.9)	---
Oligocera violacea (1)	Bahamas	-	---	---
X. muta (2)	Belize	-	---	---
Aplysina fishilaris (1)	Belize	+	crenarchaeote JTB167 (96.5)	---
Aplysina sp. (2)	La Paz, Baja	-	---	---
A. archeri (1)	Bahamas	-	---	---
N. mordens (1)	Indonesia	-	---	---
Order: Dyctioceratida (4)	Belize	weak + (1)	archaea clone SBAR5 (89.3)	---
Ant1	Antarctica	+	*Cenarchaeum symbiosum* (100.0)	---
Ant2	Antarctica	+	*Cenarchaeum symbiosum* (100.0)	
Ant3	Antarctica	+	---	---
Ant4	Antarctica	+	---	---
Ant5	Antarctica	+	---	---
Ant6	Antarctica	-	---	---

Table A2-1 (continued)

Sponge Species or Specimen[a]	Location Collected[b]	Archaeal rDNA[c]	Sponge Archaea Most Similar to[d]	% Archaeal rRNA[e]
Ant7	Antarctica	+	---	---
Ant 8	Antarctica	+	---	---
Ant 9	Antarctica	+	archaea clone Antarctic12 (99.3)	1.3 (1.3)
Ant 10	Antarctica	+	*Cenarchaeum symbiosum* (100.0)	1.6 (1.5)
Ant 11	Antarctica		archaea clone Antarctic12 (97.5)	3.0 (2.2)
Ant12	Antarctica	+	---	---
Ant13	Antarctica	+	---	---
Ant14	Antarctica	-	---	---
Ant 15	Antarctica	+	archaea clone Antarctic12 (99.6)	2.4 (2.4)

[a]Numbers in parentheses indicate the number of individuals that were tested.

[b]All tropical sponges were collected and identified by C. Diaz. Antarctic sponges unidentified. See appendix 1 for photographs of spicule types. Antarctic sponges were collected in Arthur Harbor, off of Palmer Station, Antarctica.

[c]The presence of archaeal rDNA was determined in a PCR assay using archaeal specific-primers. The number in parenthesis indicates the number of individuals that gave a positive result with the archaeal specific rDNA primers. Bacterial rDNA was amplified from all individuals using PCR with bacterial specific primers. See Chapter 2 for methods.

[d]Amplified archaeal 16S rDNA genes were directly sequenced (see chapter 2) and compared to marine crenarchaea including *C. symbiosum*. The rRNA gene clone and % similarity of the most closely related rRNA gene sequence are given in parentheses. Nucleotide positions between 172 and 407 (E. coli numbering) were used in the analysis of archaeal rDNA from the Antarctic sponges and positions 130 to 322 for archaeal rDNA from the tropical sponges.

[e]% archaeal rRNA was determined by rRNA hybridization using Domain-specific oligonucleotide probes.
--- indicates experiments not performed.

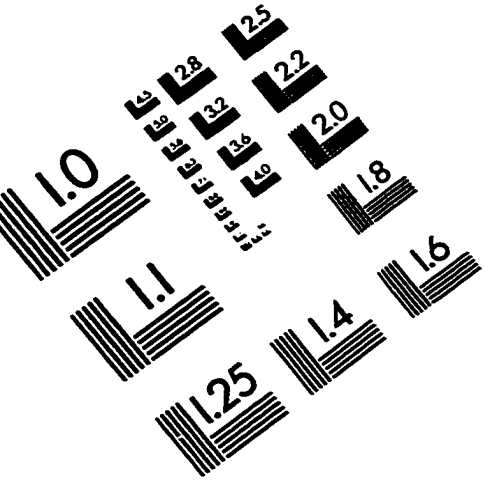
Table A2-2. Similarity matrix comparing the archaeal 16S rRNA genes from sponges Ant1 and Ant2 collected from Antarctica to *C. symbiosum* and planktonic archaea.

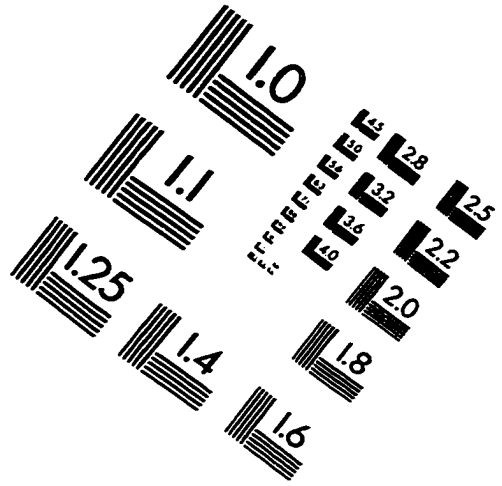
	Ant1	Ant2
Ant1		
Ant2	100	
101G10	100	100
60A5	99.2	99.2
ms1	99	99
ms2	99.2	99.2
ms4	99.5	99.5
clone 33	92.8	92.8
WHARQ	91.6	91.6
Ts235c310	90	90
Antarctic12	92.5	92.5
4b7	92.5	92.5
sbar5	91.4	91.4
sbar12	91.5	91.5

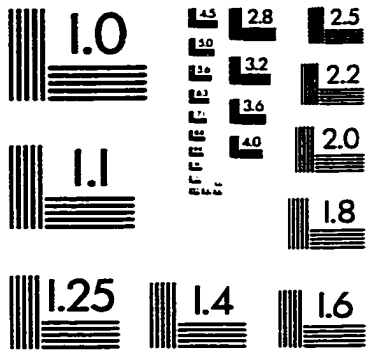
Line delineates the archaeal 16S rRNA genes from sponge-associated and marine planktonic archaea. Ant1 and Ant2 represent two different specimens of the same species of sponge collected near Palmer Station, Anvers Island Antarctica (see appendix 1). 101G10 and 60A5 represent the two archaeal 16S rRNA genes of *C. symbiosum* from the Santa Barbara Channel. Archaeal rRNA genes from *A. mexicana* collected from Monterey, CA (prefix ms) Clone 33, WHARQ, TS235C310, Antarctic12, 4b7, sbar5, and sbar12 are crenarchaeal 16S rRNA phylotypes recovered from seawater (van der Maarel *et al.*, 1998; Stein *et al.*, 1996; DeLong, 1992; Mullarkey *et al.*, unpuplished). The region compared corresponds to nucleotide positions 134 to 876 in the 16S rRNA gene of *E. coli*..

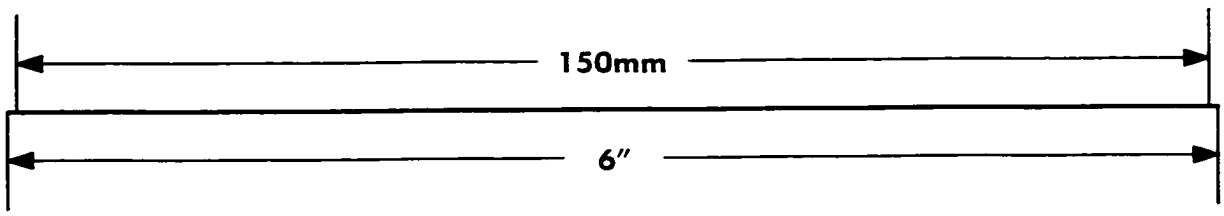
The 16S rRNA gene sequence of both Ant1 and Ant2 shared the same nucletides at the signature positions (see table 3-1) with *C. symbiosum* variant A (represented by 101G10 above).

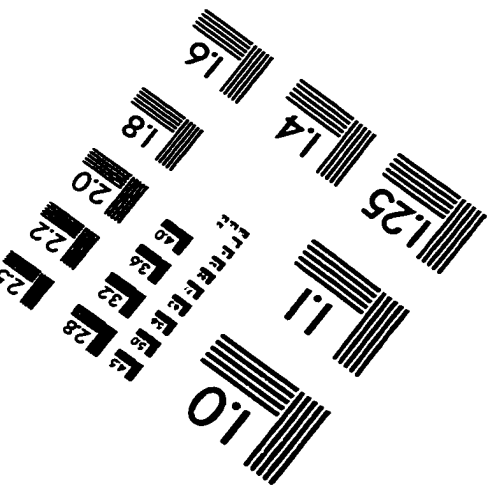
IMAGE EVALUATION TEST TARGET (QA-3)

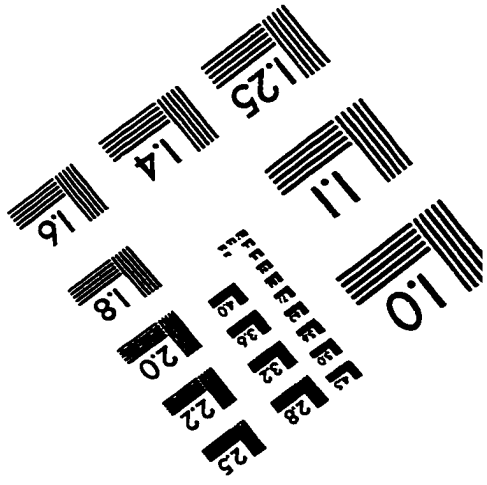












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