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The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium

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Abstract The microbial community cultured from the marine sponge *Rhopaloeides odorabile* Thompson et al. is dominated by a single bacterium, designated strain NW001. Sequence analysis of 1212 bp of the16S rRNA gene of strain NW001 indicates that it is a member of the α-subgroup of the class Proteobacteria. The association between this bacterium and its host sponge was observed in healthy *R. odorabile* collected from six different reefs in the Great Barrier Reef representing a geographic distance of 460 km, and in four collections in different seasons in 1997–1998 at Davies Reef (18°49.6′S; 147°34.49′E). The proportion of colonies of strain NW001 in samples from *R. odorabile*, expressed as a percentage of the total heterotrophic bacterial colony count, showed no significant spatial (range: 81–98%) or temporal differences (range: 81–99%), although colony counts of strain NW001 varied by up to two orders of magnitude between reef sites and sampling periods. The location of strain NW001 within the sponge mesohyl was visualized by in situ hybridization, using fluorescently labeled probes based on the 16S rRNA gene sequence of this strain. Cells of strain NW001 surround the choanocyte chambers, suggesting that these bacteria may play a role in nutrient uptake by the sponge. The absence of strain NW001 from corresponding seawater samples indicates that it has a specific, intimate relationship with *R. odorabile* and is not being utilized as a food source. A unique cyanobacterium related to the genera *Leptolyngbya* and *Plectonema* was also isolated from *R. odorabile* and characterized by 16S rRNA gene sequencing.

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Introduction

Sponge–microbe associations involve a diverse range of heterotrophic bacteria, cyanobacteria, facultative anaerobes, unicellular algae, and Archaea. Suggested benefits that these symbionts may provide to the sponge include: nutrition through direct incorporation of dissolved organic matter in the seawater (Wilkinson and Garrone 1980), nutrition through translocation of photosynthate from symbiotic cyanobacteria (Wilkinson 1983), transportation of metabolites throughout the sponge mesohyl (Borowitzka et al. 1988), contribution to sponge structural rigidity (Wilkinson et al. 1981), and assistance in chemical defense (Unson et al. 1994). Facultative anaerobic symbionts metabolize a wide range of compounds and may be involved in removing waste products whilst the sponges are not circulating water (Wilkinson 1978a).

Sponges are widely recognized as rich sources of novel compounds and bioactive secondary metabolites (e.g. Conte et al. 1994; Perry et al. 1994; Shigemori et al. 1994; Brantley et al. 1995; Hirota et al. 1996; compounds recently isolated from sponges are reviewed by Faulkner 2000). Traditionally these compounds have been attributed to the sponges, but there is growing interest in the possibility that, in some cases, compounds are being synthesized by symbiotic bacteria within the sponge tissue (Stierle et al. 1988; Bultel-Poncé et al. 1997; Bewley and Faulkner 1998; Schmidt et al. 2000). There is good evidence for this in only a few cases, based on studies where sponge and symbiont cells are physically separated and analyzed. Unusual polychlorinated compounds were located in a cyanobacterial symbiont of the sponge *Dysidea herbacea* found in Australia (Unson and Faulkner 1993), whereas in specimens of the same sponge collected in Palau, the symbiotic cyano-

bacteria contained a brominated biphenyl ether (Unson et al. 1994). Two bacterial cell fractions were isolated from the sponge *Theonella swinhoei*, and the bioactive compounds swinholide A and theopalauamide were localized in symbiotic unicellular bacteria and filamentous δ-Proteobacteria, respectively (Bewley et al. 1996; Schmidt et al. 2000). Because of the difficulty in defining a particular heterotrophic bacterium as the source of a bioactive compound using techniques based on cell separation, Faulkner et al. (1999) concluded that, 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 secondary metabolites include consistent yield and large scale production without the need for harvesting sponges from their natural environment.

Rhopaloeides odorabile was selected as a model sponge in which to investigate the diversity and spatial and temporal changes in a sponge-culturable microbial community. *R. odorabile* is a dictyoceratid sponge common throughout the Great Barrier Reef region. The chemistry of this sponge is distinctive, being characterized by a rare group of C_{20} diterpene furanodiols, triols, and their peracetates. Substantial variation in the yield and composition of the diterpenes for different collections of *R. odorabile* have been shown to reflect the range of environmental conditions under which they live (Thompson et al. 1987). The readily cultivated microbial symbionts associated with *R. odorabile* were isolated as a first step in ascertaining whether any of these microbes are implicated in the production of bioactive compounds. However, the primary goal of the study was to investigate whether the culturable microbial assemblage of *R. odorabile* was constant or varied markedly among individuals in a single location, between widely dispersed geographic locations, or at different sampling periods.

Materials and methods

Sampling

Specimens of the marine sponge *Rhopaloeides odorabile* Thompson et al. (class Demospongiae; order Dictyoceratida; family Spongiidae) were collected at 8–12 m by SCUBA between December 1997 and December 1999 from Palm Island (18°44.63′S; 147°40.34′E), Trunk Reef (18°18.51′S; 146°51.79′E), Britomart Reef (18°11.73′S; 146°48.54′E), Myrmidon Reef (18°15.93′S; 147°22.60′E), Davies Reef (18°49.53′S; 147°38.45′E), and Lizard Island (14°41.40′S; 145°27.94′E), in the northern Great Barrier Reef region. All samples were collected in channels of moderate to high current, which is the only place where this species occurs. All sponges for the temporal analysis were collected from Davies Reef on 20 August 1997, 8 November 1997, 9 January 1998, and 5 May 1998. Sections of the sponge were removed using sterile scalpel blades, and the tissue transferred directly into a plastic sampling bag containing seawater. Sponge tissue was processed for microbiology within 15 min of collection. Three individual sponges were sampled, wherever possible within a 20 m radius, and water samples were collected adjacent to each sponge in sterile 50 ml polypropylene tubes.

Bacterial isolation and enumeration

Heterotrophic bacteria

All isolation procedures were performed aseptically. A 1 cm^3 portion of sponge tissue was excised, rinsed briefly in 70% ethanol and rapidly transferred to sterile artificial seawater (ASW). The tissue was removed from the seawater, cut into thin sections using a sterile scalpel, and finely ground with a mortar and pestle. This material was suspended in 9 ml of sterile ASW and mixed by vortexing for 10 min. Tenfold serial dilutions of the suspension were prepared to a dilution of 10^{-4}, and 100 µl of each dilution was spread-plated in triplicate on Bacto Marine Agar 2216 (Difco Laboratories, Detroit, USA), a medium designed for isolation and enumeration of heterotrophic marine bacteria. All plates were incubated at 27 °C for 72 h, and representatives of each colony morphotype were serially streak-plated on Marine Agar 2216 until pure cultures were obtained. Plates were retained for an additional 2 months to allow for the isolation of slow-growing bacteria. Counts of total numbers of culturable bacteria were recorded as colony forming units (CFU) ml^{-1} of sponge tissue. Total bacterial counts were determined by epifluorescent microscopy as described by Porter and Feig (1980). Briefly, formaldehyde-preserved cell suspensions, stored at 4 °C until processing, were filtered onto 0.2 µm filters, stained with 4′6-diamidino-2-phenylindole (DAPI), and enumerated by fluorescent microscopy.

Cyanobacteria

Cell suspensions, prepared as described above, were inoculated into 10 ml MN + B12 broth (Waterbury and Stanier 1978), for culture of oxygenic phototrophs (including cyanobacteria). Clear polypropylene vials containing MN + B12 were incubated under natural light conditions at room temperature for up to 8 weeks. Vials showing turbidity were serially diluted until uni-cyanobacterial cultures were obtained, as indicated by microscopic examination.

Bacterial identification by 16S rRNA sequence analysis

The heterotrophic bacterium strain NW001 and cyanobacterium strain NW4194 were identified by 16S ribosomal RNA (rRNA) sequence analysis. Isolate NW001 was grown overnight in 100 ml Marine Broth 2216 (Difco Laboratories) and NW4194 was grown in 1000 ml MN + B12 under fluorescent light for 21 days. Total DNA was extracted using a method based on that of Ausubel et al. (1987). Eubacterial-specific oligonucleotide primers [forward primer 8-27: 5′-GAGTTTGATCCTGGCTCAG-3′ (Weisburg et al. 1991) and reverse primer 1492: 5′-GGTTACCTTGTTACGACTT-3′ (Reysenbach et al. 1992)] were used to amplify 16S rRNA gene fragments from NW001. Cyanobacterium and plastid-specific primers (Nübel et al. 1997) were used to amplify the 16S rRNA gene fragment from NW4194 [CYA106F: 5′-CGGACGGGTGAGTAACGCGTGA-3′; CYA359F: 5′-GGGGAATYTTCCGCAATGGG-3′; CYA781R(a): 5′-GACTACTGGGGTATCTAATCCCATT-3′; CYA781(b): 5′-GACTACAGGGGTATCTAATCCCTTT-3′]. PCR fragments were purified using the Microcon 30 system (Amicon, Beverly, Mass.), and sequenced on an ABI 310 automated sequencer using the PRISM Ready Reaction Kit (PE Applied BioSystems). Sequence data were analyzed by comparison to 16S rRNA genes in the Ribosomal Data Base Project (Maidak et al. 1999) and the Genbank database. All sequences were manually aligned to *Escherichia coli* using Phydit software (Chun 1995). The nearest relatives of each organism were obtained by BLAST searches (Altschul et al. 1990). Phylogenetic trees were then inferred by comparing homologous nucleotides using the neighbor-joining (Saitou and Nei 1987), Fitch–Margoliash (Fitch and Margoliash 1967), and maximum parsimony (Kluge and Farris 1969) algorithms in the PHYLIP package (Felsenstein 1993). Evolutionary distance matrices for the neighbor-joining and Fitch–Margoliash methods were generated as described by Jukes and

Cantor (1969). Tree topologies were evaluated after 1000 bootstrap re-samplings of the neighbor-joining data.

Sponge cell separation

Cell separation procedures were performed aseptically on freshly collected sponge material. Ficoll solutions of 26.3, 21.7, 19.0, 14.3, 10, and 5% (w/v) were prepared in Ca/Mg-free seawater (CMF-ASW). Sponge tissue (500 g) was rinsed in aerated CMF-ASW to remove natural seawater. CMF-ASW prevents re-aggregation of dissociated sponge cells (Pavans de Ceccatty 1974). Sponge tissue was sectioned into small fragments (< 0.5 cm diameter), placed in 250 ml CMF-ASW solution containing 50 mg proteinase K, and held at 28 °C for 30 min. Ficoll gradients were prepared in 50 ml polypropylene centrifuge tubes and stored on ice until use. Cells were further dissociated by squeezing through sterile cheesecloth. This cell suspension was centrifuged at 600*g* for 5 min, and cells were resuspended in ca. 100 µl of CMF-ASW and loaded onto Ficoll gradients. Gradients were centrifuged at 600 *g* for 5 min. Bands of cells between the Ficoll layers were individually removed and placed in sterile 30 ml centrifuge tubes on ice. The cells were rinsed in sterile CMF-ASW to remove traces of Ficoll. A small quantity of each cell fraction was fixed in 2.5% glutaradehyde (in 0.1 M sodium cacodylate buffer) for electron microscopy and the remainder stored at 4 °C until use. In an attempt to increase the number and diversity of culturable bacteria obtained from sponge tissue, 100 µl of each cell fraction was spread on Marine Agar 2216. Plates were incubated and processed as described above for isolation of heterotrophic bacteria.

Transmission electron microscopy

R. odorabile sections were cut into small pieces (ca. 1 mm diameter) and fixed for 20 h in 0.1 M sodium cacodylate buffer, pH 7.4, prepared in ASW and containing 2.5% (v/v) glutaraldehyde. Fixed samples were removed, placed in fresh 0.1 M sodium cacodylate buffer, and stored at 4 °C until further processing. Fixed tissue was placed in a 1% (w/v) osmium tetroxide solution (prepared in 0.2 M potassium phosphate buffer, pH 7.4) for 3.5 h and subsequently dehydrated in a graded ethanol series [15, 35, 55, 75, 85 and 95% (v/v) ethanol]. Tissue was embedded in Spurr's resin, sectioned with an ultramicrotome, and stained with 2% (w/v) uranyl acetate followed by 0.2% (w/v) lead citrate. Sections were mounted on 200 mesh copper grids coated with carbon and Formvar. Samples were visualized by transmission electron microscopy (Jeol 2000 FX).

Probe design and synthesis

The NW001 specific probe "NW442" (5′-AGTTAATGTCATT-ATCTTCACTGC-3′) was designed by comparative analysis of all closely related sequences for regions which allowed discrimination from other reference sequences. The specificity of this oligonucleotide probe sequence was confirmed by BLAST searches and tested using whole cell hybridizations with strains *Brucella suis*, *Vibrio parahaemolyticus*, *Moraxella* sp. (AJ000645), *Paracoccus* sp. KS1 (U58015), and two taxonomically unidentified α-Proteobacteria MBIC3368 and MBIC3865 (AB012864 and AB015896, respectively). The α-Proteobacteria MBIC3368 has identical sequence to NW001 and was used as a positive control during verifications.

All oligonucleotide probes were 5′ end-labeled with the indocarbocyanine fluorochrome Cy3 and synthesized by MWG Biotech (Ebersberg, Germany). Probes were resuspended in dH_2O to a working concentration of 50 ng μl^{-1}. The domain-level bacterial probe, EUB 338 (5′-GCTGCCTCCCGTAGGAGT-3′, Amann et al. 1990) was used to determine the total density of bacteria residing within *R. odorabile*. A negative control probe with the antisense sequence to EUB338 was used to check for non-specific hybridization. Probe ALF1b [5′-CGTTCG(C/T)TCTGAGCCAG-3′, Manz et al. 1992] was used as a group-specific probe for detection of α-Proteobacteria.

Fluorescence in situ hybridization (FISH)

Tissue from three individual sponges was fixed in 4% paraformaldehyde for 8 h at 4 °C and transferred to 70% ethanol until further processing. Sections of sponge tissue (ca. 1 cm^3) were immersed in a 15% sucrose solution for 3 h and then displaced in a 15% sucrose: OCT (Tissue-Tek, Sakura Finetek USA, Torrance, Calif., USA) series of 3 sucrose: 10 OCT, 1 sucrose: 10 OCT, 1 sucrose: 30 OCT and 100% OCT for a minimum of 2 h at each step. OCT moulds (Sakura Finetek USA) were placed on dry ice and a small quantity of OCT allowed to freeze in the bottom of the moulds prior to the addition of samples. Moulds were allowed to set on dry ice and subsequently stored at − 20 °C. Tissue sections were cut to a thickness of 10 µm using a cryomicrotome (Leitz 1720C) and transferred to silane-coated glass slides (2% Aminosilane: 3-aminopropyltriethoxy-silane, Sigma Pharmaceuticals). Tissue of *R. odorabile* was found to be highly autofluorescent under red, green, and blue filter sets, making dual probe labeling impossible. Prior to hybridization, sections were photo-bleached under a 100 W mercury lamp for 60 s to reduce the autofluorescence of the sponge tissue. Hybridization solution [8 µl 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 20% (v/v) formamide] was mixed with 1 µl of the appropriate fluorescently labeled oligonucleotide and applied to each section of sponge tissue. Samples were incubated in 50 ml polypropylene tubes at 46 °C for 3 h. After hybridization, slides were carefully removed and rinsed immediately in prewarmed wash buffer (20 mM Tris-HCl, 0.01% SDS, 0.225 M NaCl) at 48 °C for 10 min. Slides were rinsed in fresh water to remove excess salts, air-dried, and mounted in the anti-fading glycerol medium, Citifluor (Citifluor, London, UK).

FISH preparations were visualized on a Zeiss Axiophot2 using a 63 × oil immersion objective (Zeiss Plan-Apochromat). Images were captured with a cooled charge-coupled device using Kontron software (KS2000) in the red and green channels using Zeiss filter sets 10 and 15. Final images were merged and manipulated in Adobe Photoshop so that autofluorescence would appear yellow and probe-conferred signal would appear red.

Data analysis

Spatial and temporal variation in bacterial numbers was examined with a one-way analysis of variance using the program Statistica (Statsoft, Tulsa, Okla.).

Results

Isolation and enumeration of bacteria

A single predominant colony morphotype was consistently observed on medium for heterotrophic bacteria (Marine Agar 2216), isolated from healthy *Rhopaloeides odorabile* sponges. This bacterium formed characteristic flattened, brown, highly mucoid colonies and was therefore easily distinguishable. The isolate was designated strain NW001, and counts of this bacterium ranged from 1.0×10^4 to 3.3×10^6 CFU ml^{-1} in 44 individual sponges sampled, while total culturable bacterial counts ranged from 1.9×10^4 to 5.2×10^6 CFU ml^{-1} in the same sponges. Colony counts of strain NW001 exceeded counts of all other morphotypes combined (Fig. 1). During the entire study, a total of 223 different colony morphotypes were observed. Colonies of strain NW001 were never observed on plating of water samples taken adjacent to sponges. There was no significant difference in the proportion of NW001 in

the culturable community among sampling sites ($F = 1.27$, $df = 5$, $P = 0.30$, range = 81–98%) and between sampling periods ($F = 0.611$, $df = 4$, $P = 0.657$, range = 81–99%) (Fig. 1).

On two occasions, *R. odorabile* individuals were observed that were clearly diseased, with the sponge tissue visibly necrosed and the pinacoderm covered with fouling organisms. Strain NW001 was not isolated from either of these diseased sponges, and these were the only *R. odorabile* samples in which strain NW001 was not present as the predominant culturable heterotrophic bacterium.

A cyanobacterium (designated NW4194) was isolated from *R. odorabile* in 100% of samples from Davies and Myrmidon reefs ($n = 4$). It was not possible to enumerate this cyanobacterium since the isolation procedure included an enrichment step in a liquid medium.

Total bacterial counts were determined by epifluorescence microscopy and ranged between 1.5×10^8 and 8.3×10^9 CFU ml^{-1} ($n = 27$). Total bacterial counts in seawater samples collected adjacent to sponges ranged between 1.4×10^7 and 2.7×10^7 CFU ml^{-1} ($n = 6$). The culturable heterotrophic bacterial assemblage comprised only 0.1% (range: 0.001–0.8%) and 0.23% (range: 0.003–0.9%) of the total microbial community in sponge and seawater samples, respectively.

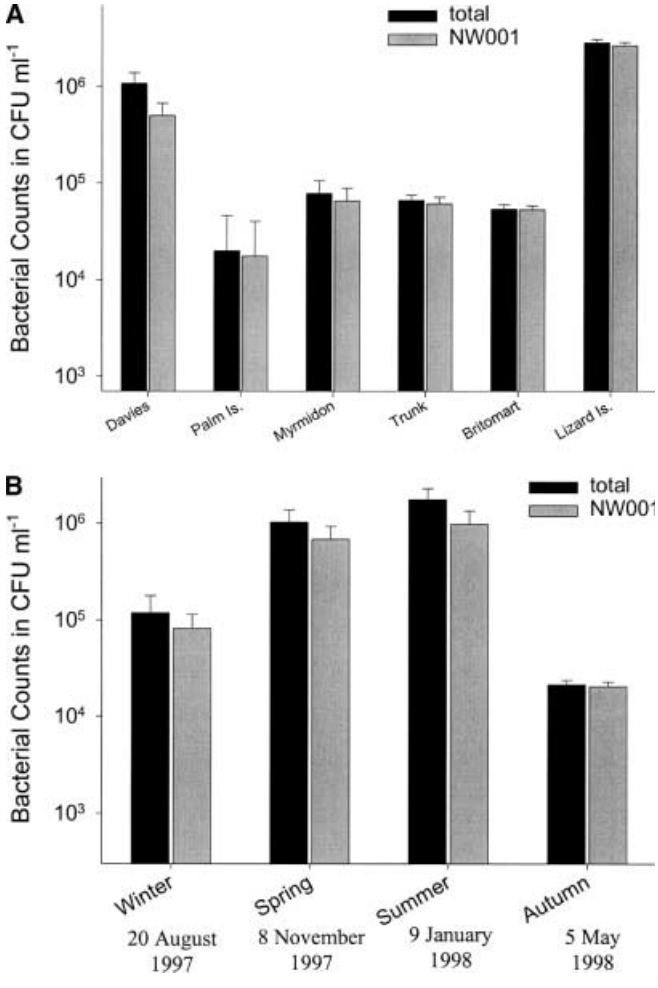

Fig. 1A, B *Rhopaloeides odorabile*. Counts ($n = 3$) of strain NW001 and total heterotrophic bacteria in sponge samples at different reef sites representing a geographic distance of 460 km **A** and at different times at Davies Reef **B**

Phylogenetic analysis

NW001 was found by 16S rRNA sequence analysis to be a member of the α-Proteobacteria. BLAST analysis revealed that all the close relatives of strain NW001 are α-Proteobacteria. Strain NW001 was only distantly related to previously classified members of the α-Proteobacteria that have been assigned to genus and species level, but had identical 16S rRNA sequence to a bacterium previously isolated from an unspecified sponge (Genbank accession number AB012864) and high similarity to a bacterium isolated from *Aplysina aerophoba* (Genbank accession number AF218241) (Fig. 2).

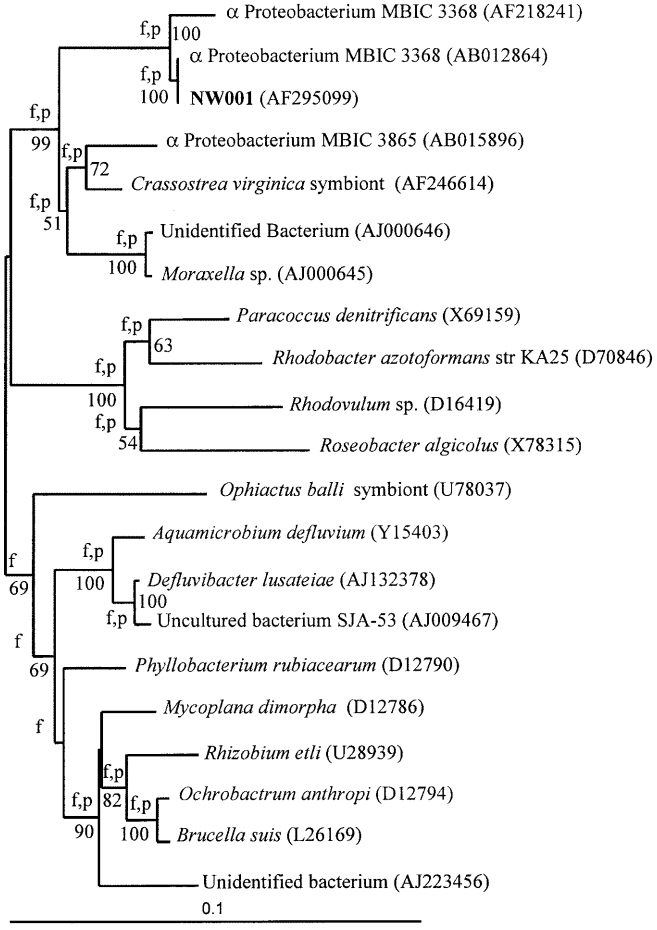

Fig. 2 Unrooted phylogenetic tree of strain NW001 and the corresponding partial sequence of 16S rRNA for related α-Proteobacteria. Neighbor-joining tree based on nearly complete (1212 bp) 16S rRNA gene sequence (*f*, p branches were also found using Fitch–Margoliash or maximum parsimony methods, respectively). *Numbers at nodes* are percentages indicating levels of bootstrap support, based on neighbor-joining analysis of 1000 re-sampled data sets. *Scale bar* = 0.1 substitutions per nucleotide position

NW4194 was confirmed to be a cyanobacterium, most closely related to Oscillatoriales in the genera *Leptolyngbya* and *Plectonema* (Fig. 3).

Localization of strain NW001 by cell separation

Strain NW001 was absent in the least dense and most dense fraction of Ficoll gradients. Despite efforts to separate sponge cells from bacterial cells using centrifugation, NW001 was evenly distributed between the three fractions of intermediate density (counts of 2.1×10^3, 3.3×10^3 and 3.1×10^3 CFU ml^{-1}), indicating that strain NW001 is present within or attached to sponge cells.

Transmission electron microscopy

Preliminary evaluation of sponge tissue by transmission electron microscopy revealed a large diversity of bacterial cell morphotypes and a high density of bacterial cells throughout the sponge mesohyl (Fig. 4). Examination of electron micrographs from an individual sponge revealed at least 29 different bacterial morphotypes within the tissue.

Fluorescence in situ hybridization

FISH studies showed that strain NW001 was localized within the mesohyl region of the sponge tissue, in particular surrounding the choanocyte chambers (Fig. 5A). The α-proteobacterial-specific probe (ALF1b) hybridized with bacterial cells in the same regions of the sponge tissue as strain NW001 (Fig. 5B). In addition, hybridization with a general bacterial-specific probe (EUB338) revealed that *R. odorabile* was host to a high density of bacterial cells which were not recovered using traditional cultivation techniques (Fig. 5C). Although strain NW001 dominates the culturable bacterial community associated with *R. odorabile*, α-Proteobacteria clearly do not dominate the total bacterial community associated with this sponge. Regions of a single sponge specimen which contained cells that were clearly probe-positive were assessed by counting the number of cells per field of view (0.01 mm^2, $n = 6$). Fluorescent cell counts using the NW001 probe averaged 22.0 ± 2.5 per field of view compared with an average of 23.1 ± 4.5 using the ALF1b probe. This indicates that NW001 is the predominant (or possibly the sole) α-Proteobacterium within *R. odorabile*. The negative control probe gave no fluorescent signal from bacterial cells, confirming the absence of non-specific hybridization.

Discussion

The culturable heterotrophic bacterial community associated with *Rhopaloeides odorabile* is dominated by a single bacterium, the α-Proteobacterium strain NW001. This is a novel finding since previous cultivation studies examining bacterial–sponge associations have demon-

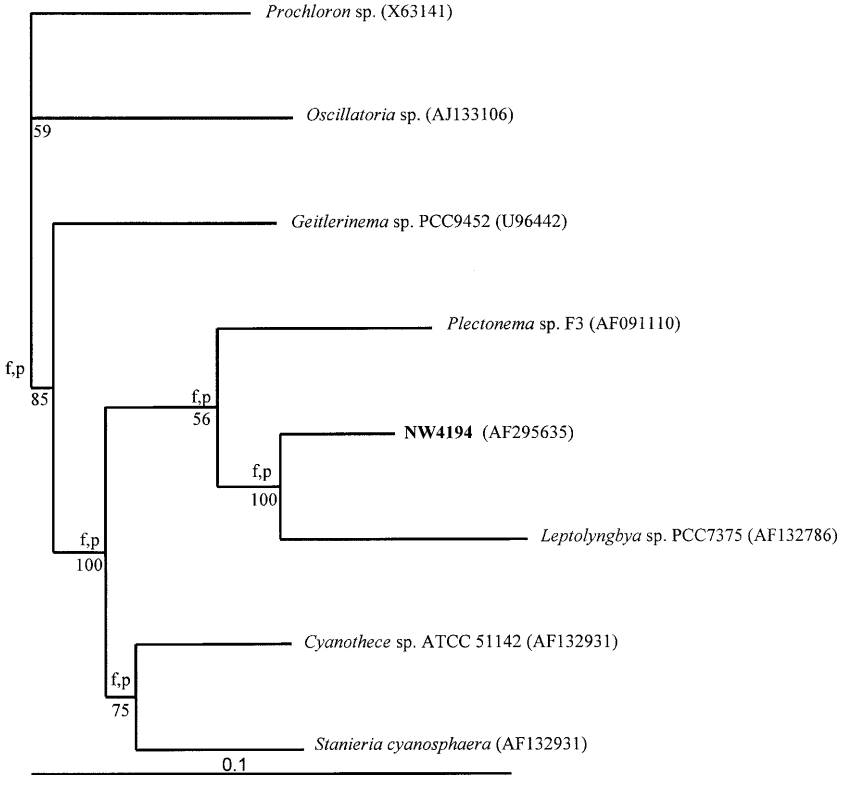

Fig. 3 Unrooted phylogenetic tree of strain NW4194 and corresponding partial sequence of 16S rRNA for related cyanobacteria. Neighbor-joining tree based on 560 bp of 16S rRNA gene sequence (*f*, *p* branches were also found using Fitch–Margoliash or maximum parsimony methods, respectively). *Numbers at nodes* are percentages indicating levels of bootstrap support, based on neighbor-joining analysis of 1000 re-sampled data sets. *Scale bar* = 0.1 substitutions per nucleotide position

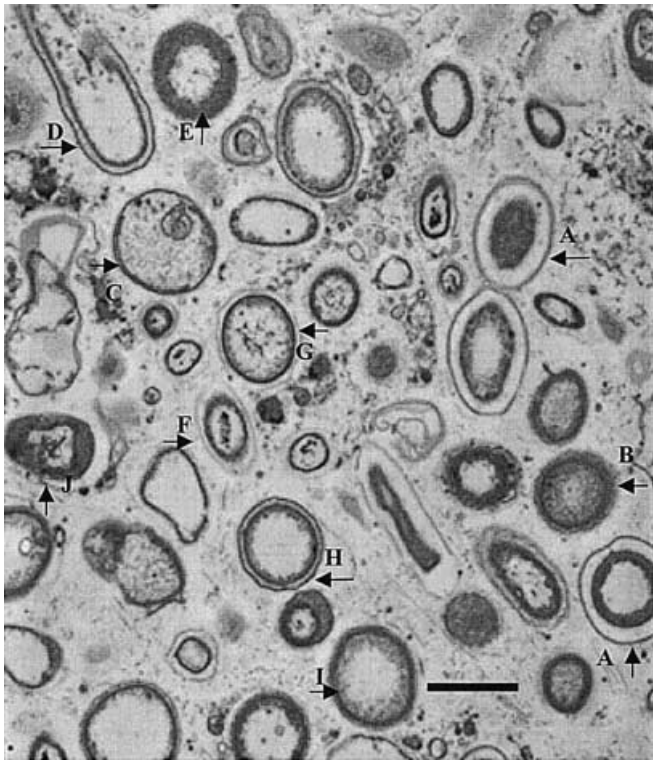

Fig. 4 *Rhopaloeides odorabile*. Transmission electron micrograph of sponge mesohyl showing diversity of bacterial morphotypes present. *A–J* indicate distinct bacterial morphotypes within a single micrograph. A high density of bacterial cells is evident and no sponge cells are present in this micrograph. *Scale bar* = 1 μm

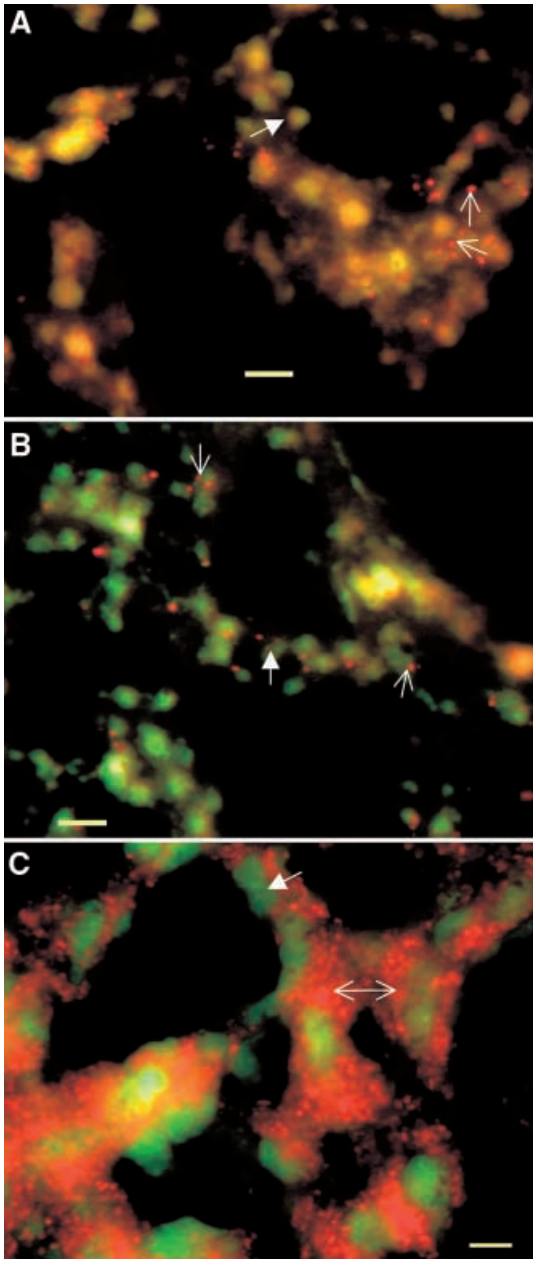

Fig. 5A–C *Rhopaloeides odorabile*. Epifluorescence micrograph of cryosections visualized by FISH with **A** Cy3-labeled NW001 probe; **B** Cy3-labeled Alf1b probe which is specific for α-Proteobacteria; **C** Cy3-labeled EUB338 general bacterial probe. *Scale bar* in all panels = 10 μm. *Solid arrows* indicate individual sponge choanocyte cells which form choanocyte chambers (centers of these aquiferous channels are the dark regions where no autofluorescent cells can be detected). *Outlined arrows* indicate probe-conferred fluorescence where bacterial cells have hybridized with their respective oligonucleotide probes

strated a diverse assemblage of heterotrophic bacteria in the matrix of several sponge species (Wilkinson 1978b; Santavy and Colwell 1990; Santavy et al. 1990). In a study on culturable bacteria from marine sponges from the Mediterranean and the Great Barrier Reef, one symbiont was found to occur in sponges from both regions and a second symbiont was specific to a single sponge species; both symbionts were part of a large, mixed bacterial population associated with the sponges (Wilkinson et al. 1981). The fact that the culturable bacterial community of *R. odorabile* was dominated by a single bacterial strain with characteristic colony morphology provided an excellent opportunity to assess temporal and geographic variations in this component of the community, as well as variations between individual *R. odorabile* sponges at a single location. The occurrence of strain NW001 in the culturable bacterial community from the host sponge was found to be relatively unchanging, being present in *R. odorabile* sampled over a geographic range of 460 km, and on four different occasions in 1997–1998 at Davies Reef. Strain NW001 was ubiquitous in all healthy individuals of *R. odorabile*. The apparent stability of the relationship between strain NW001 and *R. odorabile*, the abundance of strain NW001 in the culturable bacterial community, and the fact that strain NW001 was never isolated from water samples taken adjacent to sponges very strongly suggest that strain NW001 is a true symbiont of *R. odorabile*.

The existence of a close symbiotic relationship is further supported by cell separation studies, in which strain NW001 was always found in fractions containing cells of *R. odorabile*, indicating that strain NW001 is likely to be intracellular within *R. odorabile* sponge cells

or tightly associated with the surface of these cells. The localization of NW001 within the sponge tissue was confirmed using fluorescence in situ hybridization probes. However, it was not possible to determine unequivocally whether strain NW001 was inter- or intracellular.

The mode whereby *R. odorabile* individuals acquire the putative symbiont strain NW001 is unclear. Although attempts to culture strain NW001 from the water column were unsuccessful, it is possible that this bacterium is present in the water column at a low concentration. The detection limit of the procedure used to check for the presence of strain NW001 in water samples is estimated at 10^1–10^2 CFU ml^{-1}. The bacterium could then be sequestered by *R. odorabile* through the aquiferous system of the sponge. Bacteria living symbiotically in sponges were previously shown to pass through their feeding chambers without being digested, suggesting an encapsulation or recognition process (Wilkinson 1987). Specific selection by *R. odorabile* of strain NW001, or rapid growth of a small number of strain NW001 cells that are taken up by the sponge, would then be required in order to obtain the high numbers (10^4–10^6 CFU ml^{-1} sponge tissue) found within *R. odorabile*. Another possibility is that strain NW001 may be acquired by *R. odorabile* via vertical transmission of symbionts from parent sponge to larvae. No gravid sponges were observed during the study period so it was not possible to obtain *R. odorabile* larvae in order to test this hypothesis.

The physiological nature of the putative symbiosis between strain NW001 and *R. odorabile* is not known. An earlier study demonstrated that this microbe utilized none of the carbon substrates (including many carbohydrates) provided to it in an extensive biochemical testing program (Burja et al. 1999). This is in contrast to the bacterial symbionts isolated from *Ceratoporella nicholsoni* which were shown to catabolize a wider variety of substrates than microbes isolated from the surrounding seawater (Santavy et al. 1990). Previous nutrient transfer between symbiotic bacteria and sponges include collagen degradation (Wilkinson et al. 1979) and utilization of dissolved amino acids (Wilkinson and Garrone 1980). Amino acids and small polypeptides are the primary carbon source in Marine Agar 2216, on which strain NW001 was isolated and grows well. This suggests that NW001 may be able to utilize dissolved amino acids in seawater. The ability of strain NW001 to degrade collagen has not been tested. The presence of NW001 around the choanocyte chambers suggests that these bacteria may play a role in nutrient uptake by the sponge since the choanocyte and archaeocyte cells forming these chambers are directly involved in nutrient uptake by phagocytosis.

Strain NW001 is a member of the α-Proteobacteria, and there are two previous reports of closely related α-Proteobacteria from sponges. An α-proteobacterium with an identical 16S rRNA sequence to strain NW001 was previously isolated from an unspecified sponge (Hamada, Genbank accession number AB012864). An α-proteobacterium closely related to strain NW001 was previously isolated from the sponge *Aplysina aerophoba* (Hentschel and Hacker, Genbank accession number AF218241), suggesting that α-Proteobacteria closely related to strain NW001 may be important members of the sponge–microbial community. However, Friedrich et al. (1999) found the δ-Proteobacteria to be most abundant in the microbial community associated with *Aplysina cavernicola*, and were not able to detect α-Proteobacteria by FISH. The importance of α-Proteobacteria in the marine environment is becoming increasingly apparent. Members of the α-Proteobacteria accounted for 40% of the microbes associated with some coastal seawater samples (Gonzalez and Moran 1997). Burnett and McKenzie (1997) described a α-proteobacterial symbiont associated with the subcuticular region of the brittle star *Ophiactus balli*. An α-proteobacterium, possibly belonging to the genus *Rhodobacter*, was found in the marine sponge *Halichondria panicea* (Althoff et al. 1998). However, in both these cases the α-Proteobacteria were not cultured. The predominance of strain NW001 in *R. odorabile* and the ease of cultivation of this strain provide an excellent "model system" in which to further examine the relationship between α-Proteobacteria and marine sponges.

In the two cases in which diseased *R. odorabile* individuals were examined, strain NW001 was found to be absent from the sponge tissue. This indicates that strain NW001 may be required for the health of *R. odorabile* or that the normal microbial community associated with this sponge is disrupted in diseased *R. odorabile*.

Cyanobacteria often play a major role as symbionts of marine sponges and can, via carbon fixation, contribute significantly to the sponges' nutrition, with the rate of carbon production being sufficient in some cases to provide at least 50% or >100% of the combined carbon requirements of sponge and symbionts (Wilkinson 1983). However, in other cases, the contribution of photosynthetic carbon was found to be minor (Beer and Ilan 1998). Attempts to culture phototrophs from *R. odorabile* produced a single cyanobacterium, designated strain NW4194.

Only ~0.1% of the total bacterial community was amenable to culture under the conditions used in this study. It is not unusual for <1% of cells in marine samples to be culturable. However, Santavy et al. (1990) reported culturable counts of 3–11% of the total bacterial population for the sponge *Ceratoporella nicholsoni*, indicating that there may be considerable variability in the proportion of sponge bacteria that are readily cultivated. Innovative culture techniques should be explored in order to obtain additional sponge symbionts in culture. These potentially novel and diverse isolates would be a useful resource for screening for bioactive compounds. Schmidt et al. (2000) succeeded in propagating the filamentous bacterial symbionts containing theopalauamide from the sponge *Theonella swinhoei* in a

mixed culture on agar plates containing aqueous sponge extract. There is also increasing interest in the examination of sponge bacterial communities by molecular techniques which circumvent the requirement to culture bacteria. Molecular approaches have revealed the presence of an uncultured archaeon (Preston et al. 1996) and an uncultured α-proteobacterium (Althoff et al. 1998) as sponge symbionts. Lopez et al. (1999) showed a wide phyletic diversity of heterotrophic bacteria associated with a deep-water *Discodermia* sp., and Friedrich et al. (1999) revealed a diverse microbial community in the sponge *Aplysina cavernicola*. On-going molecular characterization of the total bacterial community associated with *R. odorabile* using 16S rRNA sequencing indicates that the uncultivated microbial community associated with this sponge is highly novel and diverse. For this reason, simple genetic fingerprinting techniques such as restriction fragment length polymorphism analysis would be ineffective for adequately describing the diversity of the microbial community associated with this sponge species. The stability of microbial communities associated with sponges has been little investigated and has important implications for production of symbiont-derived bioactive compounds and for the use of sponges as source material for microbial diversity in programs screening for natural products.

The culture-based approach taken in the current study proved to have two benefits over molecular approaches. The presence of a single predominant culturable heterotrophic bacterium with characteristic colony morphology enabled demonstration of the consistent presence of this component of the microbial community in many sponges more simply and rapidly than would be possible by molecular approaches. In addition, a bacterium–sponge association has been defined that provides a useful model system for studies on the nature of the symbiotic relationship between α-Proteobacteria and marine sponges.

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