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ORIGINAL ARTICLE

An oligarchic microbial assemblage in the anoxic bottom waters of a volcanic subglacial lake

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In 2006, we sampled the anoxic bottom waters of a volcanic lake beneath the Vatnajökull ice cap (Iceland). The sample contained 5×10^5 cells per ml, and whole-cell fluorescent *in situ* hybridization (FISH) and PCR with domain-specific probes showed these to be essentially all bacteria, with no detectable archaea. Pyrosequencing of the V6 hypervariable region of the 16S ribosomal RNA gene, Sanger sequencing of a clone library and FISH-based enumeration of four major phylotypes revealed that the assemblage was dominated by a few groups of putative chemotrophic bacteria whose closest cultivated relatives use sulfide, sulfur or hydrogen as electron donors, and oxygen, sulfate or CO_2 as electron acceptors. Hundreds of other phylotypes are present at lower abundance in our V6 tag libraries and a rarefaction analysis indicates that sampling did not reach saturation, but FISH data limit the remaining biome to $<$10–20% of all cells. The composition of this oligarchy can be understood in the context of the chemical disequilibrium created by the mixing of sulfidic lake water and oxygenated glacial meltwater.

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Introduction

Perennial lakes under the ice sheets of Antarctica (Siegert *et al.*, 2005) and Iceland (Björnsson, 2002) are potential habitats for chemotrophic microbial communities tolerant of oligotrophic conditions, low temperatures and high pressures. They are plausible analogs of habitats on Mars and the icy satellites of the outer planets (Gaidos *et al.*, 1999; Kennicut, 2000; Farr, 2004). In Iceland, volcanic heat maintains three lakes beneath the Vatnajökull ice cap (Björnsson, 2002). The largest ($\sim 20\,km^2$) lies within the Grímsvötn caldera and two smaller ($1\,km^2$) lakes (western and eastern Skaftá lakes) occupy the glacial divide separating Grímsvötn and the Báðarbunga volcano (Figure 1). Geothermal heat melts the overlying ice, creating a depression in the glacier surface that drives the restoring inward flow of ice. These lakes drain episodically in jökulhlaups (outburst floods) when rising water levels open a channel under an ice barrier.

Subglacial volcanic lakes differ markedly from their subaerial counterparts; the former are diluted by melting glacial ice while the latter experience evaporation and sunlight (Pasternack and Varekamp, 1997). The Grímsvötn lake is a cold ($-0.2\,^{\circ}C$), oligotrophic ($\sim 0.3\,mg\,l^{-1}$ particulate organic carbon), acidic (pH 5.7–7.0) and fresh (total dissolved solids $= 200\,mg\,l^{-1}$) body of water with little apparent geothermal influence (Ágústsdóttir and Brantley, 1994; Gaidos *et al.*, 2004). An analysis of samples from the water column and volcanic

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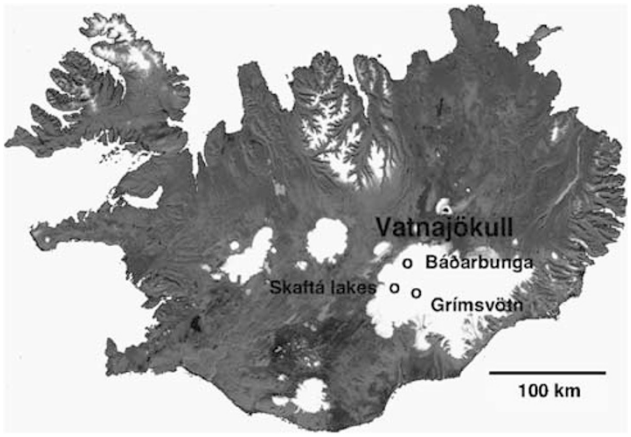

Figure 1 Location of the subglacial Grímsvötn and Skaftá lakes and the Báðarbunga volcano under the Vatnajökull ice cap, Iceland. Map based on a NASA image.

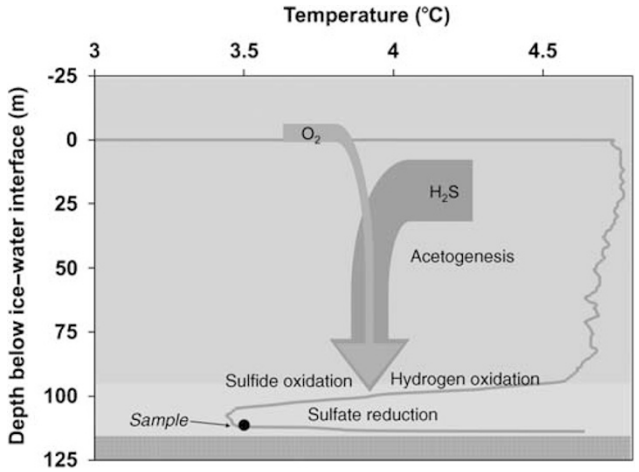

Figure 2 Diagram of the western Skaftá lake, showing the temperature profile on 19 June 2006 (Jóhannesson *et al.*, 2007), and the overlying ice (light blue), anoxic water column (red) and the bottom water (magenta). The proposed mechanism of bottom water formation and important microbial metabolism are illustrated (see text for details). The location of the water sample is marked. The color reproduction of this figure is available on the html full text version of the manuscript.

tephra sediment found a diverse assemblage of microorganisms (that is, bacteria), some of which were cold tolerant or autotrophic, but with no evidence for adaptation to geothermal habitats (Gaidos *et al.*, 2004). However, the chemistry and biology of subglacial volcanic lakes should reflect the relative input of oxygenated glacial melt and reducing geothermal fluid, and other systems may differ from Grímsvötn.

In June 2006, we obtained temperature profiles and a single water sample from the western Skaftá lake. At the location of our borehole (66.4963°N, 17.6174°W), the glacier was 300 m thick and the lake was 115 m deep. Temperatures were close to 4.7 °C throughout the water column except for a 15-m thick, colder (3.5 °C) bottom water mass overlying a warmer (6 °C) 1- to 2-m thick layer (Figure 2) (Jóhannesson *et al.*, 2007). The sample, collected from 3 m above the lake bottom, was anoxic (<2 μM O_2) and contained 1.08 mM total sulfide ($H_2S + HS^-$), 0.16 mM sulfate (SO_4^{2-}), 30 mM dissolved CO_2 and unquantified micromolar H_2 (Jóhannesson *et al.*, 2007). Using the temperature profile and thermodynamic constraints, Jóhannesson *et al.* (2007) concluded that the bottom water mass was a 6:1 mixture of glacial meltwater and sulfidic geothermal fluid. Here, we describe an assemblage of bacteria in the sample which was dominated by a few groups, some of which appear well adapted to this unique environment.

Materials and methods

Drilling

Hot water drilling was performed with the system described by Thorsteinsson *et al.* (2008). The design and operation of the drill minimized the potential for forward contamination of the lake and reverse contamination of samples, and included using snow 50 m upwind of the rig for drilling fluid, disposal of the upper meter of snow, a 50 μm filter, an ultraviolet sterilization system, heating of the snowmelt to >100 °C and flushing with several volumes of 70% ethanol before operation. Relative to a diH_2O blank, direct counts of cells in samples decreased from 1.6×10^4 in snowmelt at the entrance to the filter to 4×10^3 per ml in the drilling water. Colony-forming units on plate count agar (3–7 d of incubation at 22 °C) decreased from 900 per ml at the entrance to the filter to undetectable (<3 per ml) at the drill head (Thorsteinsson *et al.*, 2008). The total concentration of cells in the drilling water was comparable with that in Vatnajökull ice (Table 3 in Gaidos *et al.* (2004)) and the number of colony-forming units was much less. About 1 m^3 of borehole water may have entered the lake at penetration, less than the mass of basal ice melted by volcanic heat per second (Jóhannesson *et al.*, 2007). Thus, any contamination of the lake was extremely minimal and consistent with the recommendations of the US National Research Council (2007).

Sampling and chemical analyses

We obtained a single 400 ml sample using a custom-built gas-tight sampler (Gaidos *et al.*, 2007). The sampler was washed with 10% NaHClO (30 min) immediately before borehole insertion. To check for sample contamination, 250 ml of filtered, sterilized, 20% Rhodamine WT dye (Keystone Aniline, Chicago, IL, USA) was added to a concentration of 25 p.p.m. in the borehole. This low-ecotoxicity dye (Jensen and Kristensen, 1999; Behrens *et al.*, 2001) would eventually mix into the lake to a concentration of 1 p.p.t. and thus its use was consistent with the recommendations of the US National Research Council (2007). Negative visual detection of dye (<25 p.p.b.) confirmed that any

borehole contamination of our sample was <1:1000. Supporting electrovoltammetry used a flow cell attached directly to the sampler (Supplementary Information). Particulate organic carbon and nitrogen were measured as described earlier (Gaidos *et al.*, 2004).

Cell counts

Samples for cell counts and fluorescent *in situ* hybridization (FISH) were immediately mixed with 3 volumes of 4%, 0.2-μm filtered paraformaldehyde solution (pH 7). After transport to the lab, two 4 ml aliquots of preserved samples were collected onto separate 25 mm black polycarbonate 0.2-μm pore size Isopore membrane filters (Millipore, Billerica, MA, USA) and washed with diH_2O. Filters were stained with 200 μl of 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) solution for 10 min in the dark, washed with 18.2 MΩ H_2O and examined with a BX51 epifluorescence microscope (Olympus, Center Valley, PA, USA). Twenty fields were counted on each filter by three investigators. The minimum and maximum values for each filter were removed before averaging.

Enrichment cultures

Five-milliliter aliquots of sample were removed in gas-tight syringe and stored in sealed, N_2-flushed serum bottles. Enrichments of 0.5 ml sample in 4.5 ml 0.2 μm-filtered lake water were prepared in Hungate tubes with two conditions: (a) addition of 100 × yeast extract–acetate medium (0.1% yeast extract, 0.1% Na acetate, 32.5 mM $(NH_4)_2SO_4$, 2 mM KCl, 1 mM K_2HPO_4, 0.7 mM $MgSO_4$, 0.06 mM KNO_3 and 0.06 mM $CaSO_4$) to 0.01% concentration, vitamin solution, Balch element solution (Balch *et al.*, 1979), S^0 (a few grains) and resazurin (0.0045%), and incubation with N_2 headspace; (b) same as (a) but under aerobic conditions (ambient headspace). Enrichments of 0.5 ml sample plus 4.5 ml sterile water were performed under two other conditions: (c) addition of 100 × yeast extract–acetate medium to 1% and incubation with H_2/CO_2 (80/20%); and (d) same as (c) but incubation under aerobic conditions (ambient headspace). All incubations were performed at 3 °C in the dark. Serial dilutions were made (0.9 ml sterile H_2O, 0.1 ml yeast extract–acetate medium + S^0) under both aerobic and anaerobic conditions at 3 °C. Enrichments prepared as in (a) and (b) were also incubated at 60 °C. Enrichment and medium for incubation at 80 °C were prepared as described by Marteinsson *et al.* (2001a) with pH adjusted to 7.0.

DNA-based clone library construction and 16S rRNA gene sequencing

Samples (250 ml) or enrichment cultures (5 ml) were filtered through 25 mm polycarbonate 0.2-μm pore size Nucleopore polycarbonate membrane filters (Whatman, Maidstone, Kent, UK). Filters were stored in DNA extraction buffer (0.1 M pH 8.0 Tris buffer, 0.1 M pH 8.0 Na-EDTA, 0.1 M phosphate buffer, 1.5 M NaCl and 0.5% cetyl trimethyl ammonium bromide) at −80 °C. DNA extraction from filters was performed as described by Marteinsson *et al.* (2001b). DNA was also extracted from 1.5 ml of sample that was centrifuged, the pellet re-suspended in lysis buffer consisting of Tris buffer (50 mM, pH 7.6), EDTA (1 mM, pH 8.0), 0.5% Tween-20 (Sigma-Aldrich, St Louis, MO, USA) and 200 μg ml^{-1} proteinase K, the buffer incubated for 2 h at 55 °C and then heated to 95 °C for 5 min. PCR was performed with the following primers: 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1544R (5′-AGAAAGGAGGTGATCCA-3′) for bacteria, and 23FLP (5′-GCGGATCCGCGGCCGCTGCAGAYCTGGTYGATYCTGCC-3′) and 1391R (5′-GACGGGCGGTGTGTRCA-3′) for archaea using the protocol of Skírnisdóttir *et al.* (2000). PCR products were cloned directly using a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Plasmid DNA from single colonies was isolated and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI sequencer (Applied Biosystems, Foster City, CA, USA) using the reverse primer 805R (5′-GACTACCCGGGTATCTAATCC-3′). Taxonomic assignments and identification of closest cultivated relatives were performed with the Ribosomal Database Project Classifier (Wang *et al.*, 2007) and NCBI BLASTn. These sequence data have been submitted to the GenBank database under accession numbers FJ229496–FJ229759 and F232072–FJ232141.

Pyrosequencing

Approximately 0.15 μg of DNA was extracted from each of the two halves of a filter using the same protocol as above. Pyrosequencing of 70 bp of the V6 variable region in the 16S rRNA gene was performed with a GS-FLX (Roche, Basel, Switzerland) at the Marine Biological Laboratory, Woods Hole, USA. The V6 region contains sufficient sequence information to make taxonomic assignments to the genus level, provided certain quality criteria are met and there are closely related sequences in a database (Huse *et al.*, 2008). Presence of suitable template was first verified by amplification using the primers 967F (5′-CAACGCGAAGAACCTTACC-3′) and 1046R (5′-CGACAGCCATGCANCACCT-3′). These were the bacteria-specific primers used for pyrosequencing minus the linker sequence and 4-base 'barcode' for parallel sample processing. Tags with one or more ambiguous base calls, those shorter than 60 bases and those encountering the proximal primer were discarded by the pipeline analysis software (Sogin *et al.*, 2006). Unique tags may represent distinct phylotypes and were assigned taxonomic affiliations (down to genus level,

if possible) by the process described in Sogin *et al*. (2006). However, tags derived from the same phylotype might be separately enumerated because they are of different length or cover slightly different regions of the V6 region. A Perl script written by EG constructed a minimum set of ribotypes for each taxon by grouping tags with identical overlapping sequences. A tag that could be assigned to more than one ribotype was assigned to the one with the greatest number of reads. Ribotypes with the same taxonomic affiliation may represent different strains or species, depending on resolution. These data have been submitted to the NCBI Short Read Archive as SRA002254.

FISH probes

We used bacterial and archaeal probes EUBI/338, EUBII, EUBIII (Daims *et al*., 1999), ARC-915 (Stahl and Amann, 1991) and ARC-344 (Raskin *et al*., 1994). Probes targeting the four most abundant phylotypes in our clone libraries (*Acetobacterium*, *Paludibacter*, *Sulfuricurvum* and *Sulfurospirillum*) were developed using PRIMER3 (Rozen and Skaletsky, 2000), checked for specificity with PRIMROSE (Ashelford *et al*., 2002) and ProbeCheck (Loy *et al*., 2007) using the SILVA database (Pruesse *et al*., 2007), and evaluated for access to the ribosome (Behrens *et al*., 2003) (Table 1). Positive controls (no mismatches) were prepared for all probes using actively growing pure or enrichment cultures (Table 1). As a positive control for our *Sulfuricurvum* probe, we used a formaldehyde-preserved subculture from a chloroethene-degrading enrichment that had produced a perfectly matching sequence (GenBank accession no. EU498374). Negative controls (one mismatch) were used for the *Acetobacterium* and *Sulfurospirillum* probes (Table 1). Probes were assayed over the stringency range 0–40% in 5% intervals. Stringencies that maximized counts in the positive controls and minimized false positives in the negative controls are reported in Table 1.

Fluorescent in situ *hybridization*

Paraformaldehyde-preserved samples (5–23 ml) were filtered and washed (diH_2O) on 25- and 47-mm white Isopore polycarbonate membrane filters (Millipore). Filters were air dried in a laminar flow hood and stored at −20 °C. To permeabilize membranes of archaea and Gram-positive bacteria, selected filter sections were overlaid with 0.2% low melting-point agarose, incubated with 0.01% lysozyme (Acros Organics, Geel, Belgium) at 37 °C for 30–90 min, rinsed with diH_2O, stopped with 0.01 N HCl for 10 min and rinsed again with diH_2O. Sections of filters were hybridized with 18 μl of hybridization buffer (0–40% formamide, 0.9 M NaCl, 20 mM Tris-HCl and 0.01% SDS) and 2 μl of Cy-3-labeled oligonucleotide probe (50 ng μl^{-1}; Thermo Fisher Scientific, Ulm, Germany), sealed between glass slides and incubated in the dark at 46 °C for 2–3 h. Afterwards, the filters were immediately placed in 5 ml wash buffer (900–55 mM NaCl, 20 mM Tris-HCl and 0.01% SDS) and incubated for 15 min at 48 °C with occasional inversion. Filter sections were washed in diH_2O, air dried in the dark for 15 min, counter stained with 30 μl of DAPI solution (1 μg ml^{-1}) for 2 min, successively washed in diH_2O, 80% ethanol and diH_2O, and then air dried on filter paper for 1 h in the dark. Sections were mounted on glass slides with 10 μl of antifading solution (Citifluor, London, UK) and examined with a BX-51 epifluorescence microscope (Olympus). Probed and DAPI-stained cells were counted in at least 10 fields in sections from at least two filters. These were compared with counts from a filter section hybridized with a nonsense probe (Table 1).

Results

Direct (DAPI) cell counts were $4.7–5.7 \times 10^5$ per ml. The combination of EUBI–III probes hybridized to 94 ± 5% of DAPI cells on filters prepared 1 month after preservation. No cells (<2%) were detected

Table 1 Oligonucleotide probes for fluorescent *in situ* hybridization experiments

Name	*Sequence (5′-3′)*	*+Control*	*−Control*	*Optimal stringency (%)*
EUBI	GCTGCCTCCCGTAGGAGT			35
EUBII	GCAGCCACCCGTAGGTGT			
EUBIII	GCTGCCACCCGTAGGTGT			
ARC-915	GTGCTCCCCCGCCAATTC			10–20
ARC-344	TCGCGCCTGCTGCICCCCGT			20
Aceto125	TCCAAAGGGTAGGTTACCCA	*Acetobacterium bakii* (DSM 8239)	*Acetobacterium carbonolicum* (DSM 2925)	15
Sspirill572	TTCCAGCCTACGCATCCTTT	*Sulfurospirillum halorespirans* (DSM 13726)	*Sulfurospirillum deleyanium* (DSM 6946)	40
Scurvum731	GTATCATCCCAGCAGATCGC	Enrichment culture	None	
Palud672	CCGCTACACAACACATTCCG	*Paludibacter propionicigenes* (DSM)	None	20
Nonsense	ACTCCTACGGGAGGCAGC			

with archaea-specific probes. Only 64–76% of cells on other filters prepared after 4 months of preservation hybridized with the (bacterial-specific) EUBI–III probes, possibly reflecting ribosome degradation (Lam and Cowen, 2004), and these filters were not used further.

Bacterial-specific PCR amplification and gel electrophoresis of two independent DNA extracts for pyrosequencing generated not only a product with the expected length of about 145 bp but also a shorter doublet at around 100 bp (data not shown). These lengths include the uninformative linker, barcode and primer. Archaea-specific amplification and pyrosequencing were not attempted because archaea were not detected by full-length PCR or FISH. Pyrosequencing of the bacterial products yielded an unexpectedly small number of reads and an anomalously large fraction of short reads (<50 bases when trimmed) that were rejected by the processing pipeline. In all, 52% and 63% of reads from the first and second extractions/reactions were rejected by the pipeline filter because they were shorter than 50 bases, had at least one indeterminate base call or encountered the proximal primer. We presume that the numerous short reads were related to the presence of the doublet. Although some matched rRNA gene sequences, others consisted of one or two copies of the 967F primer, suggesting an anomalous chemistry during DNA processing or amplification. In all, 2368 and 6984 reads from the first and second reactions passed the pipeline filter. We excluded 394 reads (210 unique tags) with GAST distances >0.25 and spurious taxonomic assignments (Huse *et al.*, 2008). Many have significant BLASTn hits to non-ribosomal genomic DNA and may be a result of nonspecific amplification. The remaining 8958 reads were grouped into 1065 unique tags.

We identified 69 tags (7253 reads, 81% of the total) that appeared in both libraries and that we consider most reliable. These represent 19 genera and 6 families, plus one member of the proteobacteria of uncertain affiliation and two members of the bacteria of unknown taxonomic identity. In Table 2, we report the number of reads assigned to each taxon, the total number of tags and the number of unique ribotypes. The fractional number of reads is plotted in Figure 3. The four most enumerated

Table 2 Major bacterial phylotypes in west Skaftá lake DNA

Taxon (class)	*Reads*[a]	*Tags*	*Ribotypes*	*Clones*	*FISH (%)*
Acetobacterium (Clostridia) ❄ 98% identity with *Acetobacterium bakii* (Kotsyurbenko *et al.*, 1995)	5710 (5076)	334	21	141	10
Thermus (*Thermus-Deinococcus*)	872 (713)	116	12	0	
Paludibacter (Bacteroidetes) ✹ 89% identity with *Paludibacter propionicigenes* (Ueki *et al.*, 2006)	762 (700)	51	2	24	27
Sulfuricurvum (ε-proteobacteria) 95% identity with *Sulfuricurvum kujiense* (Kodama and Watanabe, 2004)	249 (199)	25	1	66	39
Pseudomonas (γ-proteobacteria)	126 (104)	18	1	0	
Sulfurospirillum (ε-proteobacteria) 98% identity with *Sulfurospirillum halorespirans* (Luijten *et al.*, 2003)	125 (92)	24	1	30	25
Desulfosporosinus (Clostridia)	99 (92)	22	5	0	
Geobacteraceae (δ-proteobacteria) ❄ 96% identity with *Geobacter psychrophilus* (Nevin *et al.*, 2005)	54 (49)	6	1	1	
Enterobacteriaceae (γ-proteobacteria)	47 (43)	6	2	0	
Ralstonia (β-proteobacteria)	35 (32)	4	1	0	
Thermoanaerobacter (Clostridia) ✹	34 (29)	7	2	0	
Peptococcaceae (Clostridia)	25 (20)	5	1	0	
Exiguobacterium (Bacilli) ❄	18 (16)	3	1	0	
Acidaminococcaceae (Clostridia)	17 (16)	3	2	0	
Acinetobacter (γ-proteobacteria)	17 (9)	6	2	0	
Microbacteriaceae (Actinobacteria)	14 (8)	5	1	0	
Staphylococcus (Bacilli)	12 (7)	5	1	0	
Desulfobulbaceae (δ-proteobacteria)	11 (6)	6	1	0	
Bacillus (Bacilli)	11 (7)	4	1	0	
Diaphorobacter (β-proteobacteria)	10 (10)	1	1	0	
Microbacterium (Actinobacteria)	8 (7)	2	1	0	
Desulfocapsa (δ-proteobacteria)	7 (7)	1	1	1	
Streptococcus (Bacilli)	7 (3)	4	1	0	
Gardnerella (Actinobacteria)	5 (4)	2	1	0	
Clostridium (Clostridia)	3 (2)	2	1	0	
Micrococcus (Actinobacteria)	3 (2)	2	1	0	
Phylum proteobacteria	9 (3)	6	1	0	
Domain bacteria	47 (11)	26	2	0	

Abbreviation: FISH, fluorescent *in situ* hybridization.
Dark-shaded rows correspond to groups of obligate anaerobes, whereas light-shaded rows correspond to facultative anaerobes. The ❄ indicates a relationship with a psychrotolerant or psychrophilic organism and the ✹ indicates a candidate thermophile.
[a]Number of reads assigned to ribotypes that appear in two independent libraries is in parentheses.

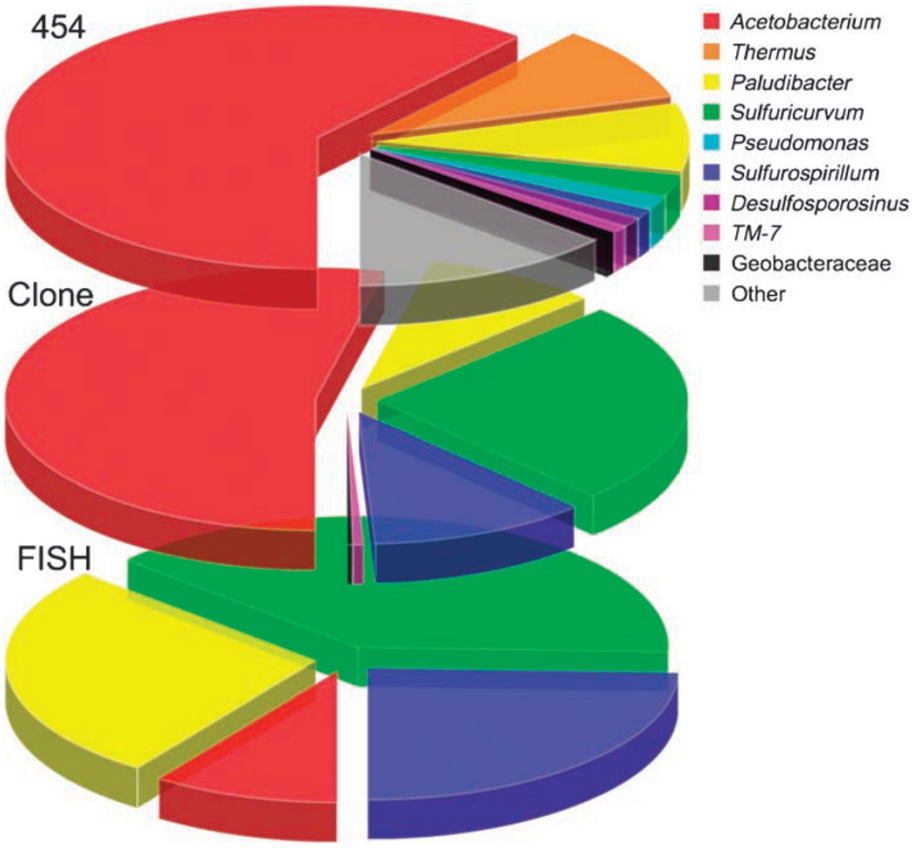

Figure 3 Distribution of 8958 reads of the rRNA gene V6 region (top), 264 partial-length rRNA clone library sequences (middle) and FISH-probed cells (bottom) in DNA libraries and filters prepared from the lake sample. FISH, fluorescent *in situ* hybridization.

phylotypes (including 36 ribotypes) account for 85% of all reads and 64% are included in a single cluster of 21 ribotypes affiliated with the genus *Acetobacterium*. Two hundred and thirty-two other tags (695 reads) were represented by at least two reads in one library, but their absence in the other is a low-significance ($>5\%$ probability) event that can be ascribed to chance rather than pyrosequencing error or contamination of one sample (Supplementary Information and Table S2.) They represent 25 genera, 11 families and taxonomically unresolved members of eight orders, three classes (α-, γ- and δ-proteobacteria) and one phylum (Bacteroidetes). Seven hundred and nineteen more tags appeared as single reads in our combined libraries (data not shown).

We sequenced 264 clones from a library of 16S rRNA gene fragments amplified using bacteria-specific primers. These sequences represent seven phylotypes (Table 2 and Figure 3) corresponding to those identified by pyrosequencing (genera *Acetobacterium*, *Paludibacter*, *Sulfuricurvum*, *Sulfurospirillum Geobacter*, *Desulfocapsa* and the TM7 division). The *Acetobacterium*-related clones are divided between two ribotypes and together account for 53% of all sequenced clones. All PCRs with archaea-specific primers failed.

Growth was observed in all enrichment cultures after 2 weeks of incubation at 3 °C. No growth was observed in the 60 °C and 80 °C incubations or in control samples. The highest growth was observed in enrichments initially prepared with filtered lake water rather than sterile H_2O. Microscopy revealed coccoid cells and both long, thin and short rods. Attempts to isolate pure strains from enrichments were unsuccessful. Libraries were constructed from DNA extracted from enrichments (b), (c) and (d), and 24, 22 and 24 clones, respectively, were sequenced. These included *Sulfuricurvum* and *Sulfurospirillum* sequences identical to those recovered from the lake sample, as well as three phylotypes not seen in the lake (Table S2).

All phylotype-specific fluorescent oligonucleotide probes hybridized to morphologically distinguishable cells (Figure 4). The *Acetobacterium*-specific probe targeted spherical cells 1 μm in diameter that were usually in attached pairs but sometimes alone or in chains of several cells. Our culture of *Acetobacterium bakii* (the closest culture relative) contained cells of similar size in doublets or chains, but the cells were more coccoid. The *Paludibacter*-specific probe targeted coccoid cells that appeared to be in linked chains, similar in morphology but longer

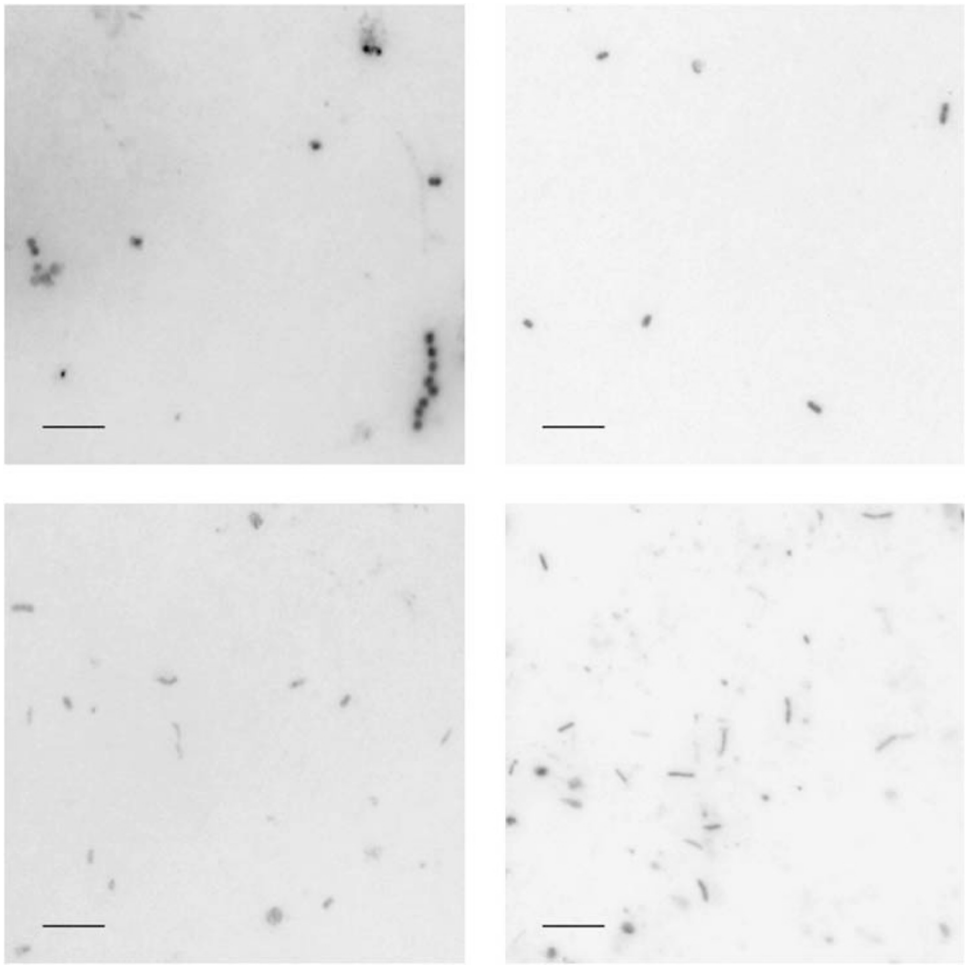

Figure 4 Images of cells hybridized by highly specific fluorescent oligonucleotide probes designed from sequences in our 16S rRNA gene clone libraries. Clockwise from top left: *Acetobacterium*-, *Sulfuricurvum*-, *Sulfurosprillum*- and *Paludibacter*-specific hybridizations. Each scale bar is 5 μm.

than the *P. kujiense*. The *Sulfuricurvum*-specific probe hybridized to 1.8-μm long rods; most were straight but a minority were curved like those of *Sulfuricurvum kujiense* (Kodama and Watanabe, 2004) and the cells detected in the enrichment culture. The *Sulfurospirillum*-specific probe hybridized to 2.3-μm long rods that were thinner than the *Sulfuricurvum*-like cells. These four phylotypes account for essentially all DAPI-stained cells but errors permit 10–20% to belong to other phylotypes (Figure 3 and Table 2).

Discussion

Our data unequivocally show that bacteria dominate in the bottom water but do not completely rule out the presence of archaea. They reveal an oligarchic assemblage of bacteria, with a few phylotypes comprising the vast majority of cells and rRNA operon pool. However, there are discrepancies between our data sets. Each phylotype in our clone library is represented by multiple V6 reads from the same genus or family, but the reverse is not true (that is, genera *Thermus*, *Pseudomonas* and *Desulfosporosinus*). Our FISH investigation limited the missing phylotypes to not more than 10–20% of cells and did not identify morphologically distinctive cells stained by DAPI or hybridized with the EUBI–III probes but not targeted by our more specific probes. The apparent paucity of *Thermus* full-length clones relative to V6 tags is unlikely to be due to inadequate PCR primer coverage or bias in clone library construction because our primers target *Thermus thermophilus* and the same procedures were used to assay *Thermus* diversity elsewhere (Skírnisdóttir *et al.*, 2000). The two cultivated strains most closely related to the *Thermus*-like V6 tags, *T. igniterrae* and *T. antranikianii*, are distinctive short rods or filaments 0.5–0.8 μm wide (Chung *et al.*, 2000). The cells belonging to these phylotypes could have been hidden on the mineral grains seen in our sample, or their DNA did not come from intact cells. These discrepancies highlight the importance of using multiple approaches to describe microbial assemblages.

Hypothetical metabolisms and physicochemical tolerances of the organisms that these major phylotypes represent can be inferred from those of cultivated close relatives, although these inferences should be viewed with caution. Many phylotypes belong to groups composed of obligate and/or facultative anaerobes (Table 2) consistent with bottom water anoxia. Several phylotypes from the sample and enrichments are related to known psychrophilic or psychrotolerant organisms (Table 2) (Kotsyurbenko *et al.*, 1995; Frühling *et al.*, 2002; Finneran *et al.*, 2003; Nevin *et al.*, 2005; Chaturvedi and Shivaji, 2006; Rodrigues *et al.*, 2006; Rodrigues and Tiedje, 2006) and suggest an adaptation to near-freezing conditions. Phylotypes affiliated with *Thermus* and *Thermoanaerobacter*, genera comprising thermophiles and including closely related isolates from Icelandic hot springs (Chung *et al.*, 2000), may have originated in hot springs elsewhere in the lake; alternatively, the genera could contain cold-tolerant species (Sheridan *et al.*, 2003).

The majorities of both V6 reads and 16S rRNA gene clones are affiliated with the genus *Acetobacterium*, but a comparatively small fraction (10%) of cells hybridized with our *Acetobacterium*-specific probe. These probes were designed using both clone ribotypes, and the distinctive morphology of acetobacteria makes it unlikely that cells were missed. Rather, their over-representation in our libraries may be a consequence of extraction/PCR bias or a high number of rRNA operons per cell. Some members of the *Clostridium* (in the order Clostridiales along with *Acetobacterium*) have $\geqslant$10 rRNA operons (Rainey *et al.*, 1996; Brüggemann *et al.*, 2003; Acinas *et al.*, 2004; Sadeghifard *et al.*, 2006).

Both *Acetobacterium* ribotypes in our clone library are closely (98% identity) related to *A. bakii*, an obligately anaerobic, psychrotolerant (growth at 1–30 °C) homoacetogen (Kotsyurbenko *et al.*, 1995) that extracts energy from the conversion of H_2 and CO_2 to acetate (CH_3COOH). The genera *Sulfuricurvum* and *Sulfurospirillum* are also abundant in our V6 tag and clone libraries, and together comprise 64% of DAPI-stained cells (Table 2 and Figure 3). *S. kujiense* (Kodama and Watanabe, 2004) and *Sulfurospirillum halorespirans* (Luijten *et al.*, 2003), the closest cultivated isolates, use sulfur compounds during chemotrophic growth; each belongs to a separate clade within the ε-proteobacteria, a class that is ubiquitous in sulfidic environments. *S. kujiense* uses elemental sulfur, sulfide and thiosulfate as electron donors during microaerobic (1% O_2) and anaerobic respiration, consistent with the presence of *Sulfuricurvum* sp. in both our aerobic and anaerobic enrichments. The Icelandic isolates related to our *Thermus* phylotypes also obtain energy from the aerobic oxidation of thiosulfate to sulfate (Skírnisdóttir *et al.*, 2001). In contrast, related *Sulfurospirillum* spp. use sulfite, thiosulfate and elemental sulfur as terminal electron acceptors and H_2 and organics as electron donors in energy-conserving reactions that reverse those catalyzed by *S. kujiense* (Luijten *et al.*, 2003).

Some members of the genera *Ralstonia*, *Pseudomonas* and Enterobacteriaceae, represented by numerous reads in our V6 tag libraries, as well as *S. kujiense* and *S. halorespirans,* are capable of microaerobic hydrogen oxidation (Aragno, 1998; Luijten *et al.*, 2003; Kodama and Watanabe, 2004). A phylotype from our aerobic enrichments (Supplementary Table S2) is closely related to *Thiobacillus plumbophilus*, an aerobe capable of growth by oxidation of H_2, as well as H_2S and PbS (Drobner *et al.*, 1992). Other metabolisms are possible: the family Geobacteraceae appears as a single phylotype in both our V6 tag and clone libraries; the latter is related to *Geobacter psychrophilus*, an Fe(III)-reducing psychrophile (Nevin *et al.*, 2005). A sequence in our aerobic enrichment cultures is closely related to *Rhodoferax ferrireducens* T118, a psychrotolerant facultative anaerobe that uses Fe(III) as a terminal electron acceptor (Finneran *et al.*, 2003) and member of the (predominantly heterotrophic) Comamonadaceae family widespread in cold environments (Skidmore *et al.*, 2005). Another abundant phylotype (27% of cells) is related to *Paludibacter propionicigenes*, an obligate anaerobe capable of fermenting sugars to propionate, acetate and succinate (Ueki *et al.*, 2006), but its potential biogeochemical role in the lake is unknown.

The inferred metabolisms of the dominant bacterial phylotypes can be understood in the context of a model of lake circulation and chemistry (Jóhannesson *et al.*, 2007). The bottom water contained 30 mM CO_2 and as much as 600 μM H_2 (A Stefansson, personal communication), and under such conditions (4 °C, pH = 5.22, and assuming $[CH_3COO^-] = 1\,\mu M$), acetogenesis is exergonic ($\Delta G = -32$ kJ per mol H_2, using constants in Kotsyurbenko *et al.* (2001)). Methanogenesis is also thermodynamically feasible ($\Delta G = -42$ kJ per mol H_2, assuming atmospheric pCH_4), and non-acetoclastic methanogens would compete with homoacetogens for H_2. However, at 4 °C and $pH_2 > 10$ Pa (0.07 μM), *A. bakii* outcompeted two psychrotolerant methanogens isolated from the same environment (Kotsyurbenko *et al.*, 2001). This could explain the abundance of acetogens and our inability to detect methanogens (exclusively archaea). Alternatively, methanogens, all strict anaerobes, might have been suppressed by the introduction of oxygen during bottom water formation. Mixing distinguishes this system from stratified, ice-covered Lake Fryxell where methanogens are abundant and active below the oxycline (Karr *et al.*, 2006).

Sulfate reduction is important in many anoxic settings and some phylotypes in our libraries are affiliated with sulfate-reducing bacteria (SRB). These are *Desulfosporosinus*, a group of mesophilic, autotrophic, spore-forming sulfate reducers that can use hydrogen (Stackebrandt *et al.*, 1997); the family Desulfobulbaceae, the members of which are strictly

anaerobic, mesophilic or psychrophilic SRB (Kuever *et al.*, 2005); and the sulfate-reducing genus *Desulfocapsa* (Table 2). The last is also represented by a 16S rRNA gene clone sequence that is related to an isolate from a meromictic alpine lake (Peduzzi *et al.*, 2003). Additional potential SRB (affiliates of *Desulfuromonas*, *Desulforhopalus*, *Desulfuromusa* and *Desulfobacula*) appear with much lower numbers of reads (Table S2). Although the reduction of sulfate to sulfide is exergonic under lake conditions (Conrad *et al.*, 1986), with the exception of *Desulfosporosinus*, SRB do not constitute a significant fraction of cells or 16S rRNA sequences. Sulfate reduction can be inhibited by a high H_2S level (Koschorreck, 2008) and/or limited by a low sulfate level (Holmer and Storkholm, 2001). Although the amount of sulfate (0.16 mM) may be above the threshold for sulfate reduction, at least some of this may be the product of recent sulfide oxidation. Inhibition of SRB, which usually outcompete homoacetogens for H_2, could explain the detection of hydrogen and the abundance of acetogens in the lake (Hoehler *et al.*, 1999).

Jóhannesson *et al.* (2007) explained lake bottom water as a mixture of overlying sulfidic lake water with oxygenated glacial meltwater (Figure 2). On the basis of their model and assuming O_2 saturation in the meltwater, bottom water would have initially contained both 1.2 mM $H_2S + HS^-$ and 100 μM O_2, a chemical disequilibrium able to support sulfide-oxidizing bacteria. In the sample, detectable ($\geqslant 2$ μM) O_2 was absent and bottom water would have evolved to sulfide/oxygen ratios $\gg 12$ within a few days by abiotic oxidation alone (Millero, 2005). At sulfide/oxygen ratios > 150, biofilms of filamentous ε-proteobacteria replace the γ-proteobacteria groups *Beggiatoa* and *Thiothrix* in cave waters (Engel *et al.*, 2004; Macalady *et al.*, 2008), and many ε-proteobacteria such as *S. kujiense* are capable of microaerophilic growth at micromolar O_2 (Kodama and Watanabe, 2004). Sulfide-oxidizing bacteria activity also explains the absence of detectable thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}), which would otherwise accumulate during abiotic sulfide oxidation (Millero, 2005).

Mixing of lake water and glacial meltwater would also have supported aerobic hydrogen oxidation by hydrogenotrophic bacteria. At $[H_2] > 0.1$ nM, H_2 oxidation is more exergonic per O_2 molecule than sulfide oxidation (based on data in Bach and Edwards (2003)). The presence of H_2-oxidizing clades has been correlated with the free energy of this reaction (Spear *et al.*, 2005), but its importance *vis-à-vis* sulfide oxidation is probably controlled by the H_2 affinities and uptake kinetics of guild members (D'Imperio *et al.*, 2008). We speculate that hydrogen-oxidizing bacteria (possibly including *Ralstonia* sp.) were active earlier at a higher oxygen concentration, but have been replaced by organisms that use sulfur species as electron acceptors such as *Sulfurospillum* sp. This could explain why the former appeared in the DNA pool but did not constitute a significant fraction of DAPI-stained cells.

Numerous additional bacterial taxa were identified by V6 tags (Supplementary Table S2) and there is evidence for greater diversity at still lower abundance, including 719 single-red tags (data not shown). A rarefaction analysis showed that phylotype sampling did not reach saturation (Figure S2). The uncultivated division TM7 (Hugenholtz *et al.*, 2001) is represented by both V6 tags and a single sequenced clone. Two phylotypes from our enrichment cultures (a *Thiobacillus*-related β-proteobacterium and a member of the Campylobacteraceae) have no counterparts in our other data and may represent failures of V6-based taxonomy, or rare, cultivable 'weeds'. These additional phylotypes cannot comprise more than 10–20% of DAPI-stained or EUBI–III-hybridized cells. Some may be dormant or slow-growing species released by melting of glacial ice but maladapted to the lake environment. Others may have dispersed from hot springs or lake sediment. Strikingly, most minor groups are proteobacteria, in contrast to the dominant groups that fall in classes Clostridia, Bacilli and Actinobacteria as well as proteobacteria (Table 2). The vast majority of sequences obtained from Grímsvötn samples were affiliated with the β-proteobacteria (Gaidos *et al.*, 2004), a group widely recovered from freshwater (Methé *et al.*, 1998), including cold oligotrophic lakes and melt ponds (Brinkmeyer *et al.*, 2004), and the accretionary ice above Lake Vostok (Priscu *et al.*, 1999).

As measured by particulate organic carbon (0.6 mg l^{-1}), the western Skaftá lake is oligotrophic and microbial autotrophy would control flows of carbon. The closest cultivated relatives of the *Acetobacteria* and *Sulfuricurvum* phylotypes are both autotrophs (Kotsyurbenko *et al.*, 1995; Kodama and Watanabe, 2004) and that of the *Sulfurospirillum* sp. can use acetate as a carbon source (Luijten *et al.*, 2003). Thus, acetogenesis may be another important entry point for carbon into this ecosystem. The bacterial oligarchy in this subglacial lake, where acetogens replaced methanogens, stands in contrast to hydrogen-dependent communities from higher-temperature subsurface habitats where (methanogenic) archaea dominate (Chapelle *et al.*, 2002). It is unclear whether the structure of this unique microbial assemblage is the result of ecological pressure or limited colonization by adapted microbes, but a comparison with the eastern Skaftá lake could prove illuminating.

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