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Consistent association of crenarchaeal Archaea with sponges of the genus *Axinella*

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Abstract The association of sponges with microorganisms has been accepted to be a common feature of the phylum Porifera. Herein we describe the association between filamentous Archaea and three Mediterranean species of sponges from the family Axinellidae (Porifera: Demospongiae). *Axinella damicornis*, *A. verrucosa* and *Axinella* sp. harbor a high concentration of filamentous Archaea in the collagen that surrounds the siliceous spicules that form their skeleton. These Archaea have been found in every axinellid specimen studied, regardless of their environment and collection time. The morphology of the filaments has been studied using transmission electron microscopy, and they all show similar characteristics. Their nature has been determined by in situ hybridization experiments and by PCR amplification and sequencing of their 16S DNA. Each sponge species contains a single filamentous archaeal phylotype. The Archaea of the three sponges are closely related to each other and to the marine "group 1" crenarchaeotes. Our findings suggest that this newly described association could be defined as a symbiosis, where biochemical and/or metabolic relationships between the sponge hosts and their symbionts remain to be determined.

Introduction

The occurrence of a large number of microorganisms associated with sponges is a common phenomenon in a wide variety of species of the phylum Porifera (Vacelet 1975; Vacelet and Donadey 1977; Wilkinson 1978a; Wilkinson et al. 1981; Santavy et al. 1990; Burja et al. 1999; Friedrich et al. 1999; Fuerst et al. 1999; Lopez et al. 1999; Manz et al. 2000). Several studies have shown that sponge microbiota differs from the ambient water microbial population (Wilkinson 1978b; Wilkinson et al. 1981; Santavy and Colwell 1990; Burja et al. 1999), suggesting that these associations are specific and therefore can be considered symbiotic. Whereas the association type seems to be constant in the species in which it occurs (Vacelet 1982), various aspects are different according to the species studied: the density of the microorganisms, their location in the sponge tissue, their morphological and genetic diversity, and probably the kind of relationships established with their host. The microorganisms are mainly found living free in the sponge mesohyl (Vacelet and Donadey 1977; Wilkinson 1978c; Vacelet 1982; Manz et al. 2000). A few sponge species contain intracellular bacteria (Vacelet and Donadey 1977; Wilkinson 1978c). Bacteria associated with the choanocyte chambers and the aquiferous system have also been described (Burlando et al. 1988). Most of the sponge microbiota belong to the Bacteria domain, with unicellular bacteria and cyanobacteria being the predominant groups (Vacelet 1975; Wilkinson 1978a; Althoff et al. 1998; Burja et al. 1999; Friedrich et al. 1999; Manz et al. 2000). Associations with dinoflagellates (Garson et al. 1998), unicellular algae (Vacelet 1982), and, recently, members of the domain Archaea (Preston et al. 1996; Fuerst et al. 1999) have also been described. The roles of these associations remain unclear. Several authors have proposed that the microbiota contributes to its host nutrition via the production and transfer of nutrients (Reiswig 1975, 1981; Wilkinson and Fay 1979; Vacelet 1982; Wilkinson et al. 1999) and the digestion and recycling of material either not available or toxic to the sponge (Vacelet 1975; Wilkinson 1978b). Phagocytosis and intracellular digestion of mesohyl bacteria has been observed (Reiswig 1975; Vacelet and Donadey 1977), suggesting a direct nutritional source for the

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sponge. Secondary metabolism of the associated microbiota could also be involved in the relationship. Some sponge compounds have often been ascribed to a microbial origin, even if few metabolites have been definitively localized in bacterial cells (Faulkner et al. 1993, 1999; Schmidt et al. 2000). The production of metabolites with antibacterial (Oclarit et al. 1994; Unson et al. 1994), antifungal (Schmidt et al. 2000), cytotoxic (Bewley et al. 1996) and feeding-deterrent (Unson and Faulkner 1993) activities has been ascribed to some symbiotic bacteria in sponges, suggesting that the microbiota secondary metabolism could play an important role in the sponge defense mechanism as well as in its evolutionary success.

These sponge–microorganism associations have often been studied using electron microscopic techniques, which can only provide morphological data about the sponge microbes. Standard isolation and cultivation methods have allowed the identification of some sponge bacteria (Wilkinson 1978b; Santavy and Colwell 1990; Burja et al. 1999; Lopez et al. 1999), but have also demonstrated that most of the sponge-associated bacteria were not readily cultivable (Lopez et al. 1999). Molecular techniques have provided new tools to genetically identify the associated microorganisms: in situ hybridization experiments with group-specific, fluorescence-labeled oligonucleotide probes and phylogenetic analysis using polymerase chain reaction (PCR) techniques allowed in situ studies of population diversity (Amann et al. 1991, 1995; Althoff et al. 1998; Friedrich et al. 1999; Lopez et al. 1999; Manz et al. 2000; Webster et al. 2001). In most of the bacteria–sponge associations studied, a variety of microorganisms has been found. However, only in a few cases was the constant presence of a species demonstrated. A remarkable example is *Cenarchaeum symbiosum*, a non-cultivable archaeon discovered inhabiting the Pacific sponge *Axinella mexicana* (Preston et al. 1996; Schleper et al. 1997, 1998). This archaeon is closely related to the marine "group 1" crenarchaeotes, a newly found group of non-cultivable Archaea that are significant components of marine picoplankton assemblages (DeLong 1992; DeLong et al. 1999; Massana et al. 1997). Herein we describe the association of "group 1" crenarchaeote-related filamentous Archaea with three Mediterranean sponge species of the family Axinellidae. The Archaea have been characterized using microscopy, in situ hybridization, and PCR sequencing of their 16S rDNA.

Materials and methods

Sample collection

The specimens of *Axinella damicornis*, *A. verrucosa* and *Axinella* sp. were collected by scuba diving from different sites on the Spanish Mediterranean coast: the islands of Menorca, Majorca, Ibiza and Formentera, and the Bay of Calabardina in Murcia. In order to obtain a wide range of specimens, the sponge samples were collected at different depths (ranging from 10 to 35 m), from different habitats (exposed rocky cliffs, caves and silty bedrock) and at different times of the year (September in Menorca and Murcia, October in Majorca, February in Ibiza and June/July in Formentera). Criteria used in sponge specimen identification included external aspect, skeletal architecture and spicule composition (Uriz 1982). Differences in spicule size between the three species were not statistically significant. *Axinella* sp. probably corresponds to the species described by Solé-Cava et al. (1991): it is an intermediate morphotype, with the color and texture of *A. verrucosa*, but with coalescing branches like *A. damicornis*, forming an irregularly shaped mass. The three species were found at all collection sites. Some of the sponges were transferred to the aquaria in our laboratories and maintained alive for further experiments. The specimens for the microscopy work and hybridization experiments were fixed immediately upon collection, and others frozen at –20°C at the time of collection.

Preparation of samples for microscopy

Two specimens of each *Axinella* species from every collection site (but collected in different diving areas and at different depths) were cut into small cubes of 0.5 cm, and fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.1 M cacodylate buffer in seawater with 14 g of sucrose per 100 ml, and then rinsed in the same buffer. The samples for transmission electron microscopy (TEM) were post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at 4°C. Samples were then rinsed in cacodylate buffer, dehydrated in an ethanol-propylene oxide series, and embedded in epoxy resin using the EPON 812 embedding kit (Tousimis Research, USA). Thin and ultrathin sections were cut with a rotary ultramicrotome. Thin sections for light microscopy were stained with toluidine blue. Ultrathin sections for TEM were mounted on grids and stained with uranyl acetate and lead citrate, and examined with a JEOL 1010 electron microscope.

In situ hybridization

The samples of the three axinellids showed a high autofluorescence at the fluorescein and rhodamine/Texas red wavelengths; however, no autofluorescence was detected at the Cy5 wavelength. Therefore, all oligonucleotide probes were 5′ labeled with Cy5. We used the Archaea-specific probe Arch-915 (GTGCTCCCCCGCCAATTCCT) and the universal Bacteria probe Eub-338 (GCTGCCTCCCGTAGGAGT), both from Amann et al. (1995). We also used the marine crenarchaeon-specific probe SY-554 (TTAGGCCCAATAATCCTCCT) from Preston et al. (1996), modified in one nucleotide (underlined) to match the 16S DNA sequences obtained from the sponge symbionts. The Cy5-labeled oligonucleotide probes were provided by Genotek S.A., Spain. The experiments were first performed on sponges freshly collected from the sea, and then on specimens that had been maintained in an aquarium for 3 and 6 months, in order to check the consistency of the association with the filamentous prokaryote (see below). In situ hybridization was performed on 0.5 cm thin slices cut from live sponges that were fixed in 4% formaldehyde in seawater at 4°C for 1 h, washed in seawater twice for 10 min, dehydrated with an ethanol series and air dried. The hybridization conditions were as described by Distel et al. (1991), but without the addition of bovine serum albumin and polyadenylic acid. Eighteen microliters of hybridization buffer (5× SET with 10% dextran sulfate), with 2 µl of Cy5-labeled oligonucleotide probe (50 ng μl^{-1}), was added to the sample. The samples were incubated at 40°C for 12 h in a moist chamber. Post-hybridization washes were made with 1× SET (150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 1 mM Na_2 EDTA) at 45°C for 20 min. The samples were then air dried, placed on a slide, mounted in Citifluor AF1 anti-fade media (Citifluor, London), covered with a coverslip and examined under a BioRad Radiance 2000 confocal microscope coupled to a Zeiss Axiovert S100 TV.

DNA extraction

DNA was extracted from five specimens of each sponge species, one from each collection site. Small slices (~0.1 g) of each sponge

were ground in liquid N_2, 500 µl of extraction buffer (0.5 M NaCl, 0.05 M Tris-HCl, pH 9.35, 0.01 M EDTA, 2% SDS, 0.4% Triton-X100) was added, and the mixture was vigorously agitated using a vortex mixer. Immediately, 500 µl of phenol:chlorophorm:isoamylic alcohol (25:24:1) was added, and the extracted DNA was purified by a standard phenol method (Sambrook et al. 1989). Total genomic DNA from each sample was stored at –20°C.

16S rDNA gene characterization

Five DNA samples from each sponge species were studied separately. The primers used to amplify the 16S rDNA from the Archaea symbiont were Arch 21F (5-TTCCGGTTGATCCTGCCGGA-3) (Giovannoni et al. 1988) and ME 1354R (5-TGACGGGC GGTGTGCAAG-3) (Shinzato et al. 1999). Hot Star *Taq* DNA polymerase from Qiagen was used with the standard reaction mix protocol with a final concentration of 2.5 mM $MgCl_2$. PCR was conducted with 10 cycles of 94°C (1 min), 60°C (1.5 min) and 72°C (2 min); 20 cycles of 92°C (30 s), 60°C (1 min) and 72°C (2.5 min); and a final elongation step of 72°C for 10 min. Prior to sequencing, the amplified fragments were washed, using a Microcon PCR centrifugal filter device (Millipore, USA), and resuspended in ultrapure H_2O. The fragments were sequenced using the Archaea-specific primers Arch 21F, Arch 480F (GCCGCGGTAAAACCAG-CACC) and ME 855F (TTAAAGGAATTGGCGGGGGA) (Shinzato et al. 1999). The sequences were analyzed with Chromas version 1.44 software (C. McCarthy, Queensland, Australia) and aligned using Clustal W version 1.7. The sequences were then submitted to the online GenBank database, using the BLAST program from NCBI (The National Center for Biotechnology Information).

Results and discussion

Microscopy studies

All specimens of the three species of sponges shared similar characteristics as determined by light microscopy. A notable common characteristic is the presence of a thick collagen matrix that maintains the structure of the siliceous skeleton. In every specimen studied we observed a high number of long filaments included in the collagen and surrounding the siliceous spicules (Fig. 1). The nature of those filaments could not be determined with the light microscope.

Ten specimens of each species of *Axinella* (two from each collection site) were analyzed by TEM. We observed a high diversity of microbial morphotypes associated with the mesohyl of the sponges, but we found no evidence of a common morphotype among the ten specimens in any of the three species of *Axinella*. No cyanobacterial or intracellular symbionts were found in any of the sponges. There was a noticeable difference in the appearance of the collagen of the sponges' mesohyl and the collagen that immediately surrounds the spicules' skeleton: the first material is formed by thick, loose fibers, while the latter is formed by thin fibers that are densely packed, forming a perispicular matrix. Examination of the collagen matrix that surrounds the spicules revealed the presence of a large number of unicellular and short filamentous microorganisms with no eukaryotic nuclei (Fig. 2a–c). They were observed in every specimen of the three species, regardless of the collection site and habitat. The individual prokaryotic cells were assumed to be cross sections of the long filaments observed under the light microscope. Thus, the filaments are formed by single, rod-shaped prokaryotic cells enveloped in a thick cell wall. Each cell is 1–1.5 µm long and 250–300 nm wide. Some of the cells contain large vacuoles, but no other well-defined intracellular structure is visible (Fig. 2d). The cell wall of the microorganisms appears to be tightly bound by the collagen.

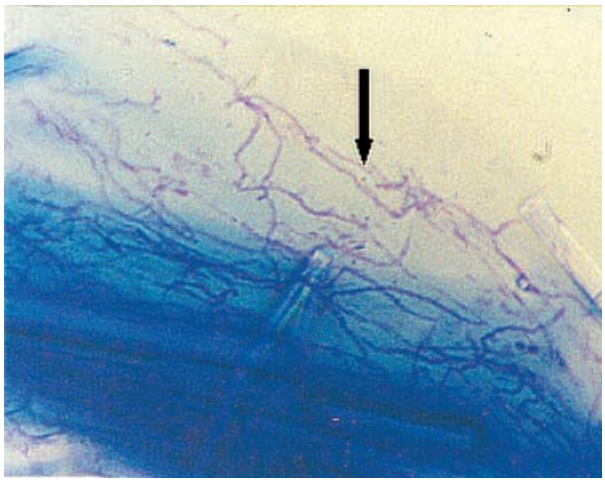


Fig. 1 *Axinella verrucosa*. Light micrograph (100×) of a toluidine-blue-stained *A. verrucosa* section showing the filamentous symbionts (*arrow*) inside the collagen matrix (*light blue*)

The constant presence of filamentous microorganisms closely associated with the sponge collagen in each sample studied suggests a consistent symbiosis in these three *Axinella* species. Moreover, this association seems to be specific of these axinellids, as no microorganisms showing similar morphology and location have been found in any of the 15 non-axinellid sponge species from the same collection areas that were analyzed by TEM and light microscopy (data not shown).

In situ hybridization

We used Cy5-labeled universal oligonucleotide probes for the domains Bacteria and Archaea to try to determine the nature of the filamentous prokaryotic symbionts. The three species of *Axinella* show autofluorescence at the fluorescein and rhodamine/Texas red wavelengths, but not at the Cy5 wavelength. Autofluorescence is extremely high in the sponge tissue and in the filaments. As the samples are not autofluorescent at the Cy5 wavelength, we chose to label the hybridization probes with Cy5, and to perform hybridizations with both probes. We used the autofluorescence of the samples at the rhodamine/Texas red wavelength to visualize the symbiont and the sponge tissues (Fig. 3d, e, f). The results of the hybridization experiments are similar in the three *Axinella* species. Hybridization with the Archaea-specific probe yielded a strong signal in the filamentous symbiont (Fig. 3a), but nowhere else in the

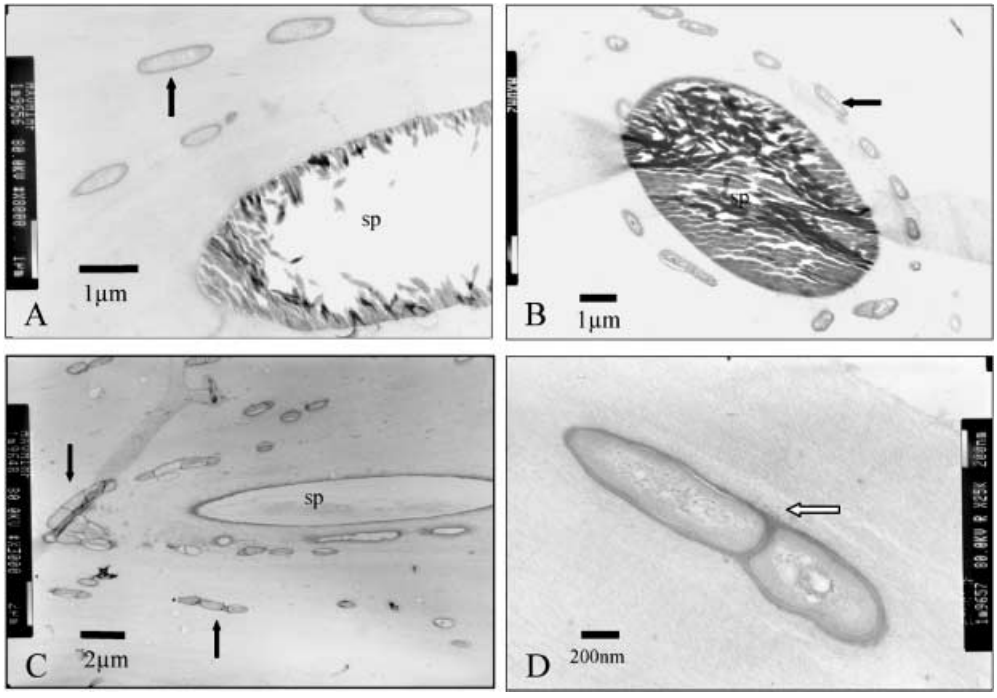
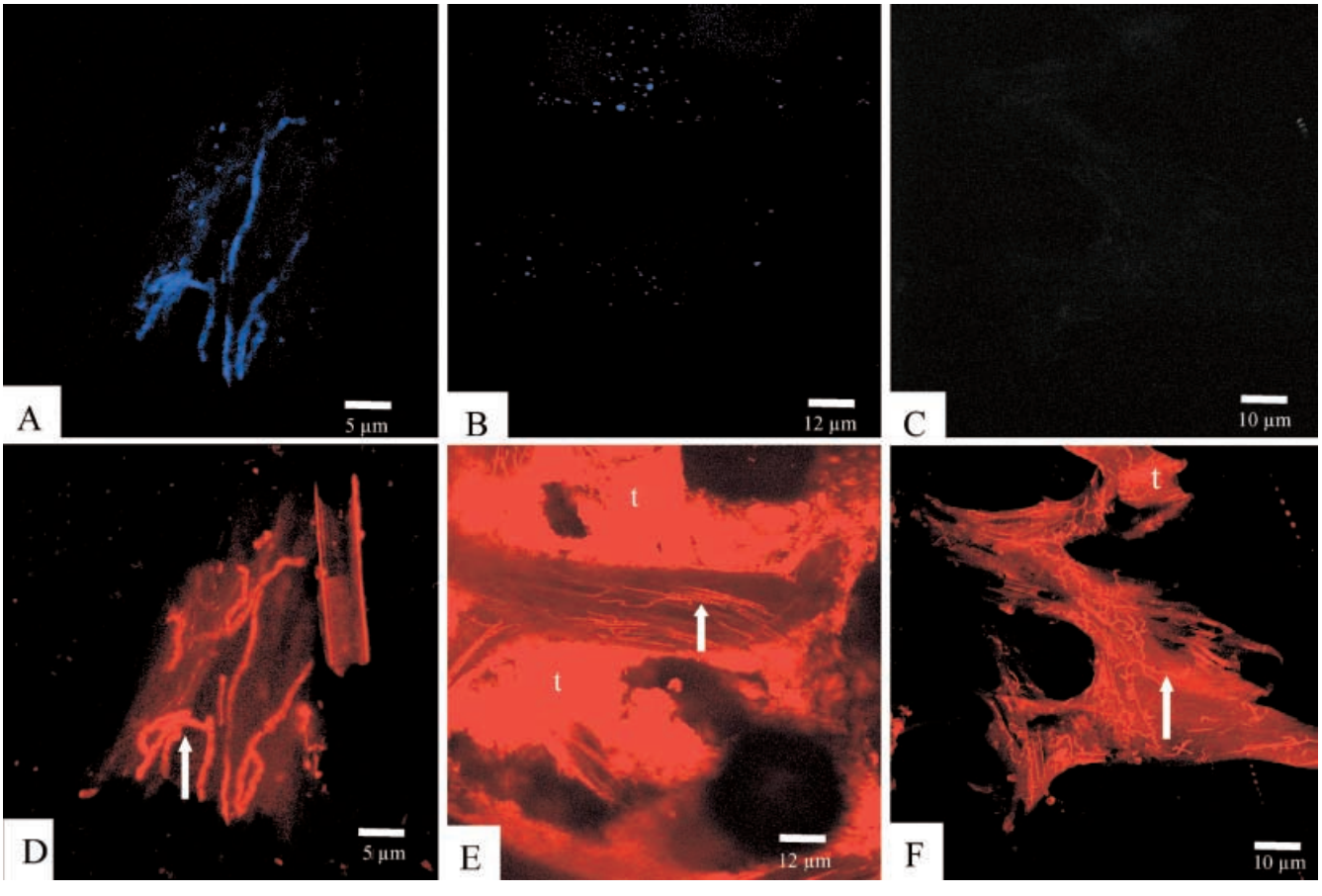


Fig. 2a–d TEM micrographs of filamentous symbionts (*arrows*) of *Axinella* in the collagen that surrounds the siliceous spicules (*sp*). **a** *A. verrucosa* symbiont; **b** *A. damicornis* symbiont; **c** *Axinella* sp. symbiont; **d** detail of a filamentous symbiont. Note the densely packed collagen fibrils (*white arrow*) around the cell wall



sponge tissue, suggesting that no other archaeon is present in a significant amount in the sponges. Hybridization with the Bacteria-specific probe yielded a signal in the sponge mesohyl, but not in the filaments (Fig. 3b). Control hybridization with no probes yielded no signal at all (Fig. 3c). We obtained the same results with

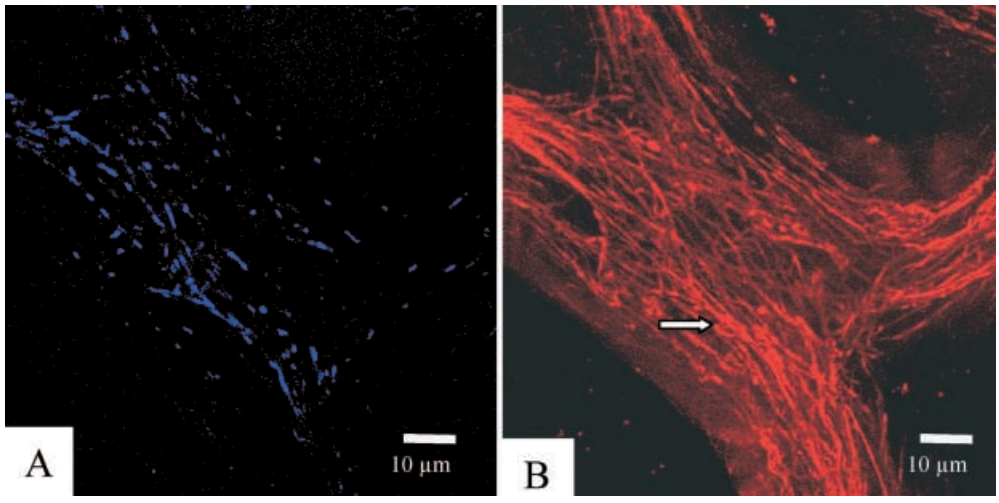


Fig. 4a, b *Axinella damicornis*. **a** Hybridization with Cy5-labeled SY-554, specific for the archaeal symbiont. The signal appears in the filaments. **b** Autofluorescence of the same field at the Texas red wavelength. The filaments are clearly visible (*arrow*)

sponges fixed immediately after collection from the sea and with samples maintained in aquaria for 3 and 6 months, confirming the consistency of the association. We therefore conclude that the filamentous microorganisms found in the three species of *Axinella* are Archaea. Hybridization was repeated using the marine crenarchaeon-specific probe SY-554, chosen to match the archaeal 16s rRNA sequences once they were obtained by PCR amplification (see below). This probe yielded a strong signal only in the filamentous symbiont (Fig. 4a, b). Again, the Bacteria-specific probe yielded a signal only in the sponge mesohyl, and control hybridization with no probe led to no immediate signal (data not shown). These experiments confirmed that the archaeal 16S DNA sequences obtained correspond to the filamentous Archaea.

16S rDNA sequencing

Because the in situ hybridization experiments indicated that the filamentous symbionts are Archaea, PCR amplification was performed with the Archaea-specific primers Arch 21F and ME 1354R on five DNA samples from different specimens of each *Axinella* species, one from each collection site, in order to check the possible variations over space and time. We obtained a 1300 bp product from each of the five DNA samples of each species. Direct sequencing of these PCR products yielded clean sequences: we found no ambiguities when analyzing each chromatogram. This result indicates, as the in situ hybridization results suggested, that a single archaeal phylotype could be present in each sample. Moreover, when aligning the sequences, the ones obtained from DNA samples of the same *Axinella* species were identical, independent of the specimen collection site or time, and they were different from the ones obtained from the other two species. These results confirm that a single phylotype of archaeon is associated with each *Axinella* species and that this association is species specific and stable over space and time.

The three archaeal sequences obtained from the three *Axinella* species were compared to each other and to the GenBank database using BLAST. They are closely related, showing a similarity of 99% between *A. damicornis* and *Axinella* sp. symbionts, 97% between *A. damicornis* and *A. verrucosa* symbionts and 97% between *A. verrucosa* and *Axinella* sp. symbionts. They are related to the marine "group 1" Crenarchaeota with a similarity of 91–92% (DeLong 1992; DeLong et al. 1999) and their nearest known relative, with a similarity of 94%, is *Cenarchaeum symbiosum* (Preston et al. 1996), a crenarchaeal symbiont from the Pacific sponge *Axinella mexicana*. This Pacific *Axinella* species harbors two phylotypes of symbionts, while the three species of Mediterranean *Axinella* from this study contain a single phylotype each, as we conclude from the chromatograms and the fact that all the sequences obtained from the same sponge species are identical.

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Fig. 3a–e *Axinella damicornis*. In situ hybridizations with Cy5-labeled probes (**a**, **b**) and the corresponding fields at the Texas red wavelength (**d**, **e**), where the filaments (*white arrow*) and sponge tissues (*t*) are clearly visible. **a** Hybridization with the Archaea-specific probe Arch-915. The signal only appears in the filaments. **b** Hybridization with Eub-338, universal eubacterial probe. The signal appears in the sponge tissue, where associated unicellular eubacteria are found. There is no signal in the filaments. **c** Control hybridization with no probes. **f** Corresponding field for c at the Texas red wavelength

Conclusions

In this study we have demonstrated the consistent presence of a high concentration of filamentous Archaea in the perispicular collagen of three species of sponges from the family Axinellidae. Their morphology and location have not been described in any of the sponge–microorganism association studies published previously (Vacelet 1975; Vacelet and Donadey 1977; Wilkinson 1978c; Fuerst et al. 1999), nor have they been described

in 15 non-axinellid sponge species studied from the same collection areas. These Archaea have been found in every species of *Axinella* studied, regardless of habitat, site or time of collection. Moreover, the in situ hybridization experiments with specimens that have been maintained alive in our aquaria for 3 and 6 months suggest that the Archaea retain their metabolic activity over time. Molecular studies have revealed that the filamentous Archaea from the three *Axinella* are closely related and are species specific, with a single phylotype inhabiting each sponge species. They are closely related to *C. symbiosum*, the archaeon found in a sponge from the same genus, *A. mexicana*, although this sponge harbors two phylotypes of the archaeon and they seem to be unicellular (Preston et al. 1996; Schleper et al. 1998). Several attempts have been made to cultivate these Archaea, with no success, suggesting that they may have metabolic needs perhaps only provided by their host sponges. The results of this study suggest a novel example of a species-specific symbiosis between several *Axinella* sponge species and filamentous Archaea within the sponge perispicular collagen. Further studies are needed to determine the biological, biochemical and ecological interactions in the symbiotic associations.

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