Denitrification likely catalyzed by endobionts in an allogromiid foraminifer

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Abstract

Nitrogen can be a limiting macronutrient for carbon uptake by the marine biosphere. The process of denitrification (conversion of nitrate to gaseous compounds, including N_2) removes bioavailable nitrogen, particularly in marine sediments, making it a key factor in the marine nitrogen budget. Benthic foraminifera reportedly perform complete denitrification, a process previously considered nearly exclusively performed by bacteria and archaea. If the ability to denitrify is widespread among these diverse and abundant protists, a paradigm shift is required for biogeochemistry and marine microbial ecology. However, to date, the mechanisms of foraminiferal denitrification are unclear and it is possible that the ability to perform complete denitrification is due to symbiont metabolism in some foraminiferal species. Using sequence analysis and GeneFISH, we show that for a symbiont-bearing foraminifer, the potential for denitrification resides in the endobionts. Results also identify the endobionts as denitrifying pseudomonads and show that the allogromiid accumulates nitrate intracellularly, presumably for use in denitrification. Endobionts have been observed within many foraminiferal species, and in the case of associations with denitrifying bacteria, may provide fitness for survival in anoxic conditions. These associations may have been a driving force for early foraminiferal diversification, which is thought to have occurred in the Neoproterozoic when anoxia was widespread.

Introduction

Over the past decade, our understanding of the nitrogen cycle has changed drastically (Francis *et al.*, 2007) with the discovery of anaerobic ammonium oxidation (anammox, Dalsgaard *et al.*, 2005)), archaeal ammonia oxidation (Könneke *et al.*, 2005), and the ability of foraminiferal eukaryotes to perform complete denitrification (Risgaard-Petersen *et al.*, 2006). The first report of benthic foraminifera having the ability to perform complete denitrification was exciting because denitrification was thought to be a process facilitated only by prokaryotes and some fungi. Additional studies suggest that the ability of foraminifera to denitrify is widespread (Høgslund *et al.*, 2008; Piña-Ochoa *et al.*, 2010). Dozens of foraminiferal species from a wide taxonomic range have been shown to store nitrate, and of the ten foraminiferal species analyzed for denitrification rates, nine perform complete denitrification. However, our understanding of the mechanisms of denitrification in these protists is incomplete.

We addressed uncertainties about foraminiferal denitrification in a symbiont-bearing allogromiid foraminifer. While allogromiid foraminifera, which are tectinous and unilocular, differ in morphology and test (shell) chemistry from the multilocular calcareous *Globobulimina pseudospinescens*, which was the first foraminiferal species determined to perform denitrification (Risgaard-Petersen *et al.*, 2006), the species live in similar habitats. The Santa Barbara Basin allogromiid lives in relatively high abundance in sediments of the deepest part of the Santa Barbara Basin (Bernhard *et al.*, 2006), and is the only allogromiid common in this part of the basin where denitrification might be expected because bottom-water oxygen concentrations in this area can be very low (typically <2 μM) (Bernhard *et al.*, 1997) or undetectable (Bernhard *et al.*, 2006). At times, sulfide concentrations can be considerable in this area (Bernhard *et al.*, 2003). The species was selected due to a number of its attributes, including

its ecology, abundant endobionts, and copious large cytoplasmic vacuoles (Bernhard *et al.*, 2006) (Fig. 1). The abundant endobionts of this SBB allogromiid appear to be one morphotype in the many specimens examined previously (Bernhard *et al.*, 2006). The copious large cytoplasmic vacuoles in this allogromiid are easily observed, and were noted in both fixed and live specimens in a prior cell-ultrastructural study (Bernhard *et al.*, 2006). Although this foraminiferal species is not formally described, it is morphologically distinct and its small subunit ribosomal RNA (SSU rRNA) gene sequence (GenBank accession number AY818728) was found to group consistently in the basal foraminiferal lineage "Clade L" (Bernhard *et al.*, 2006).

In the earlier study of this Santa Barbara Basin allogromiid, the identity of the endobiont was unknown but hypothesized to be a sulfur-oxidizing bacterium (Bernhard *et al.*, 2006). The reports of denitrification in foraminifera spawned two new hypotheses, however: 1) that the allogromiid's large cytoplasmic vacuoles contained nitrate and 2) that the endobionts were denitrifiers. Using a variety of geochemical and molecular approaches, we address these hypotheses and consider the results with respect to chemocline biogeochemistry and ecology, as well as early foraminiferal diversification.

Materials and Methods

Sample Collection. Samples were collected from water depths of 580-590 m in Santa Barbara Basin (SBB), which is a silled basin with restricted water circulation located off Southern California USA (centered on 34˚13.5N, 120˚02'W (e.g., Reimers *et al.*, 1990; Reimers *et al.*, 1996)). Sediments were collected on five occasions (9/07, 6/08, 10/08, 6/09, 4/10) with a Soutar boxcorer, from which various samples were obtained. Those intended as sources for live material

were placed along with bottom water in tightly sealed HDPE bottles, kept chilled, and transported to our lab where they were maintained at 7 ˚C, which is near ambient temperature.

Nitrate Content and Isotopic Composition. To determine intracellular nitrate concentrations, live specimens were isolated from sediments by gently sieving over a 90-µm screen using chilled bottom water as soon as possible (within a few days) after returning to the laboratory, rinsed twice in 0.2 µm-filtered, nitrate-free seawater, measured for length and diameter using an ocular micrometer mounted on a Nikon SMZ 2B stereomicroscope, and individually air dried in 0.2 ml acid-cleaned polypropylene tubes. Nitrate content and isotopic composition were measured using isotope ratio mass spectrometry (IRMS) with the denitrifier method (Sigman *et al.*, 2001), which relies on conversion of NO_3^- to N_2O and sensitive detection of N_2O via IRMS. Air dried individuals were dissolved in 100 µl acetic acid and replicate 50 µl aliquots were analyzed for nitrate content against low-level (0.05-1.0 nmol) nitrate standards using the peak area of the major ion beam (m/z = 44). Intracellular nitrate concentrations were determined using recorded dimensions and volume calculations for a cylinder prolate spheroid ($\pi[\frac{1}{2}W]^2L$, where W = width and L = length). When nitrate content was sufficient (> 0.5 nmol), the $\delta^{15}N$ of the nitrate ($\delta^{15}N_{NO3} = (({}^{15}N/{}^{14}N)_{NO3} \div ({}^{15}N/{}^{14}N)_{AIR} - 1) * 1000$) was determined for the individual. $\delta^{15}N_{NO3}$ was normalized to the AIR reference scale by analysis of nitrate reference materials USGS32, USGS34, and USGS35 at 0.1 – 1.0 nmol levels.

DNA extraction, PCR amplification, alignment and phylogenetic analysis. Nucleic acids were extracted from individual allogromiids obtained from sediments by picking cells under a dissecting microscope from sediments in a Petri dish resting on ice with a pulled Pasteur pipette. Picked cells were washed briefly by transferring them three times into sterile seawater to remove most loosely attached bacteria from the test surface, and DNA was extracted using either a

standard CTAB extraction protocol (Winnepenninckx *et al.*, 1993) or the Qiagen DNeasy Plant DNA Extraction kit. There were 10-35 individuals pooled for each extraction method. Amplification of 16S rRNA gene fragments were performed using each of these DNA extracts using combinations of the bacterial primers 8F, 357F, 1542R (Lane, 1991), and 341F (Muyzer *et al.*, 1993), the universal reverse primer 1492R (Longnecker and Reysenbach, 2001) and the archaeal primer 1100F (Reysenbach and Pace, 1994). PCR conditions were: 95 ^{o}C for 5 min, followed by 35 cycles of 95 ^{o}C for 1 min, 45 ^{o}C for 1 min, and 72 ^{o}C for 90 s, with a final incubation of 72 ^{o}C for 7 min for primer sets 357F/1542R, 1100F/1492R, 8F/1492R, and 8F/1542R. PCR conditions for primer set 341F/1492R were 95 °C for 5 min followed by 35 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min followed by 72 °C for 10 min.

PCR amplifications of the dissimilatory nitrite reductase genes *nirK* and *nirS* were attempted using extracted DNA and published primers: Cunir3/Cunir4 (Casciotti and Ward, 2001) and FlaCu/R3Cu (Hallin and Lindgren, 1999) or nirS1F/nirS6R (Braker *et al.*, 1998) and cd3aF/R3cd (Michotey *et al.*, 2000; Throback *et al.*, 2004), respectively. Touchdown PCR was used with an annealing temperature beginning at 50 ˚C and decreasing by -0.5 ˚C/cycle for the initial 10 cycles followed by 30 cycles at an annealing temperature of 45 ˚C. The positive controls were *Alcaligenes faecalis* for *nirK* and *Pseudomonas stutzeri* for *nirS*. Allogromid specimens were also screened for sulfur oxidation and sulfate reduction genes (*soxB* and *dsrAB*, respectively), with *Thiomicrospira* sp. strain L-12 and *Desulfovibrio vulgaris* as positive controls, respectively, using established primers and protocols (Petri *et al.*, 2001). Dissimilatory (bi)sulfite gene amplifications were run using the DSR1Fmix and DSR4Rmix primer sets (Loy *et al.*, 2004).

PCR products from all positive amplifications were gel purified using the Qiaquick Gel Extraction Kit (Qiagen) and cloned into the pCR4 vector in the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions (for 357F/1492R, 4 separate amplification products were pooled prior to cloning). Selected clones (74 from 357F/1542R and 16-24 clones from all other primer pairs) were sequenced using an Applied Biosystems 3730XL capillary sequencer. Sequences were then edited and assembled into contigs using Sequencher (Gene Codes Corporation). Chimeric sequences were removed from further analyses by visual inspection and the CHECK_CHIMERA program (Cole *et al.*, 2003).

Clone sequences were aligned using the autoaligner function within the software ARB (Ludwig *et al.*, 2004) using the SILVA Reference database, and then the alignment was manually corrected using secondary structure information. Only reliably aligned sites were included in subsequent phylogenetic analyses. Bootstrapping and determination of the best estimate of the ML tree topology were conducted with the Rapid Bootstrapping algorithm of RAxML (1000 bootstrap replicates) version 7.0 under the GTR+I model running on the CIPRES portal (Stamatakis, 2006; Stamatakis *et al.*, 2008) (www.phylo.org).

FISH and CARD-FISH. On the ship, sediments were preserved in 2.0% paraformaldehyde for 1 h, rinsed three times in phosphate buffered saline (PBS), and then stored in PBS/ethanol (1:1) for later analysis by Fluorescent In Situ Hybridization (FISH). Specimens for FISH were hand picked under the dissecting microscope, washed briefly by transferring them three times into sterile seawater to remove most loosely attached bacteria from the test surface, and transferred to a 0.2-µm-pore size, 25-mm Isopore GTTP filter (Millipore). The filters were subsequently overlaid with 0.2% (w/v) Metaphor agarose at 37 °C and then dried at 50 °C. FISH protocols followed those published in Pernthaler *et al*. (2001), and Catalyzed Reporter Deposition

(CARD)-FISH protocols followed Edgcomb *et al*. (2010) as modified from Pernthaler *et al*. (2002). The only variations to the CARD-FISH protocol was the use of a multi-step permeabilization routine when targeting intact allogromiid cells that incorporated a 1 h incubation at 37 °C in lysozyme solution (10 mg/ml final concentration), followed by a wash in 50 ml of sterile PBS, a 1 h incubation in ProteinaseK (50 µl of 1064 U/ml in 10 ml Tris EDTA), deactivation in 0.01 M HCl for 20 min, a wash in 50 ml sterile PBS, a 30 min incubation in Triton X (0.5% in PBS), and a final wash in 50 ml PBS, 50 ml of MilliQ-H_20, and 50 ml 200-proof ethanol. The probes used include EUB338 I-III (Amann *et al.*, 1990; Daims *et al.*, 1999), NON338 (Wallner *et al.*, 1993), Arch915 (Stahl and Amann, 1991), DELTA495a, b, and c and the corresponding competitor probes for each, cDELTA495a, b, and c (Lucker *et al.*, 2007), BET42a (Manz *et al.*, 1992), BET42a competitor (Yeates *et al.*, 2003), Gam42a (Manz *et al.*, 1992), Gam42a competitor (Yeates *et al.*, 2003), and a general *Pseudomonas* probe PS1284 (Gunasekera *et al.*, 2003). All probe hybridizations were conducted with 35% formamide and 0.080 M NaCl; all were conducted at 46 °C and washes at 48 °C following published optimized protocols, confirmed in our laboratory with positive control pure cultures (delta-, beta-, and gamma-proteobacteria) for each probe. For regular FISH hybridization studies targeting the allogromiid endobionts, the hosts were lysed to expose endobionts by vortexing for 15 s prior to depositing lysate on filters. All 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, as described below under the GeneFISH protocol.

GeneFISH. GeneFISH uses multiple digoxigenin (Dig)-labeled polynucleotide probes to target genes, followed by the binding of horseradish peroxidase (HRP)-conjugated antibodies and

CARD to amplify and visualize the signal (Moraru *et al.*, 2010). Published protocols were followed, except that a FISH probe (vs. a CARD-FISH probe) was used for the 16S rRNA gene. For our application, the FISH probe targeting the ribosomal RNA gene was the general *Pseudomonas* probe (Gunasekera *et al.*, 2003) and the 437 bp Dig-labeled probe targeting the *nirK* gene was generated using primers specific to the allogromiid *nirK* sequence. The forward primer was FLaCu 5'-ATCATGGTSCTGCCGCG-3'; the reverse was R3Cu 5'-GCCTCGATCAGRTTGTGGTT-3' (Hallin and Lindgren, 1999). The construction of this Dig-labeled probe used the Roche PCR DIG Probe Synthesis Kit (Roche Diagnostics) and 25 pg of template DNA. PCR cycling consisted of a 1 min denaturation at 95 °C, followed by 30 cycles of 95 °C for 1 min, 45 °C for 1 min, and 72 °C for 90 s, followed by a final single cycle of 72 °C for 7 min. It was not possible to test this probe on a separate positive control organism because, by definition, in this protocol, the probe is unique to this particular *nirK* sequence, and would not be expected to hybridize optimally to another slightly different *nirK* sequence. With GeneFISH, Moraru *et al*. (2010) reported that background fluorescence can come from false signals that are as bright as the true gene signal, and from background fluorescence that is much weaker than the gene signal, such as that generated by tissue autofluorescence. As discussed at great length in Moraru *et al*. (2010), with the current GeneFISH protocol it is not yet possible to completely eliminate false positive hybridization signal of the first type described above, which can occur at random on both host material and on bacterial cells. A hybridization result is judged to be positive, therefore, when false positives (scored by hybridization of the gene probe to DNAse-treated samples) are significantly outnumbered by positives (hybridization of the gene probe to samples not treated with DNAse) (Moraru *et al.*, 2010). Gene-specific hybridization is also assessed against a simultaneously hybridized 16S rRNA probe – in this case the *Pseudomonas*-

specific 16S rRNA probe. By applying the gene probe during the GeneFISH protocol at a concentration of 25 pg/µl and using a 22 h hybridization, acceptable percentages of false positive signals (1-4%) and hybridization efficiencies (50-65%) were obtained. These results were repeated with a minimum of 20 allogromiid smear preparations. While disruptive of the allogromiid cell organization, lysing individuals and depositing several spots of the lysate on each filter reduced interference from background and the three-dimensional shape of the host, making it easier to distinguish the co-location of 16S rRNA and *nirK* hybridization than with intact allogromiids. As noted in Moraru *et al*. (2010), hybridization efficiency for the gene probe is often ~45%, and false positives ~4-7%. GeneFISH images were collected using a Zeiss Axioplan 2 epifluorescence microscope equipped with a Zeiss AxioCam camera and 20×-100× objectives.

Results

Nitrate. Intracellular nitrate contents of the SBB allogromiid foraminifer were variable. Some individuals had high NO_3^- contents (up to 1172 pmol per specimen; mean = 570 ± 354 pmol per individual, n=10) while others contained no NO_3^- (n=17). When normalized per unit volume, nitrate concentrations varied substantially (70 ± 49 mmol/l; n = 10), but were as high as 165 mmol/l in one individual. In those individuals with sufficient NO_3^- to measure $\delta^{15}N_{NO3}$, specimens had consistently high $\delta^{15}N_{NO3}$ values (17.6 ± 0.2 ‰; n = 7).

16S rRNA Sequencing. All bacterial primer pairs tested (see Materials and Methods) produced a positive PCR amplification from the SBB allogromiid. Archaeal 16S rRNA genes were not detected by PCR. The bacterial 16S rRNA gene clone libraries included multiple taxonomic groups, including delta-proteobacteria, beta-proteobacteria (*Delftia*), and members of the

223 gamma-proteobacteria. Although it is not possible to reliably correlate abundance of a particular
224 sequence in clone libraries to abundance in a sample, gamma-proteobacteria represented the
225 highest percentage of clones in libraries generated with different bacterial primer sets (20-50%
226 depending on primer combination). Among the 17 gamma-proteobacterial sequences in our
227 different 16S rRNA gene clone libraries, 16 were most closely related (90% bootstrap support
228 under maximum likelihood) to an uncultured *Pseudomonas* (AY987841), with its next closest
229 relatives being *Halomonas* sp. and *Marinobacter* sp. (Fig. 2). The gamma-proteobacterial
230 sequence affiliated with *Pseudomonas* is deposited in GenBank under the accession number
231 JF414803.
232 **Functional gene sequencing.** The nitrite reductase gene *nirK* was also detected in DNA
233 extracts from the SBB allogromiids. We recovered only one *nirK* sequence variant. The *nirK*
234 sequence was most closely related to an uncultured bacteria (DQ182218) isolated from a
235 denitrifying community in an activated sludge sample (Hallin *et al.*, 2006) and also
236 *Mesorhizobium* sp. 4FB11 based on phylogeny and BLAST analysis (data not shown). The
237 single *nirK* sequence recovered was deposited in GenBank under the accession number
238 JF414804. A *nirS* gene was not detected. Sulfur metabolism was not evident in the SBB
239 allogromiid because (1) genes for sulfur oxidation (*soxB*) and sulfate reduction (*dsrAB*) were not
240 found and (2) spectra and elemental maps did not indicate presence of elemental S in the
241 endobionts aligning the large vacuole peripheries (data not shown).
242 **CARD-FISH.** DAPI staining and CARD-FISH with a universal eubacterial probe confirmed the
243 presence of endobionts in our material (Fig. 3a,b). CARD-FISH with a general gamma-
244 proteobacterial probe was also positive (results not shown), while CARD-FISH using the general
245 delta- and beta-proteobacterial probes both produced negative results (~20 allogromiids tested

per probe, 3 separate hybridization trials), as were the NON probe (Fig. 3c,d) and allogromiids subject to the CARD-FISH procedure with no probe (both negative controls run on ~20 allogromiid cells each). Altogether, CARD-FISH results suggested that the endobiont was a gamma-proteobacterium.

FISH. FISH using a FITC-labeled general *Pseudomonas* probe and a published optimized protocol (Gunasekera *et al.*, 2003) was positive (Fig. 3e,f). Smear preparations of individual allogromiid cells provided clear images of the hybridization results with this probe, free of ambiguity caused by the faint background interference from the allogromiid (using optics for FITC/Cy3). The exact percentage of endobiont cells that affiliate with the pseudomonads could not be calculated because we lacked sufficient allogromiid specimens to conduct a dual hybridization experiment using the universal bacterial probe and the *Pseudomonas*-specific probe. However, comparison of DAPI and FITC images of the same preparations, revealed only occasional (< 5%) endobiont cells that did not hybridize to both the gamma-proteobacterial and *Pseudomonas*-specific FISH probes. As with CARD-FISH, hybridizations with a NON probe and cells put through the same FISH procedure with no probe were both negative (~20 SBB allogromiid cell preparations each).

GeneFISH. GeneFISH indicates that this *nirK* gene sequence is located within the endobionts (Fig. 3e-h). A comparison of the signal for the gene probe labeled with Texas Red on the negative control filter (DNAsed filter, Fig. 3f) to the same field of view under FITC showing hybridization of the 16S rRNA probe for *Pseudomonas* (Fig. 3e), illustrates that there are very few false positives (~1%) with the *nirK* gene probe. For further explanation of the issues regarding false positives with the GeneFISH procedure, see the Materials and Methods section. The low percentage of false positives was confirmed with a minimum of 10-15 fields of view on

each of two different negative-control filters. When comparing the signal for the *nirK* gene probe on the positive filters (not DNAsed, e.g., Fig. 3g,h) to the signal on the negative controls (Fig. 3e,f), a significant increase in signal is observed on the positive filters. The hybridization efficiency of the *nirK* gene probe (i.e., percent of positive cells for the *nirK* gene out of positive cells for the 16S rRNA gene) ranged from ~50-65%, depending on the filter and field of view (6 filters observed, 10-15 fields per filter). This difference between the percent of false positives and the percent of true positives gives us confidence that the GeneFISH results are reliable.

Discussion

Intracellular nitrate contents and concentrations in the SBB allogromiid were high and comparable to those of other foraminifera reported to store nitrate and/or catalyze denitrification (Høgslund *et al.*, 2008; Piña-Ochoa *et al.*, 2010; Risgaard-Petersen *et al.*, 2006). Thus, our inference that the abundant large cytoplasmic vacuoles (Fig. 1a) are filled with nitrate is likely. High intracellular nitrate concentrations compared to surrounding waters (Sigman *et al.*, 2003) suggest that the SBB allogromiid (and other foraminifera reported in the literature) are intentionally transporting NO_3^- into their cells or are producing it intracellularly. Given that no known nitrifying microorganisms were detected in our clone libraries and that the allogromiids inhabit anoxic sediments (Bernhard *et al.*, 2006), it is unlikely that NO_3^- is being produced within the allogromiid cells. Instead, we hypothesize that NO_3^- is transported inside the foraminiferal cell for use by the endobionts. While it was not a primary goal of this work, the $\delta^{15}N$ value of intracellular NO_3^- was also determined for a subset of the individual allogromiids with sufficient intracellular NO_3^-. The observed allogromiid $\delta^{15}N_{NO3}$ values were higher than for NO_3^- in bottom water (8-12‰; Sigman *et al*. 2003), but should be compared to porewater $\delta^{15}N_{NO3}$ in order to determine whether NO_3^- is fractionated during transport and/or consumption inside the cell.

Unfortunately, porewater samples were not collected here, but remain an avenue of active research.

An earlier TEM study showed that the SBB allogromiids shows no evidence of ectobionts, but have endobionts of a single morphotype of coccoid to shortened rod-shaped cells found as individuals or in short chains (Bernhard *et al.*, 2006). Individual allogromiids, which are fragile and have a thin (~1µm) test of clay particles (Bernhard *et al.*, 2006), were difficult to completely clean of "contaminating" extracellular bacteria prior to nucleic acid isolation, even after rinsing specimens in several washes of sterile seawater. As a result, the obtained 16S rRNA gene clone libraries contained sequences from a variety of genotypes, most likely including some 'contaminating' bacteria. The washing procedures were sufficient, however, to remove a significant portion of contaminant bacteria from the surface of the tests, and similar to Bernhard *et al*. (2006), that used the same procedure, we only observed occasional, mostly filamentous external prokaryotes attached to the tests of this allogromiid during microscopy. Our attention was initially drawn to the gamma-proteobacteria because they represented a significant portion of the recovered gene sequences (described above), which we infer to belong to the numerically-dominant endobiont. Subsequent FISH experiments that showed positive hybridization with the general bacterial, general gamma-proteobacterial, and general *Pseudomonas* probes support the conclusion that the SBB allogromiid endobionts are pseudomonads. To our knowledge, these FISH results are the first ever reported in the literature for foraminiferal endobionts.

Marine pseudomonads capable of denitrification or nitrate reduction to ammonia are known (Gruntzig *et al.*, 2001). Further, we recovered a single *nirK* sequence from the allogromiid, and by generating a probe unique to this *nirK* sequence, were able to show with GeneFISH that this probe hybridized to the same endobiont cells that hybridized to the general

315 *Pseudomonas* probe. The purpose of applying GeneFISH in this study was to determine whether
316 the *nirK* gene sequence would localize to the endobionts or to the allogromiid host; we only
317 observed hybridization to the endobionts. As noted above, although lysing individual allogromiid
318 cells for the GeneFISH procedure destroys topological information, this process was required for
319 unambiguous interpretation of GeneFISH results due to interference from slight autofluorescence
320 of the allogromiid in the FITC channels and from its 3-dimensional shape. Figures 3e-f show that
321 the GeneFISH probe designed from our *nirK* sequence hybridizes to the same cells as does the
322 general *Pseudomonas* probe applied in this study. The connection between this *nirK* sequence
323 and the specific *Pseudomonas*-related rRNA sequence recovered from the allogromiid is only
324 indirect, as GeneFISH was not attempted with a 16S rRNA probe unique to the specific
325 *Pseudomonas*-related rRNA sequence. However, we conclude that the allogromiid endobionts
326 are likely a species of denitrifying pseudomonad because (1) *nirK* was detected in DNA extracts
327 from the SBB allogromiid, (2) this *nirK* gene was localized to the endobionts, (3) the SBB
328 allogromiid endobionts are spatially associated with the peripheries of vacuoles (Bernhard *et al.*,
329 2006), which presumably contained the high observed concentrations of nitrate. The failure to
330 detect genes for elemental sulfur oxidation and sulfate reduction is consistent with our
331 conclusion inasmuch as *Pseudomonas* species are not recognized to carry out such sulfur
332 metabolisms. Although the 16S rRNA gene sequence of the SBB allogromiid endobiont is most
333 closely related to an uncultured *Pseudomonas* detected in a hypersaline saltern (Maturrano *et al.*,
334 2006), the exact phylogenetic affiliation of this allogromiid endobiont within the
335 Pseudomonadales should be interpreted with caution until more sequences of uncultured
336 pseudomonads are available in public data bases.

Risgaard-Petersen *et al*. (2006) calculated that 6,000-23,000 denitrifying bacteria were necessary to account for the measured denitrification rates in their foraminiferal species. Using previously published TEM images crossing a transect of a SBB allogromiid and average allogromiid length and width data presented in Bernhard *et al*. (2006) as well as average endobiont dimensions, we estimate the abundance of endobionts in an average SBB Clade L allogromiid to be >250,000 bacteria, taking up approximately 17.6% of the cell volume. While we lack denitrification rates for the SBB allogromiid, the allogromiid endobiont abundance per foraminifer is 1-2 orders of magnitude greater than needed to account for denitrification rates reported for other foraminiferal species (Piña-Ochoa *et al.*, 2010; Risgaard-Petersen *et al.*, 2006).

Denitrification is almost undoubtedly carried out by bacterial endobionts in this Clade L allogromiid species, even though no Clade L allogromiids have been shown to denitrify (nor tested for denitrification activity). Thus, our data suggests that denitrification in some species may be dominated by prokaryotic associates. Given that bacterial symbionts in benthic foraminiferal species from anoxic to micro-oxic habitats are not uncommon (Bernhard, 2003; Bernhard *et al.*, 2000; Bernhard *et al.*, 2010) and many or most of the foraminiferal species reported to denitrify (Piña-Ochoa *et al.*, 2010) have not been examined for endo- and/or ectobionts, the role of prokaryotes in these associations deserves further study to resolve the enigma of foraminiferal denitrification. It is possible that other foraminiferal species, especially those lacking symbionts, may indeed perform complete denitrification but additional comparative studies using symbiont bearing and non-symbiont bearing species as well as antibiotic treatments are required to shed more light on the ability of these eukaryotes to denitrify.

The activity of the endobionts apparently allows the host to survive in anoxic habitats. The mechanism of this interaction is currently unknown, but may occur through removal of hydrogen or other metabolic intermediates. From a different perspective, non-fossilizable unilocular foraminifera similar to the SBB allogromiid evolved before more commonly known multilocular calcareous forms; such evolution likely occurred in the Neoproterozoic (Pawlowski *et al.*, 2003). Convincing evidence exists that anoxic marine habitats were extensive at this time (Frei *et al.*, 2009). Thus, acquisition of denitrifying bacterial symbionts would have expanded the foraminiferal habitat range early in their history.

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References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990). Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* **56:** 1919-1925.

Bernhard JM (2003). Potential symbionts in bathyal foraminifera. *Science* **299:** 861-861.

Bernhard JM, Buck KR, Farmer MA, Bowser SS (2000). The Santa Barbara Basin is a symbiosis oasis. *Nature* **403:** 77-80.

Bernhard JM, Goldstein ST, Bowser SS (2010). An ectobiont-bearing foraminiferan, *Bolivina pacifica*, that inhabits microxic pore waters: cell-biological and paleoceanographic insights. *Environmental Microbiology* **12:** 2107-2119.

Bernhard JM, Habura A, Bowser SS (2006). An endobiont-bearing allogromiid from the Santa Barbara Basin: Implications for the early diversification of foraminifera. *Journal of Geophysical Research-Biogeosciences* **111**.

Bernhard JM, Sen Gupta BK, Borne PF (1997). Benthic foraminiferal proxy to estimate dysoxic bottom-water oxygen concentrations: Santa Barbara basin, US Pacific continental margin. *Journal of Foraminiferal Research* **27:** 301-310.

Bernhard JM, Visscher PT, Bowser SS (2003). Submillimeter life positions of bacteria, protists, and metazoans in laminated sediments of the Santa Barbara Basin. *Limnology and Oceanography* **48:** 813-828.

Braker G, Fesefeldt A, Witzel KP (1998). Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology* **64:** 3769-3775.

Casciotti KL, Ward BB (2001). Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. *Applied and Environmental Microbiology* **67:** 2213-2221.

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA *et al* (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31:** 442-3.

Daims H, Bruhl A, Amann R, Schleifer KH, Wagner M (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology* **22:** 434-444.

Dalsgaard T, Thamdrup B, Canfield DE (2005). Anaerobic ammonium oxidation (anammox) in the marine environment. *Research in Microbiology* **156:** 457-464.

Edgcomb VP, Breglia SA, Yubuki N, Beaudoin DJ, Patterson DJ, Leander BS *et al* (2010). Identity of epibiotic bacteria on symbiontid euglenozoans in O_2-depleted marine sediments: evidence for symbiont and host co-evolution. *ISME Journal***:** 1-13.

Francis CA, Beman JM, Kuypers MMM (2007). New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *ISME Journal* **1:** 19-27.

Frei R, Gaucher C, Poulton SW, Canfield DE (2009). Fluctuations in Precambrian atmospheric oxygenation recorded by chromium isotopes. *Nature* **461:** 250-U125.

Gruntzig V, Nold SC, Zhou JZ, Tiedje JM (2001). *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. *Applied and Environmental Microbiology* **67:** 760-768.

Gunasekera TS, Dorsch MR, Slade MB, Veal DA (2003). Specific detection of *Pseudomonas* spp. in milk by fluorescence *in situ* hybridization using ribosomal RNA directed probes. *Journal of Applied Microbiology* **94:** 936-945.

Hallin S, Lindgren PE (1999). PCR detection of genes encoding nitrile reductase in denitrifying bacteria. *Applied and Environmental Microbiology* **65:** 1652-1657.

Hallin S, Throback IN, Dicksved J, Pell M (2006). Metabolic profiles and genetic diversity of denitrifying communities in activated sludge after addition of methanol or ethanol. *Applied and Environmental Microbiology* **72:** 5445-5452.

Høgslund S, Revsbech NP, Cedhagen T, Nielsen LP, Gallardo VA (2008). Denitrification, nitrate turnover, and aerobic respiration by benthic foraminiferans in the oxygen minimum zone off Chile. *Journal of Experimental Marine Biology and Ecology* **359:** 85-91.

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437:** 543-546.

Lane D (ed.) (1991) *16S/23S rRNA sequencing*. John Wiley & Sons: New York, 115-175pp.

Longnecker K, Reysenbach AL (2001). Expansion of the geographic distribution of a novel lineage of epsilon-Proteobacteria to a hydrothermal vent site on the Southern East Pacific Rise. *FEMS Microbiology Ecology* **35:** 287-293.

Loy A, Kusel K, Lehner A, Drake HL, Wagner M (2004). Microarray and functional gene analyses of sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal cooccurrence of recognized genera and novel lineages. *Applied and Environmental Microbiology* **70:** 6998-7009.

Lucker S, Steger D, Kjeldsen KU, MacGregor BJ, Wagner M, Loy A (2007). Improved 16S rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence *in situ* hybridization. *Journal of Microbiology Methods* **69:** 523-528.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363-71.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria - problems and solutions. *Syst Appl Microbiol* **15:** 539-600.

Maturrano L, Santos F, Rossello-Mora R, Anton J (2006). Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. *Applied and Environmental Microbiology* **72:** 3887-3895.

Michotey V, Mejean V, Bonin P (2000). Comparison of methods for quantification of cytochrome cd(1)-denitrifying bacteria in environmental marine samples. *Applied and Environmental Microbiology* **66:** 1564-1571.

Moraru C, Lam P, Fuchs BM, Kuypers MMM, Amann R (2010). GeneFISH - an *in situ* technique for linking gene presence and cell identity in environmental microorganisms. *Environmental Microbiology* **12:** 3057-3073.

Muyzer G, Dewaal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S robosomal RNA. *Applied and Environmental Microbiology* **59:** 695-700.

Pawlowski J, Holzmann M, Berney C, Fahrni J, Gooday AJ, Cedhagen T *et al* (2003). The evolution of early Foraminifera. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 11494-11498.

Pernthaler A, Pernthaler J, Amann R (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Applied and Environmental Microbiology* **68:** 3094-3101.

Pernthaler J, Glockner FO, Schonhuber W, Amann R (eds) (2001) *Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes*. Academic Press: San Deigo, 207-226pp.

Petri R, Podgorsek L, Imhoff JF (2001). Phylogeny and distribution of the *soxB* gene among thiosulfate-oxidizing bacteria. *FEMS Microbiology Letters* **197:** 171-178.

Piña-Ochoa E, Høgslund S, Geslin E, Cedhagen T, Revsbech NP, Nielsen LP *et al* (2010). Widespread occurrence of nitrate storage and denitrification among Foraminifera and Gromiida. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 1148-1153.

Reimers CE, Lange CB, Tabak M, Bernhard JM (1990). Seasonal spillover and varve formantion in the Santa Barbara Basin, California. *Limnology and Oceanography* **35:** 1577-1585.

Reimers CE, Ruttenberg KC, Canfield DE, Christiansen MB, Martin JB (1996). Porewater pH and authigenic phases formed in the uppermost sediments of the Santa Barbara Basin. *Geochimica et Cosmochimica Acta* **60:** 4037-4057.

519 Reysenbach AL, Pace NR (eds) (1994) *Reliable amplification of hyperthermophilic Archaeal*
520 *16S rRNA genes by the polymerase chain reaction*. Cold Spring Harbor Laboratroy Press:
521 Cold Spring Harbor.
522
523 Risgaard-Petersen N, Langezaal AM, Ingvardsen S, Schmid MC, Jetten MSM, Op den Camp
524 HJM *et al* (2006). Evidence for complete denitrification in a benthic foraminifer. *Nature*
525 **443:** 93-96.
526
527 Sigman DM, Casciotti KL, Andreani M, Barford C, Galanter M, Bohlke JK (2001). A bacterial
528 method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Analytical*
529 *Chemistry* **73:** 4145-4153.
530
531 Sigman DM, Robinson R, Knapp AN, van Geen A, McCorkle DC, Brandes JA *et al* (2003).
532 Distinguishing between water column and sedimentary denitrification in the Santa
533 Barbara Basin using the stable isotopes of nitrate. *Geochemistry Geophysics Geosystems*
534 **4**.
535
536 Stahl DA, Amann R (eds) (1991) *Development and application of nucleic acid probes in*
537 *bacterial systematics*. Wiley & Sons Ltd.: Chichester, England, 205-248pp.
538
539 Stamatakis A (2006). RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with
540 thousands of taxa and mixed models. *Bioinformatics* **22:** 2688-2690.
541
542 Stamatakis A, Hoover P, Rougemont J (2008). A Rapid Bootstrap Algorithm for the RAxML
543 Web Servers. *Systematic Biology* **57:** 758-771.
544
545 Throback IN, Enwall K, Jarvis A, Hallin S (2004). Reassessing PCR primers targeting *nirS*, *nirK*
546 and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS*
547 *Microbiology Ecology* **49:** 401-417.
548
549 Wallner G, Amman RI, Beisker W (1993). Optimizing fluorescent *in situ* hybridization with
550 rRNA-targeted oligonucleotide probes for flow cytometric identification of
551 microorganisms. *Cytometry* **14:** 136-143.
552
553 Winnepenninckx B, Backeljau T, Dewachter R (1993). Extraction of high molecular weight
554 NDA from mollusks. *Trends in Genetics* **9:** 407-407.
555
556 Yeates C, Saunders AM, Crocetti GR, Blackall LL (2003). Limitations of the widely used
557 GAM42a and BET42a probes targeting bacteria in the Gammaproteobacteria radiation.
558 *Microbiology* **149:** 1239-1247.
559

Figure Legends:

Figure 1. TEM micrographs of the SBB allogromiid. **a**, Low magnification view showing abundant vacuoles (v) in the endoplasm vs. ectoplasm; e = environment outside foraminifer. **b**, View of coccoid endobionts showing their tendency to form short chains (⁎). Scales: a=5µm; b=1µm.

Figure 2. Phylogenetic analysis of the 16S rRNA gene from the endobiont of SBB allogromiid showing its placement within gamma-proteobacteria. Maximum likelihood tree is based on an alignment of 1400 nucleotides. Scale is given as substitutions per site. See Methods for details.

Figure 3. Paired FISH images of the SBB allogromiid. **a-b,** Paired images of whole specimen showing DAPI (**a**) and DAPI/CARD-FISH with Cy3-labeled universal Eubacterial probe EUB338I-III (**b**). **c-d**, Paired images of whole specimen showing DAPI (**c**) and CARD-FISH with Cy3-labeled negative control probe NON338 (**d**). **e-f**, Paired GeneFISH images of lysed allogromiid hybridized to the FITC-labeled *Pseudomonas*-specific 16S rRNA gene probe **(e)** and negative control of Texas-Red labeled *nirK* gene (**f**) showing some non-specific background hybridization to cellular debris found in close association with some endobiont cells. **g-h**, Paired GeneFISH images of another lysed allogromiid hybridized to the FITC-labeled *Pseudomonas*-specific 16S rRNA gene probe (**g**) and Texas-Red labeled *nirK* gene (**h**). Arrows indicate short chains of coccoid cells in FITC image **(g)** and coccoid cells in the GeneFISH image (**h)**. Scales: **a,c**=50µm; **g**=20µm (e-h all same magnification).

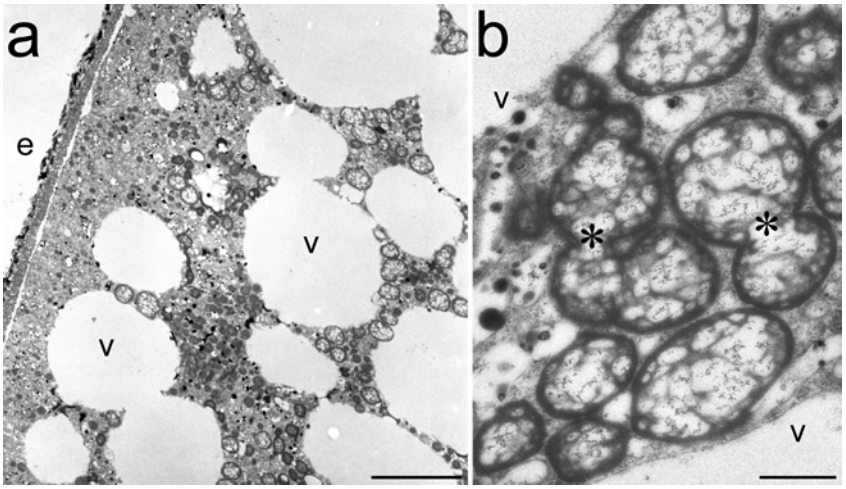

Figure 1.

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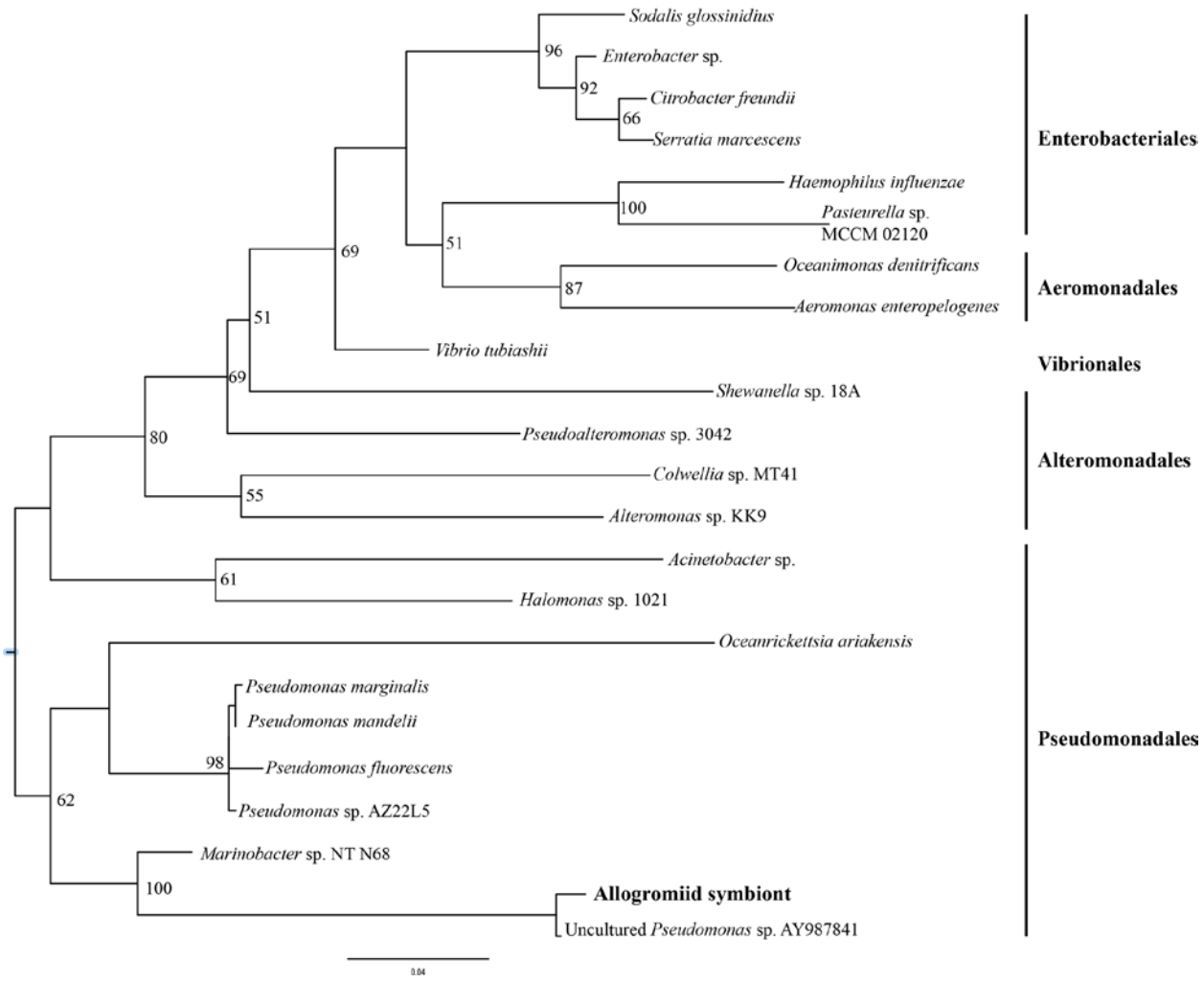

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587 Figure 2.

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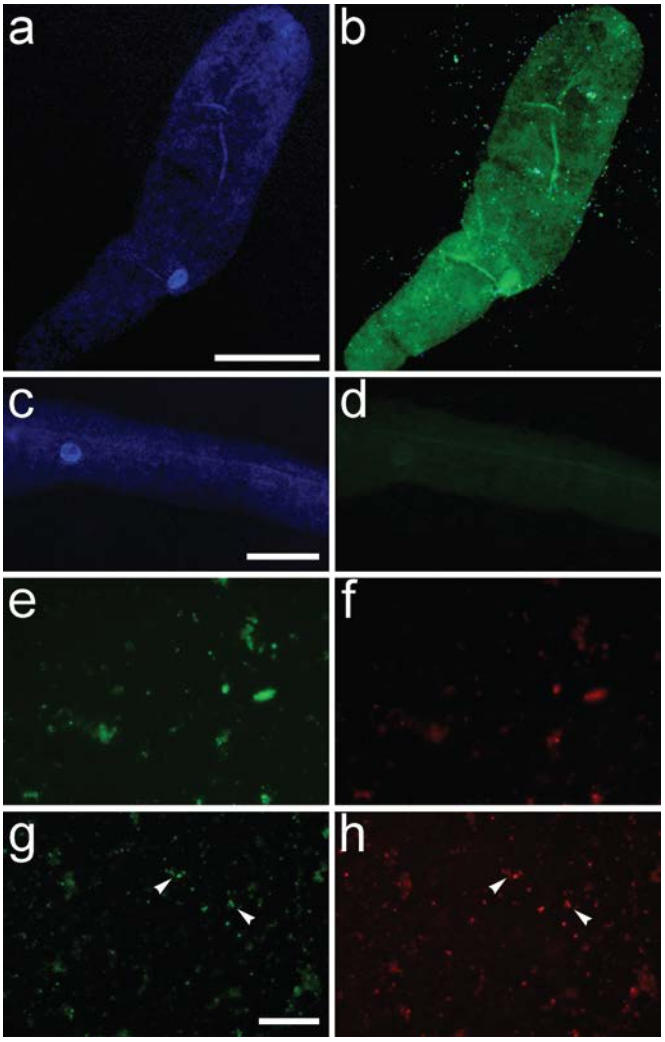

Figure 3.