

DNA signature of thermophilic bacteria from the aged accretion ice of Lake Vostok, Antarctica: implications for searching for life in extreme icy environments

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Abstract: We have used 16S ribosomal genes to estimate the bacterial contents of Lake Vostok accretion ice samples at 3551 m and 3607 m, both containing sediment inclusions and formed 20 000–15 000 yr ago. Decontamination proved to be a critical issue, and we used stringent ice chemistry-based procedures and comprehensive biological controls in order to restrain contamination. As a result, up to now we have only recognized one 16S rDNA bacterial phylotype with confident relevance to the lake environment. It was found in one sample at 3607 m depth and represents the extant thermophilic facultative chemolithoautotroph *Hydrogenophilus thermoluteolus* of beta-Proteobacteria, and until now had only been found in hot springs. No confident findings were detected in the sample at 3551 m, and all other phylotypes revealed (a total of 16 phylotypes, 336 clones including controls) are presumed to be contaminants. It seems that the Lake Vostok accretion ice is actually microbe-free, indicating that the water body should also be hosting a highly sparse life. The message of thermophilic bacteria suggests that a geothermal system exists beneath the cold water body of Lake Vostok, what is supported by the geological setting, the long-term seismotectonic evidence from ⁴He degassing and the '18O shift' of the Vostok accretion ice. The seismotectonic activity that seems to operate in deep faults beneath the lake could sustain thermophilic chemolithoautotrophic microbial communities. Such a primary production scenario for Lake Vostok may have relevance for icy planets and the approaches used for estimating microbial contents in accretion ice are clearly relevant for searching for extraterrestrial life.

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Introduction

Lake Vostok is the Earth's largest subglacial lake among more than 70 subglacial water bodies so far detected in Antarctica (Kapitsa *et al.* 1996; Siegert 2000, 2001). It has been buried beneath a thick ice sheet for at least 15 million years (Barrett 2003) and by this raises exceptional interest for a search for organisms in a location that has been isolated from the Earth's surface for a very long period. The lake represents

an extreme environment with a pressure close to 400 atmospheres, an ultra-low nutrient supply from the glacier melt, an ambient water temperature close to freezing (i.e. -2.5°C), and a possible excess of oxygen and nitrogen (Lipenkov & Istomin 2001; McKay *et al.* 2003). Subglacial environments are expected on the Jovian satellite Europa, but as an ocean covered by an ice shell (Carr *et al.* 1998; Pappalardo *et al.* 1999) making Lake Vostok a terrestrial analogue.

A 3623 m Vostok ice core was drilled, mainly for paleoclimate studies (Petit *et al.* 1999). The bottom 80 m of the core is within a 215 m thick ice accretion layer, and the 3538–3623 m

* Deceased 8 September 2002.

Table 1. *Ice core samples studied*

Sample (#)	Depth interval (m)	Physical characteristics	Initial weight (g)	Amount removed (%)	rDNA clone library
Glacial ice core #3001 (F)*	3000.00–3000.50	Poly-crystalline ice	103.0	53.4	3001
Accretion ice core #3551 (F)	3550.49–3550.89	Mono-crystal with 2–3 mm sediment inclusion	35.0	38.0	3551
#3607 (F)	3606.00–3606.54	Mono-crystal with 5–6 mm sediment inclusion	22.0	55.0	3607
#3607b (F)	3606.00–3606.54	Three-crystal boundaries	40.3	61.0	3607b
#3607c (RU)	3606.545–3606.995	Mono-crystal	137.0	37.2	3607c

* French (F) or Russian (RU) allotment.

horizon is derived from the lake water (Jouzel *et al.* 1999; Masolov *et al.* 2001), which provides ice core samples that currently offer the best template of the lake water contents.

Previous studies by microscopic observation, organic chemistry, DNA amplification, metabolic activity detection or culturing suggested that Lake Vostok might host life (Karl *et al.* 1999; Priscu *et al.* 1999; Abyzov *et al.* 2001; Christner *et al.* 2001). However, a major uncertainty in Lake Vostok geomicrobiological studies is the extent of ice core contamination by the drilling fluid and core handling (Vincent 1999, 2000). Due to the very low biomass in Vostok ice samples (Bulat *et al.* 2002, 2003), searching for life there has proved to be constrained by a high chance of contamination in a similar manner to the forward-contamination¹ which can occur when searching for life in very poor media in terms of the planetary protection policy (Space Studies Board 2000; Rummel 2001).

Therefore, confidence in biological ice core findings increases in value as long as: (i) the ice chemistry meets the standard for low-concentration measurements of polar ice; (ii) the molecular biology meets the standard of forensic science; (iii) the biological findings differ from those from the various environments in contact with the ice core; (iv) the biological findings are discussed with respect to the expected conditions of subglacial environments. A multidisciplinary approach to such a study is therefore the obvious imperative.

The stringent decontamination techniques currently used in ice core chemistry allow for reliable measurements of major ions as well as the trace elements (Boutron *et al.* 1994; Legrand & Mayewski 1997) present in ice for central Antarctica at concentration levels of 10^{-9} and 10^{-12} g/g, respectively. In this work, we have applied a rigorous ice-chemistry-based decontamination procedure for Antarctic ice core samples to produce the most reliable chemical composition for major ions. For the samples that have satisfactorily passed the chemical control, we have implemented numerous and comprehensive biological controls to avoid misleading results.

We report here the results of a 16S ribosomal gene study in estimating the bacterial contents of two samples of the aged Lake Vostok accretion ice at 3551 and 3607 m.

¹ Accidental contamination of other worlds with microbes brought from Earth.

Materials and methods

Vostok ice core samples

The Vostok ice core samples studied were taken from accretion ice (at 3551 and 3607 m) and one sample was taken from glacial ice (at 3001 m) for comparison. The accretion ice horizon was found to contain small visible inclusions of sediment trapped when the glacier moved across a shallow embayment upstream of the Vostok site (Jouzel *et al.* 1999; refer also to Fig. 3 below).

The age of the glacial ice sample is about 300 000 yr (Petit *et al.* 1999). The accretion rate is about 1 cm annum⁻¹ (Petit 2003; Petit *et al.* 2003), and at Vostok the total accretion ice is about 215 m. These results in the samples #3607 and #3551, which are located at ca. 150 and 200 m from the water–ice interface, are ~15 000 and 20 000 years old, respectively.

Table 1 indicates the physical and decontamination characteristics of the selected samples and the generated rDNA clone libraries.

Ice core decontamination procedure

As a first step, ice cutting and surface cleaning were performed in a cold room (–15 °C) using an ethanol treated saw. Then, pre-treated ice samples were washed in triple step-by-step with exceptionally pure water <2 ppb C, ELGA PURELAB Maxima[®] with a 5 kDa cutoff ultra filter (Vivendi Water Systems Ltd, UK) and melted in clean sterile polycarbonate straight-sided wide-mouth jars (Nalgene) at room temperature. Core washing was carried out within a class 100 laminar hood (EU-ISO-5) in a class 10 000 (EU-ISO-7) dust-free laboratory and the laboratory personnel used surgical supplies (sterile cloths, gloves, masks and shields). Surfaces and tools were cleaned with decontamination solutions (e.g., Proline Biocontrol, Biohit).

Water aliquots were analysed by ion chromatography (Legrand *et al.* 1993). For dissolved organic carbon (DOC) measurements, ice core sub-samples were decontaminated by controlled fractional melting and consecutively analysed using a dedicated flow injection system coupled to a UV-based combustion/CO₂ monitor unit (Ultra TOC Astrolab), which featured a detection limit of less than 5 ppb C.

The ice samples were prepared in a different building to that used for DNA extraction and PCR analyses in order

to avoid any impact of PCR products on samples. The class 10 000 dust-free laboratory was never used for DNA work.

DNA extraction, 16S rDNA amplification, cloning, and sequencing

The melt water of samples #3001 and #3607b were concentrated 780 and 2000 times, respectively, by centrifugal filtering through 3 kD membranes (Pall Gelman and Millipore) and DNA was extracted (QIAamp DNA Mini kit, Qiagen). For samples with inclusions (#3551 and #3607), DNA extraction was performed directly from the sediment material containing melt water that was collected by centrifugation.

A semi-nested broad-range bacterial 16S ribosomal DNA PCR was performed for 28 cycles with the 6Fb–787Rb primer pair (10 s at 93 °C, 40 s at 55 °C, 60 s at 72 °C with the first DNA melting at 93 °C for 3 min) followed by 25 cycles with the 338Fb–787Rb primer pair under the same conditions (except for the final elongation step, 10 min at 72 °C) using 0.3–0.5 µl aliquots of primary PCR amplification. The universal primers were adapted from Godon *et al.* (1997), Teske *et al.* (1996), Muyzer *et al.* (1993) and McGregor *et al.* (1996) to cover representatives of most bacterial phylums: 6Fb (5'-GAAGAGTTTGATCCTGGCTC-3'), 338Fb (5'-ACHCCTACGGGDRGCGCA-3') and 787Rb (5'-WGGACT-ACCIGRGTHTCTAAT-3').

The clones were only 405–430 bases in length because it was impossible to amplify full-sized 16S rRNA genes using different DNA polymerases and primers. This may be indicative of a very small amount of DNA or its ancient status (Cooper & Poinar 2000) due to possible damage by, e.g., oxygen content in the lake (Lipenkov & Istomin 2001). It is worth noting that the DNA was extracted from melt water concentrated about ~1000 times and we were nevertheless forced to use a very sensitive semi-nested PCR scheme to be able to generate signals. One-round PCR did not work, whilst PCR inhibitor tests performed with *E. coli* genomic DNA added to the original ice DNA samples were always negative.

PCR products embracing the V3 region of 16S rRNA genes were cloned into the TOPO TA (Invitrogen) or pGEM-T Easy (Promega) cloning vectors and the corresponding rDNA libraries were constructed (Table 1). After plating, up to 100 positive transformants per library were screened for inserts by PCR using flanking vector primers. The PCR products were cleaned with a kit (QIAquick PCR Purification Kit, Qiagen) and sequenced. A total of 128 405–431 base pair sized amplicons from ice sample libraries were sequenced. From these, seven chimeric sequences were detected both by visual inspection of corresponding alignments and by the Ribosomal Database Project II Chimera Check online tool (<http://rdp.cme.msu.edu/cgiis/chimera.cgi?su=SSU>).

Preliminary control experiments performed by *E. coli* cell counting followed by PCR extinction showed that the detection limit of our PCR schemes was equivalent to ca. 5–8 cells ml⁻¹ of melt water.

In testing for Archaea, the same semi-nested ribosomal DNA PCR approach was implemented. The primers Ar4Fb (5'-TTCYSGTTGATYCYGCSRGA-3'), Ar347Fb

(5'-GGYGCASCAGGCGCGAA-3'), 958Rb (5'-YCCG-GCGTTGAMTCCAATT-3') and 1114Rb (5'-GGGTCTCG-CYYGTTDCC-3') were adapted to cover representatives of the four known phylums of Archaea (Delong 1992; Burggraf *et al.* 1997; Huber *et al.* 2002) and tested in two different schemes: Ar4F–1114R primer combination followed by Ar4F–958R; and Ar4F–958R primer combination followed by Ar347F–958R. None of the schemes gave PCR products.

The sequences reported in this paper have been deposited in the GenBank database (accessions AF532058, AF532060, AF532061).

Precautions and controls

The five types of control for contaminant cells and dissolved DNA, inspected with PCR and DNA sequencing, were as follows. (i) Sham (negative) DNA extraction: (a) all reagents, spin filters and tubes (DNA extraction performed with PCR grade water (Invitrogen)); (b) TE buffer (ready-to-use, Sigma) made with Molecular Biology grade water (MoBio) and used for DNA elution. (ii) Negative PCR: PCR reagents including DNA polymerases that generate PCR products with no template DNA added. (iii) Ice core wash water. (iv) Original Vostok drilling fluid from different depths in the borehole. (v) Laboratory environment: dust collected with sterile tampons and soaked with ice core wash water or Molecular Biology grade water (MoBio) (in both the French and Russian biological laboratories).

For all five controls, corresponding rDNA libraries were constructed as described above and a total of 224 expected-size clones were sequenced (of these, nine sequences were discarded as chimeric). By this procedure, a contaminant database was established. Separate contaminant datasets included 21 from the washing water, 118 from the laboratory environment, 31 from the drilling fluid, and 27 and 18 from negative PCR and sham DNA extraction, respectively. In addition, any known contaminant clones deposited into public databases (e.g., by Norman Pace) or published elsewhere (e.g., Tanner *et al.* 1998; Cisar *et al.* 2000; Kawai *et al.* 2002; Kulakov *et al.* 2002) were employed.

Bacterial species delineation was conducted more strictly than recommended (Stackebrandt & Goebel 1994) because we worked on one-third of the rRNA gene sequence and a maximum of 2% sequence divergence was used to restrict overestimation of species limits.

Phylogenetic analyses

Neighbouring sequences were determined with the BLAST (Altschul *et al.* 1997) and FASTA (Pearson 1990) programs from public databases (GenBank). Sequences were aligned using the Clustalw program (Higgins *et al.* 1994).

Phylogenetic trees were estimated using the maximum parsimony, the minimum evolution (LogDet/paralinear distance parameter correction) and the maximum likelihood criteria in PAUP (version 4.0b10) (D. Swofford, Sinauer Associates). The trees obtained were analysed with the Kishino–Hasegawa test in order to select the best, which were then consequently optimized under the maximum likelihood criterion.

Bootstrap values were obtained by the maximum-parsimony method on 100 replicates with random-order sequence additions.

For phylogeny reconstruction, aligned sequence blocks were fully cleaned for deletions or insertions, unassigned bases and ambiguous local alignments. Species limits depicted in a tree were defined using 3% sequence divergence (0.03 substitutions per site) (Stackebrandt & Goebel 1994).

Results and discussion

The ice chemistry

Major ion and light carboxylic acid concentrations are sensitive to gaseous contamination from the laboratory environment (Legrand *et al.* 1993) and served as a first control for our decontamination procedures for the samples prior to molecular biological studies. For the lower part of the Vostok ice core (>3520 m), an initial reference profile of major ion and light carboxylic acid concentrations of glacial and accretion ice samples revealed very low contents of NH_4^+ (0.1–4 ppb), NO_2^- (<0.1 ppb), acetate (0.5–2 ppb) and formate ions (<0.5 ppb), which are consistent with those previously found in other Antarctic cores (Legrand & Saigne 1988). In addition, the concentration of DOC, which is influenced by the presence of kerosene from the drilling fluid, was shown to be low and similar in both glacial (24 ± 14 ppb C) and accretion (17 ± 7 ppb C) ice, indicating clean samples. These data are consistent with the low carbon contribution from the light carboxylic acids and the values lie well below other data reported for accretion ice (80–500 ppb C) (Karl *et al.* 1999; Priscu *et al.* 1999). The samples studied here for biology contained 10.7 ppb C (glacial ice at 3001 m) and 12.5 ppb C (accretion ice at 3607 m). The DOC content of the accretion ice at 3551 m was not determined.

The biological aspect

As shown earlier, the decontamination procedure that we followed is reliable for ice core chemical analyses (e.g., major ions). However, even when using ultra-pure water the core washing may be not efficient enough for removing all biological aliens (e.g., Kawai *et al.* 2002; Kulakov *et al.* 2002). In addition, commercial DNA extraction and PCR enzymes, reagents and disposable plastic articles suitable for use under low biomass conditions are not yet always certified DNA-free (e.g., Corless *et al.* 2000; Millar *et al.* 2002; Grahn *et al.* 2003). That is why we have established our own contaminant database and used DNA sequencing approach in order to decipher potential contaminants from the original ice signals as confidently as possible.

Ice core findings against contaminant database

The water used for ice washing, the original drilling fluid, the biological laboratory environment, sham DNA extraction and negative PCR controls were all monitored using the established contaminant database. Bacterial taxa identified by 16S ribosomal DNA sequences in ice samples were indexed on the basis of six criteria, of which the contaminant database forms

Table 2. Indexing contaminant criteria for bacteria recorded in Vostok ice core

Key Score	Regulation
A*****	Taxa recorded in our contaminant database (refer to Methods). A1 – Ice core wash water, A2 – Original Vostok drilling fluid, A3 – Laboratory environment.
B****	Taxa proved to be contaminants (e.g., Tanner <i>et al.</i> 1998; Cisar <i>et al.</i> 2000; Kawai <i>et al.</i> 2002; Kulakov <i>et al.</i> 2002).
C***	Bacteria able to metabolize aliphatic and aromatic hydrocarbons (present in e.g. kerosene).
D***	Human/animal/plant saprophytes, commensals and pathogens (and those associated with human activity including food, etc.).
E*	Taxa revealed in wastewater, activated sludge, contaminated aquifer etc. (linked to human activity).
F*	Taxa revealed in other glaciers. In the present study F1 – GenBank accession No. AF479378, Taylor Dome, Antarctica, F2 – No. AF479381, Taylor Dome, Antarctica, F3 – No. AF479368, Sajama, Bolivia.

* low Confidence, *** confident, **** high confidence, ***** highest confidence.

a powerful but singular decisive factor (Table 2). Since the biological composition of Lake Vostok is hard to presume, a degree of confidence was introduced into the table allowing us to discard obvious cases with the highest score as contaminants, while those with lowest score refer to likely contaminants.

A total of 215 rDNA clones from various controls and 121 clones from both accretion (#3551:6 and #3607:26, #3607b:53, #3607c:16) and glacial (#3001:20) ice were kept for further analyses. The ice clones showed high redundancy resulting in the recognition of twenty 16S rDNA phylotypes, of which 16 were seen in the accretion ice and three of these were also found in the glacial ice. We only discuss the question of accretion ice findings here. Most phylotypes in accretion ice had close GenBank homologues (>98%), i.e. were identified or assigned to known sequences, but three were distinctly different from their nearest neighbours (<96% similarity). Thirteen of the 16 phylotypes in accretion ice were indexed as contaminants and two extra phylotypes were regarded as additional likely contaminants because of their uncertain status (Table 3).

No species passing the contamination test were found in both the glacial and the accretion ice, suggesting a small probability of microbes transiting from the 1 000 000 year old (Salamatina *et al.* 2003) glacier melt water into the lake and becoming refrozen in accretion ice (Siegert *et al.* 2001). In addition, it is worth noting that we have no confident findings from glacier ice sample #3001 (Bulat *et al.*, unpublished) possibly due to the presence of dissolved oxygen in the ice matrix (about $1.0 \text{ O}_2 \text{ mg l}^{-1}$) (Ikeda *et al.* 2000; Lipenkov *et al.* 2002).

It is of interest that amongst the contaminants that we found in the ice (as proved by our contaminant database and N. Pace negative DNA extraction control AF058372), (Table 3, F1–F3), some showed a high similarity (up to 100%) to a sequence with bacteria previously published from a 3593 m core (Table 2, key F).

Table 3. Bacterial 16S rDNA clones recovered from Vostok ice core and presumed to be contaminants

Number of allelic clones ^a in rDNA clone library					Closest neighbours in GenBank	Contamination criterion	Total score ^b
3607	3607b	3607c	3551	3001			
Alpha-Proteobacteria							
3			1		100% <i>Sphingomonas paucimobilis</i> (U37337)	A3, C, F1	9
	3				98–100% <i>Sphingobium yanoikuyae</i> (AY047219)	A3, C	8
1	8				99–100% <i>Sphingomonas natatoria</i> (AB024288)	A2, F2	6
3				4	99–100% Uncultured bacterium D from beetle larva intestine (AJ459874)	D	3
	2				99.8% Uncultured bacterium clone E6 from shower curtain biofilms (AY268237)	D	3
Beta-Proteobacteria							
	2				97% rDNA clone pACH94 (AY297809) 96% <i>Rhodocyclus sp.</i> R6 (AJ224937), rDNA clones (AF204245), (AF502232), (AF204244)	Linked to E Likely contaminant (AF532058)	NA ^c
Gamma-Proteobacteria							
1	4		1		98–99% <i>Acinetobacter johnsonii</i> (X89775)	A3	5
		2			99% <i>Pseudomonas rhodesiae</i> (AF064459)	B	4
		6			98% <i>Haemophilus parainfluenzae</i> (M75081)	D	3
Delta-Proteobacteria							
	1		1	1	92–93% <i>Chondromyces apiculatus</i> (AJ233938), <i>C. robustus</i> (AJ233941)	A3	5
Actinobacteria							
	1			3	98% <i>Micrococcus luteus</i> (AJ409096)	B, F3	5
1					100% <i>Brevibacterium iodinum</i> (X83813), <i>B. epidermidis</i> (X76565)	D	3
		1			99–100% <i>Microbacterium sp.</i> oral clone AV005b (AF385527)	D	3
Firmicutes							
	2				99% <i>Abiotrophia defectiva</i> (D50541)	D	3
OP11 Candidate division							
3					91.7% Uncultured soil bacterium C011 (AF507682)	Linked ^d to A1 and E ^e Likely contaminant (AF532061)	NA ^c

^a Differs by 1–5 nucleotides within a phylotype.

^b Refer to Table 2.

^c Non applicable.

^d Both, ice clones and 'A1' clone, showed the same low level similarity to soil bacterial rDNA clones of one location (Dunbar *et al.* 2002; Kuske *et al.* 1997; Swaty *et al.* 1998).

^e Contaminated aquifer (N. Pace, unpublished).

Confident findings

Only one 16S rDNA phylotype (12 clones; two sequence types differing by one nucleotide substitution) successfully passed our contaminant database and other contaminant

screening criteria (Table 2). The sequences were all found in only one sample at a depth of 3607 m (#3607b). No confident findings were detected in ice core samples #3551, #3607 and #3607c.

Table 4. Taxonomic identification of clone LV3607bR-40A^a recovered from ice sample #3607b

Closest neighbours and their features						
GenBank ^b similarity (%)	Acc. No.	Representative	Sampling site	Temperature profile	Source	Refs
100	AB009828	<i>Hydrogenophilus thermoluteolus</i> TH-1	Hot springs, Izu district, Japan	52 °C ^c	Crustal fluid, adjacent soil	1, 2
99.5	AJ131694	<i>Hydrogenophilus hirschii</i> yel5a	Angel Terrace Spring, Yellowstone National Park, USA	50–67 °C	Crustal fluid	3
99.1	AB009829	<i>Hydrogenophilus thermoluteolus</i> TH-4	Hot springs, Izu district, Japan	50 °C ^c	Crustal fluid, adjacent soil	1, 2
96.3	AF026979	Unidentified beta-proteobacterium OPB30 (rDNA study only)	Obsidian Pool, Yellowstone National Park, USA	75–95 °C	Sediments	4

^a Representative of phylotype (AF532060, 12 clones, two sequence types differing by one nucleotide substitution).

^b Only full-sized and published 16S rDNA sequences are included in the analysis.

^c Temperature profile for culturing.

[1] Goto *et al.* (1977); [2] Hayashi *et al.* (1999); [3] Stohr *et al.* (2001); [4] Hugenholtz *et al.* (1998).

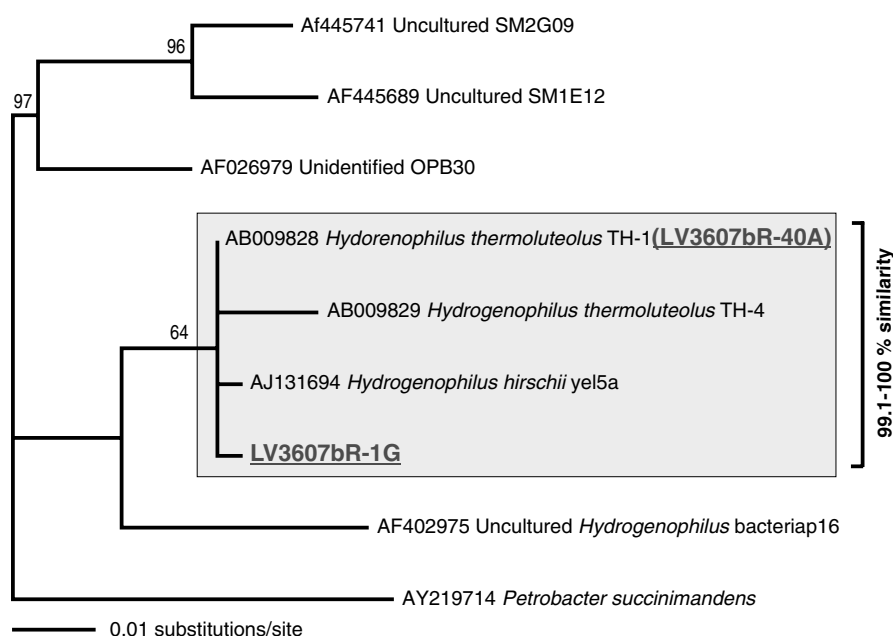


Fig. 1. Phylogenetic analysis of the bacterial rDNA clone LV3607bR-40A (a 428 nucleotide mask of unambiguously aligned positions) recovered from accretion ice sample #3607b (Table 4). The clone LV3607bR-1G represents the 2nd sequence type of the phylotype differing by one nucleotide substitution. Bootstrap values of more than 50% are shown at the nodes. Ice clones are in bold and underlined font styles. Species limit is depicted with transparent boxes. Clone 3607bR-40A displays a sequence identical to AB009828. (The image can be viewed in colour at <http://lgge.obs.ujf-grenoble.fr/axes/glaciobiology>.)

The phylotype depicted by the clone LV3607bR-40A represents the extant thermophilic bacterium *Hydrogenophilus thermoluteolus* (beta-Proteobacteria) that until recently was only encountered in hot springs in Izu district (Japan) with closely related species *H. hirschii* known for the hot springs of Yellowstone National Park, USA (Fig. 1, Table 4). Potentially, it is capable of growing as a chemolithoautotroph by oxidizing H₂ and reducing CO₂ (Goto *et al.* 1977; Hayashi *et al.* 1999; Stohr *et al.* 2001).

A sequence of rDNA ascribed to the genus *Hydrogenophilus* was also recently recovered by López-García *et al.*

(2003) from hydrothermal sediment at the Mid-Atlantic ridge. It was reported that this was the first time that members of this genus have been detected in deep-sea vents. In addition to the absence of *Hydrogenophilus sp.* in our contaminant database as well as from other known contaminant data (Table 2), the limited distribution of closest relatives of this species at the world scale (Table 4) provides an additional indication supporting its non-contaminant status.

It is worth noting that thermophilic bacterium *Hydrogenophilus thermoluteolus* was detected in a sample with crystal boundaries (#3607b), while analysis of extra mono-crystal

samples with (#3607) and without (#3607c) sediment inclusions did not give any confident signal. Because the bacterium we have found is a thermophilic species with an optimal growth temperature of around 50 °C (Goto *et al.* 1977; Hayashi *et al.* 1999), we explain its occurrence in the ice as an indication of the long-term concentration of microbes at the crystal boundaries by a freezing process, rather than the bacterium thriving in ice veins as suggested by Price (2000).

Unclassified findings

As mentioned above, three of the 16S rDNA phylotypes recovered in the Vostok ice core samples could not be identified because of their low similarity to sequences in GenBank. One species related to *Chondromyces sp.* of delta-*Proteobacteria* (Table 3) was also detected in our contaminant database, thus representing obvious contamination. However, two other phylotypes from different samples of accretion ice at 3607 m showed no clear counterparts amongst contaminants, but nevertheless they were regarded as likely contaminants (Table 3).

Phylogenetic analysis showed that the first phylotype (two clones; two sequence types differing by three nucleotide substitutions) is most related by a sequence to one uncharacterized clone detected in waterlogged archaeological wood (AY297809, 97% similarity) and polyphosphate-accumulating strains (96% similarity) of the *Rhodocyclus* group with phototrophic beta-*Proteobacteria* affiliation (Crocetti *et al.* 2000), but demonstrates no confident clustering with these strains (Fig. 2(a)).

Most phototrophic beta-*Proteobacteria* of the genera *Rhodocyclus*, *Rubrivivax* and *Rhodoferax* are mesophilic and neutrophilic freshwater bacteria with optimal growth temperatures of between 25 and 30 °C. The species of *Rhodocyclus*, *Rhodoferax* and *Rubrivivax* are facultative anaerobic bacteria that preferably grow under anoxic conditions by anoxygenic photosynthesis. All of the species grow photoheterotrophically and most of the species are able to grow photoautotrophically with hydrogen as a reducing power for CO₂ fixation. They all are able to grow aerobically to microaerobically in the dark by oxygen-dependent respiration (Imhoff 2003). However, because the phototrophic beta-*Proteobacteria* are common in stagnant waters (freshwater ponds, sewage ditches and activated sludge) that are enriched by organic compounds and nutrients and exposed to light (Imhoff 2003), it is unlikely that the phylotype recovered in ice is representative of lake biota although it has lower DNA G+C contents when compared with all tested relatives (about 53 and 56%, respectively) and by this may represent a separate lineage.

The second phylotype (three clones; three sequence types differing by two nucleotide substitutions each) provide a more complex story. It showed rather distant relationships with an uncultured bacteria of the OP11 candidate division and is confidently clustered with an rDNA PCR clone from a hot, dry, nutrient-poor sandy-loam (sandstone and limestone) soil environment near the Sunset Crater, Arizona, USA (91.7% sequence similarity) (Kuske *et al.* 1997; Swaty

et al. 1998; Dunbar *et al.* 2002; see also Fig. 2(b)). All of the closest representatives from GenBank are uncultured and distant relatives of each other and ice clones, thus leaving this bacterial finding unresolved in terms of its taxonomy, features and living conditions.

However, in our contaminant database (the ice core wash water dataset), one clone-phylogroup (E2-27) was detected which also demonstrated a low-score (85%) similarity to the same set of soil bacterial clones (Sunset Crater, Arizona, USA) and OD1 clones sampled in a contaminated aquifer (N. Pace, unpublished) while showing no evident relatedness to the ice phylotype (76–77% sequence similarity) (Fig. 2b). Nevertheless, because we are now increasingly aware of possible bacterial contamination, the presence of a contaminant phylotype that shares the same set of soil bacterial clones may be indicative of a contaminant status for the ice phylotype (Table 3).

Of the two distantly related phylotypes, the latter related to OP11 candidate division has a better chance of originating from the ice. However, due to the largely uncharacterized nature of microbial habitats in the biosphere, the environmental versatility of closely related species and the ubiquity of some microbial genera predictions of physiology based solely on 16S rDNA similarity, especially low similarity values, must be interpreted very cautiously.

Deliberations

Various bacteria previously discovered in Vostok accretion ice (Karl *et al.* 1999; Priscu *et al.* 1999; Abyzov *et al.* 2001; Christner *et al.* 2001) and identified by terminal restriction fragment length polymorphism (Priscu *et al.* 1999) and by 16S rRNA gene sequencing (Christner *et al.* 2001) have suggested the presence of life in Lake Vostok. In our approach we identified only one bacterium (12 clones; two sequence types differing by one nucleotide substitution) which successfully passed stringent contaminant criteria and, hence, can be considered as relevant to the accretion ice contents. This indicates that contamination, most from the laboratory environment (dust) as well as human sources (Table 3), even after rigorous decontamination procedures, is still an important source of foreign bacteria. This fact along with the inability to generate PCR products in one round of PCR indirectly suggests a very low number of cells and/or DNA amount in the Vostok accretion ice.

Our findings are of special interest because the bacterium we discovered represents a genuine thermophilic species. Taking into consideration the limits of any PCR approach (e.g., PCR bias in estimating species composition and diversity (Dunbar *et al.* 2002; Lueders & Friedrich 2003)) and despite the fact that we do not know enough about the correlation between rRNA sequence type and phenotype (though we obtained 100% sequence similarity for the region studied) we regard our finding of *Hydrogenophillus thermoluteolus* as an indication of the geothermal environment beneath Lake Vostok. It is known that in such an environment, Archaea may be present (e.g., Barns *et al.* 1996). However, until now we have obtained no indication of them whilst the

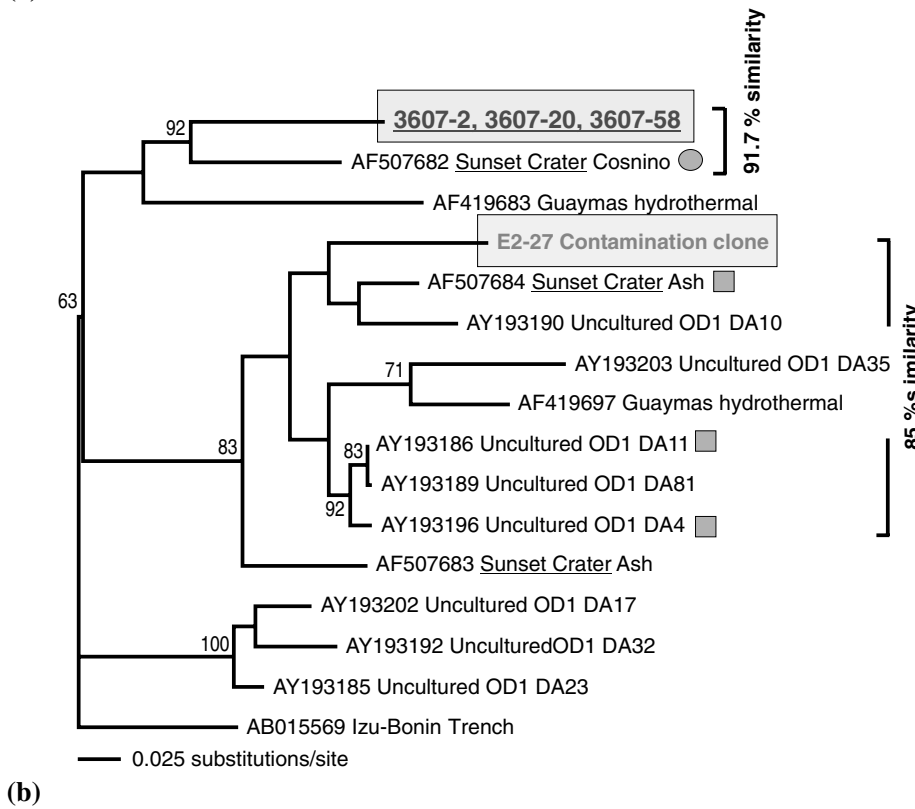
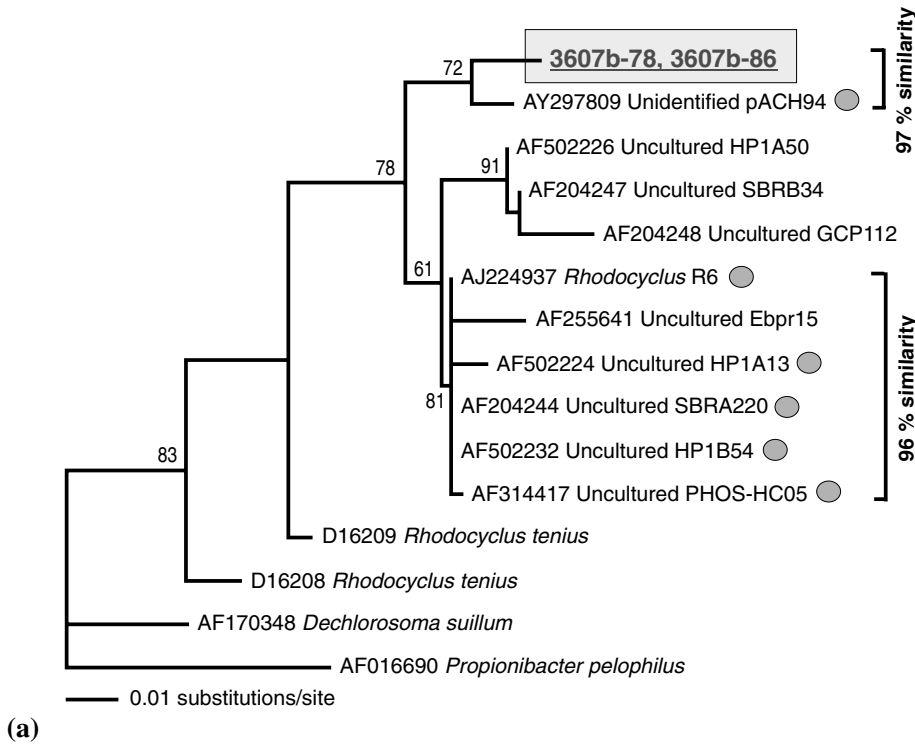


Fig. 2. Phylogenetic analysis of the bacterial rDNA clones recovered in 3607 m accretion ice and recorded as likely contaminants (Table 3). Bootstrap values of more than 50% are shown at the nodes. Species limits are depicted with transparent boxes. Sequences from GenBank with the highest similarity score (%) are labelled with full circles (concerning ice clones) or squares (concerning contaminants). Ice clones are in bold and underlined font styles. (a) Phylogenetic tree (a 428 nucleotide mask of unambiguously aligned positions) for ice clones related to *Rhodocyclus* sp. (b) Phylogenetic tree (a 403 nucleotide mask of unambiguously aligned positions) for ice clones related to OP11 candidate division soil strains. OD1 clones were sampled from a contaminated aquifer (N. Pace, unpublished). Contaminant clone is in bold font style. Soil clones from Sunset Crater are in underlined font style. (The image can be viewed in colour at <http://lgge.obs.ujf-grenoble.fr/axes/glaciobiology>.)

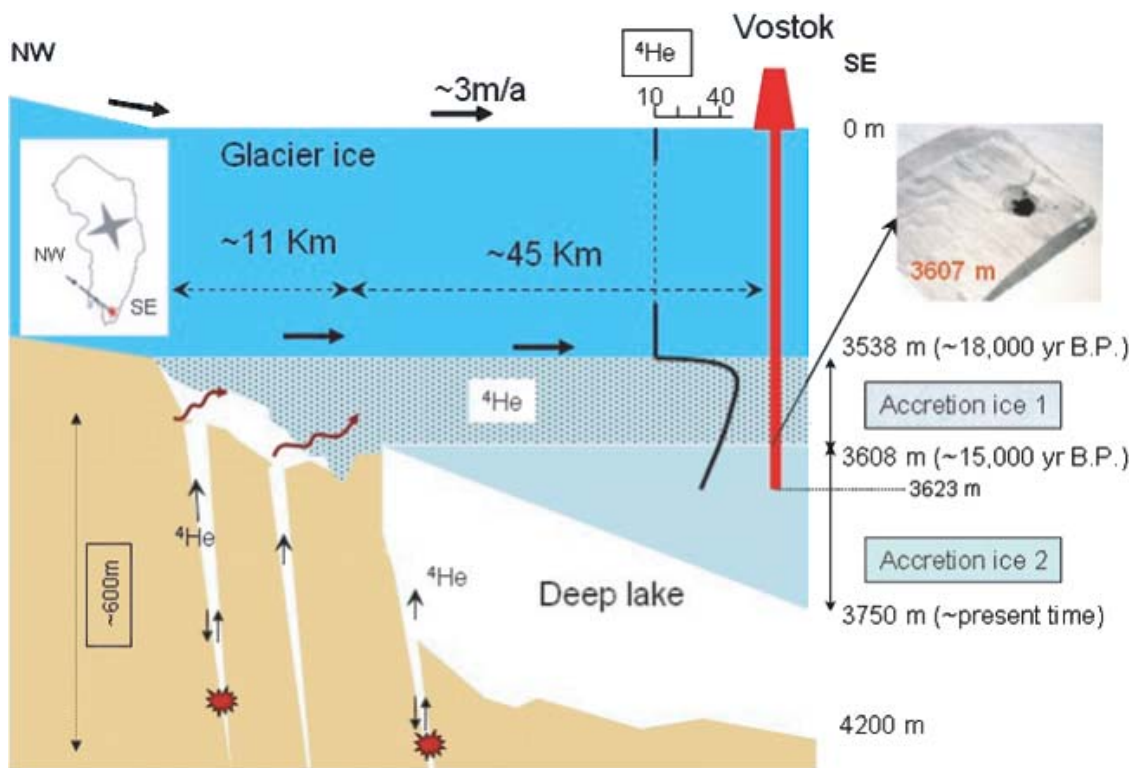


Fig. 3. Sketch of the glacier and lake basement along the Vostok ice flowline (adapted from Jouzel *et al.* 1999). On the 3623 m Vostok ice core, the accreted ice interval from 3538 to 3608 m depth (accretion ice 1) contains visible sediment inclusions (clay and mica aggregates) (insert on the right). Deeper ice and likely that ice all the way down to the glacier–water interface (accretion ice 2) are clean. Glacier flows are at about 3 m per year from the North–West to South–East (Bell *et al.* 2002) and the accreted ice 1 was formed approximately 15 000–20 000 years ago. A rise in relief is located at about 11 km enfolding a shallow depth embayment (Bell *et al.* 2002) where sediments could become incorporated into accreted ice (Jouzel *et al.* 1999). The rock basement is represented by escarpments, where deep faults allow water to seep to depth, become heated and then circulate. Fault activity and ^4He degassing from the rocks are depicted by an explosion symbol and vertical arrows, respectively. A sketch of down core ^4He concentration (Jean Baptiste *et al.* 2001) shows a constant value for glacial ice and a change by a factor of three at the glacier–accretion ice boundary. In accretion ice, a decreasing trend is observed from $38.5 \text{ nmole kg}^{-1}$ at a depth of 3551 m to $35.5 \text{ nmole kg}^{-1}$ at a depth of 3608 m suggesting ^4He contribution from the shallow embayment area upstream of Lake Vostok. (The image can be viewed in colour at <http://lgge.obs.ujf-grenoble.fr/axes/glaciobiology/>.)

updated primers and more sophisticated PCR schemes were tested. It is worth noting that Priscu and co-workers did not detect Archaea in accretion ice as well (Priscu *et al.* 1999).

The geophysical aspect

The apparent geothermal environment beneath Lake Vostok is counterintuitive for such a site. The absence of ^3He isotope enrichment in accretion ice with respect to the glacial ice excludes the possibility of a contribution from mantle-derived hydrothermal fluids (e.g., black smokers) (Jean Baptiste *et al.* 2001, 2003). However, three indices lend support to geothermal activity, just not necessarily mantle derived.

A first indicator is the geological setting. The lake is ~1200 m deep and lies in a depression within the thick continental basement: its sharp shores (Masolov *et al.* 2001) and likely U-shaped cross-section imply deep faults (Fig. 3) where water could seep to a great depth, heat up ($\sim 20^\circ\text{C km}^{-1}$) and rise back to the lake causing hydrothermal circulation.

The second indicator is the long-term tectonic activity. The earthquakes observed from a fault within the lake have been used to support the possible introduction of energy into the

lake, thereby fuelling hydrothermal circulation (Studinger *et al.* 2003). Past activity is supported by ^4He isotope measurements. A three-fold ^4He excess in the ca. 15 000 year old accretion ice is observed with respect to the overlying glacial ice (Jean Baptiste *et al.* 2001, 2003). ^4He , a by-product of uranium series decay, remains entrapped within the rock matrix but can be released by tectonic activity. The sediments at the bottom of the lake may also be the source of ^4He , but in this case the down core ^4He content of the accreted ice would increase with depth as a response to steady-state and upward He diffusion within the ice. In fact the opposite is observed, and ^4He concentration is higher in the upper accretion layer (Jean Baptiste *et al.* 2003) (Fig. 3). This suggests the possibility of a ^4He degassing contribution from fault vents which could be in the shallow bay located upstream of the Vostok ice core site (Fig. 3). The excess of ^4He found in the ca. 15 000–20 000 year old accretion ice suggests that tectonic activity was already operating at that time.

Third, hydrothermal area spring waters are known to be enriched with the ^{18}O isotope with respect to precipitation supplying the system (Craig 1963; Clayton & Steiner 1975).

This 'oxygen shift' with respect to the meteoritic water line (MWL) results from the isotope exchanges with ^{18}O rich rocks and silicates at high temperatures (Craig 1963). Lake Vostok is continuously renewed and its turnover time (τ) is 5000–100 000 years (Kapitsa *et al.* 1996; Jean Baptiste *et al.* 2001, 2003; Bell *et al.* 2002) which is small with respect to the likely age of the Lake ($\sim 1\,000\,000$ years or more). Assuming a mass balance, the input (i.e. melting ice) equals the output (i.e. accreted ice exported by glacier movement) and the equilibrium holds for the isotope composition of ice and water (i.e. deuterium δD and oxygen $\delta^{18}\text{O}$). The main melting area is located ~ 250 km north of Vostok, where the glacier flows down the slope from ridge B and it is thickest over the lake (Bell *et al.* 2002). This scenario satisfies the deuterium balance (Souchez *et al.* 2003). However, when compared to MWL from the Dome B ice core (Vimeux *et al.* 2001), a site located upstream of the melting area in Lake Vostok, accretion ice composition appears enriched in $\delta^{18}\text{O}$ by $\sim 0.5\%$ and this can be regarded as an imprint from a hydrothermal water contribution to the lake. This enrichment may explain the unusually low deuterium excess ($d = \delta\text{D} - 8 \cdot \delta^{18}\text{O} \sim 6\text{‰}$) (Jouzel *et al.* 1999) of the accretion ice with respect to ice compositions from the Dome B and Vostok areas (minimum value $\sim 10\text{--}13\text{‰}$) (Vimeux *et al.* 2001 and references therein), which likely supply the lake.

Deliberations

Thus, the geological setting, the long-term seismotectonic evidence from ^4He degassing and the ' ^{18}O shift' of accretion ice, all support the proposal that a geothermal environment exists beneath Lake Vostok.

Assuming that Lake Vostok is a vestige of an old continental rift system (Leitchenkov *et al.* 2003), seismotectonic activity has been operating locally for a long time. Local and distant tectonic events could activate fluid circulation (Dziak & Johson 2002) and promote chemical reactions between rocks and water. Among other chemicals, hydrogen could be released (Wakita *et al.* 1980), and this powerful reducer could be consumed by microorganisms (Chappelle *et al.* 2002). Our finding of *Hydrogenophilus thermoluteolus* in Lake Vostok accretion ice containing sediments is consistent with such a scenario.

Conclusions

The present study attempts to estimate the microbial content of Lake Vostok accretion ice and clearly shows that in the case of low biomass, the forward-contamination of samples represents a very substantial problem. It seems that the accretion ice is essentially germ-free indicating that the water body should also be hosting a highly sparse life, if any, unless the lake water lost its biological content during the accretion process. We should also note that some terrestrial areas have already been assumed to be lifeless (Navarro-González *et al.* 2003).

Of the 16 bacterial phylotypes initially recovered from the accretion ice, only one was confidently thought to be relevant

to the lake while the other 15 were presumed to be contaminants. The one relevant phylotype represents a single print of life in the lake environment. The best way to avoid contamination in future studies appears to use stringent ice-chemistry-based decontamination procedures and comprehensive biological controls including establishing a contemporary contaminant database as a prerequisite to identifying and categorizing sources of extraneous cells and nucleic acids.

The very low organic carbon and nutrient content measured in accretion ice suggests conditions that are more favourable for sustaining chemoautotrophic rather than heterotrophic biota. Our finding of *Hydrogenophilus thermoluteolus*, a well-known thermophilic facultative chemolithoautotroph usually inhabiting hot springs, can be regarded as a strong indication for such biota. In addition, we consider its recognition exclusively from an ice interval that contains sediment inclusions as an indicator for a geothermal environment underneath the lake. Such organisms are unlikely to thrive in the open lake, where the high concentration of dissolved oxygen (up to $0.7\text{--}1.3\text{ O}_2\text{ g l}^{-1}$) (Lipenkov & Istomin 2001; McKay *et al.* 2003) is expected to be a significant constraint for at least heterotrophic life (Mikell *et al.* 1986). This suggests that the bacteria like *H. thermoluteolus* perhaps live at depth in 'hot' basement faults filled with sediment. It can also be speculated that such faults may have been colonized long ago, soon after their formation and/or possibly before the onset of the first Antarctic glaciation around 34 000 000 years ago (Barrett 2003).

The hydrothermal plumes boosted by seismotectonic events could flush out bacteria and sediments from faults towards vents in the freezing zone where the microbes may have been trapped in the ice by the process of accretion (Jouzel *et al.* 1999). In Lake Vostok, seismotectonic influences exist (Studingier *et al.* 2003) and probably sustain the geothermally fuelled biological environment beneath the lake. Such influences could provide an alternative explanation to that based on black smokers (McCullom 1999) for biological primary production.

The results of the present study have distinct implications in the search for life forms on Jovian icy moons and Mars where low microbial biomass is expected, thus, making forward-contamination by, e.g., spacecraft terrestrial bioload a rather crucial issue (Space Studies Board 2000; Chyba & Phillips 2001). Specifically, the ice decontamination protocol developed as well as the contaminant database established in the present study represent 'lessons' for exobiology purposes.

As a guide for searching for life in subglacial environments like Lake Vostok, our criteria which are the very image of those developed for ancient DNA research (Cooper & Poiner 2000) can be summarized as follows.

- Apply stringent ice decontamination procedures to meet the chemistry and forensic science (DNA-free) standards.
- Document the biological content of various environments (including humans) in contact with ice samples (contaminant databases).

- Ensure that relevant biological tools (microscopy, culturing and PCR-hybridization) are used to cover all known and expected biodiversity.
- Verify microbial findings through their possible metabolic (redox) profiles taking into account the known physical and chemical features of icy environments (e.g., in Lake Vostok, no light, close to freezing point temperature, low DOC and other nutrients contents, excess of oxygen and nitrogen).

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