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ORIGINAL ARTICLE

Microbial communities in the subglacial waters of the Vatnajökull ice cap, Iceland

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Subglacial lakes beneath the Vatnajökull ice cap in Iceland host endemic communities of microorganisms adapted to cold, dark and nutrient-poor waters, but the mechanisms by which these microbes disseminate under the ice and colonize these lakes are unknown. We present new data on this subglacial microbiome generated from samples of two subglacial lakes, a subglacial flood and a lake that was formerly subglacial but now partly exposed to the atmosphere. These data include parallel 16S rRNA gene amplicon libraries constructed using novel primers that span the v3–v5 and v4–v6 hypervariable regions. Archaea were not detected in either subglacial lake, and the communities are dominated by only five bacterial taxa. Our paired libraries are highly concordant for the most abundant taxa, but estimates of diversity (abundance-based coverage estimator) in the v4–v6 libraries are 3–8 times higher than in corresponding v3–v5 libraries. The dominant taxa are closely related to cultivated anaerobes and microaerobes, and may occupy unique metabolic niches in a chemoautolithotrophic ecosystem. The populations of the major taxa in the subglacial lakes are indistinguishable ($>$99% sequence identity), despite separation by 6 km and an ice divide; one taxon is ubiquitous in our Vatnajökull samples. We propose that the glacial bed is connected through an aquifer in the underlying permeable basalt, and these subglacial lakes are colonized from a deeper, subterranean microbiome.
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Introduction

Investigations have described microorganisms in the seafloor (Sogin *et al.*, 2006; Mason *et al.*, 2010), in continental crust (Davidson *et al.*, 2011) and beneath ice sheets (Lanoil *et al.*, 2009). A deep microbial biosphere may be a vast reservoir of diversity (Whitman *et al.*, 1998), a refuge for life (Sleep and Zahnle, 1998) and the setting of the origin of life itself (Martin *et al.*, 2008). Subglacial lakes, such as those under East Antarctica, are analogs to possible extraterrestrial habitats (Siegert, 2005). In the absence of sunlight or organic matter from the surface, subglacial life must rely on ambient geochemical energy (Hoehler, 2004). In subglacial volcanic settings, mixing of volcanic gases, fluids and minerals, as well as oxygenated glacial meltwater, creates chemical disequilibrium (Giggenbach, 1980; Arnórsson *et al.*, 1983; Oelkers and Gíslason, 2001; Symonds *et al.*, 2001), and the available energy can be harvested by microorganisms in coupled reduction–oxidation reactions (Gaidos *et al.*, 1999). Populations of such microbes may descend from the original inhabitants of the unglaciated landscape, but may also be released into a lake during basal melting of the glacier in which they were originally entombed (Christner *et al.*, 2000; Miteva *et al.*, 2004).

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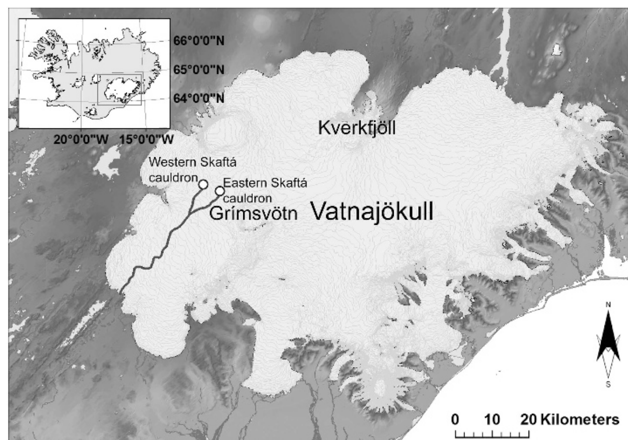

Figure 1 Map of the Vatnajökull ice cap in Iceland showing locations of the Grímsvötn, Skaftárkatlar and Kverkfjöll lakes, and the subglacial conduit through which the Skaftárkatlar lakes drain into the Skaftá river.

In Iceland, the Vatnajökull ice cap covers several active volcanoes. Meltwater drains to minima in the glaciostatic potential at its bed and fills three subglacial lakes: one in the Grímsvötn caldera and two beneath depressions or 'cauldrons' (Icelandic *katlar*, singular *ketill*) in the glacier surface to the northwest of Grímsvötn (Björnsson, 2002). The latter two lakes periodically discharge in subglacial floods (*jökulhlaups*) to the Skaftá river 40 km away and are called the Skaftárkatlar lakes. Forty km to the northeast, glacial melt from a hydrothermal field feeds Kverkfjallalón lake, which is hydraulically disconnected from the Skaftá system. This lake was formerly subglacial, but is now exposed to the atmosphere (Figure 1).

Previous investigations of the Grimsvotn and west Skaftárketill lakes found their waters to be dilute (total dissolved solids <200 p.p.m.), cold ($<5\,^{\circ}$C) and low in organic matter ($\ll 1\,mg\,l^{-1}$ C) owing to melting of the overlying, nutrient-poor ice (Ágústsdóttir and Brantley, 1994; Gaidos *et al.*, 2004; Jóhannesson *et al.*, 2007; Gaidos *et al.*, 2009). The Grímsvötn lake was sampled in June 2002, 3 months after a jökulhlaup, and consisted of oxygenated glacial melt, but the west Skaftárketill lake, explored in June 2006, was anoxic and sulfidic, with hydrothermal input (Jóhannesson *et al.*, 2007). The pelagic microbial communities are also distinct: Grímsvötn contains a low abundance (2×10^4 cells ml^{-1}) of diverse heterotrophic bacteria (Gaidos *et al.*, 2004), whereas west Skaftárketill has a higher biomass (5×10^5 cells ml^{-1}) dominated by a few bacterial taxa related to anaerobes and microaerobes, with no evidence for archaea (Gaidos *et al.*, 2009). Differences in chemistry can be explained by varying inputs of meltwater, volcanic gases and hydrothermal fluid. Differences in the microbial communities can be ascribed to environmental selection, provided cells affiliated with these groups are carried from other anoxic sites to the glacier surface and remain viable during passage to the lake. Vatnajökull ice contains $\sim4\times10^3$ cells cm^{-3}, but these appear neither to be viable nor related to the Skaftárketill taxa (Gaidos *et al.*, 2004).

Here we present results from additional sites under Vatnajökull, including the east Skaftárketill lake, a jökulhlaup in the outlet to the Skaftá river and the Kverkfjallalón lake. These molecular data include the first paired, long-read pyrosequence libraries of the 16S rRNA gene based on different primer sets. Our findings indicate that the dominant bacterial taxa are widely distributed beneath the ice of Vatnajökull, suggesting a subterranean source for this subglacial microbiome.

Materials and methods

Drilling, sampling and temperature data

The east Skaftáketill lake was sampled during a 3-day interval in June 2007 by two boreholes drilled through the 280-m-thick ice sheet by a sterilizing hot water drill (Thorsteinsson *et al.*, 2008). Boreholes A and B, separated by 170 m, were at 64.48702°N 17.50583°W (1593 m above sea level (a.s.l.)) and 64.48757°N 17.50915°W (1588 m a.s.l.), respectively. The depth of the lake was 103 m at both locations. Multiple temperature profiles were obtained in each borehole using a Starmon mini-temperature recorder with an estimated accuracy of $\pm 0.02\,^{\circ}$C after zero-point adjustment. Depth and bottom temperatures were obtained with a Geokon 4500SHSR-7.5 MPa vibrating wire pressure sensor and thermometer connected to a Campbell CR-10X data logger on the glacier surface. Samples were collected in a 1 l gas-tight sampler (Mount Sopris 2FSA-1000). Contamination of the lake and reverse contamination of samples were avoided using the techniques described in Gaidos *et al.* (2009). Samples A1–A3 were retrieved from 284, 331 and 379 m below the ice surface and B1–B4 from 284, 336, 377 and 388 m. Samples were kept under anaerobic conditions at 4 °C during transport to the laboratory and processed within 3–4 days. In June 2009, 750 ml of the Kverkfjallalón lake (64.669°N, 16.685°W, 1675 m a.s.l.) was collected from the surface and depths of 4, 6 and 9 m using the same sampler. This was reduced by Na_2S solution (0.05% (w v^{-1}) final concentration) and kept at 4 °C during transport to the laboratory. A jökulhlaup sample was collected from the Skaftá river near its outlet point at the margin of the Skaftárjökull outlet glacier of Vatnajökull (64.256°N, 18.136°W) on 11 October 2008, close to the time of maximum discharge (1290 m^3 s^{-1}).

Chemical analysis

Samples for determination of major and trace elements were filtered on-site through a 0.2-μm cellulose acetate membrane with a Teflon filter or, using polypropylene syringes, into polypropylene

bottles and acid-washed amber glass bottles. The water pH was analyzed on site using a pre-calibrated combination glass electrode (Metrohm, Riverview, FL, USA). The measurements were carried out at $\sim 25\,^{\circ}C$ to prevent errors related from pH dependence on temperature. Samples for major anions (CO_2, Cl, F, SO_4, S_2O_3) were analyzed on-site within ~ 20 min of sampling using ion chromatography (Kaasalainen and Stefánsson, 2011). Dissolved H_2S was measured using precipitation titration on-site with Hg-acetate solution (Kaasalainen and Stefánsson, 2011). Samples for major elemental analysis (Si, B, Fe, Al, Na, K, Ca, Mg) were collected into polypropylene bottles and acidified with Suprapur HNO_3 (0.5 ml acid to 100 ml sample) and analyzed using inductively coupled plasma emission spectrometry (Spectro, Kleve, Germany) (Skoog and Leary, 1992). Dissolved inorganic nutrients NO_2^-, NO_3^- and NH_4^+ were collected into high-density polyethylene bottles and preserved by freezing ($-18\,^{\circ}C$) until analyzed, to prevent any organic growth or decay. Dissolved nutrients were analyzed colorimetrically. Before analysis of NH_4, the samples were acidified using H_2SO_4 (0.5 ml of 1% H_2SO_4 to 100 ml sample) (APHA, 2005).

Enrichment cultures

Media for enrichments of chemolithotrophic and chemo-organotrophic organisms were prepared as described in Gaidos *et al.* (2009) using 0.5 ml sample and 4.5 ml 0.2-μm filtered water from the subglacial lake (the latter alone as a negative control). Cultures were incubated under aerobic (ambient headspace) and anaerobic conditions at $3\,^{\circ}C$, $60\,^{\circ}C$ and $80\,^{\circ}C$. Each enrichment was prepared in a Hungate culture tube. WN2 enrichments contained 0.01% yeast extract, vitamin solution, Balch element solution (Balch *et al.*, 1979), S^0 and resazurin, and were incubated under pure N_2 and 0.025% final $wt\,v^{-1}$ $Na_2S{\bullet}9H_2O$. WO2 enrichments were the same as WN2, except incubated aerobically with ambient headspace. C–J enrichments had addition of 50 μl of 100 × yeast-acetate medium and were incubated with 80%/20% H_2/CO_2 and 0.025% final $wt\,v^{-1}$ $Na_2S{\bullet}9H_2O$. A–J enrichments were the same as C–J, but incubated aerobically with ambient headspace. Additional enrichments used R_2A medium and 162 Thermus medium (Degryse *et al.*, 1978), both aerobically with ambient headspace, and Thermotoga ('Toga') medium (Marteinsson *et al.*, 1997) and YPS medium (Marteinsson *et al.*, 2001) under pure N_2 headspace. Growth in enrichments was confirmed with phase-contrast microscopy (Olympus BX51). Growth was observed every 2 days for 5 weeks and then every 2–6 days for several months. Where growth occurred, serial dilutions were performed in complement medium to isolate strains.

Cell counts and fluorescent in situ hybridization

For flow cytometry, samples were fixed with 1% ($v\,v^{-1}$) glutaraldehyde for 20 min at room temperature before staining with 1:1000 solution of Sybrgreen I (Molecular Probes, Eugene, OR, USA). Cells were enumerated by a FACS (Aria II) flow cytometer and samples were delivered at a calibrated rate for 2 min. Forward- and right-angle light scattering and DNA fluorescence data were collected. Fluorescent *in situ* hybridization (FISH) hybridizations and enumeration by microscopy were as described in Gaidos *et al.* (2009). We used three universal probes for bacteria (EUB I/II/III), two universal probes for archaea (ARC344 and ARC 944) and four probes previously designed to target the dominant lineages in the west Skaftá lake: *Acetobacterium*, *Sulfuricurvum*, *Sulfurospirillum* and *Paludibacter* (Supplementary Table S1). Enumeration of each taxon was normalized by total bacterial counts with EUB I/II/III. We determined the efficiency of our probes by comparing them with our pyrosequence libraries (described below). The *Sulfuricurvum*, *Sulfurospirillum* and *Paludibacter* probes matched 95%, 96% and 96%, respectively, of v3–v5 sequences with the same taxonomic assignment. For the v4–v6 libraries, the respective fractions are 97%, 76% and 93%, respectively. The *Acetobacterium* probe does not target a part of the ribosomal gene that was pyrosequenced.

Clone library construction and sequencing

DNA was extracted as described previously (Gaidos *et al.*, 2009). Polymerase chain reaction amplification was performed according to the protocol in Skírnisdóttir *et al.* (2001), with primers targeting either the archaeal or bacterial domains (Supplementary Table S1). Only reactions with bacterial primers yielded detectable product; all amplification attempts with archaea-specific primers using gradients, different dilutions and alternative cycling protocols were unsuccessful. All positive controls using the archaeon *Thermococcus barophilus* produced detectable product. Polymerase chain reaction product was cloned by the TA method using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmid DNA from single colonies was isolated and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI sequencer (PE Applied Biosystems, Foster City, CA, USA). All clones were sequenced using the reverse primer 805R (Supplementary Table S1). Chimera detection was performed using Greengenes (DeSantis *et al.*, 2006). Sequences were aligned and taxonomically classified using neighbor-joining and parsimony trees constructed by the ARB package (Ludwig *et al.*, 2004). These sequence data have been submitted to the GenBank database under accession numbers JQ697981–698334.

Pyrosequencing

Pyrosequencing of 70–100 nucleotides (nt) of the v6 variable region in the 16S ribosomal gene was performed with a 454 GS-FLX (Roche, Basel,

Switzerland) on samples B1, B3 and B4 from the east Skaftárkatlar lake, a Kverkfjallalón sample from 4 m and the Skaftá river sample. Template was first verified by amplification using primers 967F and 1046R, the same used for our study of the west Skaftá lake (Supplementary Table S1). The primers were subsequently used for $5' \rightarrow 3'$ pyrosequencing, with the addition of the linker sequence plus a unique 4-base 'barcode' to the forward primer for parallel sample sequencing. Tags shorter than 60 nt were discarded when trimmed by the GS FLX software. The resulting v6 libraries comprise 59 772 reads. Two sets of reactions targeting the v3–v5 and v4–v6 regions of the bacterial 16S rRNA gene were performed, and pyrosequencing carried out on a 454 GS-FLX with Titanium chemistry as part of the Census of Deep Life (Sogin *et al.*, 2010). Details of library construction are given in the Supplementary Information. New primers (Supplementary Table S1) were designed to capture $>95\%$ of known eubacterial diversity based on the SILVA version 106 database (Pruesse *et al.*, 2007). They match 50–100% of GenBank 16S sequences from members of all known phyla, with the exception of a few small phyla such as OP11 and SR1. Sequencing was from the v3 and v6 ends of the respective libraries. Raw sequences were filtered, trimmed and matched to a reference sequence database constructed from SILVA by the VAMPS pipeline (Huse *et al.*, 2010). The distribution of v3–v5 read lengths has peaks at 415 and 440 nt, and that of v4–v6 reads peaks at 475 and 487 nt. After quality control and removal of likely non-ribosomal interlopers, pyrosequencing runs on east lake extracts yielded 150 586 v3–v5 reads and 147 979 v4–v6 reads. Filtering of chimeras reduced these to 150 586 and 147 959 reads, respectively. Statistics of individual libraries are provided in Supplementary Table S2. These data have been submitted to the NCBI Short Read Archive as SRA 050216, 050305, 050636 and 050638.

Pyrosequence analysis

Further sequence library analysis used MOTHUR version 1.22 (Schloss *et al.*, 2009, 2011), custom Perl scripts and the R statistical package (R Development Core Team, 2008). Raw v6 libraries were filtered of reads with lengths shorter than 50 nt, lengths longer than 110 nt (to remove dimers), ambiguous base calls or average call quality factor <30. The v3–v5 or v4–v6 sequences with GAST distances >0.25 are probably non-ribosomal and were removed. Unique v3–v5 and v4–v6 sequences were aligned to the Greengenes core alignment (DeSantis *et al.*, 2006). The v6 sequences were aligned to the SILVA bacterial alignment (Pruesse *et al.*, 2007) and those that started after position 31 189 or ended before position 3255 were removed. Missing or incompletely covered ends were filtered and non-unique sequences were again removed from the resulting alignment. Sequences were 'pre-clustered' to account for amplification and sequencing errors (Huse *et al.*, 2010). On the basis of an average 454 GS-FLX error rate of 1% (Gilles *et al.*, 2011) and the need to reduce sequences to a practical number, pre-clustering criteria of 1 nt difference for v6, 2 nt for v3–v5 and 4 nt for v4–v6 were adopted. Chimeras were identified and removed using Chimera Slayer (Haas *et al.*, 2011) and UCHIME (Edgar *et al.*, 2011), with the data sets themselves as the references. Calculation of distances between sequences penalized gaps by -1 and excluded end gaps. The v3–v5 and v4–v6 sequences were clustered into operational taxonomic units (OTUs) using a 3% average distance cutoff; we adopted a 10% cutoff for v6-based OTUs (Gaidos *et al.*, 2009). To compare α-diversities without regard to sampling effort, we constructed 1000 bootstrap libraries of each real library with the size of the smallest library, and the average and standard deviation of the abundance-based coverage estimator of species richness (Lee and Chao, 1994) were computed. The distinctiveness of libraries was determined using the integral form of the Cramer–von Mises test. Dissimilarity distances between libraries were calculated using UPGMA trees based on either Czekanowski–Bray–Curtis distances (Bray and Curtis, 1957; Czekanowski, 1909) or Horn–Morisita distances (Horn, 1966); bootstrap support was based on 1000 Monte-Carlo simulations of the data and a majority consensus tree constructed using PHYLIP (Felsenstein, 1989). Taxonomic assignments used the Ribosomal Database Project Bayesian classifier (Wang *et al.*, 2007) and bacterial and archaeal subsets of the reference database of Huse *et al.* (2010). We required 80% consensus of members for OTU classification.

Results

Physiochemical conditions

Temperature profiles of the east Skaftárketill lake are near 4 °C and about 0.2 °C above the maximum density temperature of pure water (Supplementary Figure S1). There is no indication of a colder bottom water mass as in the west lake (Jóhannesson *et al.*, 2007), but a single profile beneath borehole A contains some suggestion of this phenomenon. Like the west lake, all east lake samples were anoxic, with average sulfide (H_2S) of 16 p.p.m., and average dissolved CO_2 of 105 p.p.m. Dissolved solids totaled 250–300 p.p.m., with SiO_2 and Na the dominant constituents (mean concentrations of 100 and 30 p.p.m., respectively) (Supplementary Figure S2 and Supplementary Table S3). Iron and sulfate (SO_4) concentrations were lower than in the west lake, with mean values of 0.2 and 1.7 p.p.m., respectively. NO_2 was marginally detected (sample average of 46 p.p.b. above blank levels), but neither NO_3 nor PO_4 were detected above blank levels of $\sim$10 p.p.b.

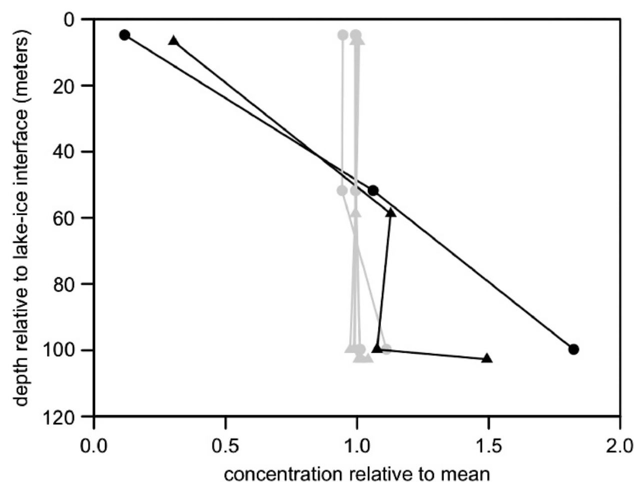

Figure 2 Profiles of sulfate concentration normalized to mean values (black lines) in the east Skaftárketill water column under boreholes A (circles) and B (triangles). Gray lines are profiles of normalized SiO_2, Na^+ and Cl^- concentrations, indicating that these biologically inert species are well mixed in the water column, and that sulfate is being rapidly produced and/or consumed. The mean concentrations of sulfate are 2.58 p.p.m. (**a**) and 0.96 p.p.m. (**b**).

The east lake appears uniform for most species (variation of a few percent), except for SO_4, H_2S and CO_2, which vary by 95%, 32%, and 14%, respectively. H_2S and CO_2 de-gas from samples retrieved from depth, but SO_4 does not. Sulfate concentration increases with depth under both boreholes and is higher at borehole A (Figure 2).

Biomass, cell and cultivable diversity
Cell counts and FISH results are summarized in the upper left panel of Figure 3. The bacterial cell concentration in the east lake samples average $4.4 \pm 2.2 \times 10^5\,ml^{-1}$ by microscopy ($4.5 \times 10^5\,ml^{-1}$ by flow cytometry). Exclusion of sample B4 (with only $1.5 \times 10^4\,ml^{-1}$) yields an average of $5.1 \pm 1.3 \times 10^5\,ml^{-1}$. Taken together, *Acetobacterium*-, *Sulfurospirillum*- and *Sulfuricurvum*-specific probes hybridized with 34–100% of all cells in each sample. Hybridizations with the *Paludibacter* probe were detected only in sample B4 and at low significance (average 1 cell per field). Growth was observed in all enrichments incubated at 3 °C but not in the negative control (Supplementary Table S4). Ribosomal sequences in clone libraries derived from enrichments of two samples (A3 and B2 in WN2 and Toga media, respectively) had 80–99% homology to sequences in clone libraries from the east lake samples. None of 17 isolates from the 3 °C lake enrichments (Supplementary Table S4) was affiliated with clone sequences from lake samples (Supplementary Table S4 and Supplementary Figure S3). Growth at higher temperature was observed only for enrichments from sample B4 (in C–J, A–J and 162-1 media at 60 °C, and in Toga, 162-1, C–J, A–J, WN2 and YPS media at 80 °C). Sequences in a clone library from the hot enrichment are related to cultivated or uncultivated thermophiles (Supplementary Table S5); 74% show 99% homology to the uncultivated bacterium SRI-280 originally detected in an Icelandic hot spring (Skírnisdóttir *et al.*, 2001) and affiliated with *Caldiserica* (formerly OP5) (Mori *et al.*, 2009).

Molecular (DNA-based) diversity
No polymerase chain reactions with archaeal primers yielded detectable product. Bacterial clone libraries from the east Skaftárkatlar lake are dominated by *Acetobacterium*- and *Sulfuricurvum*-related sequences, with a smaller number of clones related to *Geobacter*, *Sulfurospirillum* and *Desulfosporosinus* (upper right panel of Figure 3). The vast majority of pyrosequencing reads are also taxonomically affiliated with these five taxa (Figures 3 and 4 and Table 1). Community richness and evenness statistics based on our pyrosequence libraries are reported in Supplementary Table S6. Clustering with a cutoff of 3% average sequence difference assigns reads from our east lake v3–v5 and v4–v6 libraries to 321 and 1500 OTUs, respectively. Sequences from the aggregated v6 libraries from three east lake samples cluster into 154 OTUs (cutoff of 10%). More than 90% of reads in all of these libraries are assigned to the five dominant taxa (Figure 4 and Table 1). This is analogous to the statistics of the v6 library from the west Skaftárketill lake (Gaidos *et al.*, 2009), which was re-analyzed alongside the other v6 libraries. However, not all major taxa are the same (Figure 4). *Thermus*- and *Paludibacter*-related sequences constitute 9.7% and 8.3%, respectively, of the west lake library, but «1% of the east lake libraries, while *Geobacter*-related sequences are comparatively rare ($\sim$0.6%) in the west lake library.

The lake community is extremely uneven, as demonstrated by a rank abundance plot of the long-read OTUs (Supplementary Figure S4). This is also indicated by low values of the Shannon's H index (1–1.8) and high values of Shannon's D index (0.28–0.49) (Supplementary Table S6). Both ACE and Chao1 estimators predict that the actual OTU richness is several thousand, that is, 3–4 times the observed values, but rarefaction analysis shows that, despite the depth of sequencing, in none of the libraries has sampling reached saturation (Supplementary Figure S5). This is a result of the extreme unevenness of the taxonomic distribution: continued sequencing only yields more reads of the same dominant taxa.

The fractional abundances of the major OTUs in the pyrosequence libraries are in quantitative agreement (Supplementary Figure S6). However, the observed and inferred numbers of OTUs in all v4–v6 libraries (and their aggregate) are higher than in the respective v3–v5 libraries (Supplementary Figure S5 and Supplementary Table S6), despite

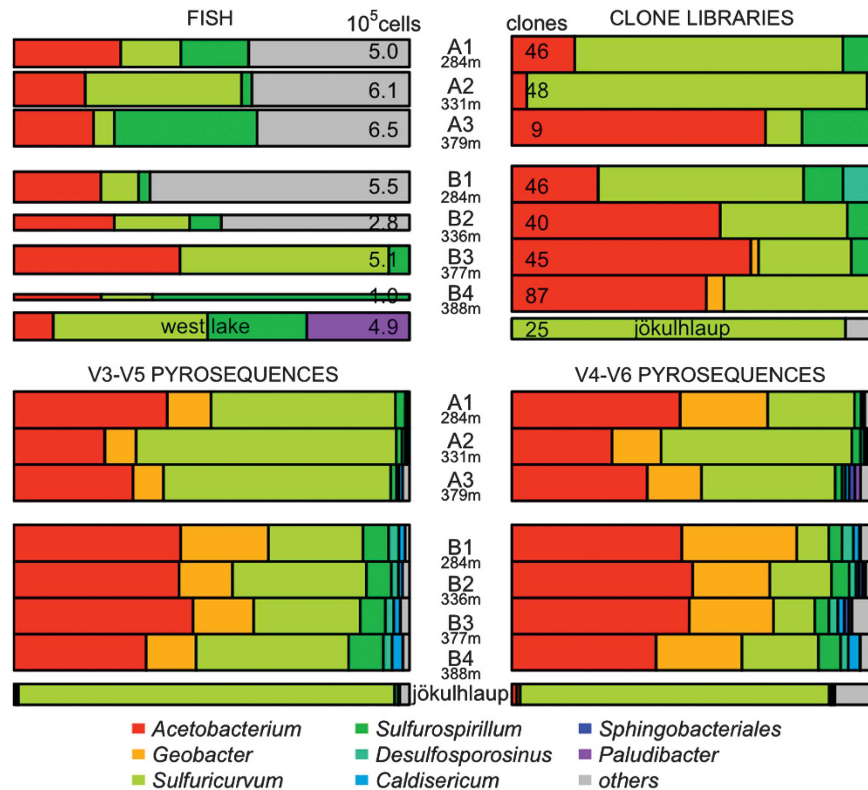

Figure 3 Taxonomic affiliations in seven east Skaftárketill lake samples (A1–B4) and a jökulhlaup sample. Clockwise from upper left: cells hybridizing with taxon-specific FISH probes relative to universal bacterial (EUB I-II-III) probes, OTUs in clone libraries, OTUs in v4–v6 pyrosequencing libraries and OTUs in v3–v5 libraries (the latter two defined by a 3% average sequence distance cutoff). Bars are labeled by the sample and the number below each label is its depth below the glacier surface (in meters). The number to the right of the FISH bars is the total cell counts in $10^5\,ml^{-1}$, and the height of each bar is proportional to the count. The number to the left of the clone bars is the size of the library. FISH was not performed on the jökulhlaup sample and data from the west Skaftárketill lake (Gaidos *et al*., 2009) are shown instead.

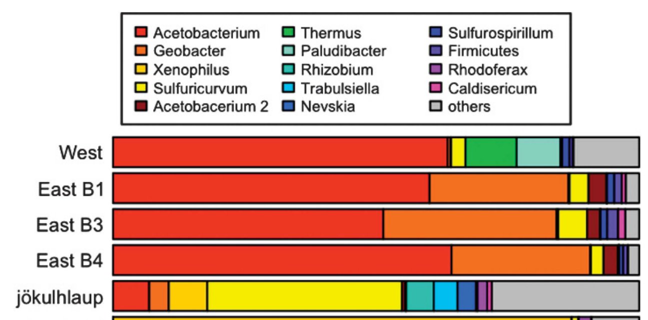

Figure 4 Composition of v6 pyrosequencing libraries for samples from the west and east Skaftárkatlar subglacial lakes, a jökulhlaup in the Skaftá river and the Kverkfjallalón subaerial lake. OTUs used was defined by a 10% average difference clustering criterion and their Bayesian classification used the VAMPS v.6 references database (Huse *et al*., 2008) based on the Silva taxonomy (Pruesse *et al*., 2007).

similar library sizes and a uniform clustering criterion. The dashed line in the inset of Supplementary Figure S4 shows that the v4–v6 excess is in low-abundance OTUs. However, richness estimates are sensitive to clustering criterion (Kim *et al*., 2011), and increasing the cutoff to 4% in the aggregate v4–v6 library decreases the number of

Table 1 Major taxa in summed analyses of east Skaftárketill lake samples (%)

Genus (Class)	*v3–v5*	*v4–v6*	*v6*	*Clones*	*FISH*
Acetobacterium (Clostridia)	36.3	42.7	62.6[a]	41.8	25.3
Sulfuricurvum (ε-proteobacteria)	41.7	23.1	3.6	48.0	22.0
Geobacter (δ-proteobacteria)	13.0	21.3	28.0	1.0	—[b]
Sulfurospirillum (ε-proteobacteria)	4.7[a]	3.4	1.1	7.9	19.6
Desulfosporosinus (Clostridia)	1.5	1.6	—[c]	1.2	—[b]

Abbreviations: FISH, fluorescent *in situ* hybridization; OTU, operational taxonomic units.
[a]Clustered into two OTUs (3% difference criterion).
[b]Not probed.
[c]Not detected.

OTUs to 615, while decreasing it to 2% in the v3–v5 library increases the number of OTUs to 1179. Evidence for differences between lake samples is marginal: variation in effort-adjusted ACE* between v3 and v5 libraries is not significant ($\chi^2 = 3.9$, $N = 7$), but that between v4 and v6 libraries is significant ($\chi^2 = 95.2$, $N = 7$) (Supplementary Table S6). Pyrosequencing libraries for a given locus are indistinguishable from one another based on Czekanowski–

Bray–Curtis distances (95% confidence). UPGMA trees constructed from Morisita–Horn distances between libraries finds relationships that are congruent between v3–v5 and v4–v6, and with strong bootstrap support (Supplementary Figure S7), there is no apparent relation with depth or water chemistry, and this may be an artifact of extraction efficiency.

Clone and long-read pyrosequencing libraries from the jökulhlaup sample are dominated by a single *Sulfuricurvum*-related taxon with ⩾90% of clones/reads and the unevenness of the abundance distribution is similar to the east lake. The observed diversity is higher than in the east lake, although the predicted richness is roughly the same. *Sulfuricurvum* is not nearly as dominant in the v6 library and the observed and predicted richness are intermediate of the two long-read libraries. The Kverkfjallalón v6 library consists mostly of a single taxon affiliated with genus *Xenophilus* in the Comamonadaceae (Betaproteobacteria). A heat map and dendogram based on Bray–Curtis distances between all the v6 libraries clearly demonstrates the similarity of the west and east lake communities and the distinctiveness of the jökulhlaup and Kverkfjallalón samples (Figure 5).

As expected, cultivable diversity is only a small fraction of total diversity in these environments. Only 13.7% (3%) of v3–v5 (v4–v6) OTUs have relatives (⩾97% identity) in clone libraries from our 3 °C anaerobic enrichments, and these OTUs represent only 1.0% (1.6%) of all reads in the pyrosequence libraries. In all, 82% (97%) of these v3–v5 (v4–v6) reads belong to a single *Desulfosporosinus*-related taxon. Conversely, all but 2 of the 62 clones from our enrichments have relatives in the pyrosequence libraries; these are affiliated with *Acetobacterium*, *Desulfosporosinus*, *Geobacter*, *Sulfurospirillum* and *Sulfuricurvum*. Nine (82) OTUs in our v3–v5 (v4–v6) libraries have relatives in the clone library from our 60/80 °C B4 enrichment, but these comprise only 1.2% (3.5%) of all reads. Nearly all (99.5% (97%)) such v3–v5 (v4–v6) sequences belong to a single OTU classified by the Ribosomal Database Project as *Caldiserica*. Isolates from cold enrichments (data not shown) represent only 0.36% (0.02%) of all reads in our v3–v5 (v4–v6) libraries.

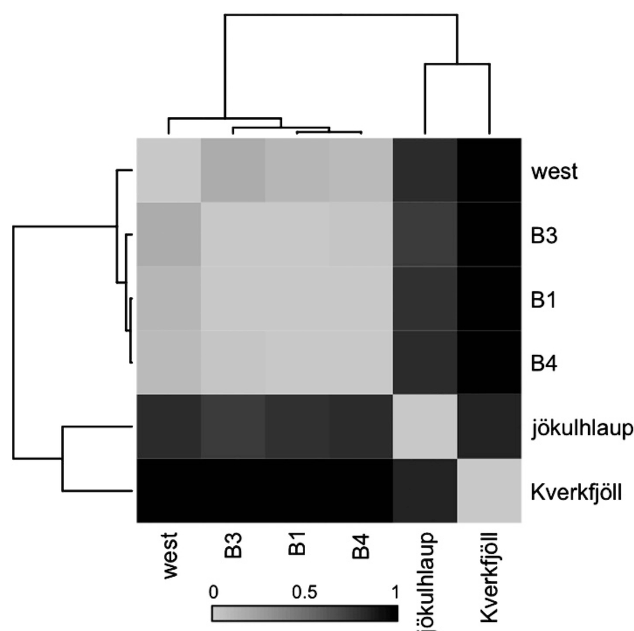

Figure 5 Heat map and relational dendogram based on Bray–Curtis distances between v6 pyrosequence libraries for three samples from the east Skaftárketill lake, the west lake, a jökulhlaup sample and the Kverkfjallalón subaerial lake. Lighter-shaded squares indicate more similar pairs of libraries.

Discussion

Paired libraries of v3–v5 and v4–v6 pyrosequences, v6 pyrosequence libraries, clone libraries and FISH give a concordant description of the bacterial community in the east Skaftárketill lake (Figure 3 and Supplementary Figure S6, as well as Table 1). Our data also demonstrate that the east and west Skaftárkatlar lakes are dominated by a few bacterial taxa affiliated with the genera *Acetobacterium*, *Geobacter*, *Sulfurospirillum*, *Sulfuricurvum* and *Desulfosporosinus* (Figures 3 and 4). *Thermus*- and *Paludibacter*-affiliated sequences are major components of the west Skaftárketill library but not the east Skaftáketill libraries, although they are present. This difference may reflect actual variation in community composition in time or space, but it is based on a single west Skaftá sample.

There are also differences between v3–v5 and v4–v6 libraries generated from the same sample: The greater taxonomic richness of the v4–v6 libraries compared with the v3–v6 libraries (Supplementary Table S6) cannot be ascribed to library size (Kemp and Aller, 2004), and Schloss (2010) found that the observed number of OTUs increased along the 16S gene (v1–v9) at the same genetic distance (but see Kim *et al.*, 2011). Moreover, our v4–v6 primers may span greater taxonomic richness than the v3–v5 primers. The uncultivated TM7 division appears in our v4–v6 but not v3–v5 libraries, although ProbeCheck (Loy *et al.*, 2008) indicates this is not an artifact of primer coverage. These results caution against interpreting richness estimates based on a single locus.

Our failure to detect a significant archaeal population in these lakes stands in contrast to other subglacial environments in which archaea have been detected (Boyd *et al.*, 2010; Stibal *et al.*, 2012), and archaeal-dominated subsurface chemolithotrophic communities (Chapelle *et al.*, 2002). In all, 9 reads out of ∼382 000 in our bacterial pyrosequence libraries assigned to the Archaea may be a result of nonspecific amplification, and it is possible that archaea are present in a benthic community we have not yet sampled. Growth in 3 °C

enrichments demonstrates that some lake taxa are cold-adapted; we speculate that differential response to low temperature (Cavicchioli, 2006) and/or level of ambient energy (Valentine, 2007) might be responsible for the bacterial dominance in these lakes.

The Skaftárkatlar community differs markedly from those in the Kverkfjallalón lake and the jökulhlaup in the Skaftá river (Figure 4). The Kverkfjallalón water column is dominated by a single affiliate to *Xenophilus* (Comamonadaceae), a group of aerobic soil betaproteobacteria that are widespread in subglacial environments (Yde *et al.*, 2010). With one exception, the Kverkfjallalón pyrosequence library is devoid of the major Skaftárkatlar taxa, while the jökulhlaup assemblage is a mix of the two communities (Figures 3 and 4). The *Sulfuricurvum*-affiliated taxon is ubiquitous in our Vatnajökull samples (Table 1). The cultivated representative of this genus is a microaerobic sulfur-oxidizing lithoautotroph (Kodama and Watanabe, 2004), traits that may promote its widespread distribution. The genome sequence should provide clues about this organism's metabolic versatility (Lucas *et al.*, 2010).

Phylogenetic analysis of sequences in respective clone libraries from our samples reveals that the major Vatnajökull OTUs are each other's closest known relatives (Figure 6). This points to efficient contact between the Skaftárkatlar and Kverkfjallalón lakes, despite their separation. Contours of the water driving potential at the glacier bed show the lakes to be separated by barriers exceeding 50 m and there is no direct contact between them at the glacier bed (Figure 7). We speculate that exchange occurs by movement of groundwater through the permeable tephra and basalt that underlies western Vatnajökull (Flowers *et al.*, 2003). Unattached cells are readily transported through basalt (Entry and Farmer, 2001; Marteinsson *et al.*, 2001). The high similarity of *Sulfuricurvum* sequences from all of our samples and enrichments suggests that it derives from a single population beneath Vatnajökull. We propose that these subglacial lakes were colonized from a deeper aquifer, rather than from the overlying ice, and that the Skaftárkatlar community was selected from this deeper biota by factors such as anoxia, low temperature, high hydrogen and the slow introduction of oxygen. Successful enrichment of thermophiles from samples and identification of thermophile relatives supports this hypothesis, but a direct investigation that samples the aquifer beneath Vatnajökull is needed.

We speculate that the dominant bacterial taxa in the oligotrophic Skaftárkatlar lakes fill niches in a chemolithotrophic ecosystem that includes sulfur cycling and is fueled by H_2, CO_2, Fe(III) and O_2 (Figure 8). On the basis of the physiologies of cultivated relatives, we propose that the *Acetobacterium*-related taxa are homoacetogens, combining CO_2 and H_2 into acetate (CH_3COOH).

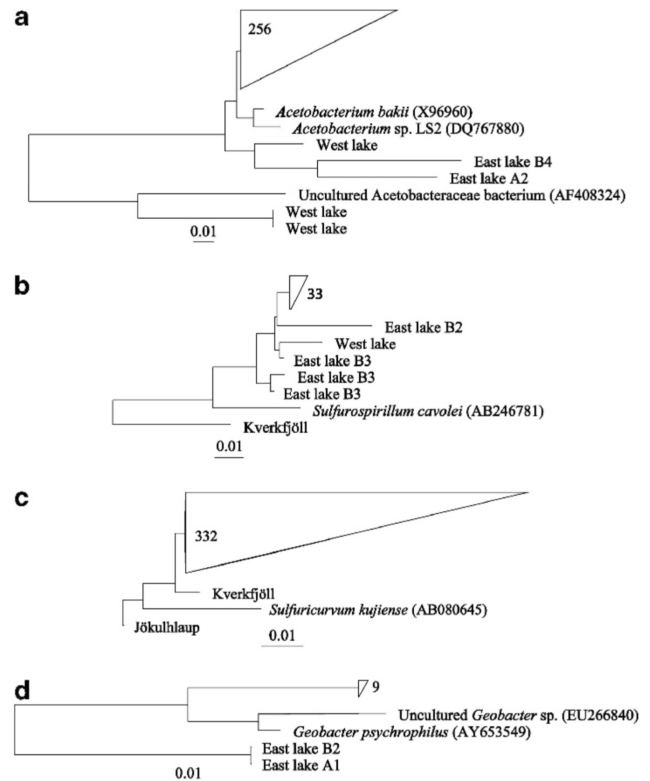

Figure 6 Neighbor-joining trees of sequences from our Iceland 16S rRNA clone libraries, databases and cultivates showing phylogenetic relationships within OTUs affiliated with (**a**) *Acetobacterium*, (**b**) *Sulfurospirillum*, (**c**) *Sulfuricurvum* and (**d**) *Geobacter*. The scale bar represents the expected % of substitutions per nucleotide position and a marine crenarchaeon was used as an outgroup. The cluster in *Acetobacterium* represents 256 clones with >99% sequence similarity (137 and 119 from the west and east Skaftárkatlar lakes, respectively). The cluster in *Sulfurospirillum* contains 25 west and 8 east lake clones with >99% similarity. The cluster in *Sulfuricurvum* contains 84 west lake (17 from enrichments at 3 °C), 196 east lake, 22 jökulhlaup and 30 Kverkfjallalón lake clones with >99% similarity. The cluster in *Geobacter* contains 1 west and 8 east lake clones with >99% similarity.

Homoacetogens can outcompete methanogens at low temperature and high H_2 concentration (Kotsyurbenko *et al.*, 2001; Drake *et al.*, 2002). We also propose that the *Geobacter* taxon carries out ferric iron reduction using H_2 as its energy source (Nevin *et al.*, 2005). Further, that the relative of *Sulfuricurvum kujiense* oxidizes sulfide, elemental sulfur (S^0) and thiosulfate (S_2O_3) at low oxygen concentrations (Kodama and Watanabe, 2004). We propose that the *Desulfosporosinus* member uses H_2 to reduce sulfate, thiosulfate or sulfite (SO_3^-), to elemental sulfur (Ramamoorthy *et al.*, 2006; Vatsurina *et al.*, 2008; Lee *et al.*, 2009; Alazard *et al.*, 2010; Abicht *et al.*, 2011), whereas the *Sulfurospirillum* member completes the sulfur cycle, reducing elemental sulfur, thiosulfate and sulfite, to sulfide (Finster *et al.*, 1997; Stolz *et al.*, 1999; Jensen and Finster, 2005; Kodama *et al.*, 2007).

Future investigations of the Vatnajökull lakes can test these hypotheses by measuring concentrations

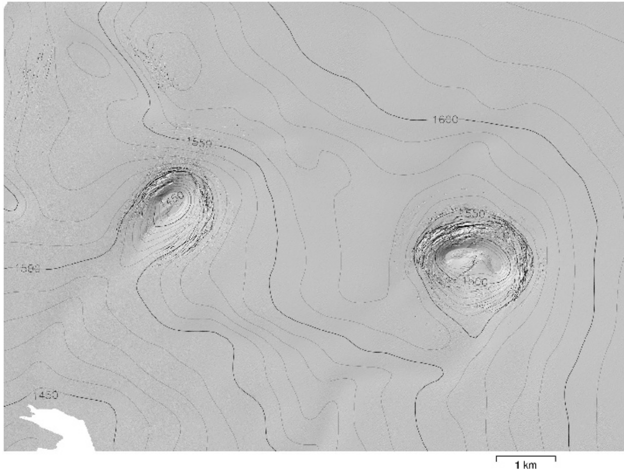

Figure 7 Contours of the water driving potential (in meters) (Cuffey and Paterson, 2010) derived from a 2010 LiDAR map of Vatnajökull in the vicinity of the Skaftárkatlar lakes superimposed on a hillshade of the ice surface shortly after both lakes were drained in jökulhlaups (data from the Icelandic Meteorological Office). North is up, the map is about 10 km across, and the whiter area is missing data. The calculations use the bedrock geometry of Björnsson and the water levels in boreholes to the lakes in 2006 and 2007 as reference values. The lakes are separated by a potential barrier exceeding 50 m in height, and independently drain through subglacial channels to the southwest. Another barrier exceeding 100 m separates these from the Kverkfjallalón lake 40 km to the northeast. The same features appear in a map constructed from a 1998 aerial SAR survey (data from the Institute of Earth Sciences) when the Skaftárkatlar lakes were nearly full (data not shown).

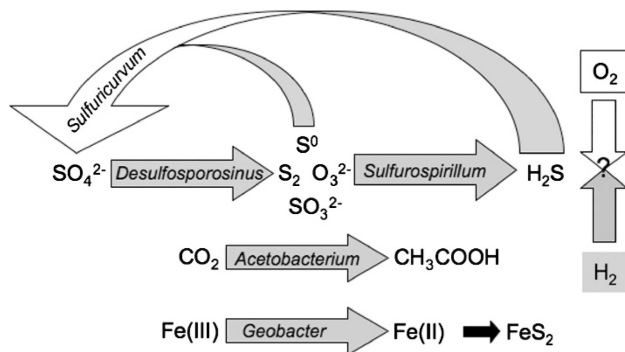

Figure 8 Schematic of possible chemolithotrophic pathways in the Skaftárkatlar lakes and proposed assignments of the major taxa based on established metabolisms of cultivated relatives. White pathways consume O_2; gray pathways consume H_2. Fe(II) will rapidly react with sulfide to form insoluble pyrite (Stumm and Morgan, 1996). No taxon has been associated with the knallgas reaction (of H_2 with O_2).

of relevant substrates, determining rates of activity and establishing the physiologies of the major taxa. Sulfate exhibits a concentration gradient in the east lake (Figure 2), indicating that it is produced and/or consumed more rapidly than the lake mixes. However, in addition to biological sulfur cycling, sulfate can be formed by abiotic sulfide oxidation and SO_2 disproportionation, and removed by dilution with glacial melt. The Skaftárketill lakes contain volcanic CO_2 and presumably Fe(III) (in mafic minerals), and we are also reasonably certain that O_2 enters the lakes as the overlying ice shelf melts but have not detected it, probably because it reacts with sulfide to sub-micromolar levels on the ~1-year residence time of the lakes (Jóhannesson *et al.*, 2007). Although, we expect H_2 from serpentinization and we detected it in the west lake, we have been unable to quantify this sparingly soluble gas because of loss to bubbles during depressurization of retrieved samples. *In situ* profiling of O_2 and H_2, the former at nanomolar sensitivity (Revsbech *et al.*, 2009), should be a high priority of future investigations.

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