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Publisher's version / Version de l'éditeur:
https://doi.org/10.1111/j.1462-2920.2008.01833.x
Environmental Microbiology, 11, 3, pp. 616-629, 2009-03

Environmental Microbiology (2009) **11**(3), 616–629 doi:10.1111/j.1462-2920.2008.01833.x

Novel sulfur-oxidizing streamers thriving in perennial cold saline springs of the Canadian high Arctic

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Summary

The perennial springs at Gypsum Hill (GH) and Colour Peak (CP), situated at nearly 80°N on Axel Heiberg Island in the Canadian high Arctic, are one of the few known examples of cold springs in thick permafrost on Earth. The springs emanate from deep saline aquifers and discharge cold anoxic brines rich in both sulfide and sulfate. Grey-coloured microbial streamers form during the winter months in snow-covered regions of the GH spring run-off channels (–1.3°C to 6.9°C, ~7.5% NaCl, 0–20 p.p.m. dissolved sulfide, 1 p.p.m. dissolved oxygen) but disappear during the Arctic summer. Culture- and molecular-based analyses of the 16S rRNA gene (FISH, DGGE and clone libraries) indicated that the streamers were uniquely dominated by chemolithoautotrophic sulfur-oxidizing *Thiomicrospira* species. The streamers oxidized both sulfide and thiosulfate and fixed CO_2 under *in situ* conditions and a *Thiomicrospira* strain isolated from the streamers also actively oxidized sulfide and thiosulfate and fixed CO_2 under cold, saline conditions. Overall, the snow-covered spring channels appear to represent a unique polar saline microhabitat that protects and allows *Thiomicrospira* streamers to form and flourish via chemolithoautrophic, phototrophic-independent metabolism in a high Arctic winter environment characterized by air temperatures commonly below –40°C and with an annual average air temperature of –15°C. These results broaden our knowledge of the physical and chemical boundaries that define life on Earth and have astrobiological implications for the possibility of life existing under similar Martian conditions.

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Introduction

Various studies describe microbial communities inhabiting diverse environments characterized by constant low or subzero temperatures, with examples including cryptoendoliths (de la Torre *et al*., 2003), polar mineral soils (Barrett *et al*., 2006; Niederberger *et al*., 2008) and lakes (Stackebrandt *et al*., 2004; Karr *et al*., 2006), saline springs (Perreault *et al*., 2007), glacier-associated systems (Stibal *et al*., 2006; Mikucki and Priscu, 2007), cryopegs (Gilichinsky *et al*., 2005), ice shelf microbial mats (Bottos *et al*., 2008) and permafrost (Hansen *et al*., 2007; Steven *et al*., 2007a). Furthermore, significant evidence suggests that these habitats harbour active microbial ecosystems rather than dormant microbial survivors (Carpenter *et al*., 2000; Christner, 2002; Junge *et al*., 2004; Steven *et al*., 2007b; Wagner *et al*., 2007; Bottos *et al*., 2008; Rodrigues and Tiedje, 2008).

Two sets of springs, Gypsum Hill (GH) and Colour Peak (CP), located at nearly 80°N in the Expedition Fjord region of Axel Heiberg Island in the Canadian high Arctic also represent potential habitats for cold-active microbial communities. The springs originate from deep saline aquifers and flow through ~600 m of continuous permafrost resulting in cold groundwater temperatures (Pollard *et al*., 1999). Despite an average annual air temperature of –15°C and with air temperatures below –40°C being common during the winter months, the springs flow throughout the entire year and maintain a constant discharge temperature ranging from –0.5 to 6.9°C, depending on the spring source (Pollard *et al*., 1999; Perreault *et al*., 2007). The geomorphology and chemistry of the springs at GH and CP have been comprehensively described by Pollard and colleagues (1999) and Omelon and colleagues (2006) respectively. The GH and CP

springs discharge waters that are moderately saline (7.5–15.8% salts), anoxic (mean ORP of −325 mV), near-neutral (pH 6.9–7.5), rich in both sulfate (2300–3724 mg l^{-1}) and sulfide (25–100 p.p.m.), low in dissolved inorganic carbon (13.1–17.2 mg l^{-1}) with undetectable dissolved organic carbon (Andersen, 2004) and display the basic signature of seawater when the dissolved ions are normalized to Na^+ (Pollard *et al*., 1999; Perreault *et al*., 2007). An initial culture-independent, 16S rRNA gene-based survey (Perreault *et al*., 2007) revealed that the microbial communities inhabiting spring sediments consisted of members of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, *Proteobacteria*, *Spirochaetes* and *Verrucomicrobia* with *Proteobacteria*, *Firmicutes* and *Bacteroidetes* related signatures accounting for 97% of the sequences recovered from spring CP-1. Archaeal signatures were also detected in both springs with sequences from GH-4 and CP-1 consisting of *Crenarchaeota* (79% and 48% respectively) and *Euryarchaeota* (21% and 52% respectively). Most sequences in the GH and CP libraries were related to potential sulfur-metabolizing bacteria, with the dominant signature of the GH-4 (19%) and CP-1 (45%) bacterial libraries being most closely related to the sulfur-oxidizing bacterium, *Thiomicrospira psychrophila.* Based on these results, it was postulated that sulfur-based metabolism is the major source of energy production and maintenance in the sediments at the source of these springs.

Investigations into microbial-based cycling of sulfur compounds has typically been focused on communities inhabiting sulfur- and sulfide-rich ecosystems such as sewer and wastewater systems (Okabe *et al*., 2005; 2007), hydrothermal vents (Teske *et al*., 2000; Brazelton *et al*., 2006), and both hot (Yamamoto *et al*., 1998; Dillon *et al*., 2007) and cold springs (Rudolph *et al*., 2001). The utilization and cycling of sulfur species also represents the major form of energy production supporting some sulfur-rich cave ecosystems (Angert *et al*., 1998; Engel *et al*., 2003; 2004; Macalady *et al*., 2006). Likewise, microbial sulfur-oxidation may be an important subglacial process in the Antarctic (Mikucki and Priscu, 2007). Altogether, the microbial assemblages involved in the above mentioned sulfur-cycling processes are typically dominated by various genera of the *Gamma-* and *Epsilonproteobacteria* (Angert *et al*., 1998; Engel *et al*., 2003; 2004; Okabe *et al*., 2005; Brazelton *et al*., 2006; Campbell *et al*., 2006; Macalady *et al*., 2006; Mikucki and Priscu, 2007).

During previous expeditions to the perennial GH springs, the removal of the snow covering the springs run-off channels revealed that copious amounts of microbial streamers had formed in the snow-covered channels during the Arctic winter months. These microbial assemblages subsequently dissipated or disappeared entirely during summer months (July, August) after the snow covering the channels had melted. The presence of these conspicuous microbial communities was surprising given the winter extreme subzero air temperatures, the lack of exposure to sunlight and the oligotrophic nature of the springs. This report describes the structure, composition and metabolism of these streamers that were collected during a late winter expedition in 2007.

Results

Streamer occurrence and distribution

In early May 2007 (average air temperature of −14°C), snow covered much of the spring sources and the run-off channels at GH. Some springs (e.g. GH-1, GH-2 and GH-3) were completely covered with ~25–50 cm of snow making them difficult to locate; however, GH-4 was clear of snow at the source, as were minor sections of its run-off channel (Fig. 1A). The snow covering the run-off channels created small snow cave-like systems (Fig. 1B) starting from ~20–30 cm above channels containing warmer spring water (~5–6.9°C; Fig. 1C) down to ~1–2 cm above the coldest spring channels (GH-2, −1.3°C; Fig. 1C), which indicated that heat released from the spring waters had a significant effect on the snow cave morphology. The removal of the snow cover revealed conspicuous amounts of streamers, particularly abundant in springs GH-2, GH-4 and GH-6 (Fig. 1C and D). Streamers were only observed in the spring channels, starting approximately > 1 m downstream from the spring source, but never observed in any of the GH spring sources (Fig. 1a). The streamers were attached to the rocks and sediments within the run-off channels of the springs and appeared as long streamers within the outflow and ranged in size from ~2–3 cm up to ~30 cm in length. The streamers were typically greyish-white in colour with some purely white forms also present in GH-4. The water temperatures at the sites of the streamers typically ranged from −1.3°C to 6.9°C, which did not differ significantly from the spring source temperatures. However, dissolved sulfide was typically lower and dissolved oxygen (DO) concentrations higher (0–20 p.p.m. sulfide and 1 p.p.m. DO) at the location of the streamers, as compared with the source (25–100 p.p.m. sulfide and 0.05–0.2 p.p.m. DO). Streamer biomass in the Arctic summer (July, August), based on six previous expeditions to the GH springs, was estimated to be ≤ 5% of that observed in April and May and always consisted of relatively short streamers (≤ 2–3 cm).

Microscopy results

Phase-contrast microscopy in the field undertaken on freshly collected samples at the McGill high Arctic Research Station (MARS) indicated high concentrations of rod-shaped microbial cells within an organic matrix containing larger diamond- and cigar-shaped objects

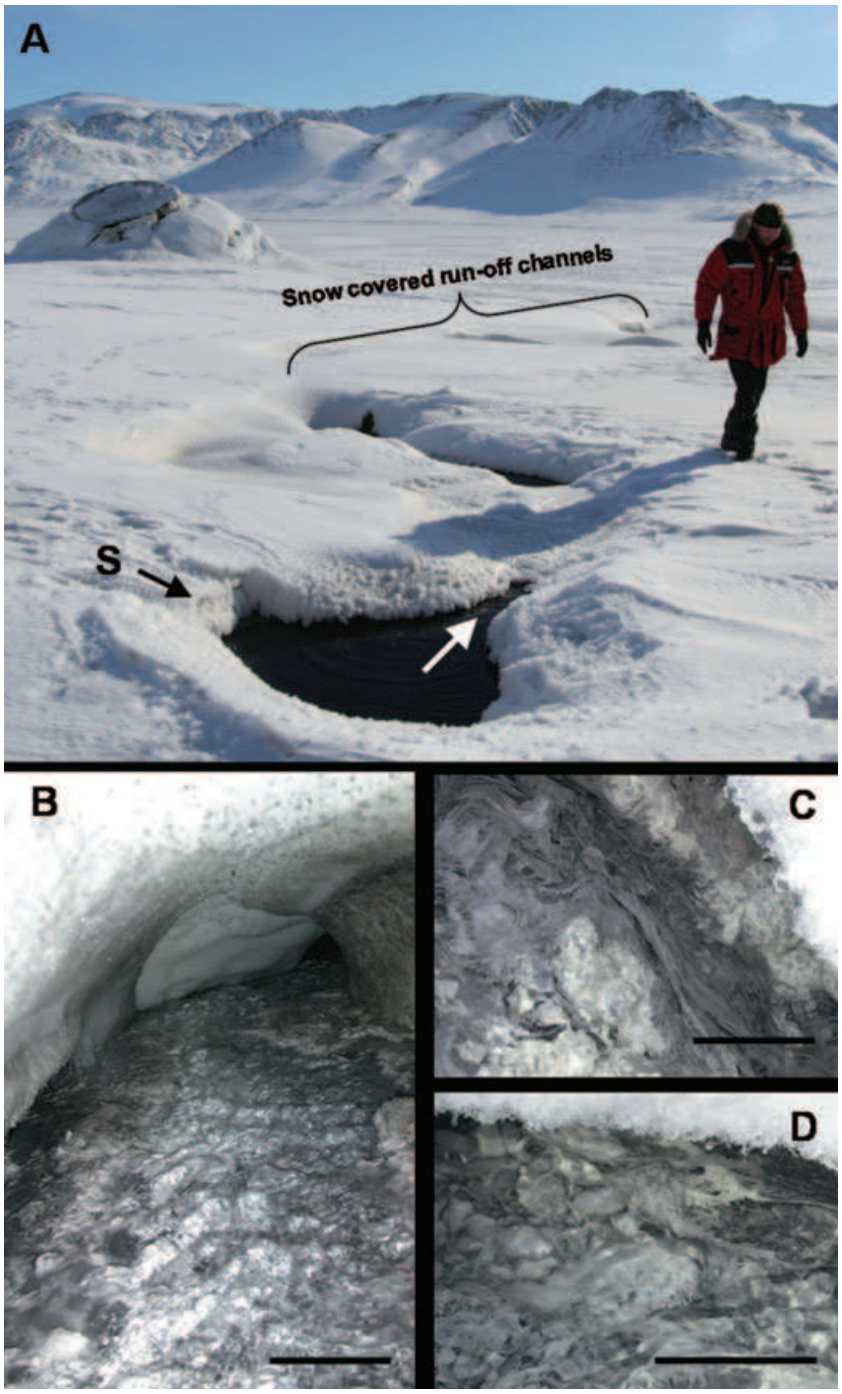

Fig. 1. Field photographs of the run-off streams and associated streamers of the GH springs.
A. Field photograph of GH-4; S indicates the spring source; the white arrow indicates the direction of the run-off discharge from the source.
B. View underneath the snow cover from a run-off channel originating from GH-4.
C. Streamers present following the removal of the snow cover from GH-4 (~5–6.9°C).
D. Streamers present following the removal of the snow cover from GH-2 (−1.3°C). Scale bar represents 10 cm for all images.

(Fig. 2A). The larger elongated (10–25 μm) structures within the streamers stained non-specifically with SYTO green and FUN 1 bacterial and fungal stains (results not shown). It was thus difficult to determine whether they represented living organisms or mineral structures. Permeabilization did not lead to a qualitative change in the labelling of the large structures, although it removed much of the organic material. SEM and EDAX analyses identified the larger elongated structures within the streamers as elemental sulfur and a variety of sulfur-containing minerals including gypsum ($CaSO_4 \cdot 2H_2O$) and halotrichite ($FeAl_2(SO_4)_4 \cdot 22H_2O$) (Fig. S1; Table S1).

Confocal Scanning Laser Microscopy (CSLM) imaging of streamer material indicated that it was composed of a globular substructure (Fig. 3A), which consisted of a matrix of exopolymeric substances binding the lectins *Banderia simplicifolia* and *Narcissus pseudonarcissus* (Fig. 3B). These lectins bind to *N*-acetyl-glucosamine and

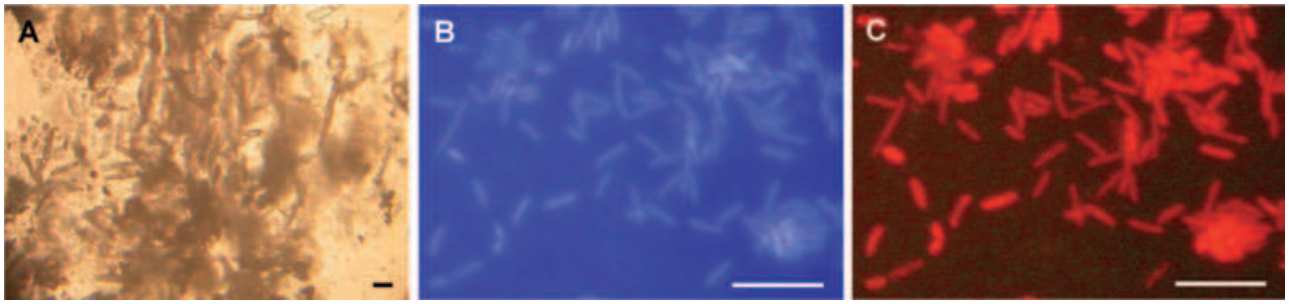

Fig. 2. Microscopic analyses of GH-4 streamer cells. (A) Phase-contrast micrograph of streamers (400×). (B) and (C) represent the same field of view (1000×) with (B) DAPI stained disrupted streamer cells (1000×) and (C) hybridization with *Thiomicrospira* probe TP1. Scale bar represents 10 μm for (A) and 5 μm for both (B) and (C).

alpha-D-mannose, with specificity for alpha-1-3 or alpha-1-6-linked mannoses, respectively, suggesting that these glycoconjugates are a major component of the EPS matrix. Within the globular structure were a high density of rod-shaped bacterial cells and highly reflective crystalline structures (Fig. 3C and D). Given the sulfide content of the environment and the chemical nature of the crystalline structures ($CaSO_4$/gypsum), it is suggested that oxidation of sulfides occurs within the globular structures with the production of sulfate.

Fluorescent *in situ* hybridization (FISH) analyses indicated that all cells within the streamers were bacterial as indicated by positive hybridization using probe EUB338. Positive hybridization signals were never observed using the eukaryotic and archaeal specific probes EUK516 and ARCH915 respectively. Imaging of positively hybridized intact streamers proved difficult due to high concentrations of hybridization signal (i.e. high fluorescence levels); however, disrupted streamers and the boundaries and thin sections of intact streamers indicated that the microbial community consisted completely of gammaproteobacterial rod-shaped cells (using probe GAM42a). Due to the dominance of molecular-based *Thiomicrospira*-related signatures within streamer DNA (see below),

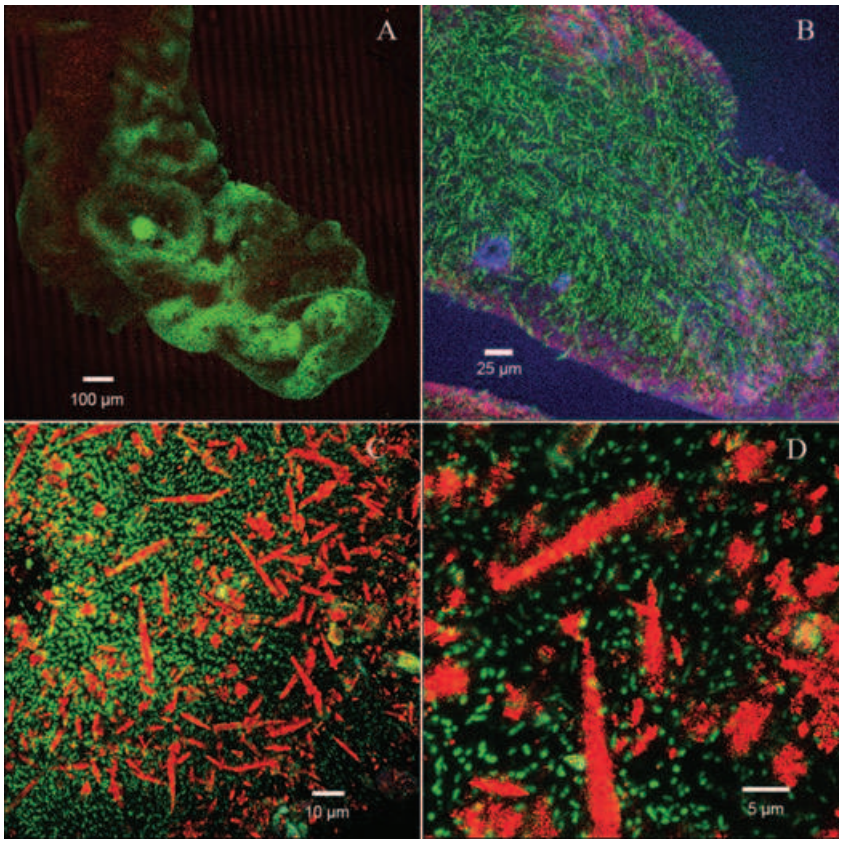

Fig. 3. Confocal micrographs of streamer materials (A) stained with the nucleic acid stain SYTO 9 (green) while crystalline structures are imaged using reflection and colour coded red, (B) stained with SYTO 9 (green), *Banderia simplicifolia*-Alexa-568 (red), *Narcissus pseudonarcissus*-Alexa-647 (blue) and reflection (green), illustrating the distribution of EPS, cells and crystalline structures; (C, D) SYTO 9 and reflection images at higher magnification showing the bacterial cells and crystals within an EPS matrix.

Thiomicrospira-specific FISH probes were constructed and tested. The utilization of an optimized *Thiomicrospira* probe (TP1) indicated that ~100% of DAPI stained cells (Fig. 2B) from disrupted streamers also stained with the *Thiomicrospira*-specific probe TP1 (Fig. 2C).

Phylogenetic analyses of streamers

Initial bacterial denaturing gradient gel electrophoresis (DGGE) comparison was undertaken between four greyish-white coloured streamer samples from GH-4, GH-2 and GH-6 as well as a white-coloured streamer from GH-4. The bacterial DGGE profiles of the streamer samples were relatively simple (1–3 dominant bands) with GH-6 and GH-2 containing an additional dominant band (bands F and H respectively) that were not present for the GH-4 samples (Fig. S2); DGGE bands were excised and sequenced to identify major microbial members. Both major (A, C, E and G) and minor bands (B and D) were near identical in sequence (99–100%, > 373 bp) to their closest NCBI BLAST relatives, namely, an uncultured clone G32 (DQ521115) and a cultured *Thiomicrospira* sp. NP51 (EU196304) both previously recovered from GH source sediment (Perreault *et al*., 2007). The DGGE band sequences were classified as *Thiomicrospira* with 100% confidence by the RDP Classifier. Attempts to sequence bands F and H failed (Fig. S2). Although FISH analyses did not indicate the presence of archaeal cells, positive archaeal DGGE amplifications were obtained from streamers collected from GH-6 and GH-2 (i.e. not GH-4). Archaeal DGGE profiles were identical between GH-6 and GH-2 (results not shown) and contained a single dominant band that contained a single nucleotide difference over 462 bp with the closest NCBI BLAST relatives (99–100%; 461–476 bp) being uncultured *Crenarchaeotal* signatures CP-A56 (DQ521209) and GH-A99 (DQ521150) previously recovered from CP and GH source pool sediments respectively (Perreault *et al*., 2007).

Subsequent 16S rRNA gene cloning was undertaken to corroborate DGGE results and to further resolve and characterize the diversity of the microbial communities within the streamers. A streamer sample from GH-2 was chosen as a representative sample for both archaeal and bacterial 16S rRNA gene clone library construction due to both a higher complexity within its bacterial DGGE profile (Fig. S2) and the detection of archaea by DGGE. A total of 24 bacterial 16S rRNA gene clones were screened with 22 clones classified within the *Thiomicrospira* genus (RDP Classifier; 100%) with the closest BLAST match (99%, > 700 bp) being *Thiomicrospira* sp. NP51 (EU196304) previously isolated from the GH sediments (Perreault *et al*., 2008). The closest validly described cultured relative (97%, > 700 bp) was *Thiomicrospira arctica* strain SVAL-E^T (AJ404731) (Knittel *et al*., 2005). DNA sequence alignment against the dominant *Thiomicrospira*-related bacterial DGGE band (i.e. A, C, E and G) proved that these sequences were 100% identical. Single bacterial clones classified within the *Desulfuromusa* genus (RDP Classifier; 100%) and the uncultured *Bacteroidetes* (RDP Classifier; 100%) were also represented in the bacterial library. The phylogenetic positions of the bacterial 16S rRNA gene clones are presented in Fig. 4. All 23 of the archaeal clones screened were classified within the *Crenarchaeota*. The archaeal library consisted of 22 clones being identical (100%, > 484 bp) to clone GH-A8 (DQ521152) previously recovered from GH source sediment (Perreault *et al*., 2007) and a single clone related to both GH-A99 (DQ521150; 93%, 470/503 bp) and CP-A56 (DQ521209; 93% 469/501) from GH and CP springs respectively (Perreault *et al*., 2007). These sequences do not have close phylogenetic relationships with any characterized archaeon and thus, no ecological role can be inferred.

Isolation of chemolithoautotrophic sulfur-oxidizing bacteria

One colony morphotype and one RFLP pattern were obtained on the thiosulfate medium and the MJ medium and a single colony was randomly selected on each medium for sequencing of the near full-length 16S rRNA gene. DNA alignment showed that both sequences were identical and had a perfect sequence match to *Thiomicrospira* sp. NP51 (EU196304) previously isolated from the GH spring waters and sediments. The *Thiomicrospira* sequences from the GH springs were also highly related (99% and 98% DNA identity over 1409 bp respectively) to *Thiomicrospira psychrophila* and *T. arctica*, two psychrophilic sulfur-oxidizing bacteria isolated from the Arctic Ocean (Knittel *et al*., 2005). The isolate grew on media used to culture both *T. psychrophila* and *T. arctica* (Knittel *et al*., 2005) and was capable of growth under aerobic and microaerophilic (6% O_2) conditions, with thiosulfate and sulfide as sole electron donors. It was capable of growth at NaCl concentrations of 2.5% at 10°C and could metabolize sulfide at concentrations as high as 125 p.p.m. in synthetic media.

In situ *activity experiments and RuBisCO detection*

In situ experiments were performed in the GH-4 spring channel to determine the capacity of the streamer microorganisms to oxidize inorganic S compounds and to fix inorganic carbon (Table 1). The lowest sulfide oxidation rate was obtained following *in situ* dark incubation (37.5 ± 4.8 μmol cm^{-3} h^{-1}) and the highest rate following light incubation at room-temperature (62.5 ± 8.8 μmol cm^{-3} h^{-1}). Thiosulfate was also used as electron donor by the streamer microbial community (Table 1) and

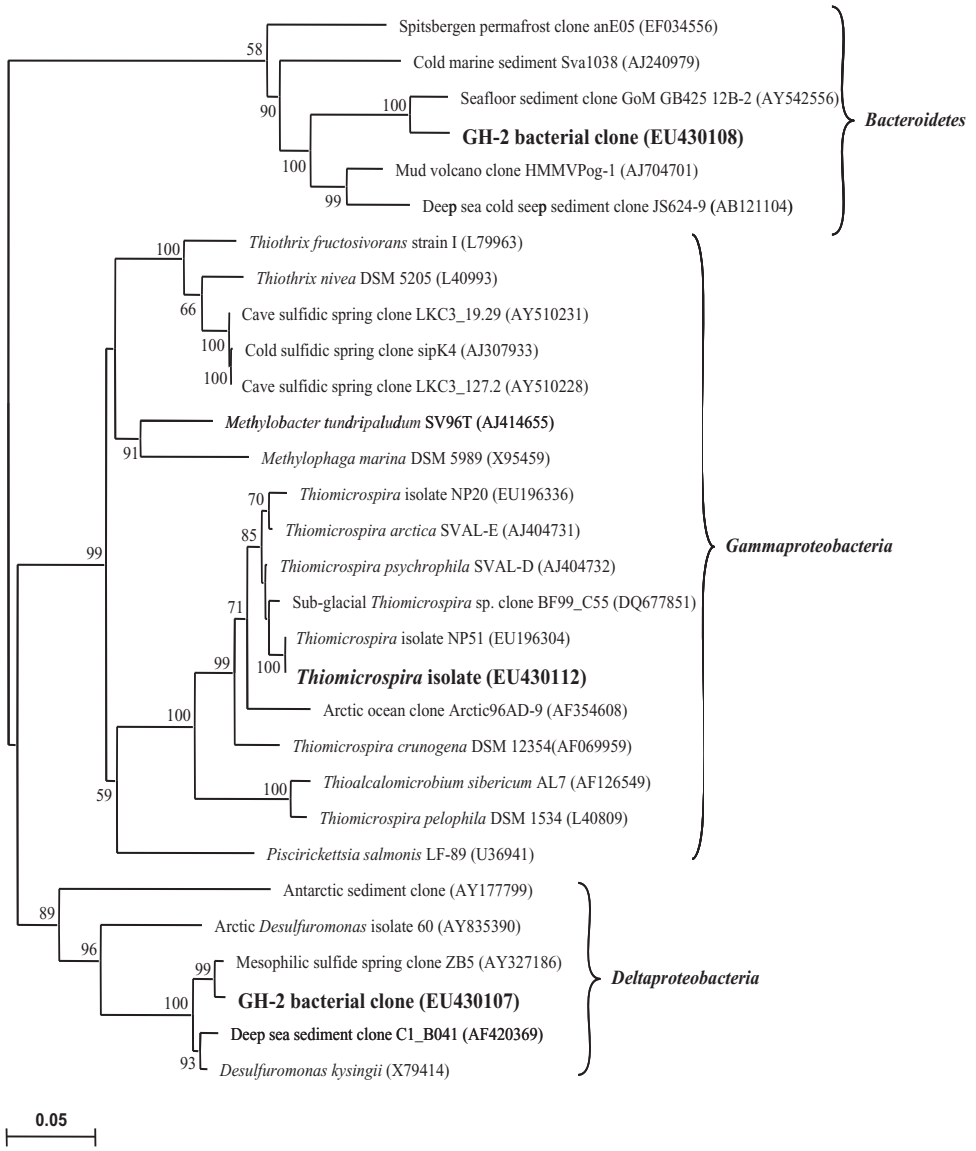

Fig. 4. Phylogenetic relationship of bacterial 16S rRNA gene sequences recovered from the streamers. The near full-length 16S rRNA gene of the *Thiomicrospira* isolate represents both the DGGE and the 22 16S rRNA gene clone library sequences classified within the *Thiomicrospira* genus, as all sequences share 100% sequence homology over 380 bp of aligned sequence. The tree is rooted to *Aquifex pyrophilus* (M83548) with the branch removed from the figure. Bootstrap values ≥ 50% of 1000 replicates are indicated at the nodes. The bar denotes the expected number of changes per nucleotide position.

Table 1. Rates of sulfide-oxidation, thiosulfate-oxidation and CO_2 fixation of the streamers.

Sulfide-oxidation rates[a]				Thiosulfate-oxidation rates[a]	CO_2 fixation[b]	
RT-light	RT-dark	*in situ*-light	*in situ*-dark	*in situ*-light	*in situ*-light	*in situ*-dark
62.5 ± 8.8	45.7 ± 5.5	51.6 ± 5.2	37.5 ± 4.8	21.2 ± 1.8	235 ± 42	317 ± 59

a. μmol cm^{-3} h^{-1}.
b. nmol C l^{-1} h^{-1}.
RT, room-temperature (17–20°C) at the McGill Arctic Research Station (MARS).

autotrophic CO_2 fixation detected by ^{14}C-bicarbonate uptake under both light (235 ± 42 nmol C l^{-1} h^{-1}) and dark incubations (317 ± 59 nmol C l^{-1} h^{-1}). Radioactivity was not detected in any of the three controls. The ability of the streamers to fix CO_2 was further corroborated by the successful PCR amplification of RuBisCO *cbbM* sequences from the community DNA of the four streamer samples and from the *Thiomicrospira* isolate. DNA sequence analysis showed that all the sequences were identical and grouped with *cbbM* sequences from *Thiomicrospira* species and other *Gammaproteobacteria* (results not shown).

Discussion

During previous summer month sampling expeditions to Axel Heiberg Island, patchy distributions of greyish streamers were observed in the run-off channels of the springs at GH. Conversely, during the winter months the concentrations of greyish microbial streamers increased markedly beneath the snow covering the perennially liquid run-off channels. Although it is not known exactly when the streamers form during the Arctic winter, similar levels of streamer biomass were observed in mid-April (average air temperature approximately –25°C) in previous years. Streamers were never observed at the source or within ~1 m from the source of the spring, even if the spring and its associated run-off channel were covered with snow. This phenomenon is hypothesized not to be due to temperature or sulfide, but rather levels of DO; as temperatures did not differ significantly between the spring source and the site of streamers and the *Thiomicrospira* isolate was shown to grow in H_2S concentrations higher (125 p.p.m.) than that measured at the spring source (25–100 p.p.m.) or in the spring run-off channel water. However, DO levels were consistently higher at the site of the streamers (1.0 p.p.m.) than at the source of the springs (0.05–0.2 p.p.m.) and it has been shown that *Thiomicrospira* sp. require aerobic or microaerophilic conditions for growth (Knittel *et al*., 2005). Therefore, the *Thiomicrospira* streamers may only develop in regions of the springs where the oxygen levels are sufficiently high enough for growth.

The construction, optimization and utilization of a *Thiomicrospira*-specific FISH probe (TP1) conclusively proved that all cells (DAPI stained) of the streamers are species of *Thiomicrospira*. Therefore, FISH confirmed the results of molecular analyses (both DGGE and clone libraries) and indicated the complete dominance of the gammaproteobacterial genus *Thiomicrospira*. Cells unrelated to the *Gammaproteobacteria* were not detected via FISH analyses, i.e. all observed cells positively hybridized with the gammaproteobacterial-specific probe. Therefore, the unknown crenarchaeote and two non-*Thiomicrospira* clones (an uncultured *Bacteroidetes* and a species of *Desulfuromonas*) detected in streamers from GH-2 by 16S rRNA gene cloning may exist below the detection level of FISH. This is also exacerbated as minor proportions of the streamers microbial community may be overlooked by FISH analyses due to the high dominance of a single type of microbial species and the subsequent bright signal of the FISH hybridization. DNA sequence homologies between the DGGE, the *Thiomicrospira*-related 16S rRNA clones and the near full-length 16S rRNA gene from the isolate were 100% identical over 380 bp of aligned sequence, indicating a high likelihood that they all represent the same strain.

The reason or function of the S-containing crystallites within the streamer matrix are currently unknown; however, they are morphologically and chemically similar to crystals in microbial mats of Miette hot springs, Jasper National Park, Canada (Bonny and Jones, 2003). Bonny and Jones (2003) presented three processes whereby these crystals form within the microbial mats of the Miette hot springs: (i) microbial extracellular polymeric substances passively concentrate crystals via a trapping effect; (ii) microbial mats may act as nucleation sites for the growth of gypsum structures; (iii) microbes within the mats influence mineral precipitation by their metabolisms, e.g. CO_2 degassing via photosynthetic pathways and the production of elemental S via sulfate reduction. These processes remain unknown in the GH streamers; however, crystal formation may occur due to the production of elemental sulfur and SO_4-minerals via S-oxidation due to the high concentrations of *Thiomicrospira* cells within the streamer matrix. The formation and role of the crystal structures within the streamers is a current avenue of further investigation, whereby sulfur isotopic analyses may determine the biogenic or abiotic origin of the crystallites.

Thiomicrospira species are obligate sulfur oxidizing bacteria (Knittel *et al*., 2005) and are typically distributed where sulfur-related metabolism is a primary energy process. They have been detected and cultured from various environments including hydrothermal vents (Brinkhoff *et al*., 1999; Brazelton *et al*., 2006; Perner *et al*., 2007), marine sediments, coastal mud flats, hypersaline ponds, sediment from a saline spring in Artern, Germany (Brinkhoff and Muyzer, 1997; Knittel *et al*., 2005) and a subglacial outflow in Antarctica (Mikucki and Priscu, 2007). Our previous culture-independent 16S rRNA gene-based study of the GH and CP sediments indicated that *Thiomicrospira* was the most abundant phylotype in both the GH and CP spring source sediments (Perreault *et al*., 2007). However, streamers have never been observed in CP springs or run-off channels. The reason for the lack of streamers at CP is not clear. The CP springs also have snow covered run-off streams; however, the increased concentration of salts at CP

(~15.5%) as compared with GH (~7.5%) may inhibit the formation of *Thiomicrospira* streamers as the tolerance of validly characterized species of *Thiomicrospira* (including the psychrophilic species, *T. arctica* and *psychrophila*) are typically between ~0–1.24 M NaCl (0–7.2% NaCl) (Sorokin *et al*., 2006). The recently described *T. halophila* can tolerate NaCl levels of up to 3.5 M NaCl (~20.5% NaCl); however, this isolate has an optimum growth temperature of 30°C and is not capable of growth at 0°C (Sorokin *et al*., 2006). The higher total discharge rate and flow rate of the CP springs (20–25 l s^{-1} and 1.5–1.8 l s^{-1} respectively) as compared with the GH springs (10–15 l s^{-1} and 0.9–10 l s^{-1} respectively) (Pollard *et al*., 1999) may also inhibit the establishment of optimal geochemical gradients (e.g. H_2S, O_2) or the attachment of cells to solid matrices that may be required for the formation of the streamers.

The microbial streamers were shown to metabolize and to proliferate extensively under the *in situ* conditions of cold (–1.3°C to 6.9°C) saline water within a microhabitat formed via snow cover protecting and insulating against the surrounding late-winter air temperatures (< –15°C). The snow cover may also allow the streamers to develop under mid-winter extreme air temperatures of –50°C as they were already extensively established by the end of winter. Natural light had a positive effect on sulfide-oxidation while it appeared to be slightly detrimental for CO_2 fixation, with CO_2 fixation rates being 25% less than for dark incubation. The same phenomenon was observed previously for CO_2 uptake in the GH spring water (Perreault *et al*., 2008). Previously rates for dark CO_2 fixation have also been described from a moderately salty (~3% NaCl) subglacial outflow (Blood Falls) from the Taylor Glacier, Antarctica (Mikucki and Priscu, 2007), although the rates were considerably lower (0.05 nmol C l^{-1} h^{-1}) than those measured in this study (235 ± 42 and 317 ± 59 nmol C l^{-1} h^{-1} for light and dark incubations respectively) which is most likely due to the low cell content (1×10^4–7.6×10^5 cells ml^{-1}) of the subglacial water. Interestingly, the dominant clone detected within the subglacial outflow water was also related to *Thiomicrospira arctica* (46% of the library) (Mikucki and Priscu, 2007); however, streamers of *Thiomicrospira* cells were not observed in this system.

Detection of both sulfide and thiosulfate oxidation and CO_2 uptake was consistent with the presence and activity of chemolithoautotrophic sulfur-oxidizing bacteria such as *Thiomicrospira* while CO_2 uptake under dark incubation confirmed that chemoautotrophic activity, and not photoautotrophy, sustains the streamer community. This suggests that *Thiomicrospira* spp. have an advantage in inhabiting cold polar environments that contain reduced sulfur-compounds and experience long-term periods of darkness during polar winters or permanent darkness as for example in Antarctic subglacial microhabitats (Mikucki and Priscu, 2007).

Form II (*cbbM*) RuBisCO is found in several photosynthetic bacteria, aerobic and facultative anaerobic, chemoautotrophic bacteria and dinoflagellates and is adapted to anoxic conditions (Portis and Parry, 2007; Badger and Bek, 2008). The anoxic *cbbM* genotype was detected in the sulfur-oxidizing streamers and the *Thiomicrospira* streamer strain, which is in accord with the hypoxic conditions in the brine of the GH spring channels. Among other *Thiomicrospira* species, *T. crunogena* (Tourova *et al*., 2006), *T. kuenenii* (Tourova *et al*., 2006) and *T. halophila* (Sorokin *et al*., 2006) also possess at least one *cbbM* gene. Altogether, the presence of a unique *cbbM* sequence, identical for both the streamer samples and the *Thiomicrospira* strain and its phylogenetic affiliation with *cbbM* sequences from other *Thiomicrospira*, support the 16S rRNA gene data, suggesting that *Gammaproteobacteria* sulfur-oxidizers of the genus *Thiomicrospira* are responsible for the autotrophic carbon fixation in the GH microbial streamers. Form II enzymes have a poor affinity for CO_2 and a low specificity factor to discriminate between CO_2 and O_2, implying that the form II enzymes operate exclusively at high CO_2 concentrations and low O_2 concentrations (Badger and Bek, 2008), hence, a large investment of energy is required for this method of CO_2 fixation. Therefore, the sulfur-oxidizing streamers theoretically have to oxidize high concentrations of reduced sulfur compounds as electron donors to generate enough energy to grow autotrophically. Therefore, the snow covering the run-off streams may trap and concentrate H_2S gas and explain the marked increase of streamers during the winter; in fact bursts of H_2S were clearly perceived following the puncturing of the snow covering the GH spring run-off streams.

An initial microbiology investigation of very low saline, sulfur springs in the Canadian high Arctic (Ellesmere Island) did not detect S-oxidizing bacteria. These springs differ to those of GH and CP as they discharge from the surface of a glacier, rather than through a thick layer of permafrost (Grasby *et al*., 2003). This is the first and only report of *Thiomicrospira* in a macroscopic streamer form and appears to be unique to the GH springs; however, the macroscopic appearance of the streamers is similar to streamers previously described in hot spring environments (Yamamoto *et al*., 1998; Nakagawa and Fukui, 2003) and cave systems (Engel *et al*., 2004; Macalady *et al*., 2006). Microbial streamer assemblages of other non-thermal spring and cave systems rich in sulfur species are composed of more than one, and sometimes quite complex microbial types. For example, a *Thiothrix*-related bacterium in association with *Archaea* forms a macroscopic structure morphologically comparable to a string of pearls in cold (10°C) sulfidic springs located in Bavaria, Germany

(Rudolph *et al*., 2001), six *Epsilonproteobacteria*-related taxonomic groups dominate (68% of 16S rRNA gene clone libraries) microbial populations of white streamer bundles in sulfurous spring cave systems in Wyoming, USA (Engel *et al*., 2003; 2004), and *Gammaproteobacteria* related to *Thiothrix* sp. and *Beggiatoa* sp. dominate white biofilms that form in sulfide-rich waters in an Italian cave system (Macalady *et al*., 2006).

The characterization of *Thiomicrospira*-dominated sulfur-oxidizing streamers, which flourish during the extreme polar winter via phototrophic-independent chemolithotrophic-based metabolism within a snow-covered microenvironment characterized by low temperatures ranging from −1.3°C to 6.9°C, depending on the spring source, high salt concentrations (~7.5%), oligotrophic and microaerophilic conditions, expands our current understanding of the limits of terrestrial life and has implications for the search for extinct or extant life on other solar system bodies. At nearly 80°N, these high Arctic cold saline springs and associated channels are among the few known examples of cold, non-volcanic springs in thick permafrost on Earth, and are considered Mars analogues as similar hydrological systems may have existed or still exist on Mars (Andersen *et al*., 2002; Andersen, 2004; Malin *et al*., 2006). For example, basal melting of Martian polar ice caps could generate significant groundwater flow systems that flow through evaporate salt deposits and finally emerge through thick permafrost as hypersaline springs (Grasby and Londry, 2007). In this respect, Mars Global Surveyor images recently detected new gully deposits, formed since 1999, providing exciting and compelling evidence that liquid water (possibly eutectic brines from the Martian subsurface) flowed on Mars during the past decade, under mean surface temperatures of −60°C and extensive permafrost (Malin *et al*., 2006). These observations fit with other models, suggesting that it may be possible for liquids to exist below the surface and be discharged under modern Martian conditions (Mellon and Phillips, 2001; Heldmann *et al*., 2005; Kraal *et al*., 2008). It is possible that such gully formations may harbour similar subsurface microenvironments (i.e. very cold, saline, shielded from sunlight and UV) to those observed at the Gypsum Hill springs and hypothetically capable of supporting chemolithotrophic, phototrophic-independent microbial life.

Experimental procedures

Site description and collection of samples

The location and setting of the perennial springs adjacent to Gypsum Hill (GH) are described in detail by Pollard and colleagues (1999). The springs from which streamer samples were collected include GH-2 and GH-4, as described in the initial investigation by Perreault and colleagues (2007), and a new spring, designated GH-6, located adjacent to GH-4. Dissolved sulfide and oxygen (DO) concentrations of water within the run-off channels were measured in triplicate via colorimetric assay (CHEMtrics, Calverton, VA, USA). Streamer samples were collected in May 2007 using sterile tweezers and were subsequently stored in spring water inside sterile polypropylene tubes (Fisher). Streamer samples were transported to McGill University at subzero temperatures and stored at both −5°C (for culture-based analyses) and −20°C (for DNA-based analyses).

DNA extraction and polymerase chain reaction (PCR)

Streamer samples were rinsed in 0.9% sterile NaCl and total community DNA extracted from 0.07 to 0.25 g of biomass using the ZR Fungal/bacterial DNA Kit™ (ZYMO Research, USA). DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA). All PCR reagents were supplied by Invitrogen Canada, Burlington, ON. Bacterial DGGE-PCR utilizing primers 341F-(GC) and 758R and the associated thermocycling conditions were undertaken as described by Steven and colleagues (2008) with the following modifications; 3 mM $MgCl_2$ and 2.5 μl each primer (10 μM concentration) for a 50 μl reaction and the inclusion of a 30 min final extension at 72°C to remedy any double-banding phenomena (Janse *et al*., 2004). Bacterial partial-length 16S rRNA gene amplification for cloning was undertaken as outlined by Steven and colleagues (2007a) utilizing primers 27F and 758R and ~50 ng of template DNA. Archaeal DGGE-PCR and partial length 16S rRNA gene PCR for cloning were undertaken using primers A344F-(GC) and A934R with ~50 ng template DNA as described previously (Perreault *et al*., 2007).

Denaturing gradient gel electrophoresis analyses

Denaturing gradient gel electrophoresis was performed as previously described by Steven and colleagues (2008) utilizing an 8% acrylamide gel with a 35–65% denaturing gradient that was run for 16 h at 60°C and 80 V and visualized by ethidium bromide staining. DGGE bands of interest were also processed and sequenced as described by Steven and colleagues (2008). Bacterial and archaeal DGGE sequences were deposited into the GenBank database as accession numbers EU430099 to EU430104 and EU430105 to EU430106 respectively.

Construction and sequencing of 16S rRNA gene clone libraries

PCR amplicons from the three partial-length 16S rRNA gene PCRs were combined and purified using a QIAquick PCR purification kit (QIAGEN Sciences, MD, USA) and quantified via ethidium bromide staining in an agarose gel and a 2:1 molar ratio of insert to vector used for both bacterial and archaeal clone libraries. The pGEM-T Easy vector system was used as per manufacturer's instructions (Promega, Madison, USA) in conjunction with subcloning efficiency DH5α competent cells (Invitrogen, CA, USA). Clones of interest were selected and vectors with the correct sized inserts

checked as described by Bottos and colleagues (2008). Inserts were sequenced with their respective forward primers used for the original partial-length 16S rRNA gene PCR. Representative bacterial and archaeal 16S rRNA gene clone sequences were deposited into the GenBank database as accession numbers EU430107 to EU430109 and EU430110 to EU430111 respectively.

DNA sequence analyses

Sequencing was undertaken at the Genome Quebec Innovation Center (McGill University) with 3730XL DNA analyser systems (Applied Biosystems). 16S rRNA gene sequences were manually edited and subjected to CHIMERA_CHECK (Cole *et al*., 2003) and the Bellerophon server (Huber *et al*., 2003). Taxonomic affiliations were determined using the Classifier tool (Wang *et al*., 2007) of the RDP II (Cole *et al*., 2007). Sequences were also compared with the GenBank database using the BLASTn algorithm (Altschul *et al*., 1990). Sequences of each clone library were aligned using ClustalW software and neighbour-joining phylogenetic trees were produced with MacVector 7.0 software package (Oxford Molecular, Oxford, UK) using Jukes-Cantor modeling with 1000 bootstrap re-samplings.

Microscopy procedures

Phase-contrast microscopy was undertaken on freshly collected streamers at the MARS, Axel Heiberg Island and micrographs taken using a Nikon COOLPIX 4500 digital camera (Nikon, Melville, NY, USA). Staining of samples with SYTO Green and FUN 1 was performed by adding the dye (Molecular Probes™, Invitrogen, Eugene, OR, USA) to a final concentration of 10 μM in a sample of streamers in a 1.5 ml Eppendorf tube containing water from the spring. The sample was mixed by inversion at ambient temperature (5–10°C) but not otherwise processed and wash steps were not performed. Permeabilization by osmotic shock (Sestak and Farkas, 2001) was undertaken as follows: the streamer sample was suspended in 10 volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM beta-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (TEGM buffer) supplemented with 33% (w/v, 3.5 M) glycerol and allowing them to stand for 10 min on ice. The suspension was then allowed to sediment by gravity (~1 min), and the sedimented cells were washed once with the same buffer without glycerol. Finally, the cells were suspended in two volumes of the same buffer and stained as outlined above. Environmental scanning electron microscopy (ESEM) was carried out using a FEG XL30 (FEI, Hillsboro, OR, USA) with an accelerating voltage of 20 kV. A gaseous secondary electron detector (GSED) was used with a Peltier cooling stage at 4.0°C and a 3.9 torr chamber pressure. The whole mount bacterial samples were rinsed in ddH_2O prior to being placed on aluminum stubs and into the ESEM. A Genesis energy dispersive X-ray (EDAX) spectrometer (Ametek, Paoli, PA, USA) was used with an ultrathin window for elemental analysis.

Fluorescent lectins with Alexa 568 and Alexa 647 labelling and the nucleic acid probe SYTO 9 were purchased (Invitrogen). The lectins *Banderia simplicifolia*-Alexa-568 and *Narcissus pseudonarcissus*-Alexa647, were employed alone, together or with SYTO 9 as described by Neu and colleagues (2001); with the exception that the staining time was extended to 12 h to allow penetration of the samples. Examination of all stained and control materials was carried out using an MRC 1024 CLSM (Zeiss, Jena, Germany) attached to a Microphot SA microscope (Nikon, Tokyo, Japan). During microscopic analyses, the following lenses were used: 60×, 1.4 numerical aperture (NA) Planapochromat (Nikon) a 20×, 0.75 NA Fluor/Ph3-DL (Nikon) and a 6×, 0.2 NA Fluorotar (Wild). Signals were recorded in the green channel (excitation 488 nm, emission 522/32), red channel (excitation 568 nm, emission 605/32) and far red channel (excitation 647 nm, emission 680/32). In addition, the reflection mode was used to detect crystal structures associated with the bacteria (Lawrence and Neu, 2007). Image stacks were projected using the software provided by Bio-Rad. During all sample handling, staining and microscopy samples were maintained at 0°C by mounting on frozen gel packs.

Streamer samples for FISH were immediately fixed in the field by the method utilized for marine sediment as described by Pernthaler and colleagues (2001) and transported frozen to McGill University and stored at −15°C. FISH analyses were performed as described by Rudolph and colleagues (2001) using gelatin/$KCr(SO_4)_2$-coated slides (Pernthaler *et al*., 2001). FISH probes and their respective hybridization and wash buffers were used according to the respective references: universal bacterial and archaeal probes, EUB338 and ARCH915 (Rudolph *et al*., 2001), labelled at the 5′-terminus with Texas Red and fluorescein isothiocyanate respectively; universal eukaryotic probe, EUK516 (Baschien *et al*., 2001), labelled at the 5′-terminus with fluorescein isothiocyanate; gammaproteobacterial specific probe, GAM42a (Macalady *et al*., 2006), labelled at the 5′-terminus with Texas Red. All FISH staining procedures included positive control samples of pure cultures of known microorganisms. Oligonucleotide FISH probes specific for *Thiomicrospira* sp. NP51 isolated from GH spring source sediment (Perreault *et al*., 2008), and the related strains *T. arctica* and *T. psychrophila* (Knittel *et al*., 2005) were designed using the program Primrose (Ashelford *et al*., 2002). Probe candidates were initially checked for specificity against the sequences in GenBank via a BLAST search with probe coverage and specificity confirmed *in silico* via probeCheck (Loy *et al*., 2008). Two oligonucleotide probes both labelled at the 5′-terminus with Texas Red, TP1 (5′-CTC TAT CGT TTC CGT CCG-3′) corresponding to *Escherichia coli* 16S rRNA nucleotide positions 63–80 and TP7 (5′-CGC CTA GAA AAG CAA GC-3′) corresponding to *E. coli* 16S rRNA nucleotide positions 91–107 were chosen for specific detection of the three *Thiomicrospira* strains. Specificity of the *Thiomicrospira* probes was tested by adjusting hybridization stringencies from 0% to 60% formamide in 10% increments. Positive controls consisted of cells of *Thiomicrospira* strain NP51 and *T. arctica* and *T. psychrophila* obtained from the DSMZ (Deutsche Sammlung van Mikroorganismen und Zellkulturen GmbH; http://www.dsmz.de). Probe TP1 provided the highest fluorescence of the tested probes on *Thiomicrospira* cells at 30% formamide and was chosen for further testing of steamer and negative control samples at 30% formamide concentration. Cross-reactivity of the TP1 probe was not observed in > 15 microorganisms

isolated from the GH springs and other high Arctic environments, including genera of gammaproteobacteria, e.g. *Pseudomonas*. All FISH stained samples were mounted with 4:1 mix (Pernthaler *et al*., 2001) of Citifluor (Citifluor, Leicester, UK) and Vectashield (Vecta Laboratories, Ontario, Canada) amended with 1 mg ml^{-1} DAPI for counterstaining purposes (Pernthaler and Pernthaler, 2007). FISH results were viewed using a fluorescent Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) with appropriate filter sets (Chroma Technology Corporation, VT, USA) and images taken using a Nikon COOLPIX 4500 digital camera.

Culture and 16S rRNA gene analyses of chemolithoautotrophic sulfur-oxidizing bacteria

A preliminary study on GH streamers collected in July 2004 identified two 16S rRNA gene phylotypes related to the sulfur-oxidizing bacteria *Thiomicrospira psychrophila* and *Sulfurimonas autotrophica* in a clone ratio of 25:1. Based on this preliminary molecular study, media were selected to target aerobic thiosulfate-oxidizers (thiosulfate medium) and microaerophilic sulfide-oxidizers (MJ medium). Ten millimetres of streamers from GH-6 was roughly disrupted in 30 ml of sterile water and 100 μl was inoculated in 60 ml serum bottles containing 20 ml of thiosulfate medium (per litre: 0.4 g NH_4Cl, 4.0 g KH_2PO_4, 4.0 g K_2HPO_4, 0.8 g $MgSO_47H_2O$, 0.03 g $CaCl_2$, 0.02 g $FeCl_36H_2O$, 0.02 g $MnSO_4H_2O$, 5.0 g $Na_2S_2O_35H_2O$, 25.0 g NaCl) or 20 ml of medium 1011: MJ medium (DSMZ). A gas mixture of $N_2/CO_2/O_2$ (77:17:6) was used in the headspace of the MJ medium. The bottles were incubated at 10°C until growth was visually detected (~10 weeks). Aliquots of the liquid cultures were dispensed on agar plates of the same medium. Ten colonies for each media were randomly selected for 16S rRNA gene restriction fragment length polymorphism (RFLP) as described previously (Juck *et al*., 2003). Two colonies (one for each culture medium) were selected for PCR and sequencing of the near full-length 16S rRNA gene (~1500 bp) using primers 8F and 1492R as described previously (Lane, 1991). The 16S rRNA gene sequence was deposited in the GenBank database as accession number EU430112.

Sulfur oxidation rates

Approximately 10 ml of streamer slurry collected from GH-4 was mixed with 30 ml of GH-4 spring water and roughly disrupted by vigorous shaking. The potential sulfur oxidation rates (SORs) of the microbial streamers were determined by inoculating 0.5 ml of the streamer slurry into sterile 120-ml serum bottles containing 50 ml of synthetic media (Asami *et al*., 2005) supplemented with NaCl (7.5%) and Na_2S (1.6 mM) or $Na_2S_2O_3$ (20 mM) as the sole electron donor. Serum bottles with Na_2S were incubated either directly in the spring (GH-4) outlet (~6.9°C) or at the MARS (17–20°C), under natural light or dark incubation (bottles covered with layers of foil). Bottles with $Na_2S_2O_3$ were incubated in the spring outlet, under natural light. Subsamples were withdrawn every 24 h for 5 days. S^- consumption was measured in the subsamples by spectrophotometric determination (Cline, 1969). $S_2O_3^{2-}$ consumption was estimated by monitoring sulfate increase by ion exchange high pressure liquid chromatography (Spectra-Physics, model SP8800). The concentrations measured during the first 48 h incubation were used to calculate SORs. Experiments were done in triplicate and controls with no biomass added were performed in parallel to determine the abiotic SORs, which was subtracted from the overall SORs.

In situ *^{14}C-bicarbonate uptake and the PCR amplification of the RuBisCO gene*

A total of 0.5 ml of the sediment slurry (as described above for the SORs assays) was added to 20 ml of spring water in sterile acid-washed 25 ml serum bottle and amended with 250 μl of ^{14}C-labelled sodium bicarbonate ($NaH^{14}CO_3$; 5 μCi) (MP Biochemicals; specific activity 53.5 mCi $mmol^{-1}$). Bicarbonate uptake was performed based on the method described by Joint and colleagues (1993) with incubation carried out in the GH-4 spring outlet (~6.9°C) for 24 h. The primer pair RuIIF1/RuIIR3 was used to target the partial gene (~800 bp) encoding the large subunit of the form II (*cbbM*) RuBisCO protein from the streamers and the *Thiomicrospira* isolate as described previously (Spiridonova *et al*., 2004).

Acknowledgements

Logistic support was provided by the Canadian Polar Continental Shelf Project (PCSP-08, 634-07, 664-06, 66) and McGill University's High Arctic Research Station. This work was supported by grants from NASA's Exobiology program (NAG5-12395), the Natural Sciences and Engineering Research Council of Canada (NSERC Discovery Program, Northern Supplements Program, Special Research Opportunities Program, and the Canadian Space Agency Canadian Analogue Research Network program. Additional funding for student research was provided by the Department of Indian and Northern Affairs – Northern Scientific Training Program, and the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT). Jay L. Nadeau acknowledges support from the Canadian Space Agency CARN program and the NSERC Individual Discovery and NanoIP programs.

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Supporting information

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

Fig. S1. ESEM images of minerals from streamers collected from GH-4. (A) and (B) are unprocessed samples. Samples presented in (C) to (F) are treated via osmotic shock. Scale bars are given on each panel. Representative minerals are labelled 'G' (gypsum), 'S' (sulfur) or 'H' (halotrichite); values for each of the labelled regions are provided in Table S1.
Fig. S2. Bacterial DGGE profiles of streamer samples. Lane 1, GH-4 (white-coloured form); lane 2, GH-4 (grey-coloured form); lane 3, GH-6; lane 4, GH-2.
Table S1. Results of energy-dispersive X-ray spectroscopy (EDAX) analyses performed on the structures within streamers as labelled in Fig. S1.

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