Low temperature S$^0$ biomineralization at a supraglacial spring system in the Canadian High Arctic

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ABSTRACT

Elemental sulfur (S$^0$) is deposited each summer onto surface ice at Borup Fiord pass on Ellesmere Island, Canada, when high concentrations of aqueous H$_2$S are discharged from a supraglacial spring system. 16S rRNA gene clone libraries generated from sulfur deposits were dominated by β-Proteobacteria, particularly Ralstonia sp. Sulfur-cycling micro-organisms such as Thiomicrospira sp., and ε-Proteobacteria such as Sulfuricurvales and Sulfurovumales spp. were also abundant. Concurrent cultivation experiments isolated psychrophilic, sulfide-oxidizing consortia, which produce S$^0$ in opposing gradients of Na$_2$S and oxygen. 16S rRNA gene analyses of sulfur precipitated in gradient tubes show stable sulfur-biomineralizing consortia dominated by Marinobacter sp. in association with Shewanella, Loktanella, Rubrobacter, Flavobacterium, and Sphingomonas spp. Organisms closely related to cultivars appear in environmental 16S rRNA clone libraries; none currently known to oxidize sulfide. Once consortia were simplified to Marinobacter and Flavobacteria spp. through dilution-to-extinction and agar removal, sulfur biomineralization continued. Shewanella, Loktanella, Sphingomonas, and Devosia spp. were also isolated on heterotrophic media, but none produced S$^0$ alone when reintroduced to Na$_2$S gradient tubes. Tubes inoculated with a Marinobacter and Shewanella spp. co-culture did show sulfur biomineralization, suggesting that Marinobacter may be the key sulfide oxidizer in laboratory experiments. Light, florescence and scanning electron microscopy of mineral aggregates produced in Marinobacter experiments revealed abundant cells, with filaments and sheaths variably mineralized with extracellular submicron sulfur grains; similar biomineralization was not observed in abiotic controls. Detailed characterization of mineral products associated with low temperature microbial sulfur-cycling may provide biosignatures relevant to future exploration of Europa and Mars.

INTRODUCTION

While uncommon on Earth at the present day, cold environments dominated by sulfur chemistry could form critical habitats for potential life at other locations in the solar system, such as Europa and Mars (Kargel et al., 2000; Zolotov & Shock, 2004; Langenau et al., 2005). At the surface of Europa, probable sulfur-rich, non-ice materials are concentrated along geologic features (McCord et al., 1998; Carlson et al., 1999; Dalton, 2007) and may reflect the chemistry of an ocean in communication with the surface. Materials carried to the surface by partial melt or mobile ice from deeper ice or ocean environments could be investigated for the presence of biosignatures by future missions (Dalton et al., 2003; Chela-Flores, 2006). H$_2$S and elemental sulfur (S$^0$) may have provided important energy sources for early metabolisms on the Earth once strong oxidants such as O$_2$ and NO$_3^-$ became available after the great oxidation event more than two billion years ago. H$_2$S accumulation and persistence in euxinic deep ocean waters may have extended into the Neoproterozoic (1.0–0.54 Gyr), as proposed by Canfield (1998). Evidence for global glaciations on the Earth in the late Neoproterozoic (Hoffman et al., 1998; Hoffman & Schrag, 2002) indicate that low temperature conditions extended over the majority of the Earth’s surface and psychrophilic sulfide oxidation may have gained increased importance under such conditions.
Despite their geobiological and astrobiological importance, micro-organisms that can conserve chemical energy from oxidation of reduced sulfur compounds under psychrophilic conditions have been little studied to date. In addition, the activities of sulfur-generating microbial communities that may produce microscopic and macroscopic mineral deposits with detectable biosignatures has until recently remained relatively unexplored (Gleeson et al., in revision). Large-scale mineral deposits can be remotely observed on Earth and other planetary bodies (Kruse et al., 1990; McCord et al., 1999; Gendrin et al., 2005; Gleeson et al., 2010), and could potentially be utilized to provide targets for in situ investigation of signs of microbial activity (Figueroedo et al., 2003; Chela-Flores, 2006). The targets could include micro-fabrics characteristic of microbialites (Grotzinger & Knoll, 1999), or isotopic signatures, chemical compositions or mineralogical structures indicative of biomineralization (Banfield et al., 2001). The primary challenge inherent in the use of $S^0$ as a biosignature is the lack of characterization of the micro-fabrics or morphologies associated with microbial $S^0$ formation, as well as an understanding of the environmental and biological conditions under which $S^0$ is stabilized and preserved.

Geologic deposits of $S^0$ form under a wide range of environmental conditions, such as in molten volcanic flows (Watanabe, 1940), at fumaroles (Ljunggren, 1960), at hydrothermal vents (Taylor et al., 1999), within marine sediments (Thamdrup et al., 1994), and over salt domes and evaporate deposits (Davis & Kirkland, 1979), with the potential for many of these to be microbially mediated. The 8-electron reduction of sulfate to sulfide does not result in the formation of $S^0$ as an intermediate (Troelsen & Jorgensen, 1982; Machel, 2001); instead, $S^0$ typically indicates the incomplete microbial or abiotic oxidation of reduced sulfur species such as $H_2S$. The complete oxidation of $H_2S$ to $SO_2^{-}$ occurs in a series of steps, frequently forming $S^0$ as an intermediate (Fuseler & Cyponka, 1995; Ehrlich, 2002).

Microbiological studies of warm sulfide springs associated with magmatic activity in locations such as Yellowstone National Park, Wyoming (Farmer & Des Marais, 1994b; Fouke et al., 2001), and Jasper National Park in Alberta, Canada (Grasby et al., 2000; Bonny & Jones, 2003) have explored the role that microbes may play in the precipitation of sulfur and carbonate minerals, while cold sulfide spring sites remain relatively understudied to date. Prior to the use of 16S rRNA technology, Thermotrix within the $\beta$-Proteobacteria were thought to be the dominant sulfur oxidizers at hot spring sites such as Mammoth in Yellowstone although clone libraries of DNA extracted from sulfur streamers at such sites were subsequently shown to be dominated by Aquificales while $\beta$-Proteobacteria were absent (Brenner et al., 2005). Reed et al. (2006) explored the microbial diversity of Bacteria and Archaea in sulfide cold-seep sediments at the base of the Florida Escarpment. Douglas & Douglas (2000) used light and electron microscopy to visually explore the microbiology and mineralogy of a cold (9 °C) anoxic sulfur spring in Ancaster, Ontario, Canada. In a stream channel, they observed colloidal sulfur-associated with unicellular, colorless, sulfur-oxidizing bacteria potentially responsible for the production of macroscopic filamentous structures. Perreault et al. (2007, 2008) found that in clone libraries constructed from cold sulfide springs emerging from permafrost on Axel Heiberg Island, Nunavut, 16S rRNA sequences from the bacterial community group with known autotrophic and heterotrophic sulfide and sulfur oxidizers. In their study, Thio- microspira arctica, Thiothrix spp. strain EBD bloom, and Halothiobacillus sp. strain RA13, accounted for 30–74% of the microbiological community. Neiderberger et al. (2009) also isolated several autotrophic sulfide and thiosulfate oxidizing Thiomicrospira sp. from samples of microbial streamers collected at the same site.

The supraglacial spring system of Borup Fiord Pass at 81°N, 81°W on Ellesmere Island in the Canadian High Arctic (Grasby et al., 2003) is a unique low temperature environment where $S^0$ is found in abundance (Fig. 1). Subsurface saline fluids rise through an estimated 200 m-thick glacier and deliver sulfur-rich waters that have mixed with glacial melt water, and deposit $S^0$, gypsum (CaSO$_4$·2H$_2$O) and calcite (CaCO$_3$) on the surface as well as along flow channels incised into the ice (Fig. 1C). The unstable CaSO$_4$ polymorph vaterite has also been observed (Grasby, 2003). Sulfide and sulfate levels within the springs have been measured as high as 142 mg L$^{-1}$ (4.2 mm) and 1786 mg L$^{-1}$ (27.9 mm), respectively, which are the highest measured dissolved sulfur concentrations across the Canadian Arctic Islands. Measured temperatures for the springs are ~0 °C while the fluid pH is alkaline between 8 and 9. The water pH rapidly decreases to ~pH 6 and H$_2$S(aq) drops below detection within meters of the spring source. Sulfur isotopic evidence suggests that the source of sulfur is most likely sedimentary sulfate (anhydrite, gypsum) that lies at depth beneath the base of the glacier (Grasby et al., 2003).

Microbial organisms have previously been detected within the spring waters and precipitates in cell densities of 1.9–2.9 × 10$^3$ cells mL$^{-1}$ (Grasby et al., 2003). Limited investigations of microbiological community composition within the precipitates have revealed the presence of $\gamma$-Proteobacteria closely related to Marinobacter sp. (98% identity match) by cloning and 16S rRNA sequencing of extracted DNA, while sequencing of bands observed in denaturing gradient gel electrophoresis (DGGE) found close matches with the $\beta$-Proteobacteria Polaromonas (99% match), Burkholderia sp., and the $\gamma$-Proteobacterium Pseudomonas fulva (Grasby et al., 2003). Due to the limited microbial community data for the site and the dynamic nature of the spring system, little is known about the microbial ecology at the spring.

The environmental and microbiological factors that lead to the formation of $S^0$ on the surface of the glacier at Borup Fiord Pass have not been previously investigated in detail. Our objective was to explore the microbial diversity of sulfur-
associated microbial communities, and to conduct targeted cultivation of psychrophilic micro-organisms that play a key role in the production of oxidized, sulfur-rich deposits on the surface ice. In particular, by combining microbiological, geochemical and mineralogical data, we expand our knowledge of biologically mediated sulfur-cycling processes in cold environments, which could inform the search for S-based microbial life and biosignatures in other locations in the solar system.

MATERIALS AND METHODS

Geochemical and mineralogical analysis of site material

Water samples and moist mineral precipitates along channels were collected from the actively flowing spring site and associated sulfur deposits during a field expedition to Borup Fiord Pass in June and July of 2006. Unstable parameters (temperature, pH, dissolved HS\(^-\)) were measured at sites along the channel, and at increasing distances from the discharge site (Fig. 2). Dissolved HS\(^-\) was measured using CHEMetrics\textsuperscript{®} (Calverton, VA, USA) colorimetric kits (±0.01 mg L\(^{-1}\)). For chemical analyses, water samples were passed through 0.45-μm filters and stored in the dark at 4 °C in high-density polyethylene bottles until analyzed. Samples for cation analyses were acidified with ultrapure nitric acid to pH <2.

Subsequent chemical analyses were carried out at the Geological Survey of Canada. Alkalinity was determined by a standard end-point titration. Anions were measured by ion liquid chromatography, and cations were measured by inductively coupled plasma emission spectrometry. Analytical error was estimated to be less than 2% (Table 1).

Mineralogy of precipitates collected from the deposits was determined by X-ray diffraction (XRD) analysis on a Philips PW1700 powder diffraction system with Cobalt x-ray source. All analyses were on powder samples, which were executed by the PANalytical X’Pert Quantify software; samples were usually scanned from 2–60° 2θ at 1° per minute with

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accelerating voltage of 40 KV and 30 mA. Mineral determination was processed by PANalytical’s X’pert Highscore program, and the quantification of mineral composition within each sample was calculated from their mineral peak intensities (or peak area). The whole rock results are semi-quantitative and they are expressed in mineral ratio percent (Table 2).

Environmental DNA clone libraries

DNA was extracted from ~0.5 g of mineral samples 4A and 5B (locations shown in Fig. 2) that were preserved in 70% ethanol using a phenol chloroform extraction technique. Polymerase chain reaction (PCR) was then conducted using protocols described by Sahl et al. (2010). The PCR primers 515F (5′-GTGACGCAAGCMGCCGCGGTAA-3′) and 1391R (5′-GACGGGCGGTGTGATCCT-3′) were selected to amplify small-subunit rRNA gene fragments for all three domains of life (Lane, 1991). After this initial screen of diversity, the Bacteria-specific forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) was used in conjunction with the universal reverse primer 1492R (5′-GGTTACCTTGTACGACTTT-3′) to obtain full-length bacterial 16S rRNA gene sequences (Lane, 1991); while PCR conducted with archaeal-specific primers was unable to amplify small-subunit rRNA gene fragments. Appropriate negative controls were employed throughout the DNA extraction and PCR processes. PCR products were gel purified with the Montage DNA Gel Extraction Kit (Millipore, Billerica, MA, USA), cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) using electroporation, Escherichia coli TOP10 cells, and sequenced on a MegaBACE 1000 dye-terminating sequencer. Sequences were assembled with Xplorseq (Frank, 2008), screened for chimeras with Mallard (Ashelford et al., 2006), and the resulting 349 non-chimeric sequences were aligned with the NAST aligner (Desantis et al., 2006b), and these alignments were manually checked in ARB (Ludwig et al., 2004). Phylogenetic relationships of sequences were determined by inserting the sequences into the ARB dendrogram by parsimony insertion using the Lanemask filter (Lane, 1991) and the most recent Greengenes (Desantis et al., 2006a) database release. Relevant sequences were exported from ARB with the Lanemask filter, and phylogenetic trees were constructed with Mr Bayes (Huelsenbeck et al., 2001).

Targeted culture of sulfide-oxidizers that produce S⁰

Sample material collected aseptically with alcohol and flame sterilized spatulas from spring deposits in the 2006 field season was used to inoculate sulfide gradient tubes on return to the laboratory. Gradient tubes were assembled using iron sulfide plugs created by adding FeS precipitate prepared according to the method of (Hanert, 2006) in a 1:1 ratio with EM media (see below) and high melt agarose (1% wt/vol) of an autoclaved, artificial seawater medium (EM) containing 27.5 g NaCl, 5.38 g MgCl₂, 0.72 g KCl, 0.2 g NaHCO₃, 1.4 g CaCl₂, 1 g NH₄Cl, and 0.05 g K₂HPO₄, dissolved above in 967 mL deionized H₂O. The medium was amended with 1 mM NaHCO₃, and sterile N₂-CO₂ was also bubbled through the agar overlay, to provide a carbon source. The initial pH was 8.5, the average pH of the spring waters, and 1 mL L⁻¹ of vitamins (Wolfe’s mineral medium, Dworkin et al., 2006) and 1 mL L⁻¹ of trace elements (containing 0.52 g EDTA,
0.15 g FeCl$_2$-4H$_2$O, 7 mg ZnCl$_2$, 10 mg MnCl$_2$-4H$_2$O, 6.3 mg H$_2$BO$_3$, 19 mg CoCl$_2$-6H$_2$O, 1.7 mg CuCl$_2$-2H$_2$O, 24 mg NiCl$_2$-6H$_2$O and 36 mg Na$_2$MoO$_4$-2H$_2$O per 100 mL deionized H$_2$O) were also added to the solution.

Samples of mineral precipitates collected on the glacier were used to inoculate the tubes, which were stored in the dark at 4 °C. Gradient tubes that contained visible yellow sulfur in the overlayer after a period of about a month of growth were then transferred to new gradient tubes to evaluate if S$^0$ production continued. In subsequent transfers the plugs were amended with 8 mM Na$_2$S, rather than FeS, to remove Fe (II) as another potential energy source for chemolithoautotrophic growth. Autoclaved sediment from the spring deposits, collected in the field, was also added at 1% wt/vol to the plugs, to ensure that potentially growth-limiting trace elements were present in the cultures.

Salinity and sulfide concentrations were also increased from initial salinities of 27–54‰ and 270‰ while sulfide concentrations were increased from 8 to 16 mM and 80 mM in gradient tubes to evaluate the optimal conditions for S$^0$ production as determined by visual inspection of tubes and confirmed by microscopy. In addition, a subset of gradient tubes was amended with pyruvate and formate (100 mM final concentration) to test for sulfide oxidation during heterotrophic growth. Evaluation of whether sulfur production increased with the addition of organic carbon was also determined by visual inspection of tubes and confirmed by microscopy.

**Simplified cultures of sulfide-oxidizing bacteria**

After 4 successive rounds of transfers in which the S$^0$ was microbiologically produced, the cultures were serially diluted in the range of 10$^{-3}$ to 10$^{-7}$ in order to isolate the most abundant members of the S$^0$-generating consortia. Dilution series were characterized by light and florescence microscopy after 1–3 months of growth. Successive rounds of serial dilutions of S$^0$ containing gradient tubes was conducted four times, and DNA was extracted at various time-points (see below) to determine the phylogeny of the consortia. In addition, some of the S$^0$ material was plated directly onto heterotrophic media containing artificial seawater with yeast and peptone and incubated in the dark at 4 °C. Plating onto autotrophic sulfide media did not result in colony growth. Colonies of varying morphology and pigmentation were then purified through successive transfers on organic-rich “K”-plates made by adding 0.5 g yeast extract, 2 g peptone and 15 g granulated agar to 980 mL artificial seawater (containing 30 g NaCl, 24 g MgSO$_4$·7H$_2$O, 3 g CaCl$_2$·2H$_2$O, 2 g KCl per liter ultrapure H$_2$O). After autoclaving, pre-sterilized solutions of 20 mL 1 M HEPES (pH 7.8) were added for a 20 mM final concentration and 100 μL 1 M MnCl$_2$ for a 100 μM final concentration. Isolated colonies were grown in liquid K-media, and DNA was extracted using the DNAEasy Kit (Qiagen, Valencia, CA, USA) for 16S rRNA sequencing of the isolates (see below).

All isolates obtained from heterotrophic plates were then reintroduced to sulfide gradient tubes to evaluate whether sulfide oxidation to S$^0$ could be carried out in pure culture. Isolates were also introduced to agar-only tubes to determine if isolates could be metabolizing the agar rather than the sulfide.

**DNA extraction, cloning, and sequencing: enrichments, co-cultures and isolates**

Once mixed-culture S$^0$-generating experiments had been through five to six rounds of transfers, and microscopic inspection had confirmed continuing sulfur production, cultures were considered to contain a semi-stable consortium. DNA was then extracted from the highest-dilution gradient tubes still producing S$^0$. Sulfur-rich material from the gradient tubes was first digested from the agar using the Qiaquick Gel Extraction Kit from Qiagen. DNA was subsequently extracted from the remaining material using the UltraClean extraction kit (MoBio Inc., Carlsbad, CA, USA).

PCR reactions were carried out on the extracted DNA using PCR primers 515F and 1074R (5’-CAGGAGCTGA-CGACAGCCAT-3’) to amplify small-subunit rRNA gene fragments for Bacteria. Due to the lack of Archaea in the environmental samples, no archaeal primers were used. Successful PCR products with bands corresponding to the correct length were cleaned with a Qiaquick PCR Purification Kit (Qiagen) and cloned as above.

To sequence the clones, colony PCR reactions were conducted using M13F (5’-GTAAAACGACGGCCAG-3’) and M13R (5’-CAGGAAACGCTATGAC-3’) primers. Successful reactions were cleaned and the DNA concentration in each sample was quantified from absorbance at 260 nm using a Beckman-Coulter UV/VIS spectrophotometer. Samples were then sent to Seqwright (Houston, TX, USA) to be sequenced using M13F primers. The resulting sequences were processed as above.

DNA was extracted from the purified isolates grown on liquid K-media using the UltraClean extraction kit (MoBio Inc.), and the DNA was amplified using 27F and 1492R primers. Full-length (~1500 bp) sequences were assembled using a complement of 3–6 forward and reverse primers submitted to Seqwright and processed as above.

**Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS)**

Scanning electron microscopy of environmental samples and minerals produced in the culturing experiments was conducted using a JSM-6480LV (low vacuum) and JSM-7401F (field emission) SEM at the Nanoscale Fabrication Laboratory at the University of Colorado at Boulder. Sulfur-rich material from the gradient tube experiments was fixed with glutaralde-
Genbank submission

16S rRNA gene sequences were submitted to Genbank under accession numbers HM141098-HM141534. Sequences were named according to sample name (BF64A and BF65B), primer pair used (U – 515F, 1391R; B – 8F, 1492R) or cultivar (C – consortia, I – isolate), and sequence number.

RESULTS AND DISCUSSION

Geochemistry and mineralogy of the springs and deposits

The sulfide springs and associated sulfur deposits are dynamic in nature, changing location and distribution from 1 year to another. Field observations in 2000 and 2006 revealed extensive areas of yellow staining across the surface of the glacier. On July 6, 2006 a large icing south of the toe of the glacier, potentially built up by spring discharge, also had yellow staining with an areal extent of ~0.12 km². Satellite imagery of the field site collected by the Hyperion instrument onboard Earth Observing Satellite-1 allowed detection of the deposits in 2006, and in 2007 was utilized to track the disappearance of these deposits over the course of the melt season (late June-early August). An onboard classification algorithm continued to detect the presence of sulfur until snow obscured the site again in late August 2007 (Gleeson et al., 2010). Field observations in other years observed only limited deposits in the form of conical structures of sulfur, calcite and gypsum (Grasby et al., 2003).

The measured spring water chemistry was saline (7400 mg/L) and dominated by Na⁺, SO₄²⁻ and Cl⁻ (Table 1). H₂S at the spring outlet was measured at 142 mg L⁻¹ (4.2 µM), the highest values yet reported in any sulfur spring in Canada. The mineralogy of the supraglacial deposits is dominated by S⁰, responsible for the yellow color of the material of the ice, and calcite (Table 2). Although quartz is also present in some of the samples, it is potentially introduced to the system as wind-blown silt.

The accumulation of extensive S⁰ deposits is not predicted to be thermodynamically stable under the highly oxygenated and high pH conditions of 8 to 9 measured at Borup Fiord (Gleeson et al., 2010). Sulfide (142 mg L⁻¹) and sulfate (1786 mg L⁻¹) levels measured in spring waters provide a lower limit on total sulfur in the system as the activities of other potential intermediate sulfur species such as sulfite (SO₃²⁻), thiosulfate (S₂O₅²⁻) and polysulfides (Sₙ²⁻) were not measured. At the pH of 8–9 measured in the spring waters, the dominant ion of sulfur is predicted to be either the HS⁻ ion or SO₄²⁻, depending upon the Eh. High dissolved Ca²⁺ concentrations of 696 mg L⁻¹ (58 µM) favor the precipitation of SO₄²⁻ as gypsum at the ambient O₂ levels if waters are saturated, but we can only detect a limited amount of gypsum in the older dry deposits measured in previous years (Grasby et al., 2003), which may result from increased saturation as the deposits dry.

Environmental community analysis

The microbial community composition of sulfur deposits associated with the supraglacial spring at Borup Fiord was analyzed with 16S rRNA gene sequencing. Clone libraries were dominated by sequences representative of the Proteobacteria. Only one archaeal sequence and no eukaryal sequences were identified after using PCR primers targeting all three domains of life. Previous microbial community analysis at the site identified members of the Proteobacteria, including microbes grouping within the Burkholderiales and Pseudomonadales and one sequence classified as a Marinobacter sp. (Grasby et al., 2003). Sequences grouping within the Burkholderiales and Pseudomonadales are also identified in the clone libraries produced from environmental samples in the current study.

The 348 bacterial 16S rRNA sequences generated from both environmental samples (Figs. 3–5) were dominated by representatives of the β-Proteobacteria (~82% of total sequences), of which ~97% were most closely related to Ralstonia sp. Sequences grouping with Ralstonia sp. have been identified in clone libraries produced from basal ice samples collected from John Evans Glacier located on Ellesmere Island (Skidmore et al., 2005). Ralstonia is a metabolically diverse genus of the Burkholderia order, often prevalent in oxic,
metal-contaminated environments (Goris et al., 2001). *Ralstonia* are known to reduce iron in association with the oxidation of organic matter (Lin et al., 2007), can oxidize Fe(II) under microaerophilic conditions (Swanner et al., 2011), and can couple nitrate reduction to H₂ oxidation (Zumft, 1997).

*Ralstonia eutropha* is a strictly respiratory lithoautotroph whose genome has recently been shown to contain sulfur oxidation (sox) genes (Cramm, 2009) although growth on thiosulfate was unsuccessful.

Other β-Proteobacteria present in clone libraries include *Comamonadaceae* and *Rhodocyclales*. Microbes classified as *Comamonadaceae* had been previously identified in samples collected from the spring site by sequencing DGGE bands (Grasby et al., 2003). *Rhodocyclales* include *Rhodocyclus*,

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Fig. 4 Phylogenetic tree of the division Proteobacteria detected in 2006 environmental data, and in cultures inoculated from 2006 samples. Sequences obtained in this study are in bold. Numbers following accession numbers indicate how many sequences grouped with each phylotype. Closely related organisms are found in many sulfur-rich and cold environments.

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which preferably grow photoheterotrophically under anoxic conditions in the light with different organic substrates as carbon and electron sources. Chemotrophic growth is also possible under microoxic to oxic conditions in the dark, and reduced sulfur compounds are not used as photosynthetic electron donors by *Rhodocyclus* sp. (Imhoff, 2005).

γ-proteobacteria representatives (≈1% of total sequences) included sequences related to *Thiomicrospira* sp. and *Shewanella* sp. One sequence was closely related (99% sequence identity) to chemolithoautotrophic sulfur-oxidizing bacteria *Thiomicrospira psychrophila* and *Thiomicrospira* sp. which have recently been shown to dominate clone libraries constructed from sulfur streamers within cold sulfur springs on the neighboring island of Axel Heiberg Island (Neiderberger *et al.*, 2009). The low abundance of *Thiomicrospira* among the environmental sequences detected in 2006 may be linked to sulfide levels at Borup Fiord Pass, which are three orders of magnitude higher than those of the springs on Axel Heiberg Island. This may significantly impact the microbial community structure. The *Shewanella* representative was closely
related to *Shewanella* sp. identified from cold, icy environments including the Blood Falls site in Antarctica (Mikucki & Priscu, 2007).

ε-proteobacteria representatives (3% of total sequences) include sulfur-oxidizing bacteria that grouped with *Sulfuricurvales* and *Sulfurovumales*. *Sulfuricurvales* representatives included sequences closely related (97% sequence identity) to *Sulfurimonas denitrificans* DSM 1251 (accession number CP000153). Filamentous ε-Proteobacteria including *Sulfuricurvales* and *Sulfurovumales*, related to those observed in environmental data, are known to dominate sulfide-oxidizing biofilms in warm sulfide-rich cave waters (Engel *et al.*, 2003; Macalady *et al.*, 2008) and the ε-Proteobacterium *Arcobacter* has been shown to oxidize sulfide, producing extracellular filaments of sulfur (Wirsen *et al.*, 2002; Sievert *et al.*, 2007).

Sequences not grouping within the Proteobacteria include representatives of the *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and *Firmicutes*. *Bacteroidetes* representatives included sequences closely related to *Flavobacterium* sp. identified at Antarctic sites, including the Blood Falls site at Taylor Glacier (Mikucki & Priscu, 2007). Sequences grouping with the *Chloroflexi* show close phylogenetic relationship to the *Chloroflexi* sp. identified in the Guerrero Negro hypersaline mats (Spear *et al.*, 2003; Ley *et al.*, 2006). Representatives of the *Actinobacteria* and *Firmicutes* also group with sequences identified at cold, saline environments.

Bacterial diversity associated with the sulfur-rich springs at Borup Fiord Pass as sampled in 2006 is broadly similar to other cold, saline environments, including those of the other High Arctic springs on Axel Heiberg Island (Perreault *et al.*, 2007, 2008) and subglacial flow from the Taylor Glacier in the Antarctic (Mikucki & Priscu, 2007). Temperature and chemistry of the spring waters likely act as the key drivers that select for microbial community ecotype. Key differences from the previous studies include the notable dominance of *Ralstonia*, for which a role in oxidative sulfur-cycling is currently unknown.

**Laboratory formation and biomineralization of S⁰**

Initial sulfide gradient tubes inoculated with Borup Fiord sulfur deposits generated macroscopically visible cloudy white clumps of S⁰ after a period of about 1 month. Mineralized zones tended to grow outward from the line of inoculation (Fig. 6) rather than in a band at a defined depth in the gradient tube. Production of S⁰ continued through successive transfers, although the total amount of S⁰ was not as abundant as found in the initial inoculations.

The sulfur-rich zones of the gradient tubes were examined using differential interference contrast and fluorescence microscopy. We typically observe abundant cells with a rod morphology and semi-transparent, curved cylindrical filaments, some of which appear hollow while others are associated with spherical globules of sulfur along their lengths, similar in appearance to the hydrated spherical colloid described by Steudel (1989). Filaments vary from a few microns to tens of microns in length (Fig. 7). Larger, rigid sheaths are also observed, commonly mineralized with sulfur. Extensively mineralized sheaths and filaments often exhibit dense clusters of rounded and angular sulfur grains, which commonly result in complete encrustation of the underlying structure. The most typical morphologies include a central mass of sulfur comprising one or more spheroidal mineral aggregates, ~5–50 μm in diameter, surrounded by narrow filaments and biomineralized sheaths attached at one end of their length and radiating out from the center of the structure (Fig. 7). The morphologies of the spheroidal mineral aggregates have also been observed in studies investigating Borup

![Fig. 6](image-url) (A) Gradient tube inoculated with Borup Fiord material shows the production of elemental sulfur in the form of cloudy, white, filamentous material. (B) False colored DIC image shows filaments and mineralized sheaths in sulfur blooms from inoculated gradient tubes. (C) Abiotic precipitates in negative controls do not show the same morphologies.
Fig. 7 Further examples of sulfur structures observed in enrichments. (A) DIC image shows that cells are observed in close association with filaments. (B) Optical image shows full extent of a sulfur structure. (C) Fluorescing cells against optical background. (D) Sulfur crystals nucleating along sheaths and filaments. (E) Sulfur globules deposited along filaments.

Fiord field samples that confirmed these materials as sulfur (Gleeson et al., in revision). Scanning electron microscopy images demonstrate that the sheaths measure ~1 μm across, while the filaments are just a few hundred nm across (Fig. 8). EDS spectra of the gold-coated samples confirm the deposited material as sulfur while the sheaths themselves are dominated by carbon.

Compositionally different but morphologically similar structures have been reported by Benison et al. (2008), who described clumps of organic bodies and sulfate crystals found in evaporite minerals from Permian and modern acid saline lakes as microbial remains. The similarities between our sulfur structures and the “hairy blobs”, despite compositional differences, provide insight on the formation of S-mineralized microbial structures, and strengthen the validity and application of these structures as combined morphological, mineralogical and organic biosignatures.

Optimal conditions for biological S\(^0\) production

During each transfer of the sulfide-oxidizing enrichments, the consortia were subjected to serial dilutions up to \(10^{-7}\). In each experiment, HS\(^-\) was provided as the sole electron donor, O\(_2\) as the sole electron acceptor (i.e., no nitrate was present), and HCO\(_3^-\) as the sole carbon source. While agar was included in early cultures to reduce the rates of oxygen diffusion, and
could have provided a carbon source for agar-degrading micro-organisms, it was phased out during the course of the experiments. In addition, agar-only tubes did not demonstrate growth, suggesting that our experiments did not select for agar-dependent bacteria. In contrast, growth was observed in all dilutions of Na\textsubscript{2}S gradient tubes, and S\textsuperscript{0} production was variably maximized in the 10\textsuperscript{−3} to 10\textsuperscript{−5} dilutions, but rarely in the most dilute. Our assumption is that full oxidation of sulfide to sulfate occurs to a greater degree when S\textsuperscript{0} is not detected even though cell growth occurs, but this hypothesis was not tested in this study. Instead, our focus was to determine the optimal conditions for microbial S\textsuperscript{0} production, and to determine the phylogeny of the organisms in the stable consortia.

Several variations in growth conditions were tested. First, gradient tubes were amended with pyruvate and lactate (100 \textmu M final concentration), but we did not observe enhanced S\textsuperscript{0} formation and these experiments were discontinued. Site spring waters with an average salinity of 7.4 g L\textsuperscript{−1} resulted in lower growth and sulfur production than our artificial EM seawater medium (27.5 g L\textsuperscript{−1} NaCl). Increased additions of NaCl to 2x and 10x EM also resulted in diminished

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growth and sulfur production, indicating that the consortia are not highly halophilic. In addition, the sulfide concentrations in the plugs were increased from 4 mM to 8, 16 and 80 mM. Decreasing amounts of sulfur production and cell growth occurred at increasing sulfide concentrations, and no growth occurred at 80 mM Na₂S.

Increasing concentrations of NaCl and sulfide increased the production of abiotic mineral precipitation within cultures, in crystalline morphologies distinct from the sulfur structures observed in our cultures. Abiotic precipitates at high sulfide concentrations were much larger in size (~100 μm across) than the structures observed in our cultures, with highly angular morphologies (Fig. 6). Previous studies of biogenic sulfur have linked the monoclinic γ-sulfur phase rosickyite with the activities of microbial sulfide oxidation (Douglas & Yang, 2002; Douglas, 2004) and XRD measurements and SEM images of Borup Fiord field samples have also revealed evidence of rosickyite (Gleeson et al., in revision). However, our cultures did not yield enough sulfur to conduct similar XRD analyses in this study.

S⁰ producing consortia

16S rRNA gene sequences of ~500 bp were obtained from extracted, cloned and sequenced DNA from 1⁰⁰ and 1⁻¹ dilutions that produced S⁰ after five to six transfers. The consortia were dominated by the γ-proteobacterium Marinobacter (Fig. 9). Other members of the consortia were closely related to Shewanella, Loktanella, Pseudomonas, Rhabdobacter, and Sphingomonas spp. Consortia sequences show close phylogenetic relationships with environmental sequences identified from the field site as well as as sequences identified from other cold, saline environments. This indicates that we have successfully enriched for stable consortia of environmentally relevant organisms.

The respective roles of each cultivar are currently unknown, but at least one organism detected has previously been implicated in sulfur-cycling. Loktanella salisilacus is an example of a heterotrophic bacterium within the Rhodobacter group isolated from Antarctic lake environments (Van Trappen et al., 2004), and closely related members of Loktanella spp. play important roles in the cycling of organic and inorganic forms of sulfur in marine environments. These roles include the degradation of dimethylsuloniopropionate (DMSP) and oxidation of sulfate and thiosulfate (Gonzalez et al., 1999, 2000), sulfur intermediates that may be present within the complex redox cycle operating within the Borup Fiord spring system.

DNA was also extracted from a seventh generation S⁰-generating gradient tube to evaluate whether the consortia had changed. Conditions had been altered by the removal of agar from the gradient tube over-layers to facilitate analysis of the sulfur. The only organisms detected in the consortia were Marinobacter sp. BF64A_C1 and Flavobacterium sp. BF64A_C33 (also included in Fig. 9). While Flavobacterium was not previously represented in the 16S rRNA gene sequences assembled for the consortia (likely due to too few sequences), closely related Flavobacteria sequences are present in the environmental data. It is possible that the change in growth conditions to a fully liquid medium with higher oxygen concentrations may have been advantageous for this organism, given its abundance in many freshwater and marine environments (Kirchman, 2002).

Several organisms in the stable S⁰-generating consortia were successfully isolated by plating the S⁰ from the gradient tubes onto organic-rich media (K-plates containing yeast and peptone), followed by the purification and sequence of colonies that varied in size and morphology. Pure cultures of Shewanella sp. BF65B_I7, Loktanella sp. BF65B_I2, and Sphingomonas sp. BF64A-I5 were obtained. In addition, Devosia sp. BF65B_I1 was also isolated (K. Wright, unpubl. data). Devosia sp. BF65B_I2 was not detected as a major member of the stable consortia during cloning and sequencing; however, closely related Devosia have been detected in environmental data. Marinobacter has not been isolated as a pure culture from heterotrophic plates to date. However, Marinobacter grows in mixed colonies with Shewanella sp. on the plates, and when reintroduced into gradient tubes together, as determined by cloning and sequencing.

Each isolate, as well as the Marinobacter sp. BF64A_I1/ Shewanella sp. BF65B_I7 co-culture, was then used to inocu-
late a set of sulfide gradient tubes to evaluate their capacity to grow via sulfide oxidation in isolation. No sulfur production or cell growth was observed in gradient tubes inoculated with any of the isolates, including *Shewanella* sp. BF6 BF6SB_17. However, the *Marinobacter/Shewanella* co-culture notably did produce $S^0$, cells, and biomineralized structures (supplemental data). DNA was extracted from the $S^0$-mineralized zones and limited cloning and sequencing again only detected *Marinobacter* and *Shewanella* spp. Isolates were also used to inoculate gradient tubes containing agar, but no sulfide, to determine whether or not agar hydrolysis could be important for growth; no cell growth was detected.

It is notable that the complex sulfur-generating consortia present in the fifth and sixth round dilution series were found to be dominated by *Marinobacter*, and that subsequently, only experiments where *Marinobacter* was present continued to generate sulfur and show both cell growth and sulfur biomineralization along sheaths and filaments. We suggest that *Marinobacter* may be the key sulfide oxidizer in the system (see below). *Marinobacter* sp. are common marine $\gamma$-Proteobacteria, widely distributed throughout the water column, in the deep ocean, and in Arctic and Antarctic ice (Brinkmeyer et al., 2003; Shivaji et al., 2005; Zhang et al., 2008). *Marinobacter* sp. are well known as facultative heterotrophs with motile rod shaped cells, and can use a wide variety of carbon sources, including hydrocarbons, as their sole source of carbon and energy (Gauthier et al., 1992). The iron oxidation capabilities of *Marinobacter aquaeolei* have recently been described (Hu et al., 1999) and closely related strains have been characterized as obligate chemolithoautotrophs (Edwards et al., 2003). *Marinobacter* is typically halotolerant or halophilic, while alkaliphilic (*M. alkalilphilus*) (Takai et al., 2005), psychrotolerant (*M. maritimus*) (Shivaji et al., 2005) and psychrophilic (*M. psychrophilus*) (Zhang et al., 2008) strains also exist. *Marinobacter* has not been shown to oxidize sulfur species although it has been observed to grow in association with sulfate reducing bacteria (Sigalevich & Cohen, 2000). Recent results by Perreault et al. (2008) have shown *Marinobacter* sp. NP40 to possess *soxB*, a gene involved in thiosulfate oxidation in many Proteobacteria. Previous preliminary sequencing carried out for Borup Fiord samples by Grasby et al. (2003) detected the presence of *Marinobacter*, and the presence of this organism has been confirmed in environmental samples from 2006 (data not presented here). From our work, it is possible that *Marinobacter* likely plays a role in the oxidation of reduced $S$ species.

While we do not have quantitative data on the abundance of each species within our cultures, the dominance of *Marinobacter* in both early clone libraries and within simplified enrichments and co-cultures points to this organism as the likely sulfide oxidizer within our cultures. The close association of *Marinobacter* and *Shewanella* both on plates and in gradient tubes may indicate that *Shewanella* plays a role in cycling sulfur generated by *Marinobacter*, such as the reduction of $S^0$ described in *Shewanella putrefaciens* by Moser & Nealon (1996). *Shewanella* and *Flavobacteria* could both utilize organic materials generated by *Marinobacter* as their heterotrophic carbon source in their respective cultures, and *Flavobacteria* are particularly well adapted to degrade biopolymers such as those in filament and sheath materials (Kirchman, 2002).

**Conclusions**

Studies of sulfide-oxidizing capabilities in the Proteobacteria have traditionally focused on environments dominated by mesophilic or thermophilic communities, such as caves and hot springs (Spear et al., 2005, 2007). Psychrophilic sulfide oxidation has been observed more recently in $\varepsilon$-Proteobacteria (Skidmore et al., 2005), $\beta$-Proteobacteria (Sattley & Madigan, 2006), and $\gamma$-Proteobacteria (Knittel et al., 2005) in a range of marine and subglacial environments. The dominance of $\gamma$-Proteobacteria related to *Marinobacter*, and $\beta$-Proteobacteria related to *Raistonia*, in our laboratory and environmental data respectively, imply that these organisms can thrive in $S$-dominated environments and their potential for directly mediating sulfur oxidation reactions should be closely evaluated.

Environmental clone libraries of the sulfur deposits are dominated by *Raistonia* sp., which are not currently known as sulfur cyclers. These libraries also contain a range of organisms known to play a role in the oxidation of sulfur compounds, including *Thiomicospira*, *Sulfuricurvales*, and *Sulfurovales*. Moreover, our cultivation experiments indicate that members of the microbial community present at Borup Fiord can grow using sulfide as an energy source and catalyze the production of $S^0$. The organisms detected in our consortia, many of which were also isolated, include *Marinobacter*, *Shewanella*, *Loktanella*, *Sphingomonas*, *Pseudomonas*, *Flavobacterium*, and *Deroia*. These organisms were well represented in the environmental clone libraries described in this paper and previous work by Grasby et al. (2003), and are considered relevant to the Borup Fiord Pass spring system.

*Marinobacter* was found to dominate simplified sulfur-generating cultures, and the generation of sulfur was not observed in any experiments where *Marinobacter* was absent. Furthermore, microscopic investigations (both in the dilution-to-extinction series and in the *Marinobacter/Shewanella* cocultures) show that the sulfur is deposited in intimate association with microbial filaments and sheaths, indicating a microbial control on morphology and distribution of the sulfur in our experiments. Abiotic controls for our experiments did not show the same accumulations of sulfur; instead, limited abiotic precipitation only occurred in some high salinity and sulfide experiments, and the precipitates were highly crystalline and distinct in size and morphology from those of the inoculated experiments. Therefore, the distinct morphology of the biogenic sulfur structures suggests that biomineralization
associated with sulfide-oxidizing bacteria has the potential to produce a morphological biosignature. S-mineralized microbial structures as morphological biosignatures could provide targets for astrobiological investigations, linked to macroscale mineral deposits that could be visualized and interpreted from distance. The existence of mineral biosignatures of this nature in a terrestrial setting informs the search for biosignatures in other locations in the solar system, such as at Mars and at the icy surface of Europa.

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REFERENCES


**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Follow-up imaging on sulfur structures within enrichments and co-cultures of Marinobacter and Flavobacteria.

**Figure S2.** Follow-up imaging of sulfur structures within co-cultures of Marinobacter and Shewanella, showing central masses comprising one or more spherical mineral aggregates, curved filaments and wholly or partially mineralized rigid sheaths.

**Figure S3.** Different stages in formation of sulfur structures by consortia. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.