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α-Proteobacteria cultivated from marine sponges display branching rod morphology

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Abstract

Most isolates recovered from marine environments are Gram-negative proteobacteria, even with the use of various media and media additions to enhance recoverability. Cultivation studies with two genera of deep-water sponges yielded nine isolates that demonstrated bulbous branching rod morphology, which is usually associated with microorganisms staining Gram-positive. Gram reactions indicated that the isolates were Gram-negative, which was confirmed by partial 16S rDNA sequencing. All nine isolates were shown to be α-proteobacteria most closely related to other α-proteobacteria isolated from various sponges.

Keywords: α-Proteobacterium; Aerobic anoxygenic phototroph; Sponge-associated microorganism; Marine sponge

1. Introduction

Branching rod morphology is usually associated with microorganisms staining Gram-positive. In Bergey's Manual of Determinative Bacteriology Ninth Edition [1], the majority of the organisms described as branching or club-like are found in the chapter covering irregular, nonsporing Gram-positive rods. It is relatively rare to find Gram-negative organisms described as club-like or branching, although the morphology has been noted previously for some members of the aerobic anoxygenic phototrophic bacteria [2].

Sponge–microbial associations have long been documented and probably date back to Precambrian times about 500 million years ago [3], but relatively little is known about the nature of the interactions. Because sponges are widely recognized as rich sources of bioactive compounds, the biology of the bacterium–sponge relationship is eliciting considerable interest as several sponge derived compounds have been found to have microbial origins (e.g., [4–7]). Studies to examine the associated microbial communities of various deep-water sponges have revealed that a morphologically diverse assortment of bacteria and fungi are amenable to cultivation (personal observations). Concurrent studies have also shown that the recovered microorganisms are not representative of the entire microbial community as determined by molecular genetic approaches (J.B. Olson and P.J. McCarthy, unpublished results).

Isolation studies using two genera of deep-water marine sponges yielded nine isolates that stained Gram-negative but displayed branching rod morphology. In this paper, we present the results of phenotypic and genetic examinations of the isolates. Morphological characteristics were determined microscopically while the phylogenetic relationships to other bacteria were analyzed by partial 16S rDNA sequence analysis. Results, from both phenotypic and genetic studies, suggest that the isolates fall into two distinct α-proteobacterial groups.

2. Methods

2.1. Strains

Nine isolates retrieved from two genera of deep-water sponges were used for these studies. Isolates H088, L333,

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and L348 were isolated from *Forcepia* sp. sponges while the remaining isolates, K786, L034, P498, P531, P669, and P708, were isolated from *Discodermia* sp. sponges. All of the strains are held within the Harbor Branch culture collection and will be made available to interested researchers for taxonomic studies.

2.2. Morphology, Gram stain, and absorption spectra

Wet mount slides were prepared from growth on Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA) and modified Emerson's YpSs plates (4 g yeast extract, 10 g soluble starch, 16 g agar, 750 ml artificial seawater (ASW), 250 ml H_2O) as well as from liquid cultures grown in Marine Broth (5 g peptone, 1 g yeast extract, 1 ml trace metal solution, 1 l ASW) and SYZ (15 g soluble starch, 2 g yeast extract, 4 g NZ Amine (enzymatic digest of casein), 2 g dextrose, 750 ml ASW, 250 ml H_2O). Slides were examined using a phase contrast 100× oil immersion objective on an Olympus BH2 microscope. Photomicrographs were taken with an Olympus 35 mm camera on Elite Chrome 160T (Kodak) slide film.

For Gram staining, approximately 10-μl aliquots of fresh growth from broth media were smeared onto clean slides and allowed to air dry. The dried slides were heat fixed and Gram stained. Stained slides were examined using a 100× oil immersion objective under bright field microscopy on an Olympus BH2 microscope.

Absorption spectra of pigments in the visible region (400–900 nm) were measured using dense suspensions of intact cells in sterile 60% sucrose solution.

2.3. Salt tolerance studies

Liquid base media (5 g peptone, 1 g yeast extract, 1 ml trace metal solution, 1 l H_2O) were prepared containing various amounts of KCl and NaCl. From these solutions, 10-ml aliquots were transferred to 16×150 mm test tubes and autoclaved. The KCl concentrations used were 0%, 1%, 2%, and 3% (w/v) while NaCl concentrations included 0%, 0.5%, 1%, 2%, and 3% (w/v). One tube of each salt concentration was inoculated with 100 μl of the bacterial suspensions grown for 24–48 h in marine broth. The inoculated tubes were incubated at ambient temperature (~22°C) and 210 rpm on a shaking table and checked daily for growth. Wet mounts slides were prepared and examined once growth was apparent. Cellular morphology and motility were noted.

Organisms (100 μl) that showed growth in liquid media containing 1% (w/v) NaCl were transferred to tubes of broth containing 3% (w/v) NaCl and incubated as described above. Conversely, aliquots of growth from 3% (w/v) NaCl broth tubes were also transferred to fresh liquid media supplemented with 1% (w/v) NaCl. Once growth was noted (via turbidity), wet mount slides were prepared and examined.

2.4. DNA extraction, PCR, sequencing, and phylogenetic analysis

A 1-ml aliquot was taken from each of the bacterial suspensions grown in Marine Broth and centrifuged for 2 min at 13 000 rpm. The supernatant was poured off and the pellet resuspended in 100 μl of a sterile 5% Chelex 100 (100–200 mesh sodium form resin; Bio-Rad Laboratories, Richmond, CA, USA) in distilled water solution. DNA extraction was achieved by incubating the tubes at 70°C for 1 h with periodic vigorous vortexing (every 15 min) followed by several freeze–thaw cycles. Universal primers 8F and 536R (8F: 5′-AGAGTTTGATCCTGGCTCAG-3′, and 536R: 5′-GWATTACCGCGGCKGCTG-3′) were used to amplify a portion of the 16S rRNA gene by PCR. Amplification conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, with a 7 min 72°C final extension following the 30th cycle. PCR products were purified by electrophoresis in a 1% (w/v) agarose gel, and bands of approximately 500 bp were excised and recovered using a gel extraction kit (Bio101, Vista, CA, USA). Recovered fragments were then ligated into vector pCR 2.1 (TA Cloning Kit; Invitrogen Corp., San Diego, CA, USA) and used to transform *Escherichia coli* according to the manufacturer's instructions. Plasmids with inserts of the correct size were sequenced at the University of Florida DNA Core Sequencing Facility using the fluorescent dideoxy terminator method on an Applied Biosystems 373A or 377 automated DNA sequencer.

Partial 16S rRNA gene sequences (~400 bp) were edited manually and aligned using ClustalW. Blastn searches of the GenBank database showed the closest matches to the submitted environmental isolate sequences. These sequences were downloaded and used as reference sequences for phylogenetic tree reconstructions. Phylogenetic analyses were performed with PHYLIP version 3.57c [8]. A bootstrap analysis of 1000 replicates was done using SEQBOOT, followed by calculation of distance matrices using DNADIST with the maximum likelihood algorithm. The NEIGHBOR program was used to construct phylogenetic trees prior to application of the CONSENSE program to yield the most supported estimate. DRAWGRAM and RETREE were used to create and label the resulting phylogenetic tree.

3. Results

The isolates were obtained from two genera of mid- to deep-water marine sponges collected over a 5 year period (five source sponges for the nine isolates). *Discodermia* sp. and *Forcepia* sp. sponges were collected at depths ranging from 163 to 188 m in the Caribbean and from 45 to 70 m in the Gulf of Mexico respectively. All but two of the isolates, one from each sponge genus, were recovered from growth on oligotrophic isolation media (>0.5 g C

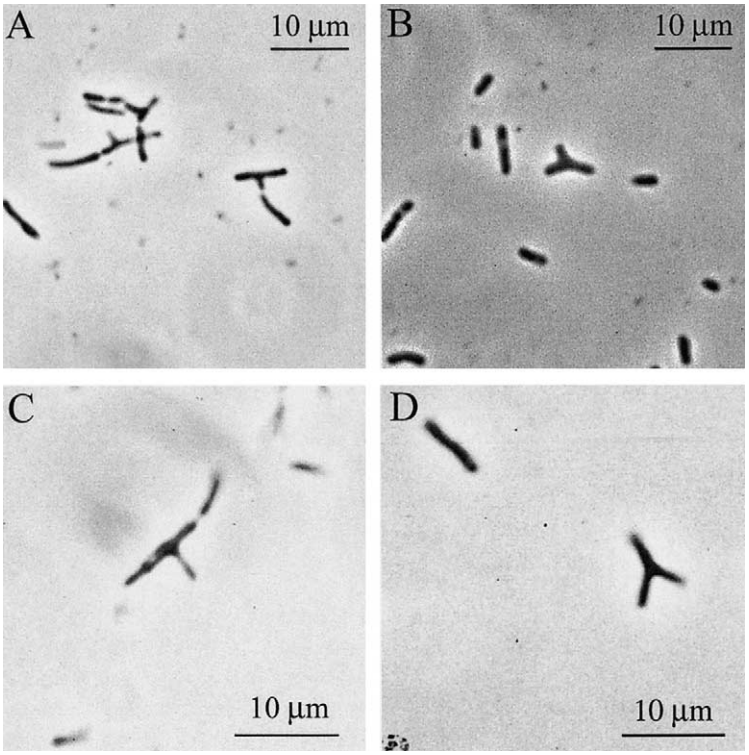

Fig. 1. Phase contrast micrographs of various isolates showing branching rod morphology. Plates A and B are isolate P708, C is P498, and D is L034.

l^{-1}) while the remaining two, K786 and L348, grew on media supplemented with moderate to high amounts of carbon (2–10 g C l^{-1}).

The nine strains tested grew on Marine Agar 2216 and YpSs plates, as well as in Marine Broth and SYZ broth, but failed to grow in freshwater nutrient broth. All samples stained Gram-negative. Bulbous rod morphology (1–2 μm width by 2–10 μm length) with occasional branching (greater than one branching cell per field of view) was noted for all strains in liquid media (see Fig. 1) but was extremely rare or absent in wet mounts prepared from growth on agar plates. The requirement for salt was tested by the addition of varying concentrations of either NaCl or KCl to liquid media. These growth results are shown in Table 1. Cellular morphology for the strains that grew at 1% NaCl (all but H088 and L333) showed two distinct patterns, which appear to be supported by the genetic affiliations suggested for the organisms. The cells for strains K786, L348, P498, and P531 were large cocci (3–5 μm diameter) with visible vacuoles, indicating that changing osmotic pressure may have an effect on the cell wall structure. This was further demonstrated by changing the salt concentration of the growth medium (from 1% to 3% and from 3% to 1%) and noting the corresponding change in cellular morphology. Conversely, the cells at 1% NaCl for strains L034, P669, and P708 were consistent with the branched rod morphology of cells seen at 2% and 3% NaCl concentrations.

Colonial morphology of the organisms on Marine Agar and YpSs plates also separated the organisms into the same two distinct groups supported by the genetic analyses. Colonies of L034, P669, and P708 were described as an opaque pale pink or peach color, with circular, slightly convex, entire, and glistening colonies. Colonies of the

Table 1
Growth in liquid Marine Broth with various concentrations of salt

Strain	0% NaCl	0.5% NaCl	1% NaCl	2% NaCl	3% NaCl	0% KCl	1% KCl	2% KCl	3% KCl	Taxonomic cluster
H088	−	−	−	++	++	−	−	−	−	MBIC 3368
K786	−	−	+	++	++	−	−	−	−	MBIC 3368
L333	−	−	−	++	++	−	−	−	−	MBIC 3368
L348	−	−	+	++	++	−	−	−	−	MBIC 3368
P498	−	−	+	++	++	−	−	−	−	MBIC 3368
P531	−	−	+	++	++	−	−	−	−	MBIC 3368
L034	−	−	++	++	++	−	−	−/+	−/+	*Roseibium*
P669	−	−	+	++	++	−	−	−	−/+	*Roseibium*
P708	−	−	++	++	++	−	−/+	−/+	−/+	*Roseibium*

−, no growth; −/+, minimal growth noted; +, good growth; ++, very good growth.

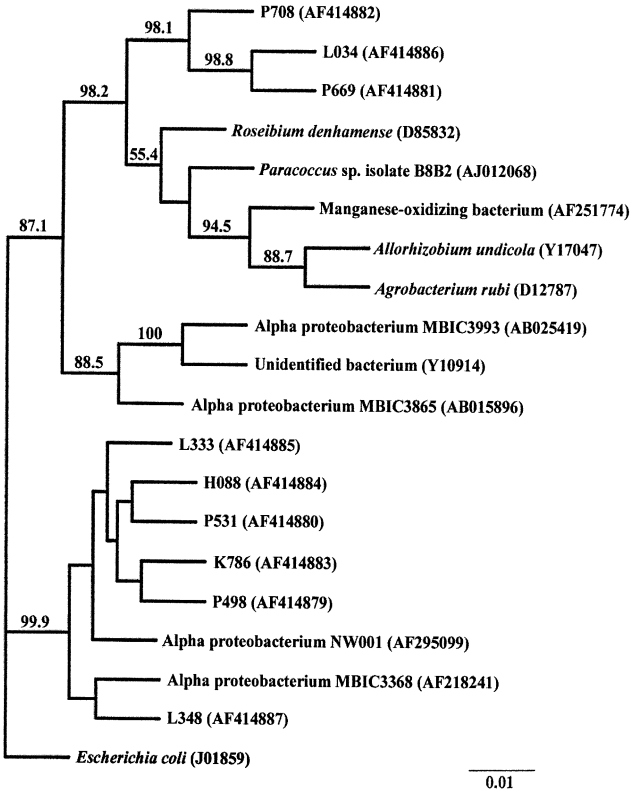

Fig. 2. Distance-based phylogenetic reconstruction using ~400 bp of the 16S rRNA gene. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resamplings. Only values over 50% are indicated. The scale bar indicates the number of substitutions per sequence position.

remaining strains, H088, K786, L333, L348, P498, and P531, ranged from translucent white to translucent beige coloration, from round to irregularly shaped spreading colonies, and were described as appearing granular around the margins with a raised center. These strains produced copious extracellular polysaccharide mucilage.

Isolates P669, P708, and L034 showed absorption spectra similar to what has been reported previously for aerobic phototrophs containing bacteriochlorophyll *a* [2,11]. Absorption maxima occurred at 400–500 nm and 800–870 nm.

Phylogenetic analyses were performed using partial 16S rRNA gene sequences since partial sequences have been shown to provide tree topologies very close to, if not identical to, that obtained by using complete sequences [9,10] and are more cost-effective. Based on the Blast results, 10 previously submitted sequences were selected to define the relationships of the isolates. All of the reference sequences have been classified as α-proteobacteria, whether obtained from isolates or retrieved as a sequence from an environmental sample. Thus, all of the isolates sequenced were also found to cluster within the α-proteobacteria. *E. coli*, a γ-proteobacterium, was used as an outgroup for the estimates of phylogeny. A distance-based phylogenetic reconstruction is shown in Fig. 2.

4. Discussion

Many mid- to deep-water sponge-associated microbial communities are dominated by Gram-negative organisms (~90%; personal observation). Isolation of environmental microbes is also often dominated by Gram-negative microorganisms. Since branching cellular morphology has traditionally been associated primarily with Gram-positive organisms, it is not a characteristic commonly found in examinations of marine environmental isolates. The discovery of a number of sponge-associated microbial isolates from different source organisms that stain Gram-negative and display bulbous branching rod morphology was unusual and interesting.

The taxonomic identifications of the source organisms and depths and locations from which they were collected vary, suggesting that the isolated microorganisms can be found across a range of marine environmental parameters. The isolates display a requirement for salt, preferentially as NaCl, indicating that they may be specifically adapted to marine environments. Additionally, seven of the nine isolates were recovered from growth on oligotrophic marine media, which replicates the low nutrient conditions found in many deep-water environments. All were retrieved from depths greater than 45 m seawater, and while significantly shallower than the depths associated with obligate barophily [12], this demonstrates that the recovered organisms can survive at higher pressures (5–20 atm).

Amplification and comparison of partial 16S ribosomal RNA genes from each of the isolates provided several interesting findings. The first is that the organisms, while sharing a common bulbous branching morphology for individual cells, fall into two distinct groups based on sequence analysis. One group is very closely related to a series of marine microorganisms recovered by numerous investigators working in Japan (MBIC series, unpublished from Marine Biotechnology Institute, Kamaishi City, Japan), Australia (NW001 [13]), and the Mediterranean (MBIC3368 [14]). This group of isolates is characterized by motility in Marine Broth, a requirement for NaCl above 1% (w/v) for growth, and production of copious extracellular polysaccharide slime. The second group is more closely related (94% similarity) to *Roseibium denhamense* (as shown in Fig. 2) and displays several distinct morphological characteristics associated with this organism. All isolates were motile and had circular, smooth, slightly convex, entire, glistening, opaque pink colonies. These organisms grew well at 1% (w/v) NaCl and yielded minimal growth when KCl was substituted for NaCl, but failed to grow at 0% NaCl. *R. denhamense* was reported to grow in the presence of 0.5–10.0% (w/v) NaCl by Suzuki et al. [15] but not at 0% NaCl, suggesting that the isolates may be closely related to this type strain.

Bacteriochlorophyll *a* was reported to be synthesized under aerobic conditions in *Roseibium* sp. by Suzuki et al. [15], who designated the new genus. The pigmented

aerobic and chemoheterotrophic isolates for the new genus were obtained from a variety of shallow water or surface-oriented marine environments around Australia. The isolates from this study that appear to be most closely related to *R. denhamense* were retrieved from deep-water sponge-associated microbial communities. These isolates showed absorption spectra similar to what has been reported previously for bacteriochlorophyll *a* containing microorganisms [2,11,15]. As a result of the depth of collection (163–188 m), even with the water clarity present in the Bahamas where the three isolates were obtained, it is surprising to recover organisms that may synthesize light absorbing pigments as relatively little light reaches these depths. Although the specific location of the microbes on/in the host sponge (or potentially in the ambient seawater) is unknown, the continued capacity for bacteriochlorophyll production is even more interesting if the retrieved isolates were specifically associated with the host sponge and were not directly exposed to light. Other researchers have recovered aerobic anoxygenic photosynthetic bacteria from waters near a hydrothermal vent at the Juan de Fuca Ridge, suggesting that they may be 'normal' inhabitants of marine environments that are below light penetration depths [16].

Webster and Hill [17] found that NW001, an α-proteobacterium closely related to the isolates in this study (see Fig. 2), appeared to be specifically associated with *Rhopaloeides odorabile*, a Great Barrier Reef sponge. Seawater samples collected adjacent to *R. odorabile* sponges failed to show growth of this organism while fluorescence in situ hybridization (FISH) studies localized NW001 to choanocyte chambers of the mesohyl region. Conversely, Hentschel et al. [14] cultivated an α-proteobacterium identical in 16S rDNA sequence to MBIC 3368 from an *Aplysina* sp. sponge which failed to be localized to sponge tissues through FISH analyses [18]. MBIC 3368 and NW001 are closely related strains based on 16S rDNA sequence homology and are the closest GenBank matches for six of the isolates in this study. These results, although contradictory regarding the specificity of the microbe–sponge association, suggest that similar α-proteobacteria are commonly found associated with marine sponges or are ubiquitous in the ambient water and are often recovered in studies of sponge–microbial interactions.

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