Identity of epibiotic bacteria on symbiontid euglenozoans in O_2-depleted marine sediments: evidence for symbiont and host co-evolution

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Abstract

A distinct subgroup of euglenozoans, referred to as the “Symbiontida,” has been described from oxygen-depleted and sulfidic marine environments. By definition, all members of this group carry epibionts that are intimately associated with underlying mitochondrion-derived organelles beneath the surface of the hosts. We have used molecular phylogenetic and ultrastructural evidence to identify the rod-shaped epibionts of two members of this group, *Calkinsia aureus* and *Bihospites bacati,* hand-picked from sediments from two separate oxygen-depleted, sulfidic environments. We identify their epibionts as closely related sulfur or sulfide oxidizing members of the Epsilon proteobacteria. The Epsilon proteobacteria generally play a significant role in deep-sea habitats as primary colonizers, primary producers, and/or in symbiotic associations. The epibionts likely fulfill a role in detoxifying the immediate surrounding environment for these two different hosts. The nearly identical rod-shaped epibionts on these two symbiontid hosts provides evidence for a co-evolutionary history between these two sets of partners. This hypothesis is supported by congruent tree topologies inferred from 18S and 16S rDNA from the hosts and bacterial epibionts, respectively. The eukaryotic hosts likely serve as a motile substrate that delivers the epibionts to the ideal locations with respect to the oxic/anoxic interface whereby their growth rates can be maximized, perhaps also allowing the host to cultivate a food source. Because symbiontid isolates and additional SSU rDNA gene sequences from this clade have now been recovered from many locations worldwide, the Symbiontida are likely more widespread and diverse than presently known.

Introduction

Examples of symbiotic relationships between prokaryotes and eukaryotes in deep sea oxygen depleted marine environments have been well documented for some groups, starting with the discovery of associations between metazoa and bacteria at hydrothermal vents (Cavanaugh *et al.*, 1981), cold seeps (Barry *et al.*, 1996) and the edges of silled basins (e.g. (Distel and Felbeck, 1988). Chemosynthetic autotrophy supports many of these associations and involves the oxidation of hydrogen sulfide or methane by endosymbiotic bacteria within the animal hosts. Similar associations have also been observed between prokaryotes and marine protists. These include a wide range of metabolic relationships observed in shallow marine, primarily reducing environments, including endosymbiotic methanogens in ciliates to epibiotic hydrogen-sulfide oxidizers on euglenids (Epstein *et al.*, 1998; Fenchel *et al.*, 1995; Ott, 1996). The first observations of episymbiotic relationships between protists and prokaryotes in the deep sea were documented in cold seeps of Monterey Bay, CA (Buck and Barry, 1998; Buck *et al.*, 2000) and in the oxygen-depleted Santa Barbara Basin, CA (Bernhard *et al.*, 2000; Bernhard *et al.*, 2010). Both sites were at water depths greater than 500 m, and had high concentrations of mat-forming chemoautotrophic bacteria. In Santa Barbara Basin bottom water, oxygen concentration rarely exceeds 5 μmol L^{-1} (~0.1 ml L^{-1}) (Kuwabara *et al.*, 1999) and sulfide concentration can exceed 50 μM between 0.5-1.0 cm depth (Bernhard, 2003; Bernhard *et al.*, 2003). In this environment, euglenozoan flagellates (including *Calkinsia aureus*) were numerically the most abundant group and most eukaryotic taxa harboured bacterial epibionts and/or endosymbionts (Bernhard *et al.*, 2000).

The Euglenozoa comprises a large group of flagellates with diverse nutritional modes, and consists of four distinct subgroups: euglenids, kinetoplastids, diplonemids, and symbiontids.

Calkinsia aureus, *Bihospites bacati*, and *Postgaardi mariagerensis* have been isolated from oxygen-depleted marine environments; each is covered with rod-shaped epibiotic bacteria (Bernhard *et al.*, 2000; Breglia *et al.*, 2010; Simpson *et al.*, 1997a; Yubuki *et al.*, 2009). All three of these species have been characterized at the ultrastructural level, and *C. aureus* and *B. bacati* have also been characterized at the molecular phylogenetic level using small subunit (SSU) rDNA sequences (Breglia *et al.*, 2010; Simpson *et al.*, 1997a; Yubuki *et al.*, 2009). The data from *C. aureus* and *B. bacati* demonstrated a distinct subgroup of euglenozoans from oxygen-depleted environments (including seven environmental DNA sequences from Northern Europe and South America) referred to as the "Symbiontida." Symbiontid isolates and additional SSU rDNA sequence representatives of the clade have now been recovered from seafloor sediments of Santa Barbara Basin, CA, coastal sediments of British Columbia, Canada, Northern Germany, and anoxic and sulfidic waters in Venezuela, Denmark and Norway (Breglia *et al.*, 2010; Yubuki *et al.*, 2009).

The rod-shaped epibionts of *C. aureus* are 3-5 μm long and 0.350 μm wide and form a tightly-packed coat over the entire surface of the host cell, with at least 128 bacterial cells observed in a transverse section through *C. aureus* (Yubuki *et al.*, 2009). Moreover, the distinctively orange color of *C. aureus* is attributable to a complex extracellular matrix. The prolate-shaped cells of *C. aureus* are around 48 μm (42.6-71.3μm) long and around 17 μm (14.2-19.5μm) wide (for a detailed ultrastructure analysis see Yubuki et al. (2009). Unlike most of their euglenozoan relatives (e.g., kinetoplastids and euglenids), *C. aureus* lacks recognizable mitochondria with cristae, and instead possesses superficially arranged double-membrane bound organelles nearly identical in morphology to the well-described hydrogenosomes found in flagellates from other anoxic environments (Fenchel and Finlay, 1995). Hydrogenosomes

function to produce molecular hydrogen, acetate, CO_2 and ATP in anoxic environments (Barbera *et al.*, 2007).

Bihospites bacati, which was recovered from oxygen-depleted sandy sediments in a shallow tidal flat in South-western British Columbia, Canada, is 40-120 µm long and 15-30 µm wide and the cell surface is covered with two different morphotypes of epibionts: (1) Spherical-shaped bacteria about 0.6µm in diameter with an extrusive apparatus and (2) rod-shaped bacteria 3-5µm long and arranged in bands along the longitudinal axis of the host (Breglia *et al.*, 2010). Longitudinal bands of rod-shaped bacteria were separated by single or double rows of spherical-shaped bacteria. Molecular phylogenetic analyses of small subunit (SSU) rRNA gene sequences demonstrated that to date *B. bacati* is positioned as the earliest diverging known representative of the Symbiontida, a position consistent with comparative ultrastructure. Several morphological features of *B. bacati* are transitional between those found in *C. aureus* and those found in phagotrophic euglenids. Although these ultrastructural data suggest that the Symbiontida is nested within the Euglenida, current molecular phylogenetic data do not shed any light on this hypothesis (Breglia *et al.*, 2010). *C. aureus* and *B. bacati*, share the feature of a coupling of rod-shaped epibionts with a superficial layer of hydrogenosome-like, mitochondrion-derived organelles having reduced or absent cristae. This appears to be a unifying characteristic of the Symbiontida, and suggests a mutualistic relationship that has enabled symbiontids to diversify within oxygen-depleted environments.

In addition to *C. aureus* and *B. bacati*, other euglenozoans from oxygen-depleted environments have been identified with epibiotic bacteria, including *Postgaardi mariagerensis* (Fenchel *et al.*, 1995; Simpson *et al.*, 1997), *Euglena helicoideus* (Leander and Farmer, 2000), *Dylakosoma pelophilum* (Wolowski, 1995), and five unidentified euglenozoans (Bernhard *et al.*,

2000; Buck *et al.*, 2000; Buck and Bernhard, 2002). Hypotheses for the biological role(s) of rod shaped epibionts of eukaryotic hosts usually involve commensalism, with the bacteria benefiting from metabolic byproducts secreted by the host (Fenchel *et al.*, 1995; Leander and Keeling, 2004; Simpson *et al.*, 1997). It has also been hypothesized that the epibionts might be chemoautotrophic sulfur- or methane-oxidizers that form a mutualistic relationship with the host, whereby the host provides a substrate for the bacteria and the bacteria detoxify the immediate environment for the host (Bernhard, 2003; Bernhard *et al.*, 2010; Bernhard *et al.*, 2003). Under certain conditions, the epibiotic bacteria may serve as food for the host (Breglia *et al.*, 2010). In order to better understand the evolutionary history of symbiotic relationships within the Symbiontida, we generated molecular data from the rod-shaped epibionts on both *C. aureus* and *B. bacati*. The resulting molecular data enabled us to more rigorously identify the epibionts, to infer co-evolutionary relationships between the bacteria and the symbiotid hosts, and to begin to ascertain biogeographical patterns of the group as a whole.

Methods

Sample Collection. The Santa Barbara Basin, which is located off California (USA), has a maximum depth of ~600m and sill depth of ~475m. The Santa Barbara samples used for this study were collected using a Soutar box corer or an MC800 multicorer from sea floor sediments (580-592 m depth) in September 2007, June 2008, October 2008, and June 2009 using the *RV Robert Gordon Sproul.* Samples bearing *Calkinsia aureus* were collected along a north-south trending transect along 120˚02’W, from to 34˚17.6’N to 34˚13.0’N. Our samples were collected from box cores that exhibited a surface covering of sulfide-oxidizing bacteria (either *Thioploca* or *Beggiatoa*, both of which require sulfide and little or no oxygen). Surface ~0-2 cm sediments

were transferred to 100-250ml high density polyethylene (HDPE) bottles with an overlayer of bottom water, and were stored at ~7°C. Oxygen concentrations of overlying bottom water on top of Soutar box cores were determined using the microwinkler method (Browenkow and Cline, 1969).

Individual cells of *B. bacati* were isolated by micropipetting from sediment samples collected in Boundary Bay, British Columbia (location details in Breglia *et al.*, (2010)) using a Leica DMIL inverted microscope. The samples were taken 30-50 m from the coastline (i.e. the high tide boundary) and at a depth of about 3 cm below the sediment surface, within a conspicuous layer of black sand.

Light Microscopy. Light micrographs of over 20 *Calkinsia aureus* living cells and fluorescence images of minimum of 60 fixed cells were taken using a Zeiss Axioplan 2 imaging microscope equipped with a Zeiss AxioCam camera. Confocal microscope images were taken with an Olympus Fluoview 300 Confocal Laser Scanning Microscope equipped with an Argon laser for FITC/Alexa488. Images of living cells of *B. bacati* were taken with a Zeiss Axioplan Microscope connected to a Leica DC500 color camera.

Electron Microscopy. Cells of *C. aureus* were prepared for SEM by mixing an equal volume of fixative solution containing 4% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (SCB) (pH 7.2) at room temperature. The fixed cells were mounted on glass plates coated with poly-L-lysine at room temperature for 1 hr. The cells were rinsed with 0.1 M SCB and fixed in 1% osmium tetroxide for 30 minutes as described in Yubuki et al. (2009). The cells of *B. bacati* were prepared for SEM by using 4% osmium tetroxide vapor for half an hour, before adding drops of

osmium 4% for around half an hour. The cells were then transferred onto a 10-µm polycarbonate membrane filter and dehydrated with a graded ethanol series as described in Breglia *et al*. (2010). Cells of *C. aureus* prepared for TEM were rinsed with 0.2 M SCB (pH 7.2) three times and then fixed in 1% (w/v) osmium tetroxide in 0.2 M SCB (pH 7.2) at room temperature for 1 hr as described in Yubuki et al. (2009). Cells of *B. bacati* were prepared for TEM using 4% (v/v) glutaraldehyde in 0.2 M SCB (pH 7.2) with the addition of 0.3 M sorbitol as described in Breglia *et al.* (2010).

DNA extraction, PCR amplification, alignment and phylogenetic analysis. The two eukaryotic hosts (and their epibionts) were independently collected from different geographic locations (Santa Barbara Basin, CA vs. Boundary Bay, British Columbia), in different oceanographic realms (600m vs. near-surface beach sand), and at different times. Cell isolation, cell washing, DNA extractions, polymerase chain reaction (PCR), cloning and sequencing were performed at different times in different laboratories on opposite sides of North America (i.e., DNA sequences were acquired from the epibionts of *C. aureus* at the Wood Hole Oceanographic Institution, MA, USA; DNA sequences were acquired from the epibionts of *B. bacati* at the University of British Columbia, B.C., Canada). Collectively, these factors make it nearly impossible that there could be cross-contamination of samples at any stage.

Single cells of *Calkinsia aureus* were picked from whole sediment samples under a dissecting microscope. In order to greatly minimize contamination, cells were rinsed three times in sterile seawater before being placed into 2.0 ml microfuge tubes and frozen at -20°C for DNA extraction. Individuals were then divided into two groups: single individuals/PCR tube for direct PCR amplification or pools of ~30 individuals for DNA extraction. DNA was extracted from

these pools using the Masterpure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) following the manufacturer's recommendations. Both bacteria- and archaea-specific primers were tested for positive amplification. Bacterial primers were Bact8F (Amman *et al.*, 1995) or Bact341F (Muyzer and Smalla, 1998) paired with U1492R (Longnecker and Reysenbach, 2001). PCR amplification for the 8F/1492R primer pair and the Arch25F/1492R pair was: 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 45°C for 1 minute, 72°C for 1.5 minutes, and a final cycle of 72°C for 7 minutes. For the 341F/1492R primer pair amplification was: 95°C for 5 minutes followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute, and 72°C for 2.5 minutes, and a final cycle of 72°C for 10 minutes. Archaeal amplification was tested with Arch25F (Urbach *et al.*, 2001) paired with U1492R. Amplified DNA was checked for quality by agarose gel electrophoresis, bands were gel purified using the Qiaquick Gel Extraction Kit (Qiagen), and cloned into the vector pCR4-TOPO using the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Plasmid DNA from 20 clones was prepared using a MWG Biotech RoboPrep2500, and inserts were sequenced bi-directionally using the universal M13 primers and an Applied Biosystems 3730XL capillary sequencer at the Keck Facility at the Josephine Bay Paul Center at the Marine Biological Laboratory (MBL), Woods Hole, MA. Processing of sequence data used PHRED, PHRAP (Ewing and Green, 1998; Ewing *et al.*, 1998), and a pipeline script. The sequences were checked for chimeras using the Bellerophon Chimera Check and the Check_Chimera utilities (Ribosomal Database Project) (Cole *et al.*, 2003).

Genomic DNA from *B. bacati* and its epibionts was extracted using the MasterPure Complete DNA and RNA purification Kit (Epicentre, WI, USA) from 30 cells that were individually isolated and washed three times in sterile seawater. PCR reactions were performed

using PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). Nearly the entire 16S rDNA gene was amplified from each isolate using the following primers: API F1 (5'-GTGCCAGCAGCMGCGGTAATAC-3') and API R1 (5'-TACGGYTACCTTGTTACGACTTC-3') (Lang-Unnasch *et al.*, 1998). PCR amplifications consisted of an initial denaturing period (95 °C for 3 minutes), 35 cycles of denaturing (93 °C for 45 seconds), annealing (5 cycles at 45°C and 30 cycles at 55 °C, for 45 seconds), extension (72 °C for 2 minutes), and a final extension period (72 °C for 5 minutes). The amplified DNA fragments were purified from agarose gels using UltraClean 15 DNA Purification Kit (MO Bio, CA, USA), and subsequently cloned into the TOPO TA Cloning Kit (Invitrogen, CA, USA). Eight clones of the 16S rRNA gene were sequenced with the ABI Big-Dye reaction mix using the vector primers oriented in both directions.

For phylogenetic analyses, we aligned the clone sequences from the symbionts of both symbiontid species to 16S rRNA sequences available in the ARB package (Ludwig *et al.*, 2004) (http://www.arb-home.de). The rRNA alignment was corrected manually according to secondary structure information. Only unambiguously aligned positions (1389 bp) were used to construct phylogenetic trees. To this alignment, we added the closest relatives of our original sequences retrieved from Genbank using BLASTn. Bootstrapping and determination of the best estimate of the ML tree topology for these datasets were conducted with the Rapid Bootstrapping algorithm of RAxML (Stamatakis, 2006; Stamatakis *et al.*, 2008) version 7.0 under the GTR+I model (selected by ModelTest (Posada and Crandall, 1998)) running on the CIPRES portal (www.phylo.org).

CARD-FISH. Catalyzed Reporter Deposition FISH (CARD-FISH) was performed with only minor modifications to the methods of Pernthaler *et al.* (2002). Individual cells were hand picked and rinsed in sterile seawater and fixed in 2% (final concentration) paraformaldehyde for one hour, then rinsed 3 times with 5 ml sterile phosphate buffered saline (PBS) by filtration onto a 0.2µm pore size, 25mm Isopore GTTP filter (Millipore, USA). After air-drying, the filters were overlaid with 37°C 0.2% (w/v) Metaphor agarose and filters were dried at 50°C. To inactivate endogenous peroxidases, filter sections were incubated in 10ml of 0.01 M HCl for 10 minutes at room temperature. Filters were washed in 50ml 1X PBS, then in 50 ml of distilled, deionized water (ddH_20). The epibiont cells were permeabilized by incubating the individual filter pieces in 2.0 ml Eppendorf microfuge tubes for 60 minutes at 37°C in a lysozyme solution (0.05 M EDTA, pH 8.0; 0.1 M Tris HCL, pH 8.0; 10 mg/ml lysozyme). The filters were washed in 50 ml ddH_20 for 2 minutes, followed by 50 ml of absolute ethanol (96%) and air-dried. Hybridization buffer and probe were mixed 300:1 in 2.0 ml Eppendorf tubes (probe at 50ng/microliter). For 50 ml of hybridization buffer we mixed 3.6 ml 5 M NaCl, 0.4 ml 1 M Tris HCl and ddH_20 depending on formamide concentration for each probe used (see Table 1). Two grams of dextran sulfate were added and the mixture heated (40-60°C) and shaken until the dextran sulfate was dissolved. After cooling, formamide was added (% formamide noted for each probe used in Table 1), 2.0 ml Blocking Reagent were added (50 ml of 100mM maleic acid in ddH_20 combined with 50 ml of 150mM NaCl and pH adjusted to 7.5 with NaOH, plus 10 g Roche Blocking Reagent (Roche Diagnostics GMbH, Germany)), and volume adjusted to 20 ml with ddH_20. Hybridization was performed at 46°C for 2 hours. Filters were washed for 5 minutes by placing them in 50ml tubes of wash buffer (0.5ml 0.5M EDTA, 1.0 ml 1M Tris HCl plus volume of 5M NaCl depending on probe used (see Table 1) and ddH_20 to make 50 ml). After washing, filters

were transferred to 50 ml 1X PBS (pH 7.6) for 15 minutes at room temperature. 1000 microliters of amplification buffer (4 ml 10X PBS, 16 ml 5M NaCl and sterile ddH_20 were mixed to a volume of 35 ml, then 4 g dextran sulfate (Sigma-Aldrich, USA) were added and mixture was heated to 40-60°C until dextran sulfate was dissolved. After cooling, 0.4 ml Blocking Reagent (see above) was added and water to a final volume of 40 ml, and the solution was filtered through a 0.2µm filter unit. This solution was mixed with 10 µl of 100X H_2O_2 stock (199 µl of 1X PBS plus 1 µl 30% H_2O_2). Filter pieces were transferred to 2.0 ml Eppendorf tubes containing amplification buffer plus 2 µl of fluorescently labeled tyramide (Alexa488-labeled from Biomers.net GmbH, Germany) and incubated at 37°C for 15 minutes in the dark on a rotary shaker. Filter pieces were washed in 50 ml 1X PBS for 15 minutes at room temperature, then 50 ml ddH_20, followed by 96% ethanol, and air-dried, all in the dark. Filters were mounted in Citifluor/Vectashield mounting solution (5.5 parts Citifluor, 1 part Vectashield, 0.5 parts 1X PBS) with 1µg/ml final concentration of DAPI, and stored at -20°C until microscopy was performed. The probes used include EUB338 I-III (Daims *et al.*, 2001), NON338 (Wallner *et al.*, 1993), Arch915 (Stahl and Amann, 1991), Alf968 (Neef, 1997), Gam42a (Manz *et al.*, 1992) and Gam42a competitor (Yeates *et al.*, 2003), BET42a (Manz *et al.*, 1992) and BET42a competitor (Yeates *et al.*, 2003), DELTA495a, b, and c and the corresponding competitor probes for each, cDELTA495a, b, and c (Lucker *et al.*, 2007), EPS549 (Lin *et al.*, 2006) and *Arcobacter* probe ARC94 (Snaidr *et al.*, 1997).

Results

Porewater oxygen concentrations. Dissolved oxygen concentrations in sediments used for recovery of *C. aureus* analyzed for CARD-FISH ranged from 0.2-0.5µM. Concentrations < 1µM

are typical for these sites (Bernhard *et al.*, 2000; Bernhard *et al.*, 2003). It should be noted that bottom water oxygen and sulfide concentrations vary considerably in Santa Barbara Basin (e.g. Bernhard *et al.*, 2003; Kuwabara *et al.*, 1999; Reimers *et al.*, 1990; Reimers *et al.*, 1996).

Light, Fluorescence and Electron Microscopy. Figure 1 presents light micrographs of *C. aureus* and *B. bacati*. In agreement with Yubuki et al. (2009), microscopic analysis revealed *C. aureus* to be on average 48.6 μm long and 16.7 μm wide, and Figure 1a-d shows that the oval-shaped *C. aureus* cells were distinctively orange in color, dorsoventrally compressed with a tapered tail that is about 10 μm long, and covered in rod-shaped epibiotic bacteria. Those bacteria were attached to a robust extracellular matrix that contains a uniform distribution of conduits that join the glycocalyx beneath the epibionts to the plasma membrane in both hosts (Figure 2 c and d). Rod shaped bacteria similar to the epibionts on *B. bacati* were observed within the host cells during TEM, but at this point we have no further evidence that these are the same cells as the epibionts. Longitudinally arranged fibrous material was present within the epibiotic bacteria of *C. aureus* (Figure 2 e and f). In agreement with Breglia *et al*. (2010), *B. bacati* ranged from 40 -120 μm long and 15-30 μm wide. Figures 1e and f show that unlike the orange color of *C. aureus*, *B. bacati* was colorless with distinctive black inclusions within the anterior half of the cell. The cell surface of *B. bacati* was covered with rod shaped epibiotic bacteria that were connected to the plasma membrane of *B. bacati* by a glycocalyx (Figures 2a and b). Spherical-shaped, extrusive epibionts were also observed on the surface (Figure 2a).

The rod shaped-epibionts of both *C. aureus* and *B. bacati* quickly became disassociated with the host cell during light microscopy. The rod-shaped bacteria can be seen floating free of the host cell in Figures 1b and c. The CARD-FISH protocol also resulted in partial to significant

loss of epibionts in spite of the protective agarose over-layer. Most of this cell loss probably occurred prior to the application of the agarose over-layer.

Sequencing. Ninety two percent of 16S clones obtained from whole DNA extracts from single cells of *Calkinsia aureus* and from pools of ~30 cells were associated with *Arcobacter*, however a few clones were affiliated with *Desulfobacterium* and uncultured alpha-proteobacteria. Sequencing of 16S ribosomal RNA genes amplified using whole DNA extracts from *Bihospites bacati* revealed four sequence types, all of which cluster together (100% bootstrap support under maximum likelihood) as a clade within the *Arcobacter* group of the Epsilon proteobacteria (Figure 3).

The *C. aureus* epibiont sequences clustered together (bootstrap support 100% under maximum likelihood) within the *Arcobacter* group. The *C. aureus* epibionts together with two sequences from uncultured epsilon proteobacteria, formed the sister group to the epibionts of *B. bacati* (bootstrap support 95% under maximum likelihood). The topology and the branch lengths on the separate phylogenetic trees for the hosts (as inferred from 18S rDNA) and the epibionts (as inferred from 16S rDNA) were very similar (compare the phylogeny of the epibionts shown in Figure 3 with the phylogeny of the eukaryotic hosts presented by Breglia *et al*. (2010). Figure 4 shows a general comparison of tree topologies and branch lengths for the epibionts and their hosts. Specifically, the number of substitutions per site from the nearest common ancestor was 0.143 for *B. bacati* (Breglia *et al.*, 2010) and 0.145 for the *B. bacati* epibionts (Figure 3); the number of substitutions per site from the nearest common ancestor was 0.060 for *C. aureus* (Breglia *et al.*, 2010) and 0.053 for the *C. aureus* epibionts (Figure 3).

CARD-FISH. DAPI staining reveals the surface epibionts on *C. aureus*, and hybridization with the Alexa488-labeled NON338 probe produced virtually no signal (Figure 5a and b). Hybridization of CARD-FISH probes to Alpha-, Beta- Delta- and Gamma-proteobacterial groups were negative on the surface of the eukaryotic host cells (data not shown). A positive hybridization was observed with the Alexa488-labeled EUB338 probe to bacteria (DAPI Figure 5c, and Alexa488 Figure 5d). No hybridization was observed with the archaeal probe ARCH915 (data not shown). A strong signal was observed with the epsilon-proteobacterial probe EPS549 (DAPI Figure 5e, Alexa488 Figure 5f and Alexa488 with confocal microscopy Figure 5h). A strong signal was also observed with the *Arcobacter*-specific probe ARC94 (Alexa488 Figure 5g).

Discussion

Sulfidic and oxygen-depleted marine habitats can occur anywhere sulfate-reducing bacteria degrade abundant organic matter and thereby produce hydrogen sulfide, such as Oxygen Minimum Zones along open ocean margins, hydrothermal-vent sediments, soft-sediment associated cold seeps, fjords and silled basins. Recent studies have revealed abundant protist populations in these environments (e.g. (Behnke *et al.*, 2006; Bernhard *et al.*, 2000; Edgcomb *et al.*, 2002; Kolodziej and Stoeck, 2007; Massana *et al.*, 2006; Not *et al.*, 2008; Stoeck *et al.*, 2009; Stoeck *et al.*, 2006). In laboratory cultures, certain flagellates have been shown to tolerate high concentrations of hydrogen sulfide (up to 30 mM (Atkins *et al.*, 2002)), which together with the recent diversity data supports the idea that protists form important components of microbial communities in anoxic, oxygen depleted and/or sulfidic marine environments. What is intriguing

is that hydrogen sulfide at micromolar concentrations is known to inhibit respiration, and therefore, the otherwise aerobic eukaryotes in these environments must have physiological adaptations that allow them to survive. The observation that the majority of observed protists in sulfidic environments have prokaryotic epibionts and/or endobionts (e.g. Bernhard *et al.*, 2000; Fenchel and Finlay, 1995; Ott *et al.*, 2008) suggests that symbiosis may be one of the most significant adaptations. In the water column of the Cariaco Basin, Edgcomb et al. (unpublished data) observed that in contrast to ciliates in the oxic upper water column where epibionts on ciliates were rarely observed, over 90% of the ciliates on SEM filters from the anoxic and sulfidic (up to 36.8μM) deeper waters (900m) had visible prokaryotic epibionts.

Although most of the observed taxa in the dysoxic and sulfidic sediments of Santa Barbara Basin harbored bacterial epibionts and/or endobionts (Bernhard *et al.*, 2000), certain taxa consistently did not, which supports the notion that the bacteria observed on the surfaces of most protists are not merely using the protists as a substrate (Bernhard *et al.*, 2000). The consistent observation of a highly specific ultrastructural affinity between *B. bacati* and *C. aureus* with their bacterial epibionts leads us to infer a symbiotic relationship. The environmental characteristics of Santa Barbara Basin and Boundary Bay, together with information on the identity and close phylogenetic relationships of the epibionts of these two members of the Symbiontida allows us to more confidently infer that this symbiosis is a mutualism and reflects a co-evolutionary history between the two symbiontid hosts isolated from different habitats. In contrast to the deeper (~600m depth) sediments of Santa Barbara Basin, Boundary Bay is a vast tidal flat. The sediments we sampled were covered with water during high tides and exposed during low tides. A common factor between the two sites is that the near-surface horizons from which the flagellates were isolated were reduced, as evidenced by a conspicuous blackish layer

of sediment/sand. Phylogenetic analyses indicate that the epibionts of *C. aureus* and *B. bacati* are affiliated with the nitrate-reducing, sulfide-oxidizing autotrophic *Arcobacter,* with the most closely related named species, Candidatus *Arcobacter sulfidicus* (Wirsen *et al.*, 2002) (Figure 3). Affiliation with *Arcobacter* is also confirmed by a positive CARD-FISH hybridization with both the general probe for epsilon-proteobacteria and the *Arcobacter*-specific probe to the epibionts of *C. aureus* (Figures 5f-h).

As indicated in the Methods section, there are five factors that collectively make it very unlikely that the epibiont sequences from either host represent contamination from non-epibiont bacteria or cross-contamination of samples of *C. aureus* and *B. bacati* during molecular procedures. First, both hosts and their symbionts were collected by different researchers from different geographic locations and depths, at different times, and their DNA was isolated, PCR amplified, cloned and sequenced by two different laboratories on opposite coasts of North America. Second, laboratories followed stringent measures to minimize contamination from other environmental bacteria and archaea by washing individually hand picked flagellate cells several times in sterile seawater prior to DNA preparation. Third, molecular phylogenetic analyses demonstrate that the vast majority (92% for *C. aureus* and 100% for *B. bacti*) of the 16S rDNA gene clones from the independent isolates of *C. aureus* and *B. bacati* are very closely related to one another and to the *Arcobacter* within the epsilon proteobacteria. Fourth, the phylogenetic topology inferred from 16S rDNA mirrors the topology for the host organisms as inferred from 18S rDNA (Breglia *et al.*, 2010), both in terms of branching order and relative branch lengths (Figure 4). Fifth, 16S rDNA gene probes applied with CARD-FISH confirm that the epibionts on *C. aureus* are affiliated with the *Arcobacter*.

Arcobacter spp. have been isolated from oil-field waters (Gevertz *et al.*, 2000), activated sludge (Snaidr *et al.*, 1997), human and veterinary sources (e.g. Van Dreiessche *et al.*, 2004), a variety of retail meats (Houf *et al.*, 2003), North Sea bacterioplankton (Eilers *et al.*, 2000), salt marsh sediments (McClung *et al.*, 1983), in association with deep-sea hydrothermal vent vestimentieran tube worms and chimney material (Naguanuma *et al.*, 1997; Cilia and Prieur, unpublished data, accession number AJ132728; uncultured eubacterium CHA3-437), Wadden Sea sediments (llobet-Brossa *et al.*, 1998), Cariaco Basin, Venezuela (Madrid *et al.*, 2001) and hypersaline cyanobacterial mats from Solar Lake (Sinai) (Teske *et al.*, 1996). *Arcobacter* spp. have an optimum growth temperature around 30°C, utilize H_2, formate, and sulfide as electron donors, and nitrate (reduced to NO_2^-), oxygen (microaerobic), and elemental sulfur (reduced to H_2S) as electron acceptors (Campbell *et al.*, 2006). The most closely related sequences to the epibiont sequences from both hosts were a sequence (D83061) isolated from the endosymbiont of a vestimentiferan tubeworm and a sequence (AB189374) from Japan Trench cold seep sediments (clade held together by 95% bootstrap support under maximum likelihood). Without any further information about the organisms from which these *Arcobacter* sequences came, we look at the closest cultured representative for insight into the metabolism of our epibionts, however, it should be noted that the bootstrap support holding our epibiont sequences together with Candidatus *Arcobacter sulfidicus* is modest (67% under maximum likelihood), and that it is, therefore, not possible to conclude that these epibionts have an identical metabolism. Candidatus *A. sulfidicus* is a chemoautotroph that utilizes sulfide (400 to 1200 µM tested in laboratory cultures) as electron donor and is capable of fixing nitrogen (Wirsen *et al.*, 2002). *Arcobacter* spp. includes chemoautotrophs and chemoorganotrophs.

Although demonstrating metabolic exchange between the host and epibionts was outside the scope of this project, we speculate that through the consumption of sulfide, the epibionts may detoxify the immediate environment surrounding the host cell membrane and provide the host with metabolic byproducts (Bernhard *et al.*, 2003). Polz et al. (Polz *et al.*, 2000) provide additional examples of animals and protists with sulfur-oxidizing chemoautotrophs growing on their surfaces in sulfide-enriched environments (nematodes, shrimp, and colonial ciliates that grow in the interstitial pores of marine sediments, at hydrothermal vents, and on decaying plant material in mangrove forests, respectively). Those authors state that the driving force behind those observed symbioses is a nutritional interaction whereby the bacterial symbionts exploit sulfide and oxygen gradients and provide the host with a constant supply of food on its body. While the proposed role of detoxification is logical given the environment in which these symbiontid hosts live, it should be noted that some eukaryotes inhabiting sulfide-enriched habitats lacking symbionts have other adaptations to sulfide exposure. For example, in some animals, sulfide oxidation occurs in mitochondria (e.g. Parrino *et al.*, 2000). Because *C. aureus* and *B. bacati* lack canonical mitochondria, we doubt that possibility here. In some metazoa, hemoglobin binds sulfide as a protective mechanism, and a sulfur-dependent anaerobic energy metabolism can be invoked (for details see reviews by Childress *et al.*, 1991; Grieshaber and Voelkel, 1998; Hagerman, 1998; Somero *et al.*, 1989; Vismann, 1991).

The epibionts, which have adapted to achieve high packing density on the surface of these flagellates (Figures 1 and 2), might benefit from the eukaryotic association in several possible ways. *C. aureus* and *B. bacati* are heterotrophs and they release metabolic byproducts that may be utilized by the epibionts as carbon substrates for chemoorganotrophy. The hosts also provide a motile substrate on which the epibionts possibly establish themselves relatively free of

competition, where they are delivered to oxic/anoxic interface environments possibly favored by the hosts for grazing activities, and ideal for sulfide oxidation activities of the epibionts. Hans *et al*. (2009) demonstrated that the sulfide-oxidizing epibionts on a filter feeding peritrich ciliate were able to sustain 100-times the sulfide uptake rates of bacteria on flat surfaces such as microbial mats. Because sulfide and oxygen are usually mutually exclusive, they typically are only both found in close proximity at oxic/anoxic interfaces. As Poltz *et al.* (2000) note, these transition zones can be quite variable in time and space, and as a result, free-living chemoautotrophic bacteria rarely live at optimal conditions. As a moveable substrate, the protist host allows for a more continuous supply of sulfide and oxygen and hence a competitive advantage. Possible advantages to the host in these oxygen-depleted and sulfidic environments include detoxification of the immediate surroundings, and the ability to farm their own food source.

Conclusion

Using both molecular phylogenetic and ultrastructural evidence, we have identified the rod-shaped epibionts of *C. aureus* and *B. bacati* as closely related sulfur or sulfide oxidizing members of the Epsilon proteobacteria, which generally play a significant role in deep-sea habitats as primary colonizers, primary producers, and in symbiotic associations (Campbell *et al.*, 2006). Because there is an intimate connection between these bacterial epibionts and the underlying organelles beneath the surface of the hosts that are likely mitochondrion-derived (the best unifying feature of the Symbiontida as a whole), the epibionts of *C. aureus* and *B. bacati* likely fulfill a role in detoxifying the immediate surroundings for hosts that live in oxygen-depleted and sulfidic environments. Moreover, the eukaryotic hosts serve as a transport vehicle

bringing the epibionts to the ideal locations along oxic/anoxic interfaces whereby their growth rates can be maximized. The nearly identical episymbiotic rod-shaped bacteria on the closely related symbiontid hosts provide evidence for a co-evolutionary history between the two sets of partners. In fact, the phylogenetic tree topologies inferred from 18S rDNA from the hosts and 16S rDNA from the bacterial epibionts are essentially identical. With such a wide geographic distribution of symbiontid isolates and additional SSU rDNA sequence representatives of the clade, it is clear that members of the Symbiontida are likely more widespread and diverse than currently known.

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Figure and Table Legends

Figure 1. DIC light microscope images of *Bihospites bacati* and *Calkinsia aureus*. **a-d** *C. aureus* showing the epibionts that disassociate with the host almost immediately upon stress. Cells can be seen floating free of host cell in **b** and **c**. **e-f** *B. bacati* showing black inclusions within the anterior part of the cell. Scale bar=20μm except panel **c** which is 10μm.

Figure 2. Electron micrographs of the epibionts on *Calkinsia aureus* and *Bihospites bacati*. **a**. Scanning electron micrograph (SEM) of *B. bacati* showing rod-shaped epibiotic bacteria and spherical-shaped, extrusive epibiotic bacteria (arrow). Note that this image is the same magnification as C. **b**. Transmission electron micrograph (TEM) of the peripheral area of *B. bacati* showing epibionts connected to the plasma membrane of *B. bacati* by a glycocalyx. Hydrogenosome-like organelles (H) were located under the plasma membrane. Note that this image is the same magnification as D. **c**. SEM of *Calkinsia aureus* showing the rod-shaped epibionts on the cell surface. **d**. TEM of the peripheral area of *C. aureus* showing epibionts connected to a robust extracellular matrix (Ex) via a glycocalyx. Arrow indicates conduits in the extracellular matrix. Hydrogenosome-like organelles (H) were located under the plasma membrane (arrow). **e**. A longitudinal section of the epibionts of *C. aureus* showing fibrous material (arrowhead). **f**. The transverse section of the epibionts of *C. aureus* showing fibrous material (arrowhead). Scale bar=2μm in **a** and **c**, 300nm in **b**, **d**, and **f**, and 500nm in **e**.

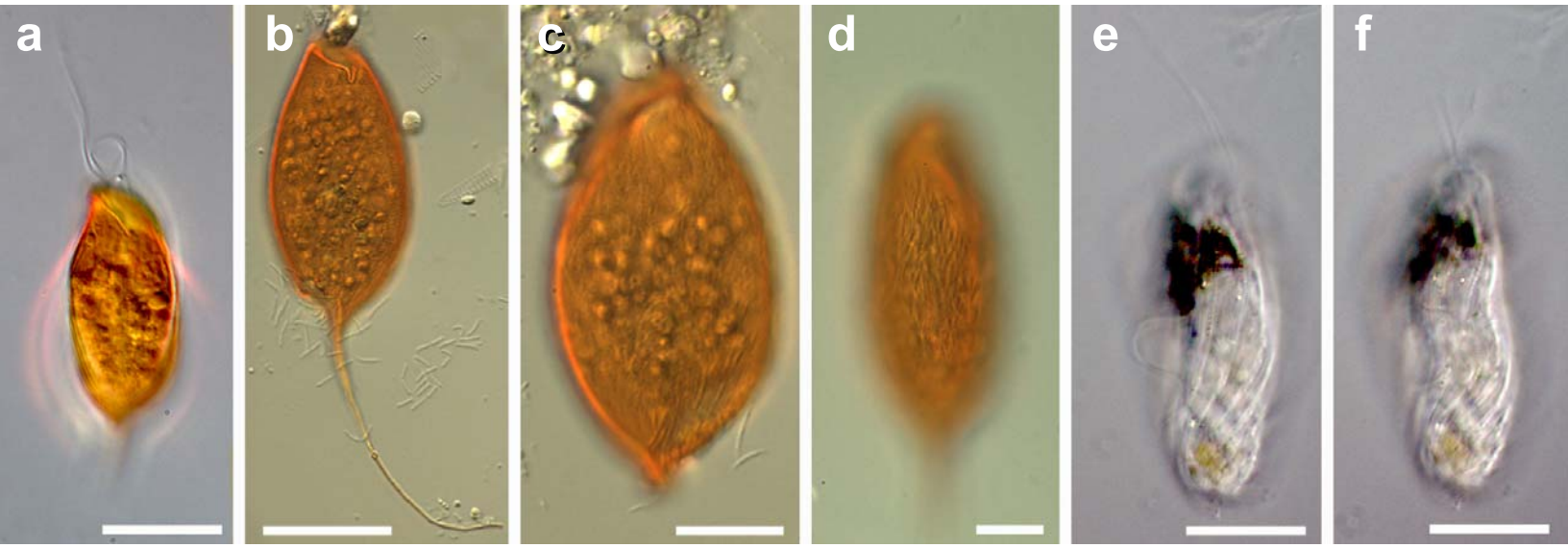
Figure 3. Phylogenetic analysis of 16S rDNA genes from the epibionts of *Bihospites bacati* and *Calkinsia aureus.* Tree is based on an alignment of 1389 nucleotides. Bootstrapping and

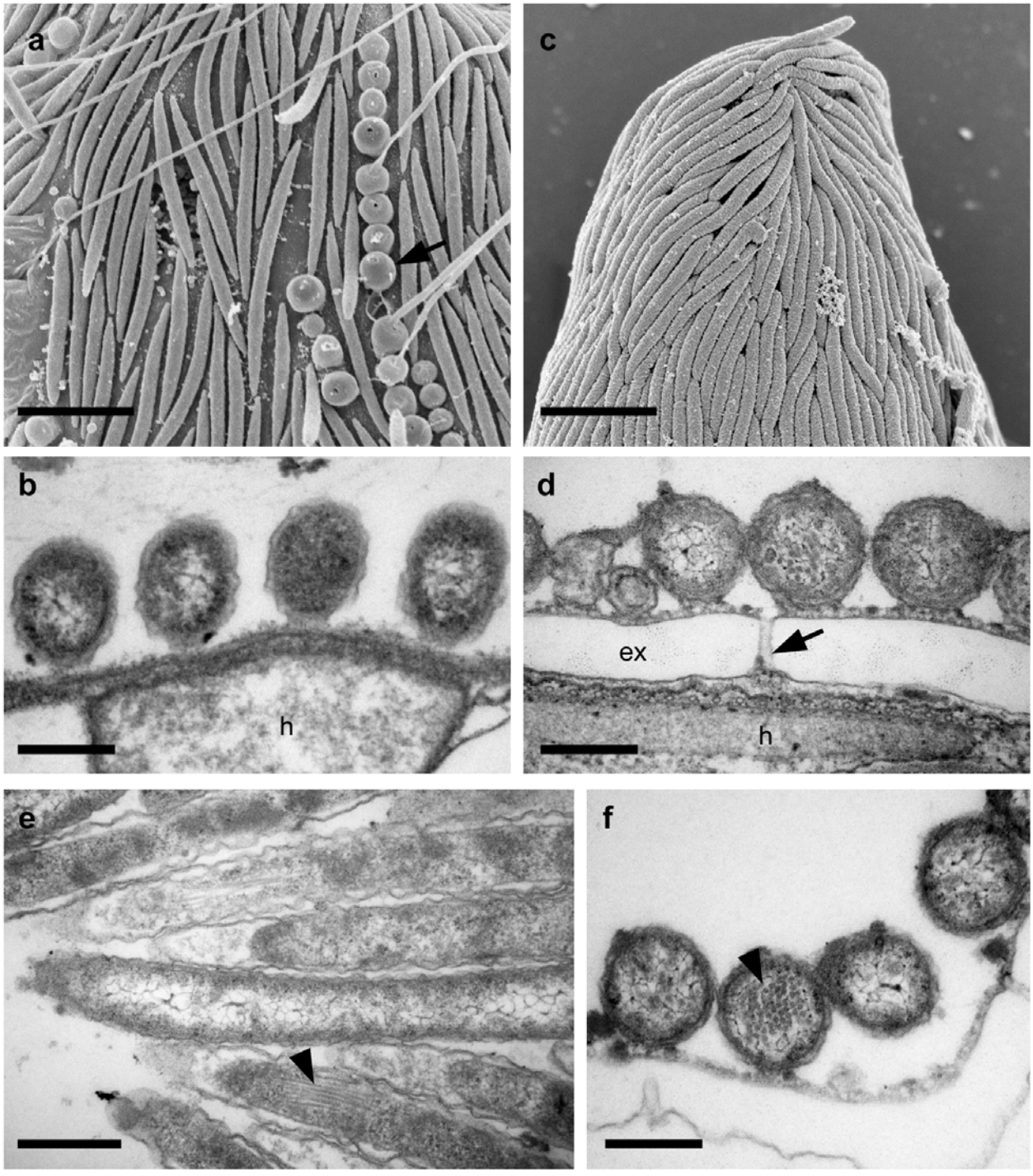
determination of the test estimate of the ML tree topology for these datasets were conducted with the Rapid Bootstrapping algorithm of RAxML version 7.0 under the GTR+I model running on the CIPRES portal (Stamatakis, 2006; Stamatakis *et al.*, 2008) (www.phylo.org).

Figure 4. General comparison of tree topologies and branch lengths in phylogenetic analyses of host and epibiont small subunit rDNA sequences.

Figure 5. CARD-FISH. **a**. Non-probe DAPI, **b**. Non-probe Alexa488, **c**. EUB338-probe DAPI, **d**. EUB338-probe Alexa488, **e**. Epsilon Proteobacteria-probe EPS549 DAPI, **f**. Epsilon Proteobacteria-probe EPS549 Alexa488, **g**. *Arcobacter*-specific probe ARC94 Alexa488, **h**. Epsilon Proteobacteria-probe EPS549 confocal FITC. Scale bars=10µm.

Table 1. CARD-FISH probes used in this study. Percent formamide in hybridization buffer and NaCl concentration in wash buffer are noted for each probe.


a
b
c
d
e
f


a
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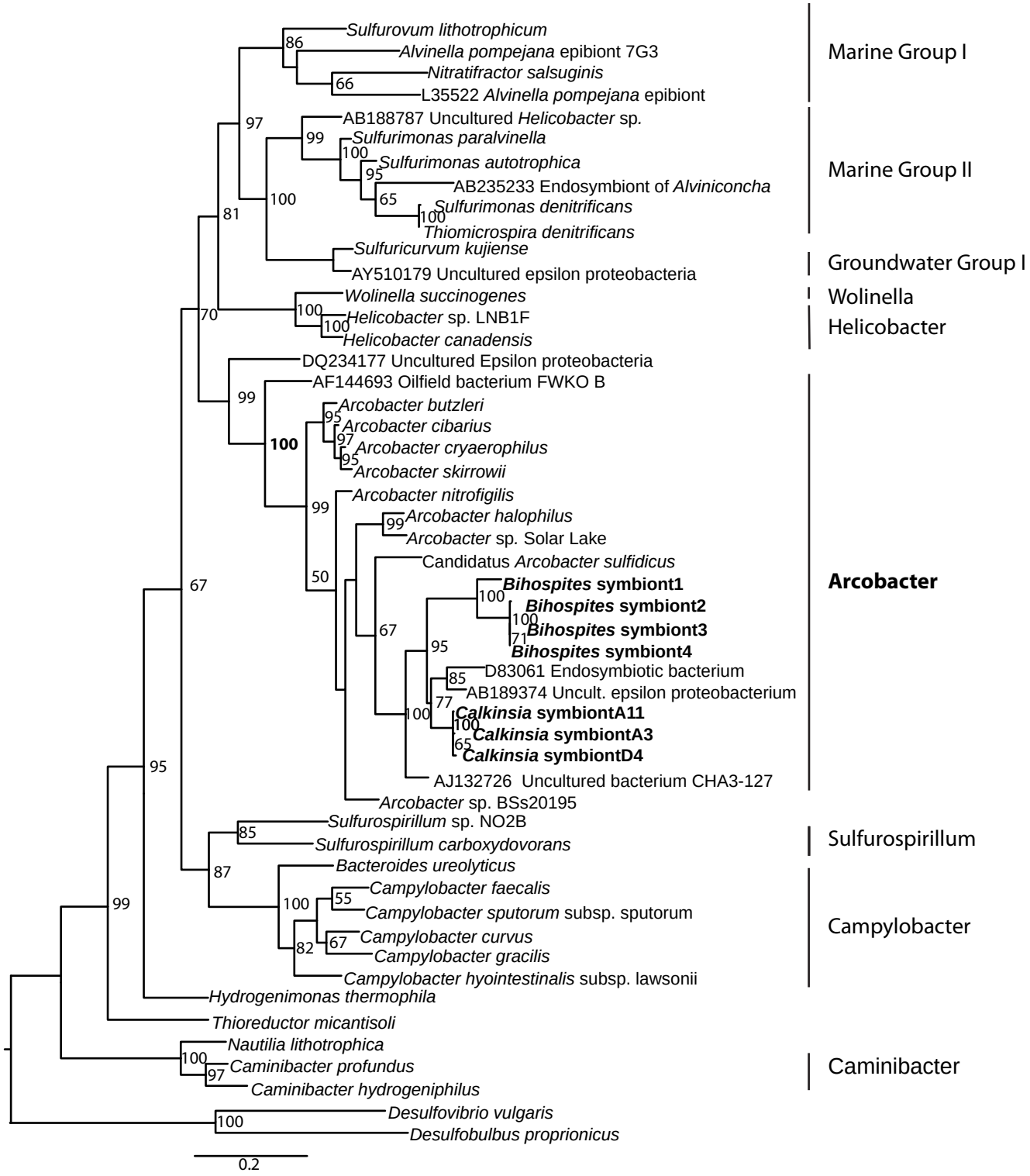
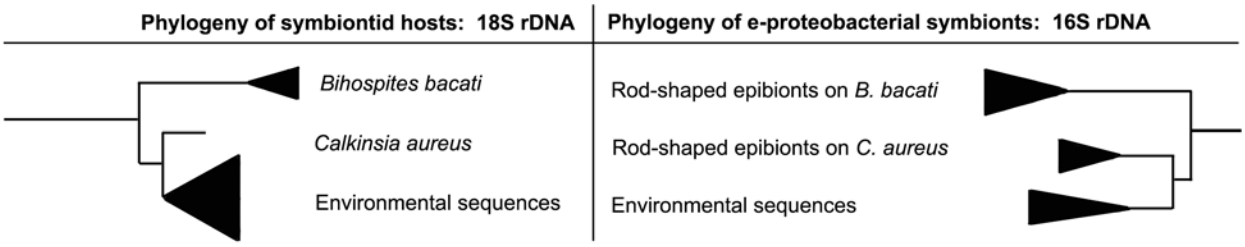
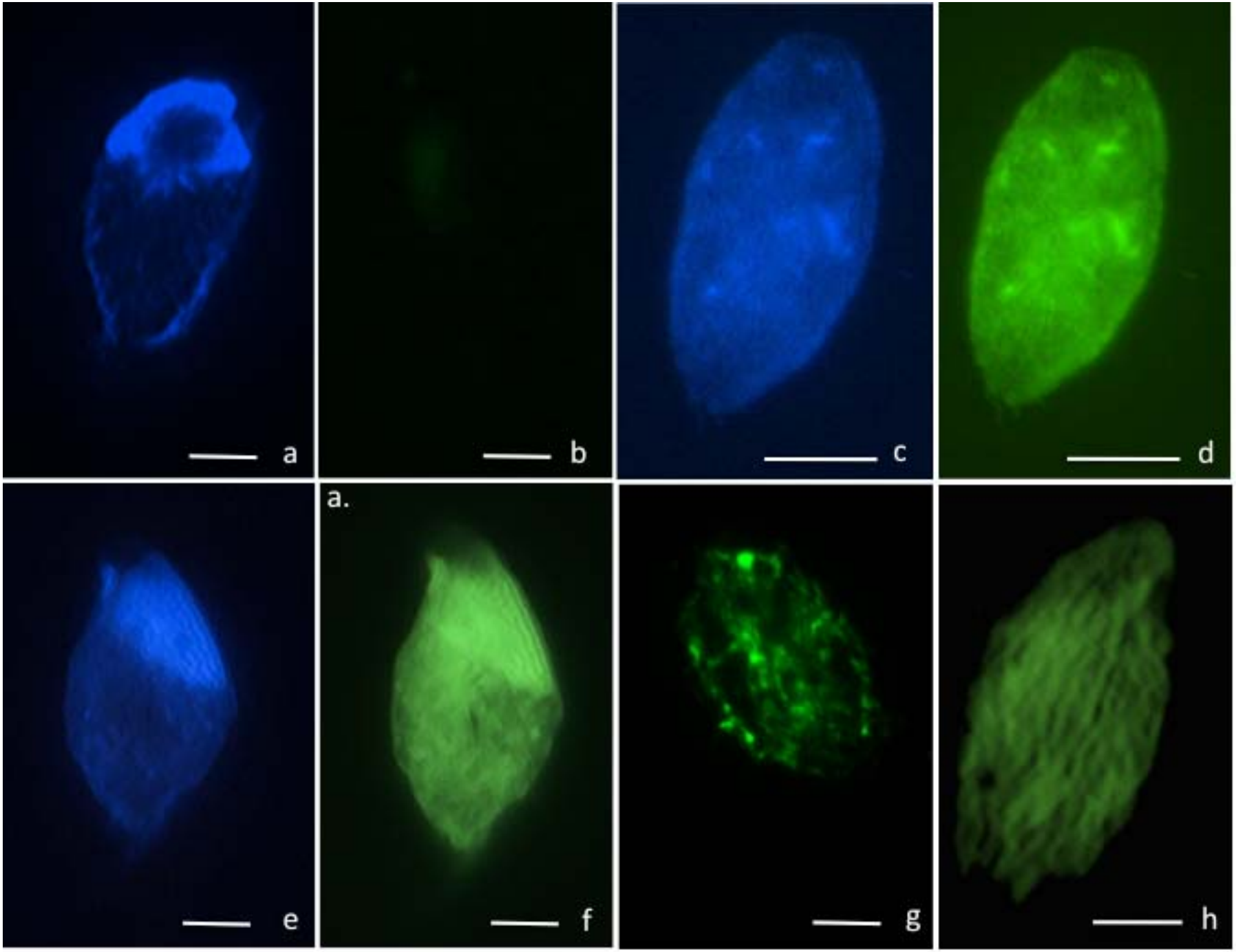

Sulfurovum lithotrophicum
Alvinella pompejana epibiont 7G3
Nitratifractor salsuginis
L35522 Alvinella pompejana epibiont
AB188787 Uncultured Helicobacter sp.
Sulfurimonas paralvinella
Sulfurimonas autotrophica
AB235233 Endosymbiont of Alviniconcha
Sulfurimonas denitrificans
Thiomicrospira denitrificans
Sulfuricurvum kujiense
AY510179 Uncultured epsilon proteobacteria
Wolinella succinogenes
Helicobacter sp. LNB1F
Helicobacter canadensis
DQ234177 Uncultured Epsilon proteobacteria
AF144693 Oilfield bacterium FWKO B
Arcobacter butzleri
Arcobacter cibarius
Arcobacter cryaerophilus
Arcobacter skirrowii
Arcobacter nitrofigilis
Arcobacter halophilus
Arcobacter sp. Solar Lake
Candidatus Arcobacter sulfidicus
Bihospites symbiont1
Bihospites symbiont2
Bihospites symbiont3
Bihospites symbiont4
D83061 Endosymbiotic bacterium
AB189374 Uncult. epsilon proteobacterium
Calkinsia symbiontA11
Calkinsia symbiontA3
Calkinsia symbiontD4
AJ132726 Uncultured bacterium CHA3-127
Arcobacter sp. BSs20195
Sulfurospirillum sp. NO2B
Sulfurospirillum carboxydovorans
Bacteroides ureolyticus
Campylobacter faecalis
Campylobacter sputorum subsp. sputorum
Campylobacter curvus
Campylobacter gracilis
Campylobacter hyointestinalis subsp. lawsonii
Hydrogenimonas thermophila
Thioreductor micantisoli
Nautilia lithotrophica
Caminibacter profundus
Caminibacter hydrogeniphilus
Desulfovibrio vulgaris
Desulfobulbus proprionicus
0.2
Marine Group I
Marine Group II
Groundwater Group I
Wolinella
Helicobacter
Arcobacter
Sulfurospirillum
Campylobacter
Caminibacter


Phylogeny of symbiontid hosts: 18S rDNA
Phylogeny of e-proteobacterial symbionts: 16S rDNA
Bihospites bacati
Calkinsia aureus
Environmental sequences
Rod-shaped epibionts on B. bacati
Rod-shaped epibionts on C. aureus
Environmental sequences


a
b
c
d
a.
e
f
g
h

Probe	Specificity	% FA	Concentration NaCl in wash buffer in Mol
EUB338-I-III	Most Bacteria	35	0.080
NON 338	Background control	35	0.080
ARCH915	Most Archaea	35	0.080
ALF968	81% Alpha-proteobacteria	35	0.080
GAM42a	Most Gamma-proteobactera	35	0.080
GAM42a competitor		35	0.080
BET42a	Most Beta-proteobactera	35	0.080
BET42a competitor		35	0.080
DELTA495a, b, and c	Most Delta-proteobacteria	35	0.080
cDELTA495a, b, and c		35	0.080
EPS549	Most Epsilon-proteobacteria	55	0.020
ARC94	*Arcobacter*	20	0.225