

ORIGINAL ARTICLE

Dimorphism in methane seep-dwelling ecotypes of the largest known bacteria

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We present evidence for a dimorphic life cycle in the vacuolate sulfide-oxidizing bacteria that appears to involve the attachment of a spherical *Thiomargarita*-like cell to the exteriors of invertebrate integuments and other benthic substrates at methane seeps. The attached cell elongates to produce a stalk-like form before budding off spherical daughter cells resembling free-living *Thiomargarita* that are abundant in surrounding sulfidic seep sediments. The relationship between the attached parent cell and free-living daughter cell is reminiscent of the dimorphic life modes of the prosthecate Alphaproteobacteria, but on a grand scale, with individual elongate cells reaching nearly a millimeter in length. Abundant growth of attached *Thiomargarita*-like bacteria on the integuments of gastropods and other seep fauna provides not only a novel ecological niche for these giant bacteria, but also for animals that may benefit from epibiont colonization.

The ISME Journal (2011) 5, 1926–1935; doi:10.1038/ismej.2011.66; published online 23 June 2011

Subject Category: microbial ecology and functional diversity of natural habitats

Keywords: *Beggiatoa*; budding; epibiont; sulfur bacteria; *Thiomargarita*; *Thioploca*

Introduction

The bacterial domain includes representatives with a variety of cell shapes (Young, 2006) and types of cell division (Angert, 2005). Bacterial cell morphologies can provide selective advantages ranging from increased motility to decreased predation susceptibility (Young, 2006). The enormous size of the sulfide-oxidizing Gammaproteobacterium, *Thiomargarita namibiensis*, is thought to provide it with the ability to store nitrate for months, persisting in environments in which availability of the primary terminal electron acceptor oxygen, or the alternative acceptor nitrate, can be temporally and spatially variable (Schulz and Jørgensen, 2001; Schulz, 2006). However, the influence of cell morphology on the ecophysiology of these giant sulfur bacteria may not be limited to their large size.

Thiomargarita was first described in 1999 from diatom-rich oozes in the Benguela upwelling system off West Africa (Schulz *et al.*, 1999). The originally described species *T. namibiensis* occurs primarily as linear chains of spherical cells enclosed in a mucous

sheath (Schulz *et al.*, 1999), though other morphotypes have since been described (Schulz, 2006; Gutzke, 2007). In 2005, *Thiomargarita*-like bacteria that undergo reductive cell division resulting in symmetrical clusters of up to 16 cells were discovered at hydrocarbon seeps from the Gulf of Mexico (Kalanetra *et al.*, 2005; Bailey *et al.*, 2007). Other *Thiomargarita*-like bacteria have also been obtained from mud volcanoes in the Barents Sea (de Beer *et al.*, 2006) and the Eastern Mediterranean Sea (Girnth *et al.*, 2011). During a 2009 cruise aboard the R/V *Atlantis*, we observed a population of *Thiomargarita*-like cells in cores and on carbonate rock samples collected with the DSV *Alvin* at methane seeps associated with fault scarps, landslides and mud mounds (Bohrmann *et al.*, 2002) at water depths of ~990–1600 m along the Pacific margin of Costa Rica. Within the *Thiomargarita*-like population present at Costa Rica margin seeps are canonical free-living, solitary and reductively dividing forms, as well as cells exhibiting an attached, presumptive dimorphic life cycle that we describe here. Similar populations of free-living and attached *Thiomargarita*-like cells were observed in Hydrate Ridge (off Oregon, USA) seep settings in 2010. The observation of dimorphic forms at multiple localities suggests that a dynamic morphology has an important role in the ecology of vacuolate sulfur bacteria in seep settings.

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Received 20 September 2010; revised 28 March 2011; accepted 3 May 2011; published online 23 June 2011

Materials and methods

Sample collection and microscopy

Samples were obtained during a research expedition to the Pacific margin of Costa Rica aboard the R/V *Atlantis* in February–March 2009 (AT15-44) and during a follow-up cruise to the same sites in January 2010 (AT15-59). Push cores, samples of authigenic carbonate and associated macro- and megafaunal animals were collected from methane seeps associated with mud mounds and fault scarps at Mound 11 (1000–1025 m, 8° 55.4'N, 84° 18.24'W), Mound 12 (990–1100 m, 8° 55.8'N, 84° 18.75'W), Jaco Scarp (1775–1850 m, 9° 7.00'N 84° 50.51'W) and Mound Quepos (1030–1179 m, 8° 58.09'N, 84° 37.95'W) using the DSV *Alvin*. Additional collections of living gastropods with attached sulfur bacteria were made during AT 15-68 at Hydrate Ridge North (44° 40'N 125 5.8'W) and Hydrate Ridge South (44° 34.1'N 125° 9.1'W).

During the research expedition, a Leica S8 APO stereo-microscope coupled with a Canon Powershot 9 (Leica, Buffalo Grove, IL, USA) was used to observe and image freshly collected free-living cells, as well as those cells attached to byssal threads, shells and other benthic substrates. Cells that were budding at the time of collection were followed for a 10-day duration of the cruise and imaged on a daily basis in order to document bud detachment. In the laboratory, an Olympus SZX16 stereo-microscope and an Olympus BX51 compound microscope equipped with an Olympus DP72 color camera were used to obtain higher resolution images of these cells and attachment substrates (Olympus, Tokyo, Japan).

Electron microscopy

Gastropods covered with attached *Thiomargarita*-like sulfur bacteria were preserved in Karnovsky's fixative (Karnovsky, 1965) before undergoing ethanol series dehydration (25%, 50%, 75%, 90%, 99%) in artificial seawater/ethanol mixtures for 30 min each. The bacteria-covered gastropod shells were then dried in a Tousimis Model 780A Critical-Point Dryer (Tousimis, Rockville, MD USA) before being imaged uncoated with a Hitachi TM-1000 tabletop scanning electron microscope (Hitachi, Tokyo, Japan).

For transmission electron microscopy imaging, samples were fixed with Karnovsky's fixative solution and after three times washing with artificial seawater, the samples were post fixed with 1% OsO₄ for 1 h. Following three times rinsing with H₂O, the samples were dehydrated in a graded series of ethanol (see above). The samples were embedded in Epon 812 resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Ultrathin sections (65 nm) were produced using a Reichert UltraCut S Ultramicrotome (Reichert Technologies, Depew, NY, USA) and stained with uranyl acetate and lead citrate before being examined with a JEOL 1200EX electron microscope (JEOL Inc., Tokyo, Japan).

16S ribosomal RNA (16S rRNA) gene sequencing

Free-living *Thiomargarita*-like cells from Costa Rica and Namibia were removed from the sediment (Salman *et al.*, 2011). The novel attached morphotype from Costa Rica were carefully scraped off byssal threads with two needles and the detached cells were removed from the sample as described above. Individual cells, that is, free-living cells and formerly attached cells sometimes showing distal budding, were washed in artificial seawater, photographed under the microscope and subsequently used for performing single-cell 16S rRNA PCR methods described elsewhere (Salman *et al.*, 2011). The specific primer for *Thiomargarita*-related organisms, VSO233F, 5'-CCTATGCCGGATTAGCTTG-3' (*Escherichia coli* 16S rRNA position 233–251, Salman *et al.*, 2011), was combined with the universal primer ITSReub, 5'-GCCAAGGCATCCACC-3' (*E. coli* 23S rRNA position 23–38; Cardinale *et al.*, 2004). Some attached cells did not reveal any PCR product with these primers, thus another specific forward primer was developed based on *Thiomargarita* sequences from the public databases: VSO360F, 5'-GGAATATTGGACAATGGGC-3' (*E. coli* 16S rRNA positions 361–379, developed in this study), which was used in combination with primer VSO1300R, 5'-ATCCGGA CTACGAGTAG-3' (*E. coli* 16S rRNA position 1291–1307; Salman *et al.*, 2011).

The amplification products were analyzed by agarose gel electrophoresis, bands of ~1750 bp length (and ~940 bp length with the other primer pair) were excised and DNA was purified with a gel extraction kit (Qiagen, Hilden, Germany). PCR products were sequenced directly using the Big Dye Cycle Sequencing Kit and analyzed on the ABI Prism Genetic Analyzer 3130x (Applied Biosystems, Carlsbad, CA, USA). The seven 16S RNA *Thiomargarita*-affiliated sequences have been submitted to the DDBJ/EMBL/GenBank databases. The accession numbers are FN811658–FN811664. The internal transcribed spacer region (spacer region between 16S rRNA and 23S rRNA as part of the PCR product when using primer ITSReub) was removed before further analysis of sequences.

Phylogenetic tree reconstruction

The phylogenetic affiliation of the retrieved sequences was inferred with the ARB software package (Ludwig *et al.*, 2004) based on release 95 of the SILVA SSURef database (Pruesse *et al.*, 2007). The alignment of all sequences was automatically performed with the integrated aligner and checked manually afterwards, taking into account the secondary structure of the 16S rRNA molecule. For tree reconstruction, a total of 90 nearly full-length sequences, including representatives of the Gamma- and Deltaproteobacteria, were used. Trees were calculated including nucleotide positions between 279 and 1463 (according to *E. coli* numbering) and performing neighbor joining, maximum parsimony

and maximum likelihood analyses, each with the application of 0%, 30% and 50% positional conservatory filters excluding highly variable regions. Partial sequences (AY632420, AF129012, FN811664) were inserted into each of the reconstructed trees by parsimony criteria without allowing changes in the overall tree topology. A consensus tree was manually produced based on the maximum likelihood tree calculated with a 30% filter using an approach described in (Peplies *et al.*, 2008). The branching patterns of all trees were compared and multifurcations were introduced at nodes where the exact tree topology could not be clearly resolved (Peplies *et al.*, 2008). The tree presented in Figure 5 shows a subset of the sequences originally used for calculation. Neighbor-joining and maximum likelihood trees are included in the supplement for comparison (Supplementary Figure S1 and S2).

Results and discussion

Occurrence of Thiomargarita-like bacteria

Free-living *Thiomargarita*-like cells were observed in sulfidic sediments at several mud mounds and associated methane seeps (Figure 1a). As in sediments from off Namibia (Schulz *et al.*, 1999), and in the Gulf of Mexico (Kalanetra *et al.*, 2005), free-living sulfur bacteria off Costa Rica resembling *Thiomargarita* were most abundant in white and gray mat associated sediments in the uppermost 3 cm beneath the sediment/water interface. At some localities, cell populations consisted primarily of single *Thiomargarita*-like cells mixed with large *Beggiatoa*-like filaments (Figure 1a), whereas at other locations, symmetrical cell clusters (not shown) predominated in gray flocculant material at the seabed. These cell clusters contained 2, 4, 8 and 16 cells, similar to those produced by reductive division in *Thiomargarita* sp. from the Gulf of Mexico (Kalanetra *et al.*, 2005). In the vicinity of these sediments, rich in free-living spherical *Thiomargarita*-like cells, thin films of microbial biomass were found attached to methane-derived authigenic carbonates (Figure 1b), byssal threads of the mussel *Bathymodiolus* spp. (Figure 1c), fragments of organic detritus, and most commonly, the integuments of seep-endemic invertebrates (Figures 2a–c). Microscopic examination of this benthic biofilm material revealed densely packed attached sulfur-storing vacuolated cells that closely resemble *Thiomargarita*, but with most cells elongated into a stalk-like form. Individual cells occasionally reached nearly 700 μm in length, but average elongate cells were $338 \pm 169 \mu\text{m}$ long (Supplementary Figure S3). These elongate cells were observed to bud spherical *Thiomargarita*-like cells from their unattached pole (Figures 3 and 4). The attached poles of the elongate cells ranged from rounded (Figures 3 and 4) to a heavily tapered, and sometimes extruded shape, forming a short appendage

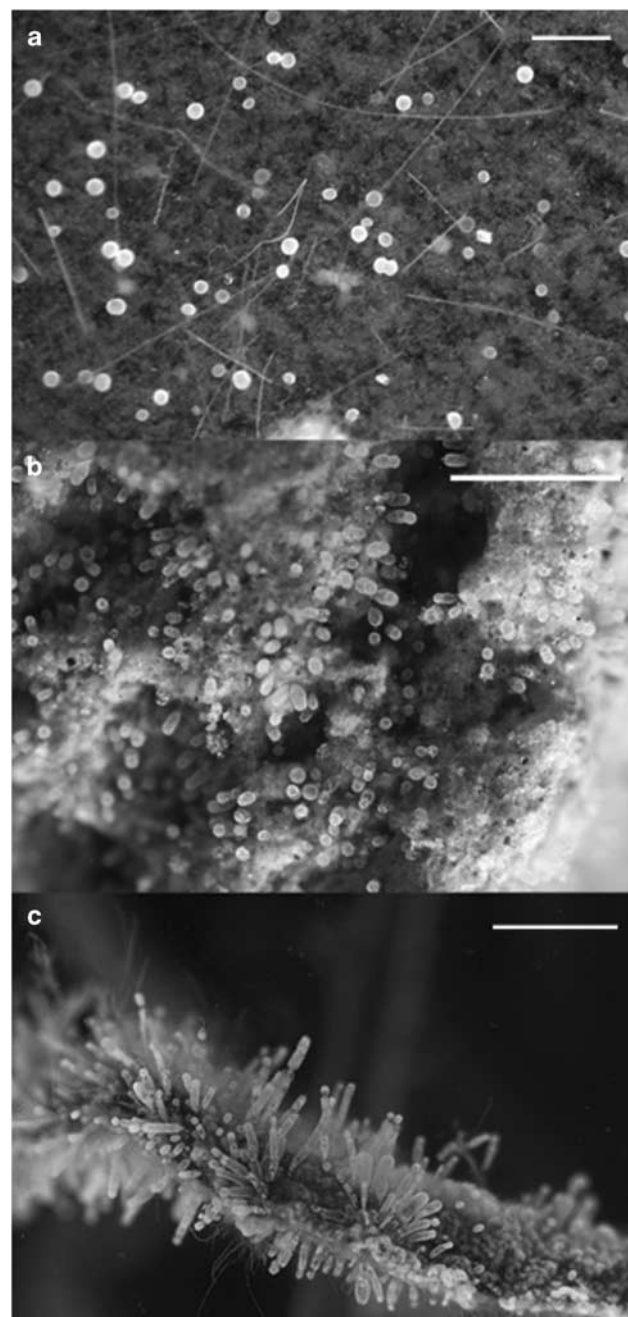


Figure 1 Vacuolate sulfide-oxidizing bacteria are abundant on a variety of substrates along the Costa Rica Margin including (a) free-living *Thiomargarita*-like cells and large *Beggiatoa*-like filaments inhabiting sulfidic sediments; (b) slightly elongate *Thiomargarita*-like morphotypes attached to authigenic carbonates; and (c) elongate *Thiomargarita*-like cells attached to *Bathymodiolus* mussel byssal threads. Scale bars represent 500 μm .

(Figure 4c). Both the attached elongate cells and the buds consisted of a single thin layer of sulfur globule-containing cytoplasm surrounding a single large vacuole (Figure 3d and transmission electron microscopy observations not shown)—a basic cell structure that they share with all previously described *Thiomargarita* ecotypes (Schulz *et al.*, 1999;

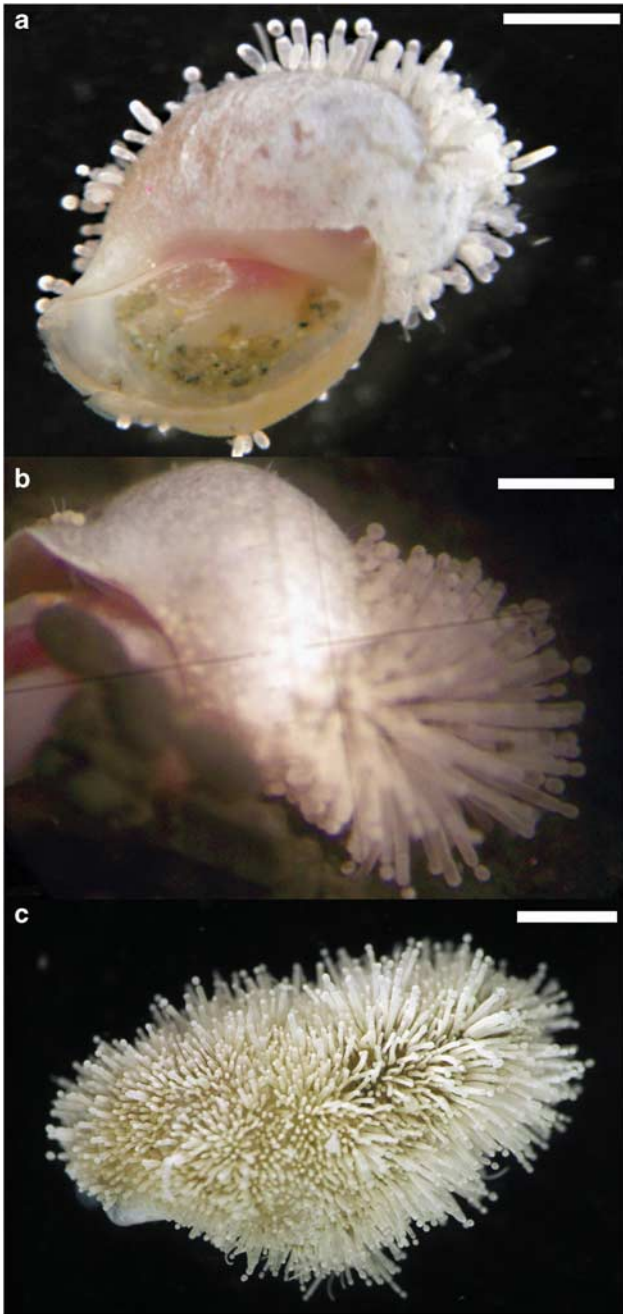


Figure 2 (a–c) The shells of living gastropods (*Provanna laevis*) from the Costa Rica margin and the Cascadia margin (Hydrate Ridge) are colonized by attached, elongate *Thiomargarita*-like cells. Scale bars represent ~ 1 mm.

Kalanetra *et al.*, 2005; Schulz, 2006). Elongate morphotypes were not observed in the unattached free-living state. We hypothesize that the attached, budding types, and at least some of the free-living cells, represent not different species, but different stages of a reproductive developmental cycle. We evaluated this hypothesis by directly observing the budding forms after collection. Furthermore, we analyzed the 16S rRNA gene sequences of the new, attached morphotypes, as well as of the free-living

canonical morphotypes. Evidence for a developmental relationship between the attached cells and free-living spherical cells was provided by direct observation of buds formed at the time of collection completely detaching from budding parent cells at a rate of approximately one detachment in 300 budding cells over a 24 h period (12 in approximately 3600 individuals observed), demonstrating that the budded daughter cells are indeed being released into the surrounding environment (Figures 3b, c, f). Fully detached, budded daughter cells were morphologically identical to free-living, spherical sulfur bacteria in surrounding sediments and both have similar cell diameters; $71 \pm 22 \mu\text{m}$, $n = 40$ versus $88 \pm 14 \mu\text{m}$, $n = 37$. The morphological similarity of the released buds to the free-living *Thiomargarita*-like cells in the surrounding sediments suggests the possibility that at least some of the latter population results from the released spherical buds. However, morphology can be misleading, particularly with respect to the phylogenetic identification of vacuolated sulfur bacteria. For example, marine filaments with a *Beggiatoa*-like morphology, that have similar filament diameters and were collected from the same locality, can be quite divergent with respect to their phylogenetic positions (Teske *et al.*, 1999; Mußmann *et al.*, 2007). The observations that the attached elongate cells are found on hard substrates that are physically separated by sediments that contain spherical, free-living cells, and that elongate cells are never observed in the free-living populations, strongly suggests that at least some of the free-living population includes the spherical offspring of the attached morphotypes. In addition, the assertion that the life cycle is initiated by the attachment of spherical cells is supported by the observation of attached spherical cells that are just beginning elongation (Supplementary Figure S4), along with the spectrum of elongation morphologies from sphere, to prolate spheroid, to increasingly longer cylindrical cells occurring in close proximity (Figure 4d). However, the percentage of free-living cells that represent the progeny of attached budding forms remains unclear.

Sequence comparisons of individual cells indicated that Costa Rica margin seeps harbor not just one, but at least three distinct phylogenetic clusters. The relatedness of the organisms in this study to *Thiomargarita namibiensis* and *Thiomargarita* sp. from the Gulf of Mexico is apparent from the phylogenetic tree produced by sequencing individual representatives from Costa Rica and additional specimens from Namibia (Figure 5). The sequences published before this study constitute the closest relatives to at least five sequences obtained in this study. Although some sequences of free-living and attached morphotypes are closely related to previously published *Thiomargarita* spp. (Kalanetra *et al.*, 2005; Schulz *et al.*, 1999), two sequences (COS001 and COS016) each formed a separate branch that did not cluster within the *Thiomargarita*

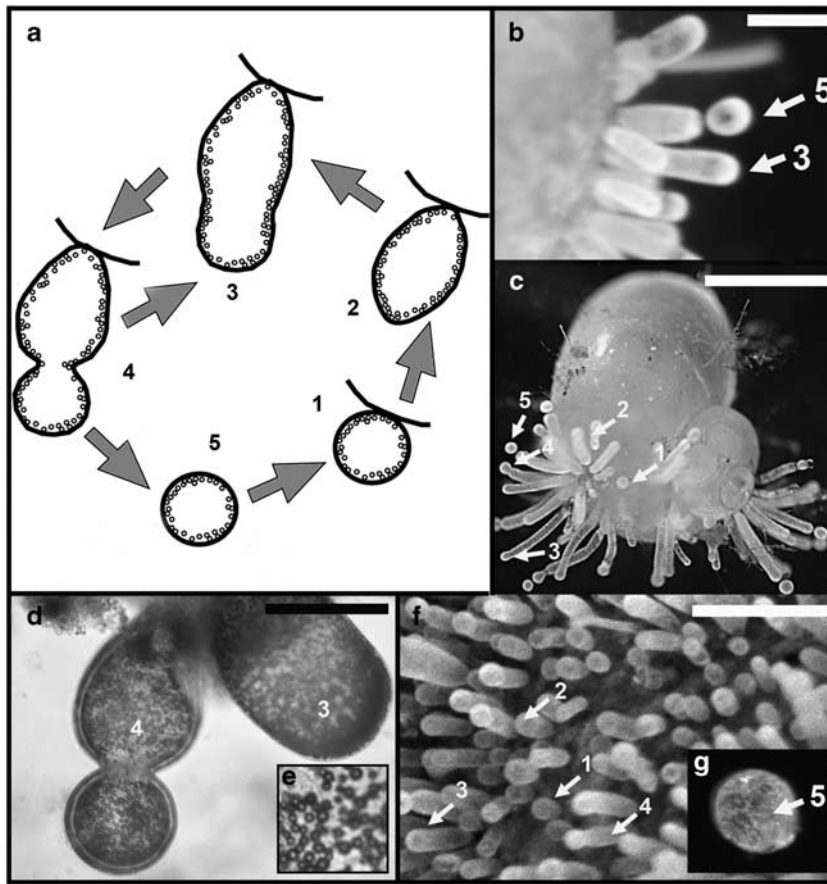


Figure 3 An apparent reproductive developmental cycle in attached sulfur bacteria (a) appears to begin with the attachment of a spherical cell to a solid substrate (arrows 1), followed by elongation to a stalked form (arrows 2), initial bud formation (arrows 3) and finally budding (arrows 4) to produce a spherical daughter cell (arrows 5). (b, c) Attached sulfur bacteria on a gastropod shell. (d, e) Detail of attached cell and intracellular sulfur globules. (f) Bacteria attached to mussel byssal threads. (g) Budded *Thiomargarita*-like cell was observed to undergo complete detachment from the mother cell between 12 and 20 h after collection. After bud detachment, the elongate mother cell (a4 → 3) can continue to produce additional buds. Scale bar in b = 350 μ m; c = 1 mm; d = 200 μ m; f = 300 μ m.

clade. The paraphyletic relationship of the retrieved 16S rRNA gene sequences surely complicates identification of cells based on morphology. Nomenclature for *Thiomargarita*-like cells and other vacuolate sulfur bacteria can be confusing, as some canonical genera include paraphyletic taxa. However, the taxonomy of the colorless sulfur bacteria, including the novel morphotypes identified in this study, was recently revisited by Salman *et al.* (2011).

Sequences in the three clusters represent either attached, free-living or paired free-living/attached morphotypes (Figure 5). Some sequences showed >99% similarity between attached and free-living morphotypes (Table 1) that may represent different developmental stages of the same organism, as the <1% difference is within the range of experimental error. Alternatively, these cells could represent closely related phylotypes that superficially resemble parts of the putative dimorphic life cycle, but do not represent the direct progeny of the attached cells. The disparity could be caused by natural variability within the seep population coupled with

the relatively low number of sequenced specimens, and the inherent limitations of sampling a deep-water site via submersible, thereby possibly missing directly corresponding mother and daughter cells. It is also unclear whether the reductively dividing free-living sulfur bacteria present in nearby sediments exhibit attachment or dimorphism as part of their life cycle(s).

Ecological significance of dimorphism

Reproductive dimorphism is known in the Alpha-proteobacteria, such as *Caulobacter crescentus*, whose parent cells are stalked, and the daughter cells are vibroid—but in those organisms the daughter cells are actively motile (Brun and Janakiraman, 2000; Poindexter, 2006). In the sulfur bacteria detected here, the daughter cells are not yet known to be actively motile—though an inconspicuous sporadic slow rolling motility has been observed in different, spherical sulfur bacteria from Namibia (Salman *et al.*, 2011). In addition, free-living daughter cells can be readily transported by

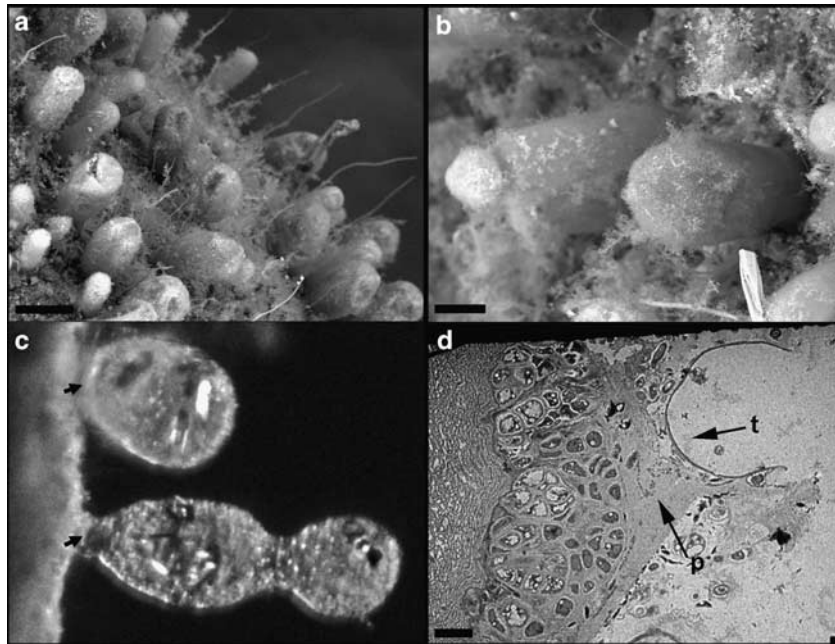


Figure 4 Attached sulfur bacteria. (a, b) Densely packed *Thiomargarita*-like cells on gastropod shell co-occur with attached filamentous bacteria. Scale bar in **a** = 30 μm ; **b** = 20 μm . (c) Particularly during early stages of attachment and elongation they have rounded bases where they are attached to benthic substrates. As the cell elongates, the base of the cells becomes tapered. In some specimens, a small appendage formed by extrusion of the cell ultrastructure is present. (d) Transmission electron micrograph of vacuolated *Thiomargarita*-like cells (t) attached to plaque (p) and mucous occupied by smaller filamentous bacteria. This material surrounds the *Bathymodiolus* mussel byssus. Scale bar = 2 μm .

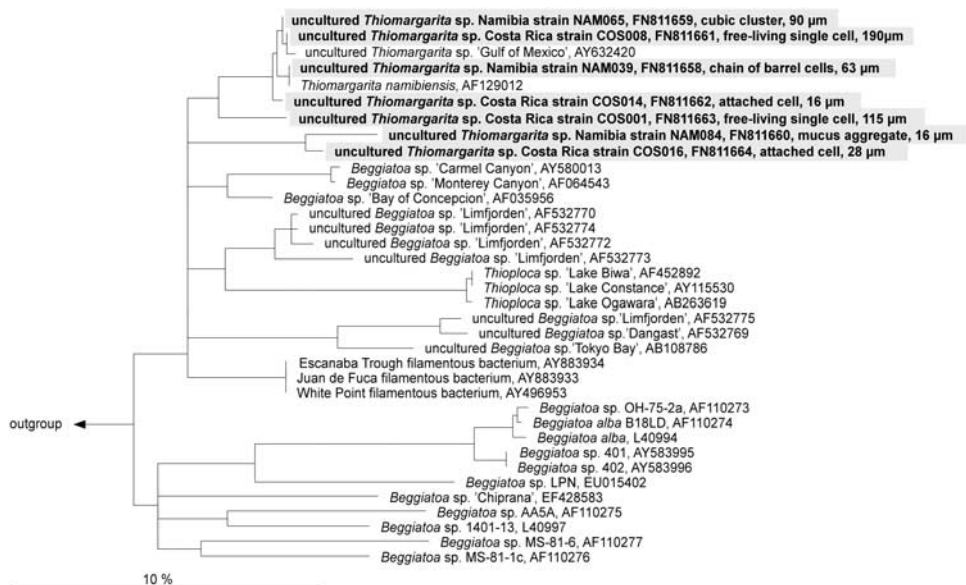


Figure 5 Phylogenetic tree of gammaproteobacterial 16S rRNA gene sequences showing sequences of different morphotypes from Costa Rica (COS) and Namibia (NAM) in gray and their affiliation to previously published *Thiomargarita* spp. These sequences are shown within the context of the broader clade of conspicuous sulfide-oxidizing Gammaproteobacteria. Of each newly sequenced cell the morphology and diameter is indicated. In attached forms, the widest diameter of the attached basal body is reported. The scale bar represents ~10% sequence divergence.

bottom currents. Broken, transported and re-deposited clasts of methane-derived authigenic carbonate edifices observed on the collection cruises, as well as evidence for local sediment winnowing, record

the presence of such currents at the sites where the attached *Thiomargarita*-like morphotypes occur. On detachment, buds may also attach to substrates directly adjacent to the mother cell. This process is

Table 1 Levels of 16S rRNA gene sequence identities of nearly full-length sequences from *Thiomargarita*-like cells from the COS and NAM along with partial sequences of formerly described *T. nam.* and *T. GOM* from the Gulf of Mexico as reference species

	NAM065	COS008	<i>T. GOM</i>	NAM039	<i>T. nam.</i>	COS014	COS001	NAM084
NAM065								
COS008	99.8							
<i>T. GOM</i>	99.7	99.8						
NAM039	99.5	99.2	99.8					
<i>T. nam.</i>	99.1	98.7	99.1	100				
COS014	99.4	99.4	99.1	99.3	98.9			
COS001	96.6	96.5	95.7	96.4	94.9	96.8		
NAM084	95.3	95.3	92.4	95.3	92.4	95.2	94.4	
COS016	95.1	95.1	95.1	95	95	94.8	93.8	99.1

Abbreviations: 16S rRNA, 16 ribosomal RNA; COS, Costa Rica margin; NAM, Namibia; *T. GOM*, *Thiomargarita* sp. Gulf of Mexico; *T. nam.*, *Thiomargarita namibiensis*.

known to occur in some prosthecate Alphaproteobacteria and would explain the observation that mats of attached *Thiomargarita*-like sulfur bacteria are often densely packed and frequently exhibit more than one developmental stage on the same substrate (Figure 3b). Alternatively, certain cells in the mat may simply have slower elongation and budding rates than others.

The factors responsible for initiating attachment, elongation and budding in these bacteria are not fully understood because *Thiomargarita* and its vacuolated relatives have yet to be cultivated in the laboratory. However, comparisons with other organisms that exhibit similar behaviors may provide clues. Attachment to solid substrates is also known in other Gammaproteobacteria, including the closely-related marine, vacuolated *Thiothrix*-like bacteria (Kalanetra *et al.*, 2004; Kalanetra and Nelson 2010). Indeed, attached filamentous *Thiothrix*-like filaments sometimes co-occur with the attached *Thiomargarita*-like cells described here. Attachment provides stabilization of a parent cell under conditions in which it can produce daughter cells for dispersal to other potential habitats via bottom currents. Presumably, stabilization of reproductively active sulfide-oxidizing cells over zones of sulfide enrichment would be more important in advective seep habitats where sulfide generated by the anaerobic oxidation of methane or vented directly from subsurface fluids is more localized than the sulfide generated by sulfate reduction in upwelling regions (Linke *et al.*, 2005).

Budding is known in *Thiothrix*-like filaments (Kalanetra *et al.*, 2004), in certain low-GC gram-positive bacteria, cyanobacteria, planctomycetes and prosthecate Alphaproteobacteria (Angert, 2005) among others. In prosthecate bacteria, it is the stalked parent cell that undergoes budding, suggesting that perhaps elongation enhances dispersal of daughter cells. However, elongation is apparently not a prerequisite to budding in attached *Thiomargarita*-like cells, as division of attached but not elongated cells, or slightly elongated cells, was observed on a handful of substrates (Figure 1b). Access to scarce metabolites may instead drive

elongation in attached sulfur bacteria. Cell elongation in *Caulobacter crescentus* is thought to enhance the uptake of phosphorous from the environment by increasing the surface area-to-volume ratio of a cell (Poindexter, 1984). *Thiomargarita namibiensis*, which lives in sediments beneath upwelling zones, sequesters phosphorous as intracellular polyphosphate granules (Schulz and Schulz, 2005). In seep settings, phosphorous is less available than in an upwelling environment (Joye *et al.*, 2004; Linke *et al.*, 2005). Thus, cell elongation in seep-dwelling sulfur bacteria may provide for enhanced phosphorous uptake as it does in the Alphaproteobacteria. Nutrient uptake and adenosine triphosphate synthesis scales with cell surface area, and *Thiomargarita* functions at a relatively low level of metabolic power per gene (Lane and Martin, 2010). An elongate cell has a higher surface area-to-volume ratio than a spherical cell of the same volume (Young, 2006). For example, if a cell was assumed to be a cylinder capped with hemispherical ends, 300 μm in length and 80 μm in diameter, the cell would have a 38.5% greater surface area-to-volume ratio (0.054) over a sphere with the same volume (0.039). The larger a *Thiomargarita* cell grows, the larger its potential store of nitrate, but at the cost of decreasing its surface area-to-volume ratio. Growth of a cell into an elongate form, as opposed to simply expanding the sphere volume, minimizes the decrease in surface area-to-volume ratio that accompanies growth. Reductive cell division, which is observed in free-living *Thiomargarita*-like cells at this site, also effectively increases surface area while maintaining a constant nitrate-storage volume, although by producing smaller cells.

The elongation of budding cells may also allow attached cells greater access to the terminal electron acceptors, oxygen and nitrate. It is not known whether attached and free-living reductively dividing *Thiomargarita*-like bacteria represent different stages within the same life cycle. However, one possibility is that elongation increases surface area in attached cells that have access to nitrate and can readily enlarge, whereas reductive division apporions stored nitrate into smaller, easily dispersible

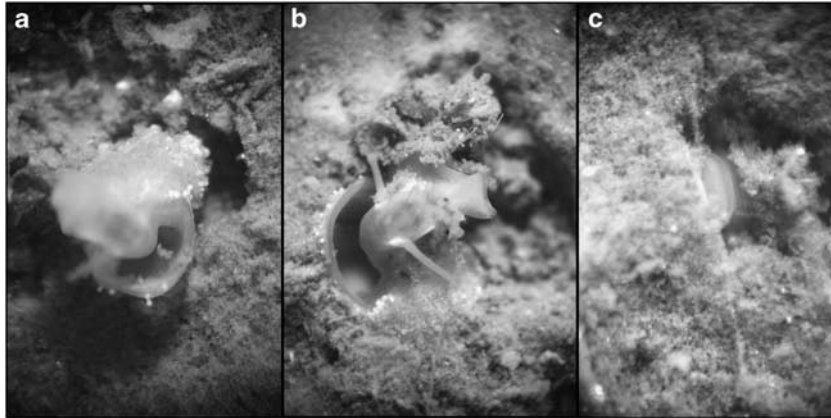


Figure 6 The gastropod *Provanna laevis* burrows into sulfidic mud in a collection container, (a) first digging a burrow, (b) then occupying the burrow with its carapace oriented downward, which exposes the population of attached sulfur bacteria to sulfidic conditions. (c) The head of the gastropod remains partially exposed above the sediment/water interface.

cells with higher surface area–volume ratios when nitrate availability is limited. Closely related vacuolate sulfur bacteria resembling *Beggiatoa* and *Thioploca* can actively move along gradients between sulfide and terminal electron acceptors (Nelson *et al.*, 1986; Huettel *et al.*, 1996). In seep settings, alternating conditions of access to electron donor and acceptor can be introduced by brine flows (Girnth *et al.*, 2011), changing currents, and heterogeneous pore fluid advection, exposing populations of sulfur bacteria to alternating conditions of high sulfide, nitrate and oxygen fluxes. Elongation in sessile bacteria may provide cells with a larger surface area for more efficient uptake of spatially segregated metabolites, under the fluctuating and heterogeneous fluxes of O_2 , NO_3^- and H_2S measured in Costa Rica margin sediments (Linke *et al.*, 2005). In some freshly collected samples, elongate cells possess a visibly marked increase in sulfur globules at the distal end of the cell. The apical, sulfur-enriched region is the site of bud formation. These sulfur resources might be incorporated into the budding offspring, providing an initial energy reserve for the free-living state. Alternatively, the irregular distribution of sulfur globules may be driven by the local geochemical environment, where the observed sulfur accumulation at the apical end of the cell is linked to close contact with either oxygenated, or sulfidic waters, depending on the orientation of the cell. However, this explanation seems less likely, as cells attached to opposite sides of byssal threads also showed apical sulfur enrichment in cells oriented in opposite directions.

Metazoan–bacterial interactions

The attachment of newly detected sulfur bacteria to benthic substrates also has potentially important implications for metazoan ecology at seeps. Elongate sulfur bacteria were found attached to byssal threads of *Bathymodiolus* spp. (Mytilidae, Bivalvia), the carapace of a Yeti crab specimen (*Kiwa* sp.), the

shells of several species of gastropod including *Provanna laevis*, *Hyalogyrina* sp. and *Pyropelta* spp., and the limpet *Lepetodrilus* sp. These gastropod shells were commonly covered with thick mats of attached elongate sulfur bacteria (Figure 2). Interestingly, bacteria-covered *Provanna* collected from Hydrate Ridge in 2010 exhibited an unusual behavior that may benefit the attached sulfide-oxidizing bacteria. When placed in a container with seawater overlying sulfidic sediments, they were observed to either climb to the uppermost portion of the jar and maintain a position adjacent to the small air headspace ($n=4$), or actively dig a burrow into the sulfidic sediments ($n=8$) and subsequently inverting themselves, so that their shell and attached bacteria were positioned within the freshly dug burrow (Figure 6). Gastropods removed from the burrows with forceps repeatedly re-established their burrows and inverted positions ($n=6$). Epibiont-bearing invertebrates are known to migrate between oxygenated and sulfidic sediment pore waters as a means of supplying symbionts with the metabolic substrates required for sulfide oxidation (for example, Ott *et al.*, 1991), and a similar behavior may be on display here. However, this behavior and association with seep invertebrates is apparently not a requirement for growth of the attached *Thiomargarita*-like cells, as these bacteria are sometimes found on stabilized mineral and detrital substrates such as carbonate rocks. The impetus for gastropods to exhibit this behavior that potentially exposes them to predation while lying inverted with their soft parts exposed remains unclear. The bacteria may reduce the animal's exposure to hydrogen sulfide as is thought to occur in other invertebrates (Alain *et al.*, 2002). These species of gastropods are not known to contain sulfide-oxidizing endobionts. Attached bacteria may also serve as food source for grazing gastropods, as occurs in some other animal/epibiont symbioses (for example, Ott *et al.*, 1991; Zbinden and Cambon-Bonavita, 2003; Gillian and Dubilier, 2004; Dattagupta *et al.*, 2009), although

these gastropods are seemingly incapable of consuming the bacteria attached to their own shells.

At Costa Rica seep settings, attached sulfur bacteria are rare on rocks and shells with large surface areas. The substrates most well colonized by the cells are those with low surface areas such as small shells and mussel byssal threads. When they do occur on larger substrates, they are found in small isolated patches or within small crevices. The apparent absence of attached bacteria on *Bathymodiolus* mussel shells, *Lamellibrachia* tubes, and most carbonate rocks suggests that they may be limited to surfaces that are not subject to intense shearing forces or grazing by gastropods. The shells and carapaces of the grazers themselves and cryptic habitats, such as mussel byssal threads, may offer a refuge from consumers. Alternatively (or additionally), the availability of sulfide, nitrate and oxygen may make certain microenvironments more conducive to colonization by attached sulfur bacteria. High oxygen and nitrate fluxes, primarily driven by bacterial oxidation of sulfide (781 and 700 $\mu\text{mol cm}^{-2}$ per year, respectively), have been reported at the collection sites, but these fluxes vary by up to three orders of magnitude over distances of tens of centimeters as a result of heterogeneities in seep fluid flow (Linke *et al.*, 2005). Whether these attached bacteria show preferential attachment to specific substrates that guarantee access to sulfide or other metabolites, or if their distribution is influenced by the very abundant microbial grazers in the system remain open questions.

Examination of photographs and specimens of seep fauna collected during 2005 and 2006 cruises to the Eel River Basin and Hydrate Ridge (off the coast of California and Oregon, USA), and observations of gastropods collected from Hydrate Ridge in 2010 both show inhabitation by morphologically similar elongate *Thiomargarita*-resembling cells (Figure 2b), suggesting that certain ecotypes of attached sulfur bacteria are geographically widespread in seep settings. The difficulty in collecting these specimens and maintaining them under enrichment conditions that allow for observation of their development renders our understanding of these organisms incomplete. However, the dimorphic life cycle in seep-associated cells closely related to *Thiomargarita* spp. described here reveals a complex reproductive ecology for these giants of the bacterial world that further confounds the generalization that bacteria are small and simple.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was funded by the Agouron Institute, the University of Minnesota Department of Geology and Geophysics, the Max Planck Society, and the National

Science Foundation (OCE-0826254 and OCE 09-39557). Parts of this work were carried out in the Characterization Facility, University of Minnesota, a member of the NSF-funded Materials Research Facilities Network (<http://www.mrfn.org>) via the MRSEC program with assistance from Chris Frithem and Fang Zhou. Scanning electron microscopy was performed in the University of Minnesota, Twin Cities, Limnological Research Center with the support of Amy Myrbo. Furthermore, we thank Tony Rathburn, Shana Goffredi, Anders Waren, Danwei Huang, Anne Dekas, Olivia Mason, Ben Harrison, the crew of the R/V *Atlantis* and pilots of the DSV *Alvin* (15–44 and 15–59) for assistance at sea, and Ben Grupe for providing live gastropods from Hydrate Ridge. We thank the reviewers of this manuscript for valuable comments that helped improve the manuscript.

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