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Heterotrophic and Autotrophic Microbial Populations in Cold Perennial Springs of the High Arctic▽†

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The saline springs of Gypsum Hill in the Canadian high Arctic are a rare example of cold springs originating from deep groundwater and rising to the surface through thick permafrost. The heterotrophic bacteria and autotrophic sulfur-oxidizing bacteria (up to 40% of the total microbial community) isolated from the spring waters and sediments were classified into four phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) based on 16S rRNA gene analysis; heterotrophic isolates were primarily psychrotolerant, salt-tolerant, facultative anaerobes. Some of the isolates contained genes for thiosulfate oxidation (*soxB*) and anoxygenic photosynthesis (*pufM*), possibly enabling the strains to better compete in these sulfur-rich environments subject to long periods of illumination in the Arctic summer. Although leucine uptake by the spring water microbial community was low, CO_2 uptake was relatively high under dark incubation, reinforcing the idea that primary production by chemoautotrophs is an important process in the springs. The small amounts of hydrocarbons in gases exsolving from the springs (0.38 to 0.51% CH_4) were compositionally and isotopically consistent with microbial methanogenesis and possible methanotrophy. Anaerobic heterotrophic sulfur oxidation and aerobic autotrophic sulfur oxidation activities were demonstrated in sediment slurries. Overall, our results describe an active microbial community capable of sustainability in an extreme environment that experiences prolonged periods of continuous light or darkness, low temperatures, and moderate salinity, where life seems to rely on chemolithoautotrophy.

Perennial springs are extremely rare in areas underlain by deep, continuous permafrost, because of the limited opportunity for exchange between sub- and suprapermafrost groundwater systems. The perennial springs found at Gypsum Hill (GH) on west-central Axel Heiberg Island in the Canadian high Arctic originate from deep saline groundwater and rise to the surface through ~600-m-thick continuous permafrost in a region with a mean annual temperature of −15°C (38). They discharge oligotrophic brines (7.5 to 7.9% salt) that are rich in inorganic sulfur compounds, saturated with dissolved gasses (primarily N_2), and maintain a constant temperature (−1.3°C to 6.9°C) throughout the year despite air temperatures that drop below −40°C during the winter (36, 38). The springs' location at nearly 80°N exposes them to long periods of continuous illumination or total darkness during the Arctic summer and winter, respectively.

Recent reports have examined mesophilic (15) and cold sulfur (14, 22, 23) springs, including cold springs in Germany that harbor a string-of-pearls-like community consisting of microbial filaments of *Archaea* in close association with sulfide-oxidizing bacteria related to *Thiothrix* (33). Sulfur springs usually support a diversity of sulfur-oxidizing bacteria, including anaerobic anoxygenic phototrophs (purple and green sulfur bacteria) that use reduced sulfur compounds as electron donors during photosynthesis (14, 15). Our initial culture-independent study, based on 16S rRNA gene analysis, suggested that the GH springs are also dominated by sulfur oxidizers (36), and abundant grayish-white microbial streamers have been observed in the spring channels during the late winter. However, despite the high sulfide concentration in the GH springs and the continuous illumination during the sampling period, neither anaerobic anoxygenic phototrophs nor any other photoautotrophic microorganisms were detected in our initial study (36).

Sulfur-based chemolithoautotrophy, mainly performed by *Epsilonproteobacteria*, was shown to sustain microbial ecosystems devoid of light, such as hydrothermal vents (28) and aphotic (cave) sulfidic springs (16, 17, 45). Chemolithoautotrophic *Thiomicrospira* species are believed to be important primary producers in Antarctic subglacial outflows (32). As the GH springs undergo ~3 months of total darkness during the Arctic winter, non-photosynthesis-based primary production was hypothesized to be important in the GH springs' microbial ecosystem (36).

In this study, we build upon our earlier work (36) with an examination of the heterotrophic and autotrophic compositions of the GH springs' bacterial isolates and measured metabolic activities (leucine and bicarbonate uptakes, sulfur metabolism, carbon isotope signatures for methane [CH_4] and dissolved inorganic carbon [DIC]) in sediments and outflow waters. The objectives were to identify phylogenetic and physiological characteristics that enable microorganisms to inhabit

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and sustain this unique and still poorly characterized Arctic ecosystem.

MATERIALS AND METHODS

Site description and sampling. The GH site is located at 79°24′30″N, 90°43′05″W. Forty springs and seeps discharge along a band that is nearly 300-m long and 30-m wide at the base of a steep slope formed by the Expedition Diapir (GH) (38). The discharged saline waters (7.5 to 7.9% salt) have temperatures ranging from −1.3°C to 6.9°C and a pH of 7.4. Oxidoreduction potential values from −283 to −375 mV indicate that the spring outlets are highly reducing environments, although not anaerobic, as low dissolved-oxygen concentrations (0.05 to 0.2 ppm) were detected in the water layer just overlying the sediments. The waters were shown to contain 25 to 50 ppm of sulfide and 3,724 mg/liter of sulfate (34). The four main springs (GH-1, GH-2, GH-3, and GH-4) (see Fig. S1 in the supplemental material), examined in our initial investigation, were also chosen for this study. As physicochemical parameters are similar for all the springs and bacterial and archaeal community profiles were shown to be similar by denaturing gradient gel electrophoresis, one spring (GH-4) was selected for activity analyses (leucine and bicarbonate uptakes, sulfur oxidation, and sulfate reduction in microcosms) and for most probable number (MPN) determination. Microbial counts by epifluorescence microscopy and enumeration of culturable bacteria by the spread plate method were performed for all four springs' waters and sediments. In July 2005, 50-ml composite sediment samples (top 10 cm) were collected; they were composed of 5 to 10 subsamples, depending on the size of the springs, to ensure that the samples were representative of the composition of the springs. One liter of water was also collected from each of the same four GH springs. These samples were brought back to the McGill High Arctic Research Station (MARS) where they were processed for microbial enumeration (epifluorescence microscopy and culturing) as described below.

Total microbial counts by epifluorescence microscopy. Total microbial numbers were determined by epifluorescence microscopy for the four springs' waters and sediments, as described by Bloem (9). In brief, 10-ml volumes of the water samples were fixed with filtered formalin (2% final concentration), stained with acridine orange (100 μg/ml final concentration) for 2 h in the dark, and filtered onto 25-mm 0.22-μm-pore-size black polycarbonate filters (Osmonics, Inc., Minnetonka, MN). For the sediments, 5 g were mixed with 45 ml filtered deionized water and vortexed for 1 min at maximum speed. Nine milliliters of the sediment suspension was fixed with filtered formalin (3.7% final concentration). Ten microliters of the suspension was evenly distributed in a specific area of 113 mm^2 defined by a hole with a diameter of exactly 12 mm on an adhesive plastic tape attached to the surface of the slide. The slide was covered with 5-(4,6-dichlorotriazinyl)aminofluorescein, and the evenness of the suspension was verified by examining cell distribution by microscopy. For each sample, at least 10 fields were counted at random with a Nikon Eclipse E600 microscope at 1,000× magnification on triplicate slides. Counts are reported as the mean of the results for triplicate assays with the standard deviation for each sediment sample.

Culture media and enumeration of culturable bacteria by the spread plate and MPN methods. Based on our initial culture-independent study, culture media for microbial enumeration and isolation were selected to target heterotrophic, sulfur-oxidizing, and sulfate-reducing bacteria (SRB), as well as haloarchaea. Heterotrophic bacteria were cultured on Difco marine medium 2216 (0.6% carbon) and an oligotrophic medium (designated AH-H; 0.03% carbon) specifically designed for the GH springs. AH-H medium was supplemented with 2.5% NaCl (AH-H2) or 8% NaCl (AH-H8) and contained the following components (per liter): 0.05 g each of yeast, peptone, casein, glucose, Na-acetate, Na-formate; 4.0 g Na_2SO_4; 0.72 g KCl; 0.27 g NH_4Cl; 0.09 g $Na_2HPO_4 \cdot 7H_2O$; 5.0 g $MgCl_2 \cdot 6H_2O$; 1.0 g $CaCl_2 \cdot 2H_2O$; and 1.12 g tampon Tris-EDTA-saccharose. A thiosulfate medium (AH-S2; 0.4 g NH_4Cl, 4.0 g KH_2PO_4, 4.0 g K_2HPO_4, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.03 g $CaCl_2$, 0.02 g $FeCl_3 \cdot 6H_2O$, 0.02 g $MnSO_4 \cdot H_2O$, 5.0 g $Na_2S_2O_3 \cdot 5H_2O$, and 25.0 g NaCl per liter) was used for cultivating chemolithoautotrophic sulfur oxidizers. Postgate's medium B (39), supplemented with 2.5% NaCl, was used for SRB. DSMZ medium 372 (http://www.dsmz.de/microorganisms/html/media/medium000372.html) supplemented with two antibiotics (ampicillin and kanamycin; 75 μg/ml final concentration) was used for haloarchaea. All media were supplemented with 1 ml trace element solution SL-10 (5) and 10 ml Balch's vitamin solution (5) and adjusted to pH 7.0 to 7.5; solid media were prepared by adding 15 g/liter of agar. Liquid media used for anaerobic studies (all except AH-S2) were made anaerobic by degassing, followed by charging with N_2 three times. Controls with resazurin were prepared in parallel to ensure that anaerobic conditions were obtained and maintained throughout the experiment.

Culturable aerobic bacteria were enumerated by the spread plate method (GH-1, GH-2, GH-3, and GH-4 waters and sediments). The number of CFU was determined by spreading 100 μl of sediment dilutions or 500 μl of spring water onto the surface of the solid media. Aerobic and anaerobic culturable bacteria (GH-4 sediment) were enumerated by the MPN method using triplicate serial dilutions to 10^{-7}, as described previously (49). Plates and tubes were incubated at 5°C for 2 months and were checked again periodically for up to 6 months. MPN tubes were marked as positive by visual observation, i.e., cloudiness and color change after the addition of 1 drop of bromothymol blue 1% as a pH indicator. Ambiguous tubes and the first negative tubes of each set of dilutions were checked by microscopy after DAPI (4′,6-diamidino-2-phenylindole; Sigma) staining of a 1-ml subsample as described by Junge et al. (27). Plate count results are presented as the mean of the results for triplicate assays. MPNs were calculated using the MPN calculator VB6 (http://www.i2workout.com/mcuriale/mpn/index.html) (25).

Isolation and growth characterization of the bacterial isolates. More than 200 colonies were selected on the agar media, based on morphology, pigmentation, and size (more than one identical colony when possible) and obtained as pure isolates. Aliquots (100 μl) of the most dilute MPN tubes of each medium were also plated onto agar media, and the corresponding isolates were identified as the most abundant culturable bacteria. The ability of each heterotrophic isolate to grow at different temperatures and salt concentrations was determined on R2A agar (43) supplemented with 0%, 2.5%, 5%, 7.5%, and 10% NaCl and incubated at 5°C, room temperature (RT) of 23 ± 2°C, and 37°C for up to 1 month. Anaerobic growth was assessed at 5°C on R2A agar 2.5% NaCl in an anaerobic jar flushed with N_2 and containing a dry anaerobic indicator strip (BBL, MD).

Genomic DNA extraction, PCR amplification, and analyses of the 16S rRNA, *pufM*, and *soxB* genes. The genomic DNA of the bacterial isolates was extracted from cells grown in 2 ml broth cultures by the phenol-chloroform-isoamyl alcohol method (4). PCR amplifications were performed on ~20 ng of DNA. All isolates were identified by sequencing of ~600 bp of the 16S rRNA gene with primers 341F and 926R. Strains with identical sequences were assigned to the same group (phylotype), and one representative of each phylotype (49 strains) was chosen for near-full-length (~1,500 bp) 16S rRNA gene amplification using the primers 8F and 1492R (44). A 229-bp portion of the *pufM* gene was amplified using the primers pufM.557F and pufM.750R as described previously (1). The partial *soxB* gene was amplified using the primers soxB432F and soxB1446B (37). The amplicons were sequenced on both strands at the McGill University Genome Quebec Innovation Centre. The 16S rRNA, *pufM*, and *soxB* DNA sequences were submitted for comparison to the GenBank databases using the BlastN algorithm (2). The DNA sequences were aligned with their closest relatives by using ClustalW, and phylogenetic trees (neighbor-joining algorithm with Jukes-Cantor corrections) were constructed using the MacVector 7.2 software package (Accelrys). The robustness of inferred topologies was tested by 1,000 bootstrap resamplings of the neighbor-joining data.

Radiolabeled leucine and bicarbonate uptake. The uptake of radiolabeled substrates was performed in the field on freshly collected GH-4 spring water samples. Leucine uptake was performed in July 2005 and bicarbonate uptake in July 2006 as indicators of heterotrophic and autotrophic activity, respectively. The reaction bottles (sterile acid-washed serum bottles sealed with butyl rubber stoppers and aluminum crimps) were incubated at the MARS at a temperature close to the spring water in situ temperature (~6.9°C). Leucine uptake was determined in triplicate by the addition of L-[4,5-^{3}H]leucine (10 nM final concentration) (specific activity, 168 Ci/mmol; Amersham Biosciences) to 10 ml of spring water. Formaldehyde-killed controls were treated in parallel. The reaction was terminated after 3 h by the addition of 10% trichloroacetic acid, and the samples were heated at 80°C for 15 min before filtration on 25-mm 0.2-μm-pore-size mixed cellulose ester filters (Millipore). Bicarbonate uptake was performed based on the method described by Joint et al. (26). ^{14}C-labeled sodium bicarbonate (10 μCi) (specific activity, 53.5 mCi/mmol; MP Biochemicals, Inc.) was added to 60 ml of spring water. Three bottles were used as experimental replicates for light and three as experimental replicates for dark. Three controls were prepared: a killed control (with 1% formaldehyde final concentration), a no-^{14}C control (no radiolabel added), and a no-cell control (prepared with deionized water). After 30 h, 30 ml was removed from each bottle by using a sterile needle, and the volume removed was replaced by air filtered through a 0.2-μm-pore-size syringe filter. The remaining 30 ml was incubated for a total of 54 h, and particulate material was collected on mixed cellulose ester filters. The filters were acidified with concentrated (fuming) HCl to eliminate adsorbed and abiotically precipitated inorganic ^{14}C.

Filters from both experiments were air dried, placed in a scintillation vial, and frozen for later processing. The filters were then dissolved with 0.5 ml of ethyl acetate, 10 ml of scintillation cocktail was added to each vial, and the radio-

activity of the particulate material was determined in a Beckman LS-6500 scintillation counter (Beckman-Coulter Inc., Fullerton, CA). No uptake was observed in the experimental controls.

Sulfur oxidation and sulfate reduction in microcosms. S-metabolizing activities were determined in laboratory microcosms by monitoring the change in the sulfate concentration of GH-4 sediment slurries. A first set of experiments performed in 2006 consisted of a 15-ml sample of sediment slurries prepared with filtered GH-4 spring water (1 part sediment to 1 part filtered spring water) in 60-ml glass serum bottles. The microcosms were amended with selected substrates (20 mM thiosulfate, 20 mM molybdate, 20 mM lactate), and sterilized controls (autoclaved) were used to determine abiotic S transformations. Sulfate reduction was assessed in anaerobic microcosms as described in Purdy et al. (40) examining Antarctic sediments. Lactate was used as the substrate as Knoblauch et al. (30) demonstrated that, unlike acetate, lactate was used by their five psychrophilic SRB strains from Arctic sediments. The microcosms for S reduction were prepared anaerobically under sterile N_2 in a glove bag (Cole-Parmer Canada Inc., Montreal, Canada). The bottles were sealed with butyl rubber stoppers and aluminum crimps, and the microcosm bottle headspaces were further flushed with N_2 three times for 5 min each, as described above. The 2006 microcosms were incubated at 5 to 8°C in the dark. A second set of experiments (2007) consisted of a 15-ml sample of sediment slurries prepared with the same thiosulfate medium (20 mM thiosulfate) as for cultivation (1 part sediment to 2 parts thiosulfate medium). The 2007 microcosms were incubated at 5 to 8°C (in the dark) or at RT (23 ± 2°C) (in the dark and light).

The microcosms were mixed on a rotary shaker (150 rpm) for 1 h every day. Liquid aliquots (200 μl) were withdrawn at time zero and at different time intervals for 30 days and centrifuged at 10,000 × *g* for 5 min. The aliquots were diluted with distillated water, and their sulfate concentrations (ppm) were determined by ion exchange high-pressure liquid chromatography (model SP8800; Spectra-Physics). The sulfate concentrations measured during the 30-day incubation were used to calculate the sulfur oxidation rates (SORs), as nanomoles of sulfate produced per cm^3 of sediment per day. Sterile controls were performed in parallel to determine the abiotic SORs, which were subtracted from the overall SORs. The sediments were equally distributed in each bottle of the same assay (7.5 g of sediment for 7.5 ml of spring water in 2006; 5 g of sediment for 10 ml liquid medium in 2007), and the rates were normalized between samples by taking into account the different dilution factors (i.e., by multiplying by 2 for the 2006 rates and by 3 for the 2007 rates). The results are presented as the mean of the results for triplicate assays. Standard errors (SE) were calculated with the equation $SE = SD/\sqrt{n}$, where SD is the standard deviation and n is the number of experimental replicates.

Gas compositional and isotopic analysis. Dissolved gas samples discharging from the springs were collected in July 2005 and 2006 and in May 2007, as in the work of Ward et al. (51). Compositional analyses of gas-phase samples arising from the springs were performed on a Varian 3400 gas chromatograph equipped with a flame ionization detector to determine the concentrations of CH_4, C_2H_6, C_3H_8, and C_4H_{10}. A thermal conductivity detector and molecular sieve 5A PLOT column (25 m by 0.53-mm internal diameter) were used to determine concentrations of the inorganic gas components (H_2, He, O_2, CO_2, and N_2). All analyses were run in duplicate, and the mean values are reported. Reproducibility for duplicate analyses was ±5%. Analyses for $\delta^{13}C_{CH4}$ values were performed by continuous-flow-compound-specific carbon isotope ratio mass spectrometry with a Finnigan MAT 252 mass spectrometer interfaced with a Varian 3400 capillary gas chromatograph and a Poraplot Q column (25 m by 0.32-mm internal diameter). Total error incorporating both accuracy and reproducibility was ±0.5‰ with respect to the Vienna-Pee Dee Belemnite international isotopic standard. Detailed analytical methods can be found in the work by Ward et al. (51).

Samples for quantitative and isotopic analysis of DIC were analyzed on an OI analytical TIC-TOC analyzer model 1010 according to the method of St-Jean (50). The TIC-TOC analyzer was connected via an interface to a Finnigan Mat DeltaPlus isotope ratio mass spectrometer. Data were normalized by an internal standard. The total analytical uncertainty for each sample is 5% for the quantitative analysis and ±0.5‰ for the isotopes, including both accuracy and reproducibility (50).

Nucleotide sequence accession numbers. The DNA sequences obtained in this study have been deposited in the GenBank database under accession numbers EU196298 to EU196353.

RESULTS

Microbial abundance. The total microbial numbers (cells ml^{-1}) in the GH spring waters were as follows: GH-1, 8.4 ± 0.3 × 10^3; GH-2, 3.4 ± 0.4 × 10^4; GH-3, 7.5 ± 0.8 × 10^3; and GH-4, 2.6 ± 0.2 × 10^4. Total microbial numbers (cells g^{-1}) in the GH spring sediments were as follows (average based on triplicate assays on a single composite sediment sample): GH-1, 8.3 ± 1.1 × 10^7; GH-2, 4.0 ± 1.0 × 10^7; GH-3, 8.2 ± 0.4 × 10^7; and GH-4, 3.5 ± 1.5 × 10^7. Plate count numbers were too low in the waters to be significant, except with the thiosulfate medium where the numbers reached 3.0 ± 0.5 × 10^3 CFU/ml for GH-3, which represented 40% of the total microbial count. The highest plate counts (1.9 × 10^5 to 2.5 × 10^5 CFU/g) in sediments were obtained with the thiosulfate medium (AH-S2) (Fig. 1). Since some heterotrophic bacteria can use agar as the sole carbon source, 16S rRNA gene sequencing (~600 bp) of 10 random colonies growing on the thiosulfate medium (all the colonies had the same morphology [shape, size, and color]) was performed, and the obtained sequences were all 99% related to the chemolithoautotrophic sulfur oxidizer *Halothiobacillus*. SRB were not detected in Postgate's medium B with anaerobic cultivation even though SRB phylotypes of *Desulfobacteriaceae* were retrieved in our initial culture-independent study (36). SRB were detected in the same batch of MPN tubes inoculated with sediment from a similar cold saline spring at Colour Peak, located 11 km away from the GH site, indicating that our conditions were adequate for the culture of at least some SRB species. Haloarchaea were not cultured either; microorganisms that grew in the DSMZ medium 372 were related to *Marinobacter*, despite the high salt concentration (20%) and the presence of two antibiotics. The MPN results for GH-4 were similar to the GH-4 spread plate counts; i.e., the highest MPN count (3.7 × 10^5 cells/g) was obtained with the aerobic thiosulfate medium (AH-S2), while the highest heterotrophic number (1.7 × 10^5 cells/g) was obtained with the M2216 medium (aerobic and anaerobic). The AH-H2 medium enumerated 3.7 × 10^4 cells/g (aerobic and anaerobic). The 8% NaCl oligotrophic medium AH-H8 gave the lowest MPN counts with 1.7 × 10^4 cells/g (aerobic) and 1.4 × 10^3 cells/g (anaerobic). The similar MPN counts in aerobic and anaerobic tubes suggested that the most abundant culturable bacterial populations were facultative anaerobes, which was subsequently confirmed by strain characterization. The resazurin controls showed that anaer-

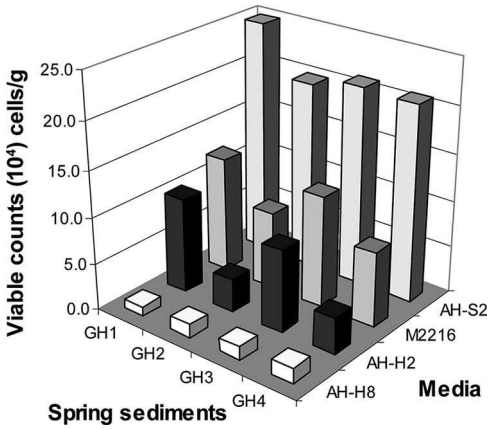

FIG. 1. Culturable microbial counts (CFU/g) obtained by the spread plate method on heterotrophic (M2216, AH-H2, and AH-H8) and thiosulfate (AH-S2) media for the GH spring sediments.

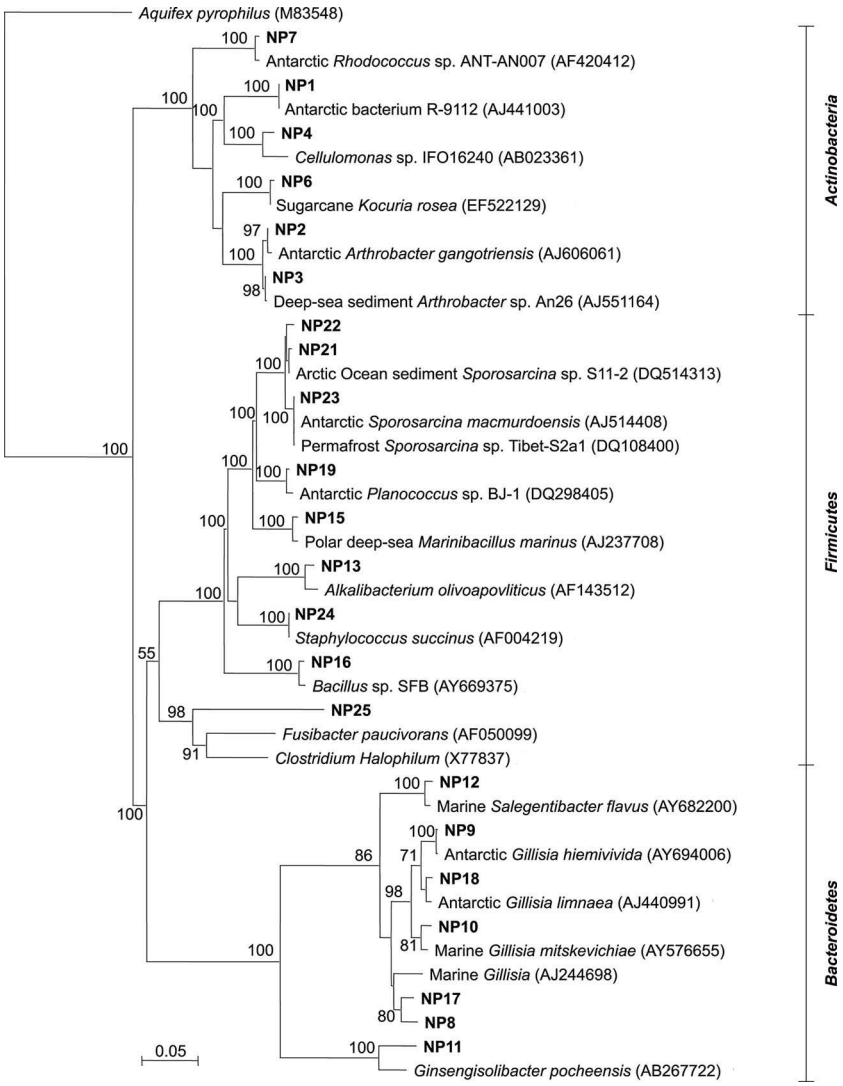

FIG. 2. Phylogenetic relationships of 16S rRNA gene sequences from the GH springs' bacterial isolates (in boldface type) related to the *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*. The tree was inferred by neighbor-joining analysis of 1,018 homologous positions of sequence from each isolate. Numbers on the nodes are the bootstrap values (percentages) based on 1,000 replicates. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

obic conditions were maintained in the MPN tubes throughout the incubation period.

Characterization of the bacterial isolates. The GH isolates divided into 49 different phylotypes that grouped into four phyla: *Actinobacteria* (12% of the phylotypes), *Bacteroidetes* (12%), *Firmicutes* (18%), and *Proteobacteria* (58%) with representatives of the *Alpha-* (29%) and *Gammaproteobacteria* subclasses (71%) (Fig. 2 and 3). The five phylotypes isolated on the thiosulfate medium were highly related (96 to 99% DNA sequence identity) to chemolithoautotrophic sulfur oxidizers of the gammaproteobacterial genera *Halothiobacillus* and *Thiomicrospira*. The most common phylotypes classified as *Gillisia*, *Psychrobacter*, *Marinobacter*, *Sporosarcina*, and *Halothiobacillus*. Most of the isolates were highly related (≥98% identity) to bacteria previously isolated from marine and permanently cold environments, including perfect matches with bacteria from Antarctica. Four isolates had ≤95% sequence identity to their closest NCBI match and likely represent novel bacterial genera. NP25 was related only distantly to any cultured bacteria, the closest bacterium being *Fusibacter paucivorans* (86% identity), an anaerobic thiosulfate reducer isolated from an oil-producing well (42).

The most-abundant culturable bacteria were related to *Marinobacter* spp. (strains SYO J55 and BSi20041 and *Marinobacter salsuginis*), *Alkalibacterium olivoapovliticus*, and *Halothiobacillus* sp. strain RA13. All the isolates tested were psychrotolerant as they grew at 5°C and at RT of 23 ± 2°C, and six isolates were also able to grow at 37°C (Table 1). All isolates grew with 2.5%, 5%, and 7.5% NaCl, with 7.5% NaCl being the salinity of the natural environment. Isolates NP15, NP21, and NP35 were able to grow without NaCl at 5°C but not at RT. These strains needed NaCl in the medium to grow at RT, which may be an example of osmotic reversal of temperature sensitivity, in which bacteria are able to grow at nonpermissive higher tem-

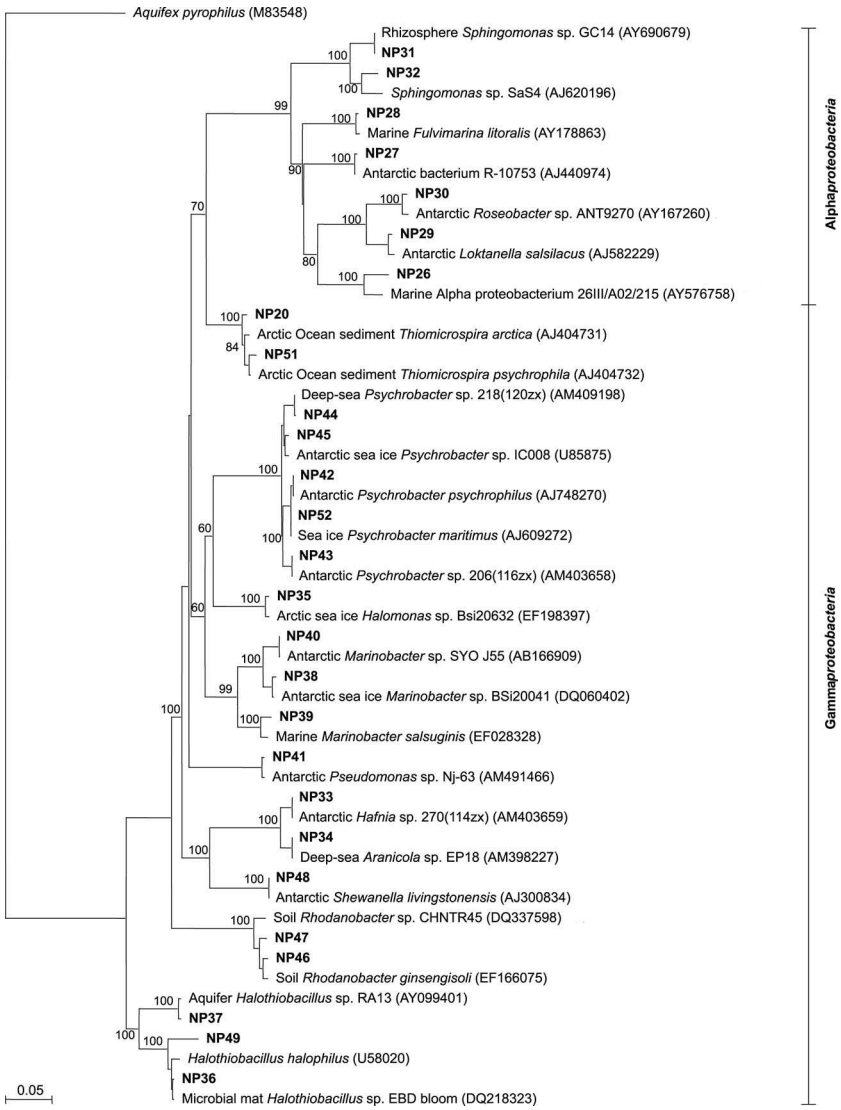

FIG. 3. Phylogenetic relationships of 16S rRNA gene sequences from the GH springs' bacterial isolates (in boldface type) related to the *Proteobacteria*. The tree was inferred by neighbor-joining analysis of 827 homologous positions of sequence from each isolate. Numbers on the nodes are the bootstrap values (percentages) based on 1,000 replicates. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

peratures on media of sufficiently high osmolality (8). Four isolates related to *Gillisia* (NP9, NP10, NP17, and NP18), NP13, and NP16 were true halophiles, requiring salt in the medium for growth. With the exception of NP1, NP16, and the five isolates classified as *Gillisia*, all isolates grew anaerobically.

All the isolates were screened by PCR for the presence of *soxB* and *pufM*, which are involved in thiosulfate oxidation and anoxygenic phototrophy, respectively. A *soxB* sequence (Fig. 4) was found in NP40 (related to gammaproteobacterium *Marinobacter* sp. strain SYO J55 [Fig. 3]) and in NP29 and NP30 (related to the alphaproteobacteria *Loktanella* and *Roseobacter*, respectively [Fig. 3]). A *soxB* gene has previously been found in *Marinobacter* sp. HY-106 and *Roseobacter denitrificans*, but this is the first report of a putative *soxB* gene in a species of *Loktanella*. The *Loktanella* sequence branched with other *soxB* sequences from *Alphaproteobacteria*. Sequences of *pufM* (Fig. 5) were amplified from isolates NP13, NP29, and NP30, related to *Alkalibacterium* (Fig. 2), *Loktanella*, and *Roseobacter* (Fig. 3), respectively. Interestingly, the 229-bp partial *pufM* DNA sequences from NP13 and NP29 were 100% identical, suggesting horizontal gene transfer. Hu et al. (24) reported sequences of *pufM* from *Gammaproteobacteria* clustering with sequences from *Alphaproteobacteria*, but here we report possible horizontal gene transfer of *pufM* between bacteria of two different phyla, *Firmicutes* and *Proteobacteria*. In agreement with the 16S rRNA gene groupings, all the isolates assigned to the same phylotype showed the same growth patterns and the same *pufM* and *soxB* genotypes.

Potential activities of the GH springs' microbial community. The rate of leucine uptake by the spring heterotrophic microorganisms was 0.30 ± 0.03 pmol liter^{-1} h^{-1}. The dark CO_2 uptake by autotrophic microorganisms was 2.78 ± 0.48 nmol C

TABLE 1. Growth characteristics of the GH spring heterotrophic isolates on R2A agar[a]

Isolate	Growth under indicated incubation condition							
	5°C/RT[b] at NaCl concn (%) of:						37°C at 2.5% NaCl concn	AN at 2.5% NaCl concn
	0[c]	0[d]	2.5	5	7.5	10		
NP1	+	+	+	+	+	+	−	−
NP2	+	+	+	+	+	−	−	+
NP3	+	+	+	+	+	−	−	+
NP4	+	+	+	+	+	+	−	+
NP6	+	+	+	+	+	+	+	+
NP7	+	+	+	+	+	−	−	+
NP8	+	+	+	+	+	−	−	−
NP9	−	−	+	+	+	−	−	−
NP10	−	−	+	+	+	−	−	−
NP11	+	+	+	+	+	+	−	+
NP12	+	+	+	+	+	+	−	+
NP13	−	−	+	+	+	+	−	+
NP15	+	−	+	+	+	+	−	+
NP16	−	−	+	+	+	+	−	−
NP17	−	−	+	+	+	−	−	−
NP18	−	−	+	+	+	−	−	−
NP19	+	+	+	+	+	+	+	+
NP21	+	−	+	+	+	−	−	+
NP22	+	+	+	+	+	+	−	+
NP23	+	+	+	+	+	+	−	+
NP24	+	+	+	+	+	+	+	+
NP25	+	+	+	+	+	+	−	+
NP26	+	+	+	+	+	−	−	+
NP27	+	+	+	+	+	−	−	+
NP28	+	+	+	+	+	+	−	+
NP29	+	+	+	+	+	−	−	+
NP30	ND	ND	ND	ND	ND	ND	ND	ND
NP31	+	+	+	+	+	−	−	+
NP32	+	+	+	+	+	−	−	+
NP33	+	+	+	+	+	+	−	+
NP34	+	+	+	+	+	−	−	+
NP35	+	−	+	+	+	+	−	+
NP39	+	+	+	+	+	+	+	+
NP38	+	+	+	+	+	+	+	+
NP40	+	+	+	+	+	+	−	+
NP41	+	+	+	+	+	−	−	+
NP42	+	+	+	+	+	+	+	+
NP43	+	+	+	+	+	+	−	+
NP44	+	+	+	+	+	+	−	+
NP45	+	+	+	+	+	−	−	+
NP46	ND	ND	ND	ND	ND	ND	ND	ND
NP47	ND	ND	ND	ND	ND	ND	ND	ND
NP48	+	+	+	+	+	−	−	+
NP52	+	+	+	+	+	+	−	+

[a] +, growth; −, no growth; AN, anaerobic incubation; ND, not determined.
[b] When not indicated otherwise, the results are for incubations at both 5°C and RT.
[c] Results are for incubations at 5°C.
[d] Results are for incubations at RT.

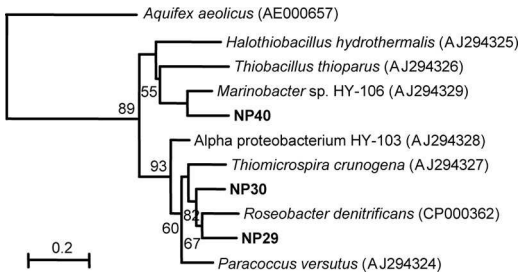

FIG. 4. Neighbor-joining tree based on 465 nucleotide positions of the *soxB* gene from the GH springs' bacterial isolates (in boldface type). Numbers on the nodes are the bootstrap values (percentages) based on 1,000 replicates. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

liter^{-1} day^{-1}, which was 22% higher than under natural light incubation. The uptake rates of the radiolabeled compounds were linear at the selected incubation times.

SORs were indirectly estimated by sulfate production in aerobic microcosms. The SOR determined in sediment slurries prepared with filtered GH spring water (Fig. 6a) were very similar in unamended microcosms and in microcosms with thiosulfate added, indicating that reduced sulfur compounds were already present in nonlimiting concentrations in the original sediment/water slurry. Unexpectedly, SOR in aerobic microcosms supplemented with thiosulfate and molybdate was 2.3 times higher (917 nmol/cm^3/day compared to 394 nmol/cm^3/day) than with thiosulfate alone. Molybdate

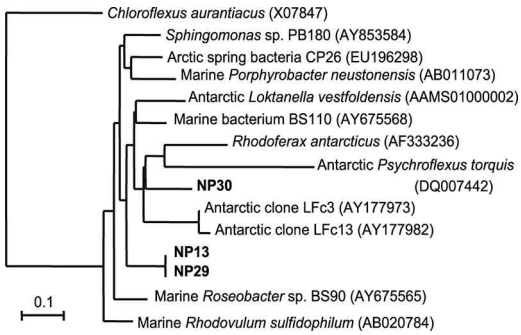

FIG. 5. Neighbor-joining tree based on 184 nucleotide positions of the *pufM* gene from the GH springs' bacterial isolates (in boldface type). The scale bar indicates the estimated number of base changes per nucleotide sequence position. The bootstrap values were lower than 50 and are not indicated on the nodes.

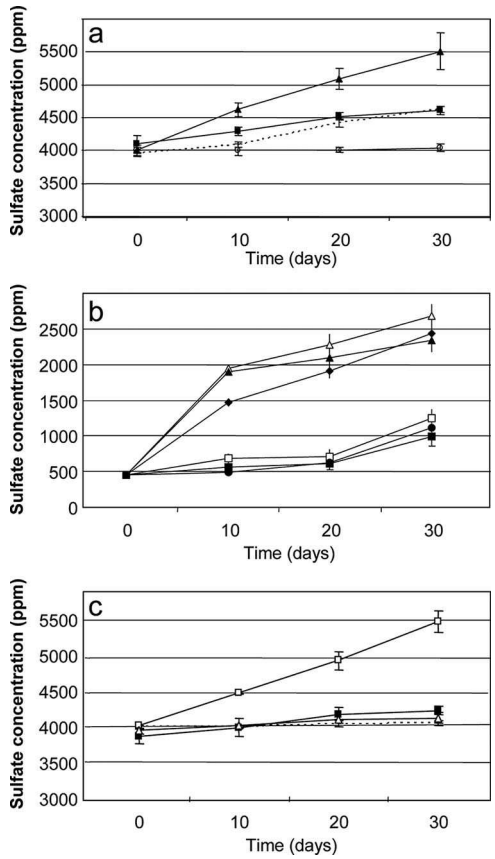

FIG. 6. Evolution of the sulfate concentration (ppm) in microcosms for the GH-4 spring sediment. (a) 2006 aerobic microcosms. ▲, S_2O_3/molybdate (Mo); ■, S_2O_3; dashed line, unamended; ○, S_2O_3, autoclaved. (b) 2007 aerobic microcosms. △, 2.5% NaCl at RT under light incubation; ▲, 2.5% NaCl at RT under dark incubation; ◆, 2.5% NaCl at 5°C under dark incubation; □, 7.5% NaCl at RT under light incubation; ●, 7.5% NaCl at 5°C under dark incubation; ■, 7.5% NaCl at RT under dark incubation. (c) 2006 anaerobic microcosms. □, lactate/Mo; ■, lactate; △, Mo; dashed line, unamended. The error bars represent the standard errors.

was used as an inhibitor of sulfate reduction and was added even though the microcosms were incubated aerobically. It is possible that sulfate reduction occurred in these aerobic microcosms in anaerobic pockets formed by insufficient aeration and that the inhibition of SRB activity with molybdate provoked a net sulfate increase due to the activity of sulfur oxidizers. The 2007 microcosms (synthetic thiosulfate medium) resulted in an SOR similar to that of the 2006 microcosms (natural spring water) when incubated under similar conditions, i.e., 5°C and 7.5% NaCl (Fig. 6b). The SOR was approximately three times higher at lower salt concentrations (2.5%) than it was at 7.5% NaCl, the salinity of the natural environment. The SORs were similar at 5°C and RT, showing that the enzymatic machinery of the sulfur-oxidizing population was well adapted to life at cold temperatures. After 10 days, the SOR was slightly higher under light incubation than it was under dark incubation, although the difference is not statistically significant since the standard error bars overlap (Fig. 6b). Sulfate concentrations remained unchanged over time in the anaerobic unamended microcosms and the microcosms amended with molybdate only (Fig. 6c). The anaerobic microcosms amended with lactate unexpectedly showed an increase in sulfate over time (a decrease would be expected for sulfate reduction), particularly in those that also contained molybdate, suggesting that heterotrophic anaerobic sulfur oxidation was occurring using the reduced sulfur compounds (such as sulfide) (36) already present in the slurry, to produce sulfate. The smaller increase in sulfate in anaerobic microcosms amended with lactate and no molybdate suggested that sulfate reduction was indeed occurring with lactate as the energy source, counterbalancing the heterotrophic anaerobic sulfur oxidation.

Carbon isotope signatures of CH_4 gas from the GH springs. Gas emissions from the springs consisted predominantly of N_2 (87 to 99%), although they also contained from 0.38 to 0.51% CH_4, ~0.3% He, and ~1% Ar (data not shown). CO_2, H_2, and higher hydrocarbon gases (ethane, propane, butane) were all below the detection limit. The lack of any significant concentrations of higher hydrocarbons associated with the CH_4 suggests that the gas originates from microbial methanogenesis (52). Values of $\delta^{13}C_{CH4}$ for the GH springs showed significant variation both from spring to spring and over time. In 2006, GH-2 had a $\delta^{13}C$ value of −71.2‰, consistent with the ^{13}C-depleted isotopic signature expected for microbial methanogenesis. Samples taken in 2005 and in 2007 for this spring, however, had $\delta^{13}C$ values of −6.1‰ and −12.4‰, respectively. Such pronounced isotopic enrichments are typical of methanotrophy, which enriches the residual methane pool in ^{13}C (resulting in a less negative $\delta^{13}C$ value) due to preferential enzymatic breakage of ^{12}C-H bonds versus that of ^{13}C-H bonds (52). Similarly, GH-4 showed very enriched $\delta^{13}C$ values in 2005 and 2007 (−10.3‰ and −6.5‰, respectively) compared to a value of −32.0‰ in 2006. The isotopic composition of methane for GH-1 and GH-3 were obtained in only 1 year, but once again, their very ^{13}C-enriched values of −12.7‰ and −12.1‰ suggest methanotrophic activity in the springs. In 2007, concentrations and carbon isotope compositions ($\delta^{13}C$) of the DIC from the GH springs were first analyzed. DIC concentrations range between 7.0 and 7.6 ppm while

DIC $\delta^{13}C$ values were −5.6‰ and −6.5‰ for GH-2 and GH-4, respectively.

DISCUSSION

Total microbial numbers in the GH springs were lower than those in the nearby Arctic Ocean (10^7 cells/ml of water and 10^9 cells/g of sediments) (41) or other cold saline aquatic environments, such as supercooled brines in permafrost (10^7 cells/ml) (20) and the hypersaline deep-sea basin Urania (10^5 cells/ml) (46), but are similar to the counts for supraglacial sulfur springs on Ellesmere Island in the Canadian high Arctic (22). The total microbial numbers in the sediments of these permafrost springs were comparable to those determined in a sedimentary permafrost sample (3.56×10^7 cells/g) from the same region of the Canadian high Arctic (49). The highest numbers of culturable bacteria were obtained with thiosulfate as the sole energy source and CO_2 as the sole carbon source. This abundance of chemolithoautotrophic sulfur-oxidizing bacteria is in agreement with the results of our initial culture-independent study (36), which found that 30% of the bacterial clones from a GH-4 clone library were related to chemolithoautotrophic sulfur oxidizers.

The bacteria isolated from the GH springs were predominantly psychrotolerant, facultative anaerobes and grew at salt concentrations at least as high as the in situ salinity. *Gillisia*, *Psychrobacter*, *Marinobacter*, and *Sporosarcina* had the highest diversity of phylotypes. However, members of these four heterotrophic genera were not detected in the previously published clone library from GH (library coverage of 84% as defined by Good's calculation) (21). While the abundance of DNA from sulfur oxidizers could have masked their presence, heterotrophic bacteria may have been successfully cultured on heterotrophic media owing to the absence of concurrent growth from sulfur-oxidizers. Only one identical phylotype related to a heterotrophic species, *Loktanella salsilacus*, was found in both studies, compared to three sulfur-oxidizing species (*Thiomicrospira psychrophila*, *Halothiobacillus* sp. strain RA13, and *Halothiobacillus* sp. strain EBD bloom). Overall, the isolates grouped into four phyla that were also identified in the clone library. Three additional divisions were found in the culture-independent study (*Verrucomicrobia*, *Gemmatimonadetes*, and *Spirochaetes*), but these phyla have few cultured members and comprised only one phylotype each in our clone library. Overall, both methods showed the prevalence of *Proteobacteria*. Three of the *Sporosarcina* spp. isolated from the GH springs were also isolated in a 9-m-deep permafrost sample from Eureka (Ellesmere Island) (49), indicating that some of the *Sporosarcina* spring strains may be permafrost inhabitants that are being picked up as the spring water rises through the 600 m of permafrost. On the other hand, the GH springs' bacterial composition was clearly different than the one found in the Eureka permafrost, which was dominated by *Firmicutes* spore formers (49). Many of the bacteria inhabiting these remote Arctic springs were phylogenetically highly related to bacteria isolated from Antarctic environments. Very similar isolates obtained from different poles have been reported elsewhere (11, 12, 48), and phylogenetic surveys of the world's oceans have strongly suggested the mixing of bacterial populations on a global scale (11). However, 16S rRNA similarity is not sufficient to establish that identical species occur at both poles. Staley and Gosink (48) proposed a number of postulates to determine if a species is cosmopolitan, including assessing DNA-DNA reassociation.

Interestingly, *Marinobacter* species, some of the most abundant culturable heterotrophs in the spring sediments, are abundant in other polar environments, such as a subglacial outflow in Antarctica (32), Arctic pack ice (11), Arctic supraglacial cold sulfidic springs (22), and a nearby methane-rich hypersaline spring on Axel Heiberg Island (L. Whyte and T. Niederberger, personal communication). *Marinobacter* spp. are moderately halophilic marine *Gammaproteobacteria* that use a wide variety of hydrocarbons as sole sources of carbon and energy (19, 47). The presence of *Marinobacter* in the GH springs could be linked to a possible marine origin of the springs (38); hydrocarbons C_{10} to C_{50} were not detected in spring sediment samples collected in 2006 (unpublished data), and shorter-chain hydrocarbons other than CH_4 were not detected in the gas emitting from the springs.

Like autotrophic bacteria, heterotrophic bacteria can also use light (photoheterotrophic) and reduced sulfur compounds, but only as a supplemental, not as the sole, energy source (31). The *pufM* gene, which encodes a pigment-binding protein for the M subunit of the anoxygenic photosynthetic reaction center (7, 24) and the *soxB* gene, encoding an enzyme essential for thiosulfate oxidation by sulfur-oxidizing bacteria of various phylogenetic groups (37), were used to detect strains capable of these metabolic activities. We found that one of the *Marinobacter* isolates and the isolates related to *Loktanella salsilacus* and *Roseobacter*, all facultative anaerobes, possessed a putative *soxB* gene. *Loktanella*-related species and some *Roseobacter* spp. are known to oxidize reduced sulfur compounds under both oxic and anoxic conditions (13); the corresponding GH strains could be involved in heterotrophic anaerobic sulfur oxidation in the spring sediments. Since the GH springs are oligotrophic and sulfidic with very low levels of oxygen, the capacity to derive energy from reduced sulfur compounds, particularly under anoxic conditions, would give a physiological advantage for competing in these ecosystems. Three isolates from the springs are putative photoheterotrophs, since they possess a *pufM* sequence; these bacteria would benefit from the long periods of summer light in this high latitude ecosystem.

While heterotrophic bacterial activity was lower than that of other polar environments (e.g., 2 to 130 pmol of leucine liter^{-1} h^{-1} in Lake Fryxell, Antarctica) (6), CO_2 fixation was relatively high under dark incubation, indicating chemolithoautotrophic activity. Similar dark CO_2 uptakes (1.2 to 3.2 nmol C liter^{-1} day^{-1}) were determined in an Antarctic subglacial outflow (32) and subglacial lake water (18). Similar to aphotic ecosystems such as deep-sea hydrothermal vents and sulfidic groundwater, chemoautotrophs may be the main primary producers in the GH springs' ecosystem. There are several lines of evidence supporting this hypothesis as follows: (i) an abundance of chemolithoautotrophic sulfur oxidizers were found in both this culture-dependent study and the initial culture-independent study (36); (ii) photoautotrophic phylotypes of sulfur oxidizers or cyanobacteria were not detected in the culture-independent study despite the high sulfide concentration and the continuous illumination during the sampling period; (iii) eukaryotic DNA

(so no phototrophic eukaryotes) was not detected in the GH spring sediments by using diverse sets of 18S rRNA gene primers, and phototrophic eukaryotic cells were not seen by microscopic observation (unpublished data); (iv) chlorophyll was not detected over the surface of the carbonates from ~100 spring locations by using a pulse amplitude modulation fluorometer (3); and (v) the only possible photosynthetic organisms detected were the heterotrophic bacterial isolates possessing a *pufM* sequence. However, such photoheterotrophs do not use carbon dioxide as their sole carbon source: they use organic compounds from the environment for their carbon requirements. Thus, unlike photoautotrophs, they do not provide net supplies of carbon to the ecosystem (29).

The predominance of CH_4 in the springs rather than a CH_4–higher hydrocarbon mixture suggests a microbial origin for these gases. The $\delta^{13}C_{CH4}$ value of −71.2‰ in 2006 is also consistent with this hypothesis. The significant variation in $\delta^{13}C_{CH4}$ values observed over time indicates, however, that the system is more complex, with methanotrophic activity as the most likely cause of the enriched $\delta^{13}C$ values (52). While $\delta^{13}C_{CH4}$ values for DIC were determined only for one spring, the very small isotopic difference between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{CH4}$ for this sample is consistent with the effects of methanotrophy (52). The highly reduced environment in the springs (−283 to −324 mV) should allow methanogenesis, and 16S rRNA gene sequences related to *Methanosarcinales* were detected in our culture-independent study (36). Methanotrophy in the GH springs may be performed by aerobic methanotrophs but could also be the result of anaerobic methane oxidation, which is catalyzed by *Archaea* phylogenetically related to *Methanosarcinales* in association with deltaproteobacterial SRB (10, 35). Isolates or 16S rRNA gene sequences directly related to known aerobic methane-oxidizing bacteria or anaerobic methane-oxidizing archaea have not yet been detected in the GH springs; on the other hand, a high proportion of sequences related to *Methanosarcinales* and deltaproteobacterial sulfate reducers were identified.

Conclusions. The springs of GH experience long consecutive periods of illumination or total darkness. These conditions have an unknown effect on the springs' phototrophic microbial populations and, accordingly, on photosynthetic primary production. Our results suggest that the springs' microbial community is primarily sustained by chemolithoautotrophic primary production performed by sulfur-oxidizing bacteria, even in the period of continuous illumination in the Arctic summer. To date, ecosystems of this type have been found only in permanently dark hydrothermal vents and sulfidic groundwater but not in illuminated ecosystems.

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