July 2001

Phylogenetic Diversity of Bacteria and Archae Associated with the Marine Sponge *Suberites ficus*

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Abstract

The microbial community associated with the marine sponge *Suberites ficus* was investigated using molecular and cultivation techniques. The present study has shown that members of the bacterial groups Cytophoga, Pseudoaltermonas, Vibrio, Photobacterium and Bacillus are associated with this sponge. The motile bacteria Pseudoaltermonas sp. SF2 showed positive chemotaxis towards the crude extract of its sponge host.

Introduction

The biology of bacterium-sponge relationships has elicited considerable interest among researches investigating marine organisms as sources of bioactive natural products. Antimicrobial compounds have been isolated from sponge-associated bacteria and has prompted the suggestion that microbial symbionts play a role in the defense of their host sponge (Webster et al. 2001). Sponge-microbe associations involve a diverse range of heterotrophic bacteria, cyanobacteria, facultative anaerobes, unicellular algae, and Archaea (Webster and Hill 2001). Marine sponges produce a wide array of bioactive natural products/ secondary metabolites (see review by Faulkner 2001). In some cases, the origin of these compounds has also been shown to be bacteria associated with sponge. For example, the antifungal peptide theopalauamide, isolated from the marine sponge *Theonella swinhoei*, was shown to be contained in a novel δ-proteobacterial symbiont (Schmidt et al. 2000).

Several suggested benefits that these symbionts may provide to the sponge include: nutrition through direct incorporation of dissolved organic matter in the seawater, nutrition through translocation photosynthates from symbiont cyanobacteria, transposition of metabolites throughout the sponge mesohyl, contribution to sponge rigidity, removal of waste products when the sponge is not circulating water, and assistance in chemical defense (Webster and Hill 2001).

Secondary metabolite production can be assigned to symbiotic microorganisms only when synthesis has been demonstrated in cultures isolated from the host species and it is still possible that the host is simultaneously producing these compounds (Webster et al 2001). Because of the difficulty in defining a particular heterotrophic bacterium as the source of a

bioactive compound in many cases, it will be essential to culture symbiotic microbes before being able to unambiguously assign secondary metabolite production to them. The advantages of culturing symbionts that produce these bioactive natural products include consistent yield and large-scale production without the need for harvesting potential pharmaceutical important sponges from their natural environment, which in some cases have limited availability (Webster and Hill 2001, Webster et al. 2001).

Suberites ficus (Demospongiae, Suberitidae) is a common Cape Cod sponge (Weiss 1995). The microbiology and natural products chemistry of this sponge species has not been investigated before. The aim of the present study was to investigate the diversity of the microbial community within this sponge through molecular and cultivation techniques.

Material and Methods

Sample collection. Specimens of the marine sponge *Suberites ficus* (Fig. 2) were collected by trawling at depth of 35 m (107 ft.) from a site 3 miles SW of Gay Head, Martha's Vineyard, MA (see Fig. 1) using the R/V Gemma (Marine Biological Laboratory (MBL), Woods Hole). Sponges were washed four times with autoclaved artificial seawater (ASW, 20g NaCl, 3 g $MgCl_2$:$6H_2O$, 0.15 g $CaCl_2$:$2H_2O$, 0.2 g KH_2PO_4, 0.5 g KCl in 1L d H_2O) to remove unattached or loosely attached bacteria. Samples for microbiological and molecular work were maintained in plastic bags containing ASW and transferred to MBL and immediately were used. Several samples were kept on ice and then placed at -80°C. The remaining samples were kept in running seawater and maintained at the MRC (Marine Resource Center, MBL) at running seawater tanks.

Bacterial isolation and enumeration. All isolation procedures were performed aseptically. A 1 cm^3 portion from the inner and surface of three replicate sponge samples were rinsed four additional times with ASW, cut into small pieces using a sterile scalpel, and macerated with 1 ml ASW with a glass rod. This material was mixed by vortexing for 3 min. Tenfold serial dilutions of the suspension were prepared to a dilution of 10^{-5} with ASW. For isolation of heterotrophic marine bacteria, 100 µl of each dilution was spread-plate in duplicates on Bacto Marine Agar 2216 (Difco). One copy of the plates was incubated aerobically at room temperature (RT) for 3-21 days. The second copy was incubated at the same conditions but anaerobically in GasPak (BBL) jars. An ASW control was prepared as

well. A representative of each colony morphotype was serially streaked on Marine Agar 2216 plates until pure cultures were obtained. For the isolation of cyanobacteria, a small (~ 5 mm^2) piece of sponge was inoculated into 100-ml SNAX (250 ml ddH_2O, 750 ml filtered seawater, 10 μM Na2CO3, 1 mM NaNO3, 100 μM NH4Cl (2 mM), 10 μM K2HPO4 (20 μM), 0.25 ml Cyano trace metals (0.222 g ZnSO4:7H2O, 1.40 g MnCl2:4H2O, 0.025 g Co(NO3)2:6H20, 0.39 g Na2MoO4:2H2O, 6.25 g citric acid hydrate, 6 g ferric ammonium citrate in 1 L ddH20), and 1.5 μM EDTA) liquid media and on SNAX plates and incubated at 22°C 15:9 Light:Dark cycle for 3 weeks.

Bacterial identification by 16S rDNA sequence analysis. DNA from the six strains SF1-SF6 (see Results) was obtained by suspending a colony into 10 μl of sterile ddH20 and incubation at 95C for 5 min. The tube was then microcentrifuged for 1 min at 14,000 rpm, and 1 μl of supernatant was used for the PCR amplification of ribosomal DNA. Eubacterial-universal oligonucleotide primers [forward primer, SDBact0008F20 (~20 pmol): 5'agagtttgatcctggctcag3' and reverse primer, SDBact1492R19 (~20 pmol): 5'ggttaccttgttacgactt3'] and archaeal-universal primers [forward primer, Arc89F: 5'gctcagtaacrcgtrg3' and reverse primer, Arc915R: 5'gtgctcccccgccaattcc3'] were used to amplify 16S rRNA gene fragments from the strains. The PCR products were checked for the presence of amplified 16S rRNA gene fragments by agarose gel electrophoresis. PCR products that contained amplified 16S rRNA gene fragments of unique sponge strains were sent out for sequencing. Sequence data were analyzed using the ARB software. All sequences were aligned to *E. coli*. The nearest relatives of each organism were obtained and phylogenetic trees were then inferred by comparing homologous nucleotides using the neighbor-joining algorithms in the ARB package and printed using the Phylodentron software.

Cloning. Genomic DNA was extracted from washed tissues of inner and surface three sponge samples using the MoBio UltraClean DNA Isolation Kit. DNA was quantified using an Eppendorf BioPhotometer, and PCR was performed using 20 ng of DNA with Eubacterial and Archaeal universal primers. PCR products were applied on an agarose gel. Since the DNA showed good amplification with Archaeal primers, these PCR products were cloned with the TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen). Plasmid inserts from 36 clones were PCR amplified using the M13 forward and

reverse primers and 5 successful (A1-A5) amplified PCR products were sent out for sequencing. Sequence data were analyzed and phylogenetic trees were made as described for the unique sponge isolates.

Chemotaxis assays. Agarose plug assays were carried out as previously described (Yu and Alam 1997, Parales et al. 2000) with slight modifications. Plugs contained 2 % low-melting-temperature agarose (SeaPlaque GTG Agarose, FMC Bioproducts) in ASW, and few µl of 0.5 % Nile blue to provide contrast. A drop (10 µl) of melted agarose with the test solution (sponge extract or control) mixed in 1:1 ratio was placed on a microscope slide, and a coverslip supported by two plastic strips was then placed on top to form a chamber. Strain SF-2 (see Results) was grown in Marine Broth 2216 (Difco) at 30C, 250 rpm, and the cells were harvested in log phase, resuspended in ASW, and flooded into the chamber to surround the agarose plug. Sponge extract were prepared by extracting sponge tissue with 1:1 dichloromethan:methanol for 24 hr at RT. The extract was then filtered with Whatman filter paper and the solvent was evaporated with N_2 gas. The crude extract was then weighed and dissolved in 0.5 % ethanol in ASW to a concentration of 1 mg/mL. Positive controls were made with 10 % Casamino Acids in ddH2O and negative controls were made as 0.5 % ethanol in ASW. Modified capillary assays were also performed essentially as previously described (Grimm and Harwood 1997, Parales et al. 2000). Capillaries (1 µl) contained sponge extract or controls in 1 % low-melting-temperature agarose dissolved in ASW. Freshly grown cells were suspended in ASW and placed in a chamber formed by a microscope slide, a glass U-tube, and a coverslip, and the capillary containing sponge extract or control was inserted into the pool of cells. Cell behavior was observed at a ×40 magnification.

Results

Isolation and enumeration of bacteria. Six colony morphotypes were isolated from the Marine Agar 2216 plates inoculated with healthy *Suberites ficus* sponges. These strains appeared at all three replicates and at the various dilution plates. One strain (SF-1) had yellow colonies and the cells were rod-shape and non-motile, another strain (SF2) was motile coccibacilli-shape and the remaining four were non-motile facultative anaerobes (SF3, SF5, SF6- rods; SF4- cocci).

No cyanobacterial growth was found on the SNAX plates and liquid media in the period of the three-week study. However, swarming and agar-degrader colonies appeared on the plates surrounding the sponge tissue.

Phylogenetic analysis of isolated strains. The six isolated strains were not amplified with the Archaeal universal primers, but did so with the bacterial ones. Based on 16S rRNA sequence analysis (Fig. 3), SF1 was found to be a member of the Cytophoga group, SF2 a Pseudoaltermonas spp., SF3 and SF4 were from the Photobacterium group, and SF5 and SF6 fell into the Vibrio species group (Fig. 3).

16S rRNA gene cloning analysis. PCR of total DNA isolated from six sponge *Suberites ficus* tissue samples using 16S rRNA primers specific for Archae yielded a band of expected size of 950 bp. These PCR products were cloned into the vector pCR2.1-TOPO, yielding 6 clone libraries with 60-100 white colonies each. 36 of these clones were run with M13 primers, but only 5 clones were amplified and subsequently sequenced. 16S rRNA sequence analysis (Fig. 4) revealed that these five "supposable" Archaeal clones reside within the Bacillus species group.

Chemotaxis assays. Using the agarose plug assays, a chemotactic response was observed in the form of band of cells of the strain SF2 that accumulate in a ring surrounding, but not touching, the sponge extract-containing agarose plug (Fig. 5).

Discussion

The culturable heterotrophic bacterial community associated with the sponge *Suberites ficus* identified in this study consisted of several groups of bacteria. These include, Cytophoga, Pseudoaltermonas, Vibrio, and Photobacterium (Fig. 3). Due to the limited time of this study not all the colonies were screened and therefore it is expected that more groups of bacteria exist in association with this sponge. However, these groups of bacteria were found to be associated with other sponges like the Great Barrier Reef sponge *Rhopaloeides odorabile* (Webster and Hill 2001).

The results of the sequence analysis of cloned 16S rDNA fragments (Fig. 4) were surprising as they fell into the gram-positive Bacillus group, instead of Archae, which they were originally been amplified. This could have been cause by unspecific amplification for some reason. The fact that clone libraries of other sponge species fell also in the Bacillus

group (Webster et al. 2001) leads to suggest that *Bacillus spp.* could well be associated with the sponge *S. ficus*. Screening of the remaining clones is therefore warranted.

It was interesting to observe that the sponge-associated *Pseudoaltermonas* sp. SF2 responded in a positive chemotaxis towards the crude extract of the sponge host. To the best of my knowledge, this is the first evidence of a chemotaxis of a microbe towards an invertebrate-host. The only evidence I found in the literature of chemotaxis of bacteria to an associated organism is of that of Paerl and Gallucci (1985), who showed that a pseudomonad microbe is chemotactic towards its associated nitrogen-fixing cyanobacterium, *Anabaena oscillarioides*. These preliminary results prompt further investigation of the chemotaxis response of a sponge-associated bacterium to its host.

Acknowledgements

I would like to thank the Directors, Faculty and Teaching Assistants of the MBL 2001 Microbial Diversity Course for their continuing guidance and help. I thank also the staff of MBL's MRC and R/V Gemma, especially E. Enos and B. Klim for their help in the sponge collection. Financial assistance to D.K. was made by The Moshe Shilo Memorial Scholarship Fund and is greatly appreciated.

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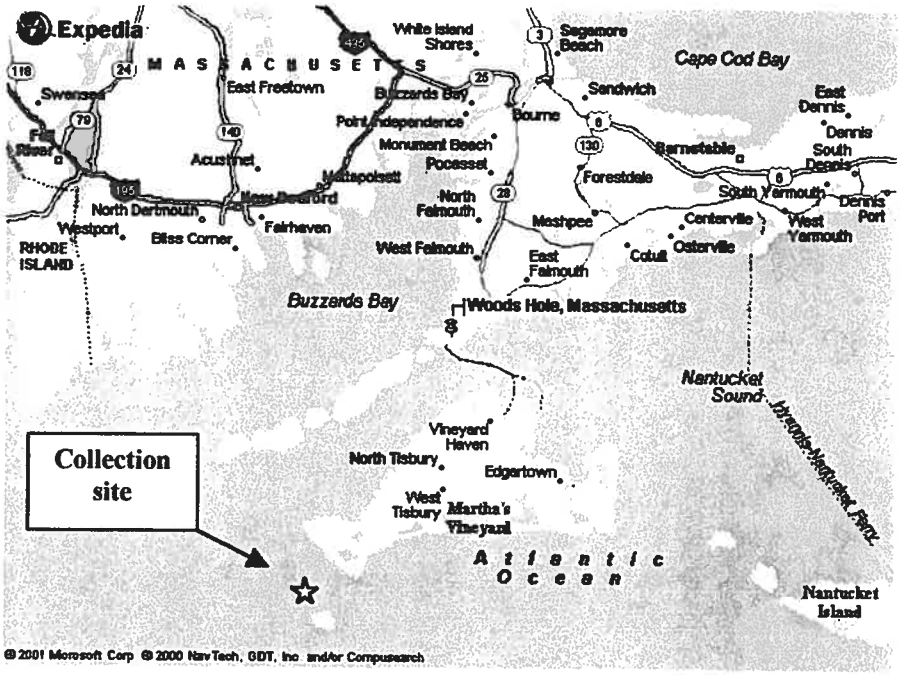


Fig. 1. Sponge collection site

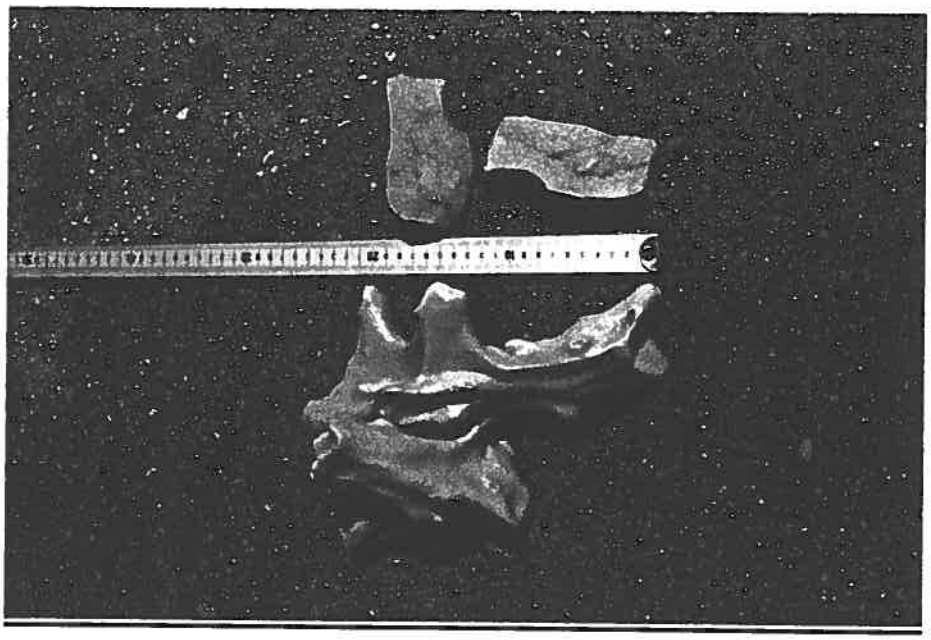


Fig. 2. The marine sponge *Suberites ficus*

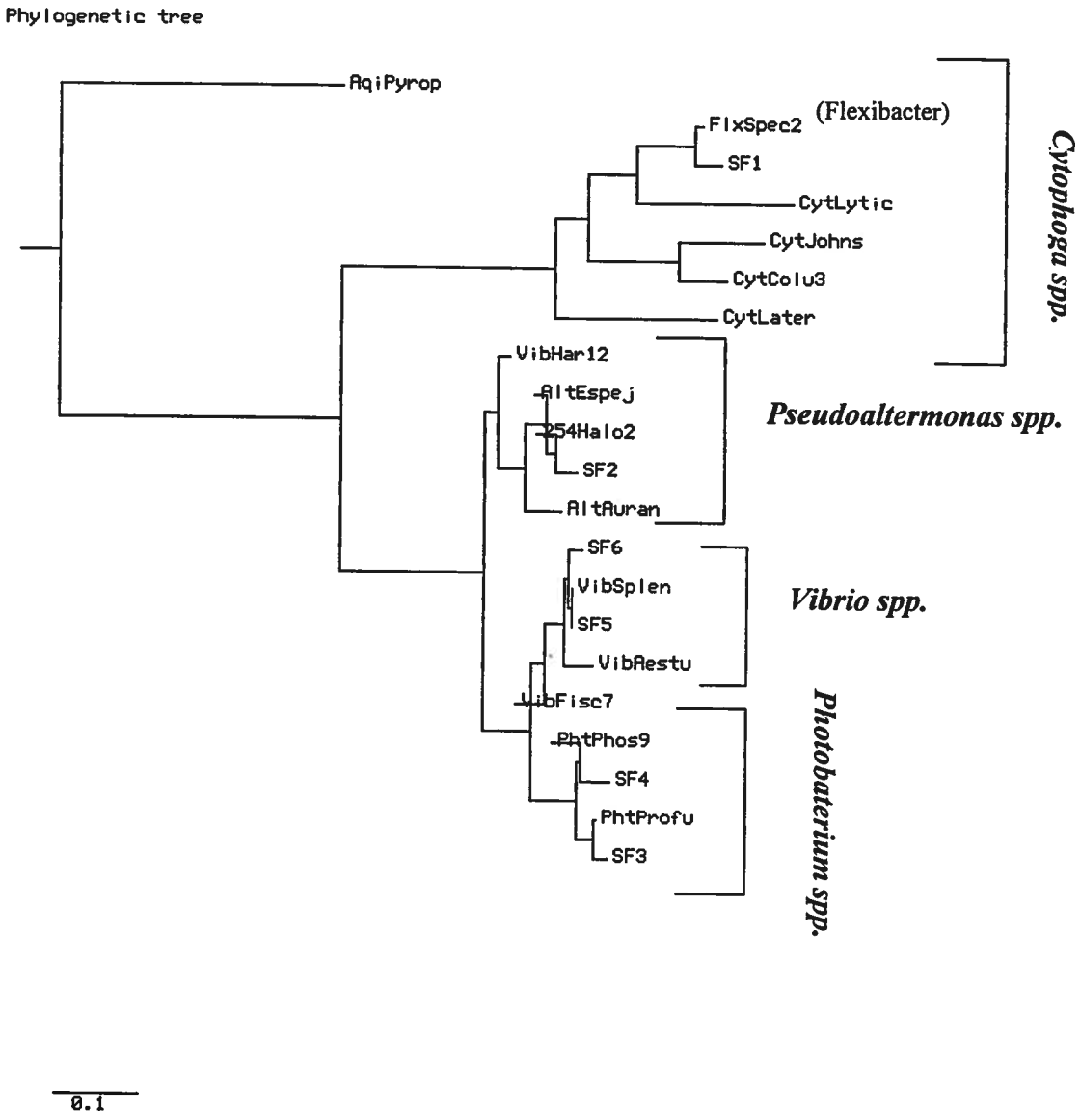


Fig. 3. Phylogenetic analysis of sponge-bacterial isolates

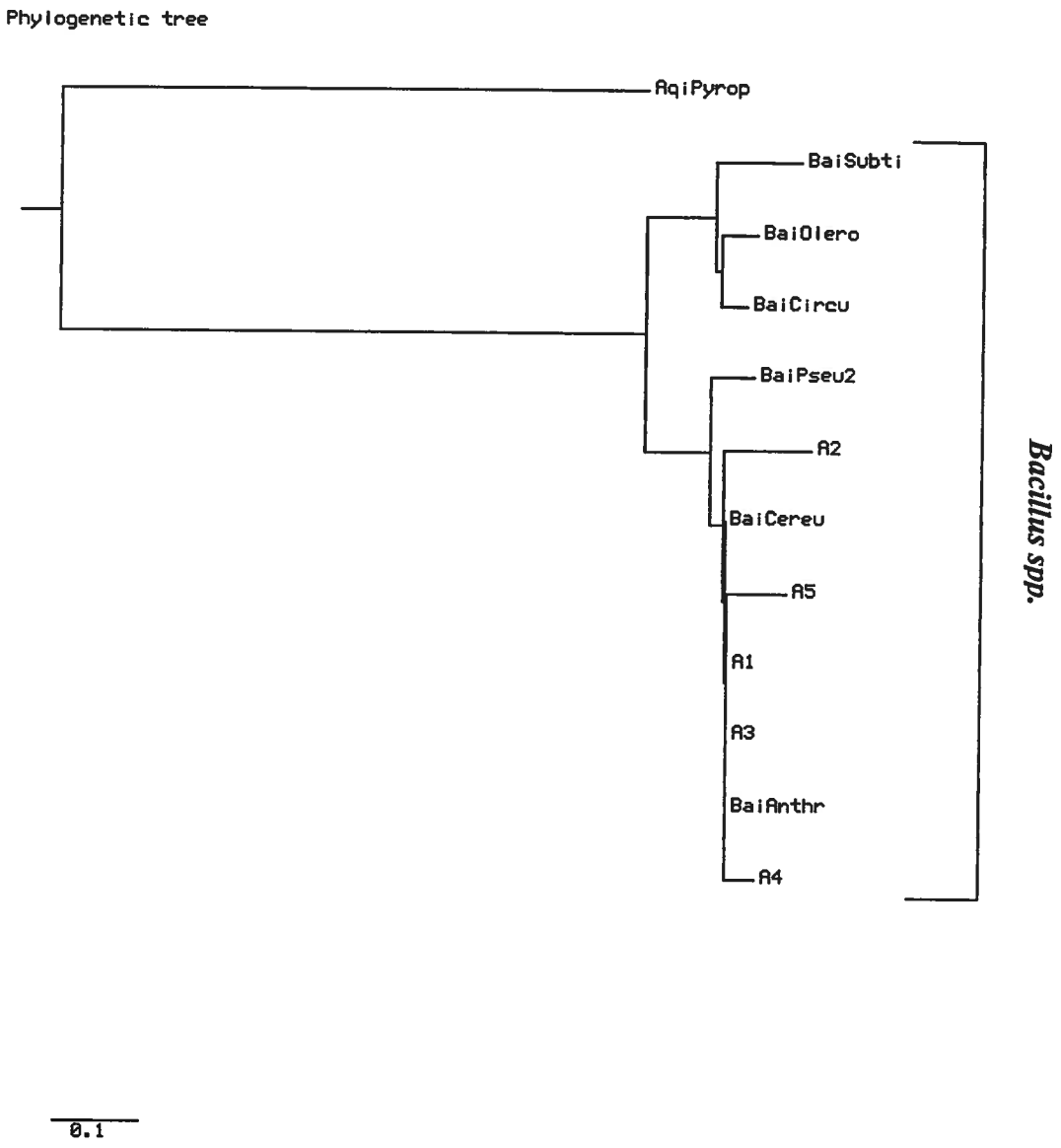
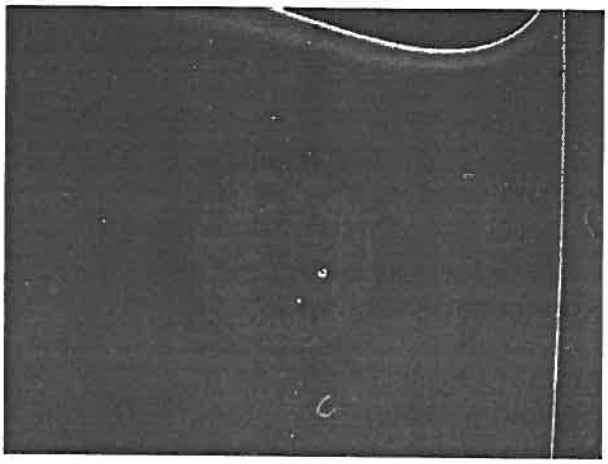


Fig. 4. Phylogenetic analysis of sponge clone sequences

A



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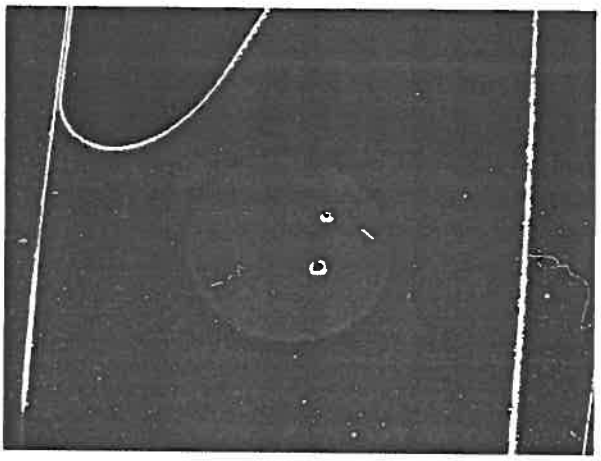

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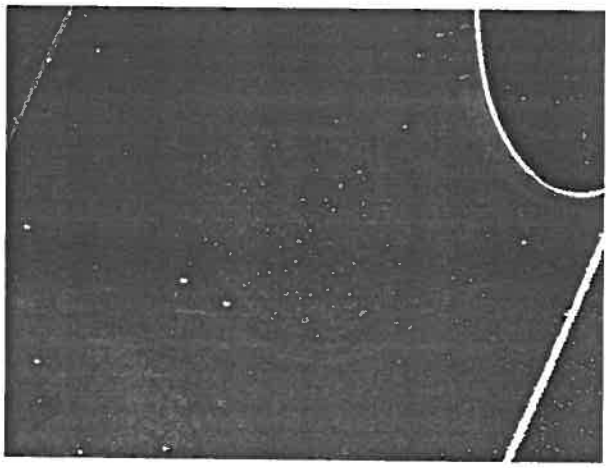

Fig. 5. Chemotaxis agarose-plug assays. (A) Positive control (with casamino acids); (B) negative control (0.5% EtOH in ASW); (C) sponge extract (1 mg/ml)